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Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and an *in vitro* vasculogenesis/angiogenesis test

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Objective

The purpose of the study was to determine the angiogenic capacity of sera in early and late pregnancy and in umbilical blood serum after childbirth, and to define how angiogenic properties assessed in a functional *in vitro* test are related to individual angiogenic proteins in six women with pre-eclampsia and in six healthy pregnant controls.

Methods

Maternal first and third trimester serum samples, and umbilical blood samples after childbirth were tested in an *in vitro* human adipose stromal cell – human umbilical vein endothelial cell (hASC-HUVEC) vasculogenesis/angiogenesis assay. The angiogenic properties of the samples were measured by quantifying tubule formation. Concentrations of total placental growth factor (PlGF), total vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) were determined by immunoassay.

Results

First-trimester maternal sera of both groups had a stimulatory effect on angiogenesis *in vitro* and levels of angiogenic proteins did not differ between the groups. Third-trimester maternal sera in the pre-eclampsia group had an inhibitory effect on tubule formation, while those from normal pregnancies remained stimulatory. Compared with the first trimester there was a significant change in the concentrations of angiogenic proteins toward an anti-angiogenic state in pre-eclampsia. Umbilical blood serum exhibited strong anti-angiogenic effects without a significant difference between groups.

Conclusions

Third-trimester serum of pre-eclamptic patients is anti-angiogenic. This phenomenon is not yet present in the first trimester. Umbilical blood serum shows inhibitory effects on angiogenesis after normal as well as pre-eclamptic pregnancy.

Keywords:
In vitro vasculogenesis/angiogenesis, angiogenic proteins, inhibition of angiogenesis, pre-eclampsia, VEGF, sFlt-1, PlGF, sEng

Introduction

There have been several studies on pro- and anti-angiogenic factors and their differences in normal pregnancies and in women with pre-eclampsia [1,2]. The most studied proteins are pro-angiogenic factors such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) and anti-angiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) [1,3]. Even in normal pregnancies changes in favour of anti-angiogenic proteins in the third trimester have been observed, but in pre-eclampsia the imbalance between pro- and anti-angiogenic proteins is stronger and a change in concentrations of these proteins toward an anti-angiogenic state has been seen to exist several weeks before the onset of clinical disease [4,5]. There are fewer studies on angiogenesis in connection with umbilical blood after pre-eclamptic pregnancy. Pre-eclampsia seems to have an effect on the umbilical cord itself and on cord blood, as structural changes have been found in endothelia of umbilical vessels and increased concentrations of s-Flt-1 in umbilical blood after childbirth [6,7].

The net angiogenic capacity of serum can be measured in *in vitro* tests. Human primary cell-based *in vitro* assays mimic the effects in humans reliably [8,9]. In *in vitro* assays the endothelial cells form capillary-like structures (tubule formation) in response to angiogenic signals from added maternal serum [10]. In contrast to quantitative tests involving measurement of concentrations of single specific pro- and anti-angiogenic factors, *in vitro* angiogenesis tests reveal functional capabilities of tested samples to promote, maintain or inhibit tubule formation [8,10]. In addition, they provide quantitative information on the extent of tubule formation [8,11]. The *in vitro* vasculogenesis/angiogenesis human adipose stromal cell – human umbilical cord vein endothelial cell (hASC-HUVEC) test offers a possibility to study the effects of serum on angiogenesis

(formation of new blood vessels from existing ones) and vasculogenesis (*de novo* blood vessel generation from vascular progenitor cells) [8,11].

In the present study we wanted to assess inter-group differences and longitudinal changes in angiogenic capacity of maternal serum during pregnancy in healthy women and in women with pre-eclampsia, utilising the hASC-HUVEC test. Another aim was to investigate how the angiogenic capacity of maternal serum is associated with known pro- and anti-angiogenic factors. Furthermore, we wanted to evaluate the angiogenic capacity of umbilical blood samples after pre-eclampsia and healthy pregnancy.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit number R11088). Written informed consent was obtained from the women agreeing to participate in the study. The use of human adipose stromal cells (hASCs) and human umbilical cord endothelial cells (HUVECs) was separately approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit numbers R03058 and R08028, respectively).

Study population

This was a cross-sectional research project where the study population consisted of six primiparous women with pre-eclampsia and six controls. All women gave two blood samples during pregnancy and an umbilical blood sample after childbirth in 2011–2014. The women were recruited to the study during the third trimester of pregnancy. The inclusion criteria for healthy controls were: blood pressure < 140/90 mmHg, urine dip stick test negative for proteinuria and previously uncomplicated singleton pregnancy. Hypertension later in pregnancy was an exclusion criterion. Early-gestation serum samples from both groups were obtained from the National Institute for Health and Welfare, where maternal sera from first-trimester screening was stored. The samples had been taken between the ninth and eleventh weeks of gestation. Gestational age was calculated on the basis of the last menstruation and corrected if necessary at first-trimester screening ultrasonography.

The definition of pre-eclampsia was that of the International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2000. Systolic blood pressure should be ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on at least two occasions 4 h apart after 20 weeks of gestation in previously

normotensive women, with proteinuria of 300 mg or more in 24 h [12]. Pre-eclampsia was defined as severe if HELLP syndrome (haemolysis, elevated levels of liver enzymes and low platelet count), eclampsia or exceptionally high blood pressure (> 160 mmHg systolic or > 110 mmHg diastolic) appeared [13,14]. In the study group maternal blood samples were taken at a maximum of three days before delivery when the patient was already hospitalized because of clinical symptoms of pre-eclampsia. Cord blood samples were taken after delivery of the placenta in both groups. There was no separation between umbilical arterial and venous blood in the cord samples. The serum samples were frozen and conserved at -70℃ until assay. Blood tests for haemoglobin level, platelet count and alanine aminotransferase level were carried out at admission for the women with pre-eclampsia. Baseline demographic details and data on pregnancy outcome were collected from the hospital maternity records.

Deviation from normal growth (mean weight of newborns at the same gestational age) was determined for all newborns according to national weight curves [15]. Small-for gestational-age (SGA) was defined as birth weight more than two standard deviations below the mean.

Immunoassays

The concentrations of sFlt-1, PlGF, VEGF and sEng were determined in all samples by using ProcartaPlex assays (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, samples were centrifuged at 1000 × *g* for 10 min before use in the assay. The samples were not diluted. Antigen standards and magnetic beads were prepared according to the manufacturer's instructions. Samples and standards were then added to the beads and incubated with shaking for 2 hours at room temperature. The detection antibodies were added and incubated for 30 min at room temperature. To stain the proteins, streptavidin-PE was added and incubated for 30 min at room temperature, after which the beads were analysed in reading buffer. The results were analysed with Bio-plex200 (Bio-Rad, California, USA) and Bio-Plex Manager™ 6.0 software (Bio-Rad). Concentrations were obtained in pg/ml. Standard concentrations for VEGF were 5.74–23,500 pg/ml, for sFlt-1 49–200,000 pg/ml, for sEng 0.61–2500 pg/ml and for PlGF 1.66–6800 pg/ml. If the result obtained was lower or higher than the range of the standards, a fixed concentration above or below the standards was used in analysis of the results. The concentration used for sEng was 2550 pg/ml when the result was over the standard range and for PlGF 1 pg/ml when the result was lower than the lowest standard. The result of the VEGF and PlGF assays can include also the bound

version of these growth factors and hence the concentration result should be considered as total concentration.

Cells

Human adipose tissue samples were obtained from waste material from surgical operations and human umbilical cords from uncomplicated pregnancies were received after Caesarean sections. Isolation of HUVECs from umbilical cord veins was performed as described earlier (Sarkanen et al. 2012) using 0.05% collagenase I (Gibco, Thermo Fisher scientific, Waltham, USA) inserted into the umbilical vein [16]. Isolation of hASCs was performed as described earlier (Sarkanen et al. 2012) using 0.15% Collagenase type I (Gibco) [16]. HUVECs were cultured in EGM-2 medium (Lonza, Basel, Switzerland) and hASCs in hASC medium (Table 1). Cells were negative for mycoplasma contamination, tested by using MycoAlert™ kits (Lonza).

***In vitro* vasculogenesis/angiogenesis test**

To study the angiogenic potency, i.e., bioactive growth factor content, of the serum samples, all three sample groups (first trimester, third trimester and umbilical blood serum samples) were studied by using the *in vitro* vasculogenesis/angiogenesis test, with a co-culture of hASCs (20,000 cells/cm^2) and HUVECs (4000 cells/cm^2). The test was performed as described earlier (Virtanen et al. 2016) [10]. Briefly, vasculogenesis/angiogenesis was induced using vasculogenesis/angiogenesis test medium (Table 1) and the co-cultures were exposed to patient serum samples at a dilution of 1:15 and cultured for six days in total with one replenishment of the growth medium. After exposure, the number of living cells was evaluated by using a WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland). Following the WST-1, the cells were fixed with ethanol and immunostained for vWf (Sigma Aldrich) to detect the endothelial cells and collagen IV (Sigma Aldrich) to detect the basement membrane of the tubules. For visualization of the tubules, fluorescent secondary antibodies against the primary antibodies were applied: anti-rabbit tetramethylrhodamine isothiocyanate (TRITC, Sigma Aldrich) for vWf and anti-mouse fluorescein 5-isothiocyanate (FITC, Sigma Aldrich) for collagen IV. The resulting fluorescent tubules were imaged and analysed using an automated image analysis platform (Cell-IQ, CM-Technologies, Tampere, Finland). Tubule formation was quantified on the basis of the intensity of the tubular network formed (tubule length and branching). The obtained result was compared with the positive tubule formation control (highest level of tubule formation induced with stimulatory factors). Tubule formation was defined as stimulatory when there was more tubule formation after exposure of the serum sample compared with the positive tubule formation control and inhibitory when there

was less tubule formation after exposure to serum. Values were expressed as percentage of positive tubule formation.

Statistical analysis

The data are expressed as medians and range. Differences in continuous variables between groups were tested by using Mann–Whitney *U*-tests and differences within the study group by using Wilcoxon's test. Spearman's correlation method was used to calculate correlation coefficients. Probabilities of less than 0.05 were considered statistically significant. Statistical analyses were performed by using the Statistical Package for the Social Sciences (IBM-SPSS), version 11.0.

Results

The characteristics of the study population are summarized in Table 2. There were two preterm births in the pre-eclampsia group, but none of the women had early-onset pre-eclampsia. Most women with pre-eclampsia had severe disease.

In the first trimester maternal sera were stimulatory, and tubule formation was equally high in the *in vitro* test in both groups (Fig. 2). Neither were there differences between groups in concentrations of the individual pro- and anti-angiogenic proteins or the sFlt-1:PlGF ratio (Table 3). In both groups the median concentrations of PlGF were higher in maternal sera in the first trimester when compared with serum samples taken in the third trimester ($p = 0.043$) or umbilical blood serum ($p = 0.028$) (Table 3).

In contrast to the first trimester, sera from women with pre-eclampsia in the third trimester exhibited an inhibitory effect on tubule formation. In comparison with healthy women, tubule formation was significantly lower in the pre-eclampsia group ($p = 0.026$) (Figs. 2 and 3). Compared with the first trimester, there was remarkably lower ($p = 0.043$) tubule formation in the third trimester in women with pre-eclampsia, whereas in the healthy control group there was no change in the capacity of tubule formation (Figs. 2 and 3). In the third trimester sFlt-1 levels were higher in the pre-eclampsia group than in the healthy control group ($p = 0.004$) (Table 3) and compared with the first trimester sFlt-1 levels had increased ($p = 0.028$) and total PlGF concentrations had decreased ($p = 0.043$) in women with pre-eclampsia. In addition, the sFlt-1:PlGF and sFlt-1/VEGF ratios increased between the first and third trimester in the pre-eclampsia group (Fig. 1). In normal pregnancies sFlt-1 concentrations and the sFlt-1/VEGF ratio were similar in the first and third trimesters.

Umbilical blood serum had an equally strong inhibitory effect on tubule formation in both groups. The inhibitory effect seen in the *in vitro* test was stronger in umbilical serum than in third-trimester maternal serum when the whole study population (i.e. including controls) was analysed as one group ($p = 0.005$). The sFlt-1/VEGF ratio was also significantly higher in umbilical than in maternal sera ($p = 0.015$). When the data was studied in separate groups, only in healthy women was the inhibitory effect on tubule formation stronger in umbilical than in maternal serum ($p = 0.028$). There were no differences between the groups in the concentrations of individual pro- and anti-angiogenic proteins, or in the sFlt-1:VEGF ratio in cord sera. In women with pre-eclampsia sFlt-1 concentrations were significantly lower ($p = 0.028$) in umbilical serum than in third-trimester maternal serum.

In the first trimester, total amount of pro-angiogenic proteins (VEGF and PlGF) did not show any correlation with the stimulatory effect seen in the *in vitro* test. Neither did any pro- or anti-angiogenic protein or tubule formation measure in the first trimester have a correlation with baseline demographic characteristics, severity of pre-eclampsia, gestational weeks at birth or weight of the newborn.

In the third trimester, when the whole study population was analysed as one group, there were significant negative correlations between tubule formation and sFlt-1 ($r = -0.902$, $p < 0.001$), sEng ($r = -0.595$, $p = 0.041$) and the sFlt-1/VEGF ratio ($r = -0.748$, $p = 0.005$). Furthermore positive correlations between the concentration of sFlt-1 and both systolic ($r = 0.741$, $p = 0.006$) and diastolic blood pressure ($r = 0.849$, $p < 0.001$) were found. A correlation between a greater inhibitory effect on tubule formation and a lower birth weight SD score reached statistical significance ($r = -0.754$, $p = 0.050$) in the pre-eclampsia group.

In umbilical blood serum samples, when the whole study population was analysed as one group, there were no correlations between the concentrations of pro- and anti-angiogenic proteins versus tubule formation, but there were correlations between a strong inhibitory effect in *in vitro* tests and low birth weight ($r = 0.790$, $p = 0.002$), low birth weight SD score ($r = 0.582$, $p = 0.047$) and earlier gestational weeks at birth ($r = 0.622$, $p = 0.031$). When the same correlations were studied in the two separate groups, the correlations with low birth weight ($r = 0.829$, $p = 0.042$) and low birth weight SD score ($r = 0.841$, $p = 0.036$) remained significant in the pre-eclampsia group.

Discussion

In the present study, we demonstrated that assay of pro- and anti-angiogenic proteins is not the only method to measure angiogenic capacity of maternal or umbilical blood serum, but that advanced *in vitro* models provide an alternative method to study angiogenesis. We longitudinally measured concentrations of pro- and anti-angiogenic proteins by immunoassay and at the same time evaluated functional angiogenic capacity in *in vitro* tests in women with pre-eclampsia and in their controls with uncomplicated pregnancies. We assessed the overall angiogenic profile not only in women with symptomatic pre-eclampsia but also in the first trimester and after childbirth.

Angiogenesis and vasculogenesis are complex processes which are regulated not only by pro- and anti-angiogenic proteins but by other factors in addition [17,18]. Immunoassays give us quantitative information on specific proteins in maternal sera, but in order to assess overall angiogenic capacity, we used an additional functional approach to angiogenesis. The human cell-based *in vitro* vasculogenesis/angiogenesis test can be described as a combination of a functional and a quantitative test. In the *in vitro* assay the endothelial cells form capillary-like structures in response to angiogenic signals from the added maternal serum and the effect of maternal serum on tubule formation can be stimulatory, neutral or inhibitory. The strength of an effect can be measured as tubule count. What we have gathered from the literature is that there is only one earlier study concerning an *in vitro* model of angiogenesis and pro- and anti-angiogenic proteins. In the study, *in vitro* tubule formation and sFlt-1 concentrations in maternal sera were assessed in women with pre-eclampsia [19]. In the present study, we used an *in vitro* model that in addition to giving a measure of the effects on angiogenesis, also shows effects on vasculogenesis.

The pro-angiogenic factors VEGF and PlGF are known to play important roles in placental development [18]. Angiogenic proteins have been widely studied in the first trimester in order to identify women destined to develop pre-eclampsia later in pregnancy, but levels of single angiogenic proteins have been observed to be of poor predictive value [2,20,21]. Our results are in concordance with these findings, since in early gestation we found no significant differences in functional test results or in the concentrations of pro- and anti-angiogenic proteins between the study groups. In our *in vitro* model of angiogenesis an equally strong stimulatory effect on tubule formation was seen in both groups in the first trimester, which reflects the pro-angiogenic state in early weeks of pregnancy. Serum levels of PlGF were higher in the first trimester than later in pregnancy in both groups, but there was wide variation in the concentrations and we found no significant correlation between stimulatory effects on tubule formation and levels of pro-angiogenic proteins. There was no inhibition of tubule formation in the first trimester even though the levels of sFlt-1 were already relatively high. The results of earlier studies concerning sFlt-1 concentrations in

the first trimester are variable, but there are more consistent findings that concentrations of sFlt-1 in women who subsequently develop pre-eclampsia are not significantly different from those in unaffected controls [22]. With a larger study population the results could be different, and it is also known that there are factors other than those we have measured involved in angiogenesis during the first trimester of pregnancy [18]. Concentrations of soluble endoglin were at the same level in both groups in the first trimester, as has been published earlier [23]. In summary, results concerning the first trimester in the present study indicate that the strong stimulatory effect seen in the *in vitro* test may better reflect the pro-angiogenic state in early gestation than levels of individual angiogenic proteins.

We have earlier shown that sera in women with symptomatic pre-eclampsia are strongly inhibitory in our *in vitro* test [10]. Our finding supported the results of the study by Maynard et al., where sera from women with pre-eclampsia inhibited and sera from women with normotensive pregnancies induced tubule formation in a HUVEC-based *in vitro* model [19]. In the present study, the decrease in tubule formation was remarkable between the first and third trimester in women with pre-eclampsia. Simultaneously there were significant changes in concentrations of sFlt-1 and PlGF towards a clearly anti-angiogenic balance. However, despite prevailing anti-angiogenesis, levels of individual pro- and anti-angiogenic proteins showed no correlation with tubule formation in the pre-eclampsia group. This could indicate that despite a steep increase in sFlt-1 concentrations there are also other factors related to anti-angiogenesis in pre-eclampsia. In the present work the small study population and wide variation in the concentrations of pro- and anti-angiogenic proteins limits the finding of true associations. As in previous studies, in our study also sFlt-1 and sEng levels were higher in the pre-eclampsia group than in the healthy control group [2,4]. Decreases in PlGF concentrations and increases in sFlt-1:PlGF ratios were significant only in the pre-eclampsia group.

The angiogenic properties of umbilical cord blood after pre-eclampsia and uncomplicated pregnancy have not been studied to the same extent as in maternal sera, and to our knowledge there are no other studies concerning the angiogenic quality of umbilical blood together with immunoassay measurements and *in vitro* test data. After childbirth, umbilical blood serum tended to be anti-angiogenic in the *in vitro* test, and it was even stronger that in maternal serum. This was surprising since there were no significant differences in the concentrations of pro- and anti-angiogenic proteins (excluding higher levels of maternal sFlt-1 in the pre-eclampsia group) between mother and fetus. The result might be affected by the structural variety of umbilical vessels when compared with vessels of the same calibre in the mother. Umbilical vessels are thought to be more susceptible to changes in haemodynamic conditions and that might have an influence on the

consistency of umbilical blood [7,24]. The results of studies on pro-angiogenic proteins in umbilical blood after pre-eclampsia and unaffected pregnancies are inconsistent [6,25]. In a recent study it was observed that median PlGF levels were lower in umbilical blood after pre-eclamptic pregnancy when compared with gestational age-matched controls [26], whereas Staff et al. reported the same finding as ourselves, that in the majority of samples levels of PlGF were below the lowest concentration standard of the immunoassay kit [6]. In the study by Sezer et al. it was found that there were no differences in sFlt-1 levels between a pre-eclampsia group and a control group as regards umbilical blood [26], as we reported, but there are also studies indicating that sFlt-1 concentrations are higher in umbilical blood after pre-eclampsia than after unaffected pregnancies [6]. In the present study, concentrations of pro- and anti-angiogenic proteins in umbilical blood sera were similar to maternal levels, with the exception of lower sFlt-1 levels after pre-eclamptic pregnancy. A correlation between high sFlt-1 concentrations and low-level tubule formation was found only in the healthy control group, as was also the case in maternal samples in the third trimester. There are probably fewer confounding factors that have an influence on angiogenesis in healthy pregnant women than in those with pre-eclampsia. In umbilical samples, there was also a correlation between a pronounced inhibitory effect in the *in vitro* test and low birth weight in the pre-eclampsia group, presumably reflecting the predominance of anti-angiogenesis present in fetal growth in pregnancies complicated by hypertensive disorders.

Strengths and limitations

To our knowledge, this is first study carried out to investigate an association between the results of an *in vitro* angiogenesis test and quantitative measurements of pro- and anti-angiogenic proteins at three different stages of pregnancy. As the proteins concerned have been extensively studied recently, we intended to achieve a new approach to the study of angiogenic changes during pregnancy in women with pre-eclampsia as well in healthy pregnant women, by using two distinct methods. In particular, the results of an advanced *in vitro* vasculogenesis/angiogenesis test give us new information about the overall balance of angiogenesis in complicated and healthy pregnancies. The study was carried out with a small number of cases, and therefore some true associations may not have reached statistical significance. Another limitation of this study was that some protein concentrations were below or above the standards in the immunoassay kits, which creates difficulties in comparing the study groups. The high level of sFlt-1 present in the studied serum samples may cross-react or interfere in the immunoassay for VEGF and PlGF.

In conclusion, in the first trimester maternal sera had a stimulatory effect on tubule formation reflecting a pro-angiogenic state in early pregnancy. In the third trimester maternal sera from women with pre-eclampsia exhibited inhibitory properties on angiogenesis, and simultaneously there was change toward a more anti-angiogenic state as regards individual proteins. The angiogenic balance in umbilical blood sera did not differ between the study groups.

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Table 1. Media used in the in vitro test

Medium name	Content	Manufacturer
hASC medium	DMEM/F12	Gibco
	10% human serum	PAA laboratories
	2mM L-Glutamine	Gibco
Vasculogenesis/angiogenesis	DMEM/F12	Gibco Invitrogen
test medium	2.56 mM L-glutamine	Gibco Invitrogen
	0.1 nM 3,3',5-triiodo-L-thyronine sodium salt	BD Biosciences
	ITS^{TM} Premix:	BD Biosciences
	6.65 µg insulin/ml	
	6.65 µg transferrin/ml	
	6.65 ng selenious acid/ml	
	1% Bovine serum albumin	PAA
	2.8 mM Sodium pyruvate	Gibco Invitrogen
	200 µg/ml Ascorbic acid	Sigma Aldrich
	0.5 µg/ml Heparin	Stemcell Technologies
	2 µg/ml Hydrocortisone	Sigma Aldrich
	2.5 ng/ml VEGF	R&D Systems
	0.25 ng/ml FGF-β	R&D Systems

Table 2. Maternal characteristics and neonatal outcome

	PE group (n=6)	Controls (n=6)	P-value
Maternal age, years[a]	29.0 (25–33)	25 (24–34)	0.240
BMI, kg/m^{2}[a]	21.3 (19.7–22.3)	26.4 (17.9–38.2)	0.065
Highest systolic BP, mmHg[a]	160 (141-171)	130 (103-138)	0.009**
Highest diastolic BP, mmHg[a]	96 (88-110)	73 (54-88)	0.002**
Severe pre-eclampsia (%)	5/6 (83.3)		
Gestational age, weeks[a]	37.5 (35.3–38.3)	40.5 (35.4–41.6)	0.065
Mode of delivery			
vaginal/CS, n (%)	4/2 (66.7/33.3)	3/3 (50/50)	
Birth weight, grams[a]	2620 (2040–3140)	3278 (2685–4535)	0.026*
Birth weight, SD[a]	-1.2 [-3.1-(-0.4)]	-0.5 (-1.7–1.5)	0.093
Umbilical arterial pH[a]	7.23 (7.13-7.36)	7.33 (7.30-7.41)	0.052

[a]Data are given as median (range). PE, pre-eclampsia; BMI, body mass index; BP, blood pressure; CS, caesarean section; SD, standard deviation. *p Value < 0,05, **p Value < 0,01, Mann-Whitney U-test

Table 3. Concentrations of angiogenic proteins in maternal serum and in umbilical blood in pre-eclampsia and control group.

	First trimester			Third trimester			Umbilical cord		
	PE	**CONTR**	P value	**PE**	**CONTR**	P value	**PE**	**CONTR**	P value
VEGF (pg/ml)	2572 (1980-25889)	2961 (1875-14003)	0.94	2196 (1417-9281)	1818 (856-17321)	0.24	6374 (5185-8619)	3893 (2238-16486)	0.18
PlGF (pg/ml)	144 (1-14155)	120 (1-1514)	0.70	23 (1-3535)	1 (1-1216)	0.24	28 (1-923)	1.6 (1-1450)	0.59
sFlt-1 (pg/ml)	3780 (2092-5878)	5914 (2489-6851)	0.18	11278 (4768-13899)	1480 (277-6339)	0.004**	1684 (397-6845)	1485 (445-7113)	0.94
sEng (pg/ml)	2550[†]	2550[†]	1.00	2550 (1009-2550)	1071 (595-2550)	0.093	1885 (846-2550)	1159 (701-2550)	0.70
sFlt-1/PlGF	35 (0.2-2092)	44 (4-6538)	0,59	614 (1.4-13064)	734 (2-6339)	0.94	52 (3-6845)	382 (1.7-7113)	0.39
sFlt-1/VEGF	1.3 (0.1-2.8)	1.6 (0.5-2.8)	0.70	5.6 (0.5-8.5)	0.5 (0.1-5.0)	0.041*	0.3 (0.1-1.3)	0.3 (0.1-2.1)	0.70
tubule formation(%)[#]	152 (126-158)	135 (70-153)	0.18	79 (22-115)	140 (72-186)	0.026*	26 (7-41)	50 (23-146)	0.13

Data are given as median (range). PE, pre-eclampsia; CONTR, control; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sEng; soluble endoglin. The concentrations of VEGF and PlGF should be considered as total concentration. [#]Tubule intensity compared to the positive tubule control. [†]All values were out of range above. *p Value < 0.05, **p Value <0.01, Mann-Whitney U-test.

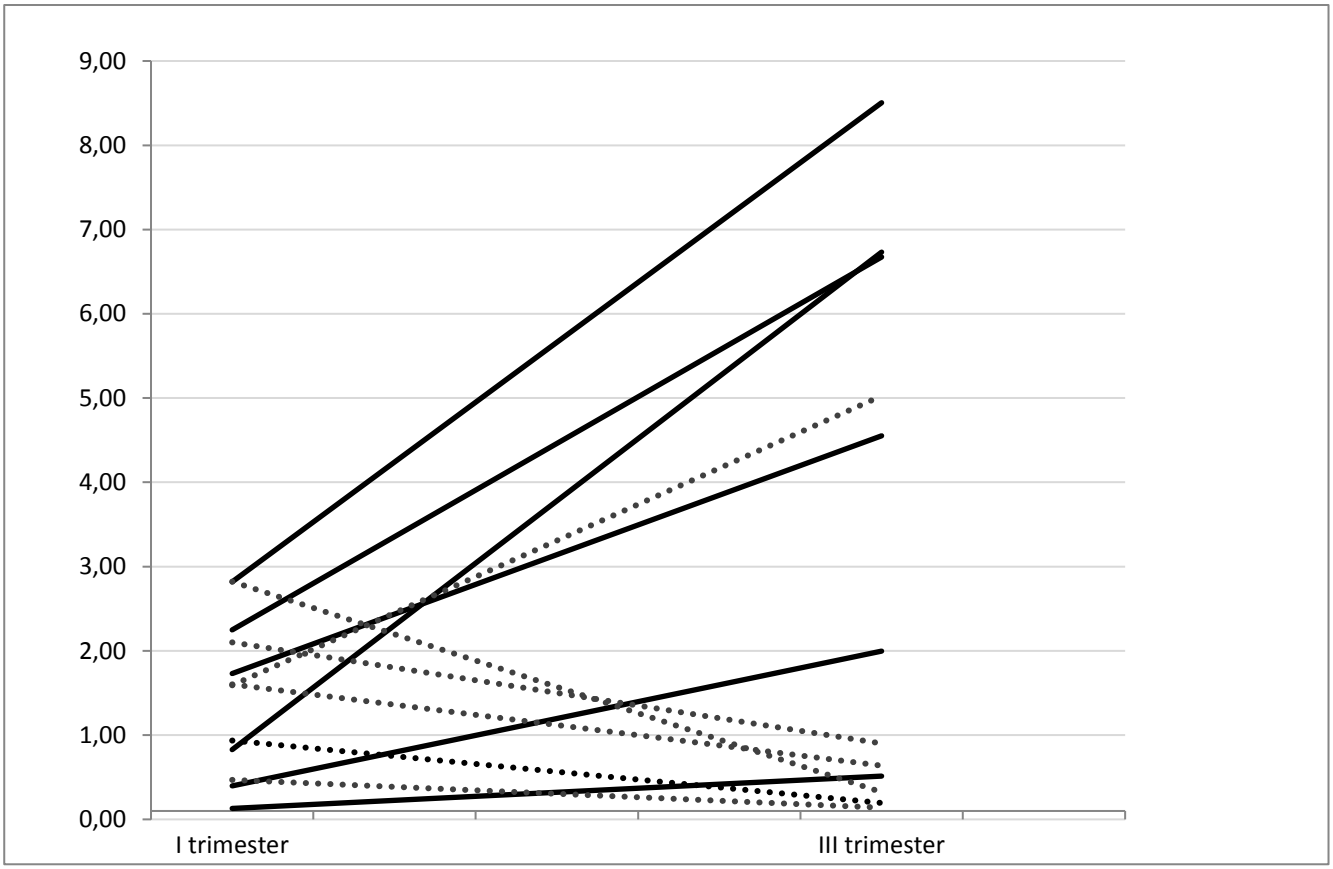

Figure 1. S-Flt-1:VEGF ratio in maternal sera in first and third trimester. sFlt-1:VEGF ratio was significantly higher in pre-eclampsia group in third trimester when compared to healthy control group (p=0.041). There was also significant increase in sFlt-1:VEGF ratio between first and third trimester in pre-eclampsia group (p=0,028). Solid lines=pre-eclampsia, dashed lines=healthy.

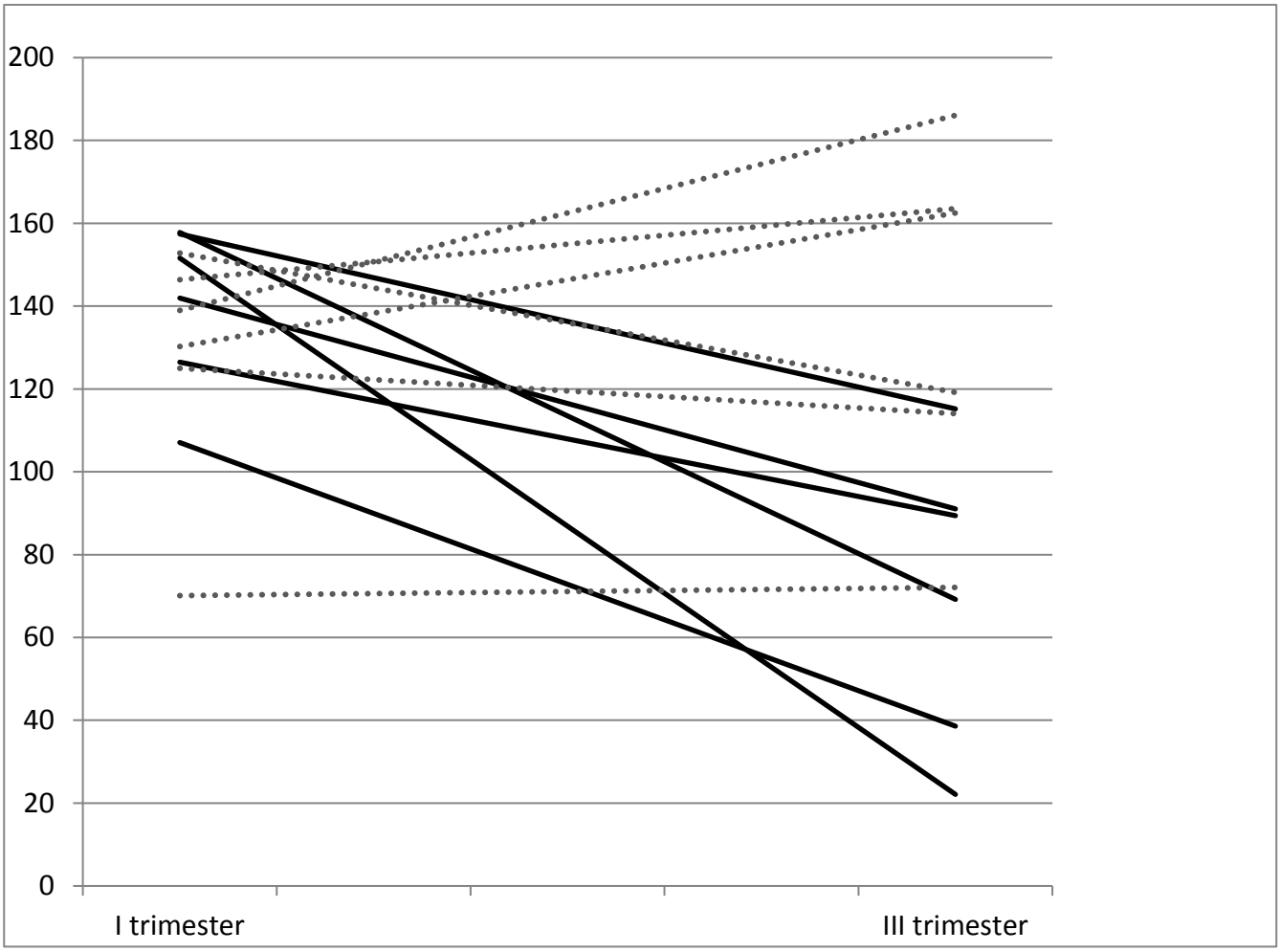

Figure 2. Tubule formation measured as tubule count in maternal serum in first and third trimester of pregnancy. The decrease in tubule formation was significant between first and third trimester in women with pre-eclampsia (p=0,043). Solid lines=pre-eclampsia, dashed lines=healthy.

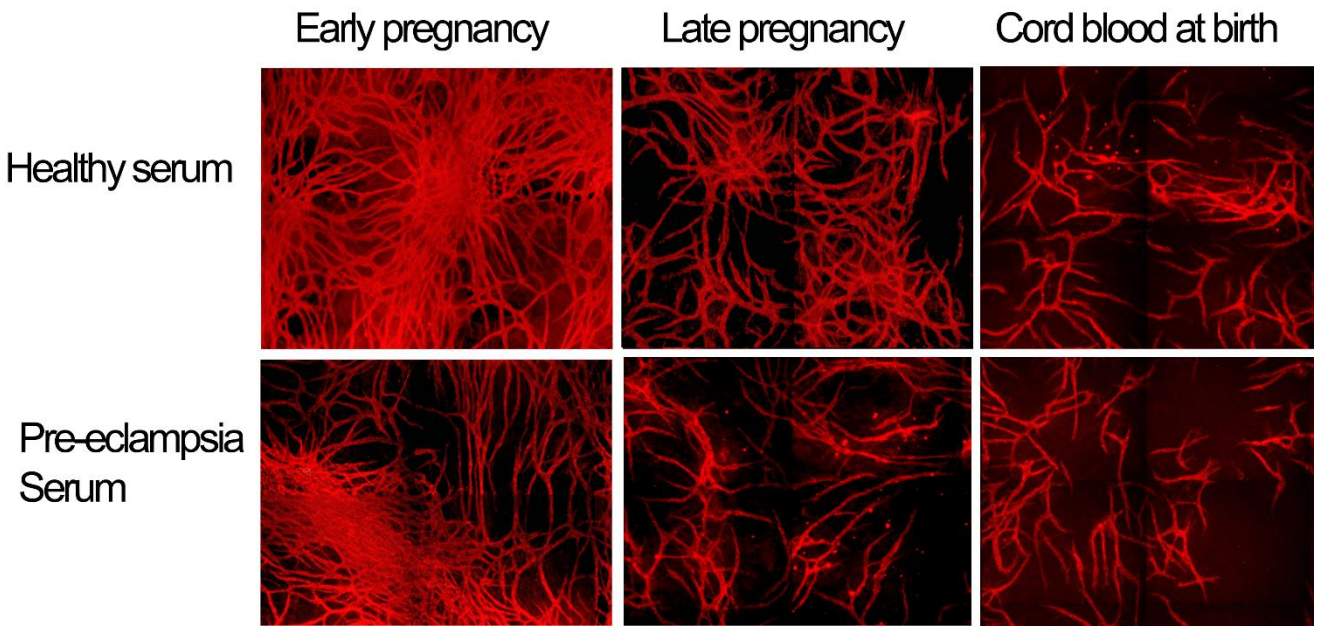

Figure 3. Fluorescence images of the von Willebrand factor -TRITC-stained tubules exposed to healthy and pre eclamptic maternal serum and cord blood. Top row: healthy serum in early pregnancy, late pregnancy and cord blood, all from the same mother and her child. Bottom row: Pre eclampic serum collected form a mother in early pregnancy and late pregnancy, and cord blood of the baby. Both early pregnancy samples induced strong tubule formation, in places forming dense tubule clumps in which the tubules are not distinguishable enough to be analyzed. Late pregnancy samples show that healthy serum induced more tubule formation. Both groups of cord blood did not show significant difference.

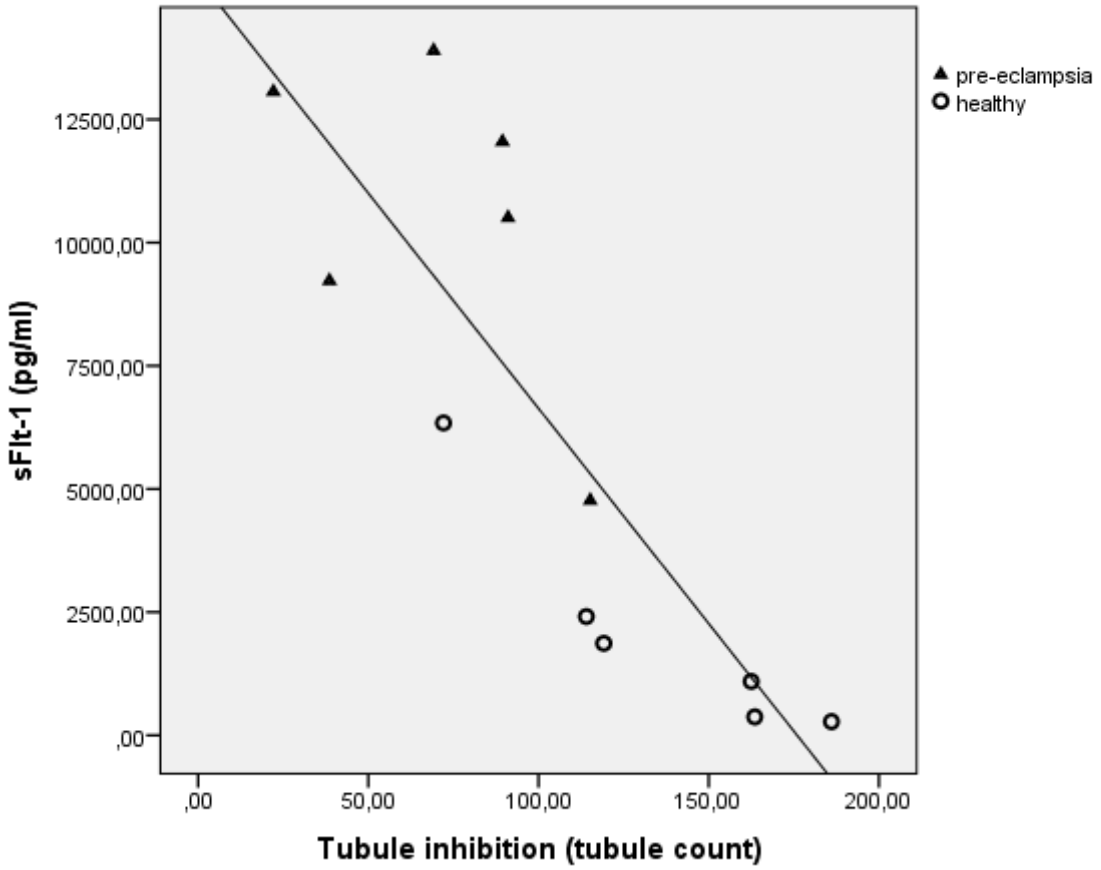

Figure 4. Correlation between sFlt-1 and tubule inhibition in maternal serum samples in pre-eclampsia and healthy control group at third trimester .Tubule inhibition was measured as tubule formation meaning the lower the tubule count, the stronger the inhibition. The correlation was significant when whole study population was analyzed as one group (r=-.902, p=0,000).