Short Communication

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Pharmacogenomics

NAMPT single-nucleotide polymorphism rs1319501 and visfatin/NAMPT affect nitric oxide formation, sFlt-1 and antihypertensive therapy response in preeclampsia

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Aim: We examined the relationships between visfatin/NAMPT and nitrite concentrations (a marker of nitric oxide [NO] formation) or sFlt-1 levels in 205 patients with preeclampsia (PE) responsive or nonresponsive to antihypertensive therapy, and whether *NAMPT* SNPs rs1319501 and rs3801266 affect nitrite concentrations in PE and 206 healthy pregnant women. **Patients & methods:** Circulating visfatin/NAMPT and sFlt-1 levels were measured by ELISA, and nitrite concentrations by using an ozone-based chemiluminescence assay. **Results:** In nonresponsive PE patients, visfatin/NAMPT levels were inversely related to nitrite concentrations and positively related to sFlt-1 levels. *NAMPT* SNP rs1319501 affected nitrite concentrations in nonresponsive PE patients and was tightly linked with *NAMPT* functional SNPs in Europeans. **Conclusion:** *NAMPT* SNP rs1319501 and visfatin/NAMPT affect NO formation, sFlt-1 levels and antihypertensive therapy response in PE.

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Preeclampsia (PE) presents with new-onset hypertension that can lead to multiple maternal organ dysfunction, which is a major contributor to maternal and fetal morbidity and mortality [1]. Despite the advances in understanding the pathogenesis of PE, the underlying mechanisms remain unclear [2,3]. However, impaired placental perfusion is thought to stimulate the release of antiangiogenic factors such as the soluble variant of the VEGFR1 (sFlt-1) into the maternal circulation, which may lead to systemic maternal endothelial dysfunction, a hallmark of PE [4]. Notably, there is clinical evidence for diminished nitric oxide (NO) formation in PE [5–7], and sFlt-1 was inversely related to NO formation in PE [6].

The role of visfatin, which is also known as NAMPT, is not fully known in pregnancy complications, including PE [8,9]. Visfatin was shown to upregulate NOS3 enzyme expression and function in endothelial cells [10], and to have

a vasodilating effect mediated via endothelium-derived NO on isolated blood vessels [11]. However, visfatin has also been shown to impair endothelium-dependent relaxation by stimulation of NADPH oxidase [12], and to produce *in vivo* endothelial dysfunction in mice, which support its role as a mediator of vascular damage [13]. Interestingly, plasma visfatin/NAMPT levels were positively related to nitrite concentrations (a marker of endogenous NO formation) and inversely related to sFlt-1 levels in healthy pregnancy, but inversely related to nitrite concentrations and positively related to sFlt-1 levels in PE [14]. Therefore, it was suggested that visfatin/NAMPT inhibits NO formation and upregulates sFlt-1 in PE [14]. However, no previous study has examined the relationships between visfatin/NAMPT levels and nitrite concentrations or sFlt-1 levels in patients classified as responsive or nonresponsive to antihypertensive drugs commonly used to treat PE.

The lack of effective pharmacological therapy for PE is a major health concern, and mechanisms and potential therapies targeting the endothelial dysfunction have been proposed for management or treatment of PE [15]. Visfatin/NAMPT arises as a novel therapeutical target for clinical conditions linked to endothelial dysfunction and vascular damage [13,16,17], and therefore, may potentially have a role in PE. We previously evaluated whether the SNPs rs1319501 and rs3801266 of the *NAMPT* gene affect plasma visfatin/NAMPT levels in PE [18] and antihypertensive therapy responsiveness in PE [19]. Notably, we found that the SNP rs1319501 in the *NAMPT* promoter was associated with PE [18] and affected visfatin/NAMPT levels only in the nonresponsive patients with PE [19]. Since visfatin was shown to modulate NO production [10], it is possible that functional *NAMPT* SNPs affecting visfatin/NAMPT levels may interfere with NO formation. However, no previous study has examined whether *NAMPT* SNPs affect NO formation during pregnancy or in patients with PE, including the groups classified as responsive or nonresponsive to antihypertensive therapy in PE.

In the present study, we examined the relationships between visfatin/NAMPT levels and nitrite concentrations or sFlt-1 levels in patients with PE classified as responsive and nonresponsive to antihypertensive therapy. We also examined whether *NAMPT* SNPs (rs1319501 T>C and rs3801266 A>G) affect nitrite concentrations in healthy pregnant (HP) and in patients with PE, including the groups classified as responsive and nonresponsive to antihypertensive therapy. In order to identify the mechanisms by which these *NAMPT* SNPs may affect plasma visfatin/NAMPT levels, we further assessed the pairwise linkage disequilibrium with other functional SNPs located in the *NAMPT* promoter.

Patients & methods

Subjects

The Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo approved the use of human subjects. All subjects were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital at the Ribeirao Preto Medical School of University of Sao Paulo, and included 205 pregnant with PE and 206 HP with uncomplicated pregnancies. PE was defined as pregnancy-induced hypertension (≥140 mmHg systolic and ≥90 mmHg diastolic on two or more measurements, at least 6 h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks post partum, and significant proteinuria (Pr; ≥0.3 g/24 h), in accordance to the American College of Obstetricians and Gynecologists report [20]. We did not include in the study women with pre-existing hypertension, with or without superimposed PE.

Maternal venous blood samples were collected into tubes containing heparin (to measure nitrite and sFlt-1 concentrations) and ethylenediaminetetraacetic acid (to measure visfatin/NAMPT concentrations) at the clinical attendance, and after the written informed consent. Plasma was obtained by centrifugation of tubes containing whole blood in heparin (at 1000 × *g* for 3 min) or ethylenediaminetetraacetic acid (at 2000 × *g* for 10 min), and stored at -70°C until assayed. Genomic DNA was extracted from the cellular fraction of 1 ml of whole blood by a standard salting-out method and stored at -20°C until analyzed.

Antihypertensive treatment & drug response evaluation

Responsiveness to antihypertensive therapy was based on the evaluation of clinical and laboratory parameters in response to the use of these drugs: methyldopa (1000–1500 mg per day) was the first antihypertensive drug of choice, followed by nifedipine (40–60 mg per day) in cases of lack of significant response to methyldopa. Hydralazine (5–30 mg) was used only in cases of hypertensive crisis. The patients included in the study were monitored with caution for signs and symptoms of PE, with fetal surveillance and laboratory tests at least once a week. The presence of at least one of the criteria stated below was considered to classify the patients with PE as nonresponsive to antihypertensive therapy [19,21–23]:

- Clinical symptoms including blurred vision, persistent headache or scotomata, persistent right upper quadrant or epigastric pain;
- Systolic blood pressure (SBP) above 140 mmHg and diastolic blood pressure (DBP) above 90 mmHg, as assessed by the blood pressure curve;
- Hemolysis, elevated liver enzymes and a low platelet count syndrome; or Pr >2.0 g per 24 h; creatinine >1.2 mg per 100 ml or blood urea nitrogen >30 mg per 100 ml; aspartate aminotransferase >70 UI^{-1} and alanine aminotransferase >60 UI^{-1};
- Fetal hypoactivity or nonreactive fetus, as revealed by cardiotocography; intrauterine growth restriction (IUGR), oligoamnio, abnormal biophysical profile score and Doppler velocimetry abnormalities, as evaluated by ultrasound.

Enzyme immunoassays of visfatin/NAMPT & sFlt-1

Visfatin/NAMPT and sFlt-1 concentrations in plasma were measured with commercially available ELISA kits (RayBio Human Visfatin EIA–VIS–1, GA, USA; and R&D Systems, MN, USA, respectively), according to manufacturer's instructions.

Measurement of nitrite concentrations

Nitrite concentrations were measured using an ozone-based chemiluminescence assay, as previously described [6]. Briefly, 200 µl of plasma aliquots analyzed in triplicate were injected into a solution of acidified triiodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer; General Electric Company, CO, USA). Approximately 8 ml of triiodide solution (2.0 g of potassium iodide and 1.3 g of iodine dissolved in 40 ml of water with 140 ml of acetic acid) were placed in the purge vessel into which plasma samples were injected. The triiodide solution reduced nitrites to NO gas, which was detected by the NO analyzer.

Genotyping

Taqman Allele Discrimination assays using probes and primers from Applied Biosystems (CA, USA) were used to determine the genotypes for the SNPs in the promoter region (rs1319501 T>C; C_7590641_30) and in intron 1 (rs3801266 A >G; C_340124_10) of *NAMPT*. PCR reactions were performed in standard conditions for thermal cycling and in a total volume of 10 µl (5 ng of template DNA, 1× TaqMan Genotyping Master Mix [Life Technologies Co., NY, USA] and 1× Taqman Allele Discrimination Assay). StepOnePlus Real-Time PCR System from Applied Biosystems was used to record the fluorescence, and results were analyzed with manufacturer's software.

Statistical analysis

The clinical characteristics were compared between PE patients and HP women, and between PE patients responsive or nonresponsive to antihypertensive therapy, using student's unpaired *t*-test, Mann–Whitney *U*-test or χ^2 as appropriate. The effects of the different genotypes for the *NAMPT* SNPs on nitrite concentrations in HP, PE patients, and responsive and nonresponsive PE patients were compared by student's unpaired *t*-test. Deviation from Hardy–Weinberg equilibrium was tested for the distributions of genotypes. The relationships between visfatin/NAMPT and nitrite or sFlt-1 concentrations were analyzed using Spearman's correlation (*r* and p-values). GraphPad Prism 5.0 was used for statistical analysis. A value of $p < 0.05$ was considered the level of statistical significance.

Identification of functional SNPs located in the *NAMPT* promoter

To identify mechanisms by which the *NAMPT* SNPs may affect visfatin/NAMPT levels, we first searched the literature for functional SNPs located in the *NAMPT* promoter that may affect *NAMPT* expression. Next, we searched the University of California Santa Cruz (UCSC) Genome Browser to examine whether these functional SNPs overlap with several data from The Encyclopedia of DNA Elements (ENCODE) Project [24], including DNase I hypersensitivity clusters, transcription factor (TF) ChIP-seq clusters (TF binding sites) and ChIP-seq data for three histone marks on seven cell lines: the acetylation of histone H3 on lysine 27 (H3K27ac), the monomethylation of histone H3 on lysine 4 (H3K4me1) and the trimethylation of histone H3 on lysine 4 (H3K4me3).

H3K27ac is often found near active regulatory elements such as enhancers [24], and distinguishes active from inactive enhancers containing the monomethylation of histone H3 on lysine 4 alone [25]. H3K4me3 is associated

Table 1. Clinical, demographic and biochemical characteristics of study subjects.

Parameter	Healthy pregnant (n = 206)	Preeclampsia (n = 205)	p-value
Age (years)	24.5 ± 0.4	26.7 ± 0.5	**0.001**
Ethnicity (% white)	67.1	69.7	0.809
Current smokers (%)	11.2	9.0	0.517
BMI (kg m^{-2})	23.3 ± 0.3	27.2 ± 0.5	**0.000**
SBP (mmHg)	111.1 ± 0.8	140.2 ± 1.5	**0.000**
DPB (mmHg)	71.8 ± 0.6	88.0 ± 0.9	**0.000**
HR (beats/min)	81.5 ± 0.7	82.6 ± 0.6	0.286
Fasting glucose (mg dl^{-1})	75.1 ± 1.0	79.2 ± 2.2	0.182
Hemoglobin (g dl^{-1})	11.9 ± 0.1	11.9 ± 0.1	0.792
Hematocrit (%)	35.7 ± 0.4	35.9 ± 0.3	0.685
Creatinine (mmol l^{-1})	61.88	61.89	0.920
24-h Pr (mg/24 h)	ND	846.5 ± 108.7	
Primiparity (%)	45.3	44.3	0.849
GAD (weeks)	39.7 ± 0.1	36.0 ± 0.3	**0.000**
Newborn weight (g)	3297 ± 39.7	2528 ± 62.8	**0.000**
GAS (weeks)	36.6 ± 0.3	34.3 ± 0.3	**0.000**
Visfatin/NAMPT (ng/ml)	21.3 ± 2.0	20.7 ± 2.5	0.290
sFlt-1 (ng/ml)	3.8 ± 0.2	11.2 ± 1.0	**0.000**
Plasma nitrite (nM)	159.8 ± 11.2	95.9 ± 4.7	**0.000**
Early-onset PE (%)	ND	24.5	
Preterm birth (%)	ND	33.8	
IUGR (%)	ND	30.4	
Maternal PE (%)	ND	88.7	
AST (U/l)	ND	26.1 ± 2.4	
ALT (U/l)	ND	19.7 ± 2.0	

Values are the mean ± SEM. $p < 0.05$ vs healthy pregnant group. Bold values are significant.
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; DBP: Diastolic blood pressure; GAD: Gestational age at delivery; GAS: Gestational age at sampling; HR: Heart rate; IUGR: Intrauterine growth restriction; NAMPT: Nicotinamide phosphoribosyltransferase; ND: Not determined (however, negative dipstick test); PE: Preeclampsia; Pr: Proteinuria; SBP: Systolic blood pressure; SEM: Standard error of the mean; sFlt-1: Soluble fms-like tyrosine kinase-1.

with promoters [26]. GeneHancer track available in the UCSC Genome Browser was also used to identify active regulatory elements (enhancers and promoters) [27] that may target the *NAMPT* gene. This approach using computational genomics to identify functional SNPs located within gene regulatory regions was recently performed elsewhere [28].

Linkage Disequilibrium (LD) analysis

We then assessed the pairwise LD calculated as D′ using the Haploview version 4.2 [29] among the functional SNPs located in the *NAMPT* promoter from the literature search (described above), and the rs1319501 in the promoter and rs3801266 in intron 1 of *NAMPT* studied here. Data from the 1000 Genomes Phase III for Africans (YRI, Yoruba in Ibadan, Nigeria), East Asians (CHB, Han Chinese in Beijing, China; and JPT, Japanese in Tokyo, Japan) and Europeans (CEU, Utah Residents with Northern and Western European Ancestry) were used for LD analysis.

Results

The characteristics of the subjects included in this study are shown in Table 1. Ethnicity (% white), % of current smokers, hemoglobin, hematocrit and creatinine were similar in HP and PE (all $p > 0.05$). PE showed higher SBP and DBP than in HP (both $p < 0.01$), despite that most PE patients were receiving antihypertensive therapy. We found higher age and BMI, but lower gestational age at delivery and at sampling, and lower newborn weight in PE than in HP (all $p < 0.01$). PE showed higher sFlt-1 and lower nitrite concentrations than HP (both $p < 0.01$), which are in line with our previous findings [6,18]. We found no differences in visfatin/NAMPT concentrations between HP and PE ($p > 0.05$).

Table 2. Clinical, demographic and biochemical characteristics of patients with preeclampsia classified as responsive and nonresponsive to antihypertensive therapy.

Parameters	PE responsive (n = 110)	PE nonresponsive (n = 95)	p-value
Age (years)	26.5 ± 0.5	26.6 ± 0.7	0.918
Ethnicity (% white)	71.4	70.5	0.953
Current smokers (%)	12.5	4.2	0.052
BMI (kg m^{-2})	28.6 ± 0.7	25.8 ± 0.6	**0.003**
SBP (mmHg)	132.2 ± 1.7	149.7 ± 2.0	**0.000**
DPB (mmHg)	83.2 ± 1.0	93.9 ± 1.3	**0.000**
HR (beats/min)	82.5 ± 0.7	82.1 ± 1.1	0.765
Fasting glucose (mg dl^{-1})	74.8 ± 2.1	84.6 ± 4.2	**0.046**
Hemoglobin (g dl^{-1})	11.9 ± 0.1	11.9 ± 0.2	0.952
Hematocrit (%)	35.9 ± 0.4	36.0 ± 0.5	0.950
Creatinine (mmol l^{-1})	61.88	70.7	**0.032**
24-h Pr (mg/24 h)	681.5 ± 144.9	1127.0 ± 174.7	**0.049**
Primiparity (%)	42.3	47.3	0.655
GAD (weeks)	38.1 ± 0.2	33.9 ± 0.5	**0.000**
Newborn weight (g)	2999 ± 67.7	2033 ± 91.7	**0.000**
GAS (weeks)	35.7 ± 0.4	32.7 ± 0.5	**0.000**
Visfatin/NAMPT (ng/ml)	21.0 ± 3.4	23.6 ± 4.7	0.884
sFlt-1 (ng/ml)	8.1 ± 1.4	16.3 ± 2.0	**0.002**
Plasma nitrite (nM)	164.3 ± 56.7	122.3 ± 14.1	0.431
Early-onset PE (%)	6.3	47.3	**0.000**
Preterm birth (%)	11.6	61.5	**0.000**
IUGR (%)	14.3	50.5	**0.000**
Maternal PE (%)	92.3	85.7	0.757
AST (U/l)	23.7 ± 3.5	29.0 ± 3.2	0.270
ALT (U/l)	15.2 ± 1.2	25.6 ± 4.3	**0.011**

Values are the mean ± SEM. $p < 0.05$ vs responsive PE patients' group. Bold values are significant.
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; DBP: Diastolic blood pressure; GAD: Gestational age at delivery; GAS: Gestational age at sampling; HR: Heart rate; IUGR: Intrauterine growth restriction; NAMPT: Nicotinamide phosphoribosyltransferase; ND: Not determined (however, negative dipstick test); PE: Preeclampsia; Pr: Proteinuria; SBP: Systolic blood pressure; SEM: Standard error of the mean; sFlt-1: Soluble fms-like tyrosine kinase-1.

The characteristics of PE patients either responsive or nonresponsive to antihypertensive therapy are shown in Table 2. Age, ethnicity (% white), % of current smokers, hemoglobin, hematocrit, visfatin/NAMPT concentrations and nitrite concentrations were similar between groups (all $p > 0.05$). Nonresponsive PE patients showed higher SBP and DBP, fasting glucose, creatinine, Pr, and sFlt-1 concentrations than responsive PE patients (all $p < 0.05$). Noteworthy, the phenotypes of early-onset PE, preterm birth and IUGR were more frequent in nonresponsive PE patients (all $p < 0.05$). Moreover, nonresponsive PE patients showed lower BMI, newborn weight, and gestational age at delivery and at sampling (all $p < 0.05$).

We examined the relationships between plasma visfatin/NAMPT and nitrite or sFlt-1 concentrations in responsive and nonresponsive PE patients. We found no significant correlations in responsive PE patients (Figure 1A & C). Conversely, plasma visfatin/NAMPT levels were inversely related to nitrite concentrations ($r = -0.376$; 95% CI: -0.643 to -0.027; $p = 0.031$; Figure 1B), and positively related to sFlt-1 levels ($r = 0.621$; 95% CI: 0.164–0.858; $p = 0.010$; Figure 1D) in nonresponsive PE patients.

Next, we evaluated the effects of *NAMPT* genotypes on plasma nitrite concentrations in HP and PE. The distribution of genotypes for the *NAMPT* SNPs showed no deviation from the Hardy–Weinberg equilibrium (all $p > 0.05$, data not shown). We found no significant effects of genotypes for the *NAMPT* SNPs on nitrite concentrations in HP or PE patients (Figure 2). We further examined the effects of *NAMPT* genotypes on nitrite concentrations in PE patients classified as responsive or nonresponsive to antihypertensive therapy. Although there were no differences in visfatin/NAMPT levels or nitrite concentrations between responsive and nonresponsive PE patients ($p > 0.05$; Table 2), we found significant effects of *NAMPT* genotypes on nitrite concentrations for the SNP rs1319501, but not for the SNP rs3801266 (Figure 3B). The TT genotype for the rs1319501 T>C SNP was

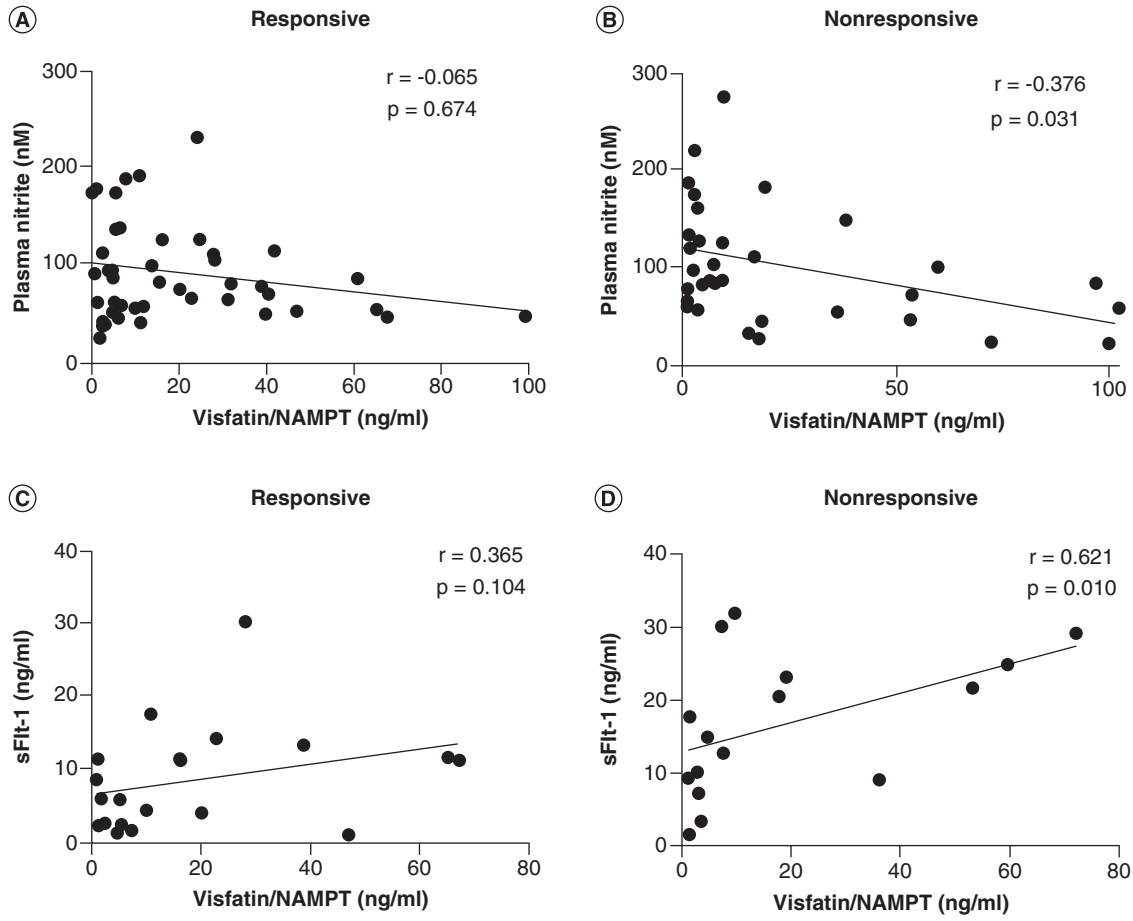

Figure 1. Correlations between plasma nitrite concentrations (A & B) or sFlt-1 (C & D) and visfatin/NAMPT levels in patients with preeclampsia classified as responsive and nonresponsive to antihypertensive therapy. The regression lines are plotted. The r- and p-values are reported.
r: Spearman's correlation.

associated with higher nitrite concentrations in nonresponsive PE patients ($p < 0.05$, compared with the TC + CC genotypes; Figure 3A).

Finally, we searched for ENCODE data at the UCSC Genome Browser to identify functional SNPs that may affect *NAMPT* expression. The *NAMPT* promoter region shows an enrichment for the active histone mark H3K27ac and H3K4me3, which is associated with active promoters, and it has a promoter element according to GeneHancer (GH07J106281; Supplementary Figure 1). We then assessed the LD among the functional SNPs in the *NAMPT* promoter and the rs1319501 and rs3801266 SNPs studied here. A short segment of high LD between the SNPs rs1319501 and rs9770242 was found in the African population (Figure 4A), which may be explained by the demographic history and higher recombination events, as compared with non-Africans. Notably, the SNP rs1319501 is in high LD with the functional SNPs rs59744560 and rs61330082 in the European population (Figure 4B). However, most of these SNPs were not found in East Asian population (Supplementary Figure 2).

Discussion

This study was the first to examine the relationships between plasma nitrite concentrations or sFlt-1 and visfatin/NAMPT levels in patients with PE classified according to antihypertensive therapy responsiveness, and the effect of *NAMPT* SNPs on nitrite concentrations in health pregnant and in PE patients. Our main findings are as follows: plasma visfatin/NAMPT levels were inversely related to nitrite concentrations and positively related to sFlt-1 levels in nonresponsive PE patients, genotypes for the *NAMPT* SNP rs1319501 affect nitrite concentrations in nonresponsive PE patients and the *NAMPT* SNP rs1319501 is in high LD with other functional SNPs located in the *NAMPT* promoter in Europeans.

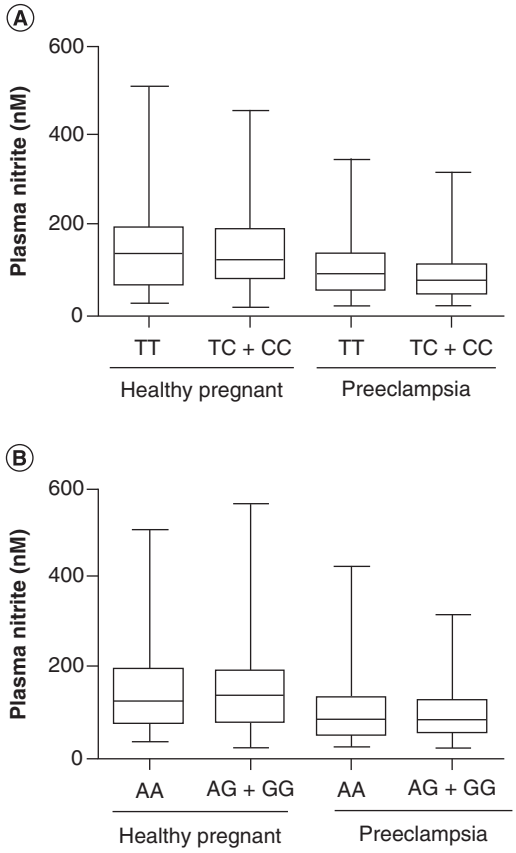

Figure 2. Plasma nitrite concentrations in healthy pregnant women & patients with preeclampsia grouped by their genotypes for the *NAMPT* SNPs: (A) rs1319501 T>C and (B) rs3801266 A>G. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and lowest values.

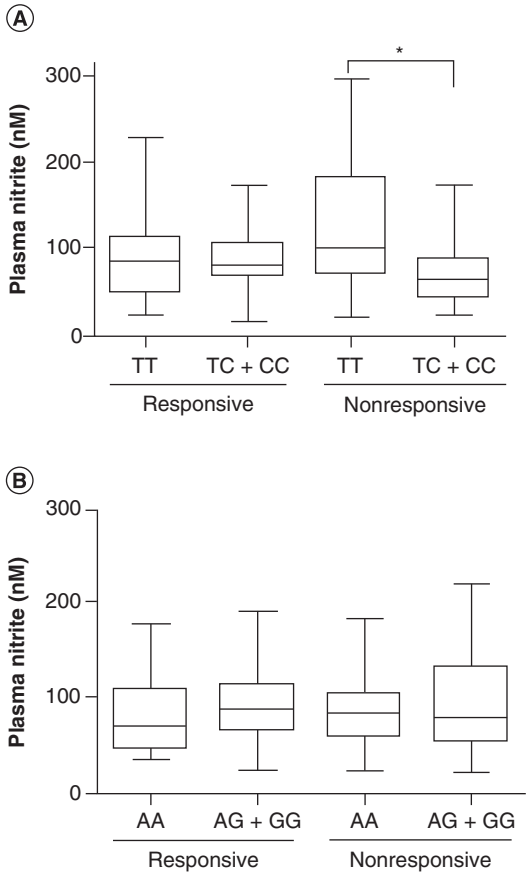

Figure 3. Plasma nitrite concentrations in patients with preeclampsia classified as responsive or nonresponsive to antihypertensive therapy and grouped by their genotypes for the *NAMPT* SNPs: (A) rs1319501 T>C and (B) rs3801266 A>G. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and lowest values.
*$p < 0.05$ versus TC + CC genotypes.

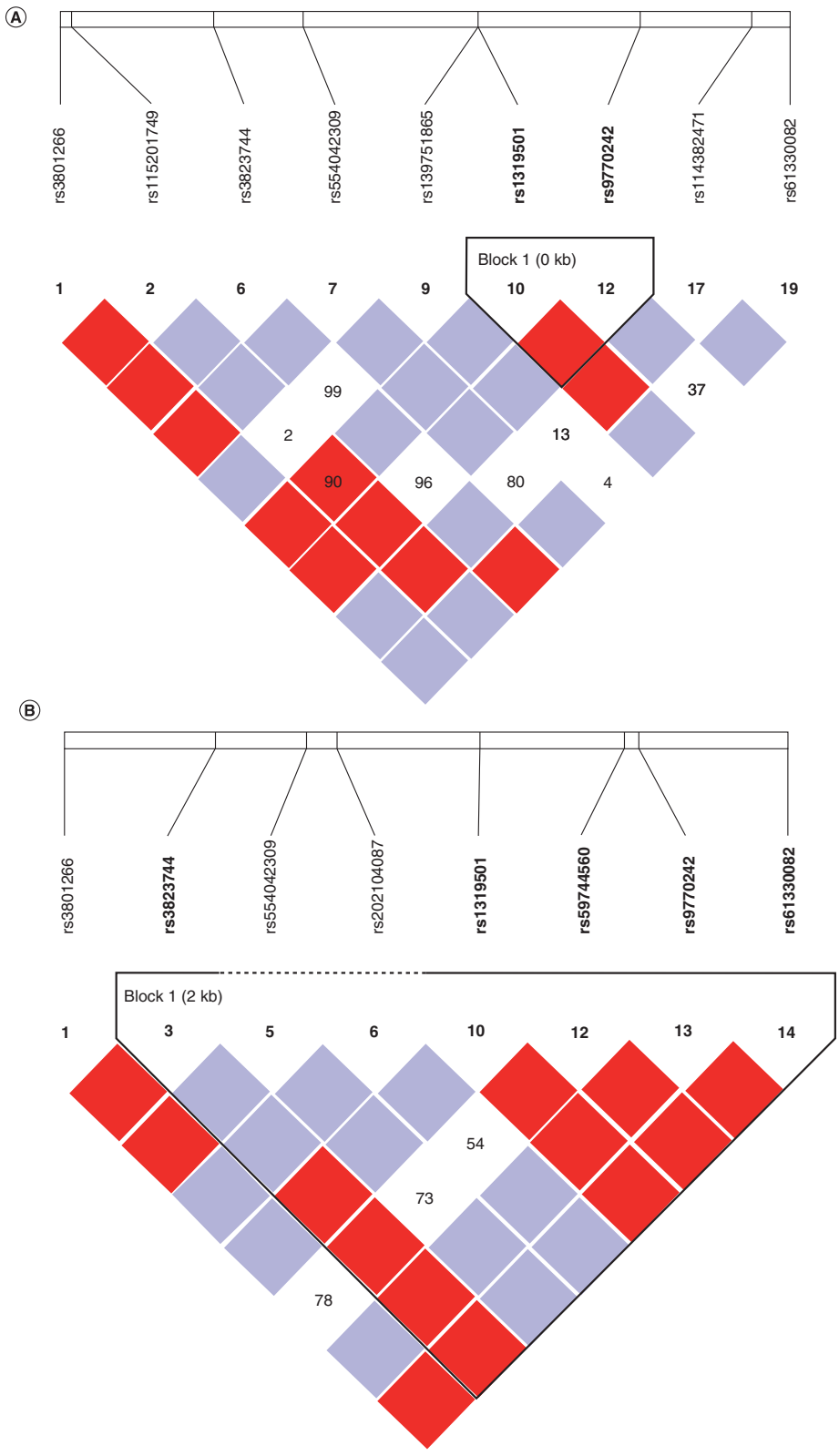

Figure 4. Linkage disequilibrium among SNPs in the *NAMPT* promoter region for (A) African and (B) European populations. The numbers below the rs IDs correspond to the number of SNPs found in this genomic region for these populations. Values for pairwise D′ are presented in each box; those without values refer to D′ = 1. Color scheme: bright red, D′ = 1 and LOD ≥2; blue, D′ = 1 and LOD <2; white, D′ <1 and LOD <2.
LOD: Logarithm of odds.

Visfatin/NAMPT may impair endothelium-dependent relaxation by activating NADPH oxidase with release of superoxide anions [12], since these scavenge NO to generate peroxynitrite [30,31]. Moreover, superoxide induces NOS3 uncoupling, leading to a reduced NO bioavailability and increased peroxynitrite production [32]. Notably, the signaling pathways related to the induction of NADPH oxidase lead to upregulation of sFlt-1, which is upregulated in PE [30]. In line with these findings, visfatin/NAMPT levels were previously shown to be inversely related to nitrite concentrations and positively related to sFlt-1 levels in PE, which suggest that visfatin/NAMPT inhibits NO formation and upregulates sFlt-1 in PE [14]. Likewise, we found here that visfatin/NAMPT levels were inversely related to nitrite concentrations and positively related to sFlt-1 levels in nonresponsive PE patients, but not significantly related in responsive PE patients. Nonresponsive PE patients presented higher creatinine, Pr and sFlt-1 levels than responsive PE patients. Noteworthy, the phenotypes of early-onset PE, preterm birth and IUGR were more frequent in nonresponsive PE patients (Table 2). Circulating factors in PE contribute to endothelial dysfunction by increasing oxidative stress, thereby decreasing NO bioavailability [33]. Specifically, sFlt-1 binds to circulating VEGF, which results in diminished production of NO [34]. In addition, sFlt-1 may also play a role in oxidative stress in trophoblasts in PE [35], and VEGF inhibition may result in hypertension through disturbance of the prooxidant/antioxidant balance [36]. Taken together, these findings suggest that visfatin/NAMPT inhibits NO formation and upregulates sFlt-1 due to the increased oxidative stress in nonresponsive PE patients, who exhibit the worst clinical outcomes [19,21–23]. However, this hypothesis remains to be proved.

Antihypertensive drug therapy during PE reduces the risk of severe hypertension [37]. Despite this therapy does not reverse the primary mechanisms of PE, the major goal is to prevent cardiovascular and cerebrovascular consequences of severe hypertension, and to prolong gestation improving both maternal and fetal outcomes [38]. There is no evidence for the antihypertensive effects of methyldopa by mechanisms involving NO production. However, nifedipine and other calcium channel blockers were shown to restore NO bioavailability and improve endothelial function [39,40]. In addition, hydralazine was shown to increase cGMP concentrations in PE patients, which may be related to NO production [41]. Therefore, these antihypertensive drugs commonly used to treat PE might produce their beneficial effects by increasing NO bioavailability, thus counteracting the diminished NO formation previously reported in PE [6,23]. However, 40% of PE patients have been classified as nonresponsive to antihypertensive therapy [19,21–23], and as such this group showed higher SBP and DBP, despite most of them were receiving more intense antihypertensive therapy (Table 2). In this context, pharmacogenomics research may help to improve antihypertensive therapy for the nonresponsive group of patients with PE [42–44].

Although no previous study has examined whether *NAMPT* SNPs affect plasma nitrite concentrations in HP and in PE, we have previously found that *NOS3* polymorphisms were associated with NO formation in healthy subjects and in PE [45,46], and with antihypertensive responses to enalapril [47,48] and in PE [23]. Moreover, while we previously found no effects of the SNP rs1319501 on visfatin/NAMPT levels in HP or PE patients [18], the TT genotype for this SNP was associated with higher visfatin/NAMPT levels in PE patients nonresponsive to antihypertensive therapy [19]. Here we found that nonresponsive PE patients with the TT genotype for the SNP rs1319501 had higher nitrite concentrations. Noteworthy, an upregulation of NOS2 has been reported in both experimental and clinical hypertension [49]. Peroxynitrite may also affect endothelial function by increasing the expression of NOS2 and ICAM-1, a marker of endothelial dysfunction due to activation of NF-κB [31]. In line with these findings, visfatin/NAMPT may activate NADPH oxidase with release of superoxide anions [12], and peroxynitrite can be formed in the presence of abnormally high NO and superoxide [50]. Taken together, we suggest that the SNP rs1319501 affects visfatin/NAMPT levels and then may alter NO bioavailability and oxidative stress in nonresponsive PE patients, which could also be increased due to NOS2 activation. Nonetheless, further studies are needed to explore this hypothesis. Gene–gene interactions in the NAMPT pathway [51,52] could also explain the effects of the *NAMPT* SNP rs1319501 on nitrite concentrations in nonresponsive PE patients.

It is possible that other functional *NAMPT* polymorphisms, mainly those located in the promoter region, may be in LD with the SNP rs1319501 and affect *NAMPT* expression. Indeed, the promoter SNPs rs1319501 and rs9770242 were in complete LD in different populations [53,54]. The 5′-upstream *NAMPT* region has several regulatory elements [55] and an *in silico* analysis of the *NAMPT* promoter revealed putative *cis*-regulatory elements, including the binding sites for the TFs NF-κβ, SP1 and STAT [56]. Notably, the SNP rs1319501 overlaps not only with these TF but also with several TF ChIP-seq data from ENCODE (Supplementary Figure 1), and it has a RegulomeDB score 2c (it is likely to affect TF binding) [57]. We further searched for functional SNPs in the *NAMPT* promoter that may affect *NAMPT* expression. Notably, the SNP rs1319501 is in high LD with the functional SNPs rs59744560 and rs61330082 in Europeans. The promoter SNP rs59744560 was shown to significantly increase

NAMPT transcription in response to cyclic stretch in endothelial cells [58]. Moreover, a variant promoter sequence containing the SNP rs61330082 resulted in an increased transcriptional activity of *NAMPT* in transfected MCF7 and HEK293T cells [59]. Taken together, these findings suggest that the SNP rs1319501 is tightly linked with functional SNPs that affect *NAMPT* expression.

The present study has some limitations. We were able to measure visfatin/NAMPT levels and nitrite concentrations only in a small number of subjects, mainly due to plasma availability and technical reasons. Despite this, we found significant correlations between visfatin/NAMPT and nitrite or sFlt-1 concentrations, and significant effects of *NAMPT* genotypes on nitrite concentrations in nonresponsive PE patients. Notably, our findings must be replicated in further studies. While there is no currently established definition of how to assess antihypertensive drug responsiveness during pregnancy, we considered the patients with PE who manifested more severe clinical symptoms as nonresponsive to the antihypertensive therapy, even when treated with a standardized antihypertensive therapy regimen.

Conclusion

We found that visfatin/NAMPT levels are inversely related to nitrite concentrations and positively related to sFlt-1 levels in patients with PE nonresponsive to antihypertensive therapy. Moreover, the *NAMPT* SNP rs1319501 affects NO formation in these nonresponsive PE patients, and it is in high LD with other functional SNPs located in the *NAMPT* promoter in Europeans. Our novel findings suggest that *NAMPT* SNP rs1319501 affects NO formation, and that visfatin/NAMPT inhibits NO formation and upregulates sFlt-1 levels in patients with PE nonresponsive to antihypertensive therapy.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pgs-2021-0006

Author contributions

All authors have made substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work. All authors have drafted the work or revised it critically for important intellectual content, and all authors have also approved the final version of the work to be published. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Ethical conduct of research

The Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (RPMS-USP) have approved the use of human subjects. In addition, informed consent has been obtained from the participants involved in this study.

Summary points

Background

- In preeclampsia (PE), the release of the antiangiogenic factor sFlt-1 into the maternal circulation may lead to systemic maternal endothelial dysfunction.
- In PE, there is clinical evidence for diminished nitric oxide (NO) formation and sFlt-1 was inversely related to NO formation.
- The role of visfatin/NAMPT is not fully known in pregnancy complications, including PE.
- Visfatin/NAMPT was shown to impair endothelium-dependent relaxation by stimulation of NADPH oxidase and to produce *in vivo* endothelial dysfunction in mice, which support its role as a mediator of vascular damage.
- Plasma visfatin/NAMPT levels were positively related to nitrite concentrations (a marker of endogenous NO formation) and inversely related to sFlt-1 levels in healthy pregnancy, but inversely related to nitrite concentrations and positively related to sFlt-1 levels in PE.

Aim

- We examined the relationships between visfatin/NAMPT levels and nitrite concentrations or sFlt-1 levels in 205 patients with PE classified as responsive or nonresponsive to antihypertensive therapy.
- Moreover, we examined whether the *NAMPT* SNPs rs1319501 and rs3801266 affect nitrite concentrations in PE and in 206 healthy pregnant, and their linkage disequilibrium (LD) with other functional SNPs in the *NAMPT* promoter.

Patients & methods

- Responsiveness to antihypertensive therapy in pregnant with PE was based on the evaluation of clinical and laboratory parameters in response to the use of these drugs: methyldopa (1000–1500 mg per day) was the first antihypertensive drug of choice, followed by nifedipine (40–60 mg per day) in cases of lack of significant response to methyldopa. Hydralazine (5–30 mg) was used only in cases of hypertensive crisis.
- Circulating visfatin/NAMPT and sFlt-1 levels were previously measured by ELISA, and nitrite concentrations using an ozone-based chemiluminescence assay.
- The UCSC Genome Browser was used to identify functional SNPs located in the *NAMPT* promoter, and their overlap with functional genomics data from the ENCODE Project, including DNase I hypersensitivity clusters, transcription factor ChIP-seq clusters and ChIP-seq data for three histone marks (H3K27ac, H3K4me1 and H3K4me3).
- Pairwise LD was calculated among these functional SNPs located in the *NAMPT* promoter and the *NAMPT* SNPs rs1319501 and rs3801266 studied here.

Results

- Pregnant women with PE who were nonresponsive to antihypertensive therapy showed higher systolic blood pressure and diastolic blood pressure, fasting glucose, creatinine, proteinuria, and sFlt-1 concentrations than responsive PE patients (all $p < 0.05$).
- Noteworthy, the phenotypes of early-onset PE, preterm birth and intrauterine growth restriction were more frequent in pregnant with PE nonresponsive to antihypertensive therapy (all $p < 0.05$).
- Plasma visfatin/NAMPT levels were inversely related to nitrite concentrations ($r = -0.376$; 95% CI: -0.643 to -0.027; $p = 0.031$) and positively related to sFlt-1 levels ($r = 0.621$; 95% CI: 0.164–0.858; $p = 0.010$) in pregnant with PE nonresponsive to antihypertensive therapy.
- The TT genotype for the *NAMPT* SNP rs1319501 (T>C) was associated with higher nitrite concentrations in pregnant with PE nonresponsive to antihypertensive therapy ($p < 0.05$, compared with the TC + CC genotypes).
- The *NAMPT* SNP rs1319501 is in high LD with the functional SNPs rs59744560 and rs61330082 in the European population from the 1000 Genomes Phase III Project.

Discussion

- This is the first study to examine the relationships between plasma nitrite concentrations or sFlt-1 and visfatin/NAMPT levels in pregnant with PE classified as responsive or nonresponsive to antihypertensive therapy, and the effect of *NAMPT* SNPs on nitrite concentrations in health pregnant and in PE patients.
- Our findings suggest that visfatin/NAMPT inhibits NO formation and upregulates sFlt-1 due to the increased oxidative stress in nonresponsive PE patients, a subgroup who exhibit the worst clinical outcomes. Moreover, the *NAMPT* SNP rs1319501 affects visfatin/NAMPT levels and thereby may alter NO bioavailability and oxidative stress in nonresponsive PE patients. However, further studies are needed to explore these hypotheses.

Conclusion

- Visfatin/NAMPT levels were inversely related to nitrite concentrations and positively related to sFlt-1 levels in patients with PE nonresponsive to antihypertensive therapy.
- The SNP rs1319501 of *NAMPT* gene affects NO formation in these nonresponsive PE patients, and it is in high LD with other functional SNPs located in the *NAMPT* promoter in Europeans.
- Our novel findings suggest that *NAMPT* SNP rs1319501 affect NO formation, and that visfatin/NAMPT inhibit NO formation and upregulate sFlt-1 levels in patients with PE nonresponsive to antihypertensive therapy.

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