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ANGIOGENIC PROFILE IN THE FINNISH GENETICS OF PRE-ECLAMPSIA CONSORTIUM (FINNPEC) COHORT

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Abstract

Objectives: To study first and second/third trimester levels of soluble fms-like tyrosine kinase 1 (sFlt1), placental growth factor (PlGF) and soluble endoglin (sEng) in (FINNPEC case-control cohort. The participants were further divided into subgroups based on parity and onset of the disease. Recommended cut-off values in aid of pre-eclampsia prediction and diagnosis were also tested.

Methods: First trimester serum samples were available from 221 women who later developed pre-eclampsia and 239 women who did not develop pre-eclampsia. Second/third trimester serum samples were available from 175 pre-eclamptic and 55 non pre-eclamptic women. sFlt-1 and PlGF were measured electro-chemiluminescence immunoassays and sEng by ELISA.

Results: In all timepoints PlGF, endoglin and the sFlt-1/PlGF ratio were increased in the PE group compared to the non-PE group. The serum concentrations of sFlt-1 were increased only at second/third trimester in PE women. Higher concentrations of s-Flt1, endoglin and higher sFlt/PlGF ratio were found at the third trimester in primiparous women compared to multiparous women. Primiparous PE women also had lower concentrations of PlGF at the third trimester. The proportion of women exceeding all cut-offs of the sFlt-1/PlGF ratio (≥33, ≥38, ≥85 and ≥110) was greater in the PE group, but there were also pre-eclamptic women who met rule-out cut-off or did not meet rule-in cut-off.

Conclusions: Primiparous pregnancies have more anti-angiogenic profile during second/third trimester compared with multiparous pregnancies. Our findings also suggest that certain maternal characteristics, e.g. BMI, smoking and pre-existing diseases, should be taken into account when different sFlt-1/PlGF ratio cut-offs are utilized.

1.Introduction

Pre-eclampsia (PE) is a complex pregnancy disorder, defined by new-onset hypertension and proteinuria after 20 weeks of gestation, or new onset preeclampsia-associated signs in the absence of proteinuria [1]. Currently there is no treatment for PE other than delivery, which often leads to premature birth due to indicated delivery. As one of the major conditions causing maternal and fetal morbidity and mortality, it is a global challenge for maternal and fetal health care providers [2]. Due to incomplete understanding of the pathogenesis and subtypes of this heterogeneous disorder a development of predictive tools, prevention and treatments poses a challenge.

An imbalance of maternal proangiogenic placental growth factor (PlGF) and antiangiogenic soluble fms-like tyrosine kinase 1 (sFlt-1) has been implicated in the prediction and outcomes of PE [2-4]. The sFlt-1/PlGF ratio is elevated in patients with PE and it is better predictor of the early-onset disease (delivery < 34 weeks of gestation) than the late-onset disease [3,5]. It has been suggested that syncytiotrophoblast stress contributes to the angiogenic imbalance especially in the early-onset disease with poor placentation [6] but angiogenic markers are released also from maternal sources [7]. With accumulating evidence for the importance of angiogenic markers as a diagnostic and prognostic marker, different cut-offs have also been defined to allow an assessment of PE [**Fig. 1**, 2,4,8,9].

In this nested case-control study we investigated first and second/third trimester levels of sFlt-1, PlGF and their ratio in addition to soluble endoglin in the Finnish Genetics of Pre-eclampsia Consortium (FINNPEC) case-control cohort. The participants were further divided into subgroups based on onset of the disease and parity. We also tested recently recommended rule-out cut-off value 33 (20 weeks to delivery), rule-in cut-offs 85 (ad 33weeks 6days) and 110 (34 weeks to delivery) for the Elecsys immunoassay sFlt-1/PlGF ratio [10]. Furthermore, we tested a cut-off for

the sFlt-1/PlGF ratio that was presented recently in the PROGNOSIS study [2]. Zeisler et al. [2] derived a single cut-off value independent of the weeks of gestation; values below 38 were considered negative and were used to rule-out PE within 1 week after assessment of the ratio.

Moreover, data on pregnancy associated placental protein A (PAPP-A) and beta human chorionic gonadotrophin (ß-hCG) were measured in the first trimester biochemical screening for fetal chromosomal abnormalities.

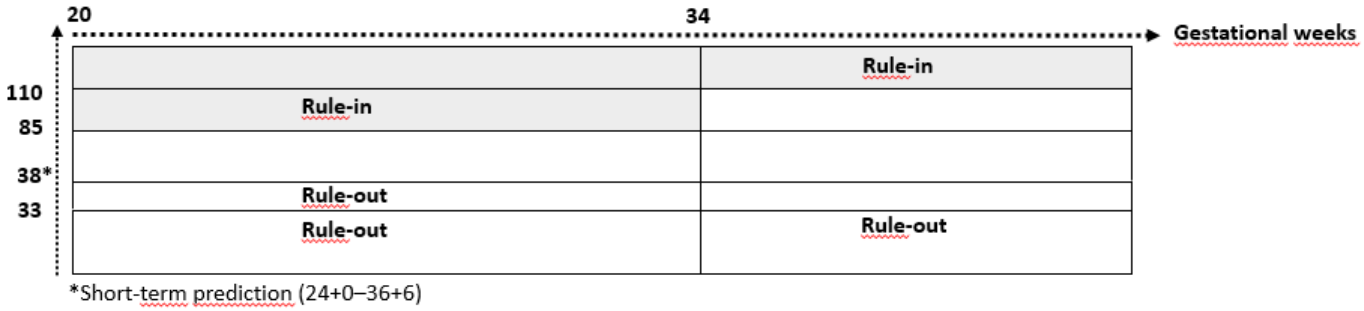

Fig. 1. The sFlt-1/PlGF ratio cut-offs recommended in aid of pre-eclampsia prediction and diagnosis.

2.Methods

2.1. Design

FINNPEC is a cross-sectional case-control multicentre study with an established nationwide clinical and DNA database on PE women and women without PE, including their partners and infants in order to identify genetic risk factors for PE. Details of the study design, methods and procedures have been described elsewhere [11]. With this study we aimed to investigate whether maternal serum concentrations of sFlt-1, PlGF, endoglin and sFlt-1/PlGF ratio available from a subset associate with PE and clinical subtypes.

2.2. Study subjects

Originally 1450 patients with PE and 1065 control women without PE were recruited at the 5 Finnish university hospitals. In this study, we focused on a prospective arm and on a subset of those women from whom first and second/third trimester serum samples were available. All PE women were already diagnosed at recruitment and second/third trimester serum samples were also drawn at this timepoint. All participants provided written informed consent, and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa.

2.2.1 Inclusion criteria

Nulliparous or multiparous women with a singleton pregnancy were eligible for the study. PE was defined as hypertension and proteinuria occurring after 20 weeks of gestation. Hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg and proteinuria as the urinary excretion of ≥0.3 g protein in a 24-hour specimen, or 0.3g/l, or two ≥1+ readings on dipstick in a random urine determination with no evidence of the urinary tract infection. Each diagnosis was ascertained based on hospital records and confirmed independently by a research nurse and a study physician.

2.2.2. Exclusion criteria

Exclusion criteria were multiple pregnancy, maternal age less than 18 years and inability to provide an informed consent based on information in Finnish or Swedish.

2.3. Background, obstetric and perinatal data

Extensive information on pregnancy complications, pregnancy outcome, proteinuria, blood pressure, laboratory measurements, delivery and baby was obtained from the hospital records and maternity cards. Information on PE in previous pregnancies was verified from the hospital records.

PE was defined as early-onset when delivery occurred before 34+0 weeks of gestation and late-onset when at 34+0 weeks of gestation or later. Birth weights below −2.0 SD units were classified as small-for-gestational age (SGA) according to Finnish standards [12].

Hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome was diagnosed when at least 2 of the following criteria were met: lactate dehydrogenase **(**LD) ≥ 235 U/l, alanine aminotransferase (ALAT) ≥ 70 U/l, aspartate aminotransferase (ASAT) ≥ 70 U/l, and thrombocytes ≤ 100 E9/l.

2.4. Serum samples and angiogenic markers

First and second/third trimester serum samples were collected from a subcohort from the Hospital District of Helsinki and Uusimaa. First trimester serum samples were obtained via first trimester biochemical screening for fetal chromosome abnormalities (range 9-15 weeks of gestation), and during the second and third trimesters (range 20-42 weeks of gestation) serum samples were collected at hospitals. The results on angiogenic markers measured from samples obtained during the second and third trimesters were further divided into early/late based on the timing of the blood sampling (early: 20-33+6 weeks of gestation and late sample ≥34 weeks of gestation).

Maternal serum sFlt-1 and PlGF concentrations were measured using sFlt-1 and PlGF electro-chemiluminescence immunoassays (ECLIA; Roche Diagnostics GmbH, Mannheim, Germany) on cobas e 601 analyzer (Hitachi High Technology Co, Tokyo, Japan). Serum concentration of endoglin (CD105) was measured using human Quantikine Endoglin ELISA kit (R&D Systems, UK) according to manufacturer's instructions.

Pregnancy associated placental protein A (PAPP-A) and beta human chorionic gonadotrophin (ß-hCG) were analyzed by time-resolved fluoroimmunoassay according to manufacturer's instructions (PerkinElmer, Wallac, Turku, Finland).

2.5. Statistical analysis

Statistical tests were performed with IBM SPSS Statistics version 24. The normality of variable distributions was verified with the Kolmogorov–Smirnov test. Logarithmic transformation was used when appropriate. Each biomarker was ln-transformed to correct for right-skewness, and estimated means were back-transformed as geometric means and 95% confidence intervals for purposes of presentation. For the continuous variables, comparisons between groups were analysed with general linear model univariate ANOVA at baseline and with linear mixed models during the pregnancy. Selected co-variables [parity, maternal age, smoking status, body mass index (BMI), gestational weeks at sampling] were included in the models as covariates. Normality was assessed by plotting the residuals.

For the categorical variables, the comparisons were performed with the Fisher's exact test. With skewed distributions, comparisons between continuous variables were performed by the Mann Whitney U-test.

3. Results

As published earlier for the whole FINNPEC population [11], in this subcohort the PE women had higher BMI and more frequently certain preexisting medical conditions (e.g. chronic hypertension, pregestational diabetes) and gestational diabetes than controls (**Table 1**). The proportion of primiparous women was also higher in the PE group **(Table 1).** At first trimester screening, both PAPP-A and ß-hCG concentrations were lower in the PE group compared to the control group.

Serum concentrations of angiogenic markers are presented in **Table 2**. In all timepoints PlGF, endoglin and the sFlt-1/PlGF ratio were increased in the PE group compared to the control group. The serum concentrations of sFlt-1 was increased only at second/third trimester in PE women compared to non-PE women. The proportion of women exceeding all cut-offs of the sFlt-1/PlGF ratio (≥33, ≥38, ≥85 and ≥110) was greater in the PE group (**Table 2**). However, there were also pre-eclamptic women who met NICE rule-out cut-off 33 at second/third trimester (n=10) or did not meet rule-in cut-off 85 (ad 33weeks 6days) (n=8) and 110 (n=4) (34 weeks to delivery). Ten PE women who did not exceed rule-out cut-off 33 had higher prepregnancy BMI, smoked more before pregnancy, suffered more from renal disease, had more often a history of PE and the relative birth weight of the newborn was higher (**Table 3a**). Moreover, eight PE women did not exceed cut-off 85. The basic characteristics of these women are presented in **Table 3b**. Also these PE women had a trend for higher prepregnancy BMI and the relative birth weight of the newborn was higher. There were four control women who exceeded the cut-off 110 and the comparison of these women with or without exceeding cut-off 110 is presented in **Table 3c**. These four women suffered more from placental insufficiency and had a trend for having SGA babies. Three of these control women exceeded the rule-in cut-off already before 34 weeks of gestation.

There where altogether 49 PE women of whom serum samples were available one week before the delivery. Of those women, only one woman did not exceed cut-off 38. In addition, there were four control women who exceeded cut-off 38 within a week of the delivery. Two of these women had gestational hypertension and SGA baby.

There were altogether 18 women with the HELLP syndrome (5.8%) of whom first and/or third serum samples were available. These women fulfilled also PE criteria, 7 of them had early- and 11 late-onset disease. PE women with HELLP syndrome had higher concentration of second/third

trimester sFlt-1 and sFlt-1/PlGF ratio when compared with PE women without HELLP syndrome (data not shown).

Onset of PE

Table 4 shows the comparison of angiogenic markers between women with early- or late-onset PE and controls. At the first trimester those women with early-onset disease had decreased concentrations of PlGF and increased concentrations of endoglin compared to women with late-onset disease. At second/third trimester women in the early-onset disease had highest concentrations of sFlt-1, endoglin and elevated sFlt-1/PlGF ratio. There was no difference in the proportions of women with early- or late-onset disease exceeding the cut-off values of 33 or 38 (**Table 4**). Both cut-offs 85 and 110 covered greater proportion of PE women with early-onset disease compared to women with late-onset disease. However, when analyses were adjusted for parity, BMI, mother's age, gestational weeks at sampling and smoking status during pregnancy, the difference was not significant.

Parity

Primiparous PE women had lower second/third trimester PlGF concentration and higher concentrations of sFlt-1 and endoglin compared to multiparous PE women (**Table 5**). Primiparous PE women had also had higher sFlt-1/PlGF ratio at the second/third trimester. Within the control group, similar differences between primi- and multiparous women were only observed for sFlt-1 and sFlt-1/PlGF ratio at the second/third trimester.

There were greater proportion of primiparous PE women exceeding cut-offs 33 and 38 at the third trimester compared to multiparous PE women. In addition, there were more primiparous women exceeding cut-offs 85 and 110 after 34 weeks of gestation than multiparous PE women. There were no similar differences within control group.

4. Discussion

In this study we found that primiparous pregnancies had more anti-angiogenic profile during second/third trimester compared with multiparous pregnancies. Our findings also suggest that certain maternal characteristics should be taken into account when different sFlt-1/PlGF ratio cut-offs are utilized in the clinic. For instance, PE women who met NICE sFlt-1/PlGF ratio rule-out cut-off 33 at second/third trimester had higher prepregnancy BMI, smoked more before pregnancy, and had more often renal disease and history of PE.

In general, our results support the increasing evidence that the pathogenesis of PE involves an imbalance between angiogenic and anti-angiogenic markers. Serum concentrations of sFlt-1, endoglin and sFlt-1/PlGF ratio levels are usually found to be higher and PlGF levels lower in the PE pregnancies, but absolute levels vary markedly between studies [2, 12, 13]. However, these factors have also limitations as PE biomarkers, especially for prediction and diagnosis of PE at term [14].

Maternal ß-hCG and PAPP-A have also gained acceptance as potential predictors of PE at the first trimester [15]. In the current study, we were able to confirm all these previously observed differences in angiogenic markers except in higher sFlt-1 concentration at first trimester.

Onset of PE

In line with the previous literature [16] we found increased serum endoglin at second/third trimester to be associated with early-onset disease. However, to our knowledge, this is the first study to show increased serum endoglin concentration in the early-onset PE already at first trimester. At the second/third trimester sFlt-1 concentration and s-Flt1/PlGF ratio were also higher in PE women with early-onset disease compared to the late-onset group. Furthermore, serum PlGF concentration was lower at the first trimester in women who later developed early-onset PE compared to women who had late-onset PE and compared to controls. PlGF is thought to exert a direct pro-angiogenic

activity, and it has been demonstrated that the levels of PlGF are decreased early in pregnancies later complicated by PE [17]. Khalil et al. [18] have also shown that maternal serum PlGF is a useful marker for PE from the first trimester onward, while the level of sFlt-1 is likely to have a predictive value from the second trimester onward. Furthermore, it has been demonstrated that serum PlGF is lower in early-onset PE than in late-onset PE, with no difference in serum sFlt-1 [19]. Our finding that the second/third trimester sFlt-1/PlGF ratio was higher in early-onset PE is in agreement with previous findings [3,20,21]. There were no differences in the number women in the early- and the late onset groups exceeding cut-offs of 33 or 38. Accordingly, in this study sFlt-1 concentration and sFlt-1/PlGF ratio were not associated with early-onset PE at first trimester which is in contrast to some of the previous studies [22,23]. Whereas there were greater proportions of women in the early- onset group exceeding cut-offs 85 and 110. These finding highlight the theory that early- and late-onset PE have different pathophysiologic pathways. In late-onset PE there is a broader spectrum of involved mechanisms possibly due to underlying maternal conditions and endothelial injury whereas early-onset PE is more closely related to placental dysfunction and angiogenic imbalance [24-26].

Early-onset PE is also associated with higher perinatal and maternal morbidity and mortality than late-onset disease, mainly due to gestational age at delivery [27]. However, very recently Christensen [28] showed that particularly gestational age at PE onset including the early-onset/late-onset distinction was associated with subclinical atherosclerosis 12 years after delivery. However, more studies on the predictive role of angiogenic markers in augmented risk for future cardiovascular disease are warranted.

The use of angiogenic markers in the first-trimester prediction of early-onset PE would be clinically relevant since low-dose aspirin started before 16 weeks' gestation may be effective in the prevention of the disease or postponing the onset of the disease [29].

Parity

Nulliparity is a well-known risk factor for PE with a reported incidence of up to 2–3 times higher than in multiparous pregnancies [30]. The mechanisms explaining this epidemiological observation have been postulated to involve e.g. immune maladaptation and greater insulin resistance [31]. There are relatively few studies on the association between parity and circulating angiogenic markers. Higher sFlt1 levels have been reported in the first and second trimester of nulliparous women [32, 33]. Furthermore, Bdolah et al. [34] have shown that nulliparous women have higher circulating sFlt1 concentration and sFlt1/PlGF ratio than multiparous women during the late third trimester. Consistent with Bdolah et al. [34], we also demonstrated higher concentration of s-Flt1 and higher sFlt/PlGF ratio at the third trimester in primiparous PE and control women compared to multiparous women. We also observed higher levels of circulating endoglin in primiparous PE women at the third trimester. Furthermore, primiparous PE women had lower concentrations of PlGF at the third trimester compared to multiparous women. It is notable that these differences were observed in analyses adjusted for most potential confounding factors (maternal BMI, age, smoking status and gestational weeks in sampling). There were also greater proportion of primiparous PE women exceeding all the cut-offs at third trimester compared to multiparous PE women, but there were no differences between primi- and multiparous PE women in cut-offs 85 or 110 if sample was obtained before 34 weeks of gestation. All these findings add further support to previous speculations that altered angiogenic profile may be a potential molecular mechanism that explains the link between PE and nulliparity. More studies are needed to understand the causal mechanism behind the phenomenon.

Cut-offs

Although there are no formal guidelines regarding the use of the sFlt-1/PlGF ratio, consensus statements have been developed by international experts on the clinical use of the Elecsys immunoassay sFlt-1/PlGF ratio. Previously it has been shown that not all women complicated with PE have altered pro- and anti-angiogenic profile [35]. Accordingly, we observed PE women (n=10) who met NICE rule-out cut-off 33 at second/third trimester. They had higher BMI, smoked more before pregnancy and suffered more from renal disease. Moreover, eight pre-eclamptic women who did not exceed cut-off 85 had a trend for higher BMI. Although the current guidelines are not based on women with clinically confirmed PE, as in our study design, we suggest that certain maternal characteristics, particularly BMI, should be taken account when different sFlt-1/PlGF ratio cut-offs are utilized in the clinic. We have recently demonstrated within the same cohort that smoking before and during pregnancy may complicate the use of angiogenic markers as a prognostic and diagnostic marker [36]. Furthermore, there are a few studies indicating that an imbalance of angiogenic markers is mild in obese pregnant women [32,37].

We conclude that the imbalance of pro- and anti-angiogenic markers is unlikely to be a primary pathophysiologic feature of PE in those women and there indeed may be angiogenic and non-angiogenic forms of PE as suggested earlier [26, 35, 38]. The findings that there were controls e.g. with placental insufficiency exceeding the cut-offs implicate that angiogenic imbalance is not exclusively limited to PE pregnancies. Obviously we cannot exclude the possibility that these women had developed PE later if the pregnancy had continued.

Zeisler et al. [2] have derived a cut-off value of 38 to serve as an aid in the short-term prediction of PE. They proposed this single cut-off rules out PE within one week. In our study, only one PE woman did not exceed cut-off 38 before the delivery. However, it was notable that there were four control women who exceeded cut-off 38 a week before the delivery.

5. Strengths and limitations

Our study has several strengths. A major strength of this study is its prospective cohort design with detailed clinical outcome information allowing to define accurately the phenotypes. In future, the combination of the angiogenic markers with clinical characteristics may substantially improve PE prediction. Detailed phenotyping enabled us to involve various adjustments for maternal characteristics and highlighted the importance of selection of clinical covariates when analysing the role of angiogenic markers in the etiology PE.

Our study has certain limitations. The sample size was limited especially when subdividing into categories. It should also be noticed that PE and control groups were not matched for gestation at sampling although this was taken account in the statistical analyses. Furthermore, the gestation at sampling for the first trimester blood samples varied from 9 to 15 weeks and this relatively wide time period might have affected e.g. the concentrations observed for sFlt-1. Moreover, there was only limited number of samples available from the second trimester. However, samples from first and second/third trimester within this study make possible to analyse changes over time.

6. Conclusions

In conclusion, we were able to confirm previously observed differences in angiogenic markers except in higher sFlt-1 concentration at first trimester. In future, special attention should be aimed to disentangle the role of angiogenic markers within the different maternal characteristics.

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Table 1. Maternal and perinatal characteristics of pre-eclamptic and control groups in a subset of the FINNPEC women.

Maternal or Perinatal Characteristics	**Pre-eclampsia (n=221)**	**Control (n=239)**	***p****	***p****
Age at delivery (y)	30.3 ± 5.3	30.5 ± 4.8	0.495***	0.661[b]
Nulliparous, n (%)	166 (75.1%)	137 (57.3%)	**<0.001**	
BMI, kg/m^2 (self-reported, pre-pregnancy)	25.5 ± 5.1	23.9 ± 3.9	**0.005*** **	**<0.001[c]**
PAPP-A (mU/l)[j]	1455 ± 1193 (214)	1952 ± 1209 (236)	**<0.001*** **	**<0.001[a]**
Beta hCG (ug/l)[j]	45.3 ± 30.1 (214)	54.4 ± 40.3 (236)	**0.038**	**0.005[a]**
Highest systolic blood pressure (mm Hg)[h]	165 ± 17	127 ± 14	**<0.001*** **	**<0.001**
Highest diastolic blood pressure (mm Hg)	110 ± 9	85 ± 10	**<0.001*** **	**<0.001**
Proteinuria (max.) (g/24 h)	3.7 ± 3.2 (211)	-	-	
Smoking before pregnancy	58 (26.9%) (216)	57 (23,9%) (238)	**0.003**	
Smoking during pregnancy	24 (11.0%) (218)	20 (8.4%) (238)	**0.005**	
Chronic hypertension[f]	33 (14.9%)	8 (3.3.%)	**<0.001**	
Gestational hypertension[g]	-	21 (8.8%)	**-**	
Gestational diabetes mellitus	28 (12.7%)	13 (5.4.%)	**0.007**	
Pregestational diabetes mellitus	12 (5.4%)	1 (0.4%)	**0.001**	
Type 1 diabetes	11 (5.0%)	1 (0.4%)		
Type 2 diabetes	1 (0.5%)	-		
Mode of delivery	133 (60.2%)88 (39.8%)		**<0.001**	
Vaginal		197 (82.7%)		
Caesarean section		41 (17.3%) (238)		
Gestational weeks at delivery	36.8 ± 3.3	39.3 ± 2.6	**<0.001**	
Birth weight, g	2792 ± 851	3476 ± 717	**<0.001*** **	**0.001[e]**
Relative birth weight (SD)	-0.9 ± 1.3	-0.2 ± 1.2	**<0.001**	**0.003[e]**
Fetal sex, female/male (%)	52.9%/47.1%	55.6%/44.4%	**0.042**	
SGA	35 (15.8%)	18 (7.5%)	**0.005**	

*unadjusted; **adjusted; ***non-parametric tests used

()number of available information/samples unless from all.

Data are presented as mean ± S.D or percentages

[a]adjusted for gestational weeks at sampling

[b]adjusted for parity

[c]adjusted for parity, mother's age at birth

[d]adjusted for parity, mother's age at birth, prepregnancy BMI

[e]adjusted for parity, mother's age at birth, gestation weeks, prepregnancy BMI, chronic hypertension, gestational diabetes, pregestational diabetes mellitus
[f]systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg detected before 20 weeks of gestation.
[g]blood pressure ≥140/90, no proteinuria
[h]when highest diastolic value recorded
[i]based on weight and height before pregnancy, self-reported at first antenatal visit.
[j]PAPP-A and beta hCG samples were obtained at 9-15 weeks of gestation.
BMI= body mass index, PAPP-A= placental protein A, ß-hCG= beta human chorionic gonadotrophin,SGA small-for-gestational age

Table 2. Concentrations of angiogenic markers in pre-eclamptic and control women of FINNPEC, geometric mean (95% CI).

	Pre-eclampsia **I trimester n=221** **II/III trimester n=175** Early sample (20-33+6), n=48 Late sample (>34), n=127	**Control** **I trimester n=239** **II/III trimester n=55** Early sample (20-33+6), n=9 Late sample (≥34), n=46	***p**unadj.*	***p*adj.***
sFlt-1 (pg/ml) I trimester II-III trimester	 1303.9 (1232.3-1379.5) (n=220) 10356.8 (9403.2-11406.1) (n=175)	 1388.1 (1322.0-1457.4) 4278.1 (3621.7-5053.8)	 0.097 **<0.001**	 0.349 **<0.001**
PlGF (pg/ml) I trimester II-III trimester	 31.3 (29.5-33.2) (n=220) 81.0 (73.5-89.4)	 41.8 (39.8-43.9) 164.6 (130.7-207.3)	 **<0.001** **<0.001**	 **<0.001** **<0.001**
Endoglin (ng/ml) I trimester II-III trimester	 5.9 (5.7-6.1) (n=220) 41.7 (37.7-46.2)	 5.6 (5.4-5.8) 16.6 (14.0-19.6)	 **0.035** **<0.001**	 **0.005** **<0.001**
sFlt-1/PlGF I trimester II-III trimester	 42 (39-45) (n=220) 188 ± 180[b]	 33 (31-35) 45 ± 47[b]	 **<0.001** **<0.001*****	 **<0.001** na

sFlt-1/PlGF ratio, ≥33 II-III trimester	165 (94.3%)	22 (40.0%)	**<0.001**[c]	**<0.001**[a]
sFlt-1/PlGF ratio, ≥38 II-III trimester	160 (91.4%)	20 (36.4%)	**<0.001**[c]	**<0.001**[a]
sFlt-1/PlGF ratio, ≥85 rule-in II-III trimester Early sample (20-33+6)	123 (70.3%) 40 (83.3%)	10 (18.2%) 3 (37.5%)	**<0.001**[c] **0.012**[c]	**<0.001**[a] **0.004**[a]
sFlt-1/PlGF ratio, ≥110 rule-in II-III trimester Late sample (≥34)	110 (62.9%) 71 (55.9%)	7 (12.7%) 4 (8.7%)	**<0.001**[c] **<0.001**[c]	**<0.001**[a] **<0.001**[a]

()number of available information/samples unless from all Data

are presented as geometric mean (95%CI) or percentages

*adjusted for parity, prepregnancy BMI, mother's age at birth, gestational weeks at sampling, smoking status during pregnancy
***Mann-Whitney U-test
[a] logistic regression adjusted for parity, prepregnancy BMI, mother's age at birth, gestational weeks at sampling, smoking status during pregnancy
[b]arithmetic mean ± SD
[c]$\chi 2$ –test
na=test not applicable

Table 3a. Characteristics of pre-eclamptic women according to the sFlt-1/PlGF ratio cut-off 33.

	II-III trim sFlt-1/PlGF ≤33 in pre-eclamptic (n=10)	II-III trim sFlt-1/PlGF >33 in pre-eclamptic (n=165)	p^a
Age	29.8 ± 5.3	31.2 ± 5.9	0.425
Prepregnancy BMI	29.2 ± 5.4	25.3 ± 5.6	**0.020**
Parity	2.1 ± 2.7	0.4 ± 1.4	**0.001**
Previous PE	5 (50.0%)	23 (13.9%)	**0.003**
Smoking before pregnancy	7 (70.0%)	37 (22.4%)	**0.001**
Smoking during pregnancy	1 (10.0%)	12 (7.3%)	0.754
Placental insufficiency	0 (0.0%)	25 (15.2%)	0.184
Renal disease	4 (40.0%)	7 (4.2%)	**<0.001**
Chronic hypertension	3 (30.0%)	33 (20.0%)	0.447
Birth weight	3445.0 ± 775.7	2546.6 ± 877.4	**<0.001**
Relative birth weight, SD	0.6 ± 0.9	-1.2 ± 1.3	**<0.001**
SGA	0 (0.0%)	42 (25.5%)	0.067
Gestational weeks of delivery	37.1 ± 3.1	36.0 ± 3.4	**<0.001**

Table 3b. Characteristics of pre-eclamptic women according to the sFlt-1/PlGF ratio cut-off 85.

	II-III trim (20-33+6 gwks) sFlt-1/PlGF ≤85 in pre-eclamptic (n=8)	II-III trim (20-33+6 gwks) sFlt-1/PlGF >85 in pre-eclamptic (n=40)	p^a
Age	30.0 ± 5.7	31.4 ± 6.1	0.562
Prepregnancy BMI	28.9 ± 5.0	25.8 ± 5.7	0.057
Parity	0.6 ± 1.4	0.5 ± 1.9	0.862
Previous PE	1 (12.5%)	4 (10.0%)	1.000
Smoking before pregnancy	3 (37.5%)	10 (25.0%)	0.664
Smoking during pregnancy	2 (25.0%)	4 (10.0%)	0.258
Placental insufficiency	2 (25.0%)	18 (45.0%)	0.440
Renal disease	1 (12.5%)	2 (5.0%)	0.429
Chronic hypertension	2 (25.0%)	14 (35.0%)	0.701
Birth weight	1976.3 ± 723.1	1469.4 ± 544.6	**0.028**

Relative birth weight, SD	-1.0 ± 0.9	-2.1 ± 1.0	**0.004**
SGA	1 (12.5%)	21 (52.5%)	0.055
Gestational weeks of delivery	32.4 ± 4.0	31.4 ± 2.7	0.134

Table 3c. Characteristics of controls according to the sFlt-1/PlGF ratio cut-off 110.

	II-III trim (>34 gwks) sFlt-1/PlGF > 110 in controls (n=4)	II-III trim (>34 gwks) sFlt-1/PlGF ≤ 110 in controls (n=42)	p^a
Age	33.0 ± 9.4	31.1 ± 4.8	0.395
Prepregnancy BMI	26.7 ± 3.4	24.2 ± 3.5	0.160
Parity	0.5 ± 1.0	0.5 ± 0.7	0.948
Previous PE	0 (0%)	0 (0%)	-
Smoking before pregnancy	2 (50.0%)	14 (33.3%)	0.602
Smoking during pregnancy	0 (0%)	3 (7.1%)	0.580
Placental insufficiency	2 (50.0%)	2 (4.8%)	**0.033**
Renal disease	0	1 (2.4%)	0.755
Chronic hypertension	1 (25.0%)	1 (2.4%)	0.168
Birth weight	2849.0 ± 1167.2	3596.8 ± 561.9	0.315
Relative birth weight, SD	-1.3 ± 1.9	0.1 ± 1.1	0.245
SGA	2 (50.0%)	3 (7.1%)	0.053
Gestational weeks of delivery	38.3 ± 3.8	39.6 ± 1.7	0.585

[a]non-parametric test

Table 4. Concentrations of angiogenic markers in pre-eclamptic (early and late onset) and control women, geometric mean (95% CI).

Maternal or Perinatal Characteristics	**Pre-eclampsia** **Early onset** (delivery ≤ 34 weeks of gestation)	**Late onset**	***p*adj. early vs. late***	**Control**	***p*adj. early vs. control***	***p*adj. late vs. control***
sFlt-1(pg/ml) I trimester II-III trimester	1274 (1076 – 1495) (33) 13227 (10509 – 16548) (37)	1313 (1233-1392) (188) 9897 (8736-10783) (138)	0.585 **0.006**	1388 (1322-1457) 4278 (3621-5053) (n=55)	0.234 **<0.001**	0.464 **<0.001**
PlGF (pg/ml) I trimester II-III trimester	25.5 (22.2-29.5) (33) 47.9 (36.7-62.9) (37)	32.5 (30.5-34.6) (187) 92.8 (85.3-102.0) (138)	**0.002** 0.097	41.8 (39.8-43.9) 164.6 (130.7-207.3) (n=55)	**<0.001** **0.020**	**<0.001** **<0.001**
Endoglin (ng/ml) I trimester II-III trimester	6.6 (6.0-7.3) (33) 64.7 (48.8-86.3) (37)	5.8 (5.6-6.0) (187) 37.0 (33.6-40.9) (138)	**0.009** **<0.001**	5.6 (5.4-5.8) 16.6 (14.0-19.6) (n=55)	**<0.001** **<0.001**	**0.046** **<0.001**

sFlt-1/PlGF I trimester II-III trimester	 49 (40-61) (33) 375 ± 271 (37) [b]	 40 (38-43) (187) 137 ± 100 (138)[b]	 **0.031** (test not available)	 33 (31-35) 45 ± 47 (n=55)[b]	 **<0.001** na	 **<0.001** na
sFlt-1/PlGF ratio, ≥33 II-III trimester	36 (97.3%)	129 (93.5%)	0.744[a]	22 (40.0%)	0.092[a]	**<0.001[a]**
sFlt-1/PlGF ratio, ≥38 II-III trimester	36 (97.3%)	124 (89.9%)	0.371[a]	20 (36.4%)	**0.010[a]**	**<0.001[a]**
sFlt-1/PlGF ratio, ≥85 II-III trimester Early sample	33 (89.2%) 33 (89.2%)	90 (65.2%) 7 (63.6%)	0.069[a] 0.196[a]	10 (18.2%) 3 (37.5%)	**0.001[a]** 0.996[a]	**<0.001[a]** 0.064[a]
sFlt-1/PlGF ratio, ≥110 II-III trimester Late sample	33 (89.2%) -	77 (55.8%) 71 (55.9%)	0.182[a] -	7 (12.7%) 4 (8.7%)	**<0.001** -	**<0.001[a]** **<0.001[a]**

**adjusted for parity, prepregnancy BMI, mother's age at birth, gestational weeks at sampling, smoking status during pregnancy
[a]logistic regression adjusted for parity, prepregnancy BMI, mother's age at birth, gestational weeks at sampling, smoking status during pregnancy
[b]arithmetic mean ± SD; [c]$\chi 2$ –test; [d]Mann-Whitney U-test
na=test not applicable

Table 5. Concentrations of angiogenic markers in pre-eclamptic and control women according to the parity status, geometric mean (95% CI).

Maternal or Perinatal Characteristics	**Pre-eclampsia**		*p**	**Control**		*p**	*p**	*p**
	Primipara	**Multipara**	(primi vs. multiparas)	**Primipara**	**Multipara**	*(primi vs. multiparas)*	*for PE primipara vs. control primipara*	*for PE multipara vs. control multipara*
sFlt-1 (pg/ml) I trimester	1318.0 (1235.3-1406.1) (166)	1262.1 (1121.3-1420.6) (55)	0.304	1392.1 (1299.2-1491.7) (137)	1382.6 (1291.9-1479.7) (102)	0.484	0.429	0.338
II-III trimester	11610.9 (10527.0-12807.7) (128)	7585.6 (6065.1-9488.2) (47)	**0.001**	5023.6 (4044.5-6239.2) (35)	3229.9 (2572.9-4044.5) (20)	**0.005**	**<0.001**	**<0.001**
PlGF (pg/ml)	30.7 (28.6-32.8)	33.5 (29.9-37.5)	0.691	41.1 (38.4-43.9)	42.8 (39.7-46.1)	0.852	**<0.001**	**0.001**

I trimester II-III trimester	(166) 75.4 (67.2-84.7) (128)	(54) 98.5 (82.5-117.6) (47)	**0.015**	(137) 147.8 (118.2-185.0) (35)	(102) 198.6 (117.7-335.2) (20)	0.097	**<0.001**	**0.033**
Endoglin (ng/ml) I trimester II-III trimester	 6.0 (5.7-6.2) (166) 47.6 (42.8-53.1) (128)	 5.8 (5.3-6.3) (54) 29.1 (23.4-36.1) (47)	 0.619 **<0.001**	 5.7 (5.4-5.9) (137) 18.5 (14.8-23.2) (35)	 5.5 (5.2-5.8) (102) 13.6 (10.8-23.2) (20)	 0.098 0.063	 **0.047** **<0.001**	 0.066 **<0.001**
sFlt-1/PlGF I trimester II-III trimester	 43 (40-44) (166) 206 ± 184[b] (128)	 38 (33-44) (54) 138 ± 160[b] (47)	 0.291 **<0.001****	 34 (31-37) (137) 51 ± 47 [b] (35)	 32 (30-35) (102) 36 ± 47 [b] (20)	 0.445 **0.022****	 **<0.001** **<0.001**	 0.042 **<0.001**
sFlt-1/PlGF ratio ≥33 II-III trimester	 126 (98.4%)	 39 (83.0%)	 **0.001[a]**	 16 (45.7%)	 6 (30.0%)	 0.179[a]	 **<0.001[a]**	 **0.004[a]**
sFlt-1/PlGF ratio ≥38 II-III trimester	 126 (98.4%)	 34 (72.3%)	 **<0.001**[a]	 14 (40.0%)	 6 (30.0%)	 0.295[a]	 **<0.001[a]**	 **0.048[a]**
sFlt-1/PlGF ratio, ≥85 II-III trimester Early sample	 100 (78.1%) 32 (84.2%)	 23 (48.9%) 8 (80.0%)	 **0.001**[a] 0.377[a]	 7 (20.0%) 2 (28.6%)	 3 (15.0%) 1 (100%)	 0.602[a] 0.375[c]	 **<0.001[a]** **0.004[a]**	 0.115[a] 1.000[c]
sFlt-1/PlGF ratio, ≥110 II-III trimester Late sample	 92 (71.9%) 61 (67.8%)	 18 (38.3%) 10 (27.0%)	 **<0.001**[a] **<0.001**[a]	 5 (14.3%) 3 (11.1%)	 2 (10.0%) 1 (5.3%)	 0.337[a] 0.448[c]	 **<0.001[a]** **<0.001[a]**	 0.183[a] 0.077[c]

*adjusted for prepregnancy BMI, mother's age at birth, gestational weeks at sampling and smoking status during pregnancy

**unadjusted

[a]logistic regression adjusted for prepregnancy BMI, mother's age at birth, gestational weeks at sampling and smoking status during pregnancy

[b]arithmetic mean ± SD

[c]$\chi 2$ –test