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ORIGINAL ARTICLE

Matrix metalloproteinase-9 polymorphisms affect plasma MMP-9 levels and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy

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Abnormal matrix metalloproteinase (MMP)-9 levels may have a role in hypertensive disorders of pregnancy. We examined whether MMP-9 genetic polymorphisms (g.−1562C>T and g.−90(CA)$_{13-25}$) modify plasma MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 levels and the responses to antihypertensive therapy in 214 patients with preeclampsia (PE), 185 patients with gestational hypertension (GH) and a control group of 214 healthy pregnant (HP). Alleles for the g.−90(CA)$_{13-25}$ polymorphism were grouped L (low) (<21 CA repeats) or H (high) (⩾21 CA repeats). Plasma MMP-9 and TIMP-1 concentrations were measured by enzyme-linked immunosorbent assay. Plasma MMP-9 concentrations were not affected by genotypes or haplotypes in HP and PE groups, except for the g.−90(CA)$_{13-25}$ polymorphism: GH patients with the LH genotype for this polymorphism have higher MMP-9 levels than those with other genotypes. The T allele for the g.−1562C>T polymorphism and the H4 haplotype (combining T and H alleles) are associated with GH and lack of responsiveness to antihypertensive therapy in GH. The H2 haplotype (combining C and H alleles) was associated with lack of responsiveness to antihypertensive therapy in PE, but not in GH. In conclusion, our results show that MMP-9 genetic variants are associated with GH and suggest that MMP-9 haplotypes affect the responsiveness to antihypertensive therapy in hypertensive disorders of pregnancy.
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Keywords: polymorphism; MMP-9; antihypertensive therapy; pharmacogenetics; preeclampsia; gestational hypertension

Introduction

Preeclampsia (PE) and gestational hypertension (GH) are relatively common hypertensive disorders of pregnancy associated with high maternal and fetal mortality and morbidity. PE is a syndrome that only develops during pregnancy and remits after delivery of the placenta, thus suggesting that its origin lies in the placenta.[1] Reduced placental perfusion leads to widespread maternal vascular endothelial dysfunction by mechanisms involving the placental release of vasopressors[2] and other factors associated with oxidative stress and inflammation into the maternal circulation.[3,4]

Matrix metalloproteinases (MMPs) are a family of structurally related enzymes that break down several extracellular matrix components.[5] Imbalanced MMP activities have been reported in many clinical conditions affecting the cardiovascular system, including hypertensive disorders of pregnancy.[6–10] Specifically, MMP-9 may be involved in placental and uterine arterial remodeling[11,12] and in the control of the vascular tone.[13,14] In addition, there is evidence that MMPs interact with oxidative stress and inflammatory mediators contributing to the endothelial dysfunction of PE patients.[15] Therefore, the involvement of MMPs in the vascular alterations of hypertensive disorders of pregnancy may aggravate the lack of responses to antihypertensive therapy in some patients.

While MMP-9 activity is regulated at different levels including activation of latent forms, by interaction with endogenous inhibitors, especially the tissue inhibitor of metalloproteinase (TIMP)-1,[16] it is also regulated at the transcriptional level. In this respect, some genetic polymorphisms in the *MMP-9* gene clearly affect MMP-9 transcription.[17] Two functional MMP-9 polymorphisms include the single-nucleotide polymorphism g.−1562C>T and the microsatellite g.−90$(CA)_{13-25}$. *In vitro* studies showed that the 'C' to 'T' substitution at −1562 position increases MMP-9 expression.[17] However, the $(CA)_{14}$ allele causes a 50% reduction in MMP-9 promoter activity as compared with the $(CA)_{21}$.[18,19] Interestingly, these polymorphisms may affect drug responses[20] and both polymorphisms have been associated with PE or GH,[21,22] thus suggesting that MMP-9 genetic variations may predispose to hypertensive disorders of pregnancy. However, no previous study has examined whether MMP-9 polymorphisms affect MMP-9 levels in hypertensive disorders of pregnancy and whether MMP-9 polymorphisms modify the antihypertensive responses in these conditions.

In this study, we aimed at comparing the circulating MMP-9 and TIMP-1 levels (and MMP-9/TIMP-1 ratio; an index of net MMP-9 activity) in patients with hypertensive disorders of pregnancy carrying different genotypes for the two MMP-9 polymorphisms described above. Moreover, we compared the distributions of MMP-9 genetic variants in GH and PE patients who respond to antihypertensive therapy with those found in GH and PE patients who do not respond to antihypertensive therapy. We have also examined whether MMP-9 haplotypes could have any effect on MMP-9 levels and on the responses to antihypertensive therapy.

Materials and methods

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo, Sao Paulo, Brazil). All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto. We studied 613 pregnants (214 healthy women with uncomplicated pregnancies, 185 women with GH and 214 women with PE). Hypertensive disorders were defined in accordance with the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy).[23] GH was defined as pregnancy-induced hypertension (⩾140 mm Hg systolic or ⩾90 mm Hg diastolic on 2 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/24 h). No women with pre-existing hypertension, with or without superimposed PE, were included in this study.

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

Genotyping

Genotypes for the g.−1562C>T. polymorphism (rs3918242) were determined by polymerase chain reaction as described previously,[24] using the primers 5′-GCCTGGCACATAGTAGG CCC-3′ (sense) and 5′-CTTCCTAGCCAGCCGGCATC-3′ (antisense). The amplified products were digested with *Sph*I restriction enzyme (New England Biolabs, Ipswich, MA, USA) overnight at 37 °C, producing fragments of 247 and 188 bp in the case of a polymorphic variant (allele T), or an undigested 435 bp band in the case of a wild-type allele (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

To determine the genotypes for the g.−90$(CA)_{13-25}$ polymorphism (rs3222264), a polymerase chain reaction was carried out as described previously,[25] using the primers 5′-GACTTGGCAGTGGAGACTGCGGGCA-3′ (sense) and 5′-GA CCCCACCCCTCCTTGACAGGCAA-3′ (antisense). The amplified products were separated in 7% polyacrylamide−8 M urea gel and visualized by silver staining. Differences in number of bases, from 144 bp (CA 13 repeats) to 168 bp (CA 25 repeats), were determined by comparison with migration of a 10 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) and with some samples from homozygotes that were sequenced. To make easier the interpretation of the bands in the gel, the alleles were classified in accordance with the biallelic distribution of this polymorphism:[26,27] 'low' (L) when the number of CA repeats was less than 21, and 'high' (H) when the number of CA repeats was 21 or more.

Antihypertensive treatment and drug response evaluation

The GH and PE patients in this study were carefully monitored for signs and symptoms of PE, with fetal surveillance and laboratory tests at least once weekly. 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) and/or hydralazine (5–30 mg per day), which were added in case of lack of significant responses to methyldopa. The following clinical laboratory outcomes were considered as reflecting a lack of response to therapy:[23,28]

(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) HELLP (hemolysis, elevated liver enzymes and a low platelet count) syndrome; proteinuria >2.0 g/24 h; creatinine >1.2 mg/100 ml or blood urea nitrogen >30 mg/100 ml; aspartate aminotransferase $>40\,U\,l^{-1}$ and alanine aminotransferase $>60\,U\,l^{-1}$; and
(4) fetal hypoactivity or non-reactive fetus, as revealed by cardiotocography; intrauterine growth restriction, oligoamnio, abnormal biophysical profile score, Doppler velocimetry abnormalities, as evaluated by ultrasound.

We excluded four GH patients and one PE patient from analysis because some laboratory tests were missing, making it impossible to classify them with certainty.

Enzyme immunoassays of plasma MMP-9 and TIMP-1

Plasma MMP-9 and TIMP-1 concentrations were measured by a commercially available sandwich enzyme-linked immunosorbent assay kit (DY911 and DY970, respectively; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out using the Stat-View (SAS Institute, Cary, NC, USA). The clinical characteristics, plasma MMP-9 and TIMP-1 concentrations and MMP-9/TIMP-1 ratio of women 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 distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype and allele frequencies among groups were assessed using χ^2 test or Fisher's exact test. A value of $P<0.05$ was considered statistically significant.

The Bayesian statistical-based program Phase 2.1 was used to estimate the haplotype frequencies in each group. The possible haplotypes including genetic variants for two MMP-9 polymorphisms studied (C or T variants for the g.−1562C>T and L or H variants for g.−90(CA)$_{13-25}$) were: H1 (CL), H2 (CH), H3 (TL) and H4 (TH). Differences in haplotype frequency were further tested using contingency tables. The minimum level of statistical significance was corrected for the number of comparisons made. Therefore, we considered significant $P<0.05$/number of haplotypes ($P<0.05/3=0.0167$). We excluded the rare haplotype (H3 frequency $<0.1\%$) from the analysis.

Linear regression analysis and nonlinear fitting routines were performed using the software JMP 5.0.1a (SAS Institute) to assess univariate relations between variables. In addition, a bivariate analysis was also performed to assess the potential confounding influence of each covariate on the relation between MMP-9/TIMP-1 levels and MMP-9 genotypes/haplotypes in HP, GH and PE groups. The variables of clinical importance were then included in multiple linear or logistic regression models. Plasma MMP-9 concentration, plasma TIMP-1 concentration, MMP-9/TIMP-1 ratio, responsiveness to methyldopa and responsiveness to therapy were considered as dependent variables. MMP-9 genotypes/haplotypes, age, ethnicity, smoking, body mass index, primiparity and gestational age at sampling were considered as independent variables.

Results

Table 1 summarizes the characteristics of pregnant enrolled in this study. HP, GH and PE women showed similar ethnicity (% white), % current smoking, hemoglobin, hematocrit and creatinine (all $P>0.05$). As expected, PE and GH presented higher systolic and diastolic blood pressure compared with HP (both $P<0.05$). It should be noted, however, that most patients were receiving antihypertensive therapy. GH and PE were slightly older than HP ($P<0.05$). Increased body mass index and fasting glucose was found in GH and PE patients compared with HP group (all $P<0.05$). We found lower gestational age at delivery in GH and PE, lower newborn weights in PE and lower % primiparity in GH (all $P<0.05$) compared with HP. Significant proteinuria was found in PE. Supplementary Tables S1 and S2 show the characteristics of pregnants in the GH and PE groups classified according to responsiveness to methyldopa and to the total therapy (Supplementary Tables S1 and S2).

Although we found higher plasma MMP-9 and TIMP-1 concentrations in GH patients compared with HP patients (Figure 1; both $P<0.05$), the MMP-9/TIMP-1 ratios were not different (Figure 1; $P>0.05$). TIMP-1 levels were higher in PE women compared with HP (Figure 1; $P<0.05$). However, MMP-9 and MMP-9/TIMP-1 ratios were similar in PE and HP (Figure 1; $P>0.05$).

The results for MMP-9 single-locus analyses are shown in Supplementary Table S3. The distribution of genotypes for the two polymorphisms studied showed no deviation from Hardy–Weinberg equilibrium (all $P>0.05$). With respect to the g.−90(CA)$_{13-25}$ polymorphism, we found no significant differences in genotype and allele frequencies when PE or GH group were compared with HP group (all $P>0.05$; Supplementary Table S3). However, the genotype and allele frequencies for g.−1562C>T polymorphism were different in the GH group compared with HP ($P<0.05$). The CT genotype and T allele were more commonly found in the GH group than in the HP group (both $P<0.05$;

Table 1 Demographic characteristics of study subjects

Parameters	*Healthy pregnant (n = 214)*	*Gestational hypertension (n = 185)*	P-*value*	*Preeclampsia (n = 214)*	P-*value*
Age (years)	24.5 ± 0.4	27.0 ± 0.5*	0.000	26.0 ± 0.5*	0.003
Ethnicity (% White)	71.5	73.3	0.704	69.9	0.718
Current Smoking (%)	12.6	11.2	0.689	8.7	0.205
BMI (kg m^{-2})	23.3 ± 0.3	29.5 ± 0.5*	0.000	27.2 ± 0.4*	0.000
SBP (mm Hg)	112.1 ± 0.7	133.4 ± 1.1*	0.000	142.3 ± 1.1*	0.000
DPB (mm Hg)	72.2 ± 0.5	84.2 ± 0.8*	0.000	88.9 ± 0.7*	0.000
HR (beats min^{-1})	82.3 ± 0.6	82.0 ± 0.5	0.749	82.7 ± 0.5	0.680
Fasting glucose (mg dl^{-1})	75.1 ± 1.0	79.2 ± 1.1*	0.005	79.2 ± 1.8*	0.047
Hb (g dl^{-1})	11.9 ± 0.1	11.9 ± 0.1	0.885	12.0 ± 0.1	0.718
Hct (%)	35.7 ± 0.4	35.8 ± 0.3	0.808	36.1 ± 0.3	0.400
Creatinine (μmol l^{-1})	58.9 ± 2.6	55.1 ± 0.8	0.190	62.6 ± 1.4	0.746
24-h Pr (mg/24 h)	ND	134.5 ± 9.3		1333.0 ± 151.0**	0.000
Primiparity (%)	50.3	39.9*	0.042	44.5	0.243
GAD (weeks)	39.8 ± 0.1	38.8 ± 0.1*	0.000	36.0 ± 0.3*	0.000
Newborn weight (g)	3316.0 ± 34.6	3202.0 ± 41.7	0.095	2546.0 ± 64.7*	0.000
GAS (weeks)	36.8 ± 0.2	36.0 ± 0.4	0.330	34.2 ± 0.4*	0.000

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

Supplementary Table S3). Conversely, we found no differences in genotype or allele frequencies for the g.−1562C>T polymorphism when the PE and the HP groups were compared (all $P>0.05$; Supplementary Table S3).

The results for MMP-9 haplotype analyses are shown in Supplementary Table S4. Although we found no differences when the PE group was compared with HP ($P>0.05$; Supplementary Table S4), the distribution of MMP-9 haplotypes in the GH group was different than that found in the HP group. We found that the H4 haplotype (T H) was more commonly found in the GH group than in the HP ($P<0.0167$; Supplementary Table S4).

To determine the influence of MMP-9 genotypes on plasma MMP-9 and TIMP-1 levels, we performed a multiple linear regression analysis adjusting for age, ethnicity, current smoking, body mass index, primiparity and gestational age at sampling (Table 2). We found that the LH genotype for the g.−90$(CA)_{13-25}$ polymorphism was significantly and positively associated with plasma MMP-9 concentration and MMP-9/TIMP-1 ratio in GH group (Table 4; both $P<0.05$). Moreover, ethnicity was associated with TIMP-1 levels in the GH group, and age and primiparity were positively associated with TIMP-1 in PE group (Table 2; all $P<0.05$).

We determined the influence of MMP-9 haplotypes on plasma MMP-9 and TIMP-1 levels. We performed another multiple linear regression analysis adjusting for the same factors cited above and we found no significant effects of MMP-9 haplotypes on plasma MMP-9 and TIMP-1 concentrations in the HP, GH and PE groups (Table 3; all $P>0.05$). Gestational age at sampling was negatively associated with MMP-9 levels in the HP group (Table 3; $P<0.05$). Ethnicity, smoking status and primiparity were positively associated with TIMP-1 in GH group (Table 3, all $P<0.05$). Ethnicity affected MMP-9 levels and MMP-9/TIMP-1 ratio in the PE group, whereas age and primiparity affected TIMP-1 levels in this group (Table 3; all $P<0.05$).

The results for MMP-9 single-locus analyses with respect to the responses to methyldopa and to the total antihypertensive therapy are shown in the Tables 4 and 5, respectively. These results were adjusted for age, ethnicity, current smoking, body mass index, and primiparity in a multiple logistic regression. Interestingly, the CT or the TT genotypes for the g.−1562C>T polymorphism were more common in GH patients non-responsive to methyldopa or to the total therapy (Tables 4 and 5, respectively; both $P<0.05$). Conversely, this polymorphism apparently has no effects on the responsiveness to therapy in the PE group (Tables 4 and 5; $P>0.05$). The g.−90$(CA)_{13-25}$ polymorphism, however, has no effects on antihypertensive therapy in both GH and PE groups (Tables 4 and 5; all $P>0.05$). It should be noted that this polymorphism tended to have some effects in the PE group ($P=0.054$ and 0.058 for the responses to methyldopa and to the total antihypertensive therapy, respectively; Tables 4 and 5, respectively).

The results for MMP-9 haplotypes analyses with respect to the responses to methyldopa and to the total antihypertensive therapy are shown in the Tables 6 and 7, respectively. These results were adjusted for the same variables cited above in a multiple logistic regression. Interestingly, the haplotype H4 (T H) was more frequent in GH patients non-responsive to methyldopa or to the total antihypertensive

Table 2 Effects of MMP-9 genotypes on plasma MMP-9, TIMP-1 and MMP-9/TIMP-1 levels after adjusting for selected variables

	Health pregnancy						*Gestational hypertension*						*Preeclampsia*					
	MMP-9		*TIMP-1*		*MMP-9/TIMP-1*		*MMP-9*		*TIMP-1*		*MMP-9/TIMP-1*		*MMP-9*		*TIMP-1*		*MMP-9/TIMP-1*	
	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*
Model	0.0326	0.3555	0.0488	0.1109	0.0328	0.3558	0.0977	0.3519	0.0763	0.0922	0.0869	0.3389	0.0405	0.3928	0.0779	0.1166	0.0483	0.4116
	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P
Ethnicity (Black)	0.0044	0.8932	0.0105	0.3019	−0.0061	0.8507	0.0152	0.6657	0.0201	0.0302*	−0.0049	0.8837	0.0576	0.1100	−0.0121	0.2586	0.0696	0.0654
Age (years)	0.3023	0.3390	0.1127	0.2538	0.1896	0.5486	0.0587	0.8549	0.0726	0.3886	−0.0139	0.9641	0.0180	0.9590	0.2442	0.0197**	−0.2263	0.5371
BMI ($>25\,kg\,m^{-2}$)	−0.0257	0.4136	−0.0017	0.8659	−0.0240	0.4446	0.0199	0.5696	0.0008	0.9297	0.0191	0.5711	0.0129	0.6941	−0.0051	0.5964	0.0180	0.5994
Smoking status (yes)	0.0106	0.7938	−0.0064	0.6146	0.0170	0.6758	0.0349	0.4516	0.02101	0.0850	0.0139	0.7556	0.0228	0.6542	0.0017	0.9092	0.0211	0.6927
Primiparity (yes)	−0.0015	0.9636	0.0058	0.5662	−0.0072	0.8223	0.0247	0.4887	0.0127	0.1742	0.0120	0.7274	0.0212	0.5833	0.0336	0.0037**	−0.0125	0.7571
GAS (weeks)	−0.0253	0.1560	0.0027	0.6205	−0.0280	0.1163	0.0017	0.8186	0.0013	0.4888	0.0004	0.9604	−0.0002	0.9807	0.0006	0.7838	−0.0008	0.9197
$C^{-1562}T$ genotypes																		
	P = 0.6970		P = 0.7172		P = 0.6157		P = 0.9670		P = 0.5893		P = 0.9172		P = 0.6222		P = 0.9120		P = 0.6606	
	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P
CC	−0.0145	0.6970	0.0042	0.7172	−0.0187	0.6157	−0.0014	0.9670	−0.0049	0.5893	0.0035	0.9172	0.0198	0.6222	0.0013	0.9120	0.0185	0.6606
CT+TT	0.0145	0.6970	−0.0042	0.7172	0.0187	0.6157	0.0014	0.9670	0.0049	0.5893	−0.0035	0.9172	−0.0198	0.6222	−0.0013	0.9120	−0.0185	0.6606
g.−90(CA)$_{13-25}$ genotypes																		
	P = 0.8792		P = 0.1086		P = 0.8445		P = 0.0049*		P = 0.8452		P = 0.0054*		P = 0.4898		P = 0.4337		P = 0.3882	
	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P
HH	0.0221	0.6128	0.0163	0.2328	0.0058	0.8942	−0.0645	0.2108	−0.0020	0.8799	−0.0625	0.2083	−0.0368	0.4420	0.0182	0.1998	−0.0550	0.2729
HL	−0.0057	0.8835	0.0169	0.1652	−0.0226	0.5617	0.1418	0.0018*	0.0067	0.5678	0.1351	0.0020*	0.0472	0.2760	−0.0027	0.8324	0.0500	0.2716
LL	−0.0164	0.7467	−0.0332	0.037	0.0168	0.7397	−0.0773	0.2318	−0.0046	0.7840	−0.0726	0.2430	−0.0105	0.8491	−0.0155	0.342	0.0051	0.9300

Abbreviations: *B*, parameter estimates for each term; BMI, body mass index; GAS, gestational age at sampling; MMP-9, matrix metalloproteinase-9; R^2, proportion of the variation in the response around the mean that can be attributed to terms in the model rather than to random error; RMSE, root mean square error; TIMP-1, tissue inhibitor of metalloproteinase-1.
*$P<0.05$ in the GH group.
**$P<0.05$ in the PE group.

Table 3 Effects of MMP-9 haplotypes on plasma MMP-9, TIMP-1 and MMP-9/TIMP-1 levels after adjusting for selected variables

	Health pregnancy						*Gestational hypertension*						*Preeclampsia*					
	MMP-9		*TIMP-1*		*MMP-9/TIMP-1*		*MMP-9*		*TIMP-1*		*MMP-9/TIMP-1*		*MMP-9*		*TIMP-1*		*MMP-9/TIMP-1*	
	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*	R^2	*RMSE*
Model	0.0304	0.3497	0.0294	0.1101	0.0296	0.3502	0.0214	0.3595	0.0734	0.0905	0.0105	0.3460	0.0340	0.3866	0.0738	0.1144	0.0390	0.4064
	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P
Ethnicity (Black)	0.0037	0.8692	0.0116	0.1005	−0.0079	0.7240	0.0305	0.2197	0.0206	0.0011**	0.0099	0.6794	0.0639	0.0106***	−0.0120	0.1038	0.0759	0.0039***
Age (years)	0.3022	0.1664	0.1106	0.1078	0.1916	0.3805	0.0667	0.7724	0.0745	0.1995	−0.0079	0.9717	0.0231	0.9238	0.2406	0.0008***	−0.2175	0.3910
BMI (>25 kg m^{-2})	−0.0258	0.2356	−0.0001	0.9869	−0.0257	0.2382	0.0088	0.7255	0.0003	0.9643	0.0085	0.7240	0.0151	0.5053	−0.0044	0.5114	0.0196	0.4131
Smoking status (yes)	0.0106	0.7072	−0.0077	0.3895	0.0183	0.5184	0.0179	0.5897	0.0197	0.0188**	−0.0018	0.9545	0.0210	0.5534	0.0028	0.7898	0.0181	0.6250
Primiparity (yes)	−0.0009	0.9663	0.0066	0.3473	−0.0075	0.7354	0.0355	0.1657	0.0131	0.0426**	0.0224	0.3629	0.0258	0.3339	0.0332	$<$0.0001***	−0.0074	0.7915
GAS (weeks)	−0.0246	0.0435*	0.0014	0.7107	−0.0260	0.0330*	0.0035	0.5034	0.0016	0.2396	0.0020	0.6983	−0.0010	0.8388	0.0008	0.5818	−0.0018	0.7274
MMP-9 haplotypes																		
	P = 0.7598		P = 0.2527		P = 0.8862		P = 0.6883		P = 0.8313		P = 0.6725		P = 0.7854		P = 0.3243		P = 0.6058	
	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P
H1 (C L)	−0.0217	0.4591	−0.0112	0.2246	−0.0105	0.7209	0.0253	0.4289	−0.0014	0.8671	0.0267	0.3868	0.0224	0.5040	−0.0129	0.1957	0.0353	0.3174
H2 (C H)	0.0009	0.9761	0.0105	0.2508	−0.0097	0.7402	−0.0154	0.6224	−0.0043	0.5886	−0.0112	0.7112	0.0057	0.8611	0.0076	0.4308	−0.0019	0.9558
H3 (T L)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H4 (T H)	0.0208	0.6113	0.0007	0.9575	0.0201	0.6236	−0.0099	0.8055	0.0056	0.5796	−0.0155	0.6887	−0.0281	0.5433	0.0053	0.7005	−0.0334	0.4924

Abbreviations: *B*, parameter estimates for each term; BMI, body mass index; GAS, gestational age at sampling; MMP-9, matrix metalloproteinase-9; R^2, proportion of the variation in the response around the mean that can be attributed to terms in the model rather than to random error; RMSE, root mean square error; TIMP-1, tissue inhibitor of metalloproteinase-1.
*$P<0.05$ in the HP group.
**$P<0.05$ in the GH group.
***$P<0.05$ in the PE group.

Table 4 Genotype frequencies of MMP-9 polymorphisms according to responsiveness to methyldopa

	Genotype	*GH—Methyldopa responsiveness*				*PE—Methyldopa responsiveness*			
		R (n = 125)	*NR (n = 56)*	*OR (95% CI)*	P-*value*	*R (n = 61)*	*NR (n = 152)*	*OR (95% CI)*	P-*value*
MMP-9 $C^{-1562}T$	CC	93 (74%)	33 (59%)	1.00 (reference)		46 (75%)	120 (79%)	1.00 (reference)	
	CT+TT	32 (26%)	23 (41%)	2.23 (1.06–4.73)*	0.036	15 (25%)	32 (21%)	0.62 (0.28–1.39)	0.240
MMP-9 $g.-90(CA)_{13-25}$	LL	21 (17%)	8 (14%)	1.00 (reference)		18 (29%)	26 (17%)	1.00 (reference)	
	LH	65 (52%)	31 (56%)	1.27 (0.49–3.33)	0.629	26 (43%)	65 (43%)	1.23 (0.53–2.92)	0.637
	HH	39 (31%)	17 (30%)	0.79 (0.25–2.40)	0.677	17 (28%)	61 (40%)	2.61 (1.00–7.16)	0.054

Abbreviations: BMI, body mass index; CI, confidence interval; GH, gestational hypertension; MMP-9, matrix metalloproteinase-9; NR, non-responsive; OR, odds ratio; PE, preeclampsia; R, responsive.
The genotype distribution was adjusted for age, ethnicity, current smoking, BMI and primiparity.
*$P<0.05$ vs responsive GH.

Table 5 Genotype frequencies of MMP-9 polymorphisms according to responsiveness to total antihypertensive therapy

	Genotype	*GH—Therapy responsiveness*				*PE—Therapy responsiveness*			
		R (n = 159)	*NR (n = 22)*	*OR (95% CI)*	P-*value*	*R (n = 114)*	*NR (n = 99)*	*OR (95% CI)*	P-*value*
MMP-9 $C^{-1562}T$	CC	114 (72%)	12 (55%)	1.00 (reference)		88 (77%)	78 (79%)	1.00 (reference)	
	CT	45 (28%)	10 (45%)	2.81 (1.00–8.10)*	0.049	26 (23%)	21 (21%)	0.66 (0.32–1.35)	0.259
MMP-9 $g.-90(CA)_{13-25}$	LL	25 (16%)	4 (18%)	1.00 (reference)		27 (24%)	17 (17%)	1.00 (reference)	
	LH	85 (53%)	11 (50%)	0.85 (0.21–3.52)	0.817	50 (44%)	41 (42%)	1.02 (0.47–2.24)	0.963
	HH	49 (31%)	7 (32%)	0.76 (0.15–3.59)	0.725	37 (32%)	41 (41%)	2.31 (0.98–5.55)	0.058

Abbreviations: BMI, body mass index; CI, confidence interval; GH, gestational hypertension; MMP-9, matrix metalloproteinase-9; NR, non-responsive; OR, odds ratio; PE, preeclampsia; R, responsive.
The genotype distribution was adjusted for age, ethnicity, current smoking, BMI and primiparity.
*$P<0.05$ vs responsive GH.

Table 6 Haplotype frequencies of MMP-9 polymorphisms according to responsiveness to methyldopa

Haplotype	*GH—Methyldopa responsiveness*				*PE—Methyldopa responsiveness*			
	R (n = 125 × 2)	*NR (n = 56 × 2)*	*OR (95% CI)*	P-*value*	*R (n = 61 × 2)*	*NR (n = 152 × 2)*	*OR (95% CI)*	P-*value*
H1 (C L)	107 (43%)	46 (41%)	1.00 (reference)		62 (51%)	116 (38%)	1.00 (reference)	
H2 (C H)	110 (44%)	41 (37%)	0.58 (0.29–1.14)	0.115	44 (36%)	154 (51%)	2.04 (1.05–3.96)**	0.034
H4 (T H)	33 (13%)	24 (21%)	2.32 (1.00–5.30)*	0.047	16 (13%)	33 (11%)	0.76 (0.32–1.92)	0.547

Abbreviations: BMI, body mass index; CI, confidence interval; GH, gestational hypertension; MMP-9, matrix metalloproteinase-9; NR, non-responsive; OR, odds ratio; PE, preeclampsia; R, responsive.
The haplotype distribution was adjusted for age, ethnicity, current smoking, BMI and primiparity.
*$P<0.05$ vs responsive GH.
**$P<0.05$ vs responsive PE.

therapy (Tables 6 and 7; both $P<0.05$). Moreover, the H2 (C H) haplotype was more frequent in PE patients non-responsive to methyldopa or to the total antihypertensive therapy (Tables 6 and 7; both $P<0.05$). Conversely, the H2 haplotype was more common in GH patients responsive to the total antihypertensive therapy (Table 7; $P<0.05$).

Discussion

Despite the fact that hypertensive disorders of pregnancy are leading causes of maternal–fetal mortality and morbidity, there is no effective drug treatment for these conditions, and current management options have limitations. In this study, we analyzed the influence of two MMP-9 polymorphisms on plasma MMP-9 and TIMP-1 levels and on the responsiveness to antihypertensive therapy. Our findings may help to understand the relevance of MMP-9 and its genetic polymorphisms to the pathophysiology and therapeutic responses of hypertensive disorders of pregnancy.

The circulating MMP-9 and TIMP-1 levels reported in this study are consistent with previous results, which indicate higher MMP-9[7] and TIMP-1[7,9,10] levels in hypertensive disorders of pregnancy compared with those found in HP.

Table 7 Haplotype frequencies of MMP-9 polymorphisms according to responsiveness to total antihypertensive therapy

Haplotype	*GH—Therapy responsiveness*				*PE—Therapy responsiveness*			
	R (n = *159* × *2*)	*NR* (n = *22* × *2*)	*OR (95% CI)*	P-*value*	*R* (n = *114* × *2*)	*NR* (n = *99* × *2*)	*OR (95% CI)*	P-*value*
H1 (C L)	134 (42%)	19 (43%)	1.00 (reference)		104 (46%)	74 (37%)	1.00 (reference)	
H2 (C H)	138 (43%)	13 (30%)	0.37 (0.14–0.96)*	0.045	96 (42%)	102 (52%)	1.90 (1.06–3.45)**	0.033
H4 (T H)	45 (14%)	12 (27%)	3.51 (1.19–9.75)*	0.018	28 (12%)	21 (11%)	0.73 (0.31–1.66)	0.454

Abbreviations: BMI, body mass index; CI, confidence interval; GH, gestational hypertension; MMP-9, matrix metalloproteinase-9; NR, non-responsive; OR, odds ratio; PE, preeclampsia; R, responsive.
The haplotype distribution was adjusted for age, ethnicity, current smoking, BMI and primiparity.
*$P<0.05$ vs responsive GH.
**$P<0.05$ vs responsive PE.

It is possible that MMP-9 released by endothelial and smooth muscle cells during hypertensive disorders leads to cardiovascular alterations associated with hypertension.[29] However, because TIMP-1 is an important endogenous MMP-9 inhibitor, the increased TIMP-1 levels found in both PE and GH has led to similar MMP-9/TIMP-1 ratios in PE and GH compared with HP, thus suggesting that similar net MMP-9 activity exist in patients with hypertensive disorders of pregnancy as compared with HP.

Although our results confirm previous findings showing that the g.−1562C>T polymorphism is associated with GH and not with PE,[22] the g.−1562C>T polymorphism had no significant effects on MMP-9 levels and MMP-9/TIMP-1 ratios in the three study groups, although an *in vitro* study showed that the T allele is associated with increased MMP-9 expression.[17] Conversely, the g.−90$(CA)_{13-25}$ polymorphism affected the circulating MMP-9 levels and MMP-9/TIMP-1 ratios in the GH group, and this finding is consistent with *in vitro* studies suggesting functional implications for this polymorphism.[18,19] Taken together, these findings may be of limited utility to understand how MMP-9 gene variations affect MMP-9 levels or MMP-9/TIMP-1 ratios. Therefore, we carried out haplotype analysis, which could be more effective in providing relevant information.

The haplotype analyses showed that H4 haplotype (combining the T allele for the g.−1562C>T and H allele for the g.−90$(CA)_{13-25}$ polymorphism) increases the susceptibility to GH, and no significant association was found with respect to PE. Moreover, MMP-9 haplotypes apparently had no effects on plasma MMP-9 levels of MMP-9/TIMP-1 ratios. These findings suggest that the possible contribution of MMP-9 to hypertensive disorders of pregnancy are not clearly reflected by the circulating levels of this MMP. Indeed, the origin of circulating MMP-9 measured in the blood is unknown, even though placental tissue may contribute to it.[11,12] There is evidence, however, that circulating activated neutrophils and monocytes may release MMP-9 as a consequence of a general inflammatory state in hypertensive disorders of pregnancy.[15] However, the placenta is mostly a fetal tissue and therefore it is possible that fetal MMP-9 genotypes contributes to MMP-9 levels and to the development of hypertensive disorders of pregnancy, although this remains to be defined.[30] Examining how the maternal MMP-9 haplotypes interact with fetal MMP-9 haplotypes is beyond the scope of this study.

Antihypertensive drugs do not prevent the pathophysiological alterations of PE. However, they allow maintenance of pregnancy and promote an increased gestational age of delivery, thus decreasing adverse maternal and fetal outcomes.[31] In this respect, while some antihypertensive drugs can downregulate MMPs' activities[32–35] and as this effect may contribute to the reduction of blood pressure, no previous study had examined whether MMP-9 polymorphisms affect the antihypertensive effects of drugs used to treat hypertensive disorders of pregnancy. In this study, we investigated for the first time the effects of MMP-9 polymorphisms in responsiveness to methyldopa or to total antihypertensive therapy in hypertensive disorders of pregnancy. Although the g.−90$(CA)_{13-25}$ polymorphism apparently had now major effects, the g.−1562C>T polymorphism was significantly associated with the antihypertensive responses. The CT/TT genotypes were associated with lack of responses to methyldopa and to the total therapy used in this study in GH, but not in PE. Interestingly, the T allele was associated with increased susceptibility to GH, and not to PE. These findings suggest that the g.−1562C>T polymorphism not only promotes GH, but also decreases the responses to antihypertensive therapy.

We have also examined whether MMP-9 haplotypes affect the responsiveness to methyldopa or to total antihypertensive therapy. Our results showed that the H4 haplotype was associated with lack of response to methyldopa and to the total therapy in the GH group, whereas the H2 haplotype decreases the responsiveness to antihypertensive therapy in the PE group. However, the H2 haplotype was more frequent in responsive than in non-responsive GH patients. Interestingly, the H4 haplotype combines the two MMP-9 variants associated with increased *in vitro* MMP-9 levels expression.[17–19] Although we have not found increased MMP-9 levels in subjects carrying the H4 haplotype, it is possible that the circulating MMP-9 levels do not reflect tissue MMP-9 levels, as discussed above. Supporting this suggestion, we found that the H4 haplotype was associated with GH.

Interestingly, we found that only the g.−90$(CA)_{13-25}$ polymorphism was associated with MMP-9 plasma levels in GH, and this polymorphism apparently does not affect

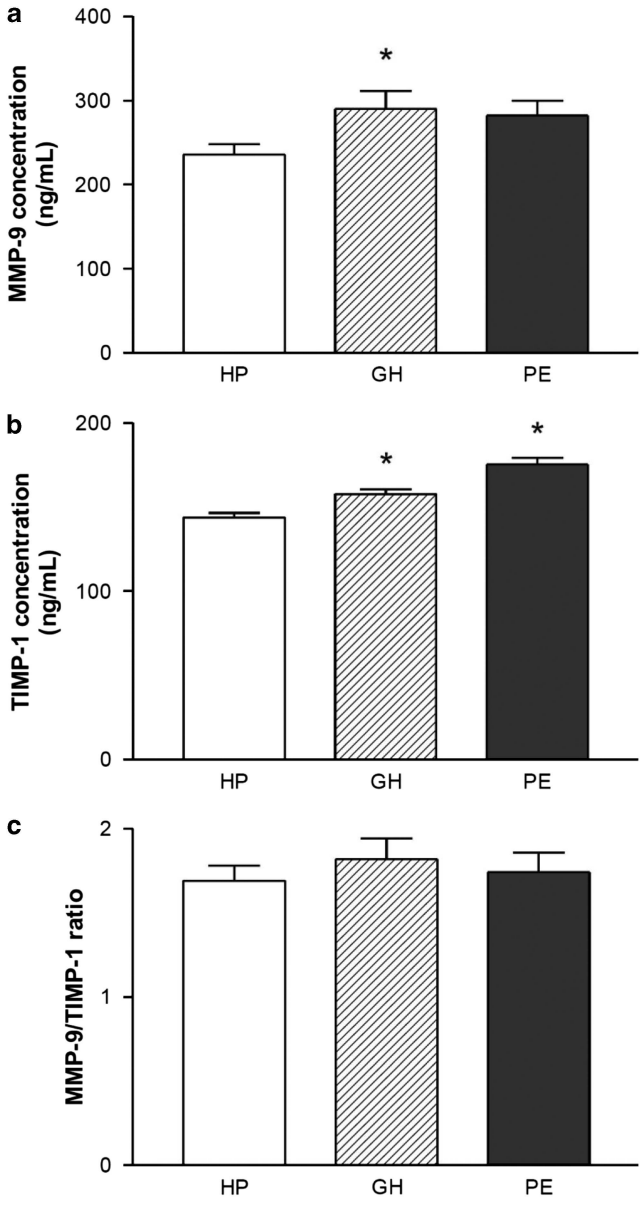

Figure 1 Plasma matrix metalloproteinase-9 (MMP-9) (**a**) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (**b**) concentrations, and MMP-9/TIMP-1 ratios (**c**) in health pregnant (HP), gestational hypertension (GH) and preeclampsia (PE). The bars show the means ± s.e.m. *$P<0.05$ vs HP.

the responsiveness to antihypertensive therapy. Conversely, the g.−1562 C>T polymorphism and the H4 haplotype, which were associated with GH and impaired the responsiveness to antihypertensive therapy, had no significant effects on plasma MMP-9 levels. These findings suggest that the circulating MMP-9 levels may have little predictive value for antihypertensive therapy. Moreover, MMP-9 levels may not reflect the possible contribution of MMP-9 genotypes or haplotypes to hypertensive disorders of pregnancy or to antihypertensive therapy. Indeed, our results do not rule out a possible contribution of MMP-9 genotypes or haplotypes to variations in MMP-9 levels in placental and uterine arterial tissues, which may be very relevant to hypertensive disorders of pregnancy.[11,12]

We found significant differences between GH and PE with respect to their possible associations with MMP-9 polymorphisms. Importantly, while the H2 haplotype was more frequent in patients with PE that do not respond to the antihypertensive therapy, the same H2 haplotype was more prevalent in GH patients who respond to the therapy. Our findings do not provide a reliable basis to explain such a difference between these hypertensive disorders of pregnancy. However, this finding is consistent with the suggestion that different mechanisms may have a role in these diseases. In addition, although we have studied a significant number of patients, it is possible that studying a greater number of subjects would allow us to detect other significant associations.

The criteria used in this study to assess the responses to antihypertensive therapy may have affected our results and conclusions. Although there is no clear definition of how to precisely assess the severity of hypertensive disorders of pregnancy, it is possible that responsiveness to therapy reflects disease severity, and additional studies are needed to improve our understanding of these syndromes. However, we could speculate that GH patients carrying the C allele for the 1562 C>T polymorphism, or with the H4 haplotype, which were associated with GH and impaired the responsiveness to antihypertensive therapy, could benefit from the use of MMPs inhibitors, such as doxycycline. It is possible that patients with these genetic markers have increased tissue levels of MMP-9, as previous studies suggest,[17–19] even though we have not found increased plasma MMP-9 level patients carrying these markers. Whether these patients would have improved outcomes after the use of MMPs inhibitors remains to be determined.

In conclusion, we found evidence indicating that the T allele for the g.−1562C>T polymorphism and the H4 haplotype are associated with GH and with lack of responsiveness to antihypertensive therapy in GH, although they do not affect MMP-9 levels. In addition, while we found increased MMP-9 levels in GH patients with the LH genotype for the g.−90$(CA)_{13-25}$ polymorphism, we found no effects of MMP-9 haplotypes on MMP-9 levels. Finally, the H2 haplotype was associated with lack of responsiveness to antihypertensive therapy in PE, but not in GH. Overall, our findings indicate that MMP-9 haplotypes affect the susceptibility to hypertensive disorders of pregnancy and the antihypertensive responses to drugs used in these conditions.

Conflict of interest

The authors declare no conflict of interest.

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