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Analysis of transcriptional activities of angiogenic biomarkers during intrauterine complications leading to preterm birth

M. RABAJDOVÁ[1], R. DUDIČ[2], P. URBAN[1], V. DUDIČOVÁ[2], P. URDZÍK[2], M. MAREKOVÁ[1]

[1]Department of Medical and Clinical Biochemistry, P. J. Šafárik University in Košice, Faculty of Medicine, Košice, Slovakia
[2]2nd Department of Gynecology and Obstetrics, P. J. Šafárik University in Košice, Faculty of Medicine, Košice, Slovakia

Abstract. – OBJECTIVE: Pre-eclampsia, growth retardation and preterm delivery are the most common reasons leading to increased maternal and perinatal mortality. The increased expression of hypoxia induced factors, such as HIF-1, triggers the overexpression of anti-angiogenic genes. The aim of this study was to determine the transcriptional activity of individual pro- and anti-angiogenic markers (VEGF, HIF-1, sEng, Flt-1, PlGF-1) in maternal blood samples from patients with spontaneous preterm labor, preterm labor in combination with pre-eclampsia and fetal growth restriction in comparison with physiologically terminated pregnancies.

PATIENTS AND METHODS: The transcriptional activity of specific genes was detected from the blood of patients using the chromatin immunoprecipitation capture method coupled with quantitative real-time PCR.

RESULTS: The maximum differences in mRNA levels of PlGF-1 and VEGF-A were detected in two groups: the group of normal-term birth with complications and the group of preterm labor with complications (both significantly lower than the control, $p < 0.001$). In contrast, a marked increase of mRNA levels was found in the same groups of patients for the HIF-1, endoglin and Flt-1 genes ($p < 0.001$).

CONCLUSIONS: According to our results, we can conclude that increased oxidative stress, increasing the expression levels of anti-angiogenic genes and reduction of the transcriptional activity of pro-angiogenic genes can provide additional information during diagnostics of pathological complications of labor.

Key Words:
Pre-eclampsia, Preterm labor, Growth retardation, Pregnancy.

Introduction

The prevalence of pathological conditions that can complicate pregnancy has recently soared. Pre-eclampsia, growth retardation and preterm delivery have been significantly involved in the increase of maternal and perinatal morbidity and mortality. According to the World Health Organization (WHO), pre-eclampsia (PE) is responsible for 70,000 maternal deaths[1] and 500,000 infant deaths per year[2].

PE in a mother can cause premature cardiovascular diseases, such as chronic hypertension, ischemic heart disease and stroke. Children born from mothers with PE have an increased risk of stroke, coronary heart disease, and metabolic syndrome in adulthood[3]. Incorrect placentation plays a central role in the etiology of the disease, causing problems in the exchange of gases, metabolic substrates[4], the abnormal invasion of trophoblast, hemodynamic changes, immunological defects, genetic predisposition[5] and the generation of oxygen radicals.

All of the mentioned processes can lead to common complications of pregnancy, including fetal growth retardation (FGR) or intrauterine growth restriction (IUGR). Both complications indicate a pathological effect on fetal growth and development, when fetal malnutrition causes a prevalent disorder of normal fetus growth. Incorrect or late identification of IUGR/FGR is a major cause of perinatal morbidity and mortality[6]. The most common causes of IUGR/FGR are: constitution (40%), uteroplacental flow (40%), genetic factors (10%) and the influence of the external environment (10%)[7].

Corresponding Author: Maria Mareková, MD; e-mail: maria.marekova@upjs.sk

The other complication during pregnancy is a preterm birth. Every year approximately 15 million babies are born preterm (prior to 37 weeks of gestation). According to WHO, in 2013 preterm birth complications were the leading cause of death amongst children under the age of five. The frequency of premature births is rising slightly in the industrial countries[8] and is at about 6-10% of births. Despite the great progress of modern medicine, every year around 4.5 million premature children are delivered. WHO classifies preterm birth into three categories, the most serious being "extremely preterm", which means birth before 28 weeks of pregnancy (with a mean fetal weight at least 500 g)[9]. Premature birth is a major etiologic factor in neonatal mortality and morbidity, causing 70-80% of perinatal deaths[10], and it remains one of the most serious problems in obstetrics.

The reasons that trigger these pathological processes remain unknown. Recent scientific papers consider a combination of factors, including hypoxia of the placental endothelium[11]. Biomolecules as antiangiogenic factors play an important role in angiogenesis during placenta development. Vascular endothelial factor (VEGF) and placental growth factor (PlGF) belong in the group of pro-angiogenic factors. VEGF in combination with other growth factors maintains the physiological function of the placental endothelium through interaction with endogenous endothelial receptors[12]. PlGF stimulates the growth of blood vessels by affecting the migration and survival of endothelial cells, vascular maturation and the stimulation of fibroblast proliferation[10]. The main factor that stimulates the up-regulation of gene expression of growth factors is placental hypoxia, which is characterized by the production of hypoxia inducible factor-1 (HIF-1). The increased expression of HIF-1 in endothelial cells positively influences the expression of PlGF in the primary cell line of cardiomyocytes[13]. The group of anti-angiogenic markers includes soluble FMS-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng)[14]. Pre-eclampsia and the combination of IUGR/FGR lead to excessive secretion of sFlt-1 and sEng, which inhibit the activity of VEGF and the transformation of growth factor-b1 (TGF-b1) in the blood vessels. These changes lead to the dysfunction of endothelial cells, reduced levels of prostacyclin, nitric oxide production and the release of procoagulant proteins[15], which can ultimately lead to premature delivery. The aim of this study was to determine the transcriptional activity of individual pro- and anti-angiogenic markers (VEGF, HIF-1, sEng, sFlt-1, PlGF) expressed in maternal blood of patients suffering from spontaneous preterm labor, pre-eclampsia in combination with IUGR/FGR and normal term and preterm birth in comparison with a control group of mothers with a physiologically terminated pregnancy.

Patients and Methods

Experimental Model

The transcriptional activity of specific genes was detected from the blood of patients using the chromatin immunoprecipitation capture method coupled with quantitative real-time PCR. The control group consisted of healthy pregnant women (n = 10) at the Department of Gynaecology and Obstetrics, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, with a normal term birth without any complications. The experimental group (n = 14) consisted of patients with confirmed pre-eclampsia together with IUGR, divided into two subgroups: normal term birth and patients with preterm birth and a group without complications but with spontaneous preterm birth. All clinical investigations were conducted according to the principles of the Declaration of Helsinki. The healthy subjects in the control group and patients in the experimental groups answered a medical questionnaire. Patients were informed by their doctor about the use of their blood for experimental – diagnostic purposes. Informed consent was signed. Ethical consent for this study was granted by the Institutional Committee on Human Research and was approved by Ethical Committee of the University Hospital of Louis Pasteur in Košice, Slovakia.

DNA and Chromatin Isolation

A 7 ml sample of whole blood was collected from all patients into Venosafe test tubes (Medistyl-Pharma, Prague, Czech Republic). The whole blood was incubated on ice for 30 minutes and then diluted with erythrocyte lysis buffer (ELB, consists of: 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.4) in a ratio of 1:4. After centrifugation at 2500 rpm/10 min/RT, the supernatant was carefully removed, and the pellet was re-suspended thoroughly in ELB. Next, centrifugation was done at 2500 rpm for 10 min at room temperature to separate the pellet of white blood cells, which was then resuspended in 1×

PBS; 1% formaldehyde was used for cross-linking the cells/for 10 min/37°C. Cross-linking was stopped by the addition of 125 mM of glycine. The cell lysates were sonicated with a Bioruptor (Diagenode, Denville, NJ, USA) at high intensity for 5 min, with 30 s ON/OFF intervals. For pre-clearing of chromatin Protein G agarose beads (Merck Millipore, Praha, Czech Republic) were used for 1 hour/4°C. Chromatin fragments with lengths of 200-500 bp were made visible on an agarose gel. Isolated cells were snap frozen and stored at −80°C. A Nanodrop LC 3000 (Thermo Scientific, Bratislava, Slovak Republic) was used for measuring the concentration of isolated chromatin.

Copy Number Variation Analysis

Analysis of gene copies was performed after the isolation of DNA using specific primers for all exon-specific gene domains of FLT-1, VEGF-A, PlGF-1 and sEng in comparison with HPRT and GAPDH. Amplification of specific genes was run for 33 cycles (95°C 5 min, 95°C for 15 seconds, 58°C-60°C for 20 seconds, 72°C for 25 seconds) using the appropriate primer sequences and a thermocycler Rotor-Gene Q-PCR thermocycler (Qiagen, Hilden, Germany).

ChIP qRT-PCR

Chromatin immunoprecipitation (ChIP) was performed using whole blood according to a previously published protocol[16]. Sonicated, precleared chromatin (115 ng) was incubated with the antibody [anti-RNAPII CTD chipGrade (ab817, Abcam, Cambridge, UK)] at 4°C overnight. The RNAse treatment was done 30 minutes before incubation using a mix of RNase A/T (Roche Slovakia, Bratislava, Slovak Republic). Protein IgG agarose beads (Merck Millipore, Prague, Czech Republic) were used for bonding with imuno-complexes and crosslinking them. The next procedure used the downward line of cleaning buffer solutions in the order buffer I and II (500 mM NaCl, 50 mM HEPES (pH 7.5), 1% Triton-X-100, 0.1% sodium deoxycholate, 1 mM EDTA (pH 7.5)), buffer III (250 mM LiCl, 0.5% NP-40, 10 mM Tris-Cl (pH 8.0), 0.5% sodium deoxycholate, 1 mM EDTA (pH 7.5)) and buffer IV (1mM EDTA, 10 mM Tris-HCl). Immunoprecipitated DNA was eluted from the beads in TE Tris-EDTA buffer with 1% SDS. For reverse crosslinking of samples a solution was used containing 5 mol/l NaCl, 5 g/ml of enzyme RNaseA (Roche Slovakia, Bratislava, Slovak Republic), 1 M TrisHCL (Sigma-Aldrich, Bratislava, Slovak Republic) and 20 g of Proteinase K (Roche Slovakia, Bratislava, Slovak Republic) which was incubated at 65°C overnight and purified using Qiagen PCR purification columns kit (28104, Qiagen, Hilden, Germany). DNA was eluted twice with 30 µl of RNAase/DNAase free water (Qiagen, Hilden, Germany). An aliquot of 2 µl of each sample was used for qRT-PCR using SensiMix (Bioline, Luckenwalde, Germany). Amplification was performed on a Qiagen Rotor-Gene Q-PCR thermocycler using the protocol: 30 cycles (95°C for 5 min, 95°C for 15 s, 60°C for 20 s and 72°C for 25 s). All primer pairs (Sigma-Aldrich, Bratislava, Slovak Republic) used for ChIP analysis were designed using Internet databases (www.genome.ucsc.edu/ and www.oligoevaluator.com/Login.jsp). The chromosomal localizations of the primer pairs are listed in Table I. Genes for GAPDH and HPRT (hypoxanthine phosphoribosyltransferase) were used as a control housekeeping gene. Each sample was measured in triplicates.

Statistical Analysis

For statistical evaluation, a One-Way ANOVA Student-Newman-Keuls test was used. Statistical analysis was processed using GraphPad INSTAT software (GraphPad Software, La Jolla, CA, USA). The same test was performed for nonparametric correlation using Spearman correlation coefficients. $p < 0.005$ was considered statistically significant.

Results

Copy Number Variation

The analysis of gene copy numbers is necessary for the phenotypic evaluation of disease activity. It is important in the evaluation of mRNA expression of the transcriptional activity of the exon portions. The number of gene copies gives us the necessary information on the translation of the mRNA expression levels of the number of copies of each gene present on the chromosome (Figure 1) clearly shows that the number of copies of all genes in the analyzed group was 1. There was no significant difference in the group of premature births with PE and the IUGR gene PlGF-1 (6% more compared to the control) and in the group with early the birth and the VEGF-A gene (by 5.5% more than the control).

Table I. Localization of the chromosome of specific genes (www.genome.ucsc.edu).

Name of gene	Chromosomal localization	Size of gene in bp including UTRs side	Analysis place of genes Ex-exon
sEndoglin	9 q34.11	130.577.291-130.617.052	Promotor Ex1, Ex3 Ex5, Ex10
PlGF-1	14q24.3	75.408.533-75.422.467	Promotor Ex3, Ex6, Ex7
FLT-1 F	13q12.2.-12.3	28.942.234-29.069.265	Promotor Ex2, Ex3, Ex5, E11
HIF-1	14q23.2	62.162.119-62.214.977	Promotor Ex2,Ex 5, Ex6, Ex 12
VEGFA	6p21.1	43.737.946-43.754.223	Ex2, Ex3, Ex4
GAPDH	12p13.31	6.643.585-6.647.537	Ex2, Ex4, Ex8

Detection of Specific Genes by Transcriptionally Active Chromatin Analysis

During the analysis of transcription activity, we found that the exon regions of each gene showed proportionately the same levels of mRNA expression. The average values of mRNA expression of individual exons in each specific gene were used in the graphs.

Gene expression changes of PlGF-1 in the blood of patients are shown in Figure 1. The mRNA levels of PlGF-1 in the group of preterm birth was non-significantly decreased in comparison with the control group. However, the maximum differences in mRNA levels of PlGF-1 were detected in the normal term birth group with the PE and IUGR complications (57% lower than the control, $p < 0.001$) and in the group of preterm birth with PE and IUGR complications (77% lower than the control, $p < 0.001$). These results suggest that the pro-angiogenic effect of PlGF-1 is decreased in all experimental groups where rapid downregulation of PlGF-1 was detected. The gene expression changes of VEGF-A in the blood of patients are shown in Figure 2.

Hypoxia as one of the main factors of preterm birth is characterized by elevated expression levels of HIF-1. The gene expression changes of HIF-1 in the blood of patients are shown in Figure 2. We detected that mRNA levels of HIF-1 are extremely high in both groups of patients with

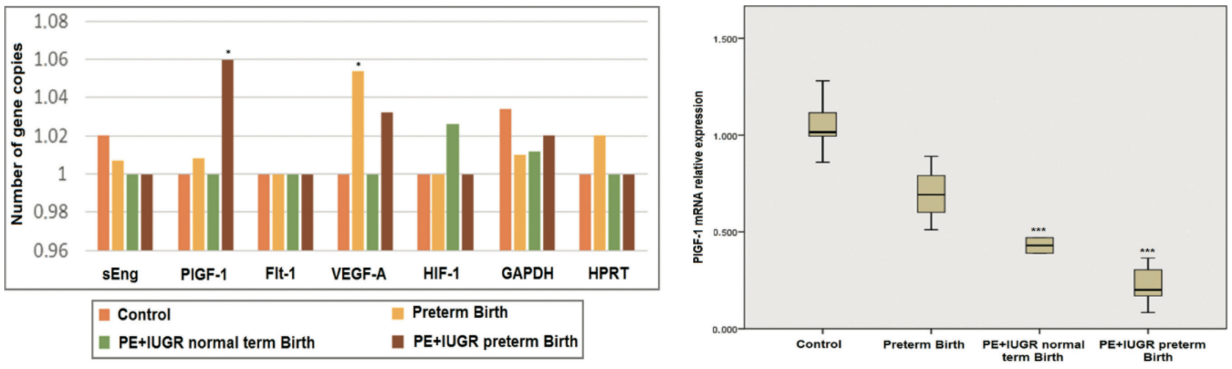

Figure 1. The gene copy number of all selected and control genes (*left*) and the gene expression of PlGF-1 (*right*). The same number of copies of all genes in the analyzed and control group, *means $p < 0.05$ change. The mRNA levels of PlGF-1 are shown as ratio PlGF-1/Control. ***means $p < 0.001$.

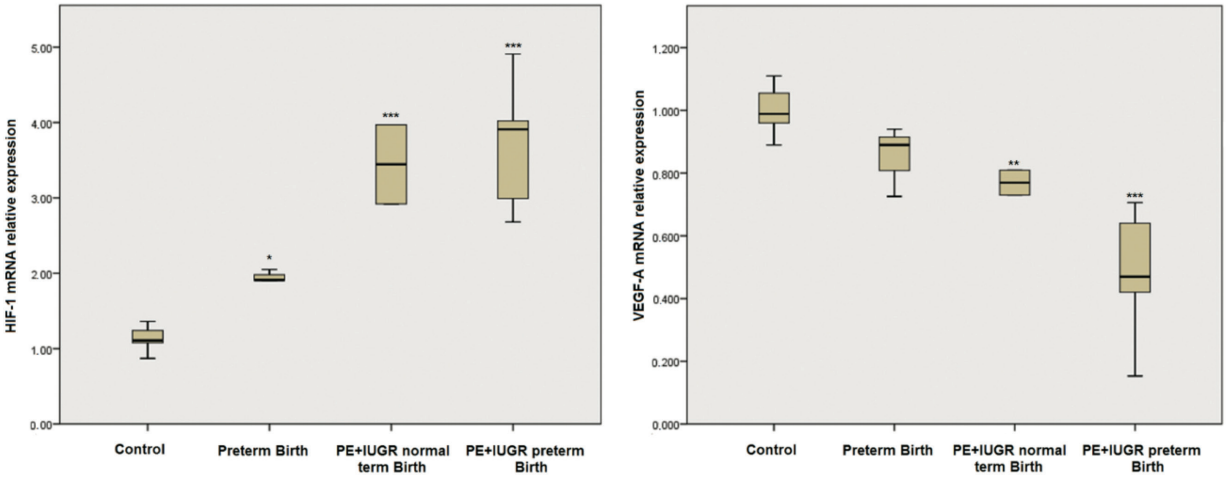

Figure 2. Gene expression of HIF-1 (*left*) and VEGF-A (*right*). The mRNA levels of HIF-1 and VEGF-A are shown as a ratio with the control group. *means $p < 0.05$, **means $p < 0.01$ and ***means $p < 0.001$.

preeclampsia and with IUGR (230% the normal term birth and 260% the preterm birth respectively, $p < 0.001$).

Transcriptional activity, and thus the level of mRNA gene expression of soluble endoglin, has been increased according to the occurrence of complications in each group (Figure 3).

Patients who were diagnosed with pre-eclampsia had the highest expression values of IUGR (340% greater than the control, $p < 0.001$). These patients were born prematurely. A significantly increasing dependency of mRNA expression was detected with the Flt-1 gene (Figure 3). The extremely elevated levels of expression of Flt-1 were detected in both groups with PE and IUGR complications (by 290% in the standard time of birth and by 416% in the pre-term labor, $p < 0.001$).

Correlations Between mRNA Expressions of Anti- and Pro-Angiogenic Genes

The level of mRNA expression of the gene Flt-1 indirectly correlates with the transcriptional activity and mRNA expression level of the PlGF-1 and VEGF-A genes. For patients with increasing levels of mRNA expression of Flt-1, we found decreasing mRNA levels of the genes VEGF and PlGF-1 (Figure 4).

The level of expression of endoglin indirectly correlates with the transcriptional activity of the PLGF-1 gene. Our results clearly showed that the increasing transcriptional activity of endoglin and sFlt1 block the angiogenic effects of PlGF-1 and VEGF.

During a comparison of the transcriptional activity of PlGF-1, we found that increasing

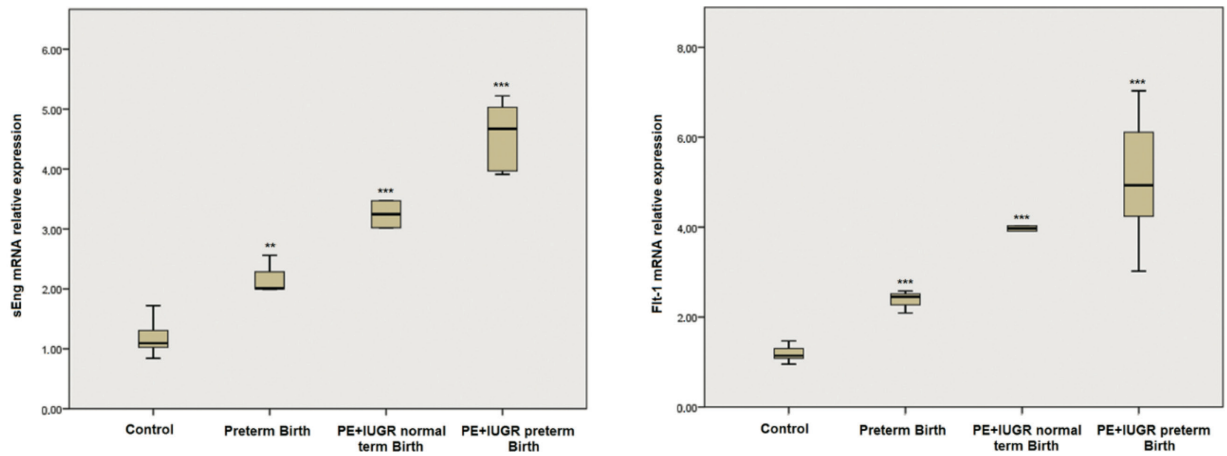

Figure 3. Gene expression of sEng (*left)* and Flt-1 (*right)*. The mRNA levels of sEng and Flt-1 are shown as a ratio with the control group. **means $p < 0.01$ and ***means $p < 0.001$.

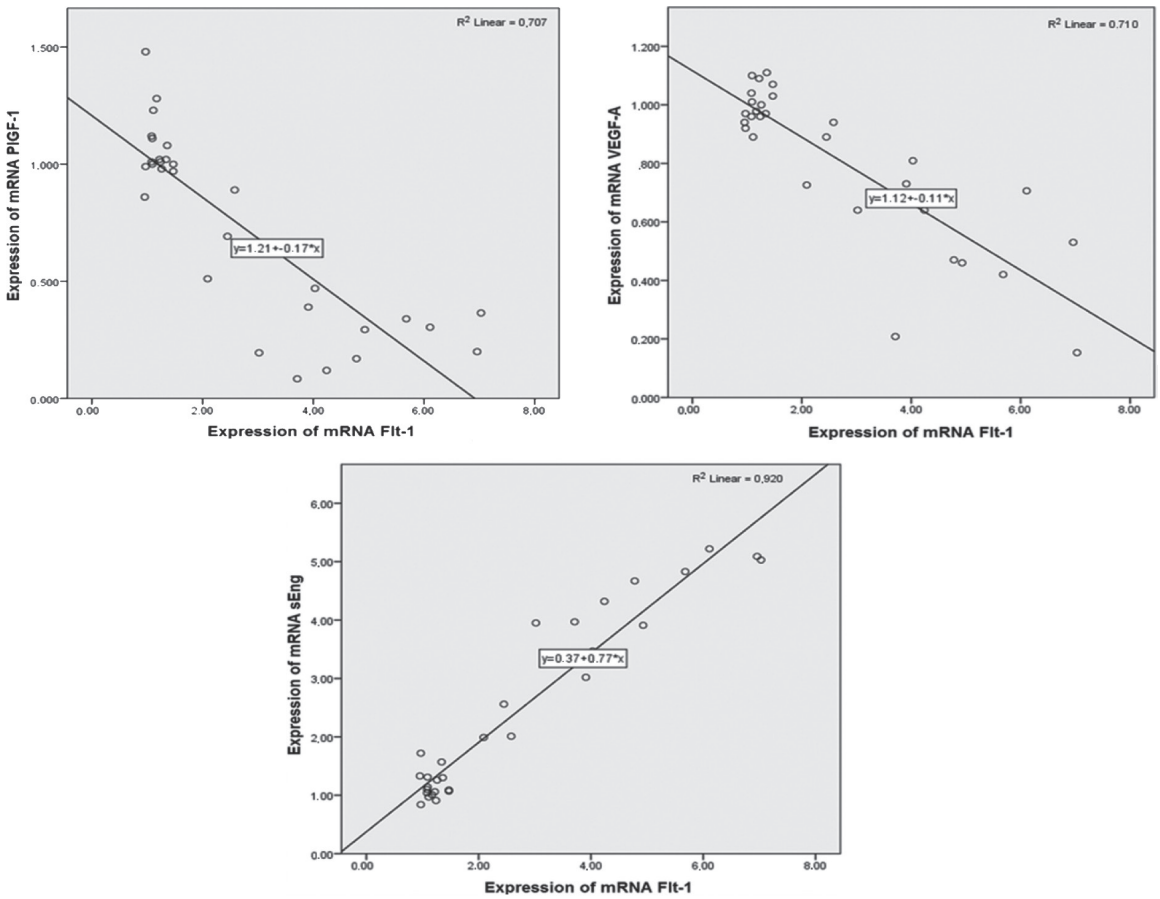

Figure 4. Correlation between Flt-1 expression and PlGF-1, VEGF-A and sEng.

expression of PlGF-1 positively correlated with the expression of VEGF-A and at the same time negatively with the expression of endoglin. From these results, it can be assumed that the pro-angiogenic effect of PlGF-1 clearly increases the expression of VEGF-A but inhibits the expression of endoglin (Figure 5).

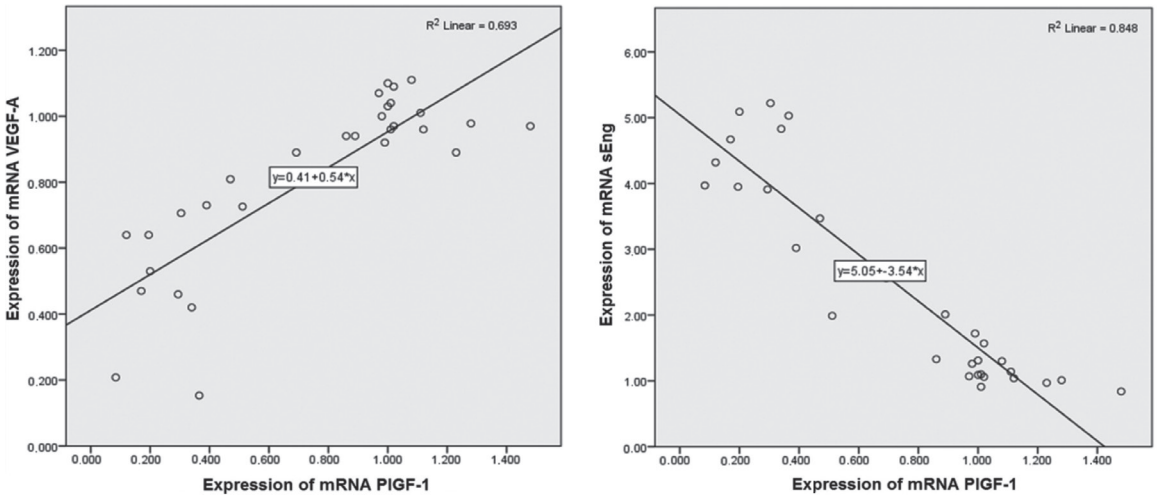

Figure 5. Correlation between PlGF-1 expression and VEGF-A and sEng.

Analysis of Transcriptional Activity According to the Weeks of Pregnancy

The downregulation of PlGF-1 expression started at 17 weeks of pregnancy. We also found a remarkable difference in the expression of PlGF-1 between the groups of patients with preterm birth without any complications and those with PE and IUGR (about a 46% decrease in the group with complications, $p < 0.01$).

We showed a general decrease in the mRNA levels of VEGF-A in all experimental groups, visible from the 16th week of pregnancy. According to our results, we suggest that pre-eclampsia and IUGR cause a decrease of VEGF-A expression and this is related to higher risk of preterm birth.

Monitoring changes of expression during the weeks of pregnancy showed early onset of a rapid increase of mRNA for HIF-1 at 16 weeks of pregnancy, with levels already two times higher in contrast to the control. Pre-eclampsia with IUGR induced the expression of HIF-1 (mRNA levels about 180% higher in the group with preterm birth and complications than with preterm birth without complications).

In the mRNA levels of sEng, we detected twofold elevated levels ($p < 0.001$) in the preterm group with complications in comparison with the preterm birth without complications. We found the early onset of a rapid increase of mRNA for sEng at 16 weeks of pregnancy, with double the levels compared with the control. The expression of Flt-1 detected in both groups with PE and IUGR complications was also high (290% and 416%, respectively, $p < 0.001$). All the results of transcriptional activity of the selected genes are summarized in Figure 6.

Discussion

Many pathological conditions can complicate pregnancy and cause it to end prematurely. Negative or positive effects of pre-eclampsia, growth retardation and preterm birth on the transcription activities of specific genes involved in angiogenesis correspond with the current state of mother and fetus. The underlying pathological mechanisms that activate these complications are still unknown. The research of Yan et al[17] showed that the incidence of pre-eclampsia correlates strongly with specific genetic factors that may be regulated via DNA methylation. Further study on the regulatory mechanisms behind the modulation of these genes should, however, be done to verify these data.

Therefore, it is necessary to study the molecular basis of the mentioned pathophysiological processes and define new strategies for earlier identification of preterm labors. The aim of this study was to determine the transcriptional activity of potential biomarkers (PlGF-1, Flt-1, sEndoglín, HIF-1a, and VEGF-A), which could be helpful in the earlier diagnosis of pre-eclampsia, IUGR, and premature birth. During the detection of the gene

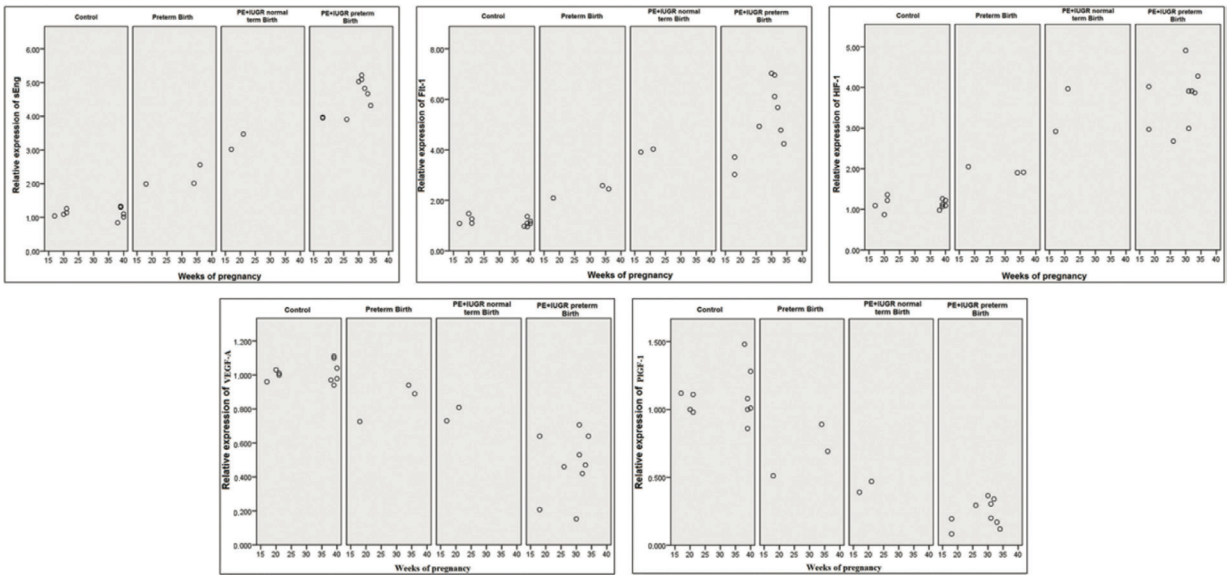

Figure 6. Transcriptional activity of selected genes according to the weeks of pregnancy.

copy number, we showed that all genes had the same gene copy number equal to 1, confirming that the gene copy number was constant in all groups and does not affect the recorded changes in the levels of gene expression. We also monitored the expression level of the mentioned genes in the different weeks of gestation.

Our results demonstrated increased transcription activity of soluble endoglin, which correlates with the results of Yinon et al[18], where they found a correlation between an increased level of endoglin mRNA in the placenta with severe growth retardation and the degree of prematurity[19]. The study of Rajakumar et al[20] using placental biopsy showed elevated levels of Flt-1 due to the induction of hypoxia in placenta and the induction of HIF1. It also confirmed the data we obtained from the analysis of mRNA expression of the endoglin specific genes, HIF-1 and Flt-1. In their study, they proposed that placental hypoxia, as a consequence of poor perfusion, is a basic mechanism for the formation of PE and IUGR, associated with the increased transcriptional activity of the genes for sEng and Flt-1 in the blood of patients[21]. Khan and Bicknell[22] indicated that endoglin gene transcription was upregulated in hypoxic damage to the specific tissue of the placenta. They also described the structure of sFlt-1 expressed into the blood stream due to the absence of the transmembrane and intracellular domain and the mutual antagonism of the VEGF and PlGF-1 proteins, which was also confirmed by our results describing the expression of VEGF and PlGF-1[23].

Chaiworapongsa et al[24] described the binding and neutralizing function of sFlt1 and the pro-angiogenic activity of VEGF and placental growth factor. High concentrations of circulating sFlt1 and low levels of VEGF and PlGF were visible not only during pre-eclampsia, but before the onset of clinical symptoms[25]. Based on cross-correlation of the transcriptional activity as well as on the level of protein encoded by endoglin and Flt-1, with the enhanced effect on endothelial dysfunction, it is obvious that these genes can be used for differentiation of the symptoms of severe pre-eclampsia, hemolysis, elevated liver enzymes, Low Platelets syndrome **(**HELLP) and its development as well as formation of IUGR. Using Spearman correlation coefficients (non-parametric correlation), we found a strong correlation between the expression of endoglin mRNA levels and the expression of the Flt-1 gene expressed in the blood of patients. Our findigs suggest that the transcriptional activity of endoglin and Flt-1 increases before the 15th week of pregnancy. Their transcriptional and translational activity showed a clear upward trend in women with pre-eclampsia and IUGR compared with patients in the control group. This is confirmed by Levine et al[26], who described that sFlt-1 levels start to rise 5 weeks before PE and remain elevated in comparison to women without PE. The mRNA levels of Flt-1 correlated directly with the severity of disease[27]. Previous studies suggest that serum levels of free PlGF decrease with the progression of PE. Karumanchi et al[28] found that under physiological conditions during the first 30 weeks of pregnancy, the concentrations of both pro-angiogenic factors were increased. The transcriptional activity of a gene encoding serum-free PlGF-1 and VEGF showed the same activity in respect to the analysis of mRNA expression of genes correlated with the particular weeks of pregnancy[29,30]. Based on our data, we conclude that increased oxidative stress, increased expression levels of anti-angiogenic genes and decreased transcriptional activity of pro-angiogenic genes may provide additional information in the evaluation of pathological processes, such as pre-eclampsia, PE and IUGR and preterm birth during pregnancy.

Conclusions

This work provides the latest knowledge on the etiology, epidemiology, and diagnosis of intrauterine retardation during the prenatal period. It discusses not only the diagnosis of the clinical conditions, such as pre-eclampsia and IUGR, but also expressional changes of potential biomolecules which play an important role in the angiogenesis of the blood vessels of the placenta. Based on an analysis of the transcriptional activity of specific genes, we found inhibiting effects of Flt-1 on the expression of pro-angiogenic growth factors (VEGF-A, PlGF-1) and an enhancing effect on the expression of soluble endoglin. During the analysis of patient blood samples, we clearly proved that the levels of mRNA expression of genes for soluble endoglin, FLT-1, PlGF-1, VEGF-A, and HIF-1 were significantly changed depending on the degree of fetal development or ischemia progression during PE and IUGR. Our results can contribute to the development of new diagnostic procedures based on the detection of changes in molecular pathological during the early stages of pregnancy and thus decrease the occurrence of preterm labors.

Acknowledgements
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Conflict of Interest
The Authors declare that they have no conflict of interests.

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