Total and Fetal Circulating Cell-Free DNA, Angiogenic, and Antiangiogenic Factors in Preeclampsia and HELLP Syndrome

Rocío Muñoz-Hernández,[1,2,*] Pablo Medrano-Campillo,[1,3,*] Maria L. Miranda,[1,4] Hada C. Macher,[5] Jose Manuel Praena-Fernández,[6] Antonio J. Vallejo-Vaz,[1,7] María J. Dominguez-Simeon,[1] Rafael Moreno-Luna,[8] and Pablo Stiefel[1,4]

[1]Laboratorio de Hipertensión Arterial e Hipercolesterolemia, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain;

[2]Laboratorio de Investigación clínica y traslacional en enfermedades hepáticas y digestivas. Instituto de Biomedicina de Sevilla (IBiS). Hospital Universitario Virgen del Rocío/ CSIC/Universidad de Sevilla, Sevilla, Spain;

[3]Universidad Autónoma de Chile, Chile;

[4]Unidad Clínico-Experimental de Riesgo Vascular (UCERV- UCAMI), Hospital Universitario Virgen del Rocío, Sevilla, Spain;

[5]Servicio de Bioquímica Clínica, Hospital Virgen del Rocío, Sevilla, Spain;

[6]Unidad de Asesoría Estadística, Metodología y Evaluación de Investigación, Fundación Pública Andaluza para la Gestión de la Investigación en Salud de Sevilla (FISEVI). Sevilla, Spain;

[7]School of Public Health, Imperial College London. London, UK; [8]Laboratorio de Fisiopatología Vascular, Hospital de parapléjicos de Toledo. Toledo, Spain.

***These authors contributed equally to this work.**

Correspondence: Pablo Stiefel (stiefel@cica.es).

ABSTRACT

BACKGROUND

Preeclampsia (PE) is a hypertensive disorder of pregnancy characterized by hypertension and proteinuria. The HELLP syndrome is the most severe form of PE. The aim of the present study was to determine different potential biomarkers that may help us perform an early diagnosis of the disease, assess on the severity of the disease, and/or predict maternal or fetal adverse outcomes.

METHODS

We measured serum levels of total and fetal circulating cell-free DNA (cfDNA), soluble endoglin, soluble form of vascular endothelial growth factor receptor, and placental growth factor in a healthy control group of pregnant women (n = 26), patients with mild (n = 37) and severe PE (n = 25), and patients with HELLP syndrome (n = 16).

RESULTS

We observed a gradual and strong relationship between all the biomarkers mentioned and the range of severity of PE, with the highest levels in patients with HELLP syndrome. Nevertheless, only the values of total cfDNA were able to significantly differentiate severe PE and HELLP syndrome (20 957 ± 2 784 vs. 43 184 ± 8 647 GE/ml, P = 0.01). Receiver operating characteristic (ROC) curves were constructed (i) for the healthy group with respect to the groups with PE
and (ii) for patients with PE with respect to the group with HELLP syndrome; sensitivity and specificity values at different cutoff levels were calculated in each case. The maximum ROC area under the curve value for PE and HELLP syndrome (with respect to controls) was 0.91 (P < 0.001)

CONCLUSIONS

The measured biomarkers of cell damage, angiogenesis, and antiangiogenesis may reflect the severity of PE, with higher levels in patients who develop HELLP syndrome. In addition, these biomarkers may also help predict adverse fetal and maternal outcomes.

Keywords: angiogenic factors; antiangiogenic factors; blood pressure; cell-free DNA; HELLP syndrome; hypertension; hypertension in pregnancy; maternal-fetal adverse outcomes; preeclampsia.

INTRODUCTION

Preeclampsia (PE) is a hypertensive disorder of pregnancy that affects 2–8% of pregnancies and is characterized bythe presence of hypertension and proteinuria, although the last is not required in the new definition of PE.[1,2] Although the physiopathology of PE is not yet well under- stood, the presence of an ineffective placentation resulting in placental ischemia seems to play a key role in the development of the disease.[3] One of the most severe syndromesrelated to PE is the so-called HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count), resulting from microangiopathic hemolytic anemia, thrombocytopenia and hepatolysis.[4] The HELLP syndrome has been related to 1% of cases of maternal mortality and about 20% of perinatal mortality.[5]

Although the diagnosis of PE is mainly based on the measurement of maternal blood pressure and proteinuria,
the sensitivity and specificity of these parameters are low to accurately predict adverse maternal and fetal outcomes in routine clinical practice.[6] Therefore, there is an urgent demand for widely applicable and affordable tests that allow clinicians to accurately identify women at risk and to predict potential maternal and fetal complications.

Different markers related to ischemia (such as circulating cell-free DNA [cfDNA]), and angiogenic/antiangiogenic factors (such as soluble endoglin [sEng], soluble vascular endothelial growth factor receptor [soluble FMS-like tyrosine kinase-1, sFlt-1], and placental growth factor [PIGF]), have been proposed as novel biomarkers in PE. In a preliminary report from our group,[7] we observed a gradual and strong relationship between cfDNA levels and the severity of PE, with the highest levels corresponding to those patients with HELLP syndrome. Thus, cfDNA levels, both fetal and total, may represent a biomarker probably related to the aforementioned placental ischemia.[5,8] In fact, its levels have been observed to be increased in pathologies involving ischemia[9–12] or situations related to hypoxia, such as experimental acute pulmonary thromboembolism[13] or obstructive sleep apnea–hypopnea syndrome.[14] sEng, an antiangiogenic factor highly expressed on endothelial cell membranes and syncytiotrophoblasts,[15] has been found to be higher in women with PE and to correlate with the severity of the symptoms.[16] Similarly, sFlt-1 levels are increased and PIGF levels are decreased throughout gestation in patients with PE.[17]

As far as we know, cfDNA, sEng, sFlt-1, and PIGF have not been previously studied together in patients with HELLP syndrome and compared with other severity stages of PE. Therefore, the main aim of our study was to measure these novel biomarkers in the third trimester of healthy pregnant women, in women with mild and severe PE and in patients with HELLP syndrome. The secondary aims of this study were (i) to determine the relationship between serum levels of cfDNA and adverse maternal-fetal outcomes in HELLP syndrome and (ii) to determine cutoff values of cell-free DNA that may predict the presence of HELLP syndrome among women with healthy pregnancy and/or PE.

MATERIALS AND METHODS

Participants

Patients and controls were recruited at the Women's Hospital of the Virgen del Rocio University Hospitals, Seville, Spain. We studied a control group of healthy pregnant women; healthy staff recruited at our hospital who consented to participate in the study and provided written informed consent (n = 26), patients with mild PE (n = 37), patients with severe PE (n = 25), and patients with HELLP syndrome (n = 16). The blood samples were collected at the time of disease onset in case of mild PE, severe PE, and HELLP groups and at gestational week 34 in the case of control group.

The diagnosis and severity of PE were established according to the 2010 National Institute for Health and Care Excellence (NICE) clinical guidelines on hypertension in pregnancy18; the HELLP syndrome was diagnosed according to the criteria described by Sibai *et al.*,19 including a platelet count ≤150.000/μl, plasma aspartate aminotransferase levels ≥70 U/l, and the presence of schistocytes, lactic dehydrogenase levels ≥600 U/l, or bilirubin levels >1.2 mg/dl. Intrauterine growth restriction was defined as a fetus with an individualized weight percentile <10% and with asymmetry in several ultrasound measurements, including a significant decrease in abdominal perimeter compared with long bone length and bi-parietal diameter. We considered preterms all newborns that were born before the 37 weeks of pregnancy. The composite of Apgar score <8, neonatal intensive care unit admission, and fetal death was defined as neonatal adverse outcome.

The exclusion criteria included patients suffering from any other disease related to ischemia and/or hypoxia; patients receiving treatment with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers or statins; coexistence of any inflammatory or autoimmune disease; evidence of any chronic illness that in the opinion of the investigator contraindicated inclusion in the study (chronic hypertension and prior diabetes mellitus were allowed in patients with PE as these conditions are well-established common risk factors for the development of PE); hypercholesterolemia; current tobacco consumption; pre-pregnancy body mass index >35 kg/m2; and women who carried fetuses with chromosomal abnormalities or congenital malformations. In the control group, women with hypertensive disorders were excluded. The present study was approved by the Committee for the Review of Human Research at the Virgen del Rocio University Hospitals. All participants provided written informed consent before inclusion. This study was performed according to the principles of the Declaration of Helsinki and the Code of Federal Regulation regarding Protection of Human Subjects.

Blood pressure measurement

Brachial systolic and diastolic blood pressure were measured in seated position according to the recommendations of the Seventh Report of the Joint National Committee on Prevention, Detection, evaluation, and Treatment of High Blood Pressure.20

sEng, sFlt-1, and PIGF measurements

Blood samples were collected into tubes containing clot activator and separator gel. The samples were centrifuged at 3,000 rpm for 10 minutes within 3 hours from collection and the serum was then immediately stored in aliquots at −80 °C until testing.

Maternal serum concentrations of sEng, sFlt-1, and PIGF were determined by high-sensitivity indirect sandwich enzyme-linked immunosorbent assay validated for serum determinations of the analytes (R&D Systems, Minneapolis, MN) using a modular analyzer power Wave XS (Biotek, Winooski, UT) following the manufacturer instructions. Each sample was analyzed in duplicate and the mean was taken as the final result.

Total and fetal cfDNA measurement

Maternal peripheral blood samples were centrifuged for 10 minutes at 3,500 rpm and the serum was frozen at −80 °C for later DNA extraction. Total cfDNA was extracted automatically from the stored serum samples (1 ml) using a Compact MagnaPure Instrument (Roche Diagnostics, Bassel, Switzerland). Total cfDNA was measured using a real-time quantitative PCR assay for the β-globin gene. Quantitative PCR analysis was performed using a LightCycler 480 Real- Time PCR Instrument (Roche Diagnostics) by 5′nuclease assay (Taqman assay).The cfDNA was resuspended in a final volume of 90 µl of RNase/DNase-free water, and the cfDNA was amplified in a total reaction volume of 20 µl, containing 2 µl of cfDNA, using 10 µl LightCycler 480 Probes Master (Roche Diagnostics), 0.4 µl 10 µM of primers (TIB MOLBIOL GmbH, Germany), and 0.2 µl 10 µM of Taqman probe (TIB MOLBIOL GmbH)
The β-globin Taqman system uses the following primers: beta-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′); beta-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′); and a dual-labeled fluorescent probe betaglobin- 402T (5′-(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3′). The thermal profile was carried out using 2-minute incubation at 95 °C, followed by an initial denaturation step at 95 °C for 5 seconds, followed by 48 cycles of 20 seconds at 62 °C. Final size of the amplicon was 102 bp. To determinate the cfDNA concentration, we ran a standard dilution curve using a known concentration of a commercially available genomic DNA (DNA Control Kit—Roche Diagnostics). We used 4 serial 10-fold dilutions, which ranged from 1.5ng/µl–1.5pg/µl.

To measure fetal cell-free DNA (cffDNA), 44 µl of template cfDNA were digested with 10U of a methylation-sensitive enzyme, *BstUI* (New England Biolabs, MA), at 60 °C for 16 hours. qRT-PCR analysis was performed using a PCR cycler CFX96 (Bio-Rad, CA). Ras-association domain family 1 isoform A (*RASSF1A)* and *β-actin* sequences were amplified simultaneously by a duplex PCR assay. The enzymedigested *RASSF1A* sequence was used to quantify cffDNA. The sequences of the primers and probes were designed as described previously.21,22 The reactions were set up in a total volume of 20 µl, containing 6.8 µl of DNA after enzyme digestion, using 10 µl TaqMan Fast Advanced Master Mix (Applied Biosystems, Waltham, MA), 0.6 µl 10 µM of primers (Sigma-Aldrich, Germany), and 0.4 µl 10 µM of Taqman probe (Applied Biosystems). PCR conditions included a first denaturation/activation step at 50 °C for 2 minutes and at 95 °C for 20 seconds, followed by 50 cycles of 95 °C for 3 seconds and at 60 °C for 30 seconds. When there was no amplification of *β-actin* after enzyme digestion the demonstrated hypomethylated *RASSF1A* gene had been digested completely. The amplification of postdigestion *RASSF1A* was performed and recorded. To determine the number of copies of *RASSF1A* in the serum sample, a calibration curve (logarithmic scale) was run in parallel with each analysis, plotting the threshold cycle against known concentrations of a serially diluted DNA reference samples (Control genomic
DNA [Human], Applied Biosystems).
All samples were analyzed in triplicated. Both in the measurement of cfDNA and in the measurement of cffDNA, a negative reaction and blank control were included to minimize the risk of contamination. A conversion factor of 6.6 pgof DNA per cell was used to express the result as number of copies or genome equivalents (GE/ml).

Statistical analysis

Categorical variables are represented by absolute frequencies and percentages (*n*, %). Noncategorical variables are shown as mean • } SEM in case of normal distribution of the variables, and as median and interquartile range otherwise.

The normality of the distributions was studied using the test of Saphiro–Willk. The Student's *t* test and *U* Mann–Whitney test were used to compare noncategorical variables in the different groups in case of normally and nonnormally distributed variables, respectively. Categorical variables were compared with Pearson $\chi 2$ tests or Fisher's exact test as appropriate. When comparing variables with more than 2 categories the analysis of variance test or the Kruskal–Wallis test were used for data normally and nonnormally distributed, respectively. A *post-hoc* analysis was performed using the test of Bonferroni. The relationships between quantitative variables were assessed by means of the Spearman test. Receiver operating characteristic (ROC) curves were created for total and fetal cfDNA, sEng, sFlt-1, PIGF and the sEng/ PIGF, and sFlt-1/PIGF ratios for the healthy control group with respect to PE (mild and severe) and HELLP syndrome, and for the PE groups (mild and severe) with respect to the HELLP syndrome. The area under the curve (AUC) and the accuracy, sensitivity, and specificity values were estimated for the different cutoff levels of the biomarkers. A *P* value <0.05 was considered significant. The 95% confidence intervals (95% CI) were calculated where appropriate. Statistical analyses were performed using IBM SPSS software (version 19.0; IBM, Armonk, NY).

RESULTS

We studied 104 mother–offspring pairs (78 with PE and 26 controls). Based on the severity of PE, these women were classified as having mild PE (*n* = 37), severe PE (*n* = 25), and HELLP syndrome (*n* = 16). Maternal and neonatal characteristics at enrolment/onset are shown in Table 1. Briefly, 6 subjects in the PE group and 2 subjects in the HELLP group had preexisting chronic hypertension. Gestational age and fetal weight at enrollment/onset were lower in patients with severe PE and HELLP syndrome. Hemoglobin levels were similar among groups but platelet counts were lower in patients with HELLP syndrome. Aaspartate aminotransferase, lactic dehydrogenase, and urine protein excretion were higher in patients with HELLP syndrome. Table 2 describes the maternal and newborn characteristics at delivery. The gestational week at delivery was lower in

those women with severe PE and HELLP syndrome compared with healthy and mild PE women. The number of cesarean delivery was higher in the PE/HELLP groups compared to controls.

Table 1. Maternal and neonatal demographic and at enrollment characteristics

	Healthy (*n* = 26)	Mild PE (*n* = 37)	Severe PE (*n* = 25)	HELLP syndrome (*n* = 16)	*P* for trend*
Age (years)	34 [29–35]	32 [29–35]	34 [29.5–37.5]	33.5 [29.2–36.5]	0.739
Primipara, *n* (%)	13 (52)	25 (69.4)	20 (80)	11 (66.7)	0.198
IVF, *n* (%)	2 (7.7)	6 (16.2)	5 (20)	5 (31.3)	NA
Singleton pregnancy, *n* (%)	24 (92.3)	27 (73)	21 (84)	13 (81.3)	0.321
Pregestational BMI (kg/m^2)	22.8 [20.5–25]	27.4 [23.3–34.1]	23.2 [21.6–29.4]	24.4 [21.8–30.5]	0.06
Previous medical history					
HT, *n* (%)	0	3 (8.1)	3 (12)	2 (12.5)	NA
DM, *n* (%)	0	1 (2.7)	1 (4)	1 (6.2)	NA
At enrollment/onset					
Gestational age (wk)	34 [30.7–39.2]	35 [33–36.5]	31 [29–33]	32 [29.5–35.7]	0.012
Gestational weight (kg)	–	2,101.5 ± 141.7	1,584.2 ± 123.2	1,866 ± 168.1	0.032
Gestational diabetes, *n* (%)	1 (3.8)	2 (5.4)	0	0	NA
IGR, *n* (%)	2 (7.1)	18 (38.3)	8 (27.6)	0	NA
Proteinuria (mg/l)	0	358 (29)	425 (98)	382 (73)	NA
Highest SBP (mm Hg)	111 [89–123]	150 [140–162.5]	166.5 [151–170]	153 [140–181]	<0.001
Highest DBP (mm Hg)	71 [58–79]	95 [83.7–100]	93.5 [90–106.2]	93 [90–103]	<0.001
Antihypertensive use					
0 drug, *n* (%)	26 (100)	0	0	0	NA
1 drug, *n* (%)	–	17 (56.7)	1 (4.5)	6 (40)	
2 drugs, *n* (%)	–	13 (43.3)	13 (59.1)	8 (53.3)	
≥3 drugs, *n* (%)	–	0	8 (36.4)	1 (6.7)	
Clinical and biochemical characteristics					
Schistocytosis, *n* (%)	–	0	0	12 (85.7)	NA
Placental abruption, *n* (%)	–	0	4 (16)	3 (18.7)	NA
LDH (U/l)[a]	–	212 [185–232]	227 [195–266.5]	304.5 [268–383.7]	<0.001
Hb (g/l)	–	109.29 ± 1.94	106 ± 3.1	103.23 ± 3.5	0.297
AST (UI/l)	–	15.5 [10.2–22.7]	12 [10–36.5]	86 [47.2–209]	0.011
Platelet (10^9/l)	–	192.8 ± 9.42	184.2 ± 8.4	103.8 ± 7.0	<0.001

Categorical variables are represented by absolute frequencies and percentages (*n*, %). Noncategorical variables are represented by mean ± SEM. Categorical variables were analyzed with Pearson x^2 tests. *P* values are from comparison of control, preeclampsia, and HELLP groups. Noncategorical variables that were normally distributed were analyzed with 1-way ANOVA test. Non categorical variables that were not normally distributed were analyzed with Kruskal–Wallis tests. Abbreviations: ANOVA, analysis of variance; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; DM, diabetes mellitus; Hb, hemoglobin; HT, chronic hypertension; IGR, intrauterine growth restriction; IVF, *in vitro* fertilization; LDH, lactic dehydrogenase; NA, not applicable; PE, preeclampsia; SBP, systolic blood pressure. **P* value for trend based on increasing severity of the samples groups.

[a]Values for LDH level are from *n* = 19 mild PE, 13 Severe PE, and 12 HELLP syndrome.

The levels of the different biomarkers for the different groups studied are shown in Figure 1 (cfDNA and cffDNA), Figure 2 (sFlt-1, sEng, and PIGF), and Figure 3 (sFlt-1/PIGF and sEng/PIGF ratios). When we carried out correlations between the different biomarkers, we found moderate positive correlations between total and fetal cfDNA with sFlt-1 and sEng levels restricted healthy patients and mild PE (all $P < 0.05$).

Table 3 shows the levels of total and fetal cfDNA, sEng, sFlt-1, PIGF and sFlt-1/PIGF and sEng/PIGF ratios in relation to the different maternal and fetal outcomes in the entire sample (n = 104). All these parameters were significantly different in women who needed a cesarean delivery, pregnancies who had a preterm labor (delivery before 37 weeks) or in newborns who suffered an adverse outcome.

With respect to maternal characteristics, we found that patients having placental abruption had significantly higher values of sEng, sFlt-1, and higher values of sFlt-1/PIGF ratio. Patients needing a cesarean section had significantly higher values of cfDNA, cffDNA, sEng, sFlt-1, sFlt-1/PIGF and sEng/PIGF ratios and lower values of PIGF. Finally, patients needing a blood transfusion had higher values of cfDNA and cffDNA but not of the other parameters studied. Regarding fetal outcomes all the studied parameters were clearly different in preterm newborns and in newborns having the composite variable named "neonatal adverse outcome". Levels of cfDNA and cffDNA were also higher in fetuses having intrauterine growth retardation. ROC curves were produced to determine the cutoff values of the studied markers that may determine the probability of having a PE or HELLP syndrome in comparison to the healthy group (Table 4), as well as the probability of having HELLP syndrome in comparison with the 2 PE groups (mild and severe) (Table 4). In the first case, the highest AUC value was that of sFLT-1 (0.91 [0.83–0.99]), followed by the level of sFLT-1/PIGF (0.91 [0.83–0.98]) and the level of cffDNA (0.89 [0.82–0.96]). The corresponding cut off values were 8,768.2 pg/ml, 20.4 and 156.4 GE/ml, respectively. In the second case, the highest AUC was that of sFlt-1/PIGF ratio (0.77 [0.66–0.88]), followed by sEng (0.74 [0.63–0.87]) and sEng/PIGF ratio (0.74 [0.63–0.86]). The corresponding cutoff values were 144.9, 43.5 ng/ml and 296.2, respectively. The levels of accuracy, sensitivity, and specificity are shown in Table 4.

Table 2. Maternal and Newborn characteristics at delivery

Maternal	**Healthy (*n* = 26)**	**Mild PE (*n* = 37)**	**Severe PE (*n* = 25)**	**HELLP syndrome (*n* = 16)**	***P* for trend***
Gestational week	39 [38–40.5]	37 [34–37]	34 [31.5–34.5]	34 [30.2–37.7]	0.012
Weight gain (kg)	16.5 [18–21.5]	14.5 [9.6–17]	13 [7.5–23.2]	10 [6.5–16]	0.195
Cesarean, *n* (%)	8 (30.8)	20 (54.1)	19 (76)	14 (87.5)	0.037
Newborn	**Healthy (*n* = 28)**	**Mild PE (*n* = 47)**	**Severe PE (*n* = 30)**	**HELLP syndrome (*n* = 19)**	***P***
Preterm, *n* (%)	1 (3.6)	18 (41.9)	21 (72.2)	16 (84.2)	<0.001
Birth weight (kg)	3,186.8 ± 107.9	2,297 ± 107.2	1,842.9 ± 115.2	1,900.7 ± 149.1	<0.001
Sex: male, *n* (%)	16 (57.1)	18 (38.3)	14 (46.6)	5 (26.3)	0.215
Fetal death, *n* (%)	0	2 (4.3)	1 (3.3)	1 (5.3)	0.824

Categorical variables are represented by absolute frequencies and percentages (*n*, %). Noncategorical variables are represented by mean ± SEM. Categorical variables were analyzed with Pearson x^2 tests or Fisher's exact test. *P* values are from comparison of control, preeclampsia, and HELLP groups. Noncategorical variables that were normally distributed were analyzed with the 1-way ANOVA test. Noncategorical variables that were not normally distributed were analyzed with Kruskal–Wallis tests. **P* value for trend based on increasing severity of the samples groups. Abbreviations: ANOVA, analysis of variance; PE, preeclampsia.

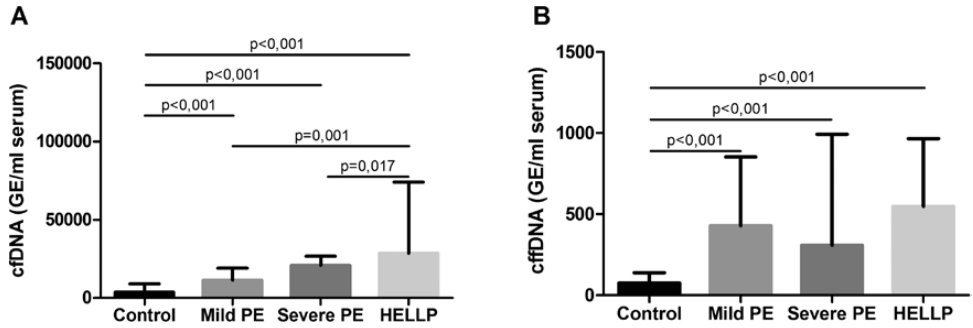

Figure 1. Total cfDNA level in control, mild PE, severe PE, and HELLP syndrome (**a**) and cffDNA in control, mild PE, severe PE, and HELLP syndrome (**b**). Data are represented as medians and inter quartile range. Abbreviations: cfDNA, cell-free DNA; cffDNA, fetal cell-free DNA; PE, preeclampsia.

DISCUSSION

The HELLP syndrome is considered the most severe form of PE, thus requiring a prompt delivery of the newborn to avoid the progression of the disease and complications.[23] This circumstance (short duration of the process) together with the low incidence of the disease result in a lack of studies assessing the physiopathology of the disease; as a consequence, many of the mechanisms underlying the onset of the syndrome remains unknown.

We have recently reported increased serum levels of total cfDNA in patients with severe PE and HELLP syndrome.[7] The present study confirms our previous results in total cfDNA and, in addition, it provides new information on fetal cfDNA and on the relationship of cfDNA with angiogenic/ antiangiogenic factors (parameters not assessed in our previous work).[7] We also report that only values of total cfDNA were able to significantly differentiate severe PE fromHELLP syndrome (20,957 • } 2,784 vs. 43,184 • } 8,647 GE/ ml, $P = 0.01$). As far as we know, there are only few studies

which have measured total and/or fetal circulating cfDNA in patients with HELLP syndrome, and their results are contradictory[24– 26] In these studies, the measurement of fetal cfDNA levels were based on the detection of Y-chromosomal sequences.[24,25] On the contrary, the analyses of fetal cfDNA levels in our study were based on the detection of hypermethylated *RASSF1A* gene. Therefore, our results may be more reliable than those from others studies that have measured fetal cfDNA in pregnant women carrying only male fetuses. On the other hand, several observations have demonstrated an imbalance in proangiogenic and antiangiogenic factors in PE, mainly arising from (i) a reduced vascular endothelial growth factor activity as a result of increased sFlt-1 levels,[27,28] (ii) a reduced transforming growth factor-β activity due to increased sEng levels,[29,30] and (iii) reduced PIGF levels.[31]

To our knowledge, the present study represents the first report that investigates the relationship between the levels of total or fetal cfDNA and sEng, sFlt-1, or PIGF in PE and to assess these levels in the HELLP syndrome as a distinct subgroup from severe PE. We observed that total and fetal cfDNA levels were significantly different amongst healthy, mild-PE, severe-PE, and HELLP syndrome pregnant women, with higher levels in those women with a more severe disease. In *post-hoc* analyses, fetal cfDNA levels in control women were significantly different from each PE/HELLP group; however, this parameter was unable to significantly differentiate the 3 pathological categories among them. On the contrary, total cfDNA levels allowed us to differentiate any category from each other, including severe PE vs. HELLP syndrome. These findings suggest that total cfDNA levels may be a promising novel biomarker to identify women with PE and assess its severity in clinical practice.

The results of the angiogenic/antiangiogenic markers are shown in Figures 2 and 3. Again the analysis of variance was clearly different ($P < 0.0001$) but the *post-hoc* analysis failed to find significant differences among groups and particularly between severe PE and HELLP syndrome. Leanos-Miranda *et al.*[32] studied a group of women with PE attended at a tertiary care hospital. Serum concentrations of sFlt-1, PIGF, and sEng, were measured. In agreement with our results, these authors showed that the risk of any adverse

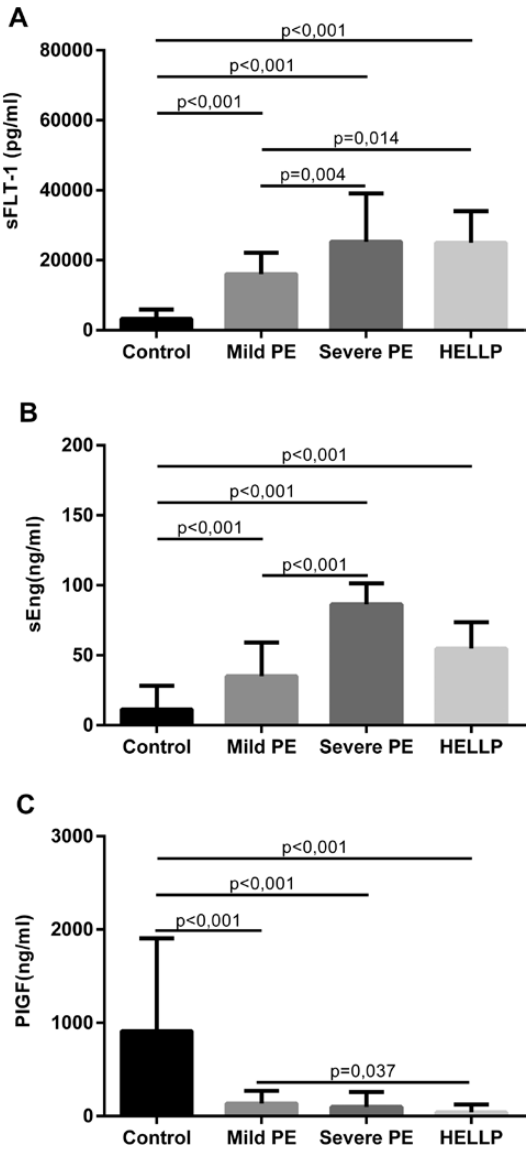

Figure 2. Values of sFlt-1 (**a**), sEng (**b**), and PIGF (**c**) in control, mild PE, severe PE, and HELLP syndrome. Data are represented as medians and inter quartile range. Abbreviations: sEng, soluble endoglin; sFLT-1, soluble FMS-like tyrosine kinase 1; PE, preeclampsia; PIGF, placental growth factor.

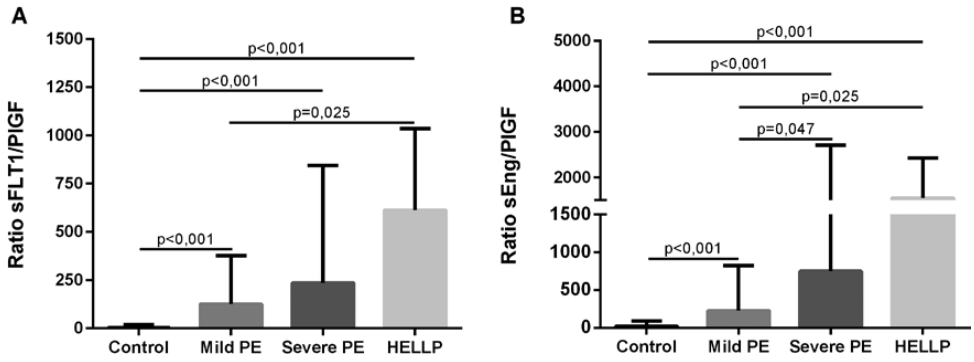

Figure 3. sFlt-1/PlGF ratio (**a**) and sEng/PlGF ratio (**b**) values in in control, mild PE, severe PE, and HELLP syndrome. Data are represented as medians and inter quartile range. Abbreviations: sEng, soluble endoglin; sFLT-1, soluble FMS-like tyrosine kinase 1; PE, preeclampsia; PlGF, placental growth factor.

Table 3. Maternal and neonatal/fetal outcome related to the levels of cell-free DNA, angiogenic and antiangiogenic factors.

	Maternal								
	Placental abruption			C-section			Transfusion		
	Yes	No	*P*	Yes	No	*P*	Yes	No	*P*
cfDNA (GE/ml) × 1,000	9.5 [8.1–2.5]	11.4 [5.4–26.4]	0.906	16.1 [9.4–28.5]	8.5 [3.2–18.3]	<0.001	28.5 [24–64.6]	10.4 [4.6–20.5]	<0.001
cffDNA (GE/ml)	278.4 [111.7–830.7]	282.1 [112.9–564.2]	0.713	355 [133.1–917.7]	241.7 [63.1–448.9]	0.016	581.7 [417.1–1066.7]	269.9 [100.4–618.1]	0.008
sEng (ng/ml)	87.6 [64.1–103.2]	38.9 [15.9–71.9]	0.006	54.9 [32.1–86.4]	24.9 [11.9–44]	<0.001	45.9 [23.8–78.9]	36.6 [18.7–74.3]	0.398
PIGF (ng/ml)	55.6 [32.4–302.8]	174.2 [63.6–477.3]	0.194	106.3 [39.8–292.3]	247.2 [123.7–914.9]	0.018	72.1 [39.2–198.1]	168.8 [54.4–482.4]	0.255
sFLT-1 (pg/ml) × 1,000	40.8 [33.6–54.6]	14.4 [5.5–23.3]	<0.001	20.5 [11.1–28]	11.2 [3.2–18]	0.001	20.7 [13.3–26.5]	14.8 [5.5–25.4]	0.118
sFLT-1/PIGF	589.9 [195.9–1178.2]	74.9 [14.9–365.9]	0.026	189.1 [47.1–660.9]	45.79 [2.4–215.3]	0.010	307.6 [75.7–656.0]	77.0 [13.8–415.8]	0.178
sEng/PIGF	1,593.9 [262.9–2,415.8]	188.8 [57.6–840.4]	0.070	458.5 [118.6–1,593.9]	100.9 [24.6–300.3]	0.002	646.6 [119.5–1,572.5]	203.3 [57–1,087.9]	0.292
	Neonatal/fetal								
	Preterm			IGR			Adverse outcome		
	Yes	No	*P*	Yes	No	*P*	Yes	No	*P*
cfDNA (GE/ml) × 1,000	21.4 [9.4–34]	9.3 [4.2–17.7]	<0.001	24.3 [9.6–46.4]	11.4 [5.4–21.6]	0.002	21.1 [9.4–34]	8.7 [3.4–18.4]	<0.001
cffDNA (GE/ml)	540.9 [263.8–1,015.3]	179.7 [81–712.2]	0.001	602 [261.7–1,215]	300.4 [103.7–870.8]	0.009	429.1 [214.1–997.6]	175.1 [80.2–601.9]	0.006
sEng (ng/ml)	54.9 [36.6–88.7]	30.5 [10.9–55.9]	<0.001	43 [32.7–92.4]	38.9 [17.1–64.6]	0.058	57 [33.5–90.1]	30.3 [11.1–46.3]	0.001
PLGF (ng/ml)	124.4 [33.7–225.6]	240.9 [78–937.3]	0.003	152 [42.4–320.5]	160.3 [64.1–468.4]	0.510	106.3 [32.4–238.6]	304.7 [117.5–1001]	0.001
sFLT-1 (pg/ml) × 1,000	22.9 [15.9–33.6]	10.8 [4.4–17]	<0.001	19.1 [12.2–24.2]	15.5 [0.6–25.8]	0.597	20.8 [12.6–25.9]	10.5 [3.4–21.3]	<0.001
sFLT-1/PLGF	189.1 [74.7–737.8]	31.7 [9.1–323.9]	<0.001	95.7 [34.8–506.9]	99.1 [16.3–468.3]	0.537	189.1 [67.9–784.9]	30.1 [2.5–184.5]	<0.001
sEng/PLGF	449.6 [166.4–2,200.9]	117.5 [26.7–549.5]	<0.001	472.3 [81.5–2,015]	188.8 [61.1–988]	0.134	537.2 [187.3–1,918.8]	97.7 [23.8–319.2]	<0.001

Data are represented as median and interquartile range. Variables were analyzed with Mann–Whitney *U* tests. Abbreviations: IGR, intrauterine growth restriction; cfDNA, cell-free DNA; cffDNA, cell-free fetal DNA; sEng, soluble endoglin; sFLT-1, soluble FMS-like tyrosine kinase 1; PLGF, placental growth factor.

Table 4. Cutoff values in the healthy group and preeclampsia groups (mild and severe) for the diagnosis of PE or HELLP syndrome

	Cutoff	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC	*P*
Healthy group						
cfDNA (GE/ml)	9,000	78.7 [68.1–86.4]	76.9 [57.9–89]	78.2 [69.2–85.2]	0.87 [0.79–0.95]	<0.001
cffDNA (GE/ml)	156.39	82.7 [72.6–89.6]	80.8 [62.1–91.5]	82.2 [73.6–88.4]	0.89 [0.82–0.96]	<0.001
sEng (ng/ml)	26.45	78.4 [67.7–86.2]	75 [55.1–88]	77.6 [68.3–84.7]	0.83 [0.72–0.94]	<0.001
PIGF (ng/ml)	<308.36	83.3 [72.7–90.1]	72 [52.4–85.7]	80.2 [71.1–86.9]	0.86 [0.77–0.94]	<0.001
sFLT-1 (pg/ml)	8,768.22	89.5 [80.6–94.6]	80 [60.9–91.1]	87.1 [79.2–92.3]	0.91 [0.83–0.99]	<0.001
sFLT-1/PIGF	20.36	88.7 [79.3–94.2]	79.1 [59.5–90.8]	86.3 [78–91.8]	0.91 [0.83–0.98]	<0.001
sEng/PIGF	97.70	82.9 [72.4–89.9]	79.2 [59.5–90.8]	81.9 [72.9–88.4]	0.89 [0.80–0.97]	<0.001
Preeclampsia groups (mild and severe)						
cfDNA (GE/ml)	12,750	77.5 [62.5–87.5]	58 [40.9–72]	68 [56.8–77.5]	0.69 [0.57–0.81]	0.003
cffDNA (GE/ml)	–	–	–	–	–	0.202
sEng (ng/ml)	43.53	79 [63.7–88.9]	69.4 [53.1–82]	74.4 [63.3–82.9]	0.74 [0.63–0.87]	<0.001
PIGF (ng/ml)	–	–	–	–	–	0.108
sFLT-1 (pg/ml)	17,542.10	72 [56.2–83.5]	60 [43.5–73.3]	65.8 [54.6–75.5]	0.72 [0.61–0.83]	<0.001
sFLT-1/PIGF	144.89	76.9 [99.7–91.8]	63.4 [52.6–73]	65.3 [55.3–74.1]	0.77 [0.66–0.88]	<0.001
sEng/PIGF	296.24	76.9 [49.7–91.8]	59.3 [48.4–69.3]	61.7 [51.6–70.9]	0.74 [0.63–0.86]	<0.001

Data of sensitivity, specificity, accuracy, and AUC are represented with the 95% confidence interval. Abbreviations: AUC, area under curve; cfDNA, cell-free DNA; cffDNA, cell-free fetal DNA; sEng, soluble endoglin; sFLT-1, soluble FMS-like tyrosine kinase 1; PIGF, placental growth factor.

maternal outcome and of having a small-for-gestational-age infant were higher among women with sFlt-1/PIGF ratios and sEng levels in the highest quartile (odds ratios ≥ 2.7), compared with the lowest quartile. Previous studies[33,34] have also analyzed the levels of sEng, sFlt-1 and PIGF, and levels of fetal cfDNA (only male fetuses) in patients with PE. In agreement with our results these studies have reported that the sFlt-1/PIGF ratio had the highest AUC for the detectionof PE but, unlike our study, they did not find a significant
cutoff value for cffDNA levels. The low sample size, the absence of female fetuses, and the lack of patients with HELLP syndrome could explain this disagreement. The existence of a relationship between placental abruption and an imbalance between angiogenic and antiangiogenic factors have been also reported by other authors.[35–37] As far as we know, the relationship between the studied parameters and the need of transfusion or cesarean section have not been analyzed before.

Regarding adverse fetal outcomes, in a study including 60 pregnant women (30 with established preterm labor) the mean cffDNA levels were approximately 6-fold higher in the pathological group compared with the control group.[38] Quezada *et al.*[39] examined cffDNA values measured at 10–19 weeks of gestation in 103 pregnancies that delivered <37 weeks, in 21 that delivered <34 weeks and in 82 that delivered between 34 and 37 weeks of gestation out of a total of 3,169 pregnancies. Conversely to the previous study these authors found no significant differences between the spontaneouspreterm delivery groups and the term delivery group.

Now, we describe that in a population including preeclamptic women and patients with HELLP syndrome the levels of cfDNA, cffDNA, and all the angiogenic/antiangiogenic factors studied are significantly different between women who had a preterm labor compared with women having a term labor. Similarly, there is contradictory evidence in the literature as to whether any increase in fetal cfDNA precedes the clinical presentation of impaired fetal growth.[40,41] As far as we know, our study is the first measuring total maternal levels of cfDNA in cases of fetal growth restriction.

Very recently, it has been published that a sFlt-1:PIGF ratio of 38 or lower could be used to predict the short-term absence of PE in women in whom the syndrome is suspected clinically.[42] Specifically, a sFlt-1/PIGF ratio of 38 or lower had a negative predictive value of 99.3% (95% CI, 97.9–99.9), with 80.0% sensitivity (95% CI, 51.9–95.7), and 78.3% specificity (95% CI, 74.6–81.7). On the other hand, the positive predictive value of a sFlt-1/PIGF ratio above 38 for a diagnosis of PE within 4 weeks was 36.7% (95% CI, 28.4 to 45.7), with 66.2% sensitivity (95% CI, 54.0–77.0), and 83.1% specificity (95% CI, 79.4–86.3). To the best of our knowledge, the predictive values of different cutoff points for sEng, cfDNA, or cffDNA levels and risk of PE or HELLP syndrome have not been published so far and, therefore, we cannot contrast our data with others studies.

There are several potential limitations of our study. For instance, the limited number of patients in the group withHELLP syndrome. This limitation is linked to the low prevalence of the syndrome and its fast clinical course. Therefore, further prospective studies are needed in order to confirm our results. Another potential limitation is the fact that cfDNA was measured in serum instead of plasma since in serum there are not coagulation factors. In conclusion, we measured plasma levels of circulating cell-free total and fetal DNA, sEng, soluble form of vascular endothelial growth factor receptor and PIGF in a control group of healthy pregnant women, patients with PE without criteria of severity, patients with severe PE and patients
with HELLP syndrome. We observed a gradual and strong relationship between all the parameters mentioned and the range of severity of PE, with the highest levels in patients with HELLP syndrome. We hypothesize that an ineffective placentation resulting in placental ischemia would play a key role in these pathologies, and that the studied parameters are biomarkers of the disease and severity. These parameters were significantly different among women who have had any maternal or fetal adverse event. The maximum ROC AUC value for PE and HELLP syndrome (with respect to controls) was 0.91 ($P < 0.001$) with a cutoff point of 8,768.2 pg/ml for sFLT-1 and 20.3 for the sFLT-1/PIGF ratio.

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