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ORIGINAL ARTICLE

Epistasis among eNOS, MMP-9 and VEGF maternal genotypes in hypertensive disorders of pregnancy

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Polymorphisms of the endothelial nitric oxide synthase (*eNOS*), matrix metalloproteinase-9 (*MMP-9*) and vascular endothelial growth factor (*VEGF*) genes were shown to be associated with hypertensive disorders of pregnancy. However, epistasis is suggested to be an important component of the genetic susceptibility to preeclampsia (PE). The aim of this study was to characterize the interactions among these genes in PE and gestational hypertension (GH). Seven clinically relevant polymorphisms of *eNOS* (T-786C, rs2070744, a variable number of tandem repeats in intron 4 and Glu298Asp, rs1799983), *MMP-9* (C-1562T, rs3918242 and -90(CA)$_{13–25}$, rs2234681) and *VEGF* (C-2578A, rs699947 and G-634C, rs2010963) were genotyped by TaqMan allelic discrimination assays or PCR and fragment separation by electrophoresis in 122 patients with PE, 107 patients with GH and a control group of 102 normotensive pregnant (NP) women. A robust multifactor dimensionality reduction analysis was used to characterize gene–gene interactions. Although no significant genotype combinations were observed for the comparison between the GH and NP groups ($P>0.05$), the combination of *MMP-9*-1562CC with *VEGF*-634GG was more frequent in NP women than in women with PE ($P<0.05$). Moreover, the combination of *MMP-9*-1562CC with *VEGF*-634CC or *MMP-9*-1562CT with *VEGF*-634CC or-634GG was more frequent in women with PE than in NP women ($P<0.05$). These results are obscured when single polymorphisms in these genes are considered and suggest that specific genotype combinations of *MMP-9* and *VEGF* contribute to PE susceptibility.
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INTRODUCTION

Hypertensive disorders of pregnancy (HDP), including preeclampsia (PE), complicate 3–10% of pregnancies and are major contributors to maternal mortality worldwide.[1] Although the etiology of PE remains unclear, impaired cytotrophoblast invasion and placental ischemia followed by systemic endothelial dysfunction are thought to represent key factors in PE development.[2,3]

During human embryo implantation and placentation, the ability of trophoblasts to infiltrate the maternal endometrium is modulated by the activity of matrix metalloproteinases (MMPs).[4] Indeed, imbalanced plasma MMP levels have been reported in HDP.[5–7] In particular, impaired trophoblast invasion may be related to altered MMP-9 expression.[8,9] Moreover, MMP-9 activity is stimulated in cytotrophoblasts cultured *in vitro* by vascular endothelial growth factor (VEGF).[10]

VEGF is an angiogenic factor essential for placental and embryonic vasculogenesis and angiogenesis.[2] Abnormal cytotrophoblastic invasion leads to placental hypoxia and the release of a splice variant of the VEGF receptor (VEGFR), soluble FMS-like tyrosine kinase-1 (sFlt-1), into the maternal circulation, promoting systemic endothelial dysfunction.[3,11,12] Importantly, an increased expression of this antiangiogenic factor reduces endothelial nitric oxide synthase (eNOS) activation, thereby contributing to endothelial dysfunction.[13,14]

Studies in PE genetics have been widely performed using the candidate gene approach. They have largely focused on maternal genotypes, and polymorphisms in genes involved in angiogenesis, endothelial function and oxidative stress have been associated with PE.[15–17] We previously studied clinically relevant polymorphisms of the *eNOS* (T-786C, 27 bp variable number of tandem repeats in intron 4 and Glu298Asp), *MMP-9* (C-1562T and -90(CA)$_{13–25}$) and *VEGF* (C-2578A and G-634C) genes in HDP. We reported associations of some genotypes and haplotypes formed by these polymorphisms with PE and/or gestational hypertension (GH).[18–21]

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These studies indicate that, at least individually, these genes may be important to the development of these disorders. Besides the study of these genes independently, epistasis has also been suggested to be an important component of complex diseases, including PE.[17,22,23] Indeed, gene–gene interactions have been successfully used to detect susceptibility to multifactorial diseases,[24–26] and it is possible that this analysis could help the genetic basis of PE and GH.

Therefore, in this study, we sought to characterize the interactions among the abovementioned polymorphisms of the *eNOS*, *MMP-9* and *VEGF* genes, which have been previously studied, by comparing normotensive pregnant (NP) women to those with GH and PE.

METHODS

Study population

This study was approved by the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto (FMRP), University of Sao Paulo, Brazil, and the subjects gave informed consent. A total of 331 pregnant women were consecutively enrolled by the Department of Obstetrics and Gynecology, University Hospital of the FMRP. Of these, 102 were NP women (NP group) with uncomplicated pregnancies, 107 were women with GH (GH group) and 122 were women with PE (PE group). Hypertensive disorders were defined in accordance to guidelines of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.[27] GH was defined as pregnancy-induced hypertension ($\geqslant$140 mm Hg systolic or $\geqslant$90 mm Hg diastolic on two or more measurements at an interval of at least 6 h) in a woman after 20 weeks of gestation, which returned to normal by 12 weeks post partum. PE was defined as GH plus significant proteinuria ($\geqslant$0.3 g/24 h). Exclusion criteria included twin or multiple pregnancies or any evidence of previous medical illness, including pre-existing hypertension.

Maternal venous blood samples were collected at the time of clinic attendance. Genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at $-20\,^{\circ}C$ until analysis.

Genotype determination

Seven clinically relevant polymorphisms were studied in *eNOS* (T-786C, rs2070744 in the promoter region; the 27 bp variable number of tandem repeats in intron 4; and Glu298Asp, rs1799983 in exon 7), in the promoter region of *MMP-9* (C-1562T, rs3918242 and -90$(CA)_{13-25}$, rs2234681), and in the promoter region of *VEGF* (C-2578A, rs699947 and G-634C, rs2010963). Genotypes for *eNOS* rs2070744 and rs1799983 and for *VEGF* polymorphisms were determined by TaqMan allele discrimination assays using probes and primers designed by Applied Biosystems (Carlsbad, CA, USA), as previously described.[20,21] Fluorescence from PCR amplification was detected using a Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using the manufacturer's software. Genotypes for the *eNOS* variable number of tandem repeat polymorphism in intron 4 were determined by PCR and fragment separation by electrophoresis in 8% polyacrylamide gels as previously described.[28] Genotypes for *MMP-9* rs3918242 were determined by PCR, digestion of the amplified products with *Sph*I restriction enzyme and separation of fragments by electrophoresis in 12% polyacrylamide gels. For *MMP-9* rs2234681, PCR and fragment separation by electrophoresis in a 7% polyacrylamide-8M urea gel was performed as previously described.[19]

Statistical analysis

The clinical characteristics of the studied groups were compared by a one way analysis of variance, followed by the Dunnett multiple comparisons test or χ^2-test when appropriate. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype and allele frequencies between groups were assessed using χ^2-tests. A value of $P<0.05$ was considered statistically significant.

Multifactor dimensionality reduction (MDR) combines multilocus genotypes into high-risk and low-risk cells, which are evaluated for their ability to classify and predict disease status through cross-validation steps and permutation testing.[26] Here, we used the robust MDR (RMDR) approach to characterize the interaction models among the seven studied polymorphisms. RMDR performs constructive induction using a Fisher's exact test rather than a predetermined threshold, and it has the advantage that only statistically significant genotype combinations are considered in the MDR analysis.[24] The cross-validation steps are similar to those of the MDR approach with the exception that a testing score is used to replace the balanced accuracy. The RMDR approach is freely available as part of the open-source MDR software package version 2.0 (http://www.epistasis.org/[24]). We considered the best interaction model to be the one that have the maximum testing score and the maximum cross-validation consistency (CVC). Permutation testing was performed with RMDR to assess the statistical significance of the testing score of the best model using the MDR Permutation Testing software package.[29]

RESULTS

Table 1 summarizes the characteristics of the 331 pregnant women enrolled in this study. We found no differences in age, ethnicity, smoking, % primiparity, heart rate, fasting glucose, Hb, hematocrit or APGAR (appearance, pulse, grimace, activity, respiration) score when GH and PE women were compared with NP women (Table 1; all $P>0.05$). As expected, higher systolic and diastolic blood pressures were found in the GH and PE groups compared with the NP group (Table 1; both $P<0.05$), despite the fact that most hypertensive patients were receiving antihypertensive drugs. Higher body mass index was found in the GH and PE groups compared with the NP group (Table 1; $P<0.05$). Lower newborn weights and gestational ages at delivery were found in the PE group compared with the NP group (Table 1; both $P<0.05$). Significant proteinuria was found only in PE women (Table 1).

The distribution of genotypes for the seven polymorphisms studied here showed no deviation from Hardy–Weinberg equilibrium (all $P>0.05$). Because significant interethnic differences exist in the distribution of *eNOS*,[28] *MMP-9*[30] and *VEGF*[31] polymorphisms, we carried out two different analyses. The first analysis included white and non-white patients, whereas the second one took into

Table 1 Demographic characteristics of study subjects.

Parameters	*Normotensive healthy pregnant*	*Gestational hypertension*	*Preeclampsia*
N	102	107	122
Age (years)	25.5 ± 5.9	26.6 ± 6.2	27.4 ± 6.7
Ethnicity (% white)	73	78.5	67
Current smoking (%)	13.7	10.3	9
Primiparity (%)	43	47	40
BMI (kg m^{-2})	23.7 ± 4.8	28.8 ± 6.4*	27.2 ± 6.4*
SBP (mm Hg)	112.1 ± 10.3	133.1 ± 17.9*	141.4 ± 18.9*
DPB (mm Hg)	72.4 ± 8.7	84.4 ± 13.3*	88.4 ± 13.4*
HR (beats per min)	83.3 ± 7.6	82.4 ± 7.8	81.7 ± 9.0
Fasting Glucose (mg dl^{-1})	74.9 ± 9.9	76.1 ± 8.7	75.7 ± 13.1
Hb (g dl^{-1})	12.1 ± 1.5	11.9 ± 1.2	11.9 ± 1.7
Hct (%)	36.3 ± 4.6	35.8 ± 3.4	36.0 ± 4.6
Newborn weight (g)	3372 ± 578	3215 ± 573	2597 ± 930*
APGAR score (1 min)	7.6 ± 2.1	7.7 ± 2.2	7.3 ± 2.4
APGAR score (5 min)	9.5 ± 0.8	9.5 ± 1.2	9.4 ± 1.4
GAD (weeks)	39.7 ± 1.4	39.1 ± 1.5	36.2 ± 3.9*
24-h-Pr (mg per 24 h)	ND	135 ± 80	1081 ± 1417#

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

consideration only white pregnant women, which corresponded to 67–79% of the subjects. Both analyses showed significant differences in the distribution of genotypes and alleles for the *VEGF* G-634C polymorphism when the PE group was compared with the NP group and for the *MMP-9* C-1562T polymorphism when the GH group was compared with the NP group, respectively (Supplementary Table 1; all $P<0.05$). The present results are in agreement with our previous findings.[19,21]

Next, we evaluated the interaction among *eNOS, MMP-9* and *VEGF* polymorphisms when the GH and PE groups were compared with the NP group. We found no statistically significant results when the GH and NP groups were compared ($P>0.05$; Table 2). Conversely, we found a significant interaction when the PE group was compared with the NP group. The best model of interaction included the G-634C polymorphism of *VEGF* and the C-1562T polymorphism of *MMP-9*, and this was observed when considering both the whole sample and only the white pregnant sample ($P=0.0115$ and $P=0.0345$, respectively; Table 2). Figure 1 depicts the specific combinations of genotypes as classified by RMDR for low and high risks. The combination of *MMP-9*-1562CC with *VEGF*-634GG was more frequent in the NP group than in the PE group, whereas the three high-risk cells contained combinations of *MMP-9*-1562CC with *VEGF*-634CC. Additionally, the *MMP-9*-1562CT with both the VEGF-634CC and -634GG genotypes were more frequent in the PE group than in the NP group (Figure 1).

DISCUSSION

This study was the first to evaluate interactions among *eNOS*, *MMP-9* and *VEGF* polymorphisms in HDP. The main findings are that significant interactions between the *MMP-9* and *VEGF* genes are associated with PE compared with normal pregnancies.

The interactions between *MMP-9* and *VEGF* polymorphisms associated with the PE group are obscured when specific genotypes of these single genes are considered (Supplementary Table 1), thus highlighting the importance of gene–gene interactions as major determinants of complex diseases.[22,25] Although epistasis is believed to be an important component of complex diseases, including HDP,[17] only a few studies have addressed this issue in PE.[32–34] Moreover, none have focused on the combinatory effects of *eNOS*, *MMP-9* and *VEGF* in the maternal genotype and its relation to HDP susceptibility.

Although we found no significant gene–gene interactions in the GH group, we found significant interaction models when the PE group was compared with the NP group. Interestingly, despite not finding an association between the *MMP-9* C-1562T polymorphism and PE during single-locus analyses (Supplementary Table 1), we observed a significant interaction of this *MMP-9* polymorphism with the *VEGF* G-634C polymorphism in women with PE (Table 2). These

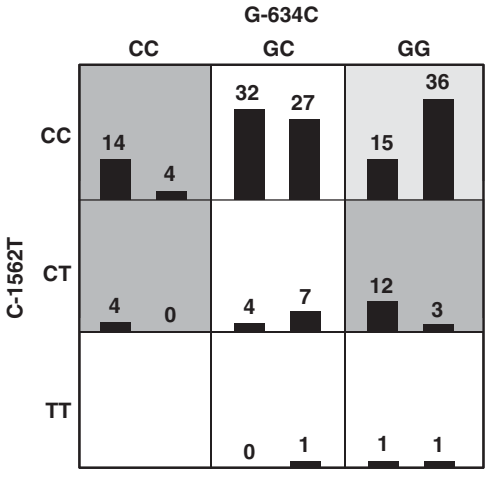

Figure 1. The best RMDR interaction model for the *MMP-9* C-1562T and *VEGF* G-634C polymorphisms when comparing PE with NP group, considering only white pregnant women. The distributions of cases (left bars) and controls (right bars) are illustrated for each multilocus genotype combinations. The white cells are labeled as unknown, light gray cells are labeled as low risk and dark gray cells are labeled as high risk.

Table 2 Robust multifactor dimensionality reduction (RMDR) interaction models among the seven polymorphisms of the three genes studied when GH and PE groups were compared with NP group (white + non-white or only white)

All pregnant (white + non-white)	*Training score*	*Testing score*	*CVC*	*P*[a]	*White pregnant only*	*Training score*	*Testing score*	*CVC*	*P*[a]
GH compared with NP group									
MMP-9 C-1562T	0.5876	0.5404	7/10	0.5380	*MMP-9 C-1562T*	0.5938	0.5360	7/10	0.5985
VEGF G-634C; MMP-9 C-1562T	0.6553	0.6248	10/10	0.0520	*VEGF G-634C; MMP-9 C-1562T*	0.6450	0.5517	6/10	0.4890
VEGF C-2578A; MMP-9 C-1562T-90(CA)$_{13–25}$	0.6760	0.5394	4/10	0.4445	*VEGF C-2578A*; *MMP-9 C-1562T*-90(CA)$_{13–25}$	0.6825	0.5830	7/10	0.2945
VEGF C-2578A G-634C; MMP-9 C-1562T-90(CA)$_{13–25}$	0.6932	0.5829	5/10	0.1730	*eNOS T-768C; VEGF C-2578A*; *MMP-9 C-1562T*-90(CA)$_{13–25}$	0.7004	0.5858	8/10	0.3060
PE compared with NP group									
VEGF C-2578A	0.5579	0.4650	5/10	0.9835	*VEGF G-634C*	0.6066	0.6111	9/10	0.1630
VEGF G-634C; MMP-9 C-1562T	0.6543	0.6472	10/10	0.0115	*VEGF G-634C; MMP-9 C-1562T*	0.6862	0.6556	10/10	0.0345
VEGF C-2578A G-634C; MMP-9 C-1562T	0.6806	0.5891	5/10	0.1285	*VEGF C-2578A G-634C; MMP-9 C-1562T*	0.7050	0.5825	5/10	0.2420
eNOS Glu298Asp; VEGF G-634C; MMP-9 C-1562T-90(CA)$_{13–25}$	0.6889	0.5023	3/10	0.7835	*eNOS T-768C* intron 4; *VEGF C-2578A G-634C*	0.7214	0.5954	6/10	0.1970

Abbreviations: CVC, cross-validation consistency; *eNOS*, endothelial nitric oxide synthase gene; GH, gestational hypertension; NP, normotensive pregnant; *MMP-9*, matrix metalloproteinase-9 gene; PE, preeclampsia; *VEGF*, vascular endothelial growth factor gene.
[a]*P*-value after 1.000 permutations.

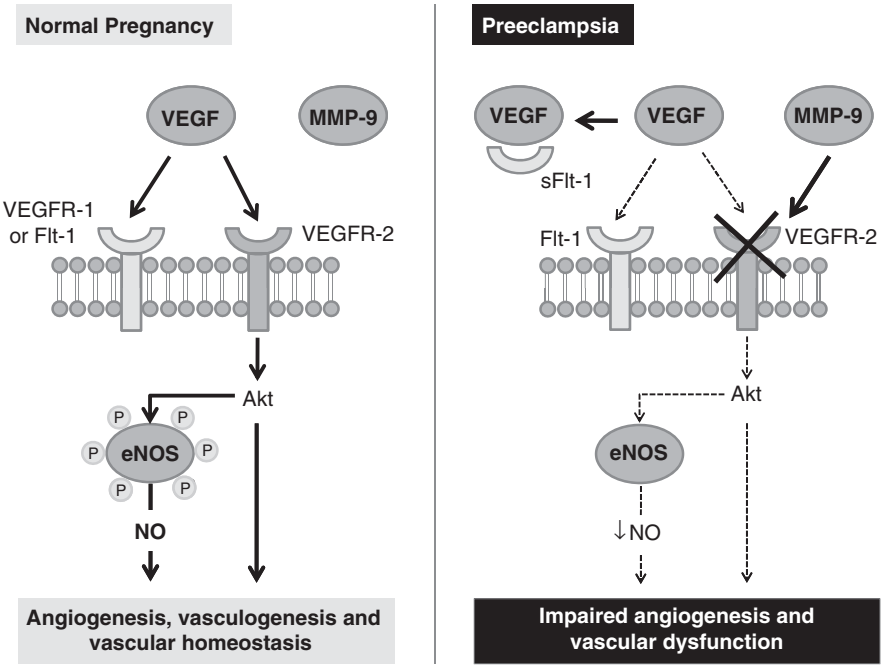

Figure 2. Interactions among *eNOS*, *MMP-9* and *VEGF* gene products in normal pregnancy and how they may be affected in PE. VEGF effects on angiogenesis, vasculogenesis and vascular homeostasis are compromised owing to VEGF binding to the excess of soluble VEGFR-1 (sFlt-1) observed in PE, as well as the degradation of the VEGFR-2 extracellular domain by MMP-9. Both mechanisms may involve diminished nitric oxide (NO) bioavailability as a result of impaired endothelial NO synthase (eNOS) phosphorylation by Akt.

discrepant results may suggest that epistasis is a ubiquitous component of the genetic architecture of common human diseases and that complex interactions are more important than independent effects of any one susceptibility gene.[22] Additionally, the best interaction model of *MMP-9* C-1562T with *VEGF* G-634C was observed both when considering the entire cohort and the cohort of only white pregnant women. Therefore, we excluded the possibility that significant interethnic differences in the distribution of these polymorphisms [30,31] drove the present results.

The specific combinations of low- and high-risk genotypes classified by RMDR are depicted in Figure 1. We found a high frequency of the *VEGF*-634GG genotype in NP. Because VEGF is an angiogenic factor essential for placental and embryonic vasculogenesis and angiogenesis,[2] our finding is in agreement with a study that showed that higher VEGF production was stimulated by mononuclear cells in carriers of the VEGF-634GG genotype.[35] Conversely, as the VEGF-634CC genotype may be related to lower VEGF production, we found a high frequency of *VEGF*-634CC genotype in the PE group. The two other high-risk cells exhibited the *MMP-9*-1562CT genotype, which was more frequent in the PE group than the NP group. Consistent with these findings, *in vitro* studies have associated the -1562T allele with increased MMP-9 expression, possibly resulting in increased circulating MMP-9 levels.[36] This is consistent with the findings that serum concentrations of MMP-9 are increased in pregnancies that subsequently develop PE.[37] However, the molecular mechanisms explaining the associations between interaction models reported herein require further studies.

Although this study has not been designed to define these underlying mechanisms, the possible interactions among the *eNOS*, *MMP-9* and *VEGF* gene products in normal pregnancy and in PE patients are presented in Figure 2. It has been shown that MMP-9 may cleave the extracellular domain of the VEGFR-2 in a spontaneously hypertensive rat model of hypertension.[38] This cleavage may prevent VEGF binding to VEGFR-2 and its downstream effects on angiogenesis, vasculogenesis and vascular homeostasis mediated through eNOS phosphorylation by Akt. Moreover, the increased expression of soluble VEGFR-1 (sFlt-1) reduces eNOS activation, contributing to endothelial dysfunction in PE.[13,14] As this study focused only on *eNOS*, *MMP-9* and *VEGF* polymorphisms, further studies should be conducted to examine whether interactions between polymorphisms in other genes affect HDP susceptibility. Moreover, the significantly associated interaction models reported in this study must be replicated in different populations with different genetic backgrounds to validate our results. These findings are relevant because the search for genetic markers associated with HDP may allow early detection of those patients with increased susceptibility to these disease conditions, which are associated with increased morbidity and mortality.[39, 40]

In conclusion, although no significant genotype combinations were associated with GH, we found specific genotype combinations of *MMP-9* and *VEGF* that are more (*MMP-9*-1562CC and *VEGF*-634CC, and combinations containing the *MMP-9*-1562CT genotype) or less common (*MMP-9-1562CC* and *VEGF*-634GG) in the PE group than in the NP group. Taken together, our results suggest an effect of these specific combinations on the genetic susceptibility of patients to PE. Moreover, our study may provide further insights into the etiology of PE and the development of early detection markers of PE.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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