

Altered angiogenesis in preeclampsia: evaluation of a new test system for measuring placental growth factor

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Abstract

Background: Decreased concentrations of the circulating angiogenic factors, free placental growth factor (PLGF) and free vascular endothelial growth factor (VEGF), and increased concentrations of the anti-angiogenic factor, soluble fms-like tyrosine kinase 1 (sFLT-1) have been observed during clinical preeclampsia. We established a new PLGF-ELISA kit for the measurement of PLGF in sera. In the present study, we demonstrated the assay characteristics by measurement of PLGF expression in normal and preeclamptic pregnancies as compared to an established research kit.

Methods: Blood samples were taken from 64 women with singleton uncomplicated pregnancies for longitudinal measurement of PLGF in the course of pregnancy. In 30 preeclamptic patients, serum levels of PLGF and sFLT-1 were measured by Human PLGF-ELISA and Human sVEGF R1 ELISA according to the described test principles. The assay characteristics of the new PLGF-ELISA were determined and the results were compared to those performed with an available research kit.

Results: The PLGF concentration in normal pregnancies showed a steady increase starting at the beginning of the second trimester with a peak at 28–32 weeks and a consistent decline thereafter. The preeclamptic pregnancies had significant lower serum concentrations of PLGF and significant higher serum concentrations of sFLT-1 as compared to the non-preeclamptic pregnancies. All the measured assay characteristics fulfilled the required specifications. Comparison of the values of the new PLGF-ELISA and the established research kit resulted in a correlation coefficient of 0.921.

Conclusions: Our results support the hypothesis that an imbalance between factors promoting angiogenesis, such as PLGF, and factors antagonizing angio-

genesis, such as sFLT-1, has a fundamental role in the pathogenesis of preeclampsia. The new established ELISA test can be considered reliable and it offers many advantages. As it is authorized for routine diagnostic testing, it may offer new possibilities in the prediction of preeclampsia in clinical routine.

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Keywords: angiogenesis; fms-like tyrosine kinase; pathophysiology; placental growth factor; preeclampsia; vascular endothelial growth factor.

Introduction

Preeclampsia is a major cause of maternal and perinatal mortality and morbidity worldwide (1, 2). It is a multisystemic disorder affecting approximately 5%–10% of pregnant women towards the end of the second trimester of gestation (3, 4).

Clinically, this disease is characterized by hypertension and proteinuria. Besides adequate and proper prenatal care (5–8), the delivery of the fetus is the only effective treatment. The decision between delivery and expectant management depends on gestational age, fetal wellbeing and severity of maternal condition at the time of assessment.

Although the pathophysiology of preeclampsia is still unknown, the placenta is considered to play a key role in this disease (9). In humans, the invasion of trophoblast cells into the decidualized endometrium and the inner third of the myometrium are of vital importance for both the anchoring of placenta and connection with the maternal vascular system (10). Extravillous trophoblast cells migrate through the uterine stroma and erode local spiral arteries to gain access to the maternal blood supply. These cells migrate actively and selectively during the first trimester into the maternal tissue of the placental bed including the maternal arteries within this tissue (11–13). Normally, the invasive trophoblast remodels the maternal vessels by replacing the vascular smooth muscle and endothelial cells and converting them to vessels with low resistance, and therefore high blood flow capacity (14). By 20 weeks, this process is more or less completed (4).

One of the most favored hypothesis is that preeclampsia is generated by shallow invasion of the extravillous trophoblast followed by an incomplete remodeling of the maternal vascular structures which leads to uteroplacental insufficiency and intrauterine growth restriction, which in turn can influence placental angiogenesis and development (14, 15).

Abnormalities in the angiogenic balance have been proposed as playing a major role in the molecular

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cascade leading to proteinuria, hypertension and endothelial dysfunction (16–18). The angiogenic factors vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) are important for effective function of endothelial cells and placental development (19, 20). Recent studies have shown that soluble fms-like tyrosine kinase 1 (sFLT-1), the soluble part of the VEGFR-1 receptor with antiangiogenic properties, is increased in the placenta (21, 22) and serum (21, 23) of women with preeclampsia. The sFLT-1 acts as a decoy receptor inhibiting the binding of the angiogenic protein VEGF to the VEGFR-2 receptor (24). In preeclampsia, these increased sFLT-1 concentrations are accompanied by decreased levels of circulating free PLGF and free VEGF suggesting that sFLT-1 binds VEGF and PLGF in the maternal circulation, and thereby blocks their angiogenic effects (23, 25, 26). A decrease in the PLGF serum level before the onset of clinical symptoms has been reported by Levine et al. (25). These published data were determined using a kit which is only authorized for research use (R&D Systems, Wiesbaden, Germany). We established a new PLGF-ELISA kit for the measurement of PLGF, which is meanwhile authorized for routine diagnostics (PLGF Enzyme-Linked Immunosorbent Assay, ELISA; DRG, Marburg, Germany). The assay characteristics were determined and PLGF was measured in sera from women with normal and preeclamptic pregnancies. The results determined from the new ELISA kit were compared with the established PLGF-ELISA for scientific purposes.

Materials, patients and methods

Human PLGF-ELISA (R&D)

This quantitative sandwich enzyme immunoassay technique (R&D Systems) employs the incubation of standards and samples into wells, pre-coated with a monoclonal antibody specific for PLGF (2 h), washing (4×) and subsequent incubation with an enzyme-linked polyclonal antibody specific for PLGF (2 h). After a further washing step (4×), the addition of substrate solution (30 min in the dark) and stopping solution, the color product was quantitated by spectrophotometry at 450 nm with a Microplate reader (AMP 400 KIN, Asys, Eugendorf, Austria), indicating the amount of PLGF bound in the initial step.

Human sVEGF R1-ELISA

A human sVEGF R1-ELISA (R&D Systems) was used according to the manufacturer's recommendations. The test principle is the same as described for the human PLGF-ELISA, it employs the use of a monoclonal antibody specific for VEGF R1 for the first incubation step and an enzyme-linked polyclonal antibody specific for PLGF for the second incubation step.

Human PLGF-ELISA (DRG)

Test principle The DRG PLGF-ELISA Kit (ELISA, DRG) is a solid phase ELISA based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a PLGF molecule.

To perform the test, 25 µL serum, controls and standards plus 250 µL dilution buffer were incubated in the coated wells for 30 min at room temperature to allow binding of the antigen by the capture antibody. After a washing cycle, 100 µL of a biotin-linked polyclonal antibody specific for PLGF was added to the wells for 60 min. After a second washing cycle to remove any unbound antibody, the amount of detector antibody bound to antigen was measured by binding with a streptavidin/horseradish peroxidase conjugate (100 µL/well for 30 min). Subsequently, the unbound enzyme complex was removed by washing and 100 µL substrate solution was added for 30 min. The reaction was stopped by the addition of 100 µL stopping solution and the colored product was quantitated by spectrophotometry at 450 nm with a Microplate reader (AMP 400 KIN, Asys). The intensity of color developed was proportional to the concentration of PLGF in the patient sample. In a study conducted with apparently normal healthy non-pregnant female adults (n=65) using the DRG PLGF-ELISA, the values observed were 20.3–85.9 pg/mL.

Measurement of assay characteristics All measurements of assay characteristics were performed according to the instruction for use. Each run included a standard curve and two control sera. All samples and the standard were measured in duplicate. The plates were measured on an E-LizaMat 3000 (DRG).

- Analytical sensitivity:** The zero standard was measured 20 times in one test to determine the analytical sensitivity for the test. The analytical sensitivity was calculated by adding 2 standard deviations (SDs) from the mean of 20 replicate analyses of the zero standard (S0).
- Intra-assay variation:** Two samples, spread over the measuring range, were measured 20 times (10 duplicates) in one test. The samples consisted of low and high control sera. The intra-assay variation (CV% intra) was calculated by dividing the SD of the 10 duplicate determinations by the mean of the 10 duplicate determinations and subsequent multiplication of the result with 100.
- Inter-assay variation:** Two samples covering the measuring range of the DRG PLGF-ELISA were assayed on 6 days in duplicate. The inter-assay variation (CV% inter) was calculated by dividing the SD of the six determinations by the mean and subsequent multiplication with 100.
- Recovery:** Three serum samples were spiked with PLGF by diluting the serum samples with Kit standards S5 (1000 pg/mL), S4 (500 pg/mL), S3 (125 pg/mL) and S2 (50 pg/mL) in a 1:1 ratio. Each sample (non-spiked and spiked) was assayed and analyte concentrations of the samples were calculated from the standard curve. The % recovery was calculated by multiplication of the ratio of the measurements and the expected values with 100. The expected values were calculated by addition of half of the values determined for the undiluted samples and half of the values known for the standards.
- Linearity:** Three serum samples were diluted in two-fold dilutions up to 1/16 in zero standard. The % recovery was calculated by multiplications of the ratio of the duplicate determinations and the expected values with 100. The expected values were calculated by dividing the values determined for the undiluted samples with the dilution quotients of 2, 4, 8 and 16.
- Method comparison:** The sera (n=65) of pregnant women were measured using the DRG PLGF-ELISA. As

a comparison kit, the R&D systems PLGF-ELISA (R&D Systems) was purchased.

Patients

A total of 94 pregnant women were enrolled in the present study, of which 64 had singleton uncomplicated pregnancies who came to our clinic for routine pregnancy care. Patients with any pregnancy complications were excluded. Serum samples were collected with informed consent from these women and the Research Ethics Committee approval for longitudinal measurement of PLGF in the course of pregnancy. Depending on the number of visitations at our clinic, five to nine serum samples were collected during pregnancy. In this group of uncomplicated singleton pregnancies, longitudinal sFLT-1 measurements were additionally performed in 28 women from 20 to 42 weeks of pregnancy. Furthermore, serum samples were collected from 30 patients coming to our clinic because of preeclamptic symptoms. In these 30 preeclamptic patients, serum levels of PLGF and sFLT-1 were measured by Human PLGF-ELISA (ELISA, DRG) and Human sVEGF R1 ELISA (R&D Systems) according to the described test principles. Preeclampsia had been diagnosed in all cases prior to the onset of labor. Preeclampsia was defined as gestational hypertension (systolic pressure of > 140 mm Hg or diastolic blood pressure of > 90 mm Hg on at least two occasions after 20 weeks of gestation) with proteinuria (> 300 mg/d or > 1+ measured by dipstick). Serum samples were obtained at 6–42 weeks of pregnancy for the singleton uncomplicated pregnancies and during 22–38 weeks of pregnancy for the preeclamptic ones. Serum samples were also collected before the onset of labor in all cases. None of the pregnant women had fetal aneuploidy and abnormalities of cord insertion. In all cases, gestational age was confirmed during early gestation by crown-rump length measurement with transvaginal ultrasonography.

Statistical analysis

The PLGF concentrations were calculated with the DRG Regression Program. Statistics were calculated with the JMP 6.02 software (SAS Institute, Cary, NC, USA). A p-value of < 0.05 was considered statistically significant.

Results

Assay characteristics

Analytical sensitivity The analytical sensitivity was < 1.062 pg/mL.

Intra-assay variation The intra-assay variations for the PLGF-ELISA were determined as 2.83% for a serum with 50.45 pg/mL and 1.7% for a serum with 478.6 pg/mL. These variations were within the required specifications of < 10%.

Inter-assay variation The inter-assay variations for the PLGF-ELISA were determined as 4.1% for a serum with 45.8 pg/mL and 7.0% for a serum with 421 pg/mL. The variations were within the required specifications of < 5%.

Recovery Recoveries of 87%–105.5% over the entire measuring range were found. The required specification of 85%–115% recovery was fulfilled for all three samples.

Linearity The % of recoveries was found between 88.1% and 112.6% over the measuring range. The required specification of 85%–115% recovery was fulfilled for all samples in all dilutions.

Method comparison Comparison of the values of the DRG PLGF-ELISA and the R&D Systems PLGF-ELISA resulted in a correlation coefficient of 0.921 (Figure 1).

Clinical data

The group of the singleton uncomplicated pregnancies consisted of 64 patients. They did not have any pregnancy complications. There was no documented hypertension or proteinuria. In this group, the mean gestational age at delivery was 39 weeks with a mean birth weight of 2980 g.

The 30 preeclamptic patients all had clinical symptoms of preeclampsia, defined as systolic blood pressure of > 140 mm Hg or diastolic blood pressure of > 90 mm Hg on at least two occasions after 20 weeks of gestation and proteinuria of > 300 mg/d or > 1+ measured by dipstick. Depending on the severity of symptoms, the patients were treated with expectant management (n = 18) or induction of delivery (n = 12) within 48 h following admission to our clinic. The mean gestational age of delivery was 30 weeks with a mean birth weight of 1540 g. The mean time of prolongation was 11 days.

PLGF and sFLT-1 expression in normal and preeclamptic pregnancies

Longitudinal serum concentrations of PLGF in normal pregnancies The gestational pattern in the PLGF level is shown in Figure 2. The PLGF concentrations

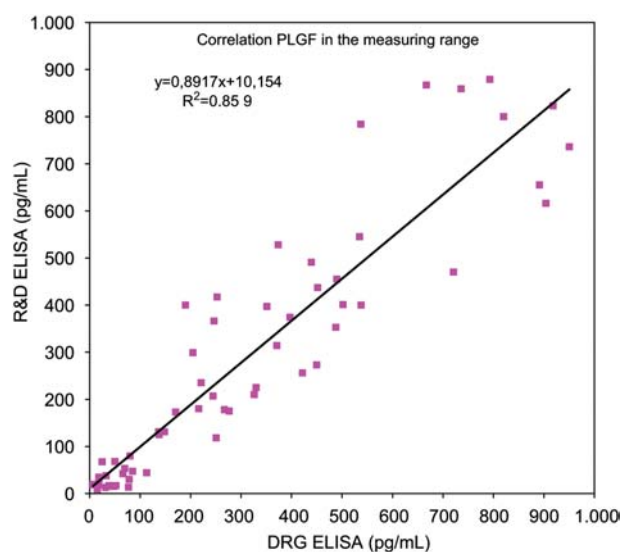


Figure 1 Method comparison of the DRG PLGF-ELISA and the R&D PLGF-ELISA.

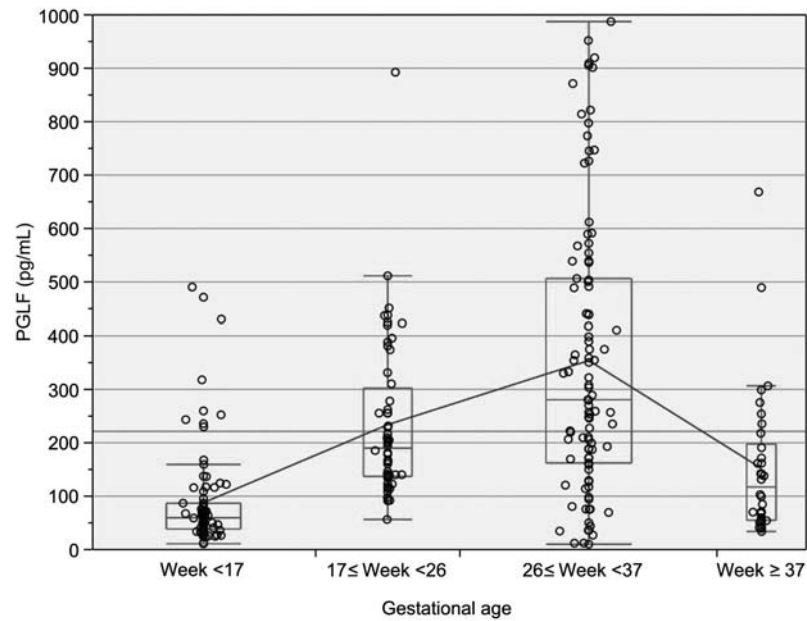


Figure 2 PGLF serum concentrations in the course of pregnancy.

A total of 275 PGLF measurements were obtained from 64 healthy pregnant women. The quantile boxes represent the 25th, 50th and 75th percentiles. The bars represent the 10th and 90th percentiles. The solid line connects the means of each group.

in normal pregnancies showed a steady increase starting at the beginning of the second trimester with a peak at 28–32 weeks and a consistent decline thereafter. The mean PLGF concentration in the first trimester was 88 pg/mL, rising up to a mean of 346 pg/mL at the beginning of the third trimester.

PLGF in preeclamptic and normal pregnancies Figure 3 depicts the PLGF expression in normal and preeclamptic pregnancies. The patients with preeclamptic pregnancies showed a significant lower serum concentration of PLGF compared to the patients with non-preeclamptic pregnancies ($p < 0.05$).

Between the 20th and 25th week, all patients with clinical signs of preeclampsia had a PLGF expression of less than 100 pg/mL. During this period all patients with non-preeclamptic pregnancies had expressions of PLGF > 100 pg/mL.

sFLT-1 in preeclamptic and normal pregnancies The expression of sFLT-1 was higher in patients with preeclamptic pregnancies; this difference was significant ($p < 0.05$). Between the 20th and 25th week, all patients with clinical signs of preeclampsia had a sFLT-1 level of $> 10,000$ pg/mL, whereas the non-preeclamptic patients had a sFLT-1 level of $< 10,000$

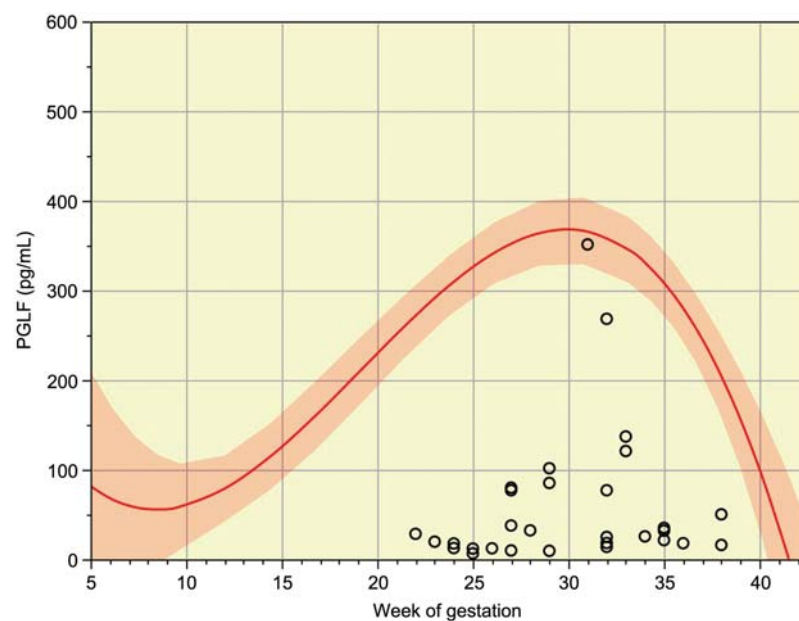


Figure 3 PGLF measurements in 30 preeclamptic pregnant women.

The reference curve was derived from 275 measurements in 64 healthy pregnant women (mean and 95% confidence interval).

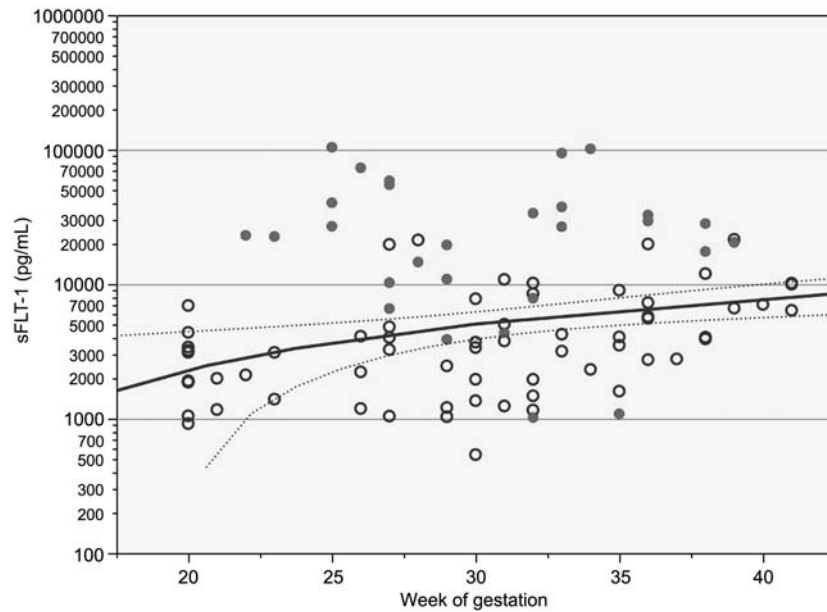


Figure 4 sFLT-1 expression in normal and preeclamptic pregnancies >20 weeks.

The solid line shows the mean sFLT-1 concentration from 68 measurements of 28 singleton uncomplicated pregnancies and the 95% confidence interval. The filled circles represent the sFLT-1 concentration of 30 preeclamptic patients ($p < 0.05$).

pg/mL during this period. The sFLT-1 levels are shown in Figure 4.

Discussion

It has been proven that an imbalance between factors promoting angiogenesis, such as VEGF or PLGF, and factors antagonizing angiogenesis, such as sFLT-1, play a fundamental role in the pathogenesis of preeclampsia (26, 27). The activity of VEGF and PLGF is, among others, regulated by the corresponding receptors FLT-1 (fms-like tyrosine kinase or VEGFR-1) (28, 29).

The sFLT1 factor as a splice variant of the FLT-1 antagonizes the angiogenic effect of VEGF by inhibiting their binding to the VEGFR-2 receptor (24). Vascularization of the placenta already starts 3 weeks after conception. At the beginning of the third week of development (day 21 p.c.), mesenchymal cells inside the villi transform into first hemangiogenic precursor cells (tertiary villi) (30). The fetoplacental angiogenesis during gestation is biphasic, the first trimester is characterized by a branching of the small vessels. From the beginning of the second trimester, the gain of size of the placenta is a consequence of a proliferation of endothelial cells. In contrast to the branching angiogenesis of the first trimester, the angiogenesis of the second and third trimester is mainly characterized as non-branching angiogenesis (31). The underlying hypothesis that preeclampsia is caused by altered angiogenesis is supported by the fact, that the PLGF serum levels in patients with preeclampsia are significantly lower than the levels in patients with non-preeclamptic pregnancies. Comparable results have been published by other authors (21, 25, 32–34). Some authors have shown that PLGF con-

centrations begin to decrease 11–9 weeks before the onset of preeclampsia, with substantial reductions during the 5 weeks before the onset of hypertension or proteinuria (25, 35, 36). Our results show that the alterations in the PLGF and sFLT-1 levels are more pronounced in women with early onset preeclampsia, especially before the 26th week of pregnancy. Similar results have been described recently (37). All these studies used the Human PLGF-ELISA by R&D Systems for scientific use. This is the first study using the new Human PLGF-ELISA by DRG. All the measured assay characteristics fulfilled the required specifications and this assay can be considered reliable. Apart from the authorization for routine diagnostics, the DRG ELISA offers some advantages compared to the R&D ELISA. Handling of the different reagents of each kit was more comfortable using the DRG kit. The DRG kit supplies “ready to use” reagents, whereas the R&D kit consists of stock solutions which have to be reconstituted. For example, the standard curve is performed out of one stock solution which first has to be reconstituted. Subsequently, dilution series have to be performed out of the stock solution by the individual user. This may result in imprecise values which may lead to a repetition of the whole assay. Furthermore, the color reagents A (stabilized hydrogen peroxide) and B (stabilized chromogen) had to be prepared 15 min prior to use, which means within a running test. In contrast, DRG supplies the user with “ready to use” solutions for all steps of the assay, which makes handling of the test not even more comfortable but also more reliable. Furthermore, the required sample amount is less and the overall incubation period of the DRG kit is shorter (2.5 h) than the R&D kit (4.5 h). Analytical sensitivity as well as intra- and inter-assay variation of the DRG kit is higher than the published test characteristics of the R&D kit. Last

but not least, the DRG kit is half the price of the R&D kit. A disadvantage of the DRG kit is the necessity of four incubation steps, whereas for the R&D kit there are three steps. The corresponding sFLT-1 assay is not yet available by DRG.

The purpose of all screening tests for preeclampsia must be the detection of a high-risk group as early as possible in pregnancy and to offer a prophylactic treatment to women at high risk. The only prophylactic regimen which may lead to a reduction of preeclampsia is low-dose aspirin (3, 38). It should start before the complete invasion of the trophoblast from the 12th to 16th week of pregnancy. Until now, there is no clinically useful screening test to predict the development of preeclampsia early in pregnancy (39). A combination of measurements of angiogenic factors and abnormal uterine artery Doppler velocimetry in the middle of the second trimester may be useful in future screening for early prediction of pregnancy complications (40, 41). Further studies need to be carried out to answer the question whether the measurement of PLGF alone or in combination with other factors in the first trimester or at the beginning of the second trimester may define a group with a high risk for preeclampsia, which then may benefit from low dosage aspirin therapy. As the new developed PLGF-ELISA is authorized for routine diagnostic testing, it may offer new possibilities in the prediction of preeclampsia in clinical routine.

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