Angiogenic imbalance in preeclampsia:

Pathogenic, **diagnostic** and **prognostic implications**

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Angiogenic imbalance in preeclampsia:
Pathogenic, diagnostic and prognostic implications

Angiogene imbalans in pre-eclampsie:
Pathogene, diagnostische en prognostische implicaties

Proefschrift

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Stellingen behorende bij het proefschrift

"Angiogenic imbalance in preeclampsia: Pathogenic, diagnostic and prognostic implications"

1. De "klassieke" diagnose pre-eclampsie is obsoleet – dit proefschrift.

2. Bepaling van de sFlt-1 en PlGF bij zwangeren met (verdenking op) pre-eclampsie geeft een betere risico-inschatting van de kans op zwangerschapscomplicaties dan de huidige diagnose – dit proefschrift.

3. De snelle en sterke daling postpartum van de anti-angiogene serumbiomarker sFlt-1 in tegenstelling tot de angiogene serumbiomarker PlGF bij patiënten met pre-eclampsie en het HELLP-syndroom bewijst dat sFlt-1 vooral door de placenta wordt geproduceerd – dit proefschrift.

4. De serumbiomarker sFlt-1 is hoger in tweeling- dan in eenlingzwangerschappen en draagt mogelijk bij aan de grotere kans op pre-eclampsie bij meerlingzwangerschappen – dit proefschrift.

5. Behandeling met een endotheline receptorantagonist van vroege, ernstige pre-eclampsie met als doel de zwangerschapsduur te verlengen, moet worden overwogen – dit proefschrift.

6. Protonpompremming geassocieerd met lagere concentraties van sFlt-1, endogline en endotheline-1 in de maternale circulatie kan bijdragen aan de behandeling van pre-eclampsie – dit proefschrift.

7. Het verbeteren van de angiogenetische disbalans in pre-eclampsie, door het elimineren van sFlt-1 en/of de toediening van PlGF, is logisch maar klinisch onbewezen – Thadhani et al., J Am Soc Nephrol 2016;27:903-913 en Spradley et al., Hypertension 2016;67:740-747.

8. In tegenstelling tot de werking van angiogenese-remmers is vroege pre-eclampsie niet genderneutraal – Schalekamp-Timmermans et al., Int J Epidemiol 2017; 46:632-642.

9. De effecten van Viagra bij pre-eclampsie zijn even teleurstellend als die van vitamine C en vitamine E – Sharp et al., The Lancet Child & Adol health 2018 in press en Conde-Ahudelo et al., Am J Obstet Gynecol 2011; 204:503-512.

10. Pre-eclampsie en diabetes gravidarum zijn cardiovasculaire risicofactoren.

11. Elk staatshoofd zou de diversiteit van een land moeten representeren en niet langer het onderlinge verschil loochenen.

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To the loving memory of my grandmother

For Kurdistan

CONTENTS

Chapter 1

General introduction

Based on:

Role of endothelin in preeclampsia and hypertension following antiangiogenesis treatment.
Langeza Saleh, A.H. Jan Danser, Anton H. van den Meiracker.
Curr Opin Nephrol Hypertens. 2016 Mar;25:94-99.

Etiology of angiogenesis inhibition-related hypertension.
Langeza Saleh *, Stephanie Lankhorst*, A.H. Jan Danser, Anton H. van den Meiracker.
Curr Opin Pharmacol. 2015 Apr;21:7-13.

GENERAL INTRODUCTION

What now is known as "preeclampsia-eclampsia" was first described by Hippocrates around 400 BC, who stated that headache accompanied by heaviness and convulsions during pregnancy was considered bad. This was the earliest suggestion that there was a specific entity associated with an unhealthy pregnancy. Practized remedies were attemped to bring the body's fluids "into balance" through altered diets, purging and blood-letting. Interestingly, the theory of an imbalance in the maternal body has survived. According to current insight an imbalance in pro-angiogenic and antiangiogenic factors underlies the manifestations of preeclampsia (PE). Mounting evidence indicates that increased placental production of the antiangiogenic factor soluble fms-like tyrosine kinase-1 (sFlt-1), resulting in a decreased activity of the pro-angiogenic vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) underlies the angiogenic imbalance in PE. These factors and their roles, which are reviewed in this thesis, are briefly described below.

Vascular endothelial growth factor (VEGF)

The mammalian VEGF family consists of five different isoforms of which VEGF-A, commonly referred to as VEGF, is best characterized.[1] VEGF exerts a variety of biological activities and is normally produced by endothelial cells (ECs), podocytes, macrophages and fibroblasts, with hypoxia inducible factor 1α as an important mediator for hypoxia-induced VEGF transcription.[2] VEGF binds three tyrosine kinase receptors (VEGFRs). VEGFR1, also known as Flt-1, and VEGFR2 are expressed on vascular ECs, while VEGFR3 is mainly restricted to lymphatic ECs.[3] VEGF has a higher affinity for VEGFR1 than VEGFR2, but most of the biological effects of VEGF are mediated by VEGFR2.[4] Besides promoting angiogenesis, i.e. the development of new blood vessel from pre-exsiting vessels, VEGF also increases vascular permeability and is required for the maintenance of a differentiated endothelial cell (EC) phenotype and EC survival.[5-8]

Placental growth factor (PlGF)

PlGF, a member of the VEGF family, binds only to VEGFR1 and is sequestered by the soluble form of VEGFR1 or sFlt-1.[9] PlGF is an proangiogenic factor: it stimulates vessel growth and maturation.[10-12] This proangiogenic activity of PlGF relies on direct effects on endothelial and mural cells, as well as on indirect effects on non-vascular cells with proangiogenic activity. PlGF enhances the proliferation, migration, and survival of endothelial cells.[9, 11, 12] By competing with VEGF for the VEGFR1, PlGF can mediate the availability of VEGF for the VEGFR2.

soluble Fms-like tyrosine kinase-1 (sFlt-1)

sFlt-1 is a splice variant of the VEGF type 1 receptor lacking the transmembrane and cytoplasmic domains, and acts as a potent VEGF and PlGF antagonist.[13] It is produced by a number of tissues, including the placenta,[13-14] but its precise physiological role is unclear. Both placental sFlt-1 expression[15-16] as well as sFlt-1 levels in amniotic fluid[8] and blood are elevated in PE.[16-17]

Aims of the thesis

- To investigate the added value of the biomarkers sFlt-1, PlGF and sFlt-1/PlGF ratio on top of the current standard of diagnosis.
- To analyze the predictive value of the sFlt-1, PlGF and sFlt-1/PlGF ratio not only for diagnosing or excluding preeclampsia, but also for the prediction of maternal and fetal/neonatal outcome in singleton pregnancies.
- To compare the predictive value of the sFlt-1/PlGF ratio thresholds of ≤38 and >85 with the predictive value of the continuous values of the individual biomarkers and their ratio.
- To examine the efficacy of sFlt-1, PlGF and various cutoff values of sFlt-1/PlGF ratio as a clinically diagnostic tool in different study groups.
- To develop a well-discriminating prediction model for the risk of maternal and fetal or neonatal complications in individual pregnant women.

STUDY SETTINGS

A few chapters in this thesis overlap concerning the patient populations. The table below serves to clarify the populations that have been used per study.

Chapter	Recruitment period	Patient population
3	March 2012 – February 2013	Healthy pregnant women, patients with suspected or confirmed clinical PE.
4	December 2013 - April 2016*	Patients with suspected or confirmed clinical PE.
5	September 2011 - August 2013	Patients with suspected or confirmed clinical PE.
6	September 2011 - August 2013	Patients with suspected or confirmed clinical PE with repetitive biomarker measurement during and, or after pregnancy.
7	September 2011 - April 2016	Twin pregnancies with suspected or confirmed clinical PE.
8	December 2013 - April 2016*	Patients with suspected or confirmed clinical PE.
9	December 2013 - April 2016*	Patients with suspected or confirmed clinical PE.

*, Studies overlap; PE, preeclampsia.

In the past several years activation of the endothelin system has emerged as an important (independent) pathway causing hypertension and proteinuria in PE. Studies in animal models representative of PE, have shown that endothelin receptor blockers prevent the development of this disease. **Chapter 2 (Part I)** critically addresses this concept, taking into consideration both clinical and preclinical data.

The consequences of a disturbed angiogenic balance on the renin-angiotensin system (RAS) and the correlation between circulating levels of sFlt-1 and ET-1 in pregnant women with and without PE are investigated in **Chapter 3**.

Due to the potential detrimental effects of PE, and the fact that the mainstay therapy remains delivery, focusing on alternative treatment strategies in order to improve pregnancy outcome is the subject of intensive experimental research. We and others have shown increased sFlt-1 levels in pregnancies complicated with PE. As a consequence, the benefit of the removal of sFlt-1 is under investigation. Interestingly, proton pump inhibitors (PPIs) that are regularly prescribed during pregnancy to combat reflux disease[18], have shown to decrease trophoblast sFlt-1 and endoglin secretion in vitro.[19] Making use of a prospective cohort study involving 430 women, **Chapter 4** investigates whether the use of PPIs affects sFlt-1, PlGF and their ratio as well as circulating endothelin and endoglin levels in women with confirmed PE or suspected of this condition.

In **Part II** we attempt to evaluate the efficacy of the biomarkers sFlt-1 and PLGF and various cutoff values of their ratio as a clinically diagnostic tool in different study groups. **Chapter 5** evaluates the utility of the sFlt-1/PlGF ratio in diagnosing PE and its agreement or disagreement with the clinical diagnosis to predict the prolongation of pregnancy and pregnancy outcome. In **Chapter 6** we determine the evolution of the biomarkers during and after pregnancy. The proposed sFlt-1/PlGF ratio ≤38 for singleton pregnancies to rule out PE is applied to twin pregnancies in **Chapter 7**.

In **Part II**, **Chapter 8**, we for the first time investigate the incremental value of the continuous concentrations of sFlt-1, PlGF and their ratio rather than dichotomized cutoffs of the sFlt-1/PlGF ratio for the prediction of maternal and fetal/neonatal outcome and pregnancy prolongation. In **Chapter 9** we developed a clinical prediction score for the risk of maternal and fetal complications in pregnant women with suspected/confirmed PE. **Chapter 10** summarizes all findings and discusses their implications.

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Chapter 2

The emerging role of endothelin-1 in the pathogenesis of preeclampsia

Langeza Saleh, Koen Verdonk, Willy Visser, Anton H. van den Meiracker and A. H. Jan Danser.

Ther Adv Cardiovasc Dis. 2016; 10:282-293.

ABSTRACT

Preeclampsia (PE) is the most frequently encountered medical complication during pregnancy. It is characterized by a rise in systemic vascular resistance with a relatively low cardiac output and hypovolemia, combined with severe proteinuria. Despite the hypovolemia, renin–angiotensin system (RAS) activity is suppressed and aldosterone levels are decreased to the same degree as renin. This suggests that the RAS is not the cause of the hypertension in PE, but rather that its suppression is the consequence of the rise in blood pressure. Abnormal placentation early in pregnancy is widely assumed to be an important initial event in the onset of PE. Eventually, this results in the release of anti-angiogenic factors [in particular, soluble Fms-like tyrosine kinase-1 (sFlt-1)] and cytokines, leading to generalized vascular dysfunction. Elevated sFlt-1 levels bind and inactivate vascular endothelial growth factor (VEGF). Of interest, VEGF inhibition with drugs like sunitinib, applied in cancer patients, results in a PE-like syndrome, characterized by hypertension, proteinuria and renal toxicity. Both in cancer patients treated with sunitinib and in pregnant women with PE, significant rises in endothelin-1 occur. Multiple regression analysis revealed that endothelin-1 is an independent determinant of the hypertension and proteinuria in PE, and additionally a renin suppressor. Moreover, studies in animal models representative of PE, have shown that endothelin receptor blockers prevent the development of this disease. Similarly, endothelin receptor blockers are protective during sunitinib treatment. Taken together, activation of the endothelin system emerges as an important pathway causing the clinical manifestations of PE. This paper critically addresses this concept, taking into consideration both clinical and preclinical data, and simultaneously discusses the therapeutic consequences of this observation.

INTRODUCTION

Preeclampsia (PE) is the most frequently encountered medical complication during pregnancy. PE is not simply *de novo* onset of hypertension and proteinuria in the last half of pregnancy, but rather a syndrome involving many organs, of which the clinical spectrum ranges from relatively mild to life-threatening [Steegers *et al.* 2010]. Currently, treatment of PE consists of treating the elevated blood pressure and prevention of seizures, but the ultimate remedy is delivery of the placenta, indicating that the placenta is a central culprit in the pathogenesis of PE [Steegers *et al.* 2010]. The etiology of PE is unknown. A large body of evidence, supported by preclinical models of PE, indicates that abnormal placentation early in pregnancy is an important initial event in the onset of PE [Roberts and Redman, 1993; Myatt, 2002]. This abnormal placentation stimulates the production of anti angiogenic factors and cytokines, resulting in generalized vascular dysfunction and the clinical manifestations of PE. In the past several years, activation of the endothelin (ET) system has emerged as an important pathway causing the clinical manifestations of PE [Makris *et al.* 2007; Maynard *et al.* 2008; Verdonk *et al.* 2014]. This paper critically addresses this concept, taking into consideration both clinical and preclinical data.

Pathogenesis and manifestations of preeclampsia

Clinically, PE is divided into 2 types: early-onset PE before 34 weeks of gestation and late-onset PE at, or after 34 weeks of gestation [Von Dadelszen *et al.* 2003]. The incidence of PE is 3–8% worldwide. The pathogenesis of PE involves two stages [Redman, 1992; Redman *et al.* 2005; Seki, 2014]. In stage one, aberrant shallow cytotrophoblast invasion in the maternal spiral arteries supplying the placenta results in poor placentation [Brosens, 1964]. This poor placentation is postulated as the root cause of stage two, consisting of repeated periods of placental hypoxia and reperfusion injury, resulting in oxidative stress and an increased production of placental factors such as soluble Fms-like tyrosine kinase 1 (sFlt-1), soluble endoglin, agonistic auto-antibodies to the angiotensin (Ang) II type 1 receptor (AT1R-AA) and inflammatory cytokines [Maynard *et al.* 2008; Naljayan *et al.* 2013; Seki, 2014]. In the maternal circulation these factors cause activation of endothelial cells and generalized endothelial dysfunction, leading to the clinical manifestations of PE. PE is a multifaceted disorder: in addition to hypertension and proteinuria, it can also affect the central nervous system, lungs, liver, and the heart [Steegers *et al.* 2010].

PE may increase the risk of eclampsia and the development of the HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome, a severe condition characterized by disseminated intravascular coagulation, acute renal failure and pulmonary edema that can end in maternal death [Haram *et al.* 2009]. PE is cured by delivery of the placenta, which to date is the only effective treatment of PE. Although beneficial for the mother, preterm delivery may compromise the health of the infant both acutely and chronically; hence treatments to prevent or alleviate PE in order to prolong pregnancy are urgently needed [Friedman *et al.* 1999].

Hemodynamics and the renin–angiotensin system in preeclampsia

Compared with normal pregnancy, PE is characterized by a rise in systemic vascular resistance with a relatively low cardiac output and hypovolemia [Hall *et al.* 2011]. This rise in systemic vascular resistance is accompanied by suppression of the renin–angiotensin system (RAS) [Powe *et al.* 2011]. The latter is somewhat unexpected in view of the reduced circulating volume. It might simply represent protection against a further rise in blood pressure, related to the fact that blood pressure itself inversely affects renin release. An alternative reason for the suppression of the RAS is the production of AT1R-AA by the placenta of PE patients [Verdonk *et al.* 2015].

Such antibodies, by activating the AT1 receptor, should indeed suppress renin release from the kidneys (the consequence of the so-called negative feedback loop between Ang II and renin release), but would also be expected to increase aldosterone synthesis in the adrenal gland. As a result, the aldosterone/renin ratio in PE should be higher as compared with the ratio in healthy pregnant women. Unexpectedly, this turned out not to be the case [Verdonk *et al.* 2015]. Moreover, AT1R-AA are not present in all PE cases and can even be detected in healthy pregnant women [Walther *et al.* 2005]. Therefore, despite preclinical studies supporting a role for AT1R-AA in the pathogenesis of PE [Faas *et al.* 1994; Zenclussen *et al.* 2004; Zhou *et al.* 2008], there is still doubt about the *in vivo* importance of AT1R-AA in PE.

It has been well established that the blood pressure rise to exogenous Ang II in PE is larger than in healthy pregnant women [Gant *et al.* 1973; Baker *et al.* 1992]. Obviously, the generalized endothelial dysfunction in PE may account for the increased Ang II sensitivity [Wenzel *et al.* 2011]. In addition, by investigating subcutaneous resistance vessels obtained from pregnant womenwith and without PE *ex vivo*, we observed that the increased vasoconstrictor response to Ang II in PE involves Ang II type 2 (AT2) receptors [Verdonk *et al.* 2015]. Normally, this receptor induces vasodilation, but often its phenotype changes under pathological conditions [Moltzer *et al.* 2010]. Possibly, therefore, the enhanced sensitivity to Ang II in PE is additionally due to an upregulation of constrictor AT2 receptors.

Recently, Gennari-Moser and colleagues proposed that vascular endothelial growth factor (VEGF) stimulates aldosterone production, both directly and indirectly, the latter by enhancing adrenal capillary density [Gennari-Moser *et al.* 2013]. PE patients display elevated levels of the VEGF-binding soluble receptor, sFlt-1, and thus would be expected to have suppressed aldosterone levels and a decreased aldosterone/renin ratio [Gennari-Moser *et al.* 2013]. However, as discussed above, although aldosterone levels are indeed diminished in PE, so are renin levels, and the aldosterone/renin ratio is unaltered [Verdonk *et al.* 2015]. This argues against VEGF being an important determinant of the suppressed aldosterone levels in PE. Rather, a factor that suppresses renin seems to be involved, which, consequently, would lower aldosterone to the same degree, simply because of RAS suppression. This factor could be the rise in blood pressure, as discussed above, although normally, the inverse relationship between blood pressure and renin release is rather modest [Danser *et al.* 1998].

Collectively, the findings summarized here indicate that RAS suppression is counter-intuitive in PE, given the hypovolemia in this disease. It might be the consequence of the rise in blood pressure. If so, remaining questions are: what causes this rise in blood pressure, if not the RAS, and, whether blood pressure is truly the only determinant of the suppression of renin release in PE. As outlined below, emerging data indicate that activation of the ET system may be the reason.

The endothelin system

ETs are a family of three 21-amino-acid peptides (ET-1, ET-2 and ET-3), each encoded by distinct genes (EDN1, EDN2 and EDN3) [Yanagisawa, 1994; Yanagisawa *et al.* 1998a]. The EDN genes encode the prepro form of ETs (prepro-ETs). Prepro-ETs are cleaved at dibasic sites to big ETs by a furin-like endopeptidase [Pollock and Opgenorth, 1993]. Big ETs are biologically inactive. They undergo further modification by one of the ET-converting enzymes (ECEs) to yield the biologically active ETs (Figure 1) [Inoue *et al.* 1989; Takahashi *et al.* 1993; Xu *et al.* 1994; Yanagisawa *et al.* 1998b]. There are three isoforms of ECE (ECE-1, ECE-2 and ECE-3), localized in endothelial and smooth muscle cells, cardiomyocytes and macrophages [Xu *et al.* 1994; Maguire *et al.* 1997; Fukuchi and Giaid, 1998]. Of the ET family, ET-1 is the predominant member. It is synthesized and secreted by a range of cells, including endothelial cells and the syncytiotrophoblasts of the placenta [Rubanyi and Polokoff, 1994]. ET secretion occurs constitutively and up activation from stores in the socalled Weibel–Palade bodies of endothelial cells [Malassine *et al.* 1993; Van Mourik *et al.* 2002].

Several stimuli like Ang II, norepinephrine, thrombin, cytokines, growth factors, hypoxia, insulin, shear stress, free radicals, but also ET-1 itself, have been reported to induce endothelial ET-1 release [Levin, 1995; Jougasaki *et al.* 2002; Romani De Wit *et al.* 2004; Marasciulo *et al.* 2006; Khimji and Rockey, 2010]. ET-1 is released towards the basolateral side of these cells, acting primarily as a paracrine or autocrine peptide [Wagner *et al.* 1992].

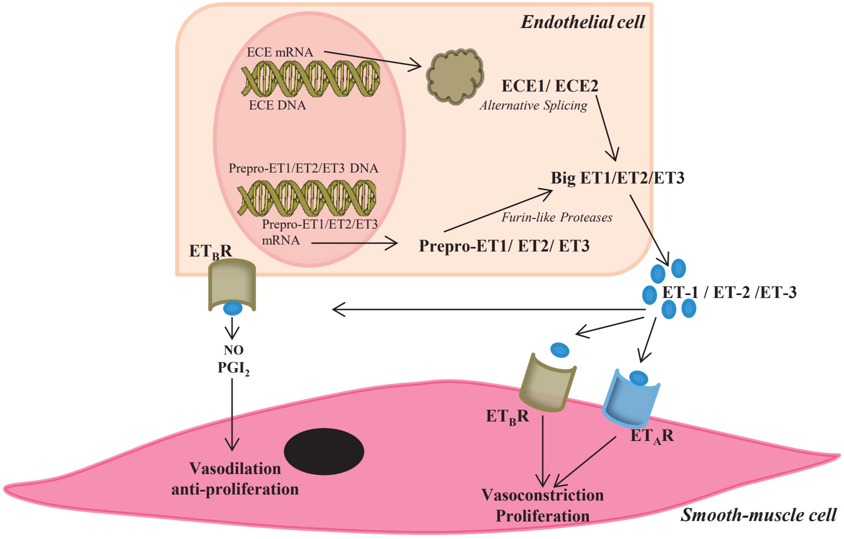

Figure 1. Endothelin (ET) synthesis and receptors in the vascular wall. ECE, endothelin converting enzyme; ET_A, ET_B, endothelin type A and type B receptor. See text for explanation.

ETs elicit their effect by binding to the cell-membrane G-protein-coupled ET type A and B (ETA and ETB) receptors, mapped on chromosomes 4 and 13 [Levin, 1995]. These receptors can be differentiated pharmacologically based on their affinity for the ETs. The ETA receptor has a 10-fold greater affinity for ET-1 and ET-2 than for ET-3, whereas the ETB receptor has similar binding affinity for all ETs [Watanabe *et al.* 1989; Sakurai *et al.* 1990]. The ETA receptor binds ET-1 almost irreversibly [Hilal-Dandan *et al.* 1997]. Furthermore, cross-talk between ETA and ETB receptors has been reported, in a way that inhibition of one receptor subtype will free the other receptor subtype from the inhibition [Fukuroda *et al.* 1996].

ETA and ETB receptors are widely distributed in various tissues, including the lungs, kidneys, liver, heart, brain, heart, eye, ovaries and adrenal glands [MacCumber *et al.* 1989, 1990; Masaki, 2004]. The majority of the ETA receptors are located on vascular smooth muscle cells (VSMC) whereas the ETB receptors are located on endothelial cells, VSMC, and epithelial cells [Masaki *et al.* 1992; Seo and Lüscher, 1995; D'orleans-Juste *et al.* 2002; Motte *et al.* 2006]. Activation of ETA and ETB receptors on VSMCs initiates vasoconstriction and cell proliferation, whereas activation of the ETB receptor on endothelial cells mediates vasodilation by releasing nitric oxide (NO) and prostacyclin [Ekelund *et al.* 1994; Lankhorst *et al.* 2013]. Binding of these receptors to different G-proteins is the most likely explanation for these diverse effects. Many factors can affect ET receptor expression. Insulin is known to cause an increase of ETA receptors in VSMCs, whereas the ETB receptor in endothelial cells is upregulated by tumor necrosis factor-α and basic fibroblast growth factor 2 [Frank *et al.* 1993; Smith *et al.* 1998; Francis *et al.* 2004].

ET-1 is rapidly cleared from the circulation. Studies revealed a plasma half-life ranging from 1.4 to 3.6 min, while the vasoconstriction has been shown to persist up to several hours [Vierhapper *et al.* 1990; Weitzberg *et al.* 1991]. The lung is able to clear more than 40% of ET-1. The kidneys and liver also play a role in the clearance of ET-1 from the circulation [Gandhi *et al.* 1993]. ETB receptors are likely responsible for this clearance, because intravenous infusion of the ETB receptor antagonist BQ788 extensively inhibited ET-1 uptake by the lungs and kidneys and increased plasma ET-1 levels while the ETA receptor antagonist BQ123 had no such effects [Fukuroda *et al.* 1994; Dupuis *et al.* 1996].

Endothelin-1 in preclinical models of preeclampsia

In the past decade, many animal models have been developed to replicate the various aspects of human PE. Here, we discuss the most important models.

Reduced uterine perfusion pressure

The reduced uterine perfusion pressure (RUPP) model in rats recapitulates many of the hallmarks of PE [Alexander *et al.* 2001a]. In this model, blood flow to the uterus is partially occluded at day 14 of gestation, resulting in placental ischemia [Alexander *et al.* 2001a; Granger *et al.* 2002]. Findings in this model demonstrate that blood pressure, proteinuria, renal expression of prepro-ET-1 in both medulla and cortex, and plasma ET-1 are significantly elevated, whereas renal function and NO production are impaired, as compared with pregnant controls [Alexander *et al.* 2001b; Kiprono *et al.* 2013]. This model is also associated with fetal growth restriction. Treating rats with an ET_A receptor blocker abol-ished the rise in blood pressure and the renal dys-function, demonstrating that the enhanced ET-1 production, via activation of the ET_A receptor, mediates the hypertension and proteinuria in this model [Alexander *et al.* 2001b]. Aside from increased ET_A receptor-mediated vasoconstric-tion, downregulation of the vasodilator microvascular ET_B receptor may also contribute to the PE-like findings in this model [Mazzuca *et al.* 2014].

Soluble Fms-like tyrosine kinase-1 elevation

An imbalance between pro- and anti-angiogenic factors, especially an increase in sFlt-1, is an important initial event in the pathogenesis of PE [Fiore *et al.* 2005; Maynard *et al.* 2008]. This has stimulated the development of rat models in which sFlt-1 has been increased in various ways [Maynard *et al.* 2003; Murphy *et al.* 2010, 2012]. Maynard and colleagues injected the tail vein with recombinant adenovirus encoding the murine sFlt-1 gene [Maynard *et al.* 2003], while Murphy and colleagues infused sFlt-1 at a rate of 3.7 µg/kg/day for 6 days [Murphy *et al.* 2010]. A three-fold increase in plasma sFlt-1 was reached in both models. The rats developed significant hypertension, proteinuria and glomerular endotheliosis [Maynard *et al.* 2003]. Moreover, increased plasma ET-1 levels and an increased prepro-ET-1 mRNA expression in both kidney and placenta were observed [Murphy *et al.* 2010]. Treatment with an ET_A receptor antagonist abolished the hypertensive responses in both studies, while having no effect in normotensive rats. This indicates that the rise in blood pressure was mediated by the ET system.

Injection of angiotensin II type 1 receptor agonistic auto-antibodies

Zhou and colleagues injected pregnant mice with 800 µg IgG isolated from sera of either normotensive pregnant women or PE patients, the latter containing AT_1R-AA [Zhou *et al.* 2008]. Mice injected with IgG from PE patients demonstrated hypertension, proteinuria, glomerular endotheliosis, elevated renal prepro-ET-1, placental abnormalities and smaller fetuses. Moreover, their sFlt-1 concentration was also significantly increased, in contrast to the sFlt-1 level in nonpregnant mice injected with IgG from either normotensive pregnant women or PE patients, which remained very low because the major source of sFlt-1 (the placenta) was missing [Herse *et al.* 2007]. Notably, pregnant mice injected with IgG isolated from normotensive pregnant women did not develop the above symptoms. Co-injecting the PE IgG-exposed mice with BQ123, an ET_A receptor blocker, abolished these features, again indicating the involvement of ET-1 production in the pathophysiology of PE [Zhou *et al.* 2011]. Identical observations were made in pregnant rats: infusion of rat AT_1R-AA resulted in hypertension and ET-1 upregulation in kidney and placenta, and treatment with the ET_A receptor antagonist ABT-627 prevented the blood pressure rise [LaMarca *et al.* 2009]. This leaves the question: how do AT_1R-AA activate the ET system? A link between AT_1 receptor activation and ET-1 release was already noted more than 20 years ago [Dohi *et al.* 1992]. Zhou and colleagues making use of human placental villous explants, recently showed that this involved the tumor necrosis factor-α/interleukin-6 signaling path-way, since antibodies against these cytokines or their receptors prevented the AT_1R-AA-induced ET-1 secretion [Zhou *et al.* 2011].

Altered anti-angiogenic state and endothelin-1 overexpression

Anti-angiogenic treatment in patients with cancer with either antibodies to VEGF or inhibitors of the VEGF receptors, so-called receptor tyrosine kinase inhibitors (RTKI), induces hypertension and renal toxicity [Hayman *et al.* 2012]. In patients treated with the RTKI sunitinib, we were the first to show that the rise in blood pressure was associated with increased circulating ET-1 levels [Kappers *et al.* 2010]. Administration of sunitinib to rats for 8 days also induced hypertension, proteinuria, renal function impairment, glomerular endotheliosis, as well as elevated circulating ET-1 levels [Kappers *et al.* 2011]. In fact, the renal histological changes observed dur-ing sunitinib exposure closely resembled those observed in PE. This is not too surprising, since VEGF inhibition (with an RTKI) will accomplish the same effect as VEGF inactivation or binding (with sFlt-1). Co-administration of the dual ET_A/ ET_B receptor blocker macitentan could prevent the hypertension and proteinuria induced by sunitinib, indicating that, like in experimental models of PE, activation of the ET axis mediates the hypertension and proteinuria induced by anti-VEGF treatment. Taken together, these data indicate that RTKI treatment induces a PE-like syndrome involving ET-1, albeit in the absence of pregnancy.

Lastly, the consequences of endothelial ET-1 excess are further emphasized by a recent study by Rautureau and colleagues [Rautureau *et al.* 2015]. They developed a mouse model of inducible endothelium-specific ET-1 overexpression. Remarkably, such endothelial ET-1 overexpression led to hypertension, in an ET_A receptor-dependent manner, but not to vascular or kidney injury, or changes in kidney perfusion or function. This suggests that renal damage, if occur-ring, depends on renal ET-1 overexpression rather than elevated circulating ET-1 levels. Our data in sunitinib-treated rats, showing that renal toxicity requires higher doses [Lankhorst *et al.* 2015], and that antihypertensive treatment with calcium antagonists, ACE inhibitors or macitentan differentially affects blood pressure and kidney damage [Lankhorst *et al.* 2014], fully confirms this view.

Endothelin-1 in clinical preeclampsia

Studies examining ET-1 in normal and PE pregnancies observed a two- to three-fold rise of circulating ET-1 in PE pregnancies compared with normal pregnancies (Table 1), with some studies indicating a positive correlation with the severity of the disease [Nova *et al.* 1991; Aydin *et al.* 2004; Baksu *et al.* 2005; Bernardi *et al.* 2008; Aggarwal *et al.* 2012; Karakus *et al.* in press; Verdonk *et al.* 2015]. In agreement with the latter, patients with the HELLP syndrome displayed even higher ET-1 levels than PE patients [Nova *et al.* 1991; Bussen *et al.* 1999; Karakus *et al.* in press]. Elevated ET-1 levels or expression in am-

Table 1. Plasma ET-1 levels in healthy pregnant women, pre-eclampsia and HELLP.

Reference	Healthy pregnancy		Pre-eclampsia		HELLP	Significance *p* value
Taylor *et al.* [1990]	6.1	± 0.7 pg/ml	11.0	± 0.9 pg/ml	–	<0.01
Nova *et al.* [1991]	3.9	± 0.3 µmol/l	5.5	± 0.3 µmol/l	8.3 ± 1.6 µmol/l	<0.001
Bussen *et al.* [1999]	0.3	± 0.3 pmol/l	–		1.8 ± 0.5 pmol/l	<0.05
Aydin *et al.* [2004]	6.0	± 0.3 ng/ml	11.5	± 0.5 ng/ml	–	<0.001
Baksu *et al.* [2005]	3.8	± 0.9 µmol/l	5.2	± 0.8 µmol/l	–	<0.001
Bernardi *et al.* [2008]	1.2	± 0.3 pg/ml	3.5	± 1.3 pg/ml	–	<0.01
Aggarwal *et al.* [2012]	0.9	± 0.4 pg/ml	1.5	± 0.6 pg/ml	–	<0.001
Verdonk *et al.* [2015]	0.69	(0.61-0.85) pg/ml	1.88	(1.19-2.49) pg/ml	–	<0.001
Karakus *et al.* [in press]	46	± 50 ng/ml	63	± 67 ng/ml	144 ± 64 ng/ml	<0.001

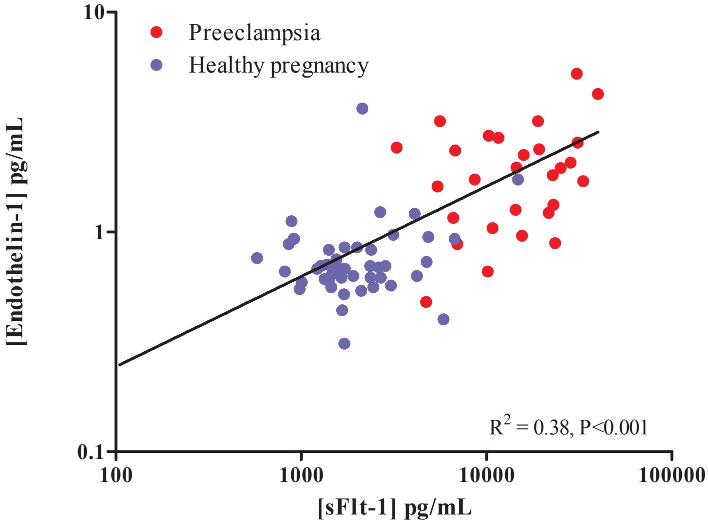

Figure 2. Relationship between soluble Fms-like tyrosine kinase-1 (sFlt-1) and endothelin-1 (ET-1) in plasma obtained from healthy pregnant women and women with preeclampsia. Data have been modified from [Verdonk *et al.* 2015].

niotic fluid and blood vessels obtained from PE patients *versus* healthy pregnant women [McMahon *et al.* 1993; Wolff *et al.* 1996; Faxen *et al.* 1997; Napolitano *et al.* 2000] confirm the concept that ET-1 levels are uniformly elevated in this disease. Importantly, multiple regression analysis revealed that ET-1 is not only an independent determinant of both the blood pressure rise and proteinuria in PE, but also a renin suppressor [Verdonk *et al.* 2015]. Animal studies support the latter [Ritthaler *et al.* 1995; Ortiz-Capisano, 2014].

It remains to be determined what causes the rise in ET-1. The strong correlation between sFlt-1 and plasma ET-1 (Figure 2) [Aggarwal *et al.* 2012; Verdonk *et al.* 2015], as well as the observation that ET-1 rises dose-dependently in rats treated with the VEGF inhibitor sunitinib [Lankhorst *et al.* 2015], suggest that the rise in ET-1 is the direct consequence of VEGF inactivation or inhibition. Since ET-1 itself triggers oxidative stress in the placenta, which in turn may result in increased production of placental factors such as sFlt-1 [Fiore *et al.* 2005], a vicious circle seems to arise which steadily raises circulating ET-1 in PE, subsequently affecting blood pressure, kidney function and RAS activity (Figure 3).

Therapeutic implications

NO, cleaved from L-arginine by NO synthase, is a well known suppressor and physiological antagonist of ET-1 [Boulanger and Lüscher, 1990; Brunner *et al.* 1995; Ohkita *et al.* 2002]. Indeed, sFlt-1-in-fused pregnant rats responded well to L-arginine administration: both the renal mRNA expression of ET-1 and maternal blood pressure decreased, while

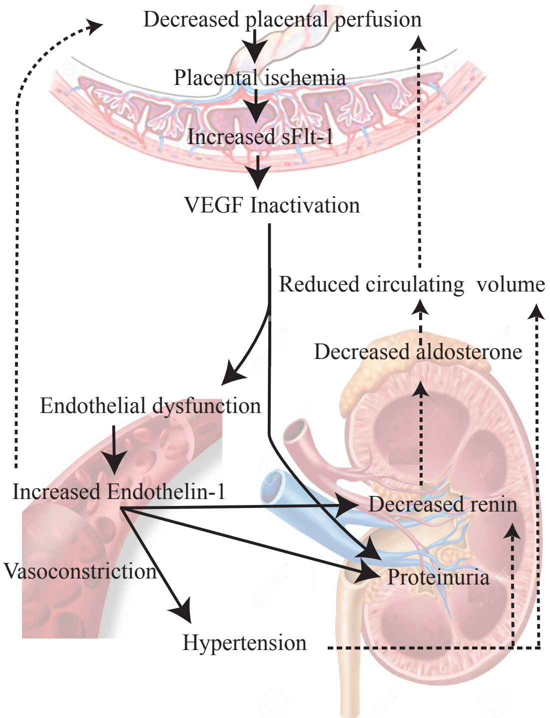

Figure 3. Unifying model depicting the central role of endothelin-1 (ET-1) in pre-eclampsia. Decreased perfusion of the placenta results in placental hypoxia and soluble Fms-like tyrosine kinase-1 (sFlt-1) release. SFlt-1 binds free vascular endothelial growth factor (VEGF), thereby inactivating this factor and inducing endothelial dysfunction. As a consequence, ET-1 production is turned on, which not only induces hypertension and proteinuria but also suppresses renin release. Such suppression will also occur due to the rise in blood pressure. The renin suppression is accompanied by a parallel aldosterone suppression, illustrating that the latter is entirely due to diminished angiotensin generation. Diminished renin–angiotensin-aldosterone system activity combined with high blood pressure results in a reduced circulating volume, thereby further decreasing placental perfusion. In addition, ET-1 induces sFlt-1 release from the placenta, thereby generating a deleterious feed-forward mechanism.

vascular function and fetal weight improved [Murphy *et al.* 2012]. A 3-week therapy of L-arginine also decreased blood pressure in women with PE, while prolonged treatment additionally improved fetal conditions and infant outcome [Rytlewski *et al.* 2005]. Facchinetti and colleagues confirmed the acute blood pressure-lowering effect of L-arginine in PE [Facchinetti *et al.* 1999], although Staff and colleagues did not observe an antihypertensive effect when giving 12 g L-arginine orally for 2 days [Staff *et al.* 2004].

Given our observation that sFlt-1 is a direct determinant of ET-1 expression [Verdonk *et al.* 2015], sFlt-1 removal might be beneficial as well. Thadhani and colleagues accomplished this by apheresis in three women with severe early-onset PE, making use of a negatively charged dextran sulfate cellulose column capable of adsorbing sFlt-1. In their study, apheresis not only decreased circulating sFlt-1, but also normalized proteinuria, stabilized blood pres-sure and prolonged pregnancy, without evident adverse outcomes in either mother or child [Thadhani *et al.* 2011]. To what degree it affected ET-1 was not investigated. Given these promising findings, the results of other ongoing (interventional) studies applying the same approach are anxiously awaited.

As discussed above, ET receptor antagonists dis-played beneficial effects in both PE animal mod-els and rats treated with sunitinib. Currently, macitentan and bosentan, oral agents with dual (ET_A and ET_B) receptor blockade function, and the ET_A receptor-selective antagonist ambrisentan, are available for clinical use. Beneficial effects of such drugs have been shown in pulmonary arterial hypertension, cancer and renal failure [Humbert *et al.* 2004]. At this stage, we do not know whether we should block both ET receptors in PE or only one subtype. Since ET_B receptors also serve as clearance receptors [Kohan, 1997], selective ET_B receptor antagonists, like ET_B receptor knockout approaches [Gariepy *et al.* 2000], will increase circulating ET-1, thereby potentially elevating blood pressure. Selective ET_A receptor antagonists therefore seem the preferred type of blocker, and additional ET_B receptor blockade may or may not have additional beneficial effects. Future studies comparing dual and selective ET_A receptor blockers head to head in appropriate models should answer this question.

Unfortunately, even when future animal studies will have yielded the best approach to block ET-1 (dual or single blockade), antagonism of ET receptors in pregnant women with PE may not be feasible, because of the potential teratogenic effects of such drugs [Clouthier *et al.* 1998; Treinen *et al.* 1999; Taniguchi and Muramatsu, 2003]. Fetal malformations have been observed in both ET_A receptor knockout mice and rats treated with ET_A receptor antagonists [Kurihara *et al.* 1994; Clouthier *et al.* 1998; Yanagisawa *et al.* 1998b]. Yanagisawa and colleagues observed that ET_A receptor antagonists given to rodents early in pregnancy resulted in craniofacial anomalies and fetal death [Yanagisawa *et al.* 1998b]. This appeared not to be the case when administration occurred late in pregnancy [Thaete *et al.* 2001; Olgun *et al.* 2008; Reichetzeder *et al.* 2014]. In humans, Bédard and colleagues have also shown that pregnant women diagnosed with pulmonary arterial hypertension treated with ET receptor antagonist developed adverse effects, like premature delivery and neonatal mortality [Bédard *et al.* 2009]. Clearly therefore, if pursuing this pathway, we need drugs that do not cross the placental barrier, or novel approaches that selectively annihilate ET-1 in the mother, by sup-pressing endothelial ET-1 synthesis (e.g., with small interfering RNA), by blocking endothelial ET-1 release, or by binding or inactivation of ET-1 in the circulation.

CONCLUSIONS

Advances in our understanding of the pathophysiology of PE confirm PE as a complex multifactorial disease potentially requiring therapeutic intervention at multiple levels. The ET system now emerges as a final pathway that may be the cause of the hypertension, renal toxicity and RAS suppression in PE. Its blockade may therefore be beneficial, although simultaneously we know that ET receptor antagonism is teratogenic. Thus, such treatment may only be feasible if started at a stage sufficiently late to no longer allow teratogenic effects, or by making use of approaches that do not affect the fetus. We also need to know which ET receptor (A or B, or both) needs to be blocked. Alternatively, one might focus on the cause(s) of the ET-1 elevation (e.g. sFlt-1). This may yield new treatment tools, for instance sFlt-1 removal by apheresis.

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Chapter 3

Association studies suggest a key role for endothelin-1 in the pathogenesis of preeclampsia and the accompanying Renin–Angiotensin Aldosterone System suppression

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ABSTRACT

Women with preeclampsia display low renin–angiotensin–aldosterone system activity and a high antiangiogenic state, the latter characterized by high levels of soluble Fms-like tyrosine kinase (sFlt)-1 and reduced placental growth factor levels. To investigate whether renin–angiotensin–aldosterone system suppression in preeclampsia is because of this disturbed angiogenic balance, we measured mean arterial pressure, creatinine, endothelin-1 (ET-1), and renin– angiotensin–aldosterone system components in pregnant women with a high (≥85; n=38) or low (<85; n=65) soluble Fms-like tyrosine kinase-1/placental growth factor ratio. Plasma ET-1 levels were increased in women with a high ratio, whereas their plasma renin activity and plasma concentrations of renin, angiotensinogen, and aldosterone were decreased. Plasma renin activity–aldosterone relationships were identical in both the groups. Multiple regression analysis revealed that plasma renin concentration correlated independently with mean arterial pressure and plasma ET-1. Plasma ET-1 correlated positively with soluble Fms-like tyrosine kinase-1 and negatively with plasma renin concentration, and urinary protein correlated with plasma ET-1 and mean arterial pressure. Despite the lower plasma levels of renin and angiotensinogen in the high-ratio group, their urinary levels of these components were elevated. Correction for albumin revealed that this was because of increased glomerular filtration. Subcutaneous arteries obtained from patients with preeclampsia displayed an enhanced, AT_2 receptor-mediated response to angiotensin II. In conclusion, a high antiangiogenic state associates with ET-1 activation, which together with the increased mean arterial pressure may underlie the parallel reductions in renin and aldosterone in preeclampsia. Because ET-1 also was a major determinant of urinary protein, our data reveal a key role for ET-1 in the pathogenesis of preeclampsia. Finally, the enhanced angiotensin responsiveness in preeclampsia involves constrictor AT_2 receptors.

INTRODUCTION

Preeclampsia is a pregnancy-related disorder, clinically characterized by the new onset of proteinuria and hypertension in the second half of pregnancy, with a great effect on maternal and fetal morbidity and mortality worldwide.[1]

A better understanding of the pathogenic mechanisms underlying preeclampsia might help identifying biomarkers that allow early diagnosis and treatment of preeclampsia. Recently, disturbances in angiogenic balance (favoring antiangiogenic over proangiogenic factors), elevated endothelin-1 (ET-1) levels, and a suppressed renin–angiotensin–aldosterone system (RAAS) have been reported.[2–4] As a consequence, the ratio of the antiangiogenic soluble Fms like tyrosine kinase-1 (sFlt-1) and the proangiogenic placental growth factor (PlGF) is now thought to be a reliable biomarker for the diagnosis of preeclampsia.[5] In fact, patients with a ratio ≥85 have a poor pregnancy outcome independent of their clinical diagnosis compared with patients with a ratio <85.[5]

Of interest, treatment of cancer patients with antiangiogenic drugs (which, like sFlt-1, prevent the actions of vascular endothelial growth factor [VEGF]) resulted in hypertension, proteinuria, renin suppression, and elevated ET-1 levels.[6] Animal studies with antiangiogenic drugs additionally revealed that the renal histological changes observed during such treatment, in particular glomerular endotheliosis, resembled the renal alterations observed in preeclampsia.[7] From the observation that the dual $ET_{A/B}$ receptor antagonist macitentan prevented both the rise in blood pressure and proteinuria during antiangiogenic treatment, it seemed that ET-1 is causally involved in these preeclampsia-like side effects.[8]

A suppressed RAAS in preeclampsia is counterintuitive given the reduced circulating volume in this disorder.[2,9] Gennari-Moser et al[10] have proposed that VEGF stimulates aldosterone production, both directly and indirectly, the latter by enhancing adrenal capillary density. On this basis, a rise in sFlt-1, via VEGF inactivation, should suppress aldosterone levels in preeclampsia. Buhl et al[11] observed that urinary plasmin levels are elevated in preeclampsia. The resulting epithelial sodium channel activation might also suppress aldosterone. Finally, the occurrence of angiotensin II (Ang II) type 1 (AT_1) receptor autoantibodies in preeclampsia, which stimulate the AT_1 receptor, should suppress renin (negative feedback loop) but increase aldosterone. Their presence may contribute to the high Ang II sensitivity in preeclampsia. Careful analysis of the plasma aldosterone/plasma renin concentration (PRC) ratio in preeclampsia might shed light on these possibilities. In the past, this ratio has been shown to be elevated in pregnant women, the highest ratio in fact occurring in preeclampsia.[4] However, given the substantial angiotensinogen rises in pregnancy (and to a lesser degree in preeclampsia), Ang I generation for a given level of renin may differ greatly between pregnant and nonpregnant women. Therefore, the plasma aldosterone/plasma renin activity (PRA) ratio might be more appropriate to investigate this relationship. Given the renal damage in preeclampsia, urinary RAAS component measurement could provide additional diagnostic information, provided that such components are truly kidney-derived (and not plasma-derived).[12]

In this study, we hypothesized that RAAS suppression in preeclampsia is the consequence of the disturbed angiogenic balance or the resulting rise in ET-1, and that urinary RAAS components provide additional diagnostic information, for example, on the degree of renal dysfunction. To address these hypotheses, we measured sFlt-1, PlGF, ET-1, and RAAS components in plasma and urine of pregnant women with sFlt-1/ PlGF ratios ≥85 or <85. We additionally investigated what Ang II receptor (type 1 or type 2) contributes to the enhanced Ang II responsiveness in preeclampsia, making use of subcutaneous arteries obtained after caesarean delivery.

METHODS

Human studies

Patients were recruited between March 2012 and February 2013 at the Erasmus MC and midwifery Rotterdam west. The Erasmus MC Medical Ethics Committee approved the study protocol, and written informed consent was obtained from all subjects before participation. Blood pressure was measured 3 times with an automated blood pressure monitor (Omron 705 CP-2 Healthcare) in sitting position. The first reading was excluded and the subsequent two were averaged. Mean arterial pressure was calculated with the formula: MAP = 2/3 (diastolic blood pressure)+1/3(systolic blood pressure). On the same day, blood and urine were collected, processed and stored at -20°C until analysis. Two groups of women were included. The first group consisted of patients with (suspected) preeclampsia, characterized by hypertension and/or proteinuria, or having preeclampsia-like complaints like headache with visual disturbances, and abdominal pain in the right upper quadrant. Exclusion criteria were coexisting diabetes (gravidarum) and inability to obtain informed consent. The second group consisted of healthy pregnant women, matched for gestational age. Exclusion criteria were a history of preeclampsia, hypertension, proteinuria, diabetes (gravidarum), or inability to obtain informed consent.

Additionally, a second cohort of patients included consisting of women with preeclampsia, defined as de novo hypertension and proteinuria in the second half of pregnancy (blood pressure ≥ 140/90 mm Hg, and proteinuria > 300 mg/24 hours after the 20th week of gestation), and healthy pregnant women, with no history of preeclampsia, hypertension, proteinuria, or diabetes. These women all delivered via caesarean section, which was performed because of fetal or maternal well-being in the case of preeclampsia, or a child presenting in breech position, or a previous caesarean section in the case of healthy pregnant women. From these women abdominal subcutaneous tissue was collected during a caesarean section for the evaluation of their microvascular function.

Rat studies

Male Wistar Kyoto rats (280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. The VEGF inhibitor sunitinib (Pfizer) was administered for 8 days by oral gavage at 3 different doses (7, 14 or 26.7 mg/kg.day; n=6-14) as described previously.[1] At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital i.p. and blood was sampled for measurement of circulating endothelin-1. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Biochemical measurements

Endothelin-1 was measured by chemiluminescent ELISA (QuantiGlo, R&D systems; detection limit 0.34 pg/mL). Plasma renin concentration and plasma prorenin (the latter after its conversion to renin by trypsin), as well as plasma renin activity (PRA), were measured by enzyme-kinetic assay as described before (detection limit 0.05 ng angiotensin I per ml/hr).[2] Plasma and urinary angiotensinogen were measured as the maximum quantity of angiotensin I that was generated during incubation with excess recombinant renin (detection limit 0.5 pmol/mL),[2] while urinary angiotensinogen was additionally measured by commercial ELISA (IBL International; detection limit of 0.01 pmol/mL). Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation; detection limit 11 pg/mL). Urinary aldosterone, but not plasma aldosterone, was extracted according to kit instructions (recovery >86%). Soluble Fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) measurements were performed on the fully automated Roche Elecsys system (Elecsys PlGF, human PlGF, and Elecsys sFlt-1, sFlt-1) as described previously,[3] and the sFlt-1/PlGF ratio was calculated for each sample. Sodium, potassium, creatinine, albumin and total protein were measured with routine laboratory methods.

Microvascular function

Arteries (diameter 1-2 mm) were isolated from abdominal subcutaneous tissue and processed either directly or after overnight storage. They were cut into segments of ≈2 mm length and mounted in a Mulvany myograph (Danish Myo Technology) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O2 and

5% CO2, and maintained at 37°C. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure, as described before.4 Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane A2 analogue U46619. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. Next, after a 30-min equilibration in fresh organ bath fluid, segments were pre-incubated for 30 min with the AT1 receptor antagonist irbesartan, the AT2 receptor antagonist PD123319 (both 1 μmol/L) or vehicle. Thereafter, concentration-response curves were constructed to angiotensin II.

Statistical analysis

The sFlt-1/PlGF ratio (≥85 or <85) was used to subdivide the patients in 2 groups. Based on a pilot study in preeclampsia patients and healthy pregnant controls, displaying urinary renin levels, respectively, of 2.1 (0.05-158) (median and range) and 0.25 (0.05-5.0) ng Ang I/ml.hr, a minimum of n=36/group was calculated to be sufficient to detect a 50% difference in urinary renin between the 2 groups with 80% power and 5% significance. Urinary renin was chosen for this power analysis given the limited knowledge about this parameter, as opposed to the substantial differences in most plasma parameters measured in this study that have already been reported by others.[5] Continuous variables are given as mean and standard deviation (SD) for normally distributed data and as median and interquartile range for non-parametrically divided data. Differences between groups were tested with the Student's t-test in case of the former and with the Mann-Whitney U-test in case of the latter. For the entire group of subjects Pearson's correlation coefficient was calculated to assess the correlation between two continuous variables. Multiple linear regression analysis was applied to determine the variables affecting circulating renin, aldosterone and endothelin-1. Non-parametrically distributed data were log-transformed before correlation or regression analysis was performed. All statistical analyses were calculated with IBM SPSS Statistics 21 (IBM Corporation).

Concentration-response curves were analyzed with Prism 5.0 for Windows (GraphPad software inc.) to obtain the maximum effect (Emax) and pEC50 ($-^{10}\log EC_{50}$). Two way ANOVA was used for comparison, followed by a Bonferroni post-hoc evaluation to correct for multiple testing.

RESULTS

Rat Studies

An 8-day treatment with the VEGF inhibitor sunitinib dosedependently increased plasma ET-1 levels in Wistar Kyoto rats (Figure 1A).

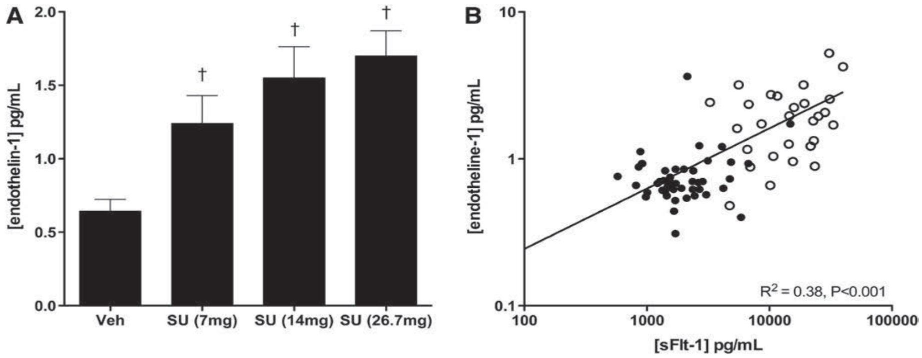

Figure 1. Plasma endothelin-1 levels in rats treated with different doses of the vascular endothelial growth factor inhibitor sunitinib (SU: 7, 14, and 26.7 mg/kg per d) or vehicle (Veh; **A**), and vs soluble Fms-like tyrosine kinase (sFlt)-1 in pregnant women (**B**), subdivided according to sFlt-1/placental growth factor (PlGF) ratio (≥85, open circles; <85, closed circles).

Human Studies

Population characteristics

Of the 103 pregnant women included, 65 had a sFlt-1/PlGF ratio <85 (negative test) and 38 had a ratio ≥85 (positive test). There were no differences in gestational age at inclusion. Patients with a positive test had a higher blood pressure and delivered on average 8 weeks earlier than patients with a negative test. As expected, treatment with methyldopa, calcium antagonists, and magnesium sulfate predominated in the group with the positive test (Table 1). Plasma ET-1 and creatinine levels were increased in patients with a positive test, whereas PRA, PRC, and plasma angiotensinogen, aldosterone and albumin were decreased in these patients (Table 1). Plasma ET-1 correlated positively with plasma sFlt-1 (Figure 1B). Plasma prorenin, the aldosterone/PRA ratio, and the PRA–aldosterone relationship were identical in both the groups (Table 1; Figure 2A).

Urinary protein, albumin, angiotensinogen, renin, and prorenin were increased in patients with a positive test, whereas aldosterone was decreased in the urine of such patients (Table 2). Urinary Na^+ levels were identical in both the groups, also after correction for K^+. For the analysis of prorenin differences, samples that yielded levels below the detection limit (11 [31.4%] in the women with a positive test and 33 [54.1%] in women

Table 1. Characteristics and Biochemical Parameters in Plasma of Women With a sFlt-1/PlGF Ratio <85 and Patients With a Ratio ≥85

Characteristic/Biochemical Parameter	**sFlt-1/PlGF Ratio <85**	**sFlt-1/PlGF Ratio ≥85**	***P* Value**
n	65	38	
SBP, mm Hg	119±14	144±13	<0.001
DBP, mm Hg	72±12	88±9	<0.001
MAP, mm Hg	87±12	106±9	<0.001
G.A. inclusion, wk+d	29+2 (26+4–34+5)	29+0 (25+3–31+4)	0.221
G.A. birth, wk+d	38+2 (37+1–39+1)	30+6 (27+2–32+0)	<0.001
sFlt-1, pg/mL	1912 (1275–3143)	15 044 (10 151–22 749)	<0.001
PlGF, pg/mL	369 (247–613)	31.6 (14.9–55.5)	<0.001
sFlt-1/PlGF ratio	4.75 (2.2–11.5)	565 (203–919)	<0.001
Creatinine, µmol/L	51 (44–58)	56 (51–66)	0.01
Albumin, g/L	36 (35–38)	31 (28–34)	<0.001
Renin, ng Ang I/mL per h	17.9 (13.0–24.7)	8.3 (5.8–11.2)	<0.001
Prorenin, ng Ang I/mL per h	172 (141–228)	198 (151–249)	0.224
Angiotensinogen, pmol/mL	6163 (5364–7008)	4433 (3719–5204)	<0.001
PRA, pmol Ang I/mL per hr	4.9 (3.7–7.2)	1.6 (1.4–2.3)	<0.001
Aldosterone, pg/mL	407 (290–822)	185 (121–378)	<0.001
Aldosterone/PRA ratio	90.3 (59.2–136.7)	103.1 (56.7–175.8)	0.414
Endothelin-1, pg/mL	0.69 (0.61–0.85)	1.88 (1.19–2.49)	<0.001
Methyldopa	9 (13.8%)	29 (76.3%)	<0.001
Calcium channel antagonists	1 (1.5%)	16 (42.1%)	<0.001
Magnesium sulfate	0	7 (18.4%)	<0.001

Ang indicates Angiotensin; DBP, diastolic blood pressure; G.A., gestational age; MAP, mean arterial pressure; PlGF, placental growth factor; PRA, plasma renin activity; SBP, diastolic blood pressure; and sFlt, soluble Fms-like tyrosine kinase.

Table 2. Biochemical Measurements in Urine of Women With a sFlt-1/PlGF Ratio <85 and Patients With a Ratio ≥85

Biochemical Parameter	**sFlt-1/PlGF <85**	**sFlt-1/PlGF ≥85**	***P* Value**
Creatinine, µmol/L	7.7 (5.2–10.9)	6.9 (4.2–11.9)	0.615
PCR, mg/mmol	10.0 (8.0–13.6)	110 (35.6–247)	<0.001
ACR, mg/mmol	0.58 (0.28–1.11)	78.6 (10.3–198)	<0.001
Angiotensinogen (EKA)/creatinine, pmol/mmol	0.45 (0.30–0.83)	0.93 (0.40–1.64)	0.003
Angiotensinogen (ELISA)/creatinine, pmol/mmol	0.08 (0.04–0.14)	0.25 (0.14–0.53)	<0.001
Renin/creatinine, ng Ang I/mL per h per mmol	0.10 (0.04–0.17)	0.20 (0.08–0.5)	0.002
Prorenin/creatinine, ng Ang I/mL per h per mmol	0.01 (0.01–0.04)	0.05 (0.01–0.23)	<0.001
Aldosterone/creatinine, pg/mmol	8522 (4163–13 151)	3012 (1605–4596)	0.001
Sodium/creatinine, mmol/mmol	11.4 (8.5–15.6)	9.6 (3.6–15.4)	0.054
Potassium/creatinine, mmol/mmol	7.7 (5.3–10.9)	4.4 (3.6–6.4)	<0.001
Sodium/Potassium ratio, mmol/mmol	1.4 (1.0–2.5)	1.7 (0.9–2.6)	0.76

ACR indicates albumin/creatinine ratio; Ang, angiotensin; EKA, enzyme-kinetic assay; PlGF, placental growth factor; PCR, protein/creatinine ratio; and sFlt, soluble Fms-like tyrosine kinase.

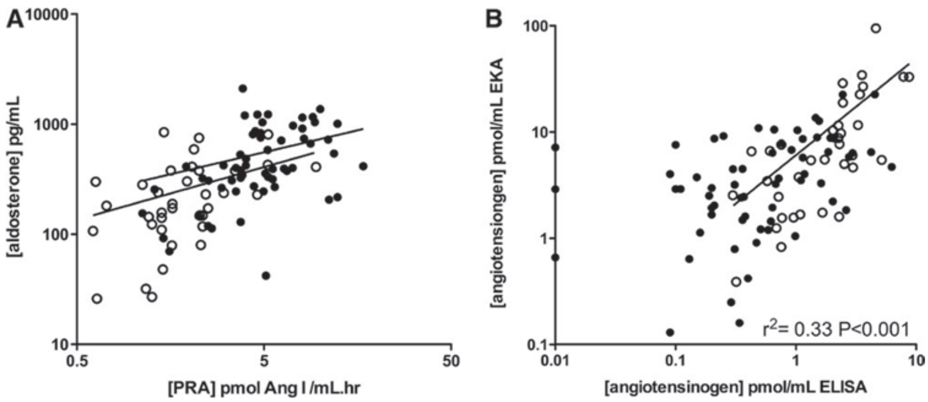

Figure 2. A, Plasma renin activity (PRA) vs aldosterone in pregnant women, subdivided according to soluble Fms-like tyrosine kinase-1/ placental growth factor ratio (≥85, open circles; <85, closed circles). The relationship was identical in both the groups. **B**, angiotensinogen measurement by ELISA vs that by enzyme-kinetic assay (EKA) in urine. Ang I indicates angiotensin I.

with a negative test) were excluded. There was no difference in urinary creatinine levels between the 2 groups, and outcomes were identical with and without correction for creatinine. Angiotensinogen measurements by enzyme-kinetic assay and ELISA were significantly correlated (Figure 2B; r=0.33; P<0.001), although levels determined with the ELISA were consistently 2- to 3-fold lower. This was most probable because of the fact that the ELISA standard, when determined twice in our enzyme-kinetic assay (applying Ang I as standard), yielded ≈2-fold higher levels than predicted (0.37 versus 0.15 pmol/mL and 1.03 versus 0.62 pmol/mL).

Determinants of plasma aldosterone, PRC, plasma ET-1, MAP, and urinary protein/creatinine ratio

Aldosterone

Plasma sFlt-1, mean arterial pressure (MAP), urinary protein/creatinine ratio (uPCR), and plasma ET-1 correlated negatively with plasma aldosterone, whereas a positive correlation was observed with plasma PlGF, gestational age at measurement, plasma angiotensinogen, PRC, and PRA (Table S1). Next, parameters displaying a significant correlation with plasma aldosterone were added into a multiple linear regression model (using PRA as a representative of both PRC and angiotensinogen, and deleting uPCR in view of its strong relationship with MAP). Under such conditions, only gestational age at measurement and PRA remained significant determinants (Table S2). Replacing PRA in the model by PRC and plasma angiotensinogen revealed that the association with PRA was because of PRC (P=0.006) and not plasma angiotensinogen (P=0.649; data not shown).

Plasma Renin Concentration

PRC correlated positively with PRA, plasma PlGF, plasma angiotensinogen, and plasma aldosterone, and negatively with plasma sFlt-1, MAP, uPCR, and plasma ET-1 (Table S1). There was no relationship with gestational age at measurement. When incorporating all significant parameters into a multiple regression model (excluding uPCR like above), only MAP and plasma ET-1 remained significantly correlated with PRC (Table S3). Aldosterone was kept out of this analysis because of its well-known strong positive correlation with renin, mediated by renin-induced angiotensin generation.

Endothelin-1

Plasma ET-1 correlated positively with plasma sFlt-1, creatinine, MAP, and uPCR, and negatively with plasma PlGF, PRC, PRA, plasma angiotensinogen, and plasma aldosterone (Table S1). There was no relationship with gestational age at measurement. Adding all independent significant parameters in a multiple linear regression model revealed that only the relationships with sFlt-1 and PRC remained significant (Table S4).

MAP and uPCR

MAP and uPCR correlated highly significantly with each other, and displayed identical positive correlations with plasma sFlt-1 and plasma ET-1, and negative correlations with PRA, plasma angiotensinogen, and plasma PlGF. In addition, uPCR correlated positively with plasma creatinine, and negatively with PRC and plasma aldosterone. Multiple linear regression analysis revealed that only plasma PlGF and uPCR correlated independently with MAP (Table S5), whereas plasma PlGF, plasma ET-1, and MAP determined 69% of uPCR variation (Table S5).

Origin of Urinary RAAS Components

At first sight, the opposite changes in urinary renin and angiotensinogen in urine versus plasma in the 2 groups (Tables 1 and 2) seem to indicate a differential regulation of the circulating and renal RAAS. However, these data should be interpreted in view of the increased albumin/protein levels in the urine of patients with a high ratio. Figures 3A–3C, therefore, compare the plasma/urine ratio of albumin with the ratios of renin, prorenin, and angiotensinogen. The highly significant positive relationships with the albumin ratio for the 3 proteins, which all have a molecular mass that is comparable to that of albumin, strongly suggest that their elevated urinary levels simply reflect the same phenomenon that underlies the elevated urinary albumin/protein levels: increased filtration. To establish the relationship for prorenin, we excluded urinary samples that yielded levels below the detection limit (n=44). Urinary aldosterone changes paralleled plasma aldosterone changes, and both parameters were highly correlated (Figure 3D). Here, we did not correct for albumin, given the much lower molecular weight of aldosterone.

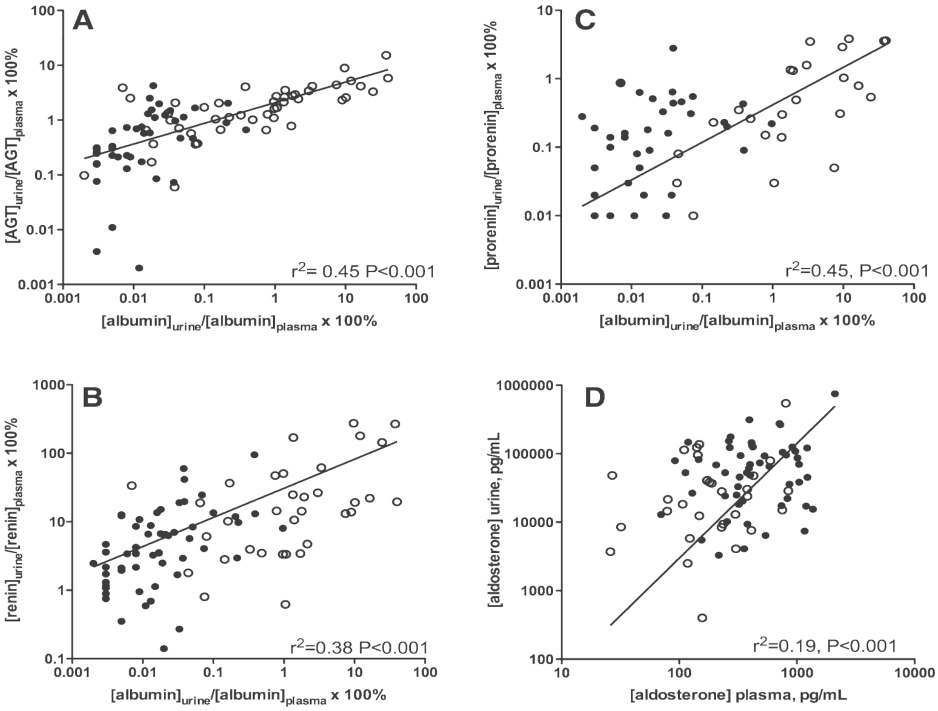

Figure 3. Correlations between the urine/plasma concentration ratio of albumin and that of angiotensinogen (AGT, **A**), renin (**B**), and prorenin (**C**) in pregnant women, subdivided according to soluble Fms-like tyrosine kinase-1/placental growth factor (PlGF) ratio (≥85, open circles; <85, closed circles). **D** compares plasma and urinary aldosterone levels.

Urinary aldosterone levels for a given level of urinary sodium (corrected for potassium, a measure for the aldosterone effect on the collecting duct) were lower for women with a positive test (Figure 4).

Microvascular Function

Ang II CRCs were obtained in subcutaneous arteries from 9 patients with preeclampsia (age, 31±4 years; gestational age, 29±3.5 weeks; MAP, 111±7 mm Hg) and 8 healthy controls (age, 35±5 years; gestational age, 39±0.5 weeks; MAP, 81±8 mm Hg; $P<0.05$ versus preeclampsia for all). Maximum constrictor responses to KCl and relaxant effects to substance P were identical in both the groups (data not shown). As expected, the Ang II E_{max} was twice as large in preeclampsia vessels as compared with healthy vessels (Figure S1), whereas Ang II potencies in both the vessel types were identical (pEC_{50}, 8.9±0.15 versus 9.0±0.13). Irbesartan completely abolished all Ang II responses (data not shown), whereas PD123319 normalized the enhanced response in preeclampsia ($P<0.05$), without having an effect in healthy vessels (Figure S1).

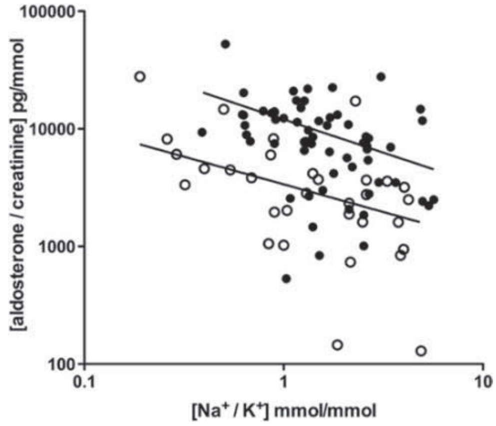

Figure 4. Urinary Na$^+$ ratio vs urinary aldosterone in pregnant women, subdivided according to soluble Fms-like tyrosine kinase-1/placental growth factor ratio (≥85, open circles; <85, closed circles).

DISCUSSION

In this study, we have used a cut-off of 85 of the sFlt-1/PlGF ratio to distinguish patients with a high and low antiangiogenic state, rather than making a subdivision based on the clinical diagnosis of preeclampsia. This was done mainly in view of our purpose to investigate the relation between the antiangiogenic state, the endothelin system, and the RAAS. It also minimizes the risk of including patients with preexisting disease such as systemic lupus erythematosus.[13] However, it should be noted here that a reanalysis on the basis of a subdivision according clinical diagnosis yielded identical results (data not shown).

A high antiangiogenic state, as reflected by elevated sFlt-1 levels, is associated with increased ET-1 levels. Given the observation that ET-1 also rises in patients with cancer and rats treated with the VEGF inhibitor sunitinib (Figure 1A),[6] the most logical explanation of this ET-1 rise is that it is the direct consequence of VEGF inhibition, either through VEGF inactivation by sFlt-1 (in preeclampsia) or through interference with VEGF signaling (with sunitinib). Elevated ET-1 levels have been reported earlier in preeclampsia.[14,15] Our study now suggests that this ET-1 elevation is a major determinant of both the blood pressure rise and the proteinuria in this disorder, as well as the RAAS suppression (Figure 5). Again a parallel may be drawn with the ET-1–mediated side effects in sunitinib-treated patients, that is, hypertension and proteinuria, which were accompanied by renin suppression.[6] ET receptor blockade prevented these side effects in sunitinib-treated rats,[8] whereas also in 2 rat models for preeclampsia (reduced uterine perfusion pressure and sFlt-1 injection) ET receptor antagonism completely blocked the hypertensive response.[16,17] Moreover, ET-1 has been reported to suppress renin release in animal models.[18,19]

The cause of the RAAS suppression in preeclampsia has always been elusive. Given the reduced circulating volume in preeclampsia, the opposite should have occurred.[2] Apparently, both the ET-1 and the rise in blood pressure overrule this response. Importantly, our data show a similar PRA–aldosterone relationship and aldosterone/PRA ratio in patients with a high and low antiangiogenic state. This implies that the decrease in aldosterone levels in preeclampsia is the simple consequence of reduced Ang I–generating activity. Gennari-Moser et al[10] have recently reported that VEGF stimulates aldosterone

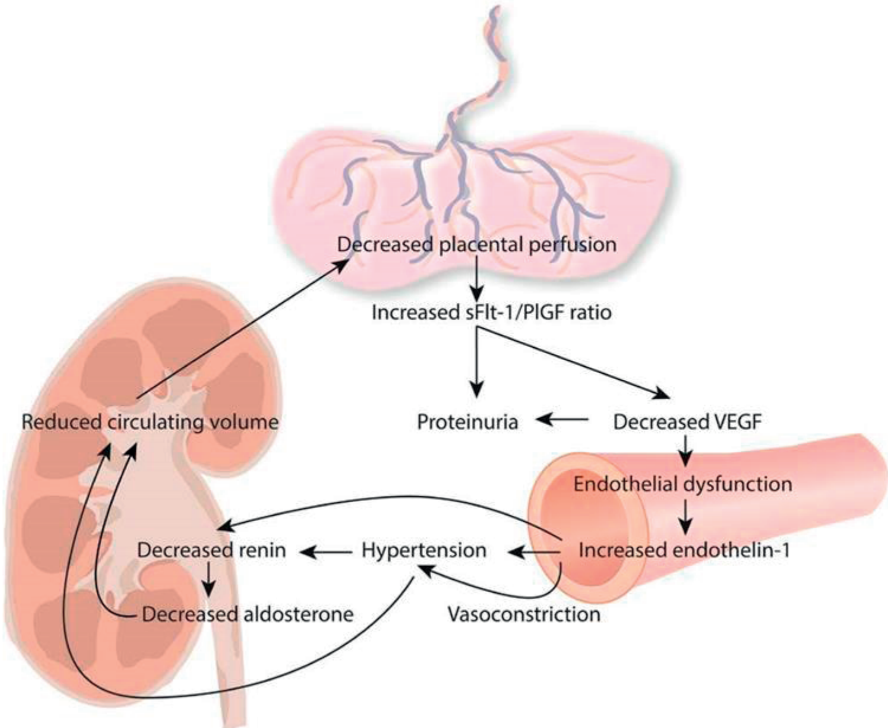

Figure 5. Unifying model summarizing all findings from this study. Decreased perfusion of the placenta (most probably resulting from impaired widening of the maternal spiral arteries[2]), results in placental hypoxia and soluble Fms-like tyrosine kinase (sFlt)-1 release. sFlt-1 binds free placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), thereby inactivating these factors, increasing the sFlt-1/PlGF ratio, and inducing endothelial dysfunction. As a consequence, endothelin-1 production is turned on, which not only induces hypertension and proteinuria but also suppresses renin release. Such suppression will also occur because of the rise in blood pressure. The renin suppression is accompanied by a parallel aldosterone suppression, illustrating that the latter is entirely because of diminished angiotensin generation. Diminished renin–angiotensin–aldosterone system activity combined with high blood pressure results in a reduced circulating volume, thereby further decreasing placental perfusion.

production by enhancing adrenal capillary density. In addition, they observed that sFlt-1 overexpression in rats reduced aldosterone levels, resulting in an inverse correlation between sFlt-1 and aldosterone. Our data fully confirm this inverse relation in humans, but multiple regression analysis subsequently revealed that it was actually because of the sFlt-1–induced rise in ET-1, which suppressed renin. Moreover, had aldosterone been selectively reduced in preeclampsia, this should have resulted in a reduced aldosterone/PRA ratio. No such reduction was observed. In fact, if anything, previous studies reported an elevated aldosterone/PRC ratio in preeclampsia versus healthy pregnant women.[4] Here, it should be considered that pregnant women display elevated prorenin and angiotensinogen levels.[2] Prorenin may interfere in the immunoreactive renin assay by cross-reacting with the antibody that recognizes renin's active site,[20] and the 4- to 5-fold elevated angiotensinogen levels will lead to a higher PRA (and, consequently, aldosterone) level for a given PRC as compared with nonpregnant women. From this point of view, it is better to compare aldosterone with PRA because this parameter takes into account the changes in angiotensinogen and is not affected by prorenin cross-reactivity. Summarizing, our data do not support a selective, reninindependent downregulation of aldosterone in preeclampsia (related to a reduced adrenal capillary density) but rather an ET-1–mediated overall RAAS suppression (Figure 5).

Buhl et al[11] found that urine of patients with preeclampsia contains high levels of plasmin, which will activate collecting duct epithelial sodium channel current, and might thus further suppress the RAAS. Because plasma sodium is kept within narrow ranges and urinary sodium is largely diet-dependent, it is difficult to obtain direct evidence for this theory in an observational study. However, in line with the theory that sodium could be retained by activation of epithelial sodium channel, we observed that urinary sodium levels for a given level of aldosterone were lower in patients with a high antiangiogenic state as compared with healthy pregnant women.

Because RAAS components in urine are thought to reflect renal RAAS activation,[21,22] and given the severe renal pathology in preeclampsia, we quantified urinary renin, prorenin, angiotensinogen, and aldosterone to investigate their biomarker value. In line with previous studies, urinary prorenin levels were often below detection limit.[23,24] When considering only the urinary prorenin levels that were detectable, it could be calculated that urinary prorenin levels were 0.21 (0.12–0.45) and 0.51 (0.19–1.47) % (P=0.007 for difference) of plasma prorenin in women with a low and high antiangiogenic state, respectively (Figure 3). In reality, when also including the samples with undetectable prorenin levels, these percentages would have been even lower, and possible as low as those observed for angiotensinogen (0.07 [0.03–0.11] and 0.16 [0.05–0.34] % of plasma angiotensinogen; P=0.001). In contrast, urinary renin levels, relative to PRC, were

≤90-fold higher (3.47 [1.15–8.79 and 14.4 [3.95–36.5 % of PRC; $P<0.001$). Because of the comparable molecular weights of all 3 proteins, at first sight these data seem to indicate selective renal renin release into urine, particularly in women with a high antiangiogenic state. However, when comparing the urine/plasma ratios of all the 3 proteins with the ratio of a protein of comparable weight, albumin (displaying urinary levels that are 0.014 [0.005–0.033] and 1.35 [0.25–5.40] % of plasma albumin in women with a low and high antiangiogenic state, respectively; $P<0.007$), strong correlations were found in all cases. In other words, renin, prorenin, and angiotensinogen enter urine, like albumin, via glomerular filtration, and their elevated levels in women with a high antiangiogenic state are the simple consequence of increased filtration. This leaves the question why urinary renin (relative to its plasma levels) is so much higher than urinary angiotensinogen or prorenin. Urinary renin levels are too low to significantly affect urinary angiotensinogen, and thus ex vivo Ang I generation cannot explain the low urinary angiotensinogen levels. Clearly, therefore, either glomerular filtration of prorenin and angiotensinogen is greatly reduced as compared with renin, or renin reabsorption in the proximal tubulus is much less efficient. Because Nielsen et al[25] found that, after blocking tubular reabsorption with lysine, the urinary clearance of prorenin was still ≈10-fold lower than that of renin, the former explanation seems the most likely. Finally, urinary aldosterone fully paralleled plasma aldosterone, in line with the concept that urinary aldosterone, like plasma aldosterone, is adrenal-derived. Thus, except for the fact that urinary aldosterone is several orders of magnitude higher than plasma aldosterone (making it easier to detect), urinary aldosterone offers no additive biomarker value for preeclampsia, nor do urinary angiotensinogen, renin, and prorenin.

Women with preeclampsia are known to display greater Ang II responses than normal pregnant women. Theoretically, such enhanced responses should result in higher aldosterone levels and a reduced renin release, via AT_1 receptors in the adrenal and kidney, respectively. Collectively, this would lead to a higher aldosterone/PRA ratio. Yet, we did not observe an altered ratio, arguing against the concept of enhanced AT_1 receptor stimulation in preeclampsia. Our data in arteries from patients with preeclampsia confirm the enhanced response, but show that it is because of constrictor AT_2 receptors. Normally, such receptors induce vasodilation, but their phenotype often changes under pathological conditions, such as hypertension and aging.[26,27] The mechanism behind this change from dilator to constrictor is unknown, but may involve a disturbed endothelial function and reduced NO availability, heterodimerization with other receptors (including the AT_1 receptor) or a different location of the AT_2 receptor (vascular smooth muscle cells versus endothelial cells).[28] If the enhanced response is indeed because of AT_2 receptors, this explains why there is no change in the aldosterone/PRA ratio.

PERSPECTIVES

Our data shed new light on the counterintuitive RAAS suppression in preeclampsia, and suggest that ET-1 has a key role not only in the pathogenesis of this disorder (contributing both to the high blood pressure and proteinuria) but also as a renin suppressor. The reduced urinary sodium/aldosterone relationship in women with a high antiangiogenic state support a second reason for RAAS suppression: the occurrence of plasmin in urine, which selectively activates epithelial sodium channel.[11] We did not find evidence for specifically reduced aldosterone levels in patients with an antiangiogenic state. Urinary RAAS components, when corrected for albumin, fully reflected the alterations in the circulating RAAS in preeclampsia, and thus offered no additive value as biomarkers. Unfortunately, despite the central role for ET-1 in preeclampsia, ET receptor antagonism currently is no treatment option in human pregnancy because of its teratogenic effects.[29] To develop treatment modalities for preeclampsia beyond ET receptor blockade, future studies should unravel why elevated sFlt-1 levels in preeclampsia, like VEGF inhibition in patients with cancer, increase ET-1 levels. Such studies might involve animal models of preeclampsia using VEGF antagonism, and should take into consideration that ET-1 has been reported to reciprocally affect the expression of VEGF and its receptors.[30,31]

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SUPPLEMENTAL DATA

Table S1. Univariable regression to assess variables affecting the plasma levels of aldosterone, renin and endothelin-1 levels, and the urinary protein/creatinine (uPCR) ratio.

Variable	Log [Aldosterone, pg/mL]	Log [Renin, ng Ang I /mL.hr]	Log [Endothelin-1, pg/mL]	Log [uPCR, mg/mmol]	MAP, mm Hg
Log [Aldosterone, pg/mL]	-	0.51*	-0.33*	-0.36*	-0.15
Log [Renin, ng Ang I /mL.hr]	0.51*	-	-0.49*	-0.43*	-0.08
Log [Endothelin-1, pg/mL]	-0.33*	-0.49*	-	0.70*	0.24*
MAP, mm Hg	-0.31*	-0.48*	0.57*	0.62*	-
Log [uPCR, mg/mmol]	-0.36*	-0.43*	0.70*	-	0.62*
Log [Plasma creatinine, μmol/L]	-0.15	-0.08	0.24*	0.28*	0.17
Log [sFlt-1, pg/mL]	-0.27*	-0.35*	0.62*	0.65*	0.60*
Log [PlGF, pg/mL]	0.52*	0.47*	-0.60*	-0.69*	-0.69*
G.A. measurement, weeks	0.36*	0.00	0.07	-0.01	0.16
Log [Angiotensinogen, pmol/mL]	0.38*	0.27*	-0.38*	-0.42*	-0.27*
Log [PRA, pmol Ang I /mL.hr]	0.60*	0.88*	-0.54*	-0.51*	-0.55*

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PRA, plasma renin activity.

Table S2. Multiple linear regression to assess variables affecting plasma aldosterone levels.

Variable	Coefficient	P-value
Log [sFlt-1, pg/mL]	0.050	0.706
Log [PlGF, pg/mL]	0.288	0.085
G.A. measurement, weeks	0.272	0.009
MAP, mm Hg	0.099	0.479
Log [PRA, pmol Ang I /mL.hr]	0.519	<0.001
Log [Endothelin-1, pg/mL]	-0.030	0.809

R Square 0.558

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PRA, plasma renin activity

Table S3. Multiple linear regression to assess variables affecting plasma renin levels.

Variable	Coefficient	P-value
Log [sFlt-1, pg/mL]	0.117	0.480
Log [PlGF, pg/mL]	0.039	0.844
G.A. measurement, weeks	0.283	0.498
MAP, mm Hg	-0.335	0.050
Log [Endothelin-1, pg/mL]	-0.306	0.039
Log [Angiotensinogen, pmol/mL]	0.052	0.695

R Square 0.307

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Table S4. Multiple linear regression to assess variables affecting plasma endothelin-1 levels.

Variable	Coefficient	P-value
Log [sFlt-1, pg/mL]	.319	.021
Log [PlGF, pg/mL]	-.061	.731
G.A. measurement, weeks	.109	.307
MAP, mm Hg	.134	.366
Log [Renin, ng Ang I /mL.hr]	-.262	.022
Log [Aldosterone, pg/mL]	-.069	.579
Log [Plasma creatinine, μmol/L]	.211	.056

R Square 0.515

MAP, mean arterial blood pressure; G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Table S5. Multiple linear regression to asses variables affecting mean arterial blood pressure (MAP) and the urinary protein/creatinine ratio (uPCR).

Variable	MAP		uPCR	
	Coefficient	**P-value**	**Coefficient**	**P-value**
Log [sFlt-1, pg/mL]	.058	.621	.144	.201
Log [PlGF, pg/mL]	-.481	.001	-.301	.032
G.A. measurement, weeks	.107	.207	.077	.350
Log [Endothelin-1, pg/mL]	.014	.904	.350	.001
Log [PRA, pmol Ang I /mL.hr]	-.137	.167	.084	.384
Log [Plasma creatinine, μmol/L]	-.134	.136	-.071	.417
Log [uPCR, mg/mmol]	.294	.024	-	-
MAP	-	-	.273	.024
R Square	**0.66**		**0.69**	

G.A., gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

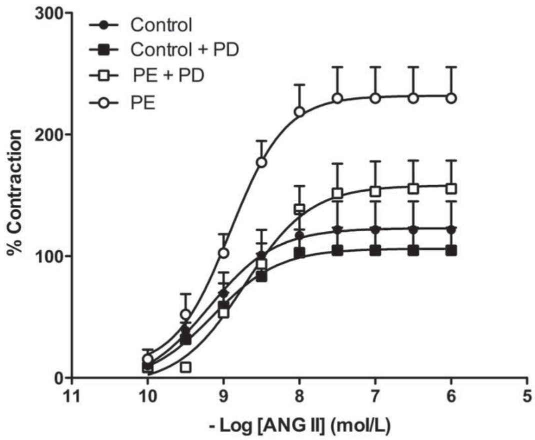

Figure S1. Angiotensin (ANG) II concentration-response curves in patients with preeclampsia (PE) and healthy pregnant controls with and without the AT_2 receptor antagonist PD123319.

Chapter 4

Low soluble Fms-like tyrosine kinase-1, endoglin, and endothelin-1 levels in women with confirmed or suspected preeclampsia using proton pump inhibitors

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ABSTRACT

Patients with preeclampsia display elevated placenta-derived sFlt-1 (soluble Fms-like tyrosine kinase-1) and endoglin levels and decreased placental growth factor levels. Proton pump inhibitors (PPIs) decrease trophoblast sFlt-1 and endoglin secretion in vitro. PPIs are used during pregnancy to combat reflux disease. Here, we investigated whether PPIs affect sFlt-1 in women with confirmed/suspected preeclampsia, making use of a prospective cohort study involving 430 women. Of these women, 40 took PPIs (6 esomeprazole, 32 omeprazole, and 2 pantoprazole) for 8 to 45 (median 29) days before sFlt-1 measurement. Measurements were only made once, at study entry between weeks 20 and 41 (median 33 weeks). PPI use was associated with lower sFlt-1 levels, with no change in placental growth factor levels, both when compared with all non-PPI users and with 80 gestational age-matched controls selected from the non-PPI users. No sFlt-1/placental growth factor alterations were observed in women using ferrous fumarate or macrogol while, as expected, women using antihypertensive medication displayed higher sFlt-1 levels and lower placental growth factor levels. The PPI use-associated decrease in sFlt-1 was independent of the application of antihypertensive drugs and also occurred when restricting our analysis to patients with hypertensive disease of pregnancy at study entry. PPI users displayed more cases with pre-existing proteinuria, less gestational hypertension, and a lower number of neonatal sepsis cases. Finally, their plasma endoglin and endothelin-1 levels were lower while sFlt-1 levels correlated positively with both. In conclusion, PPI use associates with low sFlt-1, endoglin, and endothelin-1 levels, warranting prospective trials to investigate the therapeutic potential of PPIs in preeclampsia.

INTRODUCTION

Preeclampsia, a pregnancy-related disorder, is a serious condition complicating 2% to 5% of all pregnancies, affecting both maternal and fetal morbidity and mortality.[1] Preeclampsia is not simply de novo onset of hypertension and proteinuria in the last half of pregnancy but rather a syndrome involving many organs, of which the clinical spectrum ranges from relatively mild to life threatening.[2,3] Because delivery remains the mainstay of treatment, pre-term delivery and its ensuing peri-natal morbidity continue as a challenge. Annually, hypertensive diseases of pregnancy contribute to 2.6 million stillbirths worldwide.[4] These dismal outcomes emphasize the need for therapy for preeclampsia prevention or ameliorating established disease.

The pathogenesis of preeclampsia is unknown. Recent advances in understanding the pathogenic mechanisms underlying preeclampsia suggest an angiogenic imbalance, reflected by elevated placenta-derived sFlt-1 (soluble Fms-like tyrosine kinase-1) and endoglin levels and decreased PlGF (placental growth factor) levels in the maternal circulation, along with elevated ET-1 (endothelin-1) levels.[5,6] As a consequence, sFlt-1 and PlGF are currently emerging as biomarkers for the diagnosis of preeclampsia. Moreover, novel therapies now focus on either sFlt-1 removal or PlGF supplementation to restore the imbalance. Recombinant human PlGF abolished hyper-tension in a rat preeclampsia model[7] while sFlt-1 removal by dextran sulfate apheresis in humans reduced proteinuria and prolonged pregnancy.[8]

Given that heme oxygenase-1 negatively regulates sFlt-1 secretion,[9] Onda et al[10] recently evaluated whether proton pump inhibitors (PPIs) that upregulate heme oxygenase-1 (eg, in gastric mucosa) affect sFlt-1. They observed that PPIs decreased sFlt-1 and endoglin secretion from trophoblasts, reduced ET-1 secretion from endothelial cells, and decreased blood pressure in a transgenic preeclampsia mouse model with placental sFlt-1 overexpression albeit in a heme oxygenase-1–independent manner.[10] PPIs are the most effective medical therapy for patients with symptomatic gastroesophageal reflux disease, a common condition associated with pregnancy. In the present study, we assessed to what degree PPI use associates with low sFlt-1, endoglin, and ET-1 levels in women with confirmed or suspected preeclampsia, making use of a prospective cohort study involving 430 women. As a comparator, we simultaneously assessed the effects of other drugs commonly used by such women, that is, antihypertensive drugs (α-methyldopa, nifedipine), corticosteroids, ferrous fumarate, and macrogol.

METHODS

Study design

This is a secondary analysis of a prospective cohort study that enrolled women with confirmed preeclampsia or with suspicion of preeclampsia between December 2013 and April 2016 at the Department of Obstetrics of the Erasmus MC in Rotterdam, The Netherlands, with the aim of evaluating the use of the sFlt-1/PlGF ratio for the diagnosis of preeclampsia. All subjects provided informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202). The inclusion criteria were women with singleton pregnancies from gestation age of ≥20 weeks who had to have preeclampsia or preeclampsia symptoms, such as hypertension, proteinuria, right upper quadrant abdominal pain, severe head-aches, changes in vision, decreased levels of platelets, or elevated liver enzymes. Preeclampsia was defined as de novo hypertension (systolic blood pressure of ≥140 and diastolic blood pressure of ≥90 mm Hg) and proteinuria (protein-to-creatinine ratio ≥30 mg/mmol or ≥300 mg/24 h, or 2+ dipstick) at or after 20 weeks of pregnancy.[11] Based on the clinical and laboratory measurements, preeclampsia was further divided into preeclampsia, superimposed preeclampsia, hemolysis, elevated liver enzymes and low platelets syndrome, or a combination of preeclampsia and hemolysis, elevated liver enzymes and low platelets. Superimposed preeclampsia 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 pre-existing proteinuria. The group with only suspicion of preeclampsia, but without gestational hypertension, was defined as no hypertensive disease of pregnancy. Women with multiple pregnancy or chromosomal/fetal anomalies were excluded from the study.

Data collection

Blood was taken at study entry only, and after centrifugation, plasma was stored at −80°C to be analyzed later. Analysis of sFlt-1 and PlGF was performed after all 430 samples had been obtained by an automated analyzer (Cobas 6000, e module; Roche Diagnostics, Mannheim, Germany). In case levels were above the upper limit of the assay, samples were diluted and remeasured. Plasma ET-1 and endoglin were measured in 40 PPI users and 80 gestational age-matched nonusers by ELISA (QuantiGlo; Bio-Techne, Abingdon, UK and R&D Systems, Minneapolis, respectively). Clinical findings, physical examination, laboratory test results, maternal/neonatal complications (diagnosed by the treating physicians), and pre- and post-partum data were obtained from patient's electronic medical records and ascertained by 2 independent researchers (L. Saleh and R. Samantar). Outcomes collected at the time of study entry were recorded in a database and consisted of the following: gestational age at biomarker measurement, parity, preexisting conditions, such as hypertension, and proteinuria, medication use, systolic blood pres-sure, diastolic blood pressure, protein-to-creatinine ratio, serum concentration of lactate dehydrogenase, alanine transaminase, creatinine, uric acid, and platelet count. Medications registered were α-methyldopa, corticosteroids, ferrous fumarate, macrogol, nifedipine, and PPIs. After delivery, the following pregnancy outcomes were registered: days from study entry until delivery, gestational age at birth, sex, birth weight, and maternal complications, such as pla-cental abruption, pulmonary edema, post-partum hemorrhage (blood loss ≥1 L after delivery), and acute renal failure (absolute increase in the serum creatinine concentration of ≥0.3 mg/dL [26.4 micromol/L] from baseline; ≥50% increase in serum creatinine; or oliguria with <0.5 mL/kg per hour for a period of 6 hours) during pregnancy. Fetal/ neonatal complications were defined as fetal/neonatal death, neonatal (birth) weight <10th percentile according to Perinatal Registration The Netherlands; prematurity, defined as infants born before a ges-tational age of 37 weeks; endotracheal tube; development of sepsis; admittance to the neonatal intensive care unit, and respiratory distress syndrome.

Statistical analysis

Data are reported as median (range) for continuous variables and as number (percent-age) for categorical variables. The normality of con-tinuous variables was assessed using the Shapiro–Wilk W test. For non-normally distributed continues variables, Mann–Whitney *U* test was applied, and for normally distributed continuous variables, *t* tests were applied. Fisher exact and χ^2 were used to compare categorical variables between groups. Multiple comparisons of mean ranks for all groups were performed as post hoc tests. Spearman rank order correlation was applied to calculate correlation coefficients. $P<0.05$ was considered statistically significant. Statistical analysis was performed using Prism 5.0 for Mac (GraphPad software inc) and R Studio Statistical Software.

RESULTS

Subject characteristics

A total of 430 women were included (Table 1), of whom 174 were without hypertensive disease of pregnancy, 67 with gestational hypertension, 148 with (superimposed) pre-eclampsia, 13 solely with hemolysis, elevated liver enzymes and low platelets, and 28 with both preeclampsia and hemolysis, elevated liver enzymes and low platelets . Of these women, 45% were nulliparous. The median gestational age enrollment was 33 weeks; 54% were enrolled below 34 weeks. Furthermore, 196 patients were recorded taking α-methyldopa, 72 corticosteroids (4 beclomethasone, 47 betamethasone, 5 budesonide, 2 dexamethasone, and 12 prednisolone), 98 ferrous fumarate, 64 macrogol, 72 nifedipine, and 40 PPIs (6 esomeprazole [20 or 40 mg/d, n=2 and 4], 32 omeprazole [10, 20, or 40 mg/d, n=3, 22, and 7], and 2 pantoprazole [20 or 40 mg/d, n=1 each]) at the time of blood sampling for sFlt-1/PlGF measurement. The median duration of PPI treatment was 29 days (range, 8–45) before blood sampling.

Table 1. Patient Characteristics at Study Entry

Parameter		
n (%)		430
Age, y	32	(18–48)
Pre-conception weight, kg	72	(45–152)
Pre-conception BMI, kg/m^2	25	(17–55)
Gestational age, wk	33	(20–41)
<34, n (%)	230	(54)
34–36, n (%)	110	(26)
≥37, n (%)	90	(21)
Nulliparous, n (%)	194	(45)
Current smoker, n (%)	26	(6)
Pre-existing hypertension, n (%)	119	(28)
Pre-existing proteinuria, n (%)	39	(9)
Clinical findings at time of study entry		
SBP, mm Hg	140	(130–150)
DBP, mm Hg	90	(80–95)
PCR, mg/mmol	29	(14–87)
LD, U/L	192	(167–235)
ALT, U/L	16	(11–27)
Creatinine, μmol/L	59	(51–68)
Uric acid, mmol/L	0.30	(0.24–0.37)
Platelet count, 10^9/L	217	(168–269)
sFlt-1, pg/mL	4292	(375–83 967)
PlGF, pg/mL	109	(3–1824)
sFlt-1/PlGF ratio	38	(1–3199)
Diagnosis at study entry, n (%)		
No hypertensive disease of pregnancy	174	(40)
Gestational hypertension	67	(16)
Preeclampsia	102	(24)
Superimposed preeclampsia	46	(11)
(Partial) HELLP syndrome	13	(3)
Preeclampsia and HELLP	28	(7)

Values are median (range). ALT indicates alanine transaminase; BMI, body mass index; DBP, diastolic blood pressure; HELLP, hemolysis, elevated liver enzymes and low platelets; LD, lactate dehydrogenase; PCR, protein-to-creatinine ratio; PlGF, placental growth factor; SBP, systolic blood pressure; and sFlt, soluble Fms-like tyrosine kinase-1.

sFlt-1 and PlGF concentrations according to drug use

Women were first subdivided according to the use of a certain drug (Table 2), and either compared with all women not using that particular drug or given that sFlt-1 and PlGF alter with advancing gestation, with gestational age-matched women not using that particular drug. We aimed at obtaining 2 gestational age-matched nonusers for each individual drug user, but because of the size of our population, this was not possible for a widely used drug like α-methyldopa. Nevertheless, results of the 2 statistical analyses were identical. Women using α-methyldopa displayed higher sFlt-1 levels and lower PlGF levels. The latter was also true for nifedipine users. The use of ferrous fumarate or macrogol did not affect sFlt-1 or PlGF levels. Corticosteroids tended to lower PlGF while

Table 2. sFlt-1, PlGF Levels, and sFlt-1/PlGF Ratio According to the Different Medications

Drug	sFlt-1, pg/mL		*P* Value + vs –	PlGF, pg/mL		*P* Value + vs –	Ratio		*P* Value + vs –
α-Methyldopa									
+ (n=196)	8031	(548–73 920)		55	(3–1824)		146	(1–3199)	
– (n=234)	3850	(375–83 967)	0.030	154	(11–1729)	<0.001	28	(1–1803)	<0.001
– (n=221)*	3765	(1120–83 967)	0.003*	163	(11–1632)	<0.001	28	(1–1803)	<0.001
Corticosteroids									
+ (n=72)	5484	(1034–83 967)		49	(5–1632)		97	(1–3199)	
– (n=358)	3444	(375–73 920)	0.076	138	(3–1824)	<0.001	27	(1–1899)	0.001
– (n=107)*	4226	(836–73 920)	0.426	90	(3–997)	0.019	51	(2–1899)	0.058
Ferrous fumarate									
+ (n=98)	4041	(857–31 025)		110	(3–1824)		28	(1–1899)	
– (n=332)	3885	(375–83 967)	0.482	110	(3–1729)	0.108	38	(1–3199)	0.173
– (n=189)*	3481	(836–83 967)	0.141	123	(3–1556)	0.073	28	(1–1899)	0.862
Macrogol									
+ (n=64)	4030	(1120–32 474)		121	(7–1824)		37	(1–919)	
– (n=366)	3665	(375–83 967)	0.668	116	(3–1729)	0.998	31	(1–3199)	0.777
– (n=130)*	3239	(836–83 967)	0.363	120	(3–1632)	0.996	26	(1–1899)	0.341
Nifedipine									
+ (n=72)	4026	(375–23 455)		103	(3–997)		58	(1–3199)	
– (n=358)	3572	(726–83 967)	0.677	123	(7–1824)	0.059	29	(1–1803)	0.052
– (n=126)*	3085	(836–83 967)	0.489	137	(10–1632)	0.003	22	(1–1803)	0.023
Proton pump inhibitor									
+ (n=40)	3505	(1120–25 162)		107	(3–1632)		29	(1–1899)	
– (n=390)	4361	(375–83 967)	0.013	110	(5–1824)	0.855	36	(1–3199)	0.382
– (n=80)*	10 638	(836–83 967)	<0.0001	61	(6–1650)	0.068	199	(3–1803)	<0.001

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

*Matched for gestational age at study entry.

PPIs lowered sFlt-1. As a result, the sFlt-1/PlGF ratio was higher in users of α-methyldopa, nifedipine, and corticosteroids and lower in PPI users. PPI use duration did not correlate with sFlt-1 levels (data not shown).

Grouping the patients in gestational blocks of 5 weeks (Figure 1) confirmed the precipitous sFlt-1 rise across gesta-tion and additionally revealed that the PPI effect was observed at all gestational ages. Its effects were most apparent when sFlt-1 were highest (between weeks 30 and 34).

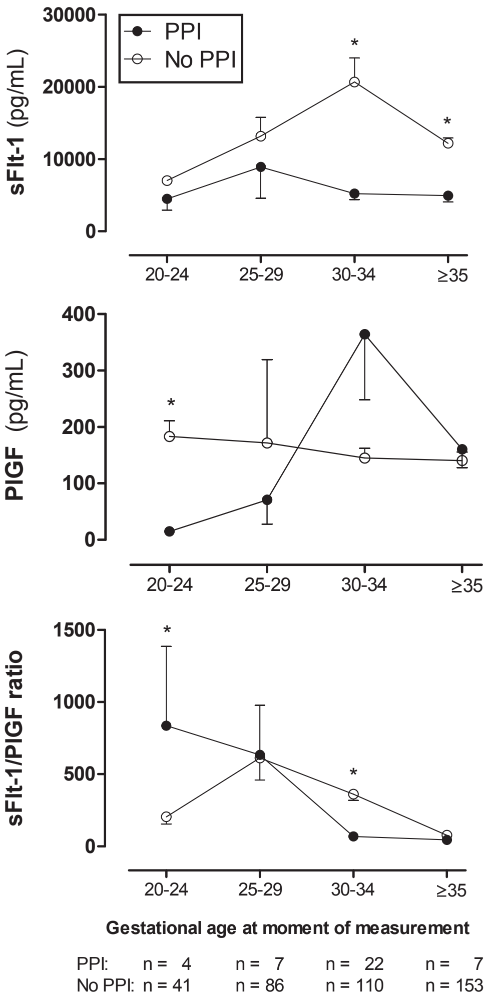

Figure 1. sFlt-1 (soluble Fms-like tyrosine kinase-1), PlGF (placental growth factor), and sFlt-1/PlGF ratio across gestation (5-week blocks) in proton pump inhibitor (PPI) users and nonusers. *P<0.05.

Table 3. Patient Characteristics According to Proton Pump Inhibitor Use

Parameter	PPI		No PPI		*P* Value
n (%)		40		80	…
Age, y	33	(21–42)	31	(19–45)	0.367
Pre-conception weight, kg	71	(50–114)	68	(52–101)	0.085
Pre-conception BMI, kg/m^2	27	(17–39)	24	(18–35)	0.069
Gestational age, wk	33	(23–41)	34	(20–41)	0.855
<34, n (%)	21	(53)	38	(48)	0.705
34–37, n (%)	12	(30)	27	(34)	0.837
≥37, n (%)	7	(18)	15	(19)	0.817
Nulliparous, n (%)	15	(38)	33	(41)	0.546
Current smoker, n (%)	5	(13)	4	(5)	0.680
Pre-existing hypertension, n (%)	14	(35)	16	(20)	0.170
Pre-existing proteinuria, n (%)	10	(26)	6	(8)	0.005
Clinical findings at time of study entry					
SBP, mm Hg	140	(129–146)	140	(130–150)	0.323
DBP, mm Hg	86	(80–95)	90	(80–96)	0.898
PCR, mg/mmol	32	(18–89)	34	(18–125)	0.777
LD, U/L	196	(162–228)	212	(179–254)	0.029
ALT, U/L	17	(12–31)	18	(12–48)	0.775
Creatinine, µmol/L	59	(51–71)	63	(55–75)	0.287
Uric acid, mmol/L	0.30	(0.26–0.37)	0.34 (0.28–0.39)		0.171
Platelet count, 10^9/L	207	(164–274)	205	(165–248)	0.700
α-Methyldopa	19	(48)	44	(55)	0.161
Corticosteroids	9	(23)	12	(15)	0.220
Ferrous fumarate	10	(25)	17	(21)	0.403
Macrogol	15	(38)	9	(11)	0.001
Nifedipine	11	(28)	11	(14)	0.151
Diagnosis at time of study entry, n (%)					
No HDP	19	(48)	19	(24)	0.020
Gestational hypertension	3	(8)	18	(23)	0.033
Preeclampsia	9	(23)	24	(30)	0.272
Superimposed preeclampsia	4	(10)	5	(6)	0.459
(Partial) HELLP syndrome	3	(8)	5	(6)	0.703
Preeclampsia and HELLP	2	(5)	9	(11)	0.322

PPI users and nonusers have been matched for gestational age at study entry. Values are median (range) or n (%). ALT indicates alanine transaminase; BMI, body mass index; DBP, diastolic blood pressure; HDP, hypertensive disease of pregnancy; HELLP, hemolysis, elevated liver enzymes and low platelets; LD, lactate dehydrogenase; PCR, protein-to-creatinine ratio; PPI, proton pump inhibitor; and SBP, systolic blood pressure.

PPI users versus gestational age-matched nonusers

PPI users did not differ from those who were not treated with these drugs (in particular with regard to the use of other sFlt-1/ PlGF-affecting drugs), with the exception of more cases with pre-existing proteinuria and no hypertensive disease of pregnancy, and less gestational hypertension among patients who were treated with PPIs (Table 3). Pre-existing proteinuria cases included diabetes mellitus type 1 (n=3), diabetes mellitus type 2 (n=3), and systemic lupus erythematosus (n=4) in the PPI users and diabetes mellitus type 1 (n=2), diabetes mellitus type 2 (n=1), systemic lupus erythematosus (n=1), and pre-existing kidney disease (n=2) in the nonusers. Adjusting for diagnosis at study entry did not change the magnitude of the observed differences in the biomarker measures. Results were also identical when restricting our analysis to patients with hypertensive disease of pregnancy at study entry (sFlt-1 4506 [1120–10 472] versus 11 600 (1286–83 967) pg/mL, PlGF 63 [3–1632] versus 56 [6–1650] pg/mL, and ratio 57 [1–1896] versus 213 [6–1803]; $P<0.001$, P=NS, and $P=0.003$, respectively) or when excluding patients with pre-existing proteinuria (data not shown). Rates of pregnancy complications for women treated with a PPI were not different from those not using these drugs, except for the number of neonatal sepsis cases, which was significantly lower in PPI users (Table 4). Furthermore, there was a strong trend toward a prolonged pregnancy duration in PPI users (ie, 16 days versus 9 days in nonusers). This most likely underlies the tendency toward an increased birthweight in PPI users.

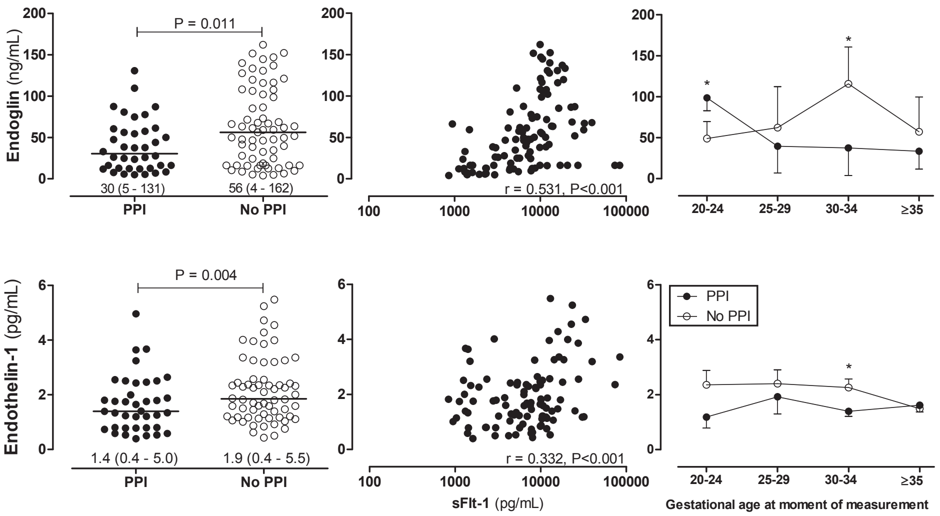

Figure 2. Left, endoglin and endothelin-1 levels in 40 women with confirmed or suspected preeclampsia using proton pump inhibitors (PPI) and 80 gestational age-matched controls not taking PPIs. **Middle**, correlations between the plasma levels of sFlt-1 (soluble Fms-like tyrosine kinase-1) and those of endoglin and endothelin-1. **Right**, endoglin and endothelin-1 levels across gestation (5-week blocks) in PPI users (n=4, 7, 22, and 7, respectively) and nonusers (n=11, 11, 25, and 33, respectively); *$P<0.05$.

Table 4. Pregnancy Outcome According to Proton Pump Inhibitor Use

Parameter	PPI		No PPI		*P* Value
n (%)		40		80	…
Gestational age at birth, wk	37	(21–41)	36	(23–41)	0.262
Prematurity, wk					
<34	10	(25)	29	(36)	0.221
34–37	9	(23)	15	(19)	0.809
Gender (M/F)	24/16	(60/40)	47/33	(59/41)	0.576
Birth weight, g	2758	(1190–4200)	2328	(900–4410)	0.264
Days until delivery	16	(2–73)	9	(0–106)	0.071
Maternal complications in all patients, n (%)					
Placental abruption		…	1	(1)	1.0
Pulmonary edema		…	1	(1)	1.0
Renal insufficiency	1	(3)	1	(1)	1.0
Post-partum hemorrhage	3	(8)	8	(10)	0.282
Fetal complications, n (%)					
Admission to NICU	19	(48)	34	(43)	0.558
Endotracheal tube	2	(5)	12	(15)	0.079
Percentile <10	7	(18)	20	(25)	0.251
Respiratory distress syndrome	4	(10)	13	(16)	0.281
Sepsis	1	(3)	12	(15)	0.029
Fetal death	1	(3)	3	(4)	0.703

PPI users and nonusers have been matched for gestational age at study entry. Values are median (range) or n (%). NICU indicates neonatal intensive care unit; and PPI, proton pump inhibitor.

Finally, PPI users displayed lower endoglin and ET-1 levels, and both parameters correlated positively with sFlt-1 (Figure 2). Grouping the patients in gestational blocks of 5 weeks revealed that these reductions were apparent at virtu-ally all gestational ages.

DISCUSSION

This study is the first to demonstrate that PPI use associ-ates with low sFlt-1 levels in women with confirmed or sus-pected preeclampsia. Mechanistically, this phenomenon can be attributed to the observation by Onda et al[10] that PPIs decrease sFlt-1 release from both primary trophoblasts and endothelial cells. The latter effect was observed, in a concen-tration-dependent manner, for lansoprazole, rabeprazole, and esomeprazole. Omeprazole (mainly used by our study popula-tion) and pantoprazole exerted more modest effects on sFlt-1 release. Nevertheless, the sFlt-1 levels in our study population were identically lowered during the use of 3 different PPIs (Figure S1 in the online-only

Data Supplement), suggesting that it is a class effect. Because PPIs selectively affected sFlt-1 in pregnant women, and not PlGF, the sFlt-1/PlGF ratio was decreased in PPI users. When subdividing the patients in ges-tational blocks of 5 weeks, it became clear that the largest PPI use-associated drop in sFlt-1 (and hence decrease in sFlt-1/ PlGF ratio) occurred in the patients with the highest sFlt-1 lev-els (ie, those between weeks 30 and 34). Finally, we observed that PPI use associated with lower endoglin and ET-1 levels, and plasma sFlt-1 levels correlated positively with the plasma levels of both endoglin and ET-1. In agreement with this observation, a previous multivariate analysis has revealed that sFlt-1 is an independent determinant of the elevated ET-1 lev-els in preeclampsia.[5] Although this might suggest that sFlt-1 determines endothelial ET-1 release, Onda et al[10] showed that PPIs directly suppress ET-1 secretion from endothelial cells in vitro (by de-creasing ET-1 mRNA expression). Thus, a likely scenario is that PPIs simultaneously lower sFlt-1, endoglin, and ET-1 synthesis and that this explains the correlations between the plasma levels of these parameters.

The effects of PPIs were observed at clinically relevant doses and following a median treatment period of 29 days. PPI treatment period did not correlate with sFlt-1 levels, suggesting that its effect on sFlt-1 synthesis might have occurred acutely. Clearly, future studies should now investigate, in a prospective manner, to what degree PPIs truly lower sFlt-1 in hypertensive disease of pregnancy and whether this has bene-ficial clinical consequences (eg, with regard to blood pressure, proteinuria, or pregnancy prolon-gation). Our data support a trend toward pregnancy prolongation and consequently an increased birthweight in PPI users. In addition, we observed more pre-existing proteinuria, no effect on blood pressure, and less gestational hypertension in PPI us-ers. However, given the simultaneous application of antihypertensive drugs, these data should be interpreted with care. In agreement with the sFlt-1/ PlGF alterations observed in preeclamptic women requiring antihypertensive treatment (ie, those with sFlt-1/ PlGF ratios above a certain cut-off level),[12–14] the use of α-methyldopa and nifedipine in our population was associated with higher sFlt-1 levels, lower PlGF levels, and a higher sFlt-1/PlGF ratio. Yet, importantly, the association of low sFlt-1 levels with PPI use was independent of the use of antihypertensive treatment and was also observed when limiting our analysis to patients with hypertensive disease of pregnancy at study entry or to patients without proteinuria.

Ferrous fumarate and macrogol did not affect sFlt-1 and PlGF while corticosteroids tended to lower the latter. This could reflect the possibility that steroids, like aldoste-rone, interfere with PlGF production.[15] However, in an earlier study in women with pre-eclampsia, Nayeri et al[16] were unable to demonstrate an effect of steroids on PlGF, and the authors concluded that the clinical improvement of steroid-exposed preeclamptic women was related to the anti-inflammatory effect of these drugs. Here, it is of interest to mention that *Helicobacter pylori* colonization is a risk factor for preeclampsia and small for gestational age birth.[17] The under-lying mechanism is unknown but may involve inflammatory factors. If true, eradication of *H. pylori* might reduce perinatal morbidity and mortality in preeclampsia, and PPI use could then possibly have beneficial effects in preeclampsia via interference with *H. pylori* colonization. A suppression of inflammation (eg, a reduction in tumor necrosis factor-α) by PPIs,[18] if occurring in pregnant women, could also underlie the reduction in neonatal sepsis cases (from 15% to 1%) in our population.

PERSPECTIVES

Alongside with potentially serious adverse consequences during pregnancy, preeclampsia associates with an increased risk for developing hypertension later in life in both mother and child.[19,20] It is, therefore, crucial to find novel therapies to maintain a healthy pregnancy in preeclamptic women, for instance aimed at normalizing the angiogenic imbalance in this disease. Our data imply that PPIs might exert such an effect. Given their excellent safety profile, an open, prospective, placebo-controlled pilot study should now be performed to investigate their efficacy. In addition, given that PPIs may lower the sFlt-1/PlGF ratio, the possibility should be considered that PPI use leads to false-negative results when using this ratio to diagnose/rule out preeclampsia.[13,21]

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SUPPLEMENTAL FIGURE

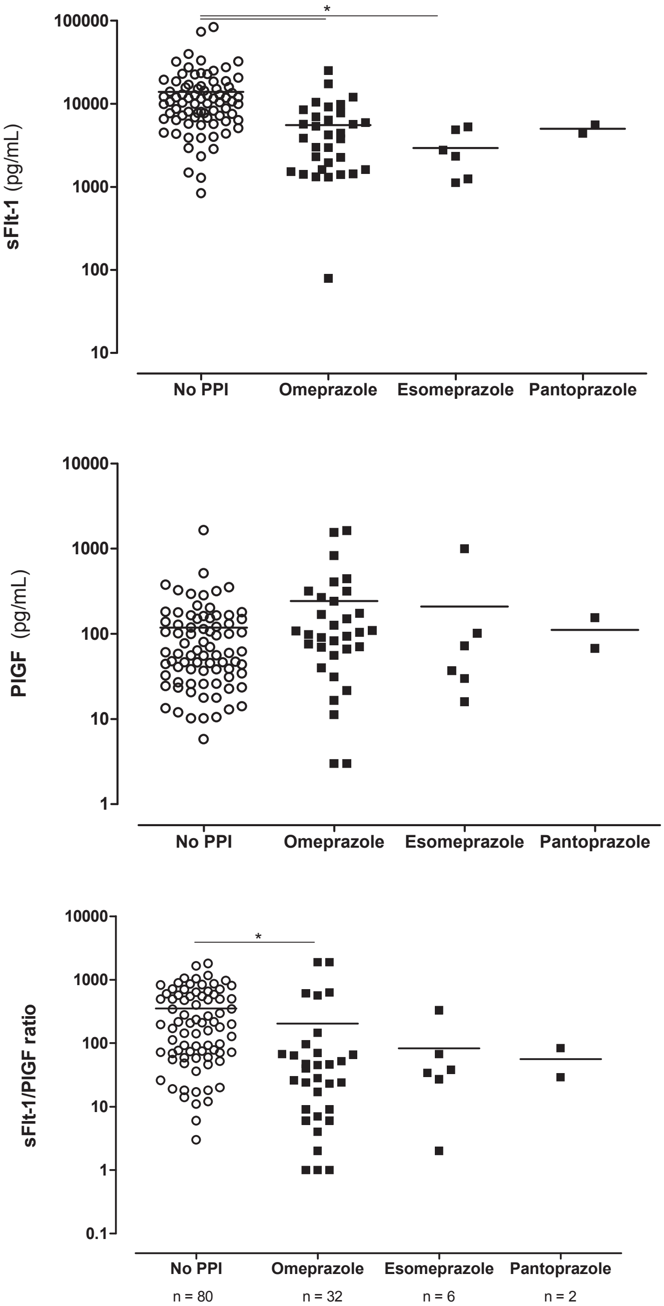

Figure S1. sFlt-1, PlGF and their ratio (individual levels and median) in pregnant women using omeprazole, esomeprazole or pantoprazole and gestational age-matched women that did not use proton pump inhibitors (PPI); *P<0.001 vs. no PPI.

Chapter 5

The sFlt-1/PlGF ratio associates with prolongation and adverse outcome of pregnancy in women with (suspected) preeclampsia: analysis of a high-risk cohort

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ABSTRACT

Objectives To evaluate the additive value of the sFlt-1/PlGF ratio for diagnosing pre-eclampsia (PE) and predicting prolongation of pregnancy and adverse outcome in a cohort of women with PE or at high risk of PE.

Study design Patients with suspected or confirmed clinical PE were recruited. At time of inclusion blood for measurement of sFlt-1and PlGF was taken. Values were determined after delivery. A cut-off ratio of 85 was defined as a positive test.

Results A total of 107 patients were included. Of the patients, 62 (58%) met the clinical criteria of PE at time of blood sampling. In 10% of these patients (n = 6) the ratio was <85 (false negative), whereas in 7% (n = 3) of patients without clinical PE the ratio was 85 (false positive), resulting in positive and negative predictive values of 95% and 88% respectively. One patient with false positive ratio developed superimposed PE and 2 developed gestational hypertension, and adverse outcome occurred in all three. An adverse pregnancy outcome was only encountered in 1 of the 6 patients with a false negative ratio. Using a binary regression model with adjustment for gestational age <34 weeks, the adverse outcome risk was 11 times increased on the basis of clinical PE, and 30 times on the basis of an elevated ratio (P = 0.036).

Conclusion The additive value of an increased ratio for diagnosing PE is limited since most patients with clinical PE also have a positive ratio. However, an elevated ratio is superior to the clinical diagnosis of PE for predicting an adverse pregnancy outcome. Furthermore, irrespective of clinical PE, a low ratio is inversely correlated with prolongation of pregnancy.

INTRODUCTION

Hypertensive disorders in pregnancy are the major cause of maternal and neonatal morbidity and mortality worldwide.[1] It is clinically important to distinguish preeclampsia (PE) from other forms of hypertension and proteinuria in pregnancy since patients with PE are at high risk for adverse pregnancy outcome.[2]. Serious events in the mother are the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, acute kidney failure, pulmonary edema, subcapsular liver hematoma, eclampsia, and cerebral hemorrhage. The most serious threats for the fetus are intrauterine growth restriction (IUGR), premature delivery, placental abruption and fetal/neonatal death.[3] Choosing between temporizing management and delivery is repeatedly a weighing of maternal versus fetal risks.

Abnormal placentation early in pregnancy is widely assumed to be an important initial event in the onset of preeclampsia. This results in the release of anti-angiogenic factors such as soluble Fms-like tyrosine kinase 1 or sFlt-1 and cytokines, leading to generalized vascular dysfunction. Elevated sFlt-1 levels bind and inactivate vascular endothelial growth factor (VEGF) and placental growth factor (PlGF).

The sFlt-1/PlGF ratio has been introduced as an aid to diagnose PE and as a valuable instrument to provide prognostic information.[4-6] This ratio of two biomarkers, produced by the placenta, has excellent test properties for diagnosing PE.[6] Before 34 weeks of gestation sensitivities and specificities of over 90% are reported.[7] However, when used after 34 weeks in pregnancy, the test performs less well due to a rise in sFlt-1 and a decreased level of PlGF at the end of normal pregnancies.[8-9] After the 34th week of gestation a sensitivity of 74% has been found.[7] We scrutinized the utility of the sFlt-1/PlGF test in the decision-making of women at high risk of PE and we analyzed the frequency of disagreement between the clinical diagnosis of PE and the sFlt-1/PlGF test for the prediction of prolongation of pregnancy and adverse outcome.

MATERIALS AND METHODS

From September 2011 until August 2013 patients with suspected or confirmed PE and a singleton pregnancy were recruited into this prospective study. Patients were suspected of PE if they presented with new onset hypertension and or proteinuria at or after 18 weeks gestation, developed an aggravation of their preexisting hypertension and or preexisting proteinuria or if they presented with symptoms such as right upper quadrant abdominal pain or headache with visual disturbances. The Erasmus MC Medical Ethics Committee approved the study protocol and written informed consent was obtained from all participants. After providing information about the aim of the study, participants were asked to donate a blood sample for measurement of sFlt-1 and PlGF at the time of admission either in the outpatient clinic or clinic because of suspicion for PE. Clinical characteristics of the patients were acquired on the same day as blood collection at inclusion. The diagnosis of PE was based on clinical judgment and routine laboratory findings at inclusion, whereas values of sFlt-1 and PlGF were determined after delivery to prevent any influence of this information on decision making of the clinicians. After inclusion, the pregnancy course of each patient was followed and clinical characteristics, prolongation of pregnancy, and pregnancy outcome were obtained from the medical records after delivery. PE was defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP).[10] We excluded twin pregnancies because differences in the biomarker concentrations between singleton and multiple gestations have been reported.[11]

We used the components of the fullPIERS predictive model, minus transfusion of blood products, for the definition of adverse maternal outcome.[12] Adverse outcome was defined as the occurrence of one or more complication(s) of PE within two weeks after blood sampling. sFlt-1 and PlGF were measured using commercially available assays on the Elecsys platform (Roche Diagnostics), as previously described.[13] After centrifugation, the samples were stored at 80 8C until analysis. For each sample the sFlt-1/PlGF ratio was calculated.[6-8, 13]

To detect a difference in prolongation of pregnancy of 10 days between a positive and a negative test we calculated a sample size of 116 patients using a power of 80% at a significance level of 5%. Comparisons were performed using the Mann–Whitney U-test for non-parametrically distributed data, or the t-test for normally distributed data. Values are reported as mean standard deviation (SD) for continuous data, as median and range (min–max) for non-normally distributed data, and as a percentage for categorical data. For the diagnosis of PE at inclusion, the diagnosis of PE as a final diagnosis and the prediction of adverse outcome, sensitivity, specificity, PPV and NPV were calculated for the sFlt-1/PlGF test using the cut-off point of 85.[14] This cut-off value was used because others have previously shown that a ratio 85 has a high sensitivity and specificity for diagnosing PE, of respectively 82% and 95%.[14] Furthermore, this ratio seems to predict the occurrence of adverse maternal and fetal outcomes related to PE in patients without a clinical diagnosis of PE.[15]

For the correlation of sFlt-1, PlGF and their ratio with the severity of the disease, Spearman's Rho correlation coefficient was used. A binary logistic analysis was performed to obtain the odds ratios for comparison of the different models to predict adverse pregnancy outcome. Statistical Package for Social Sciences (SPSS) software, version 21.0, was used for analysis. For all tests a value of $P < 0.05$ was considered to be significant.

RESULTS

A total of 107 patients were enrolled in the current study. The demographic and clinical characteristics at time of blood sampling for sFlt-1/PlGF measurement are listed in Table 1. Sixty-two (58%) patients met the clinical criteria for PE at time of blood sampling, of whom 6 with HELLP syndrome. The prevalence of 1 antihypertensive medication use was 54 (87%) in the PE and 19 (42%) in the non-PE group. Age or gestational age (GA) at study entry between patients with or without PE did not differ. Systolic and diastolic blood pressure (SBP and DBP), urinary protein-to-creatinine ratio, sFlt-1 and the sFlt-1/PlGF ratio among patients with PE were higher than in the non-PE group (Table 1). Three (3%) women developed HELLP syndrome as a complication, 2 (2%) women had pulmonary edema, and 4 (4%) developed acute renal failure (Table 2). Sixteen infants had IUGR (Table 2).

The prolongation and outcome of pregnancy stratified accord-ing to the clinical diagnosis of PE and a positive or negative ratio is given in Table 2. Out of 62 patients clinically diagnosed with PE, 56 (90%) had a ratio 85, and 42 out of the 45 patients (93%) without clinical diagnosis of PE had a ratio <85. Pregnancies of patients with a negative test were prolonged 10 days longer (P < 0.001) compared to patients with a positive test, independently of their clinical diagnosis. Six out of 48 (13%) patients with a negative test delivered within 10 days after testing. One of those patients was known with preexisting proteinuria and liver cirrhosis. She was admitted at 27.6 weeks because of severe ascites,

Table 1. Characteristics of patients at time of inclusion

	No preeclampsia		Preeclampsia		P
N	45		62		
Age, years	32	6	32	5	0.357
GA at inclusion, weeks	31	5	30	4	0.145
GA at delivery, weeks	36	4	31	4	<0.0001
SBP, mmHg	133	14	145	13	<0.0001
DBP, mmHg	83	10	89	7	0.001
PCR, mg/mmol	18	(7–1548)	99	(30–6016)	<0.0001
HELLP	–		6	(10%)	0.001
Preexisting hypertension, n	20	(44%)	17	(27%)	0.005
1 anti-hypertensives, n	19	(42%)	54	(87%)	<0.0001
Preexisting proteinuria, n	11	(24%)	6	(10%)	0.030
sFlt-1, pg/ml	2686	(696–21130)	14425	(1022–84339)	<0.0001
PlGF, pg/ml	191	(13–1708)	43	(6–853)	<0.0001
sFlt-1/PlGF ratio	13	(1–495)	407	(3–1824)	<0.0001

Values are mean SD, median (range) or number (%). GA: gestational age; SBP: systolic blood pressure; DBP: diastolic blood pressure; PCR: protein-to-creatinine ratio.

Table 2. Pregnancy outcome stratified by clinical diagnosis and sFlt-1/PlGF ratio

	Positive test with PE at inclusion	Positive test without PE at inclusion	Negative test with PE at inclusion	Negative test without PE at inclusion
N	56	3	6	42
Prolongation of pregnancy, days	4 (0–28)	5 (4–27)	18 (1–104)	28 (1–101)`
GA at delivery, weeks	30 4	28 2	35 4	37 3
Birth weight, g	1220 (325–3490)	835 (430–1250)	2665 (760–2860)	2915 (490–4510)
Fetal/neonatal complication				
IUGR, n	11 (20%)	1 (33%)	–	4 (10%)
Extreme prematurity, n	37 (66%)	3 (100%)	1 (17%)	3 (7%)
NICU stay, days	5 (0–163)	0 (0–9)	0 (0–40)	0 (0–44)
Fetal/neonatal death, n	3 (5%)	1 (33%)	–	–
Maternal complication				
HELLP syndrome, n	3 (5%)	–	–	–
Pulmonary edema, n	2 (4%)	–	–	–
Acute renal failure, n	4 (7%)	–	–	–
sFlt-1, pg/ml	15711 (4859–84339)	10495 (6388–11647)	3709 (1022–11597)	2562 (696–21130)
PlGF, pg/ml	39 (6–179)	34 (13–76)	126 (68–853)	212 (30–1708)
sFlt-1/PlGF ratio	442 (89–1824)	343 (138–495)	44 (3–78)	13 (1–83)

Values are median and ranges, mean SD, n (%). Positive test, sFlt-1/PlGF ratio 85; negative test, sFlt-1/PlGF ratio <85; GA, gestational age; IUGR, intra uterine growth retardation; NICU, neonatal intensive care unit; extreme prematurity, gestational age at delivery <32 weeks.

edema and a SBP of 140 mmHg. At 28.5 weeks, pregnancy was terminated because of deterioration of her preexisting maternal condition. The other five patients were admitted after 36 weeks gestation. According to the protocol of our hospital expectant management is not performed in women with suspected preeclampsia after 36 weeks.

Similarly, irrespective of the test, patients without the clinical diagnosis of PE were also prolonged 10 days longer compared to patients with the clinical diagnosis of PE ($P < 0.001$). Only patients with a clinical diagnosis of PE and a positive ratio developed maternal complications (Table 2). Irrespective of the clinical diagnosis of PE, fetal/neonatal complications were considerably lower (17% versus 81%, $P < 0.001$) in patients with a negative ratio compared to those with a positive ratio. Six of the 62 patients (10%) with a clinically diagnosis of PE had a ratio <85 (Table 3). GA at time of blood sampling varied from 22 to 36 weeks. Only one of these patients (no. 2 in Table 3) encountered an adverse pregnancy outcome; she delivered extremely preterm. It should be remarked that her ratio of 78 was relatively high. The other 5 pregnancies were uncomplicated,

Table 3. Characteristics of the patients with a ratio <85 and clinically diagnosed with preeclampsia at time of inclusion (false negative ratio)

Case	1	2	3	4	5	6
Maternal age, years	33	26	32	41	30	34
GA at inclusion, weeks, days	22.1	25.4	31.3	32.6	36.2	36.6
SBP, mmHg	145	140	135	180	150	150
DBP, mmHg	90	95	85	100	90	90
PCR, mg/mmol	549.2	94.6	99.3	31.1	45.5	66.7
Preexisting hypertension	No	No	Yes	Yes	No	No
Antihypertensive drug use	No	Yes	Yes	Yes	Yes	No
Preexisting proteinuria	Yes	No	No	No	No	No
Other (preexisting) diseases	–	–	SLE, LN, APS	GD	ITP	–
sFlt-1, pg/ml	1022	11597	3838	3143	3580	6797
PlGF, pg/ml	337	148	68	101	853	104
sFlt-1/PlGF ratio	3	78	56	31	4	65
Days from inclusion to delivery	104	13	36	30	1	1
GA at delivery, weeks, days	37.0	27.3	36.4	37.1	36.3	37.0
Reason for delivery	At term	Fetal distress	Maternal C	At term	PROM	SROM
Adverse outcome	–	Extr prem	–	–	–	–

GA, gestational age; SBP, systolic blood pressure at diagnosis, DBP: diastolic blood pressure at diagnosis; PCR, protein-to-creatinine ratio at diagnosis; Extr prem, extreme prematurity <32 weeks of GA; SLE, systemic lupus erythematosus; LN, lupus nephritis; APS, antiphospholipid syndrome; Maternal C, maternal condition; GD, gestational diabetes; PROM, premature rupture of membranes, SROM, spontaneous rupture of membranes, ITP; idiopathic thrombocytopenic purpura.

notwithstanding the clinical diagnosis of PE. In 3 of the 45 patients (7%) without clinical diagnosis of PE at inclusion, the ratio was >85 (Table 4). Of these, one developed superimposed PE (10 days after inclusion), and the other two gestational hypertension (GH), both 4 days after inclusion. Adverse pregnancy outcome occurred in all 3 patients. The duration between inclusion and delivery was respectively 27, 4 and 5 days (Table 4).

With a cut-off value of the ratio >85 the sensitivity for the clinical diagnosis of PE at inclusion was 90%. This value modestly increased for the final clinical diagnosis of PE (Table 5). Compared to the clinical diagnosis of PE, sensitivity and specificity for prediction of adverse outcomes were better for the ratio, using a cut-off value of >85 (Table 5).

The PlGF concentrations were considerably lower in extreme premature and in preterm than in the term deliveries, respectively 32 pg/ml (6–489) and 77 pg/mL (17–853) versus 205 pg/mL (61– 1708) ($P < 0.001$). PlGF was also lower in pregnancies complicated by IUGR (28 pg/mL (7–489)). The sFlt-1/PlGF ratio correlated positively with blood pressure, PCR, the serum concentration of uric acid, adverse outcome and stay at the NICU, but

Table 4. Characteristics of the patients with a ratio >85 without a clinically diagnosed preeclampsia at time of inclusion (false positive ratio)

Case	1	2	3
Maternal age, years	27	20	35
GA at inclusion, weeks, days	21.3	28.3	28.6
SBP, mmHg	160	140	120
DBP, mmHg	105	90	80
PCR, mg/mmol	22.7	13.1	15.7
Preexisting hypertension	Yes	No	No
Antihypertensive drug use	No	No	No
Preexisting proteinuria	No	No	No
Other preexisting diseases	–	–	SLE, APS
sFlt-1, pg/ml	6388	10495	11647
PlGF, pg/ml	13	76	34
sFlt-1/PlGF ratio	491	138	343
Days from inclusion to delivery	27	4	5
GA at delivery, weeks, days	25.2	29.0	29.4
Days from inclusion to final diagnosis	10	4	4
Final diagnosis	sup PE	GH	GH
Reason for delivery	Stillbirth	Fetal distress	Fetal distress
Adverse outcome	IUGR, Stillbirth[a] Extreme prematurity	Extreme prematurity	Extreme prematurity

[a] Stillbirth, severe IUGR early in pregnancy.

GA, gestational age; SBP, systolic blood pressure at diagnosis, DBP: diastolic blood pressure at diagnosis; PCR, protein-to-creatinine ratio at diagnosis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome; supPE, superimposed preeclampsia; GH, gestational hypertension; HELLP, hemolysis elevated liver enzymes and low platelet count; IUGR, intrauterine growth restriction.

Table 5. Sensitivity, specificity, positive and negative predictive values for the ratio cut-off point of 85 for diagnosing preeclampsia with clinical preeclampsia as reference and for adverse outcomes. Sensitivity, specificity, positive and negative predictive values for adverse outcomes based on clinically diagnosed PE are also provided

	sFlt-1/PlGF ratio cut-off point of 85			Clinically diagnosed PE
	PE at inclusion	Final diagnosis PE	Adverse outcomes	Adverse outcomes
Sensitivity (%)	90	91	86	75
Specificity (%)	93	98	93	79
PPV (%)	95	98	95	86
NPV (%)	88	83	81	75

PE, preeclampsia; PPV, positive predictive value; NPV, negative predictive value inversely with prolongation of pregnancy, gestational age at delivery and birth weight percentile corrected for GA (Table 6). When comparing the sFlt-1 and PlGF concentrations separately with variables of disease severity and outcome, it became clear that sFlt-1 correlated positively with maternal variables of disease severity, and PlGF with fetal/neonatal variables of disease severity (Table 6). In a binary regression model, patients with an established clinical diagnosis of PE had a 5 (2–12) times increased risk for having an adverse pregnancy outcome (Table 7). After correction for GA at time of measurement, this risk was 11 (4–30). Patients with a positive test had a 9 (4–21) times increased risk for adverse pregnancy outcome, and 30 (9–96) when correcting for GA at testing. When both risk factors (clinical diagnosis and ratio) were combined in a multiple regression model and corrected for GA at testing (Table 8), only a positive sFlt-1/PlGF test remained significant with an odds ratio of 22 (5–91).

Table 6. Correlations of sFlt-1, PlGF and their ratio with variables of severity of the disease and outcome

	Ratio	sFlt-1	PlGF
	**	**	**
SBP, mmHg	.409*	.324 *	.341*
DBP, mmHg	.252*	.229 **	.241
PCR, mg/mmol	.279	.314 **	.141
Serum creatinine, mmol/l	.229	.416	.042
	**	**	
Serum uric acid, mmol/l	.372**	.500 **	.144**
Prolongation of pregnancy, days	.656**	.696 **	.499**
GA at delivery, weeks	.699**	.439	.736**
Birth weight, percentile	.304**	.154 **	.318**
Apgar score 5 min	.392**	.271 **	.393**
Stay at NICU, days [a]	.565**	.401 **	.629**
Any adverse outcome	.642	.460	.587

[a] Any form of adverse pregnancy outcome as mentioned in the Methods.
* P < 0.05.
** P < 0.01.

Table 7. Odds for adverse outcome of pregnancy

R^2		Odds	P value
0.18	Clinical diagnosis	5 (2–12)	<0.001
0.29	sFlt-1/PlGF ratio 85	9 (4–21)	<0.001
0.32	Clinical diagnosis corrected for gestational age at time of measurement	11 (4–30)	<0.001
0.50	sFlt-1/PlGF ratio 85 corrected for gestational age at time of measurement	30 (9–96)	<0.001

Table 8. Odds for adverse outcome of pregnancy, combining clinical diagnosis and a sFlt-1/ PlGF ratio 85 in one model after correcting for gestational age at time of measurement

R^2		LR+	LR	Odds		P value
0.53	Clinical diagnosis	1.71	0.45	1	(0.4–6)	0.59
	Positive test	2.50	0.34	22	(5–91)	<0.001

LR+, positive likelihood ratio; LR, negative likelihood ratio.

COMMENTS

To the best of our knowledge this study is the first to compare the sFlt-1/PlGF test with the clinical PE diagnosis for the prediction of both prolongation and adverse outcome of pregnancy. We found an increased risk for delivery and adverse pregnancy outcome when the ratio was 85. This agrees with findings reported by Rana et al. showing that both imminent delivery within 2 weeks and adverse outcomes occurred significantly more in patients with an elevated ratio.[16] Although the calculated number of 116 patients was not achieved, we did find a difference in prolongation of pregnancy between women with a positive and a negative test.

Binary regression analysis revealed that a positive test is a better predicting factor for poor pregnancy outcome than the clinical PE diagnosis. This finding is important because the clinical diagnosis and test result did not always comply. As observed before, we found that even patients not meeting the classical diagnostic criteria for PE, but who did have a positive test, had a comparable incidence of adverse outcomes as patients with a clinical diagnosis of PE and a positive test (Table 2).[15-17] This suggests that either there is a shortcoming of the current definition of PE, that the diagnosis has been missed, and/or that the clinical syndrome of PE was still in its developing phase. Although experienced clinicians made the diagnosis, the high prevalence of preexisting hypertension and proteinuria in our population of respectively 35% and 16% might have hampered the diagnostic process in some of the patients. Particularly for these patients, the sFlt-1/PlGF test may be a helpful tool for diagnostic and decision-making purposes.[17] A concern is the observation that 6 patients with clinically diagnosed PE had a negative sFlt-1/PlGF test. One of these 6 experienced an adverse outcome, but had a relatively high ratio of 78. The other 5 pregnancies were uncomplicated and the women remained pregnant, in one case even until the 37th week of gestation, indicating that a negative test may predict a prolonged duration of the pregnancy despite diagnosing PE on the basis of the traditional criteria. It should be noted that 2 of these patients were tested after 36 weeks of pregnancy, when the ratio may lose its accuracy.[13]

Simultaneously, we had 3 patients with a positive test, but without clinical diagnosis of PE at inclusion. All 3 were later diagnosed with GH or superimposed PE, and delivered preterm. The high ratio in the GH patients is not in line with other reports.[18] Yet, our findings support previous findings that an elevated ratio can detect disease and adverse outcomes in some individual cases earlier than achievable by routine clinical and laboratory assessment, and that it is helpful to distinguish between preexisting conditions and superimposed PE.[17,19]

Furthermore, almost all patients with a negative test remained pregnant >10 days, except patients who were included late in their pregnancy.

Interestingly, sFlt-1 and PlGF levels correlated with well-known parameters for the severity of the disease and outcome. Indeed, sFlt-1 correlated positively with relevant maternal parameters such as blood pressure, proteinuria and uric acid, while PlGF correlated positively with fetal adverse outcomes like GA at delivery, IUGR and prolonged stay at the NICU. As reported recently, a low PlGF is strongly linked with low birth weight and therefore can add valuable information to identify pregnancies with a potential adverse fetal outcome, necessitating urgent delivery.[19]

Some limitations of our study should be mentioned. This study was performed in a single center and the sample size of this study was therefore limited and we did not reach the ideal sample size with increased risk for type 1 and 2 errors. Furthermore, we only measured the sFlt-1/PlGF ratio once. Others demonstrated the importance of repeated measurements of the ratio for prediction of pathologic pregnancy outcomes, including PE and IUGR.[20,21] In particular, Schoofs et al. found that the time-dependent slope of the ratio is predictive for future pregnancy outcome and risk of developing PE up to 4 weeks later. Hagmann et al. also emphasize in their review the additive value of repetitive measurements of the sflt-1/PlGF ratio.[21] To advocate repeated measurements of the sFlt-1/PlGF ratio, large scale studies are necessary both to define cut-off values of the slopes of the ratios in relation to pregnancy outcome and to establish their additive value as compared to the currently used traditional assessments and/or a single ratio measurement. At last, we used a single cut-off value of the ratio of < or 85 to establish PE. In a recent study it has been shown that the use of different cut-off values related to gestational phase of pregnancy enhances the diagnostic accuracy of the sFlt-1/ PlGF ratio as a diagnostic tool for PE.[22] A disadvantage of this approach, apart from its complexity, is the introduction of a gray zone at which value PE can neither be ruled in or ruled out.

CONCLUSION

In a cohort of pregnant women with clinical PE or at high risk of PE we have shown that an sFlt-1/PlGF ratio 85 is associated with higher odds for adverse pregnancy outcome of pregnancy (Tables 7 and 8) than the clinical diagnosis of PE. The additional value of the test for diagnosing purposes remains limited since most patients with a positive test do have the clinical diagnosis of PE. Yet, particularly in pregnant women with preexisting hypertension and/or proteinuria, measurement of the test may be valuable for making the correct diagnosis. Finally, due to its correlation with clinical parameters, the sFlt-1/PlGF ratio may be useful for research purposes as the clinical definition of PE is not always straightforward.[23]

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Chapter 6

sFlt-1 and PlGF kinetics during and after pregnancy in women with suspected or confirmed preeclampsia

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ABSTRACT

Objectives To assess the evolution of the sFlt-1/PlGF ratio in women with suspected or with confirmed preeclampsia and to investigate the changes in sFlt-1 and PlGF levels in preeclamptic women after delivery.

Methods In this exploratory tertiary referral university centre study, using the Roche Diagnostics Elecsys assay, sFlt-1 and PlGF were determined in two groups of patients. In the first group of 46 patients with suspected or confirmed preeclampsia, sFlt-1 and PlGF were measured at least twice during their pregnancy. Women had singleton pregnancies and a median pregnancy duration of 26 weeks (range 18– 40 weeks). In the second group, sFlt-1 and PlGF of 26 preeclamptic patients were determined before and after delivery. The median gestational age at inclusion was 29 weeks (16 – 37) and the median days between antepartum measurement and delivery was 2 days (1 – 7).

Results In the first group, 90% of patients with a sFlt-1/PlGF ratio ≤38 at baseline (n=30), ruling out PE, the sFlt-1/PlGF ratio remained stable for up to 100 days. In 16 patients with a sFlt-1/PlGF ratio >38 and in 10% of those with a sFlt-1/PlGF ratio <38 at baseline the ratio increased further. In the second group, after delivery, sFlt-1 dropped to <1% of its pre-delivery values with a half-life of 1.4±0.3 days, while PlGF dropped to ≈30% of its pre-delivery values with a half-life of 3.7±4.3 days.

Conclusions Based on this small cohort, up to 10% of women admitted with suspected or confirmed PE presenting with a sFlt-1/PlGF ratio ≤38 display a rise of this sFlt-1/PlGF ratio in subsequent weeks, implying that repeated determination of the sFlt-1/PlGF ratio is required to reject this condition definitively. Furthermore, the rapid and pronounced decline of sFlt-1 values after delivery in patients with PE/HELLP suggests that sFlt-1, in contrast with PlGF, is almost entirely placenta-derived.

INTRODUCTION

Preeclampsia (PE) is estimated to complicate 5% of pregnancies worldwide. It is a multi-system disorder defined as new onset hypertension and proteinuria at gestational week 20 or after.[1] Studies have shown that the ratio of the serum biomarkers sFlt-1 and PlGF is markedly increased in preeclamptic patients and in women with pre-existing conditions predisposing or mimicking PE as compared to healthy pregnant women.[2-4] Both biomarkers are believed to be largely derived from the placenta.[5,6] A higher sFlt-1/PlGF ratio is related to the severity of the disease as reflected by the shorter time-interval between presentation and induced labour and adverse maternal and neonatal outcomes.[7-17] To diagnose PE, the sFlt-1/PlGF ratio has been reported to perform better than the standard diagnostic work-up, involving the determination of blood pressure, proteinuria, serum uric acid, serum alanine aminotransferase, platelet count and serum creatinine.[9] In addition, a recent paper reported that women with a sFlt-1/PlGF ratio below 38 did not develop PE within one week.[18] The implication of a single measurement of the biomarkers beyond one week remains unknown and so far little is known about the change in sFlt-1 and PlGF during pregnancy. An important question is how frequently one has to repeat the measurement of these proteins, especially when women are suspected of PE early in pregnancy and hospitalized for prolonged periods. Moreover, it is generally assumed that these proteins disappear after giving birth in view of their origin in the placenta.[19] However, it is not clear how fast these levels normalize and at what stage post-partum patients with PE display the same concentrations as healthy non-pregnant women. In this exploratory study, we evaluated the evolution of these biomarkers in women with suspected or confirmed PE, and investigated the changes in sFlt-1 and PlGF levels in preeclamptic/HELLP women after delivery.

METHODS

Study design and participants

This is a secondary analysis of a prospective cohort study that enrolled women with singleton pregnancies with suspicion or with confirmed PE from 18 weeks' gestation between December 2013 and April 2016 at the Department of Obstetrics of the Erasmus Medical Center in Rotterdam. All subjects provided informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202).

Women with multiple pregnancy or chromosomal/fetal anomalies were excluded from the study.

Diagnosis

Women were suspected of PE if they presented with hypertension, proteinuria and/or symptoms associated with PE such as right upper quadrant abdominal pain or headache with visual disturbances. PE was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[20] Conforming to this, PE is defined as de novo hypertension (systolic blood pressure (SBP) of ≥140 and/or a diastolic blood pressure (DBP) of ≥90 mmHg) and proteinuria (PCR ≥30 mg/mmol or ≥300mg/24 h) at or after 20 weeks of gestation. HELLP syndrome was defined as haemolysis, elevated liver enzymes and low platelets.

Data collection and procedure

In the first subgroup we determined sFlt-1 and PlGF at minimally 2 time points during pregnancy in 46 patients with suspicion of or with confirmed PE. Blood for measurement of the biomarkers was collected when blood was drawn for routine laboratory testing of PE, i.e. for clinically indicated reasons. Therefore, the number of days between the 2 or more measurements and the gestational age at measurement varied. The aim of this part of the study was to assess the evolution of the sFlt-1/PlGF ratio in women with suspected PE or confirmed PE.

In a second subgroup, we determined sFlt-1 and PlGF levels a few days before and after delivery in 26 patients diagnosed with PE/HELLP. The majority of the pregnancies were terminated electively due to evolution of PE. The aim of this part of the study was to investigate the changes in sFlt-1, PlGF and their ratio in preeclamptic/HELLP women after delivery.

Clinical findings, physical examination, laboratory test results, maternal, fetal/neonatal complications (diagnosed by the treating physicians) were obtained from patient's electronic medical records and ascertained by two independent researches (LS and WV). After blood was taken, and centrifuged, plasma was stored at -80 °C to be analysed later. Analysis of sFlt-1 and PlGF was performed after all samples had been obtained by an automated biochemistry analyzer (Cobas 6000, **e** module; Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences 22 (SPSS, IBM Corp., Armonk, NY, USA). For continuous variables, median and range or mean±SD are given, and number (%) is given for categorical data. The course of the sFlt-1, PlGF and their ratio from admission until delivery per patient is displayed by spaghetti plots. Concentrations after delivery are expressed as a percentage of the concentration in the last sample taken during pregnancy. The half-life (t1/2) over the first week was calculated on the basis of the formula Ct = C0 x e-kt, where C0 is the concentration in the last sample taken during pregnancy, Ct the concentration at day t after delivery, and k = ln2/t1/2. Since this was an exploratory study, no power analysis to determine sample size was performed. According to a previous study, a sFlt-1/PlGF ratio ≤38 was considered to be low, excluding PE for one week.[18]

RESULTS

Subgroup one, repetitive measurements

In this subgroup we determined the sFlt-1/PlGF ratio at two or more time points in 46 patients (age 20-45 years) with suspected or confirmed PE. Gestational age at study entry was <37 weeks in 44 (96%) patients. At time of inclusion, 33 (72%) women were suspected of PE, 4 (9%) were diagnosed with gestational hypertension and 9 (20%) with preeclampsia/HELLP. Of the 46 patients, 30 had a sFlt 1/PlGF ratio of ≤38 at inclusion. Table 1 displays the characteristics and baseline values of all patients and those of the patients with sFlt-1/PlGF ratios ≤38 or >38 at the time of the first measurement.

Demographic characteristics, BP and protein-to-creatinine ratio did not differ between the groups. The changes in sFlt-1, PlGF and the sFlt-1/PlGF ratio of the individual patients during pregnancy are plotted in Figure 1. In 90% of patients with a sFlt-1/PlGF ratio ≤38 at inclusion (median 8, range 2, 37), the sFlt-1/PlGF ratio remained stable for at least 100 days with a median change per day of 0.08 (-8, 4). In three women starting with a sFlt-1/PlGF ratio ≤38 the sFlt-1/PlGF ratio increased to levels above 38 (from 21, 10 and 7 to respectively 49, 57 and 404). The intervals between the measurements were respectively 14, 64 and 90 days, and they delivered at 29.5, 36.4 and 39.0 weeks of gestation. The increase in sFlt-1/PlGF ratio was caused both by an increase in sFlt-1 (of 97%, 270% and 1212%) and a decrease in PlGF (of 17%, 36% and 78%).

In 16 (35%) out of the 46 patients with a sFlt-1/PlGF ratio of >38 at inclusion (median 98, range 42, 991), the sFlt-1/PlGF ratio increased further over time, roughly doubling every week, with a median change per day in sFlt-1/PlGF ratio of 7.1 (-5, 43). In 3 of these 16 patients the sFlt-1/PlGF ratio declined by respectively 44, 31 and 22%, but always remained >38.

Subgroup two, comparing antepartum biomarker levels with postpartum levels

In this subgroup, we determined the sFlt-1, PlGF and sFlt-1/PlGF ratio before and after delivery in 26 patients diagnosed with PE/HELLP. The demographic and clinical characteristics of these patients are provided in Table 2, and Figure 2 displays the median antepartum and postpartum levels of sFlt-1, PlGF and sFlt-1/PlGF ratio. In all but one of the patients blood was taken during the last 2 days before delivery, but the moment of postpartum measurement varied from 1 to 152 days. In 7 patients blood was drawn within one week postpartum and these data were used for the calculation of

Table 1. Characteristics of patients at time of inclusion with a sFlt-1/PlGF ratio ≤ 38 or above 38

N	**All patients**	**Ratio ≤38**	**Ratio >38**	**P-value**
N	46	30	16	
Age (yrs)	35 (20, 45)	36 (22, 45)	33 (20, 44)	0.315
Gestational age (wks)	26 (18, 40)	26 (18, 39)	30 (18, 40)	0.316
Patients included ≥37 weeks (n, %)	2 (4%)	1 (3%)	1 (6%)	0.648
Nulliparous (n, %)	26 (57)	16 (53)	10 (63)	0.550
Caucasians (n, %)	22 (48)	14 (47)	8 (50)	0.829
History of PE (n, %)	11 (24)	8 (27)	3 (19)	0.549
Pre-existing hypertension (n, %)	32 (70)	22 (73)	10 (63)	0.447
Pre-existing proteinuria (n, %)	19 (41)	14 (47)	5 (31)	0.312
Clinical findings at time of inclusion				
SBP (mmHg)	139 ± 14	136 ± 13	144 ± 12	0.765
DBP (mmHg)	88 ± 10	86 ± 10	91 ± 9	0.522
PCR (mg/mmol)	39 (5, 824)	39 (5, 764)	39 (8, 824)	0.943
ALT (U/L)	17 (6, 862)	19 (6, 862)	15 (7, 423)	0.742
Creatinine (mmol/L)	56 (36, 149)	56 (36, 133)	57 (36, 149)	0.488
Uric Acid (mmol/L)	0.29 (0.16, 0.54)	0.27 (0.16, 0.47)	0.31 (0.17, 0.54)	0.058
Platelet Count (10^9/L)	234 (4, 522)	245 (4, 316)	222 (81, 522)	0.725
sFlt-1 (pg/mL)	2987 (1022, 16041)	2037 (1022, 9902)	7042 (1880, 16041)	<0.001*
PlGF (pg/mL)	165 (10, 997)	257 (50, 997)	46 (10, 208)	<0.001*
ratio	19 (2, 991)	8 (2, 37)	98 (42, 991)	<0.001*
Diagnosis at inclusion				
Suspected of preeclampsia	33	26	7	0.002*
Gestational hypertension	4	-	4	0.004*
Preeclampsia/HELLP	9	4	5	0.230

Values are median and range or mean ± SD or number (%) where appropriate. SBP, systolic blood pressure; DBP, diastolic blood pressure; PCR, protein-to-creatinine ratio; ALT, alanine aminotransaminase. These are all subjects in whom the sFlt-1 and PlGF is measured at ≥2 time points.
*Significant at p<0.5 comparing subjects with a sFlt-1/PlGF ratio <38 and those with a sFlt-1/PlGF ratio >38.

the half-lifes. In all patients sFlt-1 rapidly and markedly decreased after delivery with an estimated half-life of 1.4±0.3 days (Figure 3), reaching steady-state levels of 77 (range: 52, 3594) pg/mL (Figure 2), i.e., corresponding with <1% of the levels before delivery. PlGF also decreased after delivery with an estimated half-life of 3.7±4.3 days, reaching steady-state levels of 9 (range: 4, 24) pg/mL, i.e., corresponding with ≈30% of the levels before delivery. The sFlt-1/PlGF ratio decreased to <38 within one day after delivery in all patients, with a halflife of 0.6±0.7 days, reaching a steady-state level of 10 (6, 274), i.e., corresponding with ≈2% of the sFlt- 1/PlGF ratio before delivery.

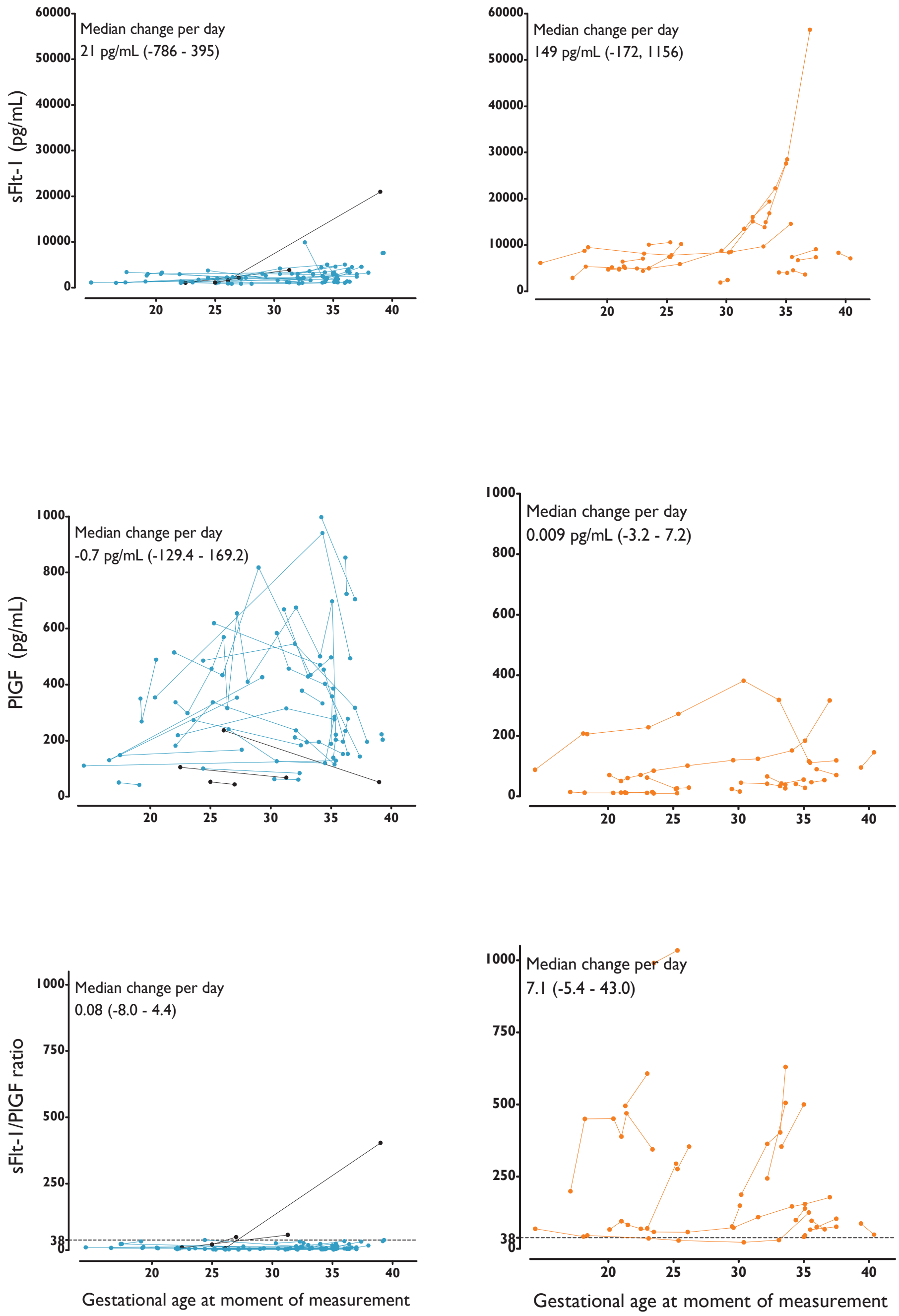

Figure 1. Repetitive levels of sFlt-1, PlGF and sFlt-1/PlGF ratio at multiple time points. Data are from patients with either a sFlt-1/PlGF ratio of ≤38 at first time of presentation (left blue graphs) or a sFlt- 1/PlGF ratio >38 at first time of presentation (right red graphs). The black graphs (left) represent the 3 patients in whom the sFlt-1/PlGF ratio increased from <38 at time of presentation to >38 later in their pregnancy.

Table 2. Baseline characteristics of preeclamptic/HELLP women with measurement of angiogenic factors pre- and postpartum

N	26
Maternal age (yrs)	31 (21, 47)
Gestational age at inclusion (yrs)	29 (16, 37)
Nulliparous (n, %)	11 (41%)
Days between antepartum measurement and delivery	2 (1, 17)
sFlt-1 (pg/mL)	10578 (4505, 85000)
PlGF (pg/mL)	26 (6, 237)
Ratio	569 (83, 1034)
Systolic Blood pressure (mmHg)	146 (95, 167)
Diastolic Blood pressure (mmHg)	90 (68, 105)
PCR (mg/mmol)	77 (11, 6016)
Gestational age delivery (wks)	31 (16, 37)
Infant birth weight (g)	1240 (60, 2150)
Infant birth weight percentile <10 (n, %)	3 (12%)

Values are median and range or number (%) where appropriate.

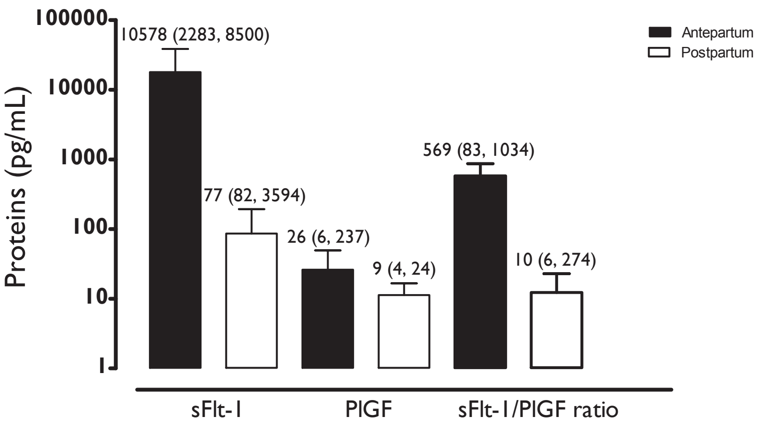

Figure 2. Circulating sFlt-1, PlGF and the sFlt-1/PlGF ratio before and after delivery. Antepartum blood was drawn in the last two days before delivery and postpartum blood was drawn from 1 to 152 days postpartum.

DISCUSSION

A sFlt-1/PlGF ratio ≤38 has been reported to rule out PE for the next 7 days18. Our study shows that in 90% of patients with suspicion of or established PE presenting with a sFlt-1/PlGF ratio ≤38 at baseline, the sFlt-1/PlGF ratio remained below this value during long-term follow up. In contrast, in patients presenting with a sFlt-1/PlGF ratio above this value, the sFlt-1/PlGF ratio rapidly increased further, roughly doubling every week in almost all patients. After delivery, sFlt-1 decreased within one week to levels corresponding with <1% of the levels prior to delivery, suggesting that the placenta is by far the major source of this biomarker. PlGF dropped more slowly, and levelled off at levels corresponding with ≈30% of its pre-delivery levels, demonstrating that this biomarker has additional important non-placental sources.

Using the proposed cut-off value of the sFlt-1/PlGF ratio of ≤38 to rule out PE18 we observed that in 27 of the 30 patients with suspected or confirmed PE, the sFlt-1/PlGF ratio did not increase during follow-up. In the 3 patients in whom the sFlt-1/PlGF ratio increased during follow-up, the pregnancies were complicated respectively by IUGR, respiratory distress syndrome, and admission to the neonatal intensive care unit, but delivery took place 33, 97 and 91 days after the first blood sampling for determination of the sFlt-1/PlGF ratio.

The evolution of the angiogenic factors in pregnancy has been investigated by a few groups.[21-23]

Schaarschmidt et al. compared the evolution of angiogenic factors in patients with early (<34 weeks) and late onset PE or HELLP syndrome with a maximal follow-up until delivery of 14 days.[21] Compared to women with late onset PE or HELLP syndrome, the increase in sFlt-1 and decrease in PlGF levels per day was 2 to 3 times greater in women with early onset PE or HELLP syndrome, in agreement with the more progressively worsening clinical course of the early onset conditions. In a recent study reported by Baltajian et al. concerning women with suspected PE a subdivision was made between women with and without adverse outcome.[22] They evaluated the evolution of the angiogenic factors in 43 women with an adverse outcome (admission sFlt-1/PlGF ratio 206) and in 57 women without an adverse outcome (admission sFlt-1/PlGF ratio 48). Based on the last minus first measurement the median increase per day in sFlt-1 was almost 3 times higher in women with an adverse outcome, whereas the decrease in PlGF was about 2 times lower, resulting in two-fold faster increase per day in the sFlt-1/PlGF ratio. Using the cut-off value of the sFlt-1/PlGF ratio of ≤38 to rule out PE, comparable results were observed in our study. Combining the results of the two mentioned studies and our study clearly demonstrates that a rise in the absolute sFlt-1 level and an increase in sFlt-1/PlGF ratio are positively associated with the admission values of these parameters (Table 3). The rapid increase of the sFlt-1/PlGF ratio in women with suspected PE already presenting with a higher sFlt-1/PlGF ratio at admission most likely reflects a continuation of the disease process, in line with the observation that removal of sFlt-1 from the maternal circulation is associated with pregnancy prolongation.[24] In addition, abnormalities in uterine artery flow and in angiogenic balance are correlated.[24-25]

The rapid decrease of the highly elevated circulating sFlt-1 level postpartum by >99% in our patients suggests that the placenta is its main source in PE.[6,26] After this initial rapid decline with a halflife of ≈1 day, the sFlt-1 value stabilized at a value around 77 pg/mL, i.e. identical to values reported for healthy non-pregnant women.[19] Of note, the soluble splice variant of the VEGF receptor 1 produced by the placenta (sFlt-1-14) and sFlt-1 present outside pregnancy may not be completely identical due to different splic-

Table 3. The absolute and percentage change of the biomarkers per day according to literature and to our study

	Condition		Absolute change per day	% Change per day
Schaarschmidt et al.	**Early onset PE (n = 13)**			
	Mean sFlt-1 at admission	12095 ± 4799	1034	11
	Mean PlGF at admission	31.9 ± 15.7	1	-4
	Mean ratio at admission	446 ± 240	89	23
	Late onset PE			
	Mean sFlt-1 at admission	10340 ± 3884	312	3
	Mean PlGF at admission	92.5 ± 33.6	1.7	-2
	Mean ratio at admission	120 ± 44	8.2	8
Baltajian et al.	**No adverse outcome**			
	Median sFlt-1 at admission	5595 (2821, 8193)	81.3 (-177.9, 449.0)	2.8 (-2.3, 7.0)
	Median PlGF at admission	125 (65, 266)	-2.4 (-13.9, 2.4)	-1.9 (-5.6, 2.8)
	Median ratio at admission	47.5 (9.7, 87.0)	2.7 (-0.6, 8.3)	7.1 (-2.2, 28.0)
	Adverse outcome			
	Median sFlt-1 at admission	9136 (5724, 12161)	491.0 (120.3, 1587.2)	7.2 (2.0, 13.1)
	Median PlGF at admission	49 (21, 94)	-2.6 (-9.8, 0.2)	-4 (-14.0, 0.6)
	Median ratio at admission	205.9 (72.5 - 453.1)	15.1 (1.8, 58.1)	14.5 (3.2, 38.3)
Saleh et al.	**Ratio ≤38**			
	Median sFlt-1 at admission	2037 (1275, 2991)	21.3 (1.5 - 64)	0.9 (0.1, 1,6)
	Median PlGF at admission	257 (146, 461)	-0.7 (-4.6, 0.4)	-0.7 (-2.0, 0.2)
	Median ratio at admission	8 (4.8, 18.0)	0.08 (-0.02 - 0.8)	0.8 (-0.3, 2.3)
	Ratio >38			
	Median sFlt-1 at admission	7042 (4554 - 8640)	148.8 (-3.6, 407.3)	1.0 (-0.1, 3.82)
	Median PlGF at admission	46 (25, 85)	0.009 (-2.1, 0.9)	0.02 (-5.5, 1.2)
	Median ratio at admission	98 (72, 335)	7.1 (0.7, 15.8)	2.5 (0.5, 4.6)
	Ratio ≤85			
	Median sFlt-1 at admission	2190 (1422 - 3533)	33.6 (3.2, 73.4)	0.9 (0.2, 1.6)
	Median PlGF at admission	221 (106 - 396)	-0.7 (-3.0, 0.4)	-0.7 (-2.3, 0.2)
	Median ratio at admission	10 (5 - 26)	0.2 (0.0, 1.0)	0.9 (0.0, 2.7)
	Ratio >85			
	Median sFlt-1 at admission	7847 (5111 - 11256)	162.5 (-52.7, 485.9)	0.9 (-1.1, 4.3)
	Median PlGF at admission	42 (13 - 51)	0.08 (-1.1, 1.1)	0.7 (-3.5, 2.0)
	Median ratio at admission	260 (99 - 461)	11.6 (-3.2, 18.3)	2.6 (-2.2, 4.3)

ing processes.[27] Contrary to the postpartum decrease in sFlt-1 observed in all patients, PlGF initially increased in some patients before it started to decrease to 1/3 of the values obtained shortly before delivery (Figure 3). This initial rise in PlGF is possibly explained by the rapid and pronounced decrease in sFlt-1, since only free PlGF is measured with the used assay. After the initial change postpartum PlGF stabilized at values around 9 pg/ml, comparable to values reported in healthy nonpregnant women.[19] The faster decline in sFlt-1 compared to PlGF also explains the even shorter half-life of the sFlt-1/PlGF ratio of 0.6±0.7 days.

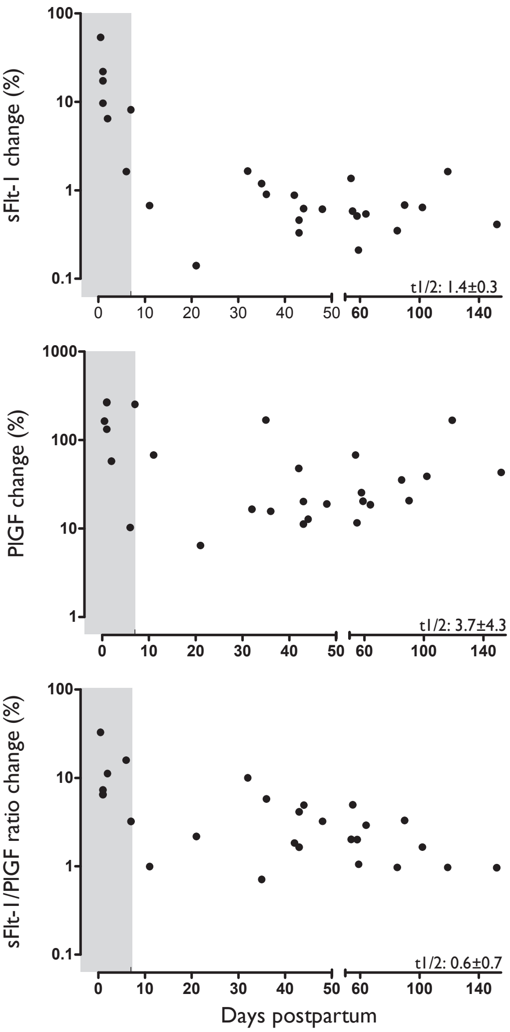

Figure 3. Remaining postpartum levels of sFlt-1 (upper), PlGF (middle) and sFlt-1/PlGF ratio (bottom panel) as a percentage of the last antepartum concentration of sFlt-1, PlGF and sFlt-1/PlGF ratio. The gray area represents week 1 postpartum and the half-lives are calculated over the first week postpartum.

The strength of our study is that it provides information of the evolution of sFlt-1 and PlGF levels over prolonged periods in a well-characterized population of patients with suspected or established PE and of the magnitude and rate of disappearance of these biomarkers after delivery. A limitation is that the markers were not measured at fixed time intervals and that the number of measurements per patient varied, related in part to variation in outpatient visits. In addition, we did not include a control group of healthy pregnant women. As a consequence, our post-delivery data obtained in preeclamptic patients can only be compared with published levels of these 2 biomarkers obtained in age-matched healthy women.

In conclusion, this study shows that in a small proportion of women admitted for evaluation of PE with a low sFlt-1/PlGF ratio at initial assessment, the sFlt-1/PlGF ratio can still increase in subsequent weeks to months, indicating that the sFlt-1/PlGF ratio has to be measured repeatedly. Furthermore, the sFlt-1/PlGF ratio doubles every week in most patients with a high sFlt-1/PlGF ratio of >38 at admission.

Our study also shows in women with PE and high sFlt-1 levels and sFlt-1/PlGF ratios that these parameters rapidly decrease postpartum to values observed in age-matched, healthy, non-pregnant women.

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Chapter 7

The predictive value of the sFlt-1/PlGF ratio on short-term absence of preeclampsia and maternal and fetal or neonatal complications in twin pregnancies

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Submitted

ABSTRACT

Objective A sFlt-1/PlGF ratio of ≤38 has been reported to predict the absence of pre-eclampsia (PE) in singleton pregnancies. We evaluated whether a sFlt-1/PlGF ratio of ≤38 could be used to predict the absence of PE in twin pregnancies and maternal and fetal/neonatal complications.

Methods This is a secondary analysis of a prospective multicenter cohort study that enrolled women with suspected or confirmed PE with the aim of evaluating the use of the sFlt-1, PlGF and their ratio to predict maternal and fetal/neonatal complications. Twin and singleton pregnancies with clinically suspected or confirmed PE were matched for gestational age and parity. Blood samples were drawn at time of study entry, but serum values of sFlt-1 and PlGF and their ratio were determined postpartum.

Results Twenty-one women with twin and 21 with singleton gestations were included at a median gestational age of 30 weeks. At inclusion PE was diagnosed in 13 twin and 15 singleton pregnancies. Compared to singleton control pregnancies sFlt-1 was higher (6377 vs 1732 pg/ml, p=0.008), whereas the sFlt-1/PlGF ratio tended to be higher (26 vs. 3) and PlGF tended to be lower (228 vs. 440 pg/ml) in twin control pregnancies. Compared to singleton preeclamptic pregnancies values of sFlt-1 (9134 vs 8625 pg/ml) were identical, whereas values of PlGF (185 vs. 33 pg/ml, p<0.001) were higher and values of the ratio (49 vs. 158, p=0.002) were lower in preeclamptic twin pregnancies. All preeclamptic patients with a singleton pregnancy had a ratio >38, but only 5 of the 13 patients with a preeclamptic twin pregnancy. Conversely, the ratio was ≤38 in 5 of the 6 control singleton, but in only 4 of the 8 control twin pregnancies. When classified according to a ratio ≤38 or >38 at inclusion, maternal complications occurred more frequently in patients with a ratio >38 both in singleton and twin pregnancies. In singleton pregnancies fetal/neonatal complications, except one admission to NICU, only occurred in patients with a ratio >38. In twin pregnancies fetal/neonatal complications occurred equally frequent in women with a ratio ≤38 or >38.

Conclusion Serum sFlt-1levels are considerably higher in twin than in singleton control gestations. A sFlt-1/PlGF ratio of ≤38 to predict short-term absence of PE is not applicable to twin pregnancies in predicting either the absence of PE or the absence of adverse pregnancy outcomes.

INTRODUCTION

Preeclampsia (PE), complicating 2-8% of all pregnancies, is associated with significant maternal and offspring morbidity and mortality.[11-13] Recent advances in understanding the mechanisms underlying PE suggest an angiogenic imbalance, reflected by elevated placenta-derived soluble Fms-like tyrosine kinase-1 (sFlt-1) and decreased placental growth factor (PlGF) levels in the maternal circulation as a dominant pathophysiological mechanism.[13-14] As a consequence, the measurement of these factors and especially the sFlt-1/PlGF ratio is emerging as a clinically valuable tool not only for diagnosing or excluding PE, but also for the prediction of pregnancy outcome in singleton pregnancies.[6, 15-20]

The Prediction of Short-Term Outcome in Pregnant Women with Suspected PE Study (PROGNOSIS) showed that a sFlt-1/PlGF ratio cut-off of 38 or lower can be used to predict the one-week absence of PE in women in whom the syndrome is suspected clinically.[21] Conversely, women with a sFlt-1/PlGF ratio >38 had a 2.9-fold greater likelihood of imminent delivery.[22]

So far, only a few studies have studied the predictive value of the sFlt-1, PlGF and their ratio in twin pregnancies,[23-30] while twin pregnancies have a 2-3 times higher risk for PE than singleton pregnancies.[31-32] Thus, the urgency of timely diagnosing or excluding PE is even more important in twin pregnancies.

With this study, we attempted to evaluate whether the sFlt-1/PlGF cut off value of ≤38 proposed for singleton pregnancies is also applicable to twin pregnancies in both predicting the one-week absence of PE as well as predicting the absence of adverse pregnancy outcomes.

METHODS

Study design

This is a secondary analysis of a prospective multicenter cohort study that enrolled women with suspected or confirmed PE at three Dutch hospitals (Erasmus MC and Maasstad Hospital in Rotterdam, Reinier de Graaf Hospital in Delft), with the aim of evaluating the use of the sFlt-1, PlGF and their ratio for the prediction of maternal and fetal or neonatal complications.[33] Identical study protocol and data collection forms were used at each center. All subjects provided written informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202).

Inclusion and exclusion criteria

Women had to have PE or clinical symptoms of PE. For the control arm, singleton pregnancies were matched with the twin pregnancies with regard to gestational age and parity. Patients who developed an acute fatty liver or were included postpartum were excluded, as well as pregnancies where fetal death occurred before inclusion.

Definitions and outcome measures

Patients were suspected of PE if they presented with new onset or aggravation of preexisting hypertension or proteinuria, right upper quadrant abdominal pain, severe headaches with visual disturbances, decreased levels of platelets and/or increased liver enzymes.

PE was defined as de novo hypertension (systolic blood pressure (SBP) of ≥ 140 and/or a diastolic blood pressure (DBP) of ≥ 90 mmHg) and substantial proteinuria (PCR ≥ 30 mg/mmol or ≥ 300 mg/24 hours) ≥20 weeks of gestation.[34] Based on the clinical judgement and laboratory measurements, PE was subdivided into PE, superimposed PE, (partial) HELLP syndrome (hemolysis, elevated liver enzymes and low platelet count), or a combination of PE and HELLP. Partial HELLP was diagnosed when two out of the three mentioned criteria of HELLP were deviant. Superimposed PE was diagnosed in women with chronic hypertension with either new onset of proteinuria, sudden increase of blood pressure, appearance of thrombocytopenia and increased liver enzymes, or a sudden increase of proteinuria in patients with pre-existing proteinuria. The group with suspicion of PE, but without gestational hypertension, was defined as *no* hypertensive disease of pregnancy (NHDP).

Maternal complications were defined as the occurrence after admission of (superimposed) preeclampsia , (partial) HELLP, eclampsia, subcapsular hematoma of the liver, pulmonary edema, placental abruption, renal insufficiency, cerebral edema, cerebral hemorrhage, and vision disorders.

Fetal and neonatal complications consisted of admission to the Neonatal Intensive Care Unit (NICU), treatment with endotracheal tube, a birth weight percentile <10, respiratory distress syndrome (RDS), bronchopulmonary dysplasia, sepsis, intracerebral abnormality such as an intracerebral bleeding, and fetal and neonatal death.

Materials

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

Follow up

At the day of study entry blood was sampled and clinical characteristics and routine laboratory data were acquired. After inclusion, the pregnancy course was followed. Pregnancy outcomes were obtained from the medical file of patient and child or children. Patient's follow-up continued until hospital discharge.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 21 was used for analysis. Normal distribution of variables was assessed by the Shapiro Wilk test. For continuous normally distributed data an unpaired t-test was performed and for continuous non-normal distributed data the Mann Whitney U-test. A Fisher's exact test was performed on categorical variables. For analyzing multiple groups, a one-way ANOVA was used for normal and a Kruskal Wallis test for non-normally distributed data, both with a Bonferroni adjustment for the post hoc analysis.

RESULTS

Demographics and characteristics

A total of 40 twin gestations were enrolled (Figure 1), of which 21 were eligible for analysis. Nineteen patients were excluded for the following reasons: inclusion at a gestational age of 15 weeks (n=1), postpartum inclusion (n=3), death of one fetus before inclusion (n=2), development of acute fatty liver (n=5), loss of follow-up (n=2), lack of laboratory measurements (n=5), and the development of a complete mola next to a normal fetus. This latter patient was excluded in view of knowledge that a molar pregnancy affects the values of the (anti-)angiogenic factors.[35-37]

The outcome of the 21 eligible twin pregnancies was compared with the outcome of 21 singleton pregnancies matched for gestational age and parity.

Baseline characteristics and laboratory findings according to the clinical diagnosis at time of study entry are shown in Table 1. Maternal age of twin gestations with PE was higher than of singleton gestations with PE. In twin pregnancies without PE at presentation the sFlt-1 level was significantly higher ($p = 0.008$), PlGF level tended to be lower and the ratio tended to be higher than in singleton pregnancies without PE. When comparing PE to non PE pregnancies in singletons, sFlt-1 levels ($p = 0.005$) and the ratio ($p < 0.001$) were higher, and PlGF lower ($p<0.001$), whereas in twins no significant differences in biomarkers were observed between PE and non PE pregnancies. When comparing twin PE with singleton PE pregnancies, sFlt-1 levels did not differ, PlGF levels were higher ($p<0.001$) and the sFlt-1/PlGF ratio was lower ($p = 0.001$). A ratio >38 was

Table 1. Baseline characteristics of patients and angiogenic serum markers at time of inclusion classified according to clinical diagnosis

	Singleton pregnancies		Twin pregnancies	
	Suspected PE	**Preeclampsia**	**Suspected PE**	**Preeclampsia**
N	6	15	8	13
Age (years)	35 (18 – 39)	29 (20 – 47)	32 (23 – 46)	36 (31 – 44)[4]
Gestational age (weeks)	31 (24 – 34)	29 (24 – 34)	29 (23 – 34)	30 (26 – 34)
Ethnicity				
White (n, %)	4 (67)	13 (87)	5 (63)	13 (100)
Black (n, %)	2 (33)	2 (13)	3 (38)	-
Gestational diabetes (*n, %)*	-	2 (13)	2 (25)	-
Preexisting proteinuria (*n, %)*	-	-	2 (25)	-
Preexisting hypertension (*n, %)*	1 (17)	3 (20)	3 (38)	-
Antihypertensive drug use (*n, %)*	5 (83)	14 (93)	6 (75)	10 (77)
Clinical findings				
Systolic blood pressure (mmHg)	140 (130 – 150)	145 (130 – 220)	141 (130 – 160)	150 (130 – 186)
Diastolic blood pressure (mmHg)	80 (70 – 95)	95 (90 – 135)[1]	90 (75 – 95)	90 (79 – 112)
Protein to creatinine ratio (mg/mmol)	13 (11 – 29)	98 (30 – 1583)[1]	15 (11 – 119)	49 (33 – 576)[2]
Creatinine (μmol/L)	55 (45 – 74)	65 (42 – 109)	51 (46 – 112)	56 (45 – 87)
Uric Acid (mmol/L)	0.21 (0.17 – 0.24)	0.33 (0.22 – 0.52)[1]	0.24 (0.19 – 0.58)	0.34 (0.24 – 0.42)
Lactate dehydrogenase (U/L)	182 (162 – 227)	212 (174 – 346)	189 (146 – 254)	202 (122 – 314)
Alanine transaminase (U/L)	11 (9 – 20)	22 (8 – 73)[1]	14 (6 – 30)	17 (10 – 110)
Platelet Count (10^9/L)	242 (202 – 304)	223 (148 – 297)	211 (148 – 274)	178 (107 – 300)
sFlt-1 (pg/mL)	1732 (836 – 6306)	8625 (1410 – 19243)[1]	6377 (2500 – 11390)[3]	9134 (1010 – 31025)
PlGF (pg/mL)	440 (97 – 883)	33 (11 – 173)[1]	228 (147 – 893)	185 (75 – 361)[4]
sFlt-1/PlGF ratio	3 (1 – 65)	158 (39 – 1605)[1]	26 (3 – 67)	49 (4 – 185)[4]
sFlt-1/PlGF ratio ≤38 (*n, %)*	5 (83)	-	4 (50)	5 (39)

Values are median (range) or number (%).The suspected PE group consists of the no hypertensive disease of pregnancy and gestational hypertension group. The preeclampsia group consists of women with (superimposed) preeclampsia and (partial) HELLP syndrome. [1] $p<0.05$ for comparison of singleton pregnancies with and without PE; [2] $p<0.05$ for comparison of twin pregnancies with and without PE; [3] $p<0.05$ fo rcomparison of singleton and twin pregnancies without PE; [4] $p<0.05$ for comparison of singletons and twin pregnancies with PE.

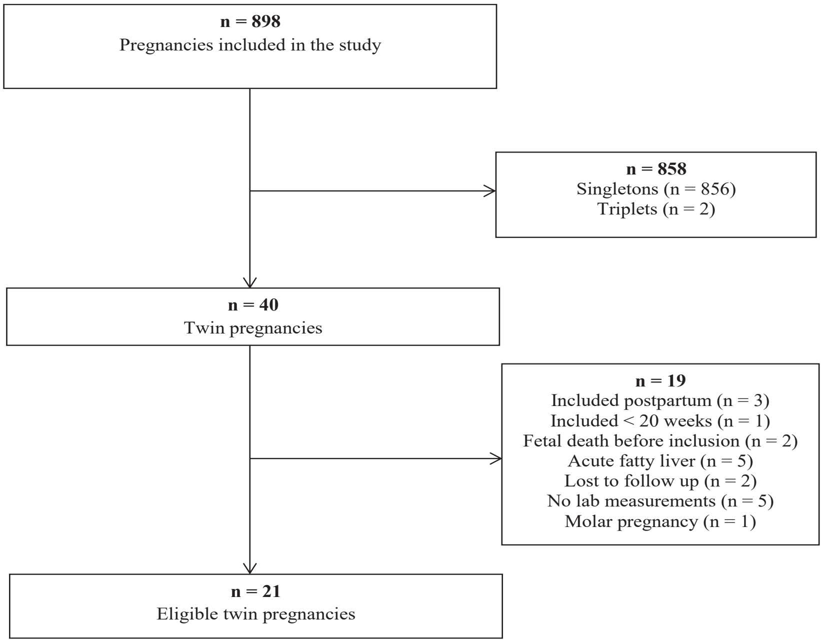

Figure 1. Flowchart.

present in all 15 singleton pregnancies with PE, but in only 8 of the 13 twin pregnancies with PE. Conversely, in 4 of the 8 twin pregnancies without PE the ratio was higher than 38 while one out of the 6 singleton non-PE pregnancies had a ratio >38. Maternal fetal/neonatal outcomes classified according to a ratio below or above 38 in singleton and twin pregnancies are provided in Table 2. Singletons with a ratio >38 had a significantly (p = 0.004) lower gestational age at delivery in comparison with singletons with a ratio ≤ 38. No significant difference in gestational age at delivery was observed in twin pregnancies with a ratio ≤38 or >38. Except for one superimposed PE, which occurred 8 weeks after inclusion, maternal complications did not occur in singleton pregnancies with a ratio ≤38 at inclusion, but were observed in 5 patients (31%) with a ratio >38. In twin pregnancies maternal complications occurred in 4 of the 12 patients (33%) with a ratio >38 at inclusion. With the exception of one NICU admission, not related to PE, fetal/neonatal complications were absent in singleton pregnancies with a ratio ≤ 38, but were commonly observed in singleton pregnancies with a ratio >38, most likely due to prematurity and low birth weight. (Table 2) In twin pregnancies, no difference in adverse fetal/neonatal outcomes were observed between patients with a ratio ≤38 or >38. Finally, delivery within 7 days related to PE did not occur in any of the 5 singleton pregnancies with a ratio ≤38 but in 4 of the 9 twin pregnancies with a ratio <38. Furthermore, no singleton pregnancies with a ratio < 38 developed PE within 7 days while 5 out

Table 2. Maternal and fetal/neonatal outcomes, classified according to sFlt-1/PlGF ratio ≤ 38 or > 38.

	Singleton pregnancies		Twin pregnancies			
	Ratio ≤ 38	**Ratio > 38**	**Ratio ≤ 38**		**Ratio > 38**	
N	5	16	9		12	
Gestation age at inclusion (weeks)	32 (24 – 34)	29 (24 – 34)	29 (23 – 34)		30 (24 – 34)	
Gestation age at delivery (weeks)	37 (34 – 39)	30 (25 – 37)[1]	35 (26 – 37)		33 (30 – 37)	
Prematurity	2 (40)	14 (88)	7 (78)		11 (92)	
< 34 weeks (n, %)	-	12 (75)	3 (33)		7 (58)	
34 – 37 weeks (n, %)	2 (40)	2 (13)	4 (44)		4 (33)	
			Twin 1	**Twin 2**	**Twin 1**	**Twin 2**
Gender (M:F, %)	1:4 (20/80)	8:8 (50/50)	2:7 (22/78)	3:6 (33/67)	4:8 (33/67)	3:9 (25/75)
Birth weight (grams)	3080 (2570 – 3995)	1329 (515 – 3790)[1]	1953 (810 – 3085)[3]	2185 (550 – 2585)[3]	1570 (1220 – 2305)[2,4]	1625 (1170 – 2755)[2,4]
Clinical findings						
Systolic blood pressure (mmHg)	140 (130 – 150)	145 (130 – 220)	145 (130 – 186)		146 (130 – 176)	
Diastolic blood pressure (mmHg)	75 (70 – 95)	95 (85 – 135)[1]	90 (80 – 112)		90 (75 – 96)	
Protein to creatinine ratio (mg/mmol)	13 (11 – 29)	85 (14 – 1583)[1]	38 (13 – 576)[3]		36 (11 – 171)[4]	
Creatinine (μmol/L)	51 (45 – 68)	66 (42 – 109)	59 (46 – 87)		53 (45 – 112)	
Uric Acid (mmol/L)	0.21 (0.17 – 0.24)	0.33 (0.21 – 0.52)	0.28 (0.23 – 042)		0.30 (0.19 – 0.58)	
Lactate dehydrogenase (U/L)	174 (162 – 192)	220 (174 – 346)[1]	199 (167 – 314)[3]		206 (122 – 249)[4]	
Alanine transaminase (U/L)	12 (9 – 20)	22 (8 – 73)	14 (6 – 35)		14 (10 – 110)	
Platelet Count (10^9/L)	253 (237 – 304)	219 (148 – 297)	183 (107 – 273)[3]		180 (107 – 300)	

Table 2. (*continued*)

	Singleton pregnancies		Twin pregnancies			
	Ratio ≤ 38	**Ratio > 38**	**Ratio ≤ 38**		**Ratio > 38**	
Maternal complications (n, %)						
(superimposed) preeclampsia	1(20)	2 (13)	-		2 (17)	
(partial) HELLP syndrome	-	1 (6)	-		1 (8)	
Renal insufficiency	-	1 (6)	-		-	
Pulmonary edema	-	1 (6)	-		-	
Vision disorders	-	-			1 (8)	
			Twin 1	**Twin 2**	**Twin 1**	**Twin 2**
			-	1(11)	-	-
			-	-	2(17)	-
			8 (89)	5 (56)	9 (75)	9 (75)
Fetal/neonatal complications (n, %)			2 (22)	1 (11)	-	-
Fetal death	-	1(6)	3 (33)	2 (22)	5 (42)	2 (17)
Neonatal death	-	1(6)	3 (30)	2 (20)	6 (50)	4 (33)
Admission to NICU	1(20)	13(81)	1 (11)	1 (11)	-	-
Endotracheal tube	-	6 (38)	1 (11)	1 (11)	-	-
Birth percentile < 10	-	3 (19)	1 (11)	-	3 (25)	-
Respiratory distress syndrome	-	9 (56)				
Bronchopulmonary disease	-	2 (13)				
Sepsis	-	5 (32)				
Intracerebral abnormality	-	6 (38)				

Values are median (range), number (%).

[1] $p<0.05$ for comparison of singleton and twin pregnancies with a ratio ≤38 vs. >38; [2] $p<0.05$ for comparison of twin pregnancies with a ratio ≤38 vs. >38; [3] $p<0.05$ for comparison of singleton and twins pregnancies with a ratio ≤38; [4] $p<0.05$ for comparison of singleton and twin pregnancies with ratio >38.

of the 9 twins who were suspected of PE at inclusion, developed PE within 7 days (data not shown).

DISCUSSION

A sFlt-1/PlGF ratio of 38 or lower has been reported to rule out PE in women with singleton pregnancies in whom the syndrome of PE is clinically suspected.[21] We examined whether this cut-off value is also applicable to twin pregnancies in women with suspected PE on clinical grounds, and to assess whether the ≤38 cut-off could be used to predict absence of adverse pregnancy outcomes in twin pregnancies. We observed that in singleton pregnancies and a ratio ≤38, no patient was diagnosed with PE either at initial evaluation or during a follow-up period of 7 days, confirming the accuracy of this cut-off value to rule out PE. The situation was different for twin pregnancies. Five of the 13 patients in this group clinically diagnosed with PE had a ratio ≤38. This finding strongly suggests that the proposed cut-off value of the ratio of ≤38 to rule out PE established for singleton pregnancies is not applicable to twin pregnancies.

A striking finding in our study was the almost four-fold higher value of the sFlt-1 level in non-preeclamptic twin compared to non-preeclamptic singleton gestations. Related to this relatively high sFlt-1 levels, the level of free PlGF, which is sequestered by sFlt-1, tended to be lower in non-preeclamptic twin than in singleton gestations, while the sFlt-1/PlGF ratio tended to be higher. Other studies have compared the circulating levels of angiostatic and angiogenic factors in singleton versus multiple gestation pregnancies.[6], [27-30] All these studies reported higher circulating sFlt-1 values in twin than in singleton gestations. These higher circulating sFlt-1 levels are most likely due to the increased placental mass in twin pregnancies. Nevertheless, they could play a role in the increased risk of PE in twin pregnancies.[30, 38]

In the current study circulating sFlt-1 levels in preeclamptic twin pregnancies did not differ from those in preeclamptic singleton pregnancies, whereas the circulating PlGF levels were considerably higher and the sFlt-1/PlGF ratio's considerably lower. Notwithstanding this higher PlGF level and lower ratio in twin pregnancies the severity of PE as reflected by blood pressure levels, degree of proteinuria and elevated serum uric acid levels, between twin and singleton preeclamptic pregnancies were identical. Another study by Dröge et al. comparing angiogenic markers between twin and singleton preeclamptic pregnancies reported that serum levels of sFlt-1 were almost two-fold and serum levels of PlGF alsmost three-fold higher in twin preeclamptic pregnancies, but no difference in the sFlt-1/PlGF ratio between twin and singleton gestations with PE was observed.[29] Expressed as multiples of the median, Boucoiron et al. reported 1.7-fold higher sFlt-1 and two-fold lower PlGF levels in twin versus singleton pregnancies who were eventually complicated by PE.[27] In that study blood for determination of the angiogenic markers was sampled at 24-26 weeks of gestation, which is much earlier than in our study or the study of Dröge et al.

When classifying our patients according to a ratio below or equal to 38 or above 38 at inclusion, maternal complications like superimposed preeclampsia and HELPP syndrome occurred more frequently in patients with a ratio above 38 both in women with singleton and twin pregnancies, confirming the association between angiogenic imbalance and maternal complications. Fetal/neonatal complications, except one admission to the NICU, were not observed in singleton pregnancies with a ratio <38 at inclusion. The latter neonate was delivered at 34 weeks because of a maternal tachycardia. The mother had no preeclampsia. In contrast, fetal/neonatal complications were frequently observed in singleton pregnancies with a ratio >38. This higher rate of complications can largely be attributed to the prematurity and low birth weight of most of the neonates. In contrast, the occurrence of fetal/neonatal complications in twin pregnancies with a ratio below or above 38 was almost identical. This absent difference in complications may be explained by the fact that most twins were born prematurely, irrespective of the ratio, posing them to an increased complication risk.

Our study has limitations. The number of twin pregnancies with and without PE was relatively small. Larger studies are required to find out whether the present findings are generalizable. Notably, previous studies already concluded that reference ranges of sFlt-1, PlGF and their ratio to exclude or diagnose PE obtained in singleton pregnancies may not be transferable to twin pregnancies.[29] Also, the markers were measured only once at different time points of gestation for different patients. Insight into the evolution of the markers in relation to the gestational age may provide additional information.

In conclusion this study confirms previous findings that circulating sFlt-1 levels are considerably higher in twin than in singleton pregnancies. It further shows that the cut-off value of the ratio below or equal to 38 suggested for singleton pregnancies, does not exclude the presence of PE in a substantial of twin pregnancies and therefore cannot be applied in this condition.

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Chapter 8

Angiogenic markers predict pregnancy complications and prolongation in preeclampsia continuous versus cutoff values

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ABSTRACT

To assess the incremental value of a single determination of the serum levels of soluble Fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF) or their ratio, without using cutoff values, for the prediction of maternal and fetal/neonatal complications and pregnancy prolongation, 620 women with suspected/confirmed preeclampsia, aged 18 to 48 years, were included in a prospective, multicenter, observational cohort study. Women had singleton pregnancies and a median pregnancy duration of 34 (range, 20–41) weeks. Complications occurred in 118 women and 248 fetuses. The median duration between admission and delivery was 12 days. To predict prolongation, PlGF showed the highest incremental value (R^2=0.72) on top of traditional predictors (gestational age at inclusion, diastolic blood pressure, proteinuria, creatinine, uric acid, alanine transaminase, lactate dehydrogenase, and platelets) compared with R^2=0.53 for the traditional predictors only. sFlt-1 showed the highest value to discriminate women with and without maternal complications (C-index=0.83 versus 0.72 for the traditional predictors only), and the sFlt-1/PlGF ratio showed the highest value to discriminate fetal/neonatal complications (C-index=0.86 versus 0.78 for the traditional predictors only). Applying previously suggested cutoff values for the sFlt-1/PlGF ratio yielded lower incremental values than applying continuous values. In conclusion, sFlt-1 and PlGF are strong and independent predictors for days until delivery along with maternal and fetal/neonatal complications on top of the traditional criteria. Their use as continuous variables (instead of applying cutoff values for different gestational ages) should now be tested in a prospective manner, making use of an algorithm calculating the risk of an individual woman with suspected/confirmed preeclampsia to develop complications.

INTRODUCTION

Preeclampsia, a multisystem disorder unique to pregnancy, is a serious and potentially life-threatening condition complicating 2% to 5% of all pregnancies.[1] The current management of patients suspected of preeclampsia is often increased monitoring and hospital admission because of the risk of liver, lung, and kidney damages, eclampsia, prematurity, intrauterine growth restriction, and maternal and fetal demise, which is also accompanied by the immense cost to society.[2,3] Although the classic diagnosis of preeclampsia is clearly defined as de novo hypertension and proteinuria in the second half of pregnancy, the clinical presentation and course vary considerably. A significant proportion of women develop preeclampsia-related complications, among others eclampsia and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, in the absence of hypertension or proteinuria.[4] However, pre-existing autoimmune and kidney disease can mimic the features of preeclampsia. Preeclampsia is characterized by an angiogenic imbalance, reflected by elevated placenta-derived soluble Fmslike tyrosine kinase 1 (sFlt-1) and decreased placental growth factor (PlGF) levels in the maternal circulation.[5–8] The measurement of these factors and especially the sFlt-1/PlGF ratio is emerging as a clinically valuable diagnostic tool not only for diagnosing or excluding preeclampsia but also for the prediction of pregnancy outcome.[9–11] Several groups have suggested various cutoff values[12–14] to predict the absence or presence of preeclampsia. The angiogenic biomarkers have also been compared with receiver-operating characteristic curve analysis with traditional laboratory variables. However, information about the value of the new biomarkers on top of simple clinical assessment and routine laboratory variables for predicting pregnancy outcome is still limited.[13,15,16] Recent studies that included sFlt-1, PlGF, or the sFlt-1/PlGF ratio on top of a model with traditional predictors[17–19] consistently restricted their outcome to the diagnosis of preeclampsia while from a clinical perspective, a more relevant question is how to identify patients at risk for adverse maternal and perinatal outcomes.

In the present study, we assessed the incremental value of sFlt-1, PlGF, and their ratio for the prediction of maternal and fetal/neonatal complications and pregnancy prolongation in a high-complication risk cohort of women with suspected or with confirmed preeclampsia. We explicitly chose to analyze the continuous values of the biomarkers rather than dichotomized values in previous studies to use as much information as possible.

METHODS

Study design

Women with singleton pregnancies from varied ethnic background with suspicion/confirmed preeclampsia were recruited into this prospective multicenter cohort study from December 2013 through April 2016 at 3 Dutch hospitals (Erasmus MC and Maasstad Hospital in Rotterdam, Reinier de Graaf Hospital in Delft). Identical study protocol and data collection forms were used at each center. All subjects 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 were singleton pregnant women who were either suspected of preeclampsia or with a confirmed diagnosis of preeclampsia from gestational age (GA) of ≥20 weeks admitted to the obstetric departments of participating hospitals. Patients were included if the treating physician deemed an evaluation of preeclampsia necessary. Indication for evaluation included: elevated blood pressure or proteinuria or symptoms associated with preeclampsia, such as right upper quadrant abdominal pain or headache with visual disturbances. Women with multiple pregnancy, chromosomal/fetal anomalies, and GA <20 weeks were excluded.

Diagnosis

Preeclampsia was defined according to the International Society for the Study of Hypertension as de novo hypertension (systolic blood pressure of ≥140 or diastolic blood pressure [DBP] of ≥90 mm Hg) and proteinuria (protein-to-creatinine ratio ≥30 mg/mmol or ≥300 mg/24 hours or 2+ dipstick) at or after 20 weeks of pregnancy.[20] The preeclampsia group was further divided into preeclampsia, superimposed preeclampsia, and HELLP syndrome. Superimposed preeclampsia 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. The group with only suspicion of preeclampsia, but without gestational hypertension, was defined as no hypertensive disease of pregnancy.

Outcome measures

Maternal complications were defined as eclampsia, development of (superimposed) preeclampsia or the HELLP syndrome after inclusion in to the study, 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). All patients diagnosed with superimposed preeclampsia, preeclampsia, or HELLP at initial inclusion (n=213) were excluded from the calculation of maternal complications.

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; 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 of inflammation and scarring, in need of artificial ventilation 4 weeks post-partum and at 36 weeks postmenstrual age; respiratory distress syndrome; necrotizing enterocolitis; intraventricular hemorrhage; periventricular leukomalacia and posthemorrhagic ventricular dilatation. All patients (n=620) were used for the calculation of fetal/neonatal complications.

Days until delivery was defined as days from study inclusion and blood sampling until delivery. Because pregnancies complicated with preeclampsia beyond 37 weeks' gestation (ie, term) are delivered to initiate resolution of preeclampsia, the additive value of the biomarkers was only calculated for women included to the study <37 weeks' gestation (n=422). The predictive and additive value of the biomarkers was also calculated for women included before 34 weeks of gestation at blood draw (n=254) in view of reports that with advancing gestation sFlt-1 and PlGF values in preeclamptic and healthy pregnancies tend to converge.[21–23]

Clinical findings, physical examination, laboratory test results, maternal and fetal/neonatal complications (diagnosed by the treating physicians), and background data were obtained from patient's electronic medical records and ascertained by three independent researchers.

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. Blood was drawn at inclusion, but values of sFlt-1 and PlGF were measured post-partum to prevent any influence of this information on decision making of the treating physicians.

Statistical analysis

Logistic regression analysis was used to study the association between the dichotomous outcomes (maternal and fetal/neonatal complications) and the novel biomarkers, traditional predictors, and traditional laboratory variables. Linear regression analysis was used for the continuous outcome number of days until delivery. The associations of the biomarkers with the outcomes were studied with restricted cubic spline functions and shown in graphs.

Traditional predictors concerned GA, parity, proteinuria (proteinto-creatinine ratio, 24-hour urine collection, or dipstick test), and DBP (model 1). Systolic blood pressure was not considered because of its high correlation with DBP. Indeed, a model with systolic blood pressure rather than DBP gave a similar fit. Traditional laboratory variables included serum concentrations of alanine aminotransaminase, creatinine, uric acid, lactate dehydrogenase, and platelets, and these were added to model 1 (model 2). sFlt-1, PlGF, or the sFtl-1/ PlGF ratio were either alone or in combination added to model 1 or 2 to assess their incremental value. Furthermore, the incremental value of dichotomous variants of the sFtl-1/PlGF ratio was assessed based on previous suggested cutoff values,[13] ≤38 or >85. Models were compared using concordance (c)-statistic for the dichotomous outcomes and R^2 for the continuous outcome. We used SPSS Statistics 21 (IBM Corporation) and R Software for the statistical analysis.

RESULTS

From January 2014 to June 2016, 620 pregnant women (age 18–48 years) were included (Table 1). GA was <34 weeks in 254 (41%) and between 34 and 37 weeks in 168 (27%) women. At time of inclusion, 288 (46%) women were suspected of preeclampsia but had no hypertensive disease of pregnancy, 118 (19%) were diagnosed with gestational hypertension, and 213 (34%) were diagnosed with (superimposed) preeclampsia/HELLP (Table 1). In total, 136 maternal complications occurred in 123 (20%) women, and 245 (40%) of the pregnancies had fetal/neonatal complications (n=506, ie, often >1 complication per patient; Table 2).

Maternal complications

Higher values of sFlt-1 and sFlt-1/PlGF ratio were linearly associated with a higher risk of maternal complications (Figure). Higher values of PlGF were associated with lower maternal risk, with low risks from 300 pg/mL onwards. All 3 markers had clear discriminative ability with C-indices between 0.73 and 0.78 in univariable analysis (Table 3). When sFlt-1, PlGF, or sFlt-1/PlGF ratio were added to a model with traditional predictors and laboratory variables (model 2), the C-index increased from 0.73 for a model without

Table 1. Characteristics of the 620 Women in Our Study

Parameter	All Patients
Age, y	31 (27–35)
Gestational age, n (%)	
<34	254 (41)
34–37	168 (27)
≥37	198 (32)
Nulliparous, n (%)	352 (57)
Current smoker, n (%)	39 (6)
Race, n (%)	
White	428 (69)
Black	99 (16)
Other	93 (15)
History of preeclampsia, n (%)	91 (15)
Pre-existing hypertension, n (%)	128 (21)
Pre-existing proteinuria, n (%)	25 (4)
Clinical findings at time of admission	
Systolic blood pressure, mm Hg	139 (127–147)
Diastolic blood pressure, mm Hg	88 (80–95)
Protein-to-creatinine ratio, mg/mmol	28 (14–72)
Lactate dehydrogenase, U/L	189 (164–226)
Alanine transaminase, U/L	15 (11–22)
Creatinine, μmol/L	58 (51–66)
Uric acid, mmol/L	0.29 (0.24–0.35)
Platelet count, 10^9/L	226 (178–273)
sFlt-1, pg/mL	3812 (1951–7215)
PlGF, pg/mL	128 (68–257)
sFlt-1/PlGF ratio	31 (9–84)
Diagnosis at inclusion, n (%)	
No hypertensive disease of pregnancy	288 (46)
Gestational hypertension	119 (19)
Preeclampsia	123 (20)
Superimposed preeclampsia	49 (8)
(partial) HELLP syndrome	17 (3)
Preeclampsia and HELLP	24 (4)

Values are median (interquartile range). HELLP indicates hemolysis, elevated liver enzymes, and low platelets; PlGF, placental growth factor; and sFlt-1, soluble Fms-like tyrosine kinase 1.

biomarkers to 0.83, 0.79, and 0.82 respectively. This indicates the incremental value of the biomarkers. If 2 of the 3 angiogenic parameters (sFlt-1, PlGF, and their ratio) were added together to model 2, the C-index increased to 0.83 in all combinations (ie, to a

Table 2. Pregnancy outcomes and occurrence of adverse maternal and fetal/neonatal outcomes, n (%) unless otherwise stated

Parameter	All Patients 620 (100)	Ratio ≤38 338 (55)	Ratio >38 282 (45)
Gestational age at birth (wk, d)	37.4 (36.0–39.1)	38 (37–39)	37 (30–38)
Prematurity			
<34	115 (19)	21 (6)	94 (33)
34–37	79 (13)	32 (9)	47 (17)
Girls	299 (48)	162 (48)	137 (49)
Birth weight, g*	3023 (2214–3512)	3235 (2833–3595)	2570 (1203–3243)
Days until delivery*	12 (3–27)	20 (7–43)	5 (2–15)
Hospitalized, d*	4 (2–8)	3 (2–6)	6 (3–11)
Maternal complications in all patients			
Eclampsia	1 (0.2)	…	1 (0.4)
(superimposed) preeclampsia	45 (7.3)	12 (3.6)	33 (11.7)
(partial) HELLP syndrome	28 (4.5)	4 (1.2)	24 (8.5)
Placental abruption	3 (0.5)	…	3 (1.1)
Pulmonary edema	7 (1.1)	1 (0.3)	6 (2.1)
Renal insufficiency	3 (0.5)	1 (0.3)	2 (0.7)
Visual disturbances	3 (0.5)	…	3 (1.1)
Postpartum hemorrhage	46 (7.4)	12 (3.6)	34 (12.1)
Fetal/neonatal complications			
Admission to neonatal intensive care		53 (15.7)	118 (41.8)
Endotracheal tube	48 (7.7)	8 (2.4)	40 (14.2)
Intracranial hemorrhage	3 (0.5)	1 (0.3)	2 (0.7)
Other intracerebral anomalies†	7 (1.1)	…	7 (2.5)
Birth weight percentile <10	94 (15.2)	28 (8.3)	66 (23.4)
Posthemorrhagic ventricular dilatation	2 (0.3)	…	2 (0.7)
Respiratory distress syndrome	82 (13.2)	10 (3.0)	72 (25.5)
Sepsis	50 (8.1)	6 (1.8)	44 (15.6)
Spontaneous intestinal perforation	1 (0.2)	…	1 (0.4)
Periventricular leukomalacia	3 (0.5)	…	3 (1.1)
Bronchopulmonary dysplasia	15 (2.4)	…	15 (5.3)
Intraventricular hemorrhage	6 (1.0)	…	6 (2.1)
Necrotizing enterocolitis	2 (0.3)	…	2 (0.7)
Death	21 (3.4)	2 (0.6)	19 (6.7)
Fetal	14 (2.3)	2 (0.6)	12 (4.3)
Neonatal	7 (1.1)	…	7 (2.5)

Values are n (percentage) or *median (interquartile range); †other intracerebral complications include stroke, cysts, developmental anomalies, meningitis, and vasculopathy. HELLP indicates hemolysis, elevated liver enzymes, and low platelets; PlGF, placental growth

Table 3. Association between maternal complications and sFlt-1, PlGF and sFlt-1/PlGF ratio in women suspected of preeclampsia (n=407)

	Univariable		Multivariable	
Model/Biomarker	Odds Ratio	C-Index	Odds Ratio	C-Index
All maternal complications				
Traditional model 1				0.59
Traditional model 2				0.73
sFlt-1	5 (3–11)	0.76	9 (4–20)	0.83
PlGF	4 (3–7)	0.73	3 (2–6)	0.79
sFlt-1/PlGF ratio	6 (3–12)	0.78	7 (3–16)	0.82
sFl-1/PlGF ratio ≤38	8 (5–14)	0.74	7 (4–14)	0.81
sFlt-1/PlGF ratio >85	7 (4–13)	0.65	5 (2–10)	0.76
Maternal complications*				
Traditional model 1				0.66
Traditional model 2				0.78
sFlt-1	4 (2–8)	0.73	11 (4–29)	0.86
PlGF	7 (4–14)	0.77	6 (3–14)	0.86
sFlt-1/PlGF ratio	4 (2–9)	0.76	11 (4–30)	0.87
sFl-1/PlGF ratio ≤38	8 (4–16)	0.74	11 (5–27)	0.85
sFlt-1/PlGF ratio >85	9 (5–18)	0.66	9 (4–19)	0.83

Traditional model 1 consists of gestational age, parity, diastolic blood pressure, and proteinuria; Traditional model 2 consists of model 1 plus serum concentrations of creatinine, uric acid, alanine transaminase, lactate dehydrogenase, and platelets. Multivariable includes the traditional predictors with either sFlt-1, PlGF, sFtl-1/PlGF ratio, sFtl-1/PlGF ratio ≤38, or sFtl-1/PlGF ratio >85. PlGF indicates placental growth factor; and sFlt-1, soluble Fms-like tyrosine kinase 1.

*Exclusion of postpartum hemorrhage as a complication. Interquartile odds ratio was calculated to aid interpretation of continuous predictors. It is defined as the ratio of the odds of a maternal complication for the 75th centile and the odds of a maternal complication for the 25th centile of the predictor.

value identical to that achieved by adding sFlt-1 alone). The incremental value of the dichotomized values for the ratio cutoffs ≤38 or >85 was lower than for the continuous values (C-indices of 0.81 and 0.76, respectively).

After exclusion of postpartum hemorrhage as a maternal complication, all 3 markers still had clear discriminative ability with C-indices between 0.73 and 0.77 in univariable analysis. When the markers were added to model 2, the C-index increased from 0.78 to 0.86 or 0.87. This again illustrates the incremental value of the biomarkers. The highest C-index (0.88) was observed for model 2 plus 2 of the 3 angiogenic parameters (sFlt-1, PlGF, and their ratio). The incremental value of the dichotomized values for the ratio cutoffs ≤38 or >85 was lower than for the continuous value (C-indices of 0.85 and 0.83, respectively).

Fetal/neonatal complications

Higher values of sFlt-1 and sFlt-1/PlGF ratio were linearly associated with higher risk of fetal/neonatal complications (Figure). Higher values of PlGF were associated with lower fetal/neonatal risk. Univariate analysis revealed that both PlGF and sFlt-1/PlGF ratio had clear discriminative ability with C-indices of 0.78 and 0.75, respectively (Table 4). Addition of sFlt-1, PlGF, or sFlt-1/PlGF ratio to a model with traditional predictors and laboratory variables (model 2) increased the C-index from 0.78 for a model without biomarkers to 0.85, 0.86, and 0.86, respectively. This indicates the incremental value of the biomarkers. If 2 of the 3 angiogenic parameters (sFlt-1, PlGF, and their ratio) were added together to model 2, the C-index increased to 0.88 in all combinations (ie, to a value higher than that achieved by any parameter alone). The incremental value of the dichotomized values for the ratio cutoffs ≤38 or >85 was lower than for the continuous value (C-indices in both cases 0.80).

Table 4. Association between fetal/neonatal complications and sFlt-1, PlGF and sFlt-1/PlGF ratio in all patients (n = 620)

	Univariable		Multivariable Model	
Model/Biomarker	Odds Ratio	C-Index	Odds Ratio	C-Index
Traditional model 1				0.75
Traditional model 2				0.78
sFlt-1	3 (2–4)	0.66	4 (3–6)	0.85
PlGF	10 (7–14)	0.78	7 (4–11)	0.86
sFlt-1/PlGF ratio	3 (2–4)	0.75	4 (2–6)	0.86
sFl-1/PlGF ratio ≤38	4 (3–6)	0.67	3 (2–5)	0.80
sFlt-1/PlGF ratio >85	8 (5–13)	0.69	5 (3–8)	0.80

Traditional model 1 consists of gestational age, parity, diastolic blood pressure, and proteinuria; Traditional model 2 consists of model 1 plus serum concentrations of creatinine, uric acid, alanine transaminase, lactate dehydrogenase, and platelets; Multivariable includes the traditional predictors with either sFlt-1, PlGF, sFtl-1/PlGF ratio, sFtl-1/PlGF ratio ≤38, or sFtl-1/PlGF ratio >85. Interquartile odds ratio was calculated to aid interpretation of continuous predictors. It is defined as the ratio of the odds of a maternal complication for the 75th centile and the odds of a maternal complication for the 25th centile of the predictor. PlGF indicates placental growth factor; and sFlt-1, soluble Fms-like tyrosine kinase 1.

Days until delivery

Higher values of sFlt-1 and sFlt-1/PlGF ratio were linearly associated with higher risk of early delivery (Figure). Higher values of PlGF were associated with lower risk of early delivery. All 3 markers only modestly predicted days until delivery with R^2 between 0.22 and 0.33 in univariable analysis (Table 5). When sFlt-1, PlGF, or sFlt-1/PlGF ratio were added to a model with traditional predictors and laboratory variables (model 2), the R^2 increased from 0.53 for a model without biomarkers to 0.60, 0.72, and 0.71, respectively.

Table 5. Association between days until delivery and sFlt-1, PlGF and sFlt-1/PlGF ratio

	Univariable		Multivariable	
Model/Biomarker	β (95% CI)	*R*2	β (95% CI)	*R*2
GA at inclusion <37 wk (n=422)				
Traditional model 1				0.39
Traditional model 2				0.53
sFlt-1, pg/mL	−22 (−24 to −20)	0.26	−44 (−46 to −42)	0.60
PlGF, pg/mL	19 (17 to 21)	0.22	32 (30 to 34)	0.72
sFlt-1/PlGF ratio	−25 (−27 to −24)	0.33	−26 (−27 to −25)	0.71
sFlt-1/PlGF ratio ≤38		0.25		0.65
sFlt-1/PlGF ratio >85		0.19		0.64
GA at inclusion <34 wk (n=254)				
Model 1				0.34
Model 2				0.53
sFlt-1, pg/mL	−30 (−34 to −27)	0.33	−46 (−48 to −42)	0.63
PlGF, pg/mL	37 (35 to 40)	0.43	29 (26 to 33)	0.79
sFlt-1/PlGF ratio	−45 (−46 to −43)	0.50	−38 (−39 to −37)	0.73
sFlt-1/PlGF ratio ≤38		0.45		0.72
sFlt-1/PlGF ratio >85		0.34		0.71

Traditional model 1 consists of gestational age, parity, diastolic blood pressure, and proteinuria; Traditional model 2 consists of model 1 plus serum concentrations of creatinine, uric acid, alanine transaminase, lactate dehydrogenase, and platelets; Multivariable includes the traditional predictors with either sFlt-1, PlGF, sFtl-1/PlGF ratio, sFtl-1/PlGF ratio ≤38, or sFtl-1/PlGF ratio >85. CI indicates confidence interval; GA, gestational age; PlGF, placental growth factor; and sFlt-1, soluble Fms-like tyrosine kinase 1.

This indicates the incremental value of the biomarkers. If 2 of the 3 angiogenic parameters (sFlt-1, PlGF, and their ratio) were added together to model 2, the R^2 increased to 0.72 in all combinations (ie, to a value identical to that achieved by adding PlGF alone). The incremental value of the dichotomized values for the ratio cutoffs ≤38 or >85 increased the R^2 to 0.65 and 0.64, respectively.

As expected, if calculations were restricted to women with a GA of <34 weeks, the predictive value of the biomarkers was substantially greater both in univariable analysis and when added to model 2. The largest increase, to 0.79, was achieved by adding PlGF. When 2 of the 3 angiogenic parameters (sFlt-1, PlGF, and their ratio) were added together to model 2, the R^2 increased to 0.79 in all combinations (ie, again to a value identical to that achieved by adding PlGF alone).

The incremental value of the dichotomized values for the ratio cutoffs ≤38 or >85 increased R^2 to 0.72 and 0.71, respectively.

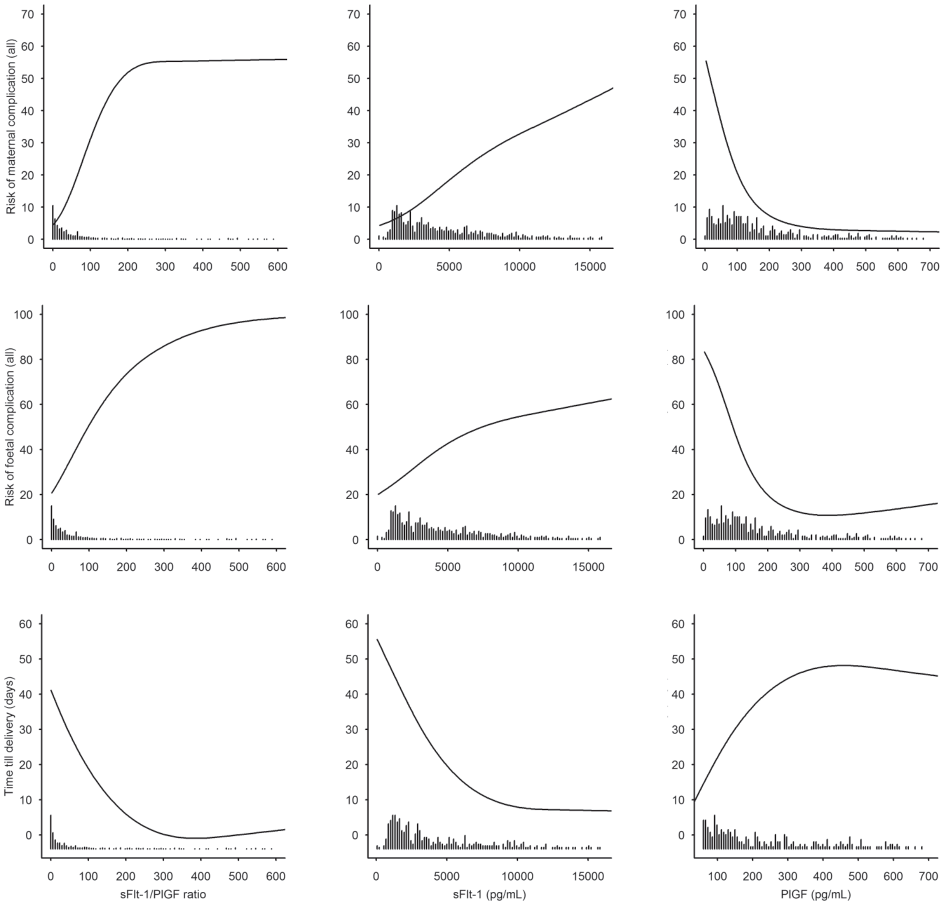

Figure 1. Associations between the levels of soluble Fms-like tyrosine kinase 1 (sFlt-1; pg/mL; **left**), placental growth factor (PlGF; pg/mL; **middle**), and the sFlt-1/PlGF ratio (**right**) with maternal (**top**), fetal/neonatal complications (**middle**), and the days until delivery (**bottom**). Curves are modeled with restricted cubic spline functions. Spikes at the bottom of the graphs indicate the distribution of the variable.

Associations of sFlt-1, PlGF, and sFlt-1/PlGF ratio with traditional laboratory measurements and blood pressure

Traditional laboratory measurements as well as DBP correlated only weakly with the individual angiogenic biomarkers and the sFlt-1/PlGF ratio, the strongest association being present between serum uric acid and the sFlt-1/PlGF ratio (*r*=0.40; Table S1). This is in line with the additive value of incorporating the various angiogenic markers in model 2.

DISCUSSION

This study, performed in a high-risk cohort of 620 women with suspected/confirmed preeclampsia, shows clear incremental value of sFlt-1, PlGF, or their ratio over clinical and traditional laboratory variables that are known to be associated with adverse pregnancy outcomes and to predict maternal and fetal/ neonatal complications, as well as pregnancy prolongation. Our findings suggest that sFlt-1, PlGF, and the sFlt-1/PlGF ratio in univariable analysis predict maternal and fetal/neonatal complications to at least the same degree as, if not better than, the combination of all clinical and traditional laboratory variables. In univariable analysis, the biomarkers were relatively weak predictors of time until delivery, but when added individually to a model with clinical and traditional laboratory variables, The traditional laboratory parameters did not show the prediction of pregnancy prolongation improved consider- incremental value on top of the traditional clinical variably. In line with previous studies,[7,21–23] the predictive value ables (GA, parity, DBP, and proteinuria) for fetal/neonatal of the biomarkers was even higher when limiting our analysis complications, suggesting that the information of the trato women with a GA <34 weeks. This was also the case for ditional laboratory parameters exclusively concerns matermaternal and fetal/neonatal outcomes (data not shown). nal health.

In the past, various cutoff values of the sFlt-1/PlGF ratio have been proposed to predict the (short-term) absence or presence of preeclampsia or pregnancy-related complications.[12,16,23-24] In our cohort, we have, therefore, compared the additional predictive value of the thresholds ≤38 and >85 of the ratio with the predictive value of the continuous values of the individual biomarkers and their ratio, either alone or in combination. For all outcome parameters, the predictive accuracy of these thresholds was lower than when the biomarkers were used as continuous variables. Our data thus illustrate that the absolute values of sFlt-1 and PlGF in combination with standard clinical and laboratory assessment have superior predictive ability compared with dichotomous versions of the biomarkers. Although we acknowledge the easier applicability of thresholds, our data suggest that loss of predictive performance may be a potential disadvantage of this approach. In part, this can be overcome by using different cutoff values for different GAs as suggested in several previous studies,[12,15] but this in turn blurs the convenience of a single cutoff point. Furthermore, when we applied the proposed sFlt-1/PlGF ratio threshold of ≤38 to predict the short-term absence of preeclampsia,[13] we still observed patients who developed preeclampsia-related complications (false negatives; Table 2).

Most studies applying models including angiogenic markers focused on the diagnosis of preeclampsia as end point instead of pregnancy outcome.[13,17,25–27] In a small cohort of

51 women with singleton pregnancies and early onset preeclampsia, a high sFlt-1/PlGF ratio was predictive for perinatal but not maternal complications.[28] Two large studies performed in women with singleton pregnancies showed that PlGF and sFlt-1 measured at, respectively, 30 to 34 weeks' and 35 to 37 weeks' gestation were poor or no predictors of stillbirth or adverse events in labor or after birth, including admission to the neonatal intensive care unit, which occurred in, respectively, 5.9% and 6.3% of newborns.[25-26] In the present study, 41.8% of newborns were admitted to the neonatal intensive care unit, reflecting the inclusion of a high-risk group in our study. Our study, therefore, is the first to allow robust conclusions on the predictive value of the angiogenic biomarkers with regard to days until delivery and maternal and fetal/ neonatal adverse events.

Several limitations of our study must be acknowledged. First, the biomarkers were not measured at fixed time points, related in part to variation in outpatient visits. Nonetheless, this reflects clinical reality. Second, we did not perform repeated angiogenic marker measurements during pregnancy. Although repeated measurements might improve the prediction of pregnancy outcomes, an important question is how frequently one has to repeat the measurement of these proteins, especially when women are suspected of preeclampsia early in pregnancy. Here, the costs of determining these markers multiple times should also be considered.

PERSPECTIVES

Our data imply that sFlt-1, PlGF, and their ratio have strong incremental value for the prediction of maternal and fetal or neonatal complications and days until delivery on top of current standard of diagnosis. Their use as continuous variables (rather than dichotomized) will facilitate the development of a well-discriminating prediction model for the risk of maternal and fetal or neonatal complications in individual pregnant women with suspected/confirmed preeclampsia.

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SUPPLEMENTAL TABLE

Table S1. Associations of sFlt-1, PlGF and sFlt-1/PlGF ratio with traditional laboratory measurements and blood pressure.

Parameter	sFlt-1 (pg/mL) *r (95CI)*	PlGF (pg/mL) *r (95CI)*	Ratio *r (95CI)*
ALT (mmol/L)	0.25 (0.18 – 0.33)	-0.24 (-0.31 – -0.16)	0.29 (0.22 – 0.36)
Creatinine (µmol/L)	0.19 (0.11 – 0.27)	-0.17 (-0.24 – -0.08)	0.22 (0.14 – 0.29)
DBP (mmHg)	0.16 (0.10 – 0.25)	-0.26 (-0.33 – -0.18)	0.18 (0.003 – 0.012)
LD (U/L)	0.27 (0.22 – 0.37)	-0.30 (-0.37 – -0.37)	0.36 (0.29 – 0.43)
PCR (mg/mmol)	0.16 (0.06 – 0.26)	-0.32 (-0.41 – -0.21)	0.29 (0.20 – 0.38)
Platelets (10^9/L)	-0.15 (-0.23 – -0.07)	0.22 (0.14 – 0.29)	-0.23 (-0.30 – -0.15)
SBP (mmHg)	0.15 (0.07 – 0.23)	-0.31 (-0.37 – -0.24)	0.29 (0.21 – 0.36)
Uric acid (mmol/L)	0.33 (0.26 – 0.40)	-0.24 (-0.41 – -0.27)	0.40 (0.33 – 0.47)

ALT, alanine transaminase; DBP, diastolic blood pressure; LD, lactate dehydrogenase; SBP, systolic blood pressure.

Chapter 9

Prediction of preeclampsia-related complications in women with suspected/confirmed preeclampsia: development and internal validation of a clinical prediction score

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ABSTRACT

Background A clinical prediction score that could reliably predict the risk of preeclampsia (PE)-related pregnancy complications does not exist.

Methods Data from our previous study, a prospective, multicenter, observational cohort study of 384 women with suspected or confirmed PE were used to develop and to internally validate a clinical score to predict PE-related maternal and foetal complications in subsequent 7, 14 and 30 days. For the development of the risk score the possible contribution of clinical and standard laboratory variables as well as the biomarkers soluble FMS like tyrosine kinase-1 (sFlt-1), placenta growth factor (PlGF) and their ratio was explored using multivariate regression analysis. We assessed the discriminative ability of the model with the concordance (c-) statistic. Bootstrapping procedure with 500 replications were used to correct the estimate of the risk score performance for optimism and to compute a shrinkage factor for the regression coefficients to correct for overfitting.

Results 96 women with suspected/confirmed PE had PE-related adverse outcomes at any time after hospital admission. Remaining significant predictors of PE-related outcomes included sFlt-1/PlGF ratio (continuous), gestational age at time of biomarker measurement (continuous) and protein-to-creatinine ratio (continuous). The c-statistic (corrected for optimism) for developing a PE-related complication within 7, 14 and 30 days was respectively 0.888, 0.881 and 0.870. There was no significant overfitting. Internal validation by means of bootstrap resampling resulted in a shrinkage factor of 0.908.

Conclusions We were successful to develop an internally validated clinical prediction score with an excellent discriminative performance to predict short-term and longer term PE-related complications. Its usefulness in clinical practice awaits further investigation.

INTRODUCTION

Preeclampsia (PE), a pregnancy-specific syndrome traditionally characterized by an elevated blood pressure and proteinuria, affects approximately 5% of pregnancies.[11] The current management of patients suspected of or with 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 foetal demise.[39 40] Despite well recognition of several risk factors including a past or family history of PE, nulliparous women, 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) that results in an anti-angiogenic state due to the inhibition of vascular endothelial growth factors, including placental growth factor (PlGF). Several models have been developed to calculate the predicted probability of preeclampsia, and a small portion of these models included sFlt-1 and PlGF as predictors.[41 42] Although the quest for a prediction model that could estimate the risk of pregnancy complications rather than diagnosing the heterogeneous PE syndrome is obvious, no such model currently exists. We recently reported that incorporation of sFlt-1, PlGF and the sFlt-1/PlGF ratio in a model based on clinical and traditional laboratory variables has substantial additive value to predict maternal and foetal complications along with pregnancy prolongation in patients suspected or with confirmed PE.[43] 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.[43] As a follow-up of these findings our aim was to develop and to internally validate a clinical score for predicting the risk of women presenting with suspected/confirmed PE for developing pregnancy complications in the subsequent 7, 14 and 30 days.

METHODS

Study design

Women with singleton pregnancies from varied ethnic background were recruited into this prospective multicentre cohort study at 3 Dutch hospitals (Erasmus MC and Maasstad Hospital in Rotterdam, Reinier de Graaf Hospital in Delft). Identical study protocol and data collection forms were used at each centre. All subjects provided written informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202).

Study population

Women with suspicion of or with confirmed PE with a gestational age of ≥20 and <37 weeks admitted to the obstetric departments of participating hospitals were included. Women with (partial)HELLP syndrome and multiple pregnancy or chromosomal/foetal anomalies were excluded.

Patients were suspected of PE if they presented with hypertension, proteinuria or symptoms associated with PE such as right upper quadrant abdominal pain or headache with visual disturbances.

PE was defined as de novo hypertension (systolic blood pressure (SBP) of ≥140 and/or diastolic blood pressure (DBP) of ≥90 mmHg) and proteinuria (PCR ≥30 mg/mmol or ≥300mg/24 h or 2+ dipstick) at or after 20 weeks of pregnancy.[34] The PE group was further divided into PE and superimposed PE (supPE), the latter diagnosed in women with chronic hypertension with new onset of proteinuria or a sudden increase of blood pressure or a sudden increase of proteinuria in patients with a pre-existing proteinuria. The group with only suspicion of PE, but without gestational hypertension, was defined as *no* hypertensive disease of pregnancy.

Outcome Measures

Pregnancy complications consisted of maternal and foetal adverse events.

Maternal complications were defined as: acute renal failure (absolute increase in the serum creatinine concentration of ≥0.3 mg/dL [26.4 micromol/L] from baseline; ≥50% increase in serum creatinine; or oliguria with <0.5 mL/kg per hour over a period of 6 hours), cerebral haemorrhage/oedema or infarction, death, eclampsia, development of the HELLP syndrome, pulmonary oedema and subcapsular liver hematoma.

Foetal complications were defined as foetal death and foetal distress requiring primary caesarean section. Days until complication was defined as days from study inclusion and blood sampling until occurrence of complication. Clinical findings, physical examination, laboratory test results, maternal and foetal 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 analyser (Cobas 6000, **e** module; Roche Diagnostics, Mannheim, Germany). Samples were stored at -80°C until analysis.

Statistical analysis

To identify candidate predictors for time to pregnancy complication, we selected variables based on clinical knowledge. These were sFlt-1, PlGF, sFtl-1/PlGF ratio, gestational age, proteinuria, DBP, lactate dehydrogenase (LD), uric acid, and platelets. Missing values were imputed 5 times using multivariate imputation by chained equations.[44] All results were pooled using Rubin rules.[45]

Table 1. Characteristics of 384 women in the PRE-RATIO study

	All patients
Age (yrs)	31 (27 – 36)
Gestational age <34 (n, %)	220 (57)
Nulliparous (n, %)	200 (52)
Current smoker (n, %)	39 (0.5)
Race (n, %)	
White	246 (64)
Black	73 (19)
Other	65 (17)
History of PE (n, %)	74 (19)
Preexisting hypertension (n, %)	101 (26)
Preexisting proteinuria (n, %)	23 (6)
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 (n, %)	
No hypertensive disease of pregnancy	186 (48)
Gestational hypertension	68 (18)
Preeclampsia	86 (22)
Superimposed preeclampsia	44 (11)

Values are median (IQ range).

A transformation with the natural logarithm was the best fit of PlGF, sFlt-1/PlGF ratio, proteinuria, DBP, platelets and the outcome, while a linear relationship between the sFlt-l, gestational age, LD, uric acid and the outcome was the best approximation. Overall percentage of missing values was low (1-3%), except for protein-to-creatinine ratio (34%) but in the latter patients the 24-hour urine protein were measured.

We used the Fine & Gray semi-parametric proportional hazards model to evaluate the effect, of predictors on the time to develop complications, using the subdistribution hazard ratios (sHRs).[46] Delivery before developing a PE-related complication, was considered a competing risk. The Fine & Gray model was applied to assess the absolute probability of developing complications using the cumulative incidence function.

Non-linearity of the continuous variables was assessed using the natural logarithm. Selection of variables was performed by means of backward selection using Wald's test based on the pooled regression coefficients and associated standard errors, we used Akaike's Information Criteria (AIC) as a stopping rule. We assessed the discriminative ability of the model with 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 resulting model was internally validated with a bootstrap procedure with 500 replications to correct the estimate of the model performance for optimism and to compute a shrinkage factor for the regression coefficients to correct for overfitting.[47] A risk chart was constructed to ease implementation of the resulting 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 the statistical analysis.

RESULTS

The study enrolled 384 singleton pregnancies that were included at 20-37 weeks' gestation, of whom 220 (57%) had a gestational age below 34 weeks (Table 1). In total, 186 (48%) women had no hypertensive disease of pregnancy, 68 (18%) had gestational hypertension, and 130 (34%) had (superimposed) PE at study entry. Table 2 lists pregnancy outcomes. The median time from inclusion to delivery was 21 days (IQR 8-41 days). Maternal complication occurred in 60 (16%) women, foetal complications requiring primary caesarean section in 55 (14%), and in total 96 (25%) of the pregnancies were developed a complication.

The final multivariable model included the following variables: sFlt-1/PlGF ratio (continuous), gestational age at time of biomarker measurement (continuous) and protein-

Table 2. Pregnancy outcomes and occurrence of adverse maternal and foetal outcomes, n (%) unless otherwise stated

	All patients
Gestational age at birth (wks, days)	37.1 (34.2 – 38.2)
Prematurity	
<34	85 (22)
34 – 37	73 (19)
Girls	186 (48)
Birth weight (gram)*	2813 (1965 – 3329)
Days until delivery*	21 (8 – 41)
Hospitalized (days)*	6 (3 – 13)
Final diagnosis	
No hypertensive disease of pregnancy	143 (37)
Gestational hypertension	64 (17)
Preeclampsia	102 (27)
Superimposed preeclampsia	53 (14)
(partial) HELLP syndrome	22 (6)
Maternal complications in all patients	
(superimposed) preeclampsia	25 (6.5)
(partial) HELLP syndrome	22 (5.7)
Placental abruption	1 (0.3)
Pulmonary oedema	7 (1.8)
Renal insufficiency	2 (0.5)
Visual disturbances	3 (0.8)
Foetal complications	
Foetal distress requiring primary caesarean section	55 (14.3)
Foetal death	9 (2.3)

*Values are median (IQ range).

to-creatinine ratio (continuous). Sub-distribution hazard ratios (sHRs) for these predictors at the day of biomarker measurement are shown in Table 3. The sFlt-1/PlGF ratio and gestational age at time of biomarker measurement were the strongest predictors ($P<0.001$) for PE-related complications. The resulting risk predictions from the model

Table 3. Subdistribution hazard ratios (sHRs) of the multivariable modelwith 95% confidence interval

	Hazard ratios (95% CI)	P-value
Gestational age at biomarker measurement (weeks, days)	0.99 (0.98 – 0.99)	<0.001
Protein to creatinine ratio (mg/mmol)*	1.23 (1.02 – 1.49)	0.03
sFlt-1/PlGF ratio*	1.79 (1.60 – 2.02)	<0.001

* Variables were transformed with the natural logarithm

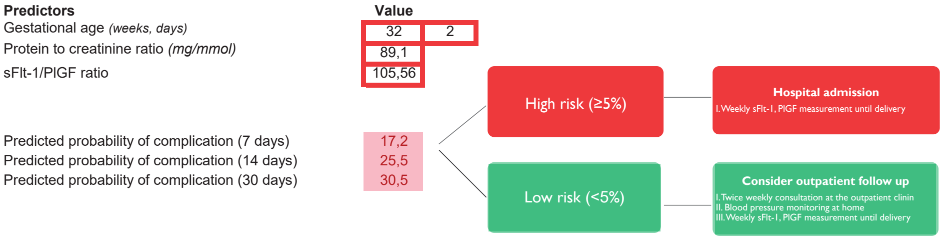

Figure 1. Risk chart

were used to develop a risk chart. For example, as illustrated in Figure 1, a patient who is suspected of PE at a gestational age of 32^{+2} weeks with a protein-to-creatinine ratio of 89.1 mg/mmol and a sFlt-1/PlGF ratio of 106 has a predicted probability of 17% for a complication within 7 days and this risk increases to 26% and 31% in the subsequent 7 and 23 days.

The goodness of fit test yielded a p-value of <0.001 confirming that the model fitted the data well. The c-statistic (corrected for optimism) for developing a PE-related complication within 7 days was 0.888 meaning that the model has an excellent ability to discriminate between patients who will develop a PE-related complication and who will not. The latter was also true for developing a PE-related complication within 14 and 30 days with c-statistics (corrected for optimism) of 0.881 and 0.870 (corrected for optimism) respectively. Internal validation by means of bootstrap resampling resulted in a shrinkage factor of 0.908.

DISCUSSION

We present the development and internal validation of a clinical score to predict the risk of PE-related complications within 7, 14 and 30 days in women with suspected/confirmed PE. Continuous sFlt-1/PlGF ratio values, protein-to-creatinine ratio and gestational age at time of blood sampling for biomarker measurement were strong predictors for the development of PE-related complications. Internal validation, using bootstrap analysis, showed that the model provides excellent discrimination between women with a high or a low risk to develop a PE-related complication. The resulting risk predictions for day 7, 14 and 30 after biomarker measurement provides support for key clinical decisions not addressed by existing single time point prognostic criteria.

The final model yielded a c-statistic of 0.888 which is superior to what has been achieved by other models. Another strength of the study is the risk chart that resulted from our model. Our model is not limited to maternal surveillance but also addresses foetal risk associated with PE that requires delivery. Obviously, to be useful in clinical decision making for patients suspected of PE presenting at the obstetric ward it is important that the biomarkers can be measured instantaneously like a point of care measurement, requiring a dedicated clinical chemistry laboratory.

A significant amount of research has been done 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.[20 21 23 48] Recently, we reported, that the prediction of the PE-related complications could significantly be improved by using continuous instead of dichotomous values of the biomarkers.[43] Based on this finding the sFlt-1/PlGF ratio is also used as a continuous variable in our prediction model. We think that application of this prediction model might be an aid for gynaecologists to better predict which patients are at high risk of serious maternal, foetal PE-related complications, i.e. those who should be admitted to the obstetric ward, and those who are at a low risk and can be monitored at home. The latter may not only reduce unnecessary preterm deliveries, antenatal admissions and foetal monitoring with false-positive diagnoses, but possibly also costs by limiting procedures due to uncertain diagnosis. Of course, this should be balanced against the additional costs related to the measurement of the biomarkers.

For better appreciation of its value application of our model to an external cohort would be worthwhile, but until now we were not successful to do so. If comparable c-statistics are achieved with external validation we can conclude with more certainty that our model is generalizable for women with suspected/confirmed PE. Of note, patients on which our clinical prediction score is based are from one academic hospital and two non-academic 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 gynaecologists in our hospital we concluded that patients with suspected or confirmed PE who have a calculated risk score < 5% are at low risk of maternal and foetal complications and likely can safely be followed at the out-patient clinic. We acknowledge that this threshold of <5% is arbitrarily chosen. To establish that this threshold is valid and safe for clinical decision making a randomized controlled intervention trial has to be conducted. In this trial patients with suspected or confirmed PE should be randomized for management according to existing guidelines or treatment based on the clinical prediction score. From such a trial, it can be learned whether introduction of the prediction score is safe and a welcome aid for gynaecologists in care of patients with (suspected)PE and whether it results in less hospital admissions, saving costs.

There are several limitations of his study. First, as already mentioned a lack of external validation. External validation may ensure that predictions based on the developed clinical prediction score are generalizable to other populations. Furthermore, 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.

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Chapter 10

Summary, discussion and future directions

SUMMARY

Chapters 1-4

Preeclampsia (PE), a hypertensive disorder associated with hypertension, proteinuria and at times edema, coagulation or liver-function abnormalities or both, occurs primarily in nulliparous women, usually after the 20th gestational week.[1] Women in whom PE is destined to develop have a marked increase in systemic vascular resistance with a relatively low cardiac output and hypovolemia, accompanied by proteinuria.[2] Despite the hypovolemia, renin–angiotensin system (RAS) activity is suppressed compared to healthy pregnancies, and aldosterone levels are declined to the same degree as renin. The latter suggests that activation of the RAS is not the explanation of the hypertension in PE, but rather that its suppression is a consequence of the rise in blood pressure. Although the pathophysiologic mechanisms that underlie PE still remain to be elucidated, it has been well established that a reduced placental perfusion plays a key role in the initial phase of PE, ultimately resulting in an angiogenic imbalance. The release of anti-angiogenic factors from the placenta [in particular, soluble Fms-like tyrosine kinase-1 (sFlt-1)] accounts for this angiogenic imbalance. In the maternal circulation, elevated sFlt-1 levels bind and inactivate vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), causing further vasoconstriction and hypertension, resulting in a vicious circle. When investigating the relationship between disturbed angiogenic balance, arterial pressure, and endothelin-1 (ET-1) in pregnant women with a high (≥85) or low (<85) sFlt-1/PlGF ratio, plasma ET-1 levels were increased in women with a high ratio. In addition, plasma ET-1 correlated positively with plasma sFlt-1 and negatively with plasma renin concentration (**Chapter 3**). Moreover, urinary protein also correlated with plasma ET-1 and mean arterial pressure. Therefore, a high antiangiogenic state induces endothelial dysfunction, reflected by activation of the

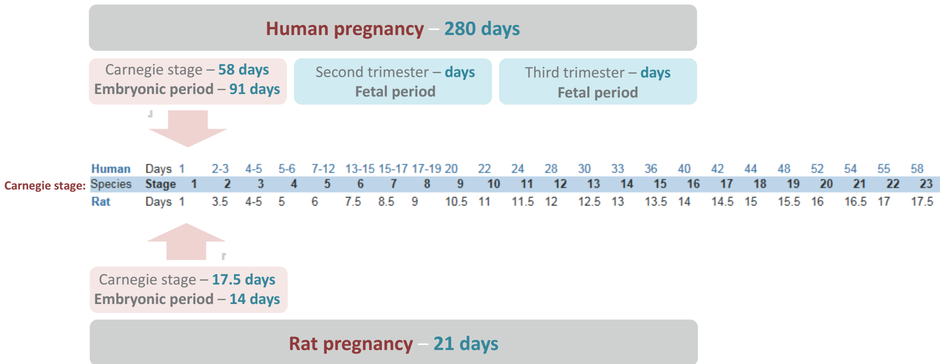

Figure 1. Developmental stages of pregnancy
Pink arrows: Carnegie stage
Grey arrows: Medication use

endothelin system, which together with the increased mean arterial pressure underlies the reduction in renin in PE.

In order to improve perinatal outcomes, the search for therapy to normalize the angiogenic state and to attenuate endothelial dysfunction and the elevated blood pressure in pregnant women with PE to prolong gestation has been extensive. Antagonizing the effects of the activated endothelin system using an endothelin receptor blocker seems straightforward, however, its use in human pregnancy is potential dangerous, because antagonizing the effects of endothelin by endothelin receptor blockers has shown to be teratogenic in animal experiments.[3] Another possibility to normalize the imbalance in PE is targeting the elevated sFlt-1 level. Proton pump inhibitors, regularly prescribed during pregnancy to treat reflux disease[4], have been shown to decrease trophoblast sFlt-1 and endoglin secretion in vitro.[5] In line with this, we demonstrated in a retrospective clinical study that PPIs lower the circulating sFlt-1, endoglin as well as ET-1 levels, suggesting that PPI prescription may be a new treatment option in PE (**Chapter 4**).

Chapters 5-7

The variable nature of PE mirrors the complex pathophysiology of the condition. Despite the clear definition, the clinical criteria alone in many instances are not specific or sensitive enough to predict the development of maternal and fetal/neonatal gestational complications. For instance, a proportion of women develop PE-related complications without being diagnosed with the disease. Conversely, those in whom the disease is diagnosed are sometimes able to carry a pregnancy to nearly full term without difficulties. In a cohort of pregnant women at high risk of or with confirmed clinical PE we have shown that a sFlt-1/PlGF ratio ≥85 is associated with higher odds for adverse pregnancy outcome than the isolated clinical diagnosis (**Chapter 5**). Nonetheless, the additional value of the elevated sFlt-1/PlGF ratio for diagnosing purposes is limited since in most patients with a high sFlt-1/PlGF ratio the diagnosis of PE could also be made on the basis of clinical grounds (**Chapter 5**). Yet, particularly in pregnant women with preexisting hypertension and/or proteinuria, measurement of the biomarkers may be valuable for making the correct diagnosis and may therefore perform better than the standard diagnostic work-up. An important question is how frequently one has to repeat the measurement of the biomarkers. We showed in a small proportion of women hospitalized for evaluation of PE with a low sFlt-1/PlGF ratio at initial assessment, that the sFlt-1/PlGF ratio can still rise in following weeks to months, indicating that the sFlt-1/PlGF ratio has to be measured repeatedly (**Chapter 6**). Our study also showed the fall of the biomarkers after delivery: sFlt-1dropped to <1% of its pre-delivery concentration with a half-life of 1.4±0.3 days, while PlGF dropped to ~30% of its pre-delivery level with a half-life of 3.7±4.3 days (**Chapter 6**).

The risk of PE is 2-3 times higher for twin than singleton pregnancies, making the urgency of timely diagnosing or excluding PE in twin pregnancies more important (**Chapter 7**) A sFlt-1/PlGF ratio of ≤38 has been reported to predict the absence of preeclampsia (PE) in singleton pregnancies. Whether this ratio of ≤38 could be used also to predict the absence of PE, and maternal and fetal/neonatal complications in twin pregnancies is unknown. In line with previous studies we showed a considerably higher sFlt-1 levels in twin than in singleton healthy gestations, implicating that a ratio of ≤38 to predict short-term absence of PE or adverse pregnancy outcomes cannot be applied to twin pregnancies (**Chapter 7**).

Chapters 8-9

As mentioned, the assessment of the biomarkers, especially the sFlt-1/PlGF ratio, is a promising clinical tool for assigning PE and the prediction of pregnancy prognosis. Different groups of investigators have proposed various cut-off values of the ratio to predict the absence or presence of PE. However, information about the value of the new biomarkers on top of simple conventional assessment and routine laboratory variables is lacking. Our data imply that sFlt-1, PlGF, and their ratio on top of the current stand of diagnosis have strong incremental value for the prediction of maternal and fetal or neonatal complications (**Chapter 8**). Their use as continuous variables (rather than dichotomized) facilitated the development of a well-discriminating prediction model for the risk of maternal and fetal or neonatal complications in individual pregnant women with suspected/confirmed PE. In women with suspected or confirmed PE, we developed a risk calculator to predict the proportional chance of adverse maternal/fetal-neonatal outcomes occurring respectively 7, 14 and 28 days after inclusion. The risk calculator includes gestational age, protein-to-creatinine ratio and sFlt-1/PlGF ratio (**Chapter 9**). Obviously, the clinical usefulness of the risk calculator should be evaluated in future studies.

FUTURE DIRECTIONS

Although the classic diagnosis of preeclampsia (PE) is clearly defined as de novo hypertension and proteinuria in the second half of pregnancy, the clinical presentation and course among patients vary considerably, and preexisting conditions, particularly autoimmune and renal diseases, can mimic the syndrome. Since symptoms and routine laboratory variables to predict the severity of PE or adverse pregnancy outcome are not very accurate, patients suspected of having PE are often hospitalized for careful evaluation, primarily as a safety measure until PE is ruled out. Nevertheless, this approach still fails to predict the most serious adverse outcomes in a substantial proportion of women.

A significant amount of research has been done on the ability of the sFlt-1/PlGF ratio to predict the absence or presence of PE or PE-related complications. In **Chapter 9** we developed a risk-calculator that consists of continuous instead of dichotomous values of the sFlt-1/PlGF ratio, gestational age and protein-to-creatinine ratio. We think that application of this calculator might be a valuable tool for gynecologists to better predict which patients are at high or low risk of serious maternal, fetal/neonatal PE-related complications, i.e. those who should be admitted to the obstetric ward, and those who can be monitored at home. To address this and to find out whether the use of risk calculator is safe we propose to start an intervention trial. In this trial patients suspected of PE will be managed according to current standards or on the basis of the risk calculated by the calculator. Our hypothesis is that introduction of the risk calculator leads to a decrease in the number and duration of hospitalizations in the obstetric ward, without compromising maternal or fetal/neonatal health outcomes.

With regard to the treatment of PE, activation of the endothelium and the consequent rise of ET-1 causing hypertension and contributing to renal dysfunction, has encouraged the use of (single and dual) endothelin receptor antagonists (ERAs) in experimental PE models. In various animal models of PE the administration of these antagonists indeed has led to an improvement of the manifestations of PE. However, the use of ERAs as a possible treatment to cure the manifestations of PE is tempered in view of its teratogenic effects observed in animals, in particular craniofacial and cardiovascular malformations. Nonetheless, sporadic cases of pregnant women with pulmonary arterial hypertension using ERAs without adverse effects have been described. Studies that examined bosentan, a dual ERA as a treatment for pulmonary hypertension inadvertently used by pregnant women, showed no major adverse effects in term and preterm neonates.[6] In animal experiments ERAs are usually administered at very early gestation. A method that allows to directly compare the timing of development of the human embryo with that of rats is the so named Carnegie staging, consisting of 23 stages. These stages are based on the morphological development of the vertebrate embryo and can be applied to all vertebrates given that the embryonic development of almost all vertebrates, including humans, is identical. The Carnegie staging system accompanied by a three-dimensional projection system, the I-space, greatly improves knowledge of normal and abnormal embryonic growth, development and morphology. As shown in the figure, in animal studies the ERAs are given before the end of the Carnegie stages. In pregnant women, these drugs can be administrated later, e.g. at the end of the second trimester when embryogenesis is complete and the symptoms of PE typically become manifest. Indeed, when dual ERAs were given later in pregnancy in rodents no congenital abnormalities were observed.[7-9] Of note, the reported prevalence of congenital abnormalities regarding ERA use of 6% in human pregnancy is not very different of the overall reported prevalence of 4%.[10] In rats the fetal drug levels of ERAs are about 2% of the maternal levels during chronic maternal administration, implying that only a minute fraction of these drugs crosses the placenta.[7] Whether this is also true for the human situation is unknown. As a first step the isolated human placenta perfusion model can be used to evaluate this further. With this model different ERAs can be tested in placenta's obtained from healthy pregnancies as well as in placenta's obtained from preeclamptic pregnancies. In addition to pharmacokinetic data this model also allows to investigate potential effects of different ERAs on the placental circulation. If these studies confirm animal pharmacokinetic data, treatment with an ERA may be considered when PE occurs at a gestational age at which the prognosis is extremely poor.

Alternative approaches, would be the development of agents that somehow interfere with the endothelin system but do not cross the placenta. Interestingly, Onda et al. observed that a variety of proton pump inhibitors (PPIs), agents that are safe to be used in pregnancy to treat gastric reflux, reduced ET-1 secretion from cultured endothelial cells, and that administration of PPIs to a transgenic PE mouse model with placental sFlt-1 overexpression decreased sFlt-1, soluble endoglin and blood pressure.[5] In line with this, we observed that PPI use in pregnant women suspected of or with confirmed PE is associated with lower sFlt-1, endoglin and ET-1 levels than in non-PPI users. Since our findings were based on retrospective data and therefore sensitive to bias, prospective randomized studies are needed to demonstrate whether early administration of PPIs to pregnant women with a high risk to develop PE can mitigate the course of the condition and indeed is associated with lower sFlt-1, endoglin and ET-1 levels.

Other investigators have reported that the angiogenic imbalance can be mitigated by using apheresis to remove elevated sFlt-1 levels from the maternal circulation, thereby restoring the angiogenic imbalance.[11-12] This has been accomplished by using a plasma-specific dextran sulfate apheresis column. With this approach circulating sFlt-1 concentrations reduced by on average 18% and the protein-to-creatinine ratio by 44%. Among the 6 women who were treated once, pregnancy continued on average for 8 days. When women were treated twice or thrice (n=5), pregnancy could be prolonged for on average 15 days. By contrast, the untreated pregnancies (n=22) continued for only 3 days. No adverse effects of apheresis treatment were observed in the mothers or their infants. Comparing women with PE who are treated with sFlt-1 apheresis with those who are not is challenging, because of the variable course of the disease. Plasma apheresis is an invasive and expensive treatment. Before considering introduction of plasma apheresis in the clinic for patients with early PE, conducting a randomized trial with a sham-control group, to demonstrate that this treatment is beneficial is mandatory. Since the angiogenic imbalance in PE is due to increased sFlt-1 and decreased PlGF as well as VEGF levels, administration of PlGF of VEGF may also be applied to mitigate or restore this imbalance. In a recent study, using the reduced uterine perfusion pressure (RUPP) rat model, administration of humanized PlGF for 5 days via intraperitoneal osmotic minipumps starting on day 14 of gestation was associated with a lower blood pressure and a higher glomerular filtration rate at day 19 of gestation, along with reduced sFlt-1 levels and reduced oxidative stress compared to vehicle treated RUPP rats.[13] These findings suggest a therapeutic potential for PlGF administration in PE. However, it is not yet clear whether PlGF administration have any unfavorable effects, especially in terms of (undetected) cancer. In tumor cells, PlGF expression is part of the angiogenic switch that supports tumour vascularisation.[14] And a positive correlation is detected between PlGF expression cancer severity in most cancer types. It seems worthwhile to explore whether such treatment in women with early PE is able to prolong gestation and improve fetal/neonatal outcome.

A recent meta-analysis showed that as compared to being more sedentary regular aerobic exercise during pregnancy starting before a gestational age of 23 weeks is associated with a significantly reduced risk of gestational hypertensive disorders overall, gestational hypertension, and cesarean delivery and a trend to a reduced incidence of PE (relative risk 0.79).[15] An intriguing question is whether regular aerobic excercise during or even before pregnancy also results in a better angiogenic profile, that may contribute to an improved outcome, especially in women who are at a relatively high risk of hypertensive disorders of pregnancy, including PE. As far as we know one cross-sectional study has reported that regular aerobic exercise in pregant women (≥ 3 hours/week) is associated with lower sFlt-1, soluble endoglin and higher PlGF in late gestation than in sedentary pregnant women.[16] Based on these findings prospective randomized studies, with different levels of exercise intensity are recommended to evaluate whether regular excercise in early pregnancy or even before conception has beneficial effects on the biomarkers of angiogenic imbalance and whether this translates in PE reduction and improved maternal and fetal/neonatal outcomes in pregnancies at high risk for this complication.

SAMENVATTING

Hoofdstuk 1-4

Pre-eclampsie (PE), een zwangerschapsgerelateerd syndroom gekenmerkt door hypertensie en proteïnurie en soms ook oedeem, trombose, en leverafwijkingen, komt voornamelijk voor bij nulliparavrouwen, na de 20^{e} zwangerschapsweek.[1] Vrouwen met PE hebben in vergelijking met gezonde zwangeren een hogere systemische vaatweerstand, een lager hartminuutvolume en hypovolemie.[2] Opvallend is dat ondanks de hypovolemie en hypertensie het renine–angiotensine aldosteronsysteem (RAAS) onderdrukt is. Activering van het RAAS lijkt dus niet verantwoordelijk voor de verhoogde vaatweerstand en hypertensie in PE en het is derhalve veel aannemelijker dat de hypertensie de RAAS-suppressie veroorzaakt. De precieze oorzaak of oorzaken van PE is (zijn) onbekend, maar in de afgelopen decennia is wel veel inzicht gekregen in de onderliggende pathofysiologie van dit syndroom. Initieel is er een verminderde doorbloeding van de placenta. Dit leidt vervolgens tot verhoogde placentaire productie van anti-angiogene factoren [in het bijzonder oplosbare Fms-achtige tyrosine kinase-1 (sFlt-1)] waardoor de sFlt-1-spiegel in de maternale circulatie aanzienlijk stijgt. De verhoogde sFlt-1 spiegel in de maternale circulatie veroorzaakt een angiogene disbalans door binding aan de vasculaire endotheliale en de placentaire groeifactoren (VEGF en PlGF). Tekort aan deze groeifactoren leidt via gegeneraliseerde endotheeldisfunctie en verhoogde productie van endotheline-1 (ET-1), de meest krachtige vasoconstrictoire factor in ons lichaam, tot vaatvernauwing en hypertensie en draagt bij aan de proteïnurie. In **hoofdstuk 3** laten we de resultaten zien van onderzoek naar de relaties tussen een verstoorde angiogene balans, uitgedrukt als een verhoogde ratio van sFlt-1 en PlGF, de arteriële bloeddruk, proteïnurie en ET-1-spiegels bij zwangeren met een relatief hoge (≥85) of relatief lage (<85) sFlt-1/PlGF ratio. Plasma ET-1-spiegels waren hoger bij vrouwen met een hoge ratio. Bovendien correleerden plasma ET-1 en plasma sFlt-1-spiegels positief, terwijl de plasma ET-1-spiegels en het plasma renine negatief gecorreleerd waren. Daarnaast waren de plasma ET-1-spiegels positief gecorreleerd met de bloeddruk en ernst van proteïnurie, terwijl bloeddruk en renine invers gecorreleerd waren. Deze gegevens ondersteunen de hypothese dat een anti-angiogene balans in de maternale circulatie via endotheeldisfunctie leidt tot een verhoogde productie van ET-1 en dat deze verhoogde ET-1-spiegel een belangrijke rol speelt bij de hypertensie, proteïnurie en suppressie van het RAAS bij PE.

Om de perinatale uitkomsten te verbeteren, wordt er gezocht naar behandelingen om de angiogene disbalans te verbeteren met als doel de zwangerschap van vrouwen met PE te verlengen. Remming van de effecten van het geactiveerde endotheline-system door een endotheline-receptorantagonist lijkt een voor de hand liggende aanpak. Helaas is het gebruik van zo'n antagonist tijdens een zwangerschap gecontra-indiceerd wegens teratogene effecten, geobserveerd in dierexperimenteel onderzoek.[3] Een andere optie is het verlagen van de verhoogde sFlt-1 spiegels. Experimenteel onderzoek heeft aangetoond dat protonpompremmers, medicijnen die de maagzuursecretie remmen en vaak voorgeschreven worden tijdens de zwangerschap om reflux te behandelen[4], de productie van sFlt-1 en endogline door de trofoblasten remmen.[5] Geheel in overeenstemming hiermee, vonden wij in een retrospectieve studie bij patiënten met PE dat het gebruik van protonpompremmers gepaard gaat met lagere spiegels van sFlt-1, oplosbaar endogline en ET-1 in de maternale circulatie. Dus wellicht kan met een protonpompremmer de angiogene disbalans in PE worden verbeterd (**hoofdstuk 4**).

Hoofdstuk 5-7

De wisselende manifestaties van PE weerspiegelen de complexe pathofysiologie van deze aandoening. Ondanks de duidelijke definitie, zijn alleen klassieke criteria vaak niet specifiek of gevoelig genoeg om de ontwikkeling van maternale en foetale/neonatale zwangerschapscomplicaties te voorspellen. Zo ontwikkelt bijvoorbeeld een deel van de zwangeren PE-gerelateerde complicaties zonder dat zij met PE gediagnosticeerd zijn. Omgekeerd zijn er zwangeren met de diagnose PE bij wie de zwangerschap vrijwel probleemloos verloopt. In een cohortstudie betreffende zwangeren met een hoog risico op PE of bij wie PE op grond van klassieke criteria werd gediagnosticeerd, hebben we aangetoond dat een sFlt-1/PlGF ratio ≥85 associeert met een grotere kans op een ongunstige afloop van de zwangerschap dan bij zwangeren met de diagnose gebaseerd op klinische criteria (**hoofdstuk 5**). De toegevoegde waarde van de verhoogde sFlt-1/PlGF ratio voor diagnostische doeleinden is beperkt, omdat bij de meeste patiënten met een hoge sFlt-1/PlGF ratio de diagnose PE gesteld kan worden op grond van klassieke criteria (**hoofdstuk 5**). Vooral bij zwangeren met reeds bestaande hypertensie en/of proteïnurie, kan het meten van de biomarkers behulpzaam zijn voor het stellen van de correcte diagnose en daarmee een essentiële aanvulling zijn op de standaarddiagnostiek. De vraag is hoe frequent het meten van de biomarkers herhaald moet worden. Bij een klein aantal patiënten die in het ziekenhuis opgenomen waren voor evaluatie van PE met initieel een relatief lage sFlt-1/PlGF ratio vonden we dat de ratio in de volgende weken tot maanden nog steeds kan stijgen. Dit impliceert dat de sFlt-1/PlGF ratio herhaaldelijk gemeten dient te worden bij verdenking op PE (**hoofdstuk 6**). In onze studie onderzochten we ook de mate en snelheid van daling van de biomarkers in de maternale circulatie na de bevalling. We vonden dat sFlt-1daalde naar <1% van de concentratie van voor de bevalling met een halfwaardetijd van 1.4±0.3 dagen, terwijl PlGF daalde naar ~30% van de waarde van voor de bevalling met een halfwaardetijd van 3.7±4.3 dagen (**hoofdstuk 6**).

Het risico op PE is 2-3 keer hoger bij een tweeling- dan bij een enkelvoudige zwangerschap. Onderzoek heeft laten zien dat een sFlt-1/PlGF ratio van ≤38 de korte-termijn afwezigheid van PE in enkelvoudige zwangerschappen met vrijwel 100% zekerheid voorspelt. Of deze afkapwaarde van ≤38 ook bruikbaar is om de afwezigheid van PE en ontwikkeling van maternale en foetale/neonatale complicaties bij tweelingzwangerschappen te voorspellen is onbekend. In overeenstemming met eerdere studies vonden we aanzienlijk hogere sFlt-1-spiegels bij tweeling- dan bij enkelvoudige, gezonde zwangerschappen aan. Dit impliceert dat een ratio van ≤38 als voorspeller van kortetermijn afwezigheid van PE of ongunstige zwangerschapsuitkomsten niet toegepast kan worden bij tweelingzwangerschappen (**hoofdstuk 7**).

Hoofdstuk 8-9

Zoals eerder is beschreven, is bepaling van de biomarkers en met name de sFlt-1/PlGF ratio, een veelbelovend hulpmiddel voor het vaststellen van PE en het voorspellen van de uitkomst van de zwangerschap. Diverse groepen hebben verschillende afkapwaarden van de ratio gerapporteerd om de aan- of afwezigheid van PE te voorspellen, maar informatie over de meerwaarde van de nieuwe biomarkers bovenop de conventionele beoordeling en routine laboratoriumbepalingen ontbreekt. Op basis van een grote cohortstudie concluderen we sFlt-1, PlGF en de ratio van deze twee biomarkers een duidelijke meerwaarde hebben voor het voorspellen van maternale en foetale/neonatale complicaties (**hoofdstuk 8**). Op grond van deze kennis hebben we gebruikmakend van multivariabele regressie- en bootstrapanalyses een risico-calculator ontwikkelt. Deze calculator heeft als inputvariabelen zwangerschapsduur, de urine eiwit-kreatinine ratio en de sFlt-1/PlGF ratio. Met behulp van deze calculator kan voor zwangeren met vermoedelijk of bevestigd PE op eenvoudige wijze de procentuele kans op zwangerschapscomplicaties, respectievelijk 7, 14 en 28 dagen na inclusie worden vastgesteld (**hoofdstuk 9**). De toegevoegde waarde van de risicocalculator voor de klinische praktijk dient in toekomstige studies geëvalueerd worden.

Richtingen voor toekomstig onderzoek

Hoewel de klassieke diagnose van PE duidelijk gedefinieerd is als de novo hypertensie en proteïnurie in de tweede helft van de zwangerschap, is er tussen patiënten veel variatie in de klinische symptomen en het verloop, en kunnen reeds bestaande aandoeningen, met name auto-immuun- en nierziekten, het syndroom imiteren. Aangezien symptomen en routine laboratoriumbepalingen om de ernst van PE of negatieve zwangerschapsuitkomsten te voorspellen niet erg accuraat zijn, worden patiënten met verdenking op PE vaak voor de veiligheid opgenomen in het ziekenhuis totdat PE is uitgesloten. Veel onderzoek is verricht naar de mogelijkheid of bepaling van de sFlt-1/PlGF ratio bruikbaar is om de aan- of afwezigheid van PE of PE-gerelateerde complicaties te voorspellen. In hoofdstuk 9 ontwikkelden we een risico-calculator met als inputvariabelen de sFlt-1/PlGF ratio, de zwangerschapsduur en de urine eiwit-kreatinine ratio.

We denken dat het toepassen van deze calculator een waardevol hulpmiddel kan zijn voor gynaecologen om beter te voorspellen welke patiënten een relatief hoog of laag risico hebben op ernstige PE-gerelateerde complicaties en behulpzaam kan zijn bij de afweging welke patiënten opgenomen dienen te worden en welke patiënten thuis gemonitord kunnen worden. Om de waarde van de risicocalculator in de praktijk te testen, zijn we voornemens te starten met een interventieonderzoek. In dit onderzoek worden patiënten die (vermoedelijk) PE hebben behandeld volgens de bestaande richtlijn of op basis van het risico berekend met de calculator. Onze hypothese is dat het toepassen van de risicocalculator leidt tot een afname van het aantal opnames en dat de duur van de opnames verkort wordt, zonder dat de gezondheid van de moeder of die van de foetus/pasgeborene in gevaar komt.

Endotheelceldisfunctie en de stijging van ET-1 met hypertensie en nierschade tot gevolg, hebben geleid tot het succesvol toepassen van endotheline-receptorantagonisten (ERA's) in experimentele PE modellen. Het gebruik van ERA's in de kliniek om PE te behandelen is gecontra-indiceerd wegens teratogene effecten (met name craniofaciale en cardiovasculaire defecten) vastgesteld in dierexperimenteel onderzoek. ERA's worden klinisch toegepast voor de behandeling van pulmonale hypertensie. Incidentele gevallen van patiënten die de ERA bosentan tijdens de zwangerschap hebben gebruikt zonder genoemde teratogene of andere afwijkingen zijn gerapporteerd.[6] In de dierexperimenten worden ERA's zeer vroeg in de zwangerschap toegediend. Een methode om een rechtstreekse vergelijking te maken tussen de ontwikkeling in de tijd van het humane embryo en die van ratten is de zogenaamde 'Carnegie stagering'. Deze stagering omvat 23 stadia gebaseerd op de morfologische ontwikkeling van gewervelde embryo's en is toepasbaar op alle gewervelden, aangezien de embryonale ontwikkeling van bijna alle gewervelden, inclusief de mens, identiek is. Door de Carnegie stagering, gekoppeld aan een driedimensionale echografisch projectiesysteem, de I-space, is de kennis van normale en abnormale embryonale groei, ontwikkeling en morfologie enorm toegenomen. Zoals in de figuur te zien is, worden in dierstudies de ERA's toegediend voor het einde van de 23 Carnegie stadia. Bij zwangeren met PE kunnen ERA's in een veel later stadium worden toegediend, bijvoorbeeld aan het einde van het tweede trimester wanneer de embryogenese compleet is en de symptomen van PE zich openbaren. Knaagdieren waaraan later in de zwangerschap bosentan werd toegediend waren gevrijwaard van congenitale afwijkingen.[7-9] Belangrijk om te realiseren is dat de gerapporteerde prevalentie van congenitale afwijkingen met betrekking tot het gebruik van bosentan van 6% bij zwangeren niet veel afwijkt van de globale gerapporteerde prevalentie van 4%.[10] Bij ratten bedraagt de ERA-concentratie in de foetus rond de 2% van die in de moeder, hetgeen erop wijst dat slechts een kleine fractie van deze middelen de placenta passeert.[7] Of dit ook voor mensen geldt, is onbekend. Een mogelijke eerste stap om dit nader te onderzoeken is het gebruik van het geïsoleerde humane placentaperfusiemodel. Met dit model kunnen verschillende ERA's getest worden in placenta's verkregen na gezonde en pre-eclamptische zwangerschappen. Naast de farmacokinetische data is het met dit model tevens mogelijk om de potentiële effecten van verschillende ERA's op placentaire circulatie te onderzoeken. Indien deze studies de farmacokinetische data in dieren bevestigen, kan behandeling met een ERA overwogen worden bij de behandeling van zeer vroege PE met slechte foetale/neonatale overlevingskansen. Een alternatief is het ontwikkelen van middelen die ingrijpen op het endothelinesysteem, maar de placenta niet passeren. Onda en collegae hebben onlangs laten zien dat diverse protonpompremmers (PPIs), in gekweekte endotheelcellen de productie van ET-1 remmen, en dat de toediening van PPIs bij een transgeen PE muismodel met overexpressie van sFlt-1 leidde tot een afname van sFlt-1 en het oplosbare endoglin een lagere bloeddruk.[5] We hebben gezien dat het gebruik van een PPI bij zwangeren met vermoedelijke of vastgestelde PE geassocieerd is met lagere sFlt-1-, oplosbaar endogline en ET-1-spiegels dan bij niet-PPI gebruikers. Onze bevindingen zijn gebaseerd op retrospectieve gegevens en daardoor gevoelig voor bias. Prospectieve gerandomiseerde studies zijn noodzakelijk om aan te tonen of vroege toediening van PPIs aan zwangere vrouwen met een hoog risico op het ontwikkelen van PE leidt tot lagere sFlt-1-, endogline- en ET-1-spiegels en betere zwangerschapsuitkomst.

Andere onderzoekers hebben laten zien dat de angiogene disbalans kan verbeteren door het toepassen van plasma-aferese met als doel sFlt-1 uit het plasma van de moeder te verwijderen.[11-12] Met deze aanpak daalden de circulerende sFlt-1-concentraties met gemiddeld 18%, nam de urine eiwit-kreatinineratio met gemiddeld 44% af en was de zwangerschapsduur significant langer dan bij de niet op deze wijze behandelde patiënten. Behandeling met plasma-aferese blijkt zowel voor moeder als kind veilig te zijn. Het blijft lastig om patiënten met PE die behandeld zijn met sFlt-1-aferese te vergelijken met onbehandelde vrouwen door het variabele ziekteverloop. Plasma-aferese is een invasieve en dure behandeling. Voordat overwogen wordt om plasma-aferese in de kliniek op grotere schaal te gaan toepassen bij patiënten met beginnende, vroege PE, is het noodzakelijk om een gerandomiseerde klinische studie met controlegroep te verrichten, om met 100% zekerheid vast te stellen dat deze behandeling nuttig is. Aangezien de angiogene disbalans in PE te wijten is aan verhoogde sFlt-1- en verlaagde - en VEGF-spiegels, is toediening van PlGF of VEGF eveneens een optie. In een recente studie, waarbij het rattenmodel met verminderde baarmoederperfusiedruk (RUPP) werd gebruikt, leidde humaan PlGF, toegediend via intraperitoneale osmotische minipompjes vanaf dag 14 van de zwangerschap tot een lagere bloeddruk, een hogere glomerulaire filtratiesnelheid, verlaagde sFlt-1-spiegels en verminderde oxidatieve stress op dag 19 van de zwangerschap in vergelijking met de controle-groep.[13] Deze bevindingen suggereren de mogelijkheid om PE te behandelen met PlGF. Onduidelijk is of de toediening van PlGF bijwerkingen heeft, in het bijzonder met betrekking tot nog niet-opgemerkte kanker. In tumorcellen speelt PlGF-expressie een rol bij de angiogene switch die de tumorvascularisatie initieert.[14] Bovendien is in diverse vormen van kanker een correlatie gevonden tussen de mate van PlGF-expressie en de ernst van de kanker.

Een recente meta-analyse heeft laten zien dat regelmatige fysieke activiteit tijdens de zwangerschap onder het niveau van de anaerobe-drempel in vergelijking met een meer sedentaire levensstijl beginnend voor de 23e week, geassocieerd is met een significant lager risico op globale hypertensieve aandoeningen, zwangerschapshypertensie en op bevallingen door middel van een keizersnede, alsook een trend voor een verminderde incidentie van PE (relatief risico 0.79).[15] Een intrigerende vraag is of regelmatige fysieke activiteit tijdens of zelfs voor de zwangerschap ook gepaard gaat met gunstiger angiogene balans. Voor zover we weten heeft één cross-sectionele studie laten zien dat zwangere vrouwen die regelmatig anaerobische oefeningen doen (≥ 3 uur per week), laat in de zwangerschap lagere circulerende sFlt-1-, oplosbare endogline- en hogere PlGF-spiegels hebben dan zwangeren met relatief weinig fysieke activiteit.[16] Op grond van deze bevindingen lijkt het zinvol om prospectieve gerandomiseerde studies op te zetten met fysieke activiteit op verschillende intensiteitsniveaus om te onderzoeken of regelmatige fysieke inspanning tijdens de vroege zwangerschap of zelfs voor de conceptie gunstige effecten heeft op sFlt-1- en PlGF spiegels en belangrijker of dit zich vertaald in een lagere PE-incidentie en verbeterde maternale en foetale/neonatale uitkomsten bij zwangerschappen met een verhoogde kans op PE-gerelateerde complicaties.

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About the author

PhD Portfolio

Dankwoord

OVER DE AUTEUR / ABOUT THE AUTHOR

Langeza Saleh is geboren op 20 oktober 1990 te Slemani, Koerdistan. Op 9-jarige leeftijd kwam ze in Nederland te wonen. Na het voltooien van de middelbare school aan de scholengemeenschap Pantarijn te Wageningen, startte zij in 2010 met de opleiding geneeskunde aan de Erasmus Universiteit van Rotterdam. Vanwege haar vroege interesse in wetenschappelijk onderzoek sloot ze in het tweede jaar zich aan bij het onderzoeksgroep van dr. Willy Visser. En zo geschiedde: binnen het onderzoeksgroep van prof. Danser resulteerde deze interesse in een promotietraject. In 2017 is ze gestart met haar coschappen en volgt ze wegens belangstelling voor zorgmanagement tevens de master "Health Care Management" aan de Erasmus Universiteit.

Langeza Saleh was born on October 20th 1990 in Sulaymaniyah, Iraqi Kurdistan. She moved to the Netherlands when she was nine years old. After completing secondary school she started Medicine in 2010 at the Erasmus University, Rotterdam. During her second year she started participating in research under the supervision of dr. Willy Visser. Thuis lead to a PhD trajectory resulting in this thesis. In 2017 she started her internships and due to her interest in the healthcare management she recently started a master in 'Health Care Management' at Erasmus University, Rotterdam.

ERASMUS MC PhD PORTFOLIO

Name of PhD student: Langeza Saleh
Erasmus MC: Dept. of Internal Medicine and dept. of Obstetrics and Gynecology
Div. of Vascular Medicine and Pharmacology
PhD period: September 2014 - June 2017
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Prof.dr. Eric A.P. Steegers
Co-promotors: Dr. Willy Visser
Dr. Anton H. van den Meiracker

Training	Year(s)	ECTS
Courses		
Biomedical English writing and communication	2015	4.0
Winterschool Dutch Kidney Foundation	2015	2.0
Coeur clinical cardiovascular epidemiology	2015	2.0
MolMed Photoshop & Illustator CS6 Workshop	2015	0.3
CPO course: patient oriented research: design, conduct, analysis and clinical implications	2015	0.3
Coeur Cardiovascular Medicine	2016	1.5
Coeur Cardiovascular Pharmacology	2016	1.5
Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK)	2016	1.5
Molmed R Statistic Course	2016	1.8
Research Integrity	2017	1.0
Congress visits – oral presentation		
Pregnancy Summit, London	2014	0.7
NVOG Dutch Gynecology congress, Amersfoort	2015	0.7
European Society of Hypertension, Milan	2015	0.7
International Society for the Study of	2015	0.7
Hypertension in Pregnancy, Budapest		
Science Days Internal Medicine, Antwerp	2016	0.7
European Society of Hypertension, Paris	2016	0.7
International Society for the Study of	2015	0.7
Hypertension in Pregnancy, Sao Paulo	2016	0.7

Dutch Hypertension Society, Amersfoort	2017	0.7
European Society of Hypertension, Milan	2017	0.7
Council on Hypertension, San Francisco	2017	0.7
Congress visits – poster presentation		
European Society of Hypertension, Milan	2015	0.7
Council on Hypertension, Washington D.C.	2015	0.7
International Society for the Study of Hypertension in Pregnancy, Budapest	2015	0.7
Science Days Internal Medicine, Antwerp	2017	0.7
European Society of Hypertension, Paris	2016	0.7
International Society for the Study of	2015	0.7
Hypertension in Pregnancy, Sao Paulo	2016	0.7
Medical curriculum and (co-)supervision of research projects		
First year medical students, Pharmacology practicum	2015-2017	3.6
Mohammed Saadulla - MSc student	2015	1.0
Omar Salem - MSc student	2015	1.0
Mona Mahjour - MSc student	2016	1.0
Roos Geensen - MSc student	2016	1.0
Ruschil Rambhadjan - MSc student	2016	1.0
Anne Koppelaar - MSc student	2017	1.0
Raaho Samantar - MSc student	2017	1.0
Sarea Tahitu - MSc student	2017	1.0
Aysel Polat-Cagli - Clinical midwife specialist	2017	2.0
Jury in the Junior Med School interviews	2017	
Madena Shareef - MSc student	2018	
Research grants		
KNAW van Walree Beurs, personal grant (€1000)	2015	
PRERISK PhD grant, co-applicant: Grant for 0.5 PhD student in our group and all blood sample measurements during the study period	2017	
Award, prizes, travel grants		
Pregnancy Summit Award	2014	
Alberto Ferrari Prize, European Society of Hypertension	2015	
Trustfonds Conference grant	2015	

ESH travel and accommodation grant	2015
Poster Prize, European Society of Hypertension	2016
Trustfonds Conference grant	2016
NVF Conference grant	2016
ESH travel and accommodation grant	2016
Travel Award, World Congress of ISSHP	2016
Roche Conference grant	2016
ESH travel and accommodation grant	2017
American Hypertension Council, best rated abstract	2017
NVF Conference grant	2017
Trustfonds Conference grant	2017

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گەورەقەڵایەکی و پشتووپەنایەکی بەهێزبوون بۆ ژیانم ،
دارعاسا بوون بۆ ماندوبوون و جامێک ئاو بوون بۆ تینوێتیم ،
چرایەک بوون بۆ ڕووناکی لەشەوە تاریکەکان بۆیە سوپاسی بێ پایانم هەیە بۆیان
ئەوبۆ کاتەی کە پێیان بەخشیم تابگەم بەم قۆناغەی تەمەنم!