The Pharmacogenomics Journal (2016), 1–8

www.nature.com/tpj

ORIGINAL ARTICLE

Gene–gene interactions in the *NAMPT* pathway, plasma visfatin/NAMPT levels, and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy

MR Luizon[1], ACT Palei[2], VA Belo[1], LM Amaral[1], R Lacchini[3], G Duarte[4], RC Cavalli[4], VC Sandrim[5] and JE Tanus-Santos[1]

Nicotinamide phosphorybosil transferase (*NAMPT*) polymorphisms affect visfatin/NAMPT levels and may affect the responsiveness to therapy in hypertensive disorders of pregnancy. We examined whether *NAMPT* polymorphisms (rs1319501 T>C and rs3801266 A>G), or haplotypes, and gene–gene interactions in the *NAMPT* pathway affect plasma visfatin/NAMPT levels and the response to antihypertensive therapy in 205 patients with preeclampsia (PE) and 174 patients with gestational hypertension. We also studied 207 healthy pregnant controls. Plasma visfatin/NAMPT levels were measured by ELISA. Non-responsive PE patients with the TC+CC genotypes for the rs1319501 T>C, and with the AG+GG genotypes for the rs3801266 A>G polymorphism had lower and higher visfatin/NAMPT levels, respectively. The 'C, A' haplotype was associated with response to antihypertensive therapy, and with lower visfatin/NAMPT levels in PE. Interactions among *NAMPT, TIMP-1* and *MMP*-2 genotypes were associated with PE and with lack of response to antihypertensive therapy in PE. Our results suggest that *NAMPT* polymorphisms affect plasma visfatin/NAMPT levels in nonresponsive PE patients, and that gene–gene interactions in the *NAMPT* pathway not only promote PE but also decrease the response to antihypertensive therapy in PE.

The Pharmacogenomics Journal advance online publication, 10 May 2016; doi:10.1038/tpj.2016.35

INTRODUCTION

Preeclampsia (PE) and other hypertensive disorders of pregnancy (HDP) affect up to 10% of pregnancies and are associated with increased maternal and perinatal mortality and morbidity.[1] Although the mechanisms of the pathogenesis of PE remain unclear,[2,3] it is thought that placental ischemia/hypoxia stimulates the release of soluble factors by the placenta into the maternal circulation leading to widespread maternal endothelial dysfunction.[4,5] Indeed, both hypertension and proteinuria implicate the endothelium as the target of PE.[4] Drugs that improve the endothelial function and directly target the systemic vascular dysfunction were proposed as potential therapies for PE.[6,7]

Visfatin, a recently described adipocytokine also known as nicotinamide phosphorybosil transferase (NAMPT), is a potential biomarker for vascular endothelial dysfunction.[8,9] Obesity increases the risk of PE,[10,11] and the dysregulation of adipocytokines may be associated with endothelial dysfunction in PE.[12] Importantly, visfatin/NAMPT has a potential role in PE,[9,13] and we have found that *NAMPT* genotypes and haplotypes affect plasma visfatin/NAMPT levels in gestational hypertension (GH) and the susceptibility to PE.[14] In that study, we focused on two *NAMPT* polymorphisms, rs1319501 T>C and rs3801266 A>G, which have been associated with obesity and cardiovascular diseases.[15,16] However, no previous study has examined whether *NAMPT* polymorphisms and haplotypes affect the responsiveness to antihypertensive therapy in HDP.

Visfatin/NAMPT has an impact on extracellular matrix remodeling,[17] as it enhances the levels and activity of matrix metalloproteinase (MMP)-2 and MMP-9, while decreasing the levels of the tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in endothelial cells.[18] MMPs degrade the components of the extracellular matrix,[19] and imbalanced MMP activities may contribute to the vascular dysfunction found in PE.[20,21] Interestingly, we have found that *MMP-9* haplotypes, formed by the combination of the g.–1562C>T and g.–90$(CA)_{13–25}$ polymorphisms, and the *TIMP-1* g.9830T>G polymorphism were associated with lack of responsiveness to antihypertensive therapy in PE.[22,23] Conversely, the *MMP-2* g.–1306C>T and g.–735C>T polymorphisms did not affect the responses to antihypertensive therapy in HDP.[24] However, gene–gene interactions must also be taken into account in pharmacogenomics.[25–27] It is possible that combinations of *NAMPT*, *MMP-2*, *MMP-9* and *TIMP-1* genotypes may be associated with the responsiveness to antihypertensive therapy in patients with GH or PE.

In the present study, we compared the distributions of *NAMPT* polymorphisms and haplotypes in GH and in PE patients who respond to antihypertensive therapy with those found in GH and in PE patients who do not respond to antihypertensive therapy. We also examined whether *NAMPT* polymorphisms and haplotypes affect plasma visfatin/NAMPT levels in these groups of GH and PE patients. In addition, we examined whether interactions among the *NAMPT* polymorphisms and the above mentioned *MMP-2*, *MMP-9* and *TIMP-1* polymorphisms were associated with

[1]Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil; [2]Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, MS, USA; [3]Department of Psychiatric Nursing and Human Sciences, Ribeirao Preto College of Nursing, University of São Paulo, Sao Paulo, Brazil; [4]Department of Gynecology and Obstetrics, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil and [5]Department of Pharmacology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, Sao Paulo, Brazil. Correspondence: Professor JE Tanus-Santos, Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Av. Bandeirantes, 3900, Sao Paulo 14049-900, Brazil.
E-mail: tanus@fmrp.usp.br
Received 5 November 2015; revised 8 February 2016; accepted 28 March 2016

the susceptibility to GH or PE and with the responsiveness to antihypertensive therapy in GH and in PE patients.

MATERIALS AND METHODS

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (RPMS-USP). All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital at the RPMS-USP. We studied 582 pregnant women (203 healthy women with uncomplicated pregnancies, 174 women with GH and 205 women with PE). Hypertensive disorders were defined in accordance to the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy).[28] GH was defined as pregnancy-induced hypertension (⩾140 mm Hg systolic or ⩾90 mm Hg diastolic at 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. PE was defined as GH plus significant proteinuria (⩾0.3 g per 24 h). No women with pre-existing hypertension, with or without superimposed PE, were included in the present study.

In the clinical attendance, written informed consent and maternal venous blood samples were collected. Genomic DNA was extracted from the cellular fraction of 1 ml of whole blood by a salting-out method and stored at −20 °C until analyzed. Plasma was obtained from whole blood in EDTA by centrifugation at 2000 *g* for 10 min and stored at −70 °C until assayed.

Antihypertensive treatment and drug response evaluation

The patients in this study were carefully monitored for signs and symptoms of PE, with fetal surveillance and laboratory tests at least once a week. Responsiveness to therapy was based on the evaluation of clinical and laboratory parameters (see below) in response to the administration of antihypertensive drugs. The initial antihypertensive drug of choice was methyldopa (1000–1500 mg per day), followed by nifedipine (40–60 mg per day), which was added in case of lack of significant responses to methyldopa. Hydralazine (5–30 mg) was used only in cases of hypertensive crisis. One of the following clinical and laboratory outcomes were considered to classify a patient as nonresponsive to the antihypertensive therapy:[22,23,28,29]

1. Clinical symptoms including blurred vision, persistent headache or scotomata, persistent right-upper quadrant or epigastric pain.
2. Systolic blood pressure above 140 mm Hg and diastolic blood pressure above 90 mm Hg, as assessed by the blood pressure curve.
3. Hemolysis, elevated liver enzymes and a low platelet count (HELLP) syndrome; or proteinuria >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}.
4. Fetal hypoactivity or nonreactive fetus, as revealed by cardiotocography; intrauterine growth restriction, oligoamnio, abnormal biophysical profile score and Doppler velocimetry abnormalities, as evaluated by ultrasound.

Genotyping

Genotypes for the g.–423T>C (rs1319501) and rs3801266 A>G polymorphisms in the promoter and intron 1 of the *NAMPT* gene, respectively, were determined by Taqman Allele Discrimination assays (C_7590641_30 and C_340124_10, respectively) using real-time PCR. Probes and primers were designed by Applied Biosystems (Foster City, CA, USA). PCR reactions were performed in a total volume of 10 µl (5 ng of template DNA, 1×TaqMan Genotyping Master Mix (Life Technologies Corporation, Grand Island, NY, USA) and 1×Taqman Allele Discrimination Assay). Thermal cycling was performed in standard conditions and fluorescence was recorded by the StepOne Plus Real-Time PCR equipment (Applied Biosystems). The results were analyzed with manufacturer's software.

Genotypes for the *TIMP-1* g.9830T>G (rs2070584) polymorphism, and for the *MMP-2* g.–1306C>T (rs243865) and g.–735C>T (rs2285053) polymorphisms were determined by Taqman Allele Discrimination Assays (Applied Biosystems) as described elsewhere.[22,24] Genotypes for the *MMP-9* g.–1562C>T (rs3918242) and g.–90$(CA)_{13–25}$ (rs3222264) polymorphisms were determined by PCR as described in detail elsewhere.[23]

Enzyme immunoassay of plasma visfatin/NAMPT

Visfatin/NAMPT concentrations were measured in EDTA-plasma using a kit (RayBio Human Visfatin EIA–VIS–1, Norcross, GA, USA), according to manufacturer's instructions.

Statistical analysis

The clinical characteristics of patients with GH or PE were compared with those found in healthy pregnant (HP) women by Student's unpaired *t*-test, Mann–Whitney *U*-test, or χ^2 as appropriate. The effects of the different genotypes for the *NAMPT* polymorphisms or different haplotypes on plasma visfatin/NAMPT levels in GH and in PE patients classified according to the responsiveness to antihypertensive therapy were compared by Student's unpaired *t*-test. All the distributions of genotypes were assessed for deviation from the Hardy–Weinberg equilibrium. The differences in genotype, allele and haplotype frequencies among groups were assessed using χ^2-test, with the corresponding *P*-values. A value of $P < 0.05$ was considered statistically significant.

Haplotype frequencies were estimated by using Haplo.stats package version 1.4.4 (http://cran.r-project.org/web/packages/haplo.stats/index.html),[30] which computes maximum likelihood estimates of haplotypes. The function *haplo.score* computes haplotype-specific score analysis to test for association, with the value of $P < 0.05$ considered statistically significant. Non-additive effects may be selected to score haplotypes. In the recessive model, haplotype effects are estimated only from subjects who are homozygous for a haplotype. Only the haplotypes with frequencies >1% were taken into consideration in the haplotype analysis, and the odds ratio and 95% confidence intervals were calculated for each haplotype. The possible haplotypes formed by the alleles of the two *NAMPT* polymorphisms rs1319501 T>C and rs3801266 A>G were: 'T, A', 'T, G', 'C, A' and 'C, G'. However, the 'C, G' haplotype was excluded from the analysis of the effects on plasma visfatin/NAMPT levels because of its low frequency.

Multifactor dimensionality reduction (MDR) identifies interactions of genotypes for their ability to classify them into high- and low-risk cells through cross-validation steps and permutation testing, and this method is also applicable to pharmacogenomic studies where the outcome variable is drug treatment response or non-response.[31] We used the robust MDR approach to characterize the interactions, which uses $\alpha = 0.1$ as the threshold for the Fisher's Exact Test rather than a threshold $T = 1$, and has the advantage that only statistically significant genotype combinations are considered in the MDR analysis.[32] The best interaction model was the model that had the maximum testing score and cross-validation consistency. Permutation testing was performed to determine the statistical significance of the best model.[22,31]

RESULTS

Table 1 summarizes the characteristics of the subjects enrolled in this study. HP, GH and PE women showed similar ethnicity (% white), % of current smokers, hemoglobin, hematocrit, and creatinine concentrations (all $P > 0.05$). As expected, PE and GH presented higher systolic and diastolic blood pressure compared with HP (both $P < 0.05$), even though most patients were receiving antihypertensive therapy. GH and PE were older than HP ($P < 0.05$). Increased body mass index and fasting glucose were found in GH and PE patients compared with HP (both $P < 0.05$). We found lower gestational age at delivery in GH and PE, and lower newborn weights in PE compared with HP (all $P < 0.05$). Significant proteinuria was found in PE. The characteristics of GH and PE patients classified according to responsiveness to methyldopa and to total antihypertensive therapy are shown in Supplementary Tables S1 and S2.

The results for *NAMPT* single-locus analyses are shown in Tables 2 and 3. The distribution of genotypes for all the polymorphisms studied showed no deviation from Hardy–Weinberg equilibrium (all $P > 0.05$, data not shown). The two *NAMPT* polymorphisms studied had no effects on the responses to methyldopa or to total antihypertensive therapy in GH or PE

Table 1. Demographic characteristics of study subjects

Parameters	*Healthy Pregnant*	*Gestational Hypertension*	P[a]	*Preeclampsia*	P[a]
	(n = *203*)	(n = *174*)		(n = *205*)	
Age (years)	24.5 ± 0.4	27.3 ± 0.5	**0.001**	26.5 ± 0.4	**0.001**
Ethnicity (% White)	69.56	69.23	0.807	70.7	0.847
Current smokers (%)	9.2	10.9	0.633	9.3	0.993
BMI (Kg m^{-2})	20.6 ± 0.6	28.8 ± 0.5	**0.000**	27.5 ± 0.5	**0.000**
SBP (mm Hg)	110.6 ± 0.8	133.1 ± 1.3	**0.000**	140.1 ± 1.4[b]	**0.000**
DBP (mm Hg)	71.7 ± 0.6	83.7 ± 0.9	**0.000**	88.1 ± 0.8[b]	**0.000**
HR (beats per min)	82.0 ± 0.6	81.6 ± 0.6	0.655	82.3 ± 0.6	0.702
Fasting glucose (mg dl^{-1})	75.2 ± 1.0	78.3 ± 1.1	**0.039**	80.9 ± 1.7	**0.008**
Hb (g dl^{-1})	11.8 ± 0.1	11.8 ± 0.0	0.901	11.9 ± 0.1	0.721
Hct (%)	35.6 ± 0.4	35.7 ± 0.2	0.936	36 ± 0.2	0.505
Creatinine (μmol l^{-1})	66.7 ± 2.8	62.5 ± 0.1	0.305	70.5 ± 1.6	0.620
24 h Pr (mg per 24 h)	ND	81.2 ± 7.9		904.6 ± 113.8[b]	**0.000**
Primiparity (%)	45.3	39.6	0.473	44.2	0.849
GAD (weeks)	39.7 ± 0.1	38.9 ± 0.1	**0.000**	35.9 ± 0.3[b]	**0.000**
Newborn weight (g)	3297 ± 39.7	3208 ± 43.1	0.129	2531 ± 68.0[b]	**0.000**
GAS (weeks)	36.5 ± 0.3	36.2 ± 0.3	0.658	34.0 ± 0.3[b]	**0.000**

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; GAD, gestational age at delivery; GAS, gestational age at sampling; ND, not determined (however, negative dipstick test); SBP, systolic blood pressure; 24 h Pr, 24 h proteinuria. Values are the mean ± s.e.m. [a]$P < 0.05$ vs healthy pregnant group. [b]$P < 0.05$ vs gestational hypertension group. Bold values are significant.

Table 2. Genotype and allele frequencies of *NAMPT* polymorphisms in GH and in PE patients grouped according to responsiveness to methyldopa

Genotype or Allele	*GH—methyldopa responsiveness*				*PE—methyldopa responsiveness*			
	R (n = *120*)	*NR* (n = *54*)	*OR (95% CI)*	P	*R* (n = *60*)	*NR* (n = *145*)	*OR (95% CI)*	P
rs1319501 T>C								
TT	70 (58%)	32 (59%)	1.00 (Reference)	—	42 (70%)	96 (66%)	1.00 (Reference)	—
TC	42 (35%)	21 (39%)	1.09 (0.55–2.13)	0.793	15 (25%)	43 (30%)	1.25 (0.62–2.50)	0.519
CC	8 (7%)	1 (2%)	0.27 (0.03–2.28)	0.202	3 (5%)	6 (4%)	0.87 (0.20–3.66)	0.854
T	182 (76%)	85 (79%)	1.00 (Reference)	—	99 (83%)	235 (81%)	1.00 (Reference)	—
C	58 (24%)	23 (21%)	0.84 (0.72–1.76)	0.557	21 (17%)	55 (19%)	1.10 (0.63–1.92)	0.728
rs3801266 A>G								
AA	59 (49%)	24 (44%)	1.00 (Reference)	—	31 (52%)	61 (42%)	1.00 (Reference)	—
AG	54 (45%)	28 (52%)	1.27 (0.65–2.46)	0.469	25 (42%)	70 (48%)	1.42 (0.76–2.67)	0.270
GG	7 (6%)	2 (4%)	0.70 (0.13–3.62)	0.672	4 (7%)	14 (10%)	1.78 (0.54–5.86)	0.339
A	172 (72%)	76 (70%)	1.00 (Reference)	—	87 (73%)	192 (66%)	1.00 (Reference)	—
G	68 (28%)	32 (30%)	1.06 (0.64–1.75)	0.804	33 (27%)	98 (34%)	1.34 (0.84–2.15)	0.213

Abbreviations: CI, confidence interval; GH, gestational hypertension; *NAMPT*, nicotinamide phosphorybosil transferase; NR, nonresponsive; OR, odds ratio; PE, preeclampsia; R, responsive. Bold values are significant.

patients (all $P > 0.05$, Tables 2 and 3). The results for *NAMPT, MMP-2, MMP-9* and *TIMP-1* single-locus analyses in the HP, GH and PE are shown in Supplementary Table S3, which are in line with our previous findings.[14,22,33,34] The TC and CC genotypes and C allele for the *NAMPT* rs1319501 T>C polymorphism were more common in the HP than in PE.[14] Conversely, the GG and TG genotypes and G allele for the *TIMP-1* g.9830T>G polymorphism were more common in PE than in the HP.[22] Regarding the *MMP-9* g.–1562C>T polymorphism, the CT genotype and T allele were associated with GH[34] (all $P < 0.05$, Supplementary Table S3). Subjects with any missing genotype data for these polymorphisms were not considered in the interaction analyses.

The distribution of *NAMPT* haplotypes are shown in Tables 4 and 5. Although we found no significant associations with response to methyldopa ($P > 0.05$, Table 4), the 'C, A' haplotype was associated with response to total antihypertensive therapy in PE ($P = 0.0123$, in the recessive model, Supplementary Table S4). The 'C, G' haplotype was found only in the nonresponsive PE patients, but the OR for this test was not significant ($P = 0.013$, Table 5).

We examined the effects of *NAMPT* genotypes and haplotypes on visfatin/NAMPT levels in the responsive and nonresponsive groups of GH and PE patients. Because of technical reasons, we were able to measure plasma visfatin/NAMPT levels only in 105 GH and 90 PE patients. We found no significant differences in visfatin/NAMPT levels between responsive and nonresponsive groups to total antihypertensive therapy in GH or PE patients ($P > 0.05$, Figure 1). However, we found significant effects of *NAMPT* genotypes on visfatin/NAMPT levels in the nonresponsive PE patients. Although the TC+CC genotypes for the rs1319501 T>C polymorphism were associated with lower levels, the AG+GG genotypes for the rs3801266 A>G polymorphism were associated with higher levels (both $P < 0.05$, Figures 2a and b). Notably, the 'C, A' haplotype was associated with lower visfatin/NAMPT levels in nonresponsive PE patients ($P < 0.05$, Figure 2c).

Table 3. Genotype and allele frequencies of *NAMPT* polymorphisms in GH and in PE patients classified as responsive and nonresponsive to total antihypertensive therapy

Genotype or allele	*GH—therapy responsiveness*				*PE—therapy responsiveness*			
	R (n = *153*)	*NR* (n = *21*)	*OR (95% CI)*	P	*R* (n = *110*)	*NR* (n = *95*)	*OR (95% CI)*	P
rs1319501 T>C								
TT	90 (59%)	13 (62%)	1.00 (Reference)	—	79 (72%)	61 (64%)	1.00 (Reference)	—
TC	54 (35%)	8 (38%)	1.02 (0.39–2.63)	0.958	24 (22%)	32 (34%)	1.72 (0.92–3.22)	0.085
CC	9 (6%)	0 (0%)	0.352 (0.02–6.42)	0.257	7 (6%)	2 (2%)	0.37 (0.07–1.84)	0.208
T	234 (76%)	34 (81%)	1.00 (Reference)	—	182 (83%)	154 (81%)	1.00 (Reference)	—
C	72 (24%)	8 (19%)	0.765 (0.33–1.72)	0.517	38 (17%)	36 (19%)	1.12 (0.67–1.85)	0.660
rs3801266 A>G								
AA	72 (47%)	10 (48%)	1.00 (Reference)	—	55 (50%)	39 (41%)	1.00 (Reference)	—
AG	74 (48%)	9 (43%)	0.87 (0.33–2.28)	0.785	47 (43%)	47 (49%)	1.41 (0.79–2.51)	0.241
GG	7 (5%)	2 (10%)	2.05 (0.37–11.3)	0.398	8 (7%)	9 (9%)	1.58 (0.56–4.47)	0.380
A	218 (71%)	29 (69%)	1.00 (Reference)	—	157 (71%)	125 (66%)	1.00 (Reference)	—
G	88 (29%)	13 (31%)	1.11 (0.55–2.23)	0.768	63 (29%)	65 (34%)	1.29 (0.85–1.97)	0.224

Abbreviations: CI, confidence interval; GH, gestational hypertension; *NAMPT*, nicotinamide phosphorybosil transferase; NR, nonresponsive; OR, odds ratio; PE, preeclampsia; R, responsive. Bold values are significant.

Table 4. *NAMPT* haplotype frequencies in GH and in PE patients classified as responsive and nonresponsive to methyldopa

Haplotype	*GH—methyldopa responsiveness*				*PE—methyldopa responsiveness*			
	R (2n = *240*)	*NR* (2n = *108*)	*OR (95% CI)*	P	*R* (2n = *120*)	*NR* (2n = *290*)	*OR (95% CI)*	P
T, A	113 (47.1%)	54 (50%)	1.00 (Reference)	—	68 (56.7%)	143 (49.3%)	1.000 (Reference)	—
T, G	69 (28.7%)	32 (29.6%)	0.97 (0.57–1.65)	0.912	31 (25.8%)	93 (32.1%)	1.43 (0.87–2.35)	0.161
C, A	58 (24.2%)	22 (20.4%)	0.79 (0.44–1.43)	0.441	19 (15.8%)	50 (17.2%)	1.25 (0.68–2.28)	0.465
C, G	0	0	NA	NA	2 (1.7%)	4 (1.4%)	0.95 (0.17–5.32)	0.954

Abbreviations: CI, confidence interval; GH, gestational hypertension; NA, not available; *NAMPT*, Nicotinamide phosphorybosil transferase; NR, nonresponsive; OR, odds ratio; PE, preeclampsia; R, responsive.

Table 5. *NAMPT* haplotype frequencies in GH and in PE patients classified as responsive and nonresponsive to total antihypertensive therapy

Haplotype	*GH—total therapy responsiveness*				*PE—total therapy responsiveness*			
	R (2n = *306*)	*NR* (2n = *42*)	*OR (95% CI)*	P	*R* (2n = *220*)	*NR* (2n = *190*)	*OR (95% CI)*	P
T, A	146 (47.7%)	21 (50%)	1.00 (Reference)	—	119 (54.1%)	94 (49.5%)	1.00 (Reference)	—
T, G	88 (28.8%)	13 (31%)	1.02 (0.49–2.15)	0.943	63 (28.6%)	60 (31.6%)	1.20 (0.77–1.88)	0.410
C, A	72 (23.5%)	8 (19%)	0.77 (0.32–1.83)	0.556	38 (17.3%)	31 (16.3%)	1.03 (0.59–1.78)	0.908
C, G	0	0	NA	NA	0	5 (2.6%)	13.9 (0.76–251.3)	**0.013**[a]

Abbreviations: CI, confidence interval; GH, gestational hypertension; NA, not available; *NAMPT*, nicotinamide phosphorybosil transferase; NR, nonresponsive; OR, odds ratio; PE, preeclampsia; R, responsive. [a]$P < 0.05$ vs responsive PE. Bold value is significant.

Next, we examined whether interactions among *NAMPT*, *MMP-2*, *MMP-9* and *TIMP-1* polymorphisms were associated with responsiveness to antihypertensive therapy in HDP. Although we found no significant interactions associated with responsiveness to methyldopa ($P > 0.05$, Supplementary Table S5), we found a significant model of interaction among *NAMPT*, *TIMP-1* and *MMP-2* genotypes associated with responsiveness to total antihypertensive therapy in PE ($P < 0.05$, Table 6). The combinations of genotypes are shown in Figure 3. The combinations of the GG genotype for the *TIMP-1* g.9830T>G polymorphism with the AA and AG genotypes for the *NAMPT* rs3801266 A>G polymorphism were more frequent in the responsive and in the nonresponsive PE patients, respectively. These combinations are significant when combined with the CC genotype for the *MMP-2* g.–735C>T polymorphism (all $P < 0.05$, Figure 3).

Finally, we examined whether interactions among *NAMPT*, *MMP-2*, *MMP-9* and *TIMP-1* polymorphisms were associated with GH or PE. Although we found no significant interactions associated with GH, we found significant interactions among *NAMPT*, *TIMP-1* and *MMP-2* genotypes associated with PE ($P < 0.05$, Table 7). The combinations of genotypes are shown in Supplementary Figure S1. The combination of the GG genotype

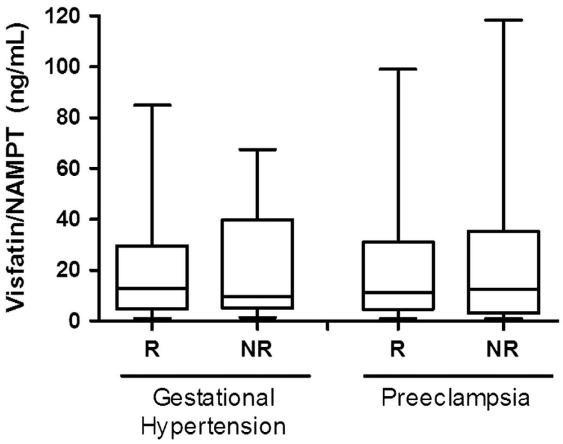

Figure 1. Plasma visfatin/NAMPT levels in gestational hypertension and preeclampsia patients classified as R and NR to total antihypertensive therapy. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. R, responsive; NAMPT, nicotinamide phosphorybosil transferase; NR, nonresponsive.

for the *TIMP-1* g.9830T > G polymorphism with the AG genotype for the *NAMPT* rs3801266 A > G polymorphism was more frequent in PE than in HP. This combination is significant when combined with the CC genotype for the *MMP-2* g.–1306C > T polymorphism ($P < 0.05$, Supplementary Figure S1).

DISCUSSION

This study was the first to evaluate the effect of *NAMPT* polymorphisms and haplotypes on plasma visfatin/NAMPT levels, and whether gene–gene interactions in the *NAMPT* pathway are associated with HDP, both in the context of responsiveness to antihypertensive therapy. Our main findings are (1) *NAMPT* genotypes affect plasma visfatin/NAMPT levels in nonresponsive PE patients; (2) the 'C, A' haplotype is associated with response to total antihypertensive therapy in PE and with lower visfatin/NAMPT levels in the nonresponsive PE patients; and (3) significant interactions among *NAMPT*, *TIMP-1* and *MMP-2* genotypes are associated with PE and with lack of response to total antihypertensive therapy in PE patients.

Some studies provide evidence for increased plasma[35,36] or serum[37,38] visfatin levels in patients with PE compared with normotensive pregnant women. However, we have previously found no significant differences in plasma visfatin/NAMPT levels between HP and PE,[14] which is in line with other findings.[39] Discordant findings for the circulating visfatin/NAMPT levels in PE may be attributed to differences between the studied populations, gestational age at sampling, and in sensitivity and specificity of different visfatin immunoassays,[40] as reviewed elsewhere.[9,14]

Here we found that *NAMPT* genotypes affect visfatin/NAMPT levels in PE patients classified as nonresponsive to total antihypertensive therapy. The TC+CC genotypes for the rs1319501 T > C polymorphism were associated with lower visfatin/NAMPT levels. Although we have earlier found no effects of this polymorphism on visfatin/NAMPT levels in HDP,[14] it is likely to affect transcription factor binding according to its score 2c at RegulomeDB.[41] Indeed, we had shown that its location in the *NAMPT* promoter region overlaps with a great number of transcription factor ChIP-seq data from The Encyclopedia of DNA Elements (ENCODE),[14] which suggests that rs1319501 may affect *NAMPT* expression. Conversely, the AG+GG genotypes for the rs3801266 A > G polymorphism were associated with higher

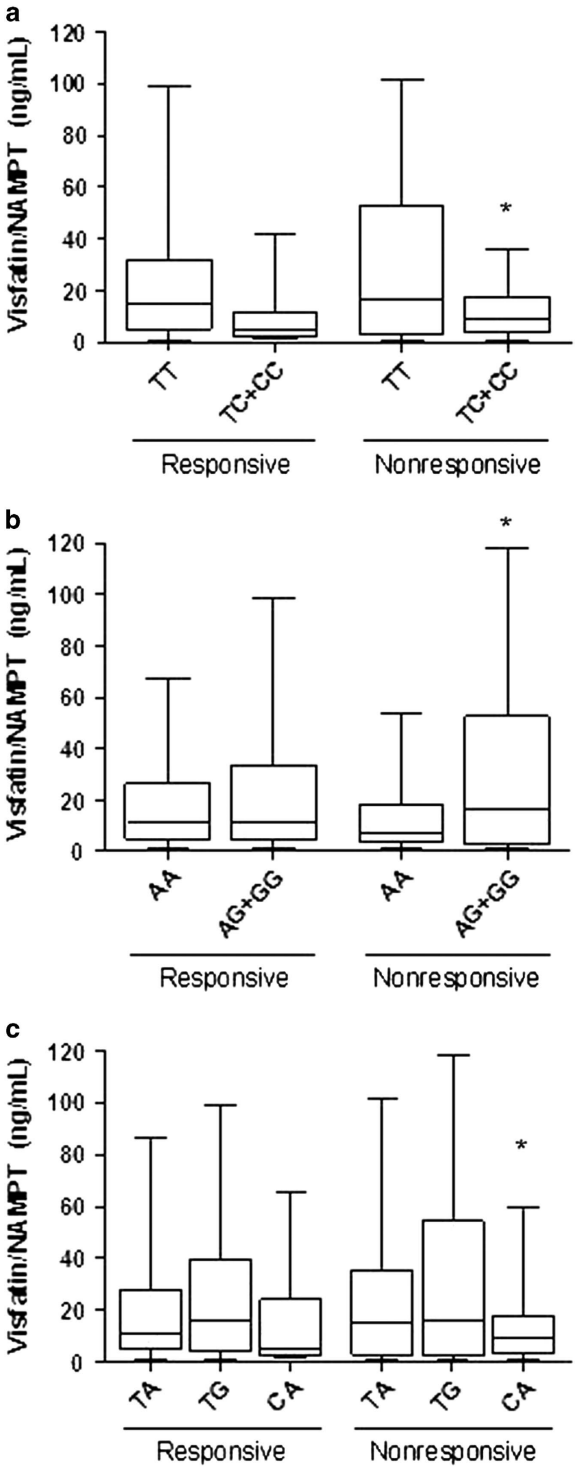

Figure 2. Plasma visfatin/NAMPT levels in preeclampsia patients classified as responsive and nonresponsive to total antihypertensive therapy and grouped by their genotypes for the *NAMPT* (**a**) rs1319501 T > C and (**b**) rs3801266 A > G polymorphisms, and (**c**) the haplotypes formed by them. The 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 the lowest values. *$P < 0.05$ versus the nonresponsive group with the TT or AA genotypes or versus subjects with TA or TG haplotypes. NAMPT, nicotinamide phosphorybosil transferase.

Table 6. Robust MDR interaction models among the *NAMPT, MMP-2, MMP-9* and *TIMP-1* polymorphisms in GH and in PE patients classified as nonresponsive and responsive to total antihypertensive therapy

Interaction Models	*Training score*	*Testing score*	*CVC*	P
GH—nonresponsive (n = 21) compared with responsive (n = 153)				
TIMP-1 g.9830T > G	0.5478	0.4850	5/10	0.8635
TIMP-1 g.9830T > G; *MMP-9 g.*–90$(CA)_{13–25}$	0.6321	0.4438	4/10	0.9735
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-9 g.*–90$(CA)_{13–25}$	0.6740	0.5706	7/10	0.4810
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-9* g.–90$(CA)_{13–25}$; *MMP-2* g.–1306C > T	0.6809	0.5380	7/10	0.6495
PE—nonresponsive (n = 95) compared with Responsive (n = 110)				
TIMP-1 g.9830T > G	0.5225	0.4597	6/10	0.9700
NAMPT rs3801266 A > G; *MMP-2* g.–735T	0.6018	0.4969	6/10	0.8075
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-2* g.–735C > T	0.6693	0.6157	10/10	**0.0425**[a]
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-2* g.–1306C > T; *MMP-2* g.–735C > T	0.7051	0.6119	9/10	0.0525

Abbreviations: CVC, cross-validation consistency; GH, gestational hypertension; MDR, multifactor dimensionality reduction; *MMP-9*, matrix metalloproteinase-9; *MMP-2*, matrix metalloproteinase-2; *NAMPT,* nicotinamide phosphorybosil transferase; PE, preeclampsia; *TIMP-1*, tissue inhibitor of matrix metalloproteinase-1.
[a]*P*-value after 1000 permutations. Bold value is significant.

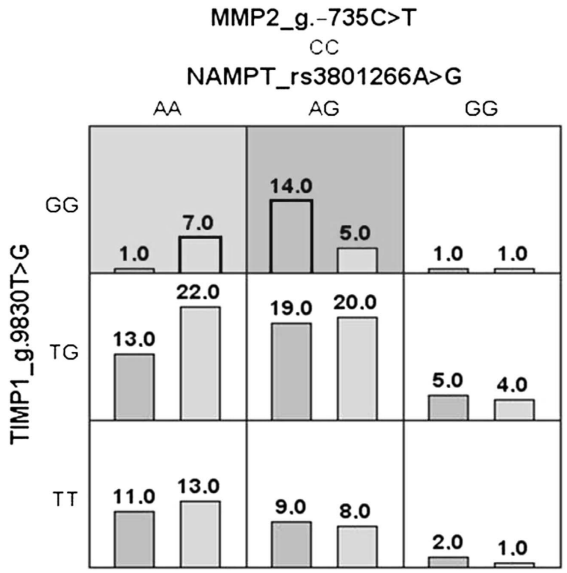

Figure 3. The best robust MDR model of interaction among *NAMPT* rs3801266 A > G, *TIMP-1* g.9830T > G and *MMP-2* g.–735C > T polymorphisms in the comparison of preeclampsia patients classified as nonresponsive and responsive to total antihypertensive therapy. The distributions of nonresponsive (left bars) and responsive are illustrated for each genotype combinations. The light gray cells are labeled as responsive, and dark gray cells are labeled as nonresponsive, and the white cells are labeled as unknown. MDR, multifactor dimensionality reduction; NAMPT, nicotinamide phosphorybosil transferase.

visfatin/NAMPT levels in nonresponsive PE patients. This result is in line with our previous findings that the AG and GG genotypes are associated with higher visfatin/NAMPT levels in GH.[14] Furthermore, we have found increased visfatin/NAMPT levels in obese children and adolescents carrying the GG genotype.[15] These findings indicate that *NAMPT* polymorphisms have important effects on visfatin/NAMPT levels, but the effects may vary under different physiological and/or disease conditions.

To our knowledge, no previous study has examined whether *NAMPT* polymorphisms or haplotypes affect the responsiveness to antihypertensive therapy in HDP. We showed here that the 'C, A' haplotype is associated with response to total antihypertensive therapy in PE. Moreover, this haplotype was associated with lower plasma visfatin/NAMPT levels in nonresponsive PE patients. It has been reported that calcium channel blockers (including nifedipine) may improve endothelial function and restore nitric oxide (NO) bioavailability.[42,43] Moreover, hydralazine increased cyclic guanosine 3′5′ monophosphate concentrations in preeclamptic pregnant women, and its effects may be related to NO production.[44] Thus, it is possible that these antihypertensive drugs used to treat PE produce their effects by enhancing NO bioavailability, thus counteracting the impaired NO formation that has been reported in PE.[29,45] Visfatin was shown to stimulate endothelial NO synthase (eNOS) expression and function in endothelial cells,[46] and to have a vasodilating effect on isolated blood vessels, which is mediated via endothelium-derived NO.[47] However, visfatin was shown to impair endothelium-dependent relaxation by mechanisms involving NADPH oxidase stimulation, and therefore arises as a potential new player in the development of endothelial dysfunction.[48] However, we have not studied the relationship between visfatin/NAMPT levels and NO bioavailability in PE patients, and this hypothesis remains to be proved.

Antihypertensive drugs are used in the clinic to improve maternal and fetal outcomes, despite they do not inhibit the pathophysiological mechanisms of PE.[49] In this context, antihypertensive drugs including Ca^{2+} channel blockers can affect circulating MMP and TIMP levels[50–52] in hypertensive patients. Moreover, visfatin/NAMPT enhances the levels and activity of MMP-2 and MMP-9, and decreases the levels of TIMP-1 and TIMP-2 in endothelial cells.[18] Although we found that the two studied *NAMPT* polymorphisms apparently had no major effects on the responsiveness to the antihypertensive therapy in GH or PE, other functional polymorphisms in the *NAMPT* gene or in the *NAMPT* pathway may be relevant to the pharmacogenetics of antihypertensive therapy in HDP.

Interestingly, we found significant interactions among *NAMPT, TIMP-1* and *MMP-2* genotypes associated with PE and with the lack of response to total antihypertensive therapy in PE patients. Although the *TIMP-1* g.9830T > G polymorphism was previously associated with lack of responsiveness to antihypertensive therapy in PE,[22] *MMP-2* [ref. 24] and *NAMPT* polymorphisms had not effects in the responses to antihypertensive therapy in HDP. These findings are obscured when single *MMP-2* and *NAMPT* genotypes alone are considered, and highlight the importance of gene–gene interactions in pharmacogenomics[25–27] and to the genetic component of PE.[53,54] Notably, the AG genotype for the *NAMPT* rs3801266 A > G polymorphism, which was associated with higher visfatin/NAMPT levels, was present in the combinations associated with PE and with the lack of response to antihypertensive therapy in PE. These findings suggest that specific combinations of *NAMPT,*

Table 7. Robust MDR interaction models among the *NAMPT, MMP-2, MMP-9* and *TIMP-1* polymorphisms when GH or PE patients were compared with HP subjects

Interaction models	*Training score*	*Testing score*	*CVC*	P
GH (n = 174) compared with HP (n = 203)				
MMP-9 g.–1562C > T	0.5531	0.4835	5/10	0.9195
NAMPT rs3801266 A > G*; MMP-9* g.–1562C > T	0.5931	0.5104	7/10	0.7210
NAMPT rs3801266 A > G*; MMP-9* g.–1562C > T; *MMP-2* g.–1306C > T	0.6052	0.5068	5/10	0.7340
NAMPT rs3801266 A > G*; TIMP-1* g.9830T > G*; MMP-9* g.–1562C > T; *MMP-2* g.–1306C > T	0.6354	0.5376	8/10	0.4315
PE (n = 205) compared with HP (n = 203)				
TIMP-1 g.9830T > G	0.5556	0.5412	9/10	0.3940
NAMPT rs3801266 A > G; *MMP-9* g.–90(CA)$_{13–25}$	0.5830	0.4755	7/10	0.9605
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-2* g.–1306C > T	0.6193	0.5837	9/10	**0.0450**[a]
NAMPT rs3801266 A > G; *TIMP-1* g.9830T > G; *MMP-9* g.–90(CA)$_{13–25}$; *MMP-2* g.–1306C > T	0.6748	0.5566	10/10	0.2510

Abbreviations: CVC, cross-validation consistency; GH, gestational hypertension; HP, healthy pregnant; *MMP-9*, matrix metalloproteinase-9; *MMP-2*, matrix metalloproteinase-2; MDR, multifactor dimensionality reduction; *NAMPT,* nicotinamide phosphorybosil transferase; PE, preeclampsia; *TIMP-1*, tissue inhibitor of matrix metalloproteinase-1. [a]*P*-value after 1000 permutations.

TIMP-1 and *MMP*-2 genotypes not only promote PE but also decrease the responses to antihypertensive therapy in PE.

It should be noted that the criteria we used to assess the responses to antihypertensive therapy may have influenced the results and conclusions present here. Currently, it is not clear how to precisely define the severity of HDP, and it is possible that our responsiveness to therapy definition denotes disease severity instead. Additional studies are required to improve our understanding of these syndromes.

In conclusion, we found evidence indicating that the *NAMPT* rs1319501 T > C and rs3801266 A > G polymorphisms, and the 'C, A' haplotype affect plasma visfatin/NAMPT levels in PE patients classified as nonresponsive to total antihypertensive therapy. Moreover, we found significant interactions among *NAMPT, TIMP-1* and *MMP-2* genotypes associated with PE and with the lack of response to total antihypertensive therapy in PE patients. Taken together, our findings suggest that *NAMPT* polymorphisms affect plasma visfatin/NAMPT levels in nonresponsive PE patients, and they support the use of genetic biomarkers to identify and benefit a subgroup of pregnant women as a result of more individualized treatment. In addition, they may help to understand the gene–gene interactions in the *NAMPT* pathway which affect the susceptibility and the responsiveness to antihypertensive therapy in PE.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This study was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil), and the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP-Brazil). MRL is a Young Talent Attraction fellow from CNPq-Brazil.

REFERENCES

1 Hutcheon JA, Lisonkova S, Joseph KS. Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. *Best Pract Res Clin Obstet Gynaecol* 2011; **25**: 391–403.
2 Palei AC, Spradley FT, Warrington JP, George EM, Granger JP. Pathophysiology of hypertension in pre-eclampsia: a lesson in integrative physiology. *Acta Physiol (Oxf)* 2013; **208**: 224–233.
3 Warrington JP, George EM, Palei AC, Spradley FT, Granger JP. Recent advances in the understanding of the pathophysiology of preeclampsia. *Hypertension* 2013; **62**: 666–673.
4 Powe CE, Levine RJ, Karumanchi SA. Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease. *Circulation* 2011; **123**: 2856–2869.
5 Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 1989; **161**: 1200–1204.
6 George EM, Granger JP. Mechanisms and potential therapies for preeclampsia. *Curr Hypertens Rep* 2011; **13**: 269–275.
7 Sasser JM, Murphy SR, Granger JP. Emerging drugs for preeclampsia - the endothelium as a target. *Expert Opin Emerg Drugs* 2015; **20**: 527–530.
8 Peiro C, Romacho T, Carraro R, Sanchez-Ferrer CF. Visfatin/PBEF/Nampt: a new cardiovascular target? *Front Pharmacol* 2010; **1**: 135.
9 Romacho T, Sanchez-Ferrer CF, Peiro C. Visfatin/Nampt: an adipokine with cardiovascular impact. *Mediators Inflamm* 2013; **2013**: 946427.
10 Roberts JM, Bodnar LM, Patrick TE, Powers RW. The role of obesity in preeclampsia. *Pregnancy Hypertens* 2011; **1**: 6–16.
11 Spradley FT, Palei AC, Granger JP. Increased risk for the development of preeclampsia in obese pregnancies: weighing in on the mechanisms. *Am J Physiol Regul Integr Comp Physiol* 2015; **309**: R1326–R1343.
12 Mori T, Shinohara K, Wakatsuki A, Watanabe K, Fujimaki A. Adipocytokines and endothelial function in preeclamptic women. *Hypertens Res* 2010; **33**: 250–254.
13 Pavlova T, Novak J, Bienertova-Vasku J. The role of visfatin (PBEF/Nampt) in pregnancy complications. *J Reprod Immunol* 2015; **112**: 102–110.
14 Luizon MR, Belo VA, Palei AC, Amaral LM, Lacchini R, Sandrim VC *et al.* Effects of NAMPT polymorphisms and haplotypes on circulating visfatin/NAMPT levels in hypertensive disorders of pregnancy. *Hypertens Res* 2015; **38**: 361–366.
15 Belo VA, Luizon MR, Lacchini R, Miranda JA, Lanna CM, Souza-Costa DC *et al.* The effects of NAMPT haplotypes and metabolic risk factors on circulating visfatin/NAMPT levels in childhood obesity. *Int J Obes (Lond)* 2015; **39**: 130–135.
16 Leander K, Gigante B, Silveira A, Vikstrom M, Hamsten A, Hogberg J. NAMPT (visfatin) and AKT1 genetic variants associate with myocardial infarction. *Clin Chim Acta* 2012; **413**: 727–732.
17 Dahl TB, Holm S, Aukrust P, Halvorsen B. Visfatin/NAMPT: a multifaceted molecule with diverse roles in physiology and pathophysiology. *Annu Rev Nutr* 2012; **32**: 229–243.
18 Adya R, Tan BK, Punn A, Chen J, Randeva HS. Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis. *Cardiovasc Res* 2008; **78**: 356–365.
19 Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003; **92**: 827–839.
20 Karthikeyan VJ, Lane DA, Beevers DG, Lip GY, Blann AD. Matrix metalloproteinases and their tissue inhibitors in hypertension-related pregnancy complications. *J Hum Hypertens* 2013; **27**: 72–78.
21 Palei AC, Granger JP, Tanus-Santos JE. Matrix metalloproteinases as drug targets in preeclampsia. *Curr Drug Targets* 2013; **14**: 325–334.
22 Luizon MR, Palei AC, Sandrim VC, Amaral LM, Machado JS, Lacchini R *et al.* Tissue inhibitor of matrix metalloproteinase-1 polymorphism, plasma TIMP-1 levels, and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy. *Pharmacogenomics J* 2014; **14**: 535–541.
23 Palei AC, Sandrim VC, Amaral LM, Machado JS, Cavalli RC, Lacchini R *et al.* Matrix metalloproteinase-9 polymorphisms affect plasma MMP-9 levels and

antihypertensive therapy responsiveness in hypertensive disorders of pregnancy. *Pharmacogenomics J* 2012; **12**: 489–498.
24 Palei AC, Sandrim VC, Amaral LM, Machado JS, Cavalli RC, Lacchini R *et al.* Effects of matrix metalloproteinase (MMP)-2 polymorphisms on responsiveness to antihypertensive therapy of women with hypertensive disorders of pregnancy. *Basic Clin Pharmacol Toxicol* 2012; **111**: 262–267.
25 Motsinger AA, Ritchie MD, Reif DM. Novel methods for detecting epistasis in pharmacogenomics studies. *Pharmacogenomics* 2007; **8**: 1229–1241.
26 Pander J, Wessels JA, Mathijssen RH, Gelderblom H, Guchelaar HJ. Pharmacogenetics of tomorrow: the 1 + 1 = 3 principle. *Pharmacogenomics* 2010; **11**: 1011–1017.
27 Silva PS, Fontana V, Luizon MR, Lacchini R, Silva WA Jr, Biagi C *et al.* eNOS and BDKRB2 genotypes affect the antihypertensive responses to enalapril. *Eur J Clin Pharmacol* 2013; **69**: 167–177.
28 Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 2000; **183**: S1–S22.
29 Sandrim VC, Palei AC, Luizon MR, Izidoro-Toledo TC, Cavalli RC, Tanus-Santos JE. eNOS haplotypes affect the responsiveness to antihypertensive therapy in preeclampsia but not in gestational hypertension. *Pharmacogenomics J* 2010; **10**: 40–45.
30 Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 2002; **70**: 425–434.
31 Motsinger AA, Ritchie MD. Multifactor dimensionality reduction: an analysis strategy for modelling and detecting gene-gene interactions in human genetics and pharmacogenomics studies. *Hum Genomics* 2006; **2**: 318–328.
32 Gui J, Andrew AS, Andrews P, Nelson HM, Kelsey KT, Karagas MR *et al.* A robust multifactor dimensionality reduction method for detecting gene-gene interactions with application to the genetic analysis of bladder cancer susceptibility. *Ann Hum Genet* 2011; **75**: 20–28.
33 Palei AC, Sandrim VC, Amaral LM, Machado JS, Cavalli RC, Duarte G *et al.* Association between matrix metalloproteinase (MMP)-2 polymorphisms and MMP-2 levels in hypertensive disorders of pregnancy. *Exp Mol Pathol* 2012; **92**: 217–221.
34 Palei AC, Sandrim VC, Duarte G, Cavalli RC, Gerlach RF, Tanus-Santos JE. Matrix metalloproteinase (MMP)-9 genotypes and haplotypes in preeclampsia and gestational hypertension. *Clin Chim Acta* 2010; **411**: 874–877.
35 Adali E, Yildizhan R, Kolusari A, Kurdoglu M, Bugdayci G, Sahin HG *et al.* Increased visfatin and leptin in pregnancies complicated by pre-eclampsia. *J Matern Fetal Neonatal Med* 2009; **22**: 873–879.
36 Zulfikaroglu E, Isman F, Payasli A, Kilic S, Kucur M, Danisman N. Plasma visfatin levels in preeclamptic and normal pregnancies. *Arch Gynecol Obstet* 2010; **281**: 995–998.
37 Fasshauer M, Waldeyer T, Seeger J, Schrey S, Ebert T, Kratzsch J *et al.* Serum levels of the adipokine visfatin are increased in pre-eclampsia. *Clin Endocrinol (Oxf)* 2008; **69**: 69–73.
38 Zorba E, Vavilis D, Venetis CA, Zournatzi V, Kellartzis D, Tarlatzis BC. Visfatin serum levels are increased in women with preeclampsia: a case-control study. *J Matern Fetal Neonatal Med* 2012; **25**: 1668–1673.
39 Mazaki-Tovi S, Romero R, Kim SK, Vaisbuch E, Kusanovic JP, Erez O *et al.* Could alterations in maternal plasma visfatin concentration participate in the phenotype definition of preeclampsia and SGA? *J Matern Fetal Neonatal Med* 2010; **23**: 857–868.
40 Korner A, Garten A, Bluher M, Tauscher R, Kratzsch J, Kiess W. Molecular characteristics of serum visfatin and differential detection by immunoassays. *J Clin Endocrinol Metab* 2007; **92**: 4783–4791.
41 Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub MA, Kasowski M *et al.* Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res* 2012; **22**: 1790–1797.
42 Ding Y, Vaziri ND. Nifedipine and diltiazem but not verapamil up-regulate endothelial nitric-oxide synthase expression. *J Pharmacol Exp Ther* 2000; **292**: 606–609.
43 Taddei S, Virdis A, Ghiadoni L, Magagna A, Favilla S, Pompella A *et al.* Restoration of nitric oxide availability after calcium antagonist treatment in essential hypertension. *Hypertension* 2001; **37**: 943–948.
44 Lopez-Jaramillo P, Narvaez M, Calle A, Rivera J, Jacome P, Ruano C *et al.* Cyclic guanosine 3',5' monophosphate concentrations in pre-eclampsia: effects of hydralazine. *Br J Obstet Gynaecol* 1996; **103**: 33–38.
45 Sandrim VC, Palei AC, Metzger IF, Gomes VA, Cavalli RC, Tanus-Santos JE. Nitric oxide formation is inversely related to serum levels of antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endogline in preeclampsia. *Hypertension* 2008; **52**: 402–407.
46 Lovren F, Pan Y, Shukla PC, Quan A, Teoh H, Szmitko PE *et al.* Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis *in vitro* and *in vivo*: translational implications for atherosclerosis. *Am J Physiol Endocrinol Metab* 2009; **296**: E1440–E1449.
47 Yamawaki H, Hara N, Okada M, Hara Y. Visfatin causes endothelium-dependent relaxation in isolated blood vessels. *Biochem Biophys Res Commun* 2009; **383**: 503–508.
48 Vallejo S, Romacho T, Angulo J, Villalobos LA, Cercas E, Leivas A *et al.* Visfatin impairs endothelium-dependent relaxation in rat and human mesenteric microvessels through nicotinamide phosphoribosyltransferase activity. *PloS One* 2011; **6**: e27299.
49 Podymow T, August P. Update on the use of antihypertensive drugs in pregnancy. *Hypertension* 2008; **51**: 960–969.
50 Martinez ML, Lopes LF, Coelho EB, Nobre F, Rocha JB, Gerlach RF *et al.* Lercanidipine reduces matrix metalloproteinase-9 activity in patients with hypertension. *J Cardiovasc Pharmacol* 2006; **47**: 117–122.
51 Zervoudaki A, Economou E, Pitsavos C, Vasiliadou K, Aggeli C, Tsioufis K *et al.* The effect of Ca2+ channel antagonists on plasma concentrations of matrix metalloproteinase-2 and -9 in essential hypertension. *Am J Hypertens* 2004; **17**: 273–276.
52 Zervoudaki A, Economou E, Stefanadis C, Pitsavos C, Tsioufis K, Aggeli C *et al.* Plasma levels of active extracellular matrix metalloproteinases 2 and 9 in patients with essential hypertension before and after antihypertensive treatment. *J Hum Hypertens* 2003; **17**: 119–124.
53 Luizon MR, Sandrim VC, Palei AC, Lacchini R, Cavalli RC, Duarte G *et al.* Epistasis among eNOS, MMP-9 and VEGF maternal genotypes in hypertensive disorders of pregnancy. *Hypertens Res* 2012; **35**: 917–921.
54 Williams PJ. Broughton Pipkin F. The genetics of pre-eclampsia and other hypertensive disorders of pregnancy. *Best Pract Res Clin Obstet Gynaecol* 2011; **25**: 405–417.

Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)