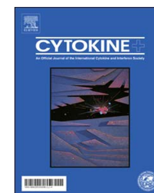
ARTICLE IN PRESS

Cytokine xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine



Angiogenic factor screening in women with mild preeclampsia – New and significant proteins in plasma

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ARTICLE INFO

Keywords:
Angiogenesis
Apoptosis
Angiogenic factors
Cytokine
Protein array
Preeclampsia
Placenta

ABSTRACT

Introduction: The aim of this study was to analyse a panel of 60 angiogenic factors (pro-angiogenic and anti-angiogenic) in the plasma of women with mild preeclampsia.
Materials and Methods: We recruited 21 women between 25 and 40 weeks gestation with diagnosed mild preeclampsia into the study group and 27 healthy women with uncomplicated pregnancies of corresponding gestational age to that of the study to the control group. We used a quantitative protein macroarray method that allowed for analysis of 60 angiogenic proteins per sample simultaneously.
Results: We showed a statistically significant increase in the concentration of 8 proteins, interferon gamma (IFN-γ), interleukin 6 (IL-6), leukaemia inhibitory factor (LIF), heparin-binding EGF-like growth factor (HB-EGF), hepatocyte growth factor (HGF), C-X-C motif chemokine 10 (IP-10), leptin and platelet-derived growth factor BB (PDGF-BB), as well as a significant decrease in the concentration of 3 proteins, vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and follistatin, in the plasma of women with preeclampsia.
Conclusion: Based on our findings, it seems that protein factors may play an important role in the pathogenesis of preeclampsia, and there are many proteins that have not been studied in PE to date. There are no previous studies assessing the LIF, follistatin, HGF, HB-EGF and PDGF-BB concentrations in the plasma of women with PE; therefore, our obtained results indicate that these proteins are new factors that can play an important role in the pathomechanisms of PE.

1. Introduction

Preeclampsia (PE) is a disorder that occurs in 3–5% of pregnancies in Western Europe and North America with almost 8.5 million cases per year recorded worldwide [1]. It is the most common cause of mortality in pregnant women. Clinically, mild preeclampsia (this type of PE will be studied in our project) is the first onset of this disease and is associated with hypertension $\geq 140/90$ mmHg and proteinuria $\geq 0.3 < 5$ g/24 h occurring after the 20th gestational week in women previously identified as normotensive with no protein in urine [2]. It is estimated that preeclampsia will develop in almost 35% of women with gestational hypertension diagnosed before the 34th week of pregnancy. The course of preeclampsia is individually specific - it may present with varying degrees of severity of hypertension and proteinuria and may be complicated by HELLP syndrome (haemolytic anaemia, elevated liver enzymes, low platelet count) as well as severe eclampsia [3]. The associated symptoms are generalized edema, headache and blurred vision, and in severe cases, it may cause liver failure, kidney disease, coagulation disorders, respiratory distress syndrome and intrauterine foetal growth restriction [2]. Despite many hypotheses, the pathogenesis of pre-eclampsia has not been clearly established, and the most effective 'remedy' is delivery [4]. There are many risk factors for

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http://dx.doi.org/10.1016/j.cyto.2017.10.020
Received 28 February 2017; Received in revised form 19 October 2017; Accepted 23 October 2017
1043-4666/ © 2017 Published by Elsevier Ltd.

preeclampsia, including obesity, abnormal lipid profile, type 2 diabetes, insulin resistance, gestational diabetes, maternal age below 20 years of age and above 35 years of age, multiple pregnancy, first pregnancy, urinary tract infection, family history of preeclampsia and cardiovascular disease occurring before pregnancy [5].

For normal growth and development of the foetus, it is essential to reconstruct the spiral arteries in the uterine wall between 10 and 16 weeks of gestation. The process is divided into three main stages according to structural criteria: (1) vascular changes independent of cytotrophoblast invasion; (2) vascular remodelling induced by perivascularly located cytotrophoblasts; and (3) cytotrophoblast infiltration into the spiral arteries [6,7]. As a result of these transformations, spiral arteries change from arteries with low blood flow and high resistance into arteries with fast blood flow and low resistance, ensuring an adequate flow of blood through the placenta and thus normal development of the foetus [4]. In preeclampsia, invasion of the vessel walls by trophoblasts is impaired, which causes the spiral arteries to narrow and results in insufficient delivery of oxygen and nutrients to the foetus for its proper development [4]. Placental ischemia triggers the release of placenta lipids and protein factors and upsets the balance of angiogenic factors, which induces endothelial dysfunction [8,9]. Therefore, measurement of angiogenic factors in women with preeclampsia could lead to a better understanding of the pathomechanisms of this disease and possibly provide new information about the processes that occur in the body of a pregnant woman with PE. The aim of this study was to profile the major angiogenesis-related factors in the plasma of patients with mild preeclampsia. The 60 proteins analysed by our team are the most important factors in human circulation and play a major role in the pro and anti-angiogenic pathways.

2. Material and methods

The recruitment of patients to the study and control groups started after 24 weeks of gestation since we attempted to have all patients undergo a 75 g Oral Glucose Tolerance Test (which is routinely conducted in Poland between the 24th and 28th week in pregnant women). We recruited patients with mild preeclampsia between 25 and 40 weeks gestation (patients with severe PE were not included because we aimed for greater homogeneity in the study group). The inclusion criteria were as follows: blood pressure between 140/90 and 160/110 mmHg in two independent measurements taken at an interval of at least 6 h, the presence of protein in the 24-h urine collection at a level above 300 mg/24 h but not more than 5 g/24 h. We excluded women with: chronic hypertension, multiple pregnancies, pre-existing diabetes or gestational diabetes, connective tissue disease, kidney disease, viral diseases, toxoplasmosis, urinary tract infection, thrombocytopenia and coagulation disorders, pregnancy diagnosed with chromosomal aberrations before or after childbirth and BMI > 30 at the time of recruitment. We obtained 20 ml of blood in EDTA tubes from each patient (fasting) who qualified for the study. In accordance with the blood fractionation procedures, we obtained plasma for protein determination. Blood taken from the patient qualified for the study group or control group was prepared for 30 minutes in an ice bath. Plasma samples were kept at −80 °C until further analysis.

Our department (Perinatology and Obstetrics of the Medical University of Bialystok) eventually recruited 21 pregnant women with mild preeclampsia (study group) and 27 pregnant women (matched for maternal age, gestational age and BMI at the moment of recruitment) with uncomplicated pregnancies (control group). The study protocol was approved by the Local Ethical Committee of the Medical University of Bialystok, Poland, and informed consent was obtained from each patient (No ethics committee approval: R-I-002/529/2013). Signed informed consent from all participants involved in the study was obtained.

To assess the concentration of angiogenic factors in blood plasma, we used a multiplex method [10–12] that allows for the simultaneous determination of 60 proteins per sample. Similar to a traditional sandwich-based ELISA, the multiplex method uses a pair of specific protein antibodies for detection. A capture antibody is first bound to the glass surface, and after incubation with the sample, the target angiogenic factor is trapped on the solid surface. A second biotin-labelled detection antibody is then added that recognizes a different isotope of the target factor. The protein factor-antibody-biotin complex is then visualized by the addition of a streptavidin-labelled Cy3 equivalent dye using a laser scanner (GenePix 4100A) and two types of software: GenePix Pro7 and Q-Analyzer. Normalization of the fluorescent signal on protein arrays was performed by the software, which consisted of subtracting the background signal so that the array of spots were recorded real signals derived from proteins.

To validate of our results from macroarrays, we used ELISA (R & D System kits) for 4 statistically significant proteins: leptin, IL-6, VEGF and HGF. ELISA is a reference method, which quantitatively measures protein concentration in biological material.

The sets (Human Angiogenesis Array 1000, RayBiotech, Inc.) consist of the following angiogenic factors: Activin A, Agouti-related protein (AgRP); Angiopoietin 1; Angiopoietin 2; Angiogenin; Angiostatin; Angiopoietin-like 4 (ANGPTL4); Basic fibroblast growth factor (bFGF); Chemokine (C-X-C motif) ligand 16 (CXCL16); Epidermal growth factor (EGF); C-X-C motif chemokine 5 (ENA-78); Fibroblast growth factor 4 (FGF-4); Follistatin; Granulocyte-colony stimulating factor (G-CSF); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Chemokine (C-X-C motif) ligand 1 (GRO); Heparin-binding EGF-like growth factor (HB-EGF); Hepatocyte growth factor (HGF); Chemokine (C-C motif) ligand 1 (I-309); Interferon gamma (IFN-gamma); Insulin-like growth factor 1 (IGF-1); Interleukin 10 (IL-10); Interleukin 12 p40 (IL-12 p40); Interleukin 12 p70 (IL-12 p70); Interleukin 17 (IL-17); Interleukin 1-alpha (IL-1 alpha); Interleukin 1-beta (IL-1 beta); Interleukin 2 (IL-2); Interleukin 4 (IL-4); Interleukin 6 (IL-6); Interleukin 8 (IL-8); C-X-C motif chemokine 10 (IP-10); Chemokine (C-X-C motif) ligand 11 (I-TAC); Leptin; Leukaemia inhibitory factor (LIF); Monocyte chemotactic protein 1 (MCP-1); Monocyte chemotactic protein 2 (MCP-2); Monocyte chemotactic protein 3 (MCP-3); Monocyte chemotactic protein 4 (MCP-4); Matrix metalloproteinase-1 (MMP-1); Matrix metalloproteinase-9 (MMP-9); Platelet-derived growth factor BB (PDGF-BB); Platelet endothelial cell adhesion molecule (PECAM-1); Placental growth factor (PLGF); Chemokine (C-C motif) ligand 5 (RANTES); Transforming growth factor alpha (TGF alpha); Transforming growth factor beta 1 (TGF beta 1); Transforming growth factor beta 3 (TGF beta 3); Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1); Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2); Metallopeptidase inhibitor 1 (TIMP-1); Metallopeptidase inhibitor 2 (TIMP-2); Tumour necrosis factor alpha (TNF alpha); Tumour necrosis factor beta (TNF beta); Thrombopoietin (TPO); Urokinase receptor (uPAR); Vascular endothelial growth factor (VEGF); Vascular endothelial growth factor 2 (VEGFR2); Vascular endothelial growth factor 3 (VEGFR3); and Vascular endothelial growth factor D (VEGF-D).

Descriptive statistics, including the mean concentration and standard error of the mean concentration, were calculated for selected angiogenic factors, henceforth called features. To detect statistically significant differences between the groups under consideration (study group versus control group), either an analysis of variance model [13] was fit or a nonparametric method (Wilcoxon rank-sum test [14]) was applied. The choice of an appropriate method was made upon fulfilling the normality and homogeneity of variances assumptions; in the case of a violation of at least one condition, a nonparametric approach was employed.

The normality of the distribution of features was checked with the Shapiro-Wilk test [15] and the homogeneity of variances with Levene's test [16]. Features that were found to be significant, that is, their distribution was statistically significantly different among the experimental groups, were taken under further investigation to determine

their prediction capability.

2.1. Sample size statistics

Based on a previously conducted pilot study, which included an investigation of the concentrations of the exact same set of proteins that were used in the present study, the following procedure was applied to calculate the required sample size. Two groups, each consisting of 12 observations, were examined in the preliminary experiment, allowing the estimation of Cohen’s d effect sizes [17]. Using the estimated values, power analysis [18] was conducted to determine the minimal sample size per group providing a 0.8 median value of the overall power. Due to the problem of multiple testing, Šidák p-value correction was applied. Therefore, the significance level, alpha = 0.05, was replaced with Šidák’s significance level [19]. It was determined that setting the sample size to 20 (per group) ensured an overall power equal to 0.79, while a sample size equal to 21 (per group) provided a power of 0.82, which led to the inclusion of 21 subjects per group.

3. Results

The clinical characteristics of the patients are presented in Table 1. Patients from both groups were matched for maternal age, number of pregnancies, gestational age at collection and BMI to ensure that the two groups were comparable and that there were no statistically significant differences between them. The values of the mean concentration and standard error of the maternal plasma angiogenic factor concentrations in each study group are presented in Table 2.

We showed that there was a statistically significant increase in the concentration of 8 proteins, Hb-EGF ($p = .0407$); HGF ($p = .0042$); IFN-gamma ($p = .0215$); IL-6 ($p = .0186$), LIF ($p = .0256$), IP-10 ($p = .0371$), leptin ($p = .0291$), and PDGF-BB ($p = .001$), and a significant decrease in the concentration of 3 proteins, VEGF ($p = .0391$), PlGF ($p = .0271$) and follistatin ($p = .0026$), in the plasma of women with preeclampsia in comparison to the plasma of women in the control group (Table 2).

Results from ELISA procedures are presented in Table 3. We validated our data from macroarrays for 4 proteins and we confirmed significance for leptin ($p = .0069$), IL-6 ($p < .0001$), VEGF ($p < .0001$) and HGF ($p = .0396$). The mean concentrations obtained from ELISA and macroarrays were different for individual proteins, but this is due to the fact that concentration measurement in macroarray procedure is based on fluorescence signal from spot on the slide. If a slide fluorescence signal is weaker or stronger than all of the spots it will also emit proportionally less or more signal which does not falsify significance but can falsify real concentration. Therefore, arrays may not always be sufficient for determining specific concentrations but are certainly capable of demonstrating the significant difference for selected factors. The traditional ELISA therefore seems to be the preferred method. Our significance studies were confirmed by ELISA.

4. Comment

In the present study, we examined the concentrations of 60 angiogenic factors in the blood plasma of pregnant patients. Of these 60 angiogenic factors, 11 were found to be statistically significantly different between the groups of patients with PE and healthy pregnant women. The concentrations of 8 proteins, IFN-gamma, IL-6, LIF, HB-EGF, HGF, IP-10, leptin, and PDGF-B above the cut-off value as well as VEGF, PlGF and follistatin below the cut-off value, indicate the risk of preeclampsia.

In our research, we found reduced levels of VEGF and PlGF in the plasma of women with PE; these results are confirmed in the scientific literature. In the described research, we treated the levels of VEGF and PlGF as reference and validation points (positive control). In the physiological environment, vascular endothelial growth factor and placental growth factor contribute to the proliferation and implantation of trophoblasts. Scientific studies demonstrate that reduced levels of these factors play a key role in PE [20]. Two placental proteins cause this reduction by antagonizing the action of VEGF and PlGF, i.e., sFLT-1 (soluble fms-like tyrosine kinase-1) and sENG (soluble endoglin) [21]. The level of sFLT-1 in the serum of women with PE has been found to be significantly higher than in women without this disease [22]. Soluble endoglin, which is a membrane endothelial and syncytiotrophoblast co-receptor, plays a role in transforming growth factor β1 and β2 [23]. As in sFLT-1, the content of soluble endoglin was shown to be increased in women with preeclampsia, and furthermore, the level of this protein positively correlates with the severity of the disease [24].

In addition to VEGF and PlGF, we found a statistically significant decreased concentration of follistatin in the plasma of women with PE. The level of this protein in the international literature has not been clearly established for PE, and there are many conflicting studies. Keelan et al. [25] showed an elevated serum level of this factor in women with PE in comparison to healthy pregnant women [25], whereas D’Antona et al. [26] found no significant differences between the same study groups [26]. Interestingly, activin A and follistatin are dynamically regulated during human pregnancy, and both are involved in the physiology of pregnancy [27]. Follistatin on its own has no effect during pregnancy, but regulates the level of activin A by binding this protein. Whereas activin A directly stimulates the release of hCG and progesterone from placental trophoblasts, scientists emphasize that follistatin has an indirect impact on embryo development and pregnancy [28]. Additionally, follistatin is a circulating binding protein for activin A, and the concentration of follistatin in PE is increased [29]. If the concentration of follistatin, which is responsible for binding activin A, in the plasma is low, the same concentration of protein that should be bound (activin A) increases. Moreover, follistatin has a strict relationship with VEGF, whose deficiency is the basis of PE. The study conducted by Oh et al [30] revealed that VEGF positively influences follistatin gene expression. In our study, both VEGF and follistatin were significantly decreased in the blood of preeclamptic mothers. Thus, reduced levels of VEGF may be responsible for the lack of expression of the follistatin gene, which results in low levels of this protein in

Table 1
Clinical characteristic of the patients.

	Group I – healthy, pregnant women (n = 27)	Group II – women with PE (n = 21)	P value
Maternal age (median ± SD)	28 ± 5.49	29 ± 5.65	0.6768
Number of pregnancies (median ± SD)	1 ± 0.85	1 ± 0.56	0.3977
Gestational age at collecting of samples in weeks (median ± SD)	34.2 ± 3.9	33 ± 4.48	0.7059
Present BMI (during pregnancy, at the moment of recruitment) (median ± SD)	25.9 ± 3.07	26 ± 2.53	0.6242

SD - standard deviation.

Table 2
Concentrations of angiogenic factors in maternal plasma.

	Group I – healthy, pregnant women (n = 27)	Group II – women with PE (n = 21)	P-value
	Angiogenic factors concentration (pg/ml) Mean ± SEM		Group I-Group II
Activin-A	260.28 ± 212.90	801.40 ± 729.47	0.4624
AgRP	439.76 ± 209.82	1034.48 ± 374.84	0.0513
Angiogenin	1028.06 ± 156.46	844.43 ± 77.40	0.4706
ANG-2	2137.63 ± 226.02	1675.97 ± 251.11	0.1667
ANGPLT4	107047.99 ± 21985.73	106346.16 ± 30089.83	0.7276
bFGF	334.92 ± 61.78	533.70 ± 76.37	0.0531
ENA-78	1868.03 ± 310.65	2009.05 ± 389.02	0.7136
GRO	324.47 ± 48.39	457.92 ± 82.30	0.1006
HB-EGF	31.00 ± 20.17	38.82 ± 9.80	0.0407**
HGF	370.59 ± 39.84	755.48 ± 129.04	0.0042**
IFN-gamma	133.63 ± 17.61	308.29 ± 96.13	0.0215**
IGF-I	15227.46 ± 3017.74	21423.106 ± 5116.30	0.4875
IL-1a	129.88 ± 20.85	275.31 ± 112.32	0.1913
IL-2	276.92 ± 64.86	472.08 ± 182.12	0.1181
IL-6	48.94 ± 7.76	80.88 ± 14.17	0.0186**
IL-8	50.68 ± 9.89	46.87 ± 5.18	0.4413
IL-17	456.37 ± 267.13	395.24 ± 93.53	0.1159
IP-10	31.61 ± 12.45	187.36 ± 153.50	0.0371**
Leptin	11088.93 ± 1730.53	25176.44 ± 4551.88	0.0291**
LIF	2074.72 ± 703.03	4207.44 ± 911.06	0.0256**
MCP-1	80.88 ± 21.73	98.54 ± 20.83	0.1409
PDGF-BB	274.00 ± 23.79	416.77 ± 32.19	0.0010*
PIGF	389.31 ± 56.84	239.75 ± 72.62	0.0271**
RANTES	3429.31 ± 796.10	2860.99 ± 612.68	0.7109
TGFb1	6255.30 ± 1630.29	10271.03 ± 2781.20	0.2373
TIMP-1	46426.84 ± 3891.19	40622.62 ± 4714.21	0.3496
TIMP-2	10209.51 ± 1440.26	13670.29 ± 2436.54	0.5109
TNF-alpha	462.67 ± 64.90	705.33 ± 156.24	0.1853
TNF-beta	150.85 ± 30.11	264.60 ± 77.56	0.1733
TPO	3478.63 ± 854.29	4955.22 ± 909.65	0.1832
ANG-1	374.77 ± 52.35	722.61 ± 176.33	0.1853
Angiostatin	263093.48 ± 12352.61	263220.02 ± 23155.62	0.4834
CXCL16	1215.79 ± 121.97	1145.10 ± 116.27	0.7575
EGF	8.65 ± 2.41	11.30 ± 4.06	0.7732
FGF-4	6050.50 ± 1211.69	13671.88 ± 8238.11	0.4715
Follistatin	33290.16 ± 3217.63	20753.30 ± 3196.87	0.0026**
G-CSF	1490.01 ± 336.22	1084.46 ± 243.42	0.9093
GM-CSF	9.70 ± 1.99	8.87 ± 1.75	0.7618
I-309	32.43 ± 4.99	63.66 ± 29.80	0.6747
IL-1b	61.22 ± 10.74	118.22 ± 46.56	0.8737
IL-4	64.15 ± 9.16	67.13 ± 24.57	0.3018
IL-10	14.04 ± 2.48	11.90 ± 3.57	0.6271
IL-12p40	868.16 ± 138.22	1999.36 ± 1074.60	1
IL-12p70	17.99 ± 2.43	14.90 ± 3.20	0.4129
I-TAC	53.90 ± 13.72	71.07 ± 37.31	0.8116
MCP-2	48.28 ± 8.29	114.81 ± 57.14	0.6654
MCP-3	45.82 ± 7.92	121.90 ± 64.32	0.9559
MCP-4	583.45 ± 96.67	786.38 ± 368.49	0.2614
MMP-1	2468.71 ± 364.36	3392.18 ± 1206.99	0.4147
MMP-9	19549.59 ± 1633.10	15562.65 ± 1511.43	0.1355
PECAM-1	2087.19 ± 249.20	1580.94 ± 281.42	0.0818
TGFa	1494.99 ± 336.23	2086.94 ± 759.80	0.9432
TGFb3	4934.80 ± 933.41	9872.26 ± 5178.52	0.8038
Tie-1	2644.88 ± 692.18	27773.73 ± 20121.11	0.4429
Tie-2	405.16 ± 54.42	990.06 ± 528.40	0.4273
uPAR	3893.61 ± 356.33	6475.25 ± 2063.20	0.7419
VEGF	522.66 ± 86.15	539.53 ± 205.91	0.0391**
VEGF-R2	2094.75 ± 178.54	1932.87 ± 427.25	0.0925
VEGF-R3	467.92 ± 106.28	1475.71 ± 675.78	0.9529
VEGF-D	234.15 ± 88.62	2546.02 ± 1830.96	0.6461

* Statistically significant value of less than 0.05 for Student's T-test.
** Statistically significant value of less than 0.05 for Mann Whitney Wilcoxon's test.

mothers' bloodstream; this may be a new pathway for PE.

It is generally accepted that placental ischemia leads to oxidative stress and induces placental apoptosis [31], stimulating the maternal immunological response [32]. Saito et al. [33] demonstrated an imbalance between the Th1 and Th2 lymphocyte fractions in the peripheral blood of preeclamptic patients in comparison to healthy women. The characteristic preeclamptic shift toward Th1-type immunity (in uncomplicated pregnancy Th2-immunity dominates) was confirmed by Szarka et al. The authors found that there is an increased concentration of cytokines that produced by Th1, namely, IL-2, IL-8, IFN-gamma, TNF-alpha, IP-10, MCP-1, ICAM-1, and VCAM-1, in maternal peripheral blood [34]. The results of our study showed that there were elevated levels of some inflammatory markers, such as IFN-gamma, IL-6, and IP-10, in agreement with other researchers [35,36].

Table 3
Concentrations of angiogenic factors in maternal plasma – ELISA validation.

	Group I – healthy, pregnant women (n = 27)	Group II – women with PE (n = 21)	P-value
	Angiogenic factors concentration (pg/ml) Mean ± SEM		Group I-Group II
Leptin	26022 ± 2794	41200 ± 4955	0.0069*
IL-6	1.18 ± 0.17	3.45 ± 0.72	< 0.0001*
VEGF	5.87 ± 0.43	4.63 ± 0.37	< 0.0001*
HGF	1544 ± 86.15	2143 ± 283.6	0.0396*

* Statistically significant value of less than 0.05 for Student's T-test.

Surprisingly, we found increased plasma levels of 3 proteins in women with PE: HB-EGF, HGF and PDGF-BB. This finding is a new contribution to the existing PE knowledge. These factors are very important in the development of pregnancy and, in particular, are involved in the proliferation, migration and invasiveness of human extravillous trophoblast cells and hence in the remodelling process of spiral arteries in the placenta of pregnant women [37,38]. Therefore, in the scientific literature, HB-EGF and HGF are often analysed in the placenta, and research has shown decreased concentrations of HB-EGF and HGF in the cytotrophoblast and mesenchymal cells [38,39], which emphasizes the role of these proteins as growth promoters of the placenta. Our results seem to be contrary to these previous findings (increased levels in blood); however, Lala et al. [38] revealed that degree of placental contribution to blood level of HGF and HB-EGF in pregnancy has not been clearly determined. As can be seen above, reduced levels of angiogenic proteins in the placenta are not necessarily reflective of the level of these proteins in the blood. Robinson et al. [40] found no significant differences in the concentrations of HGF between healthy pregnant women and women who later developed PE, which agrees with our finding of elevated levels of HGF and HB-EGF in the plasma of women with PE. Recent studies indicate that HGF is involved in placental growth and expansion, and inhibiting HGF production in animal models results in intrauterine demise due to placental insufficiency [41]. The results of these studies are consistent with our results because it can be assumed that, as revealed in our results, high levels of HGF can cause saturation of receptor-binding HGF (S-Met), which in turn causes a low level of the free fraction of s-MEt. A second protein, HB-EGF, is involved in the very early stages of embryonic development and occurs on the 4th day of pregnancy in uterine epithelial cells. Embryonic induction of HB-EGF provided insights for the first time that blastocysts send signals to prepare the maternal environment in the uterus for normal embryo growth [42]. Additionally, scientists revealed that HB-EGF expression by trophoblast cells of the developing placenta appears to regulate extravillous differentiation and provide cytoprotection in a hostile environment [43]. Therefore, there is no doubt that overexpression of HB-EGF in the mothers' blood, which plays an important role in embryonic and placental development, can lead to abnormalities underlying PE.

PDGF-BB is a mitogenic peptide, which together with oestrogen, plays an important role in regulating pregnancy-induced smooth muscle cell proliferation through activation of tyrosine kinase, calcium,

and protein kinase C [44]. PDGF receptors have been found in the uterine artery during pregnancy; PDGF mRNA and protein expression increase in myometrial tissue [45,46]. Similar to our results, Meng et al. [47] found elevated concentrations of PDGF-BB in decidual blood vessels and in serum in PE women in comparison to normal pregnant women. PDGF-BB has a role in development in normal pregnancy, but its specific role is not known. However, it can be assumed that disturbances in the level of this protein affect the development of preeclampsia.

It is difficult to clearly identify potential pathways in which the concentrations of HGF, HB-EGF and PDGF-BB could grow increase; however, it is interesting that these proteins are also involved in cell apoptosis, as has been shown in studies on the induction of cell death and muscle cell tumours [48,49]. Therefore, these three proteins may be involved in apoptosis in PE.

In our present research, we revealed increased concentrations of LIF in the plasma of preeclamptic women. Although there are no previous studies assessing the LIF concentration in the plasma of women with PE, there is a potential explanation for the elevated levels of LIF found in our research. Interestingly, Reister et al. [50] showed an increased number of NK cells in the uterus and around the spiral arteries of women with PE, and van der Meer et al. [51] found that the LIF protein was produced by NK cells in the placenta of pregnant women. LIF is a protein of the same cytokine class as IL-6, and both have a role in the establishment of pregnancy by supporting decidual and placental differentiation as well as embryo development [52]. Additionally, LIF regulates trophoblasts by promoting proliferation, invasion and differentiation, and as Zheng et al. revealed, this process mediates uPAR (urokinase-type plasminogen activator receptor) [53]. Sharkey et al. found high expression levels of LIF receptors in human trophoblasts, decidua, endothelial cells of the foetal villi and decidual leukocytes [54]. A reduced level of LIF had also been observed in infertile women with implantation failure [55]. In summary, excess and deficient LIF during pregnancy can lead to disturbances in the initial development of the foetus and placenta remodelling.

We have shown that the levels of both proteins were significantly increased, and therefore, it can be postulated that their very high concentrations inhibit the differentiation of trophoblast cells in the walls of the spiral arteries of the uterus. On the other hand, Eva Dimitriades et al. noted that LIF can inhibit spiral artery remodelling in mice mid-gestation.

Leptin was also found to be elevated in our investigation. Many studies seem to confirm our result regarding leptin levels in PE and show that this protein plays a marked role in the physiology of pregnancy [56,57]. Leptin is an adipokine that is involved in modulating satiety, energy homeostasis, and reproductive biology during pregnancy. Leptin is produced by the placenta, and its concentration is increased at the end of the second trimester of pregnancy. In addition, leptin has been shown to be important for the maternal-foetal exchange processes regulating growth and development (maternal amino acid transport by the placenta of the foetus) [57,58]. Leptin resistance occurs in obese pregnant women, thus inhibiting excessive transport of amino acids to the foetus. Therefore, researchers believe that pregnant women with preeclampsia occur due to overproduction of leptin by the placenta because preeclampsia leads to foetal growth restriction. Increased leptin could result in increased transport of amino acids to the foetus and affect foetal growth. This is a mechanism that protects the normal growth of the foetus [58].

In our study, we used a very strict recruitment criteria. In addition, the obtained results showed a decrease in the concentration of PlGF and VEGF, which are the most investigated proteins in PE. It is known that their content decreases in the plasma of women with PE. As a result, we treated them as an internal control to ensure that our groups were homogeneous. As can be seen, some of the results that we obtained are similar to those in the scientific literature, confirming their role in PE. However, a large number of significant factors (LIF, follistatin, HB-EGF, HGF and PDGF-BB) are interesting and not well-studied in the pathomechanisms of PE. These proteins have not been previously researched directly in the plasma of women with preeclampsia; therefore, the present results are illuminating in terms of information concerning the pathogenesis of this disease. These protein factors point to a potential new pathway for preeclampsia and confirm the importance of these protein levels in maternal blood, not only at the placenta level, which was previously often investigated. Due to the complexity of the pathomechanism responsible for preeclampsia, further functional experiments should be performed. Subsequent experiments confirming the importance of new proteins in PE could be the basis for the creation of diagnostic tests to detect early PE. These proteins would be tested in large groups of patients. Additionally, scientists will need to check their sensitivity and specificity in analysis of receiver operating characteristic (ROC) curves. There is still no rapid test to diagnose PE in pregnant women. Our study is the only screening test to identify essential novel proteins in PE with an emphasis on their potential role in PE and is a great starting point for research into biomarkers. In addition, our results may be the basis for pharmacological tests examining the interactions of proteins with other compounds. Perhaps block over-expression (or supplementing the low protein concentrations in the women blood) of certain proteins involved in the pathogenesis of PE would stop development of this disease and its negative effects during pregnancy.

Acknowledgements

This work was supported by grant numbers: 36/KNOW/2013 from The Leading National Scientific Centre, Medical University of Bialystok, Poland and 153-33733 from the Ministry of Higher Education, Poland. Study sponsored by a Polpharma Scientific Foundation.

The manuscript has undergone professional English editing by Elsevier editors.

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