Lipoxin A4 Is Increased in the Plasma of Preeclamptic Women

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BACKGROUND
Excessive inflammation is involved in preeclampsia (PE) pathogenesis. Lipoxin A4 (LXA4) is an eicosanoid that counter-regulates inflammation. The main objective of this study was to determine LXA4 plasma levels in PE women. The correlations among LXA4 levels, ultrasensitive C-reactive protein (us-CRP) levels, and clinical/laboratory parameters of the studied participants were also investigated.

METHODS
LXA4 plasma levels were determined by ELISA in 23 nonpregnant, 26 normotensive pregnant, and 27 PE women (early PE (N = 10) and late PE (N = 17)), according to gestational age (GA) at clinical symptoms onset). The clinical/laboratory parameters included in Spearman's correlation analysis were: systolic and diastolic blood pressure (SBP and DBP, respectively), lactate dehydrogenase (LDH) activity, platelet count, proteinuria, and white blood cell count (WBC).

RESULTS
LXA4 levels were higher in PE women than in nonpregnant and normotensive pregnant women, and similar between nonpregnant and normotensive pregnant women. LXA4 plasma levels were higher in early PE vs. normotensive pregnancy (GA < 34 weeks) and in late PE vs. normotensive pregnancy (GA ≥ 34 weeks). No significant differences were detected between early and late PE. LXA4 levels were positively correlated with us-CRP levels, SBP, DBP, and WBC. No significant correlation was detected between LXA4 levels and the other laboratory parameters.

CONCLUSIONS
Chronic inflammation in PE, in spite of increased levels of LXA4, points to a possible failure in this regulatory pathway. Further studies are necessary to clarify this issue and to evaluate the role of LXA4 and other proresolving mediators of inflammation in the pathogenesis of PE.

Keywords : blood pressure; hypertension; inflammation; lipoxin A4; preeclampsia; resolution.

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Preeclampsia (PE) is a multisystem disorder of unknown cause that occurs at ≥20 weeks of gestation. This gestation-specific syndrome affects 2–8% of pregnancies and is associated with substantial maternal and perinatal morbidity and mortality worldwide.[1] Abnormal placentation and endothelial dysfunction are thought to play crucial roles in PE pathogenesis. These events may trigger an excessive activation of the inflammatory and haemostatic systems.[2] PE can be classified according to severity or onset-time of clinical symptoms.[3] The last classification has been more appreciated lately because it has been admitted that early and late PE have different etiopathogenesis, and it allows identifying PE women with worst prognosis.[4]

There are several evidences of systemic maternal inflammatory response in PE, such as altered cytokine levels and increased complement system and neutrophil activation.[5,6] Indeed, PE women show higher levels of ultrasensitive C-reactive protein (us-CRP) than normotensive pregnant women.[7–9] Although proinflammatory mechanisms have been extensively explored in this disease, pathways involved in the counter-regulation of inflammation are still poorly understood. Lipoxins (LXs) are endogenous lipid-based autacoids that are generated from arachidonic acid via lipoxygenase-mediated biosynthesis.[10,11] Among this lipid family, lipoxin A4 (LXA4) and its analogues (aspirin triggered LXs(ATL)) act as "braking signals" for inflammation. They evoke this action by modulating the onset of inflammation and by acting as an agonists on resolution phase of inflammation.[12]

Chronic inflammation in PE women indicates that the resolution of inflammation is dysfunctional. To investigate this hypothesis, we examined LXA4 levels in PE women

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and compared with normotensive pregnant and nonpregnant women. We also investigated whether LXA4 levels were associated with us-CRP levels and clinical/laboratory parameters of the studied population.

METHODS

Subjects

This case–control study included 76 Brazilian women: 27 preeclamptic, 26 normotensive pregnant, and 23 nonpregnant. All pregnant women were at the third trimester of gestation at blood collection and were selected from public hospitals (Hospital Municipal Odilon Behrens and Maternidade Odete Valadares). Ethics approval was obtained from Universidade Federal de Minas Gerais (Institutional Review Board Project #0618.0.203.000-10) and from the participant hospitals (#0681.0.000.216-11 and CEP-FHEMIG 077/2008, respectively), and written informed consent was obtained from all enrolled women.

PE was defined as hypertension (i.e., blood pressure ≥ 140/90 mm Hg, at least on two occasions, 4 or more h apart) and proteinuria (≥300 mg in a 24-h urine collection or ≥1+ on a random urine sample) on or after 20 weeks of gestation. In the absence of proteinuria, PE was defined as new-onset hypertension plus new onset of any of the following features: thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, cerebral, or visual disturbances.[3] No cases of chronic hypertension or superimposed PE were included in this study. Preeclamptic women were stratified in two subgroups according to gestational age (GA) at clinical symptoms onset: early PE (GA < 34 weeks; $N = 10$) and late PE (GA ≥ 34 weeks, $N = 17$).[13] Blood from PE women was collected after the time of diagnosis. The normotensive pregnant group included women with healthy pregnancies and it was matched for GA at the time of blood collection (GA < 34 weeks: $N = 10$; GA ≥ 34 weeks: $N = 16$) of the PE group. The nonpregnant group included healthy women age matched. Exclusion criteria common for all groups were: obesity (grades II and III)[14]; diabetes mellitus; cancer; coagulation disorders; cardiovascular, autoimmune, hepatic, renal, and inflammatory/infectious diseases. We did not exclude patients taking any kind of medication, since polytherapy is common in PE women. The clinical data were obtained from medical records and during the recruitment interview.

Sample collection and processing

Peripheral blood samples were obtained from all women into EDTA anticoagulant-coated tubes (BD Vacutainer). The plasma was separated by centrifugation spin at 3,000*g*, at room temperature, for 15 min and stored at −80 °C until LXA4 assay.

LXA4 measurement

LXA4 levels were measured in lipids extracted from plasma by a commercial ELISA kit (Neogen Corporation, Lexington, KY) according to the manufacturer's instructions. Samples from nonpregnant, normotensive pregnant and PE women were run simultaneously in the same plate. LXA4 levels are represented as pictogram per milliliter (pg/ml) of plasma. The assay is able to detect 100% of LXA4. Its cross-reactivity is 15-epi-lipoxin (24%), 5(S),6(R)-diHETE (5.0%), lipoxin B4 (1.0%), 15-HETE (0.10%), and 5-HETE (<0.01%). The assay range was 20–2,000 pg/ml. Both intra- and inter-assay coefficients were ≤10%.

Assessment of laboratory parameters

us-CRP levels were measured by an immunoturbidimetric assay, as described previously.[7] Lactate dehydrogenase (LDH) activity, platelet count, proteinuria, and white blood cell count (WBC) data were obtained from medical records at the time of blood collection and were only available for PE women. These parameters were assessed in this study because they have been traditionally correlated with clinical outcome in PE (LDH activity, platelet count and proteinuria) and with inflammation (WBC). The normal ranges considered in this study (82–524 U/L, 146–429 × 10^3/mm^3, <0.3 g/24 h urine specimen, 5.6–16.9 × 10^3/mm^3, respectively) were based on a population of healthy pregnant women in the third trimester of gestation.[15]

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 19.0 software (SPSS IBM Corporation, Armonk, NY). The normality of continuous variables was assessed using Shapiro–Wilk's *W*-test. The comparison of continuous variables with normal distribution was performed by analysis of variance test with *post hoc* LSD test (three groups) or Student's *t*-test (two groups). The comparison of continuous variables not normally distributed was made by Kruskal–Wallis test with *post hoc* Dunn–Bonferroni's test (three groups) or Mann–Whitney *U*-test (two groups). Comparison of categorical variables was performed by Pearson chi-square (χ^2) test. The relationship between the variables which were significantly associated with Spearman coefficients was further evaluated with linear regression analysis and expressed as r^2. The correlations among LXA4 levels, us-CRP levels and the clinical parameters (systolic blood pressure (SBP) and diastolic blood pressure (DBP)) included all the participants of the study or the PE group, separately, while the correlations between LXA4 levels and the laboratory parameters (LDH activity, platelet count, proteinuria, and WBC) included only PE women. $P < 0.05$ was considered statistically significant.

A *t*-test for two independent groups was used in the sample size calculation (OpenEpi, version 3, Rollins School of Public Health, Emory University, USA), which was performed based on LXA4 values (mean ± SD) that were obtained from a similar study including normotensive pregnant women and PE women.[16] The calculated sample size for both groups was three participants (statistical power: 95%, confidence interval: 95%).

RESULTS

Clinical characteristics

The clinical characteristics of the studied participants are displayed in Tables 1 and 2. There were no significant differences in age and body mass index (BMI) before pregnancy

Table 1. | Clinical characteristics of the studied participants

Variables	NP (*N* = 23)	Norm (*N* = 26)	PE (*N* = 27)	*P* value
Age (years)[a]	26 ± 6	27 ± 6	26 ± 6	0.778[b]
BMI (kg/m^2)[c]	21.5 (20.1–23.9)	22.5 (20.6–26.1)	24.1 (20.6–26.3)	0.218[d]
GWG (kg)[c]	N/A	10.7 (7.0–13.6)	13.0 (10–20.6)	0.017[d]*
GA (weeks)[c]	N/A	36 (32–39)	34 (32–38)	0.574[d]
Primiparas (%)[e]	N/A	9 (35)	15 (56)	0.126[f]
SBP (mm Hg)[c]	120 (110–120)	110 (100–112.5)	160 (150–170)	<0.001[d]***
DBP (mm Hg)[c]	80 (70–80)	70 (68–80)	102 (100–110)	<0.001[d]***

Abbreviations: BMI, body mass index (before pregnancy for normotensive pregnant women and preeclamptic women); GWG, gestational weight gain; GA, gestational age at blood collection; SBP, systolic blood pressure; DBP, diastolic blood pressure; NP, nonpregnant women; Norm, normotensive pregnant women; PE, preeclamptic women; N/A, not applicable.

[a]Data are presented as mean ± standard deviation. [b]Analysis of variance with *post hoc* LSD. [c]Data are presented as median (25th–75th percentiles). [d]Kruskal–Wallis with *post hoc* Dunn–Bonferroni/Mann–Whitney test. [e]Data are presented as number (percentage). [f]Pearson chi-square (χ^2) test. *P < 0.05, ***P < 0.001.

Table 2 | Clinical characteristics of the subgroups of normotensive pregnant women and PE women

Variables	Norm < 34weeks (*N* = 10)	Norm ≥ 34weeks (*N* = 16)	*P* value	Early PE (*N* = 10)	Late PE (*N* = 17)	*P* value
Age (years)[a]	33 ± 7	30 ± 6	0.717[b]	29 ± 4	30 ± 7	0.787[b]
BMI (kg/m^2)[c]	22.3 (19.7–26.3)	22.5 (21.3–26.1)	0.660[d]	24.1 (21.1–26.3)	24.1 (20.4–26.3)	0.863[d]
GWG (kg)[c]	8.1 (6.0–11.1)	12.7 (9.0–15.7)	0.061[d]	12.0 (7.0–16.5)	16.4 (10.5–23)	0.049[d]*
GA (weeks)[c]	30 (29–32)	38 (36–39)	<0.001[d]***	31 (30–32)	37 (35–39)	<0.001[d]***
Primiparas (%)[e]	10 (50%)	4 (25)	0.234[f]	5 (50)	10 (59)	0.706[f]
SBP (mm Hg)[c]	105 (100–110)	110 (100–120)	0.279[d]	170 (160–180)	160 (150–170)	0.012[d]*
DBP (mm Hg)[c]	70 (63–78)	70 (70–80)	0.754[d]	110 (100–113)	100 (100–110)	0.186[d]

Abbreviations: BMI, body mass index (before pregnancy for normotensive pregnant women and preeclamptic women); GWG, gestational weight gain; GA, gestational age at blood collection; SBP, systolic blood pressure; DBP, diastolic blood pressure; NP, nonpregnant women; Norm, normotensive pregnant women; PE, preeclamptic women.

[a]Data are presented as mean ± standard deviation. [b]Student's *t*-test. [c]Data are presented as median (25th–75th percentiles). [d]Mann–Whitney test. [e]Data are presented as number (percentage). [f]Pearson chi-square (χ^2) test. *P < 0.05, ***P < 0.001.

among nonpregnant, normotensive pregnant and PE women. The GA at blood collection and number of primiparas were also similar between the pregnant groups. PE women had a greater gestational weight gain (GWG) than normotensive pregnant women (P = 0.017). As expected, both SBP and DBP were elevated in PE women compared with nonpregnant and normotensive pregnant women (all P < 0.001). In addition, normotensive pregnant women showed decreased SBP (P = 0.014) and DBP compared with nonpregnant women (P = 0.005).

Ten (37%) preeclamptic women were classified into early PE and 17 (63%) into late PE group. Early and late PE women had similar age, BMI, and DBP. Late PE women had a greater GWG than early PE women (P = 0.049), while SBP was higher in early PE (P = 0.012). The normotensive pregnant subgroups had similar age, BMI, GWG, SBP, and DBP. As expected, GA at blood collection was higher in late PE vs. early PE and in normotensive pregnancy with GA ≥34 weeks vs. normotensive pregnancy with GA <34 weeks (all P < 0.001). There were no cases of HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) or eclampsia among PE women.

LXA4 plasma levels

Plasma levels of LXA4 were increased in PE women (median (25th–75th percentiles): 290 (110–430) pg/ml) than in nonpregnant women (70 (60–120) pg/ml) (P < 0.001) and in normotensive pregnant women (90 (70–130) pg/ml) (P = 0.001). Nonpregnant women and normotensive pregnant women showed similar plasma levels of LXA4 (Figure 1).

We also evaluated LXA4 plasma levels in PE women according to the onset-time of clinical symptoms and compared to those levels in normotensive pregnant women matched for gestational age at the time of blood collection. LXA4 levels were higher in early PE women (210 (110–380) pg/ml) than in normotensive pregnant women with GA <34 weeks (80 (50–130) pg/ml) (P = 0.029). Pregnant women with late PE (280 (90–440) pg/ml) also showed increased levels of LXA4 compared to normotensive pregnant women with GA ≥34 weeks (100 (80–130) pg/ml) (P = 0.026). There was no significant difference in LXA4 levels comparing early PE vs. late PE and normotensive pregnant women with GA <34 weeks vs. normotensive pregnant women with GA ≥34 weeks.

Laboratory parameters

LDH activity (median (25–75% percentiles): 425 (309–552) U/L), WBC (mean ± standard deviation: 10.6 ± 3.6 × 10^3/mm^3) and platelet count (208 ± 70 × 10^3/mm^3) values were within the normal range in the PE group (Methods section). As expected, proteinuria values were above the normal range (2.9 ± 2.4 g/24 h) in these women (Methods section). These parameters were analyzed in PE women according to the onset-time of clinical symptoms and no statistical differences were detected comparing early PE and late PE (data not shown).

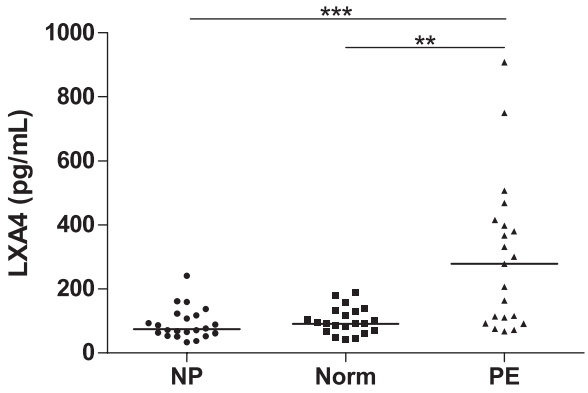

Figure 1. | LXA4 plasma levels in nonpregnant, normotensive pregnant and PE women. Horizontal bars represent median values for LXA4 (pg/ml). Plasma levels of LXA4 were higher in PE women than in normotensive pregnant and nonpregnant women. No significant differences were found comparing nonpregnant and normotensive pregnant women. Abbreviations: LXA4, lipoxin A4; NP, nonpregnant women; Norm, normotensive pregnant women; PE, preeclamptic women. $^{**}P < 0.01$, $^{***}P < 0.001$.

Correlations among LXA4, us-CRP, and clinical/laboratory parameters

In a prior study, we showed that us-CRP plasma levels were higher in PE women (5.8 (3.6–15.0) mg/L) than in nonpregnant women (0.9 (0.2–2.4) mg/L) ($P < 0.001$) and in early PE women (8.1 (2.8–13.6) mg/L) than in normotensive pregnant women with GA <34 weeks (3.2 (1.7–3.9) mg/L) ($P = 0.018$).[7] In the present study, we evaluated the correlations among LXA4, us-CRP, SBP, DBP, LDH, platelet count, proteinuria, and WBC of the studied participants. LXA4 levels showed a positive correlation with us-CRP levels, SBP and DBP only when all the participants were included in the analyses, but not in the PE group separately (Figure 2A–C). Moreover, LXA4 levels showed a positive correlation with WBC in PE women (Figure 2D). There were no statistical correlations between LXA4 levels and the other laboratory parameters analyzed in PE women.

DISCUSSION

Much evidence supports that the inflammatory response has a central role in PE pathogenesis.[5] Despite the intense investigation of proinflammatory mediators, the proresolving mechanisms have been poorly studied in the disease. In the present study, we showed that LXA4 concentration is

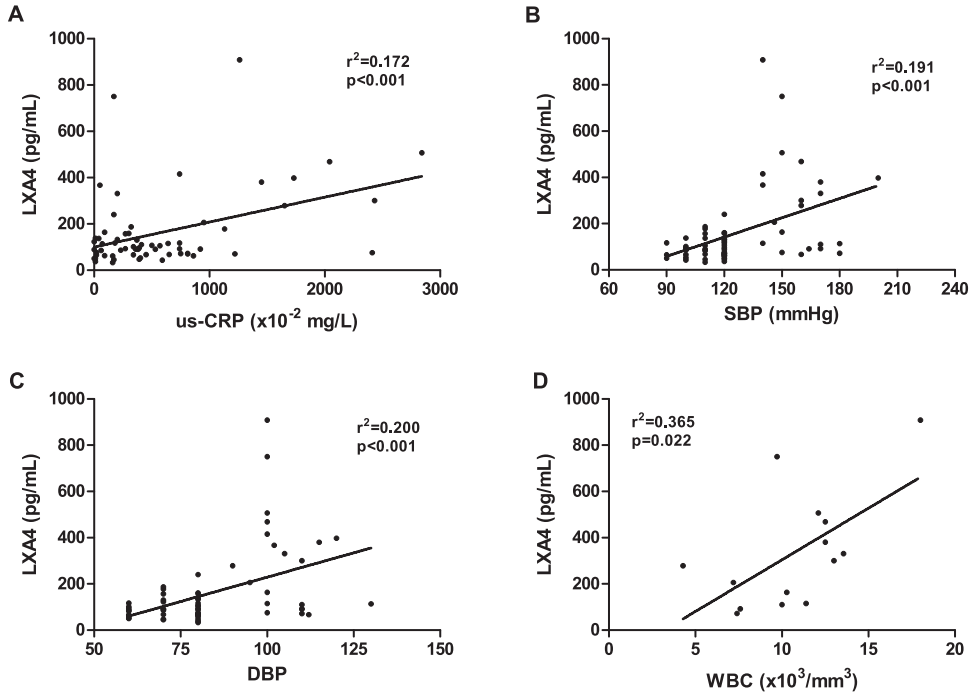

Figure 2. | Correlations among LXA4 levels, us-CRP levels, SBP, DBP, and WBC. The lines represent linear regression and the closed circles represent the participants of this study. LXA4 levels correlated positively with us-CRP levels (**A**), SBP (**B**), and DBP (**C**) when all the participants of the study were included in the analyses. LXA4 levels correlated positively with WBC in the PE group (**D**). Abbreviations: LXA4, lipoxin A4; us-CRP, ultrasensitive C-reactive protein; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell count.

increased in the plasma of PE women, regardless of the clinical form (early or late PE).

The inflammatory response is usually self-limiting and has a key role in maintaining tissue homeostasis, but it can progress to a chronic stage if the resolution process fails.[17] LXA4 is an endogenous eicosanoid that elicits counter-regulatory responses by interacting with its specific receptor, a G-protein-coupled receptor named lipoxin A4 receptor or formyl peptide receptor like-2 (ALX/FPR2, also known as FPRL1), hereafter referred as FPR2. LXA4 acts as an anti-inflammatory mediator by inhibiting cytokine and chemokine production, polymorphonuclear cell (PMN) activation and tissue infiltration. On the other hand, LXA4 prompts resolution of inflammation by increasing neutrophils apoptosis, non-phlogistic infiltration of monocytes and stimulating macrophage phagocytosis of apoptotic PMN.[12]

According to our review of the literature, two previous studies have shown increased circulating levels of LXA4 in PE women compared to normotensive pregnant women,[18,19] but an opposite finding was reported by Xu et al.[16] It is noteworthy that the studies involving proresolving molecules, like LXA4, in the context of PE are new, and aspects such as different populations analyzed, may affect the outcome of the proresolving response, due to different diet or hormones. Moreover, patients with diabetes, renal diseases and other co-morbidities were excluded in our study, but not in Xu et al. work, which may have biased their results. No difference in LXA4 levels was observed between nonpregnant and normotensive pregnant women in our study. However, Maldonado-Pérez *et al.*[20] reported decreased levels of LXA4 in nonpregnant women compared with normotensive pregnant women. LXA4 levels might be influenced by age and adiposity.[21,22] In Maldonado-Pérez *et al.* study, normotensive pregnant women had higher BMI than in our study (26.92 ± 2.12 vs. 22.5 (20.6–26.3) kg/m^2). In addition, nonpregnant and normotensive pregnant women seem diverge in age (25.33 ± 2.26 vs. 30.00 ± 2.67 years, respectively) and BMI (22.6 ± 0.55 vs. 26.99 ± 2.12 kg/m^2, respectively) in their work. Dong *et al.* and Huang *et al.* also measured LXA4 levels in mild and severe PE, but their results were contradictory, probably due to different GA at sample collection.[18,19] More studies with standardized methodologies and protocols of patient selection should be conducted in order to clarify these divergent findings.

To the best of our knowledge, no study has evaluated the differential expression of LXA4 in the placenta of normotensive and preeclamptic women. It would be also of great value the measurement of the enzymes involved in LXA4 synthesis such as 15-lipoxygenase-2. These issues are under investigation in our lab. We propose that LXA4 levels are increased in PE women in an attempt to attenuate the exacerbated inflammatory response in these women. Indeed, in our study LXA4 levels were positively correlated with WBC in PE women. In addition, there was a positive correlation between LXA4 and us-CRP, but this correlation failed to reach statistical significance in the PE group, probably due to its small sample size. An inverse correlation between LXA4 and CRP levels was reported in chronic heart failure and asthma.[23,24] Although PE, chronic heart failure and asthma are chronic inflammatory diseases, the mechanisms of immune regulation might differ among them.

Nevertheless, LXA4 concentration seems to be insufficient to attenuate inflammation in PE, because these women show features of systemic inflammatory response despite increased levels of LXA4. In a recent study, we showed that annexin A1 (AnxA1) levels are increased in PE women.[7] AnxA1 is a protein that is also endowed with anti-inflammatory and proresolving properties.[25] Chronic inflammation in PE, despite high levels of proresolving mediators, such as LXA4 and AnxA1, suggests a failure in the engagement of these resolution pathways, which might be a consequence of decreased expression of their receptors or defective engagement on them. Both LXA4 and AnxA1 activate and signal via FPR2 receptor.[26] In two previous studies, FPR2 mRNA placental expression was decreased in PE women compared with normotensive pregnant women,[16,19] although another study reported an opposite result.[18] Increased inactivation of LXA4 and AnxA1 could also interfere with their bioactions. LXA4 can be inactivated by dehydrogenases and oxidoreductases, while AnxA1 can be inactivated by proteolytic enzymes, such as proteinase 3 and neutrophil elastase, in an inflammatory milieu.[27–29] Indeed, placentas from PE women are rich of proteases, such as neutrophil elastase.[30] Based on these studies, we suggest that LXA4 and AnxA1 increase in the circulation of PE women could function as a compensatory mechanism to resolve inflammation, which may not be effective. Further studies are necessary to confirm whether and how these dysfunctional resolution mechanisms operate in PE.

Finally, LXA4 plasma levels were correlated positively with both SBP and DBP in the present study. However, these correlations failed to reach statistical significance when only the PE group was analyzed, probably due to the small sample size of this group. Hypertension, the main feature of PE, results from systemic endothelial dysfunction.[2] Besides its well-described immunomodulatory actions, LXA4 might also play an important role on vascular integrity. ATL suppress reactive oxygen species in endothelial cells and enhances oxide nitric generation.[31,32] Indeed, experimental PE rats treated with a synthetic analogue of LXA4 had their SBP and proinflammatory cytokines levels reduced.[33] Thus, LXA4 levels could be increased in PE in an attempt to attenuate not only inflammation, but also endothelial dysfunction. However, LXA4 increase seems to be insufficient to do that, probably due to inadequate LXA4 action, as previously discussed. LXA4 role in vascular integrity in PE should be better investigated in future studies. Although high LDH activity and low platelet count have been associated with a poor clinical outcome in PE, no differences were detected between early and late PE, and theses parameters were not correlated with LXA4 levels in our study. Proteinuria levels were not correlated with LXA4 levels and were also similar between early and late PE, reaffirming the weak association between proteinuria and PE prognosis.

The interpretation of our findings was limited by the relative small sample size of the study. Hence, the correlation analyses among LXA4, us-CRP, SBP, and DBP considering only PE women was probably prejudiced. Another limitation of this study was that the data about the laboratory parameters were only available for the PE group, which precluded us to evaluate the association between LXA4 levels and these

parameters in normotensive pregnant and nonpregnant women. In addition, we did not explore the potential causes (e.g., altered cellular expression of FPR2) underlying LXA4 apparently lack of effectiveness to resolve inflammation in PE women. This will be a matter of investigation in future studies of our research group.

We did not find any studies in the literature reporting the effects on LXA4 levels of the antihypertensives (nifedipine, methyldopa, and hydralazine) and anticonvulsivants (magnesium sulfate) used by PE women in our study. Glucocorticoids (GCs) may inhibit LXA4 synthesis.[34,35] GCs were prescribed only for PE women in our study, in 44% of them. LXA4 levels were analysed in PE women that had or not GC prescription and no difference was detected between them (data not shown). Moreover, LXA4 levels were higher in PE women in our study. Thus, we believe that GCs did not interfere significantly in this result.

Our data suggest that LXA4 may participate in PE pathogenesis. Increased LXA4 plasma levels in PE women, despite the exacerbated inflammatory response observed in these women, suggest a failure in the engagement of this resolution pathway. To the best of our knowledge, this is the first study that evaluated the association between LXA4 plasma levels and clinical/laboratory parameters of PE women. Moreover, this is the first report on LXA4 levels in preeclamptic Brazilian women. Further studies are necessary to investigate the role of LXA4 and other pro-resolving molecules in PE.

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DISCLOSURE

The authors declared no conflict of interest.

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