UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" INSTITUTO DE BIOCIÊNCIAS CAMPUS DE BOTUCATU

Interactions between *NOS3* and *HMOX1* on methyldopa responsiveness in preeclampsia

Programa Pós Graduação em Farmacologia e Biotecnologia

(PPG-FARMATEC)

Curso Mestrado

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Orientadora: Valéria Cristina Sandrim

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Resumo

A pré-eclâmpsia (PE) é a principal causa de mortalidade e morbidade entre as gestantes no Brasil e em vários países. A fisiopatologia desta doença é complexa e envolve vários processos. Um destes, amplamente validado na literatura, relaciona-se a um status oxidativo, onde há prevalência de produção de radicais livres e/ou redução da atividade antioxidante. Apesar destas evidências, a suplementação clínica com antioxidantes (vitamina C e E) não se demonstrou promissora em PE. Recentemente, vem sendo explorado como terapia em várias doenças, a ativação de um fator de transcrição, o NRF2 (do inglês - *nuclear factor, erythroid 2-like 2*), que atua induzindo a transcrição de diversos genes que promovem a proteção celular através da codificação de proteínas com atividade desintoxicante e antioxidante. Entre elas a heme oxigenase (HO-1) é a mais estudada, pois apresenta efeitos antiapoptóticos, antioxidantes e citoprotetor.

Além disso, o aumento do estresse oxidativo na PE pode potencialmente reduzir a biodisponibilidade de óxido nítrico (NO) que pode ser modulado por alguns polimorfismos localizados no gene da óxido nítrico sintase (*NOS3*). Notavelmente, os haplótipos formados pela combinação de polimorfismos foram associados a diferentes subgrupos de resposta à terapia anti-hipertensiva em PE.

No presente estudo, comparamos as distribuições dos polimorfismos localizados nos genes *NFE2L2* rs35652124 (C/T) e no gene *HMOX1* rs2071746 (A/T) em gestantes com PE que respondem à metildopa ou à terapia anti-hipertensiva total com aqueles encontrados em gestantes com PE que não respondem à metildopa ou a terapia anti-hipertensiva total. Examinamos se

estes polimorfismos afetam os níveis plasmáticos de HO-1 nestes grupos. Além disso, analisamos se as interações entre os polimorfismos dos *genes NFE2L2, HMOX1* e *NOS3* (rs179998 e rs2070744) estão associadas à PE, e com a capacidade de resposta à metildopa e à terapia anti-hipertensiva total. Nossos principais resultados neste estudo são: (1) o genótipo TT de rs2071746 está associado a não responsividade à metildopa em gestantes com PE; (2); O polimorfismo rs2071746 afeta os níveis plasmáticos de HO-1 em pacientes com PE responsivas à metildopa e à terapia anti-hipertensiva total; (3) interações significativas entre os genótipos dos SNPs *HMOX1* rs2071746 e *NOS3* rs1799983 foram associados à responsividade à metildopa na PE.

Abstract

Preeclampsia (PE) is the leading cause of mortality and morbidity among pregnant women in Brazil and several countries. Its pathophysiology is complex and involves several processes, including the oxidative stress (increase of free radicals and/or decrease of antioxidant defense). Although evidences, clinical supplementation with vitamins (C and E) was not promising in preeclampsia. Currently, therapeutically the activation of transcription factor, NRF2 (*nuclear factor, erythroid 2-like 2*), has been explored in several diseases. This factor promote cytoprotection by activates the transcription of several antioxidant and detoxification proteins. Hemeoxigenase-1 (HO-1) is the most studied, because has antiapoptotic, anti-inflammatory and cytoprotection activities.

In addition, the increased oxidative stress in PE can potentially reduce the bioavailability of nitric oxide (NO) which may impaired by some SNPs on endothelial nitric oxide synthase *(NOS3*) gene. Notably, haplotypes formed by the combination of polymorphisms of were associated with different subgroups of response to antihypertensive therapy in PE. Therefore, in the present study we compared the distributions of rs35652124 (C/T) *NFE2L2* and rs2071746 (A/T) *HMOX1* polymorphisms in PE patients who respond to methyldopa or antihypertensive therapy with those found in PE patients who do not respond to methyldopa or total antihypertensive therapy. We also examined whether *NFE2L2* and *HMOX1* polymorphisms affect plasma HO-1 levels in these subgroups of PE patients. In addition, we examined whether interactions among *NFE2L2, HMOX1* and *NOS3* polymorphisms were associated with PE, and with the responsiveness to methyldopa and total antihypertensive therapy in PE pregnant women.

Our main novel findings are (1) the TT genotype of rs2071746 are associated of nonresponse to methyldopa in PE; (2); rs2071746 affect the plasma HO-1 levels in the methyldopa and total antihypertensive responsive group of PE patients and (3) significant interactions between genotypes of the HMOX1 rs2071746 and NOS3 rs1799983 SNPs were found to be associated with responsiveness to methyldopa in PE.

Sumário

Introdução

A pré-eclâmpsia (PE) é uma síndrome da gravidez caracterizada por hipertensão identificada pela primeira vez após a 20ª semana de gestação, associada a proteinúria (≥ 300mg em urina de 24 horas) ou na ausência de proteinúria a associação do surgimento da hipertensão com plaquetopenia, insuficiência renal, lesão hepática, edema pulmonar e distúrbios neurológicos ou visuais (AMERICAN COLLEGE OF OBSTETRICIANS AND GYNECOLOGISTS; TASK FORCE ON HYPERTENSION IN PREGNANCY, 2013).

Durante a gestação normal são observadas diversas adaptações cardiovasculares na gestante que permitem o suprimento constante de nutrientes e metabólitos ao feto, sem comprometer as necessidades maternas. Estas alterações são resultantes de interações complexas entre hormônios, substâncias vasoativas e vários outros fatores (PRIDJIAN; PUSCHETT, 2002). A gravidez é acompanhada por aumento de todos os fluidos corporais, o volume sanguíneo (VS) aumenta aproximadamente 40-50% (HYTTEN; PAINTIN, 1963; ROVINSKY; JAFFIN, 1965) e a frequência cardíaca (FC) também apresenta elevações de 22-26% (CLAPP; CAPELESS, 1997). Desta maneira, o débito cardíaco, que é resultante do produto da FC x VS, também está aumentado em aproximadamente 30-50% (ROBSON et al., 1989). A elevação do débito permite fluxo constante e flexível conforme as necessidades do feto, além de suprir as necessidades maternas. A partir destas alterações hemodinâmicas, poderíamos esperar um aumento da pressão sanguínea durante a gravidez. No entanto o contrário é observado, ou seja, uma gravidez normal é caracterizada por diminuições da pressão sanguínea sistólica e diastólica até a 16º e 20º semana de gestação (MACGILLIVRAY; ROSE; ROWE, 1969; PAGE; CHRISTIANSON, 1976). Este contrabalanço deve-se a diminuição significativa da resistência vascular periférica devido

predominantemente a vasodilatação, a redução da resposta aos vasoconstritores e a angiogênese (CHESLEY et al., 1965; GANT et al., 1974).

Entretanto, em algumas mulheres a gravidez não é acompanhada por estas alterações hemodinâmicas que permitem a acomodação do volume corpóreo e consequente decréscimo da pressão sanguínea. Nestas mulheres é diagnosticado após a 20º semana de gestação um quadro de hipertensão, caracterizada por pressão arterial sistólica acima de 140 mmHg e a diastólica >90 mmHg em duas tomadas de pressão, com intervalo de 4h, em repouso. Nos casos onde a hipertensão é a acompanhada por proteinúria (0,3 g/L em urina 24 horas) ou outros sinais sistêmicos é caracterizado um quadro de pré-eclâmpsia. É importante ressaltar que mulheres com pré-eclâmpsia não apresentaram manifestações hipertensivas antes da gestação (PRIDJIAN; PUSCHETT, 2002).

O tratamento anti-hipertensivo estabelecido para pré-eclâmpsia envolve a administração de metildopa, nifedipina, hidralazina e labetalol (não disponível no Brasil). Baseado na resposta clínica e laboratorial aos anti-hipertensivos, nosso grupo criou um critério de responsividade, onde subagrupamos pré-eclâmpsia responsiva e não responsiva ao tratamento anti-hipertensivo. Geralmente pacientes não responsivas à terapia hipertensiva apresentam um quadro mais severo da doença (PALEI et al., 2012a, 2012b; SANDRIM et al., 2010a).

A disfunção endotelial sistêmica resultante de eventos complexos e difusos a partir de problemas na perfusão dos trofoblastos e consequente má placentação acarreta em complicações progressivas em todo organismo materno. Os principais sistemas afetados são o vascular, renal, hepático e cerebral. Todas as complicações observadas nestes sistemas podem explicar a alta incidência de mortalidade e morbidade feto-maternal entre as mulheres com pré-eclâmpsia, fazendo destas patologias uma das principais causa de morte materna no Brasil (LAURENTI;

JORGE; GOTLIEB, 2004; SOUBHI KAHHALE; ROSSANA FRANCISCO; MARCELO ZUGAIB, 2018;) e em vários outros países (SAFTLAS et al., 1990; WORLD HEALTH ORGANIZATION, 2011). Sendo assim, o entendimento da fisiopatologia desta doença, assim como seu tratamento são essenciais para diminuição destes números.

- Fisiopatologia: estresse oxidativo e disfunção endotelial

Apesar da fisiopatologia da pré-eclâmpsia ainda ser desconhecida, atualmente é amplamente aceito que a isquemia da placenta é um fator primordial. Sugere-se que a isquemia deste tecido acarrete estresse oxidativo elevado e consequente liberação na circulação materna de fatores placentários (HLADUNEWICH; KARUMANCHI; LAFAYETTE, 2007; MUTTER; KARUMANCHI, 2008; OLIVEIRA; KARUMANCHI; SASS, 2010) levando a uma disfunção endotelial vascular por todo o organismo materno. Várias evidências suportam este entendimento da doença, onde os sinais clínicos e sintomas da pré-eclâmpsia envolvendo a vasculatura materna tem ligação com fatores circulantes. Por exemplo, a incubação in vitro de soro/plasma de mulheres com pré-eclâmpsia induz a injúria de células endoteliais, verificados através de análise de expressão gênica, ensaios funcionais, entre outros (DONKER et al., 2005; MACKENZIE et al., 2012). O início da síndrome se dá pelas alterações placentárias associadas posteriormente a um endotélio materno predisposto. Os mecanismos fisiopatológicos citados acima induzem a formação de fatores que promovem um estado de estresse oxidativo, ou seja, um desequilíbrio onde prevalece a produção de radicais livres e a redução da atividade antioxidante.

Num estado sadio, mecanismos celulares são ativados no sentido de contrabalancear este estresse, produzindo, por exemplo, vários antioxidantes incluindo heme oxigenase-1 (HO-1), superoxido dismutase (SOD) e glutationa peroxidase (GPx). Assim em uma situação de estresse oxidativo, uma gama de genes codificadores de enzimas antioxidantes e citoprotetoras são

ativados, principalmente através da ativação coordenada do Elemento de Resposta aos Antioxidantes (ERA, ou em inglês - *Antioxidant Response Element*) localizado nas regiões promotoras desses genes. Um dos principais fatores de transcrição responsável pela ativação do ERA é o NRF2 (*nuclear factor, erythroid 2-like 2*). Este fator é ativado em resposta ao status oxidante celular e leva a transcrição destes genes, os quais atuam protegendo a célula contra os danos causados pelo desequilíbrio oxidativo ou contra várias toxinas (AL-SAWAF et al., 2015; CHEN et al., 2015).

Na célula endotelial, o NRF2 é considerado um regulador mestre da resposta antioxidante, exercendo papel de proteção do endotélio contra os danos causados por ROS (Espécies reativas de oxigênio do inglês *Reactive Oxygen Species*) (HEISS et al., 2009; HYBERTSON et al., 2011). Em condições normais, o NRF2 está ligado a proteína inibidora Keap-1 (*Kelch-like ECH-associated protein-1*), tornando-se alvo de ubequitinização e degradação proteossomal, mantendo seu nível baixo no citoplasma. Já em condições de estresse, oxidantes endógenos ou pró-oxidantes reagem com o grupo tiol dos resíduos de cisteína presentes na Keap-1, tornando-o oxidado e resultando na dissociação do NRF2 e Keap-1. O NRF2 agora livre é transportado para o núcleo, onde reconhece e se liga ao ERA no DNA, e atuando em sinergia com outros fatores de transcrição, promove a transcrição de genes citoprotetores incluindo a enzima antioxidante hemeoxigenase-1 (HO-1) (CHEN et al., 2015).

O sistema (HO) é um regulador da integridade das células endoteliais e do estresse oxidativo. HO-1 e HO-2, duas isoformas de HO, são vistos como tendo um papel importante na formação de monóxido de carbono (CO), bilirrubina e na quebra do heme. A HO-1 é fortemente induzida pelo estresse oxidativo e seu substrato heme, em conjunto com a capacidade robusta de HO-1, para proteger contra o insulto oxidativo sugere um sistema de compensação ao estresse

oxidativo. Os efeitos antioxidantes de HO-1 surgem da sua capacidade de degradar o heme, bem como da elaboração de biliverdina e bilirrubina, que possuem potentes propriedades antioxidantes. CO, um produto de HO, não é um antioxidante, mas tem efeito antiapoptótico. Além disso, o CO é um vasodilatador que tem demonstrado aumentar a função endotelial e desempenha um papel importante na regulação basal do tônus vascular induzido pela constrição. Assim o HO-1, pode restaurar a homeostase em muitas situações através de suas atividades anti-inflamatória, antiapoptótica e antiproliferativa (TURKSEVEN et al., 2005). Recentes estudos evidenciam o papel protetor da HO-1 e sugerem esta enzima como um novo alvo para o tratamento da pré-eclâmpsia (AHMED, 2011; AHMED; CUDMORE, 2009).

Apesar do conhecimento sobre a participação do estresse oxidativo na fisiopatologia da pré-eclâmpsia até o momento, a investigação sobre este processo foi focada em atividade de enzimas antioxidantes e oxidantes, nos níveis de moléculas endógenas antioxidantes, na quantificação de vitaminas antioxidantes (vitamina C e E), entre outros, sendo os resultados muitas vezes contraditórios (PALEI et al., 2012b, p. 9; SANDRIM et al., 2010c). Com isso o conhecimento da via do NRF-2 e HO-1 através do estudo de variantes genéticos pode contribuir para um melhor entendimento do estresse oxidativo na pré-eclâmpsia.

-Polimorfismos genéticos

A variação genética humana e nosso ambiente são os dois fatores-chave que tornam cada um de nós diferente. Esta variação assume muitas formas, embora essas variantes surjam de apenas dois tipos de eventos de mutação genética. O tipo mais simples de variante resulta de uma mutação de base única que substitui um nucleotídeo por outro (BETTS; RUSSELL, 2003). Os polimorfismos de nucleotídeos únicos (SNPs, do inglês *single nucleotide polymorphism*) são diferenças genéticas únicas entre indivíduos que contribuem de forma significativa para a determinação da

variação humana, incluindo características físicas como altura e aparência, bem como traços menos óbvios como personalidade, comportamento e susceptibilidade às doenças. Além de fatores como idade, gênero e estilo de vida, traços genotípicos à presença de polimorfismos específicos de um único nucleotídeo (SNPs) revelam-se importantes na avaliação e previsão da resposta de um paciente aos medicamentos e a reações adversas. Identificar e caracterizar eficientemente tais variantes genéticas no perfil genotípico de um paciente pode, portanto permitir a adaptação individual da intervenção terapêutica, a seleção de fármacos e a adaptação da dose. Abordagens genômicas têm recebido atenção e investimento porque oferecem potencial para proporcionar uma melhor compreensão dos fatores genéticos na saúde humana e doença, bem como definições mais precisas dos fatores não genéticos envolvidos (VOISEY; MORRIS, 2008).

O ponto de corte arbitrário entre uma mutação e um polimorfismo é de 1%. Isto é, para ser considerado como um polimorfismo, a variação deve ter uma frequência de 1 % ou mais numa determinada população. Se um alelo ocorre a uma frequência inferior a esta porcentagem, o alelo é considerado uma mutação (SRIPICHAI; FUCHAROEN, 2007).

Neste contexto, o gene que transcreve o NRF2 (*NFE2L2*) é polimórfico e apresenta vários polimorfismos. De maneira interessante, alguns SNPs já foram estudados em doenças cardiovasculares (rs35652124) e correlacionados aos níveis pressóricos e em ensaios de vasodilatação (rs6721961 e rs35652124) (KAARTOKALLIO et al., 2014; MARCZAK et al., 2012).

Marczak *et al* , revelaram em seu estudo uma associação significativa entre polimorfismos dentro da região promotora do gene *NFE2L2* e a resposta vasodilatadora em seres-humanos. Através da técnica de pletismografia de oclusão venosa e posterior aplicação de bradicinina e nitroprussiato

de sódio em indivíduos saudáveis (afro-americanos e brancos) avaliou-se o fenótipos vasculares periféricos correlacionados as variantes genéticas deste gene. Nos afro-americanos, os portadores de alelos variantes 653G (rs35652124) apresentaram fluxo sanguíneo do antebraço (FBF) significativamente mais baixo e a resistência vascular (FVR) mais elevada em condições basais bem como em resposta a bradicinina ou nitroprussiato de sódio em comparação aos indivíduos portadores do alelo (A/A) ($P < 0,05$ para cada comparação). Nos indivíduos brancos, embora não tenham sido observadas associações significativas com o genótipo - 653A/G, os portadores do alelo variante 617A (rs 6721961) apresentaram uma FVR significativamente mais elevada no início e em resposta à bradicinina e ao nitroprussiato de sódio em comparação com indivíduos portadores do alelo (C/C). Neste mesmo trabalho, foi realizado um estudo in vitro (utilizando ensaio de luciferase) e observado que culturas celulares transfectadas com o variante (A) apresentava redução de 42% na atividade transcricional em relação as culturas transfectadas com o variante (C).

Com relação ao gene da HO-1 (*HMOX1*), apesar de este apresentar mais de 20 polimorfismos, apenas um estudo avaliou polimorfismo deste gene na pré-eclâmpsia. No caso, o polimorfismo avaliado foi do tipo microssatélite, e o grupo demonstrou que um alelo (GTn longo) presente na região do promotora está associado a certos subtipos de pré-eclâmpsia (KAARTOKALLIO et al., 2014) .

Em outro estudo, Ono *et al.* ao pesquisarem as frequências genotípicas do SNP rs2071746 em uma determinada população revelaram uma frequência do genótipo (A/A) deste SNP significativamente maior nos indivíduos saudáveis (21,3%) do que nos indivíduos com infarto do miocárdio e angina de peito (16,3 e 15,7%, $P = 0,0173$) respectivamente. O ensaio de luciferase

revelou ainda uma atividade transcricional acima de 50% para o alelo (A) quando comparada ao alelo (T) ($P<0,01$) (ONO et al., 2004).

Quanto a enzima oxido nítrico sintase (eNOS), sua participação e importância nos processos fisiológicos que envolvem a produção de NO esta amplamente validada na literatura. Portanto, uma eNOS funcional é essencial para um sistema cardiovascular saudável. A interação entre genes relacionados à produção de NO, estresse oxidativo e disfunção endotelial em PE fornece suporte para hipóteses sobre os mecanismos moleculares subjacentes, que podem guiar a análise de interação entre variantes funcionais que afetam a expressão desses genes. O NO é produzido pela enzima NOS3 no sistema cardiovascular. A expressão reduzida do gene *NOS3* leva à diminuição da formação de NO, que desempenha um papel importante na disfunção endotelial associada à PE (ASIF et al., 2009). Além disso, a PE está associada à diminuição da biodisponibilidade do NO, que está inversamente relacionada aos níveis séricos dos fatores antiangiogênicos como sFlt-1 (*soluble fms-like tyrosine kinase 1*) e sEng (*soluble endogline*) (SANDRIM et al., 2008). Vários polimorfismos do gene *NOS3*, ou as combinações de seus alelos em haplótipos, foram associados com PE e relacionados à resposta a drogas cardiovasculares (SANDRIM et al., 2010a, 2010b).

TITLE PAGE:

Interactions between *NOS3* and *HMOX1* on methyldopa responsiveness in preeclampsia

Short title: *NOS3* and *HMOX1* in antihypertensive therapy

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Author Contributions:

Valeria Sandrim: I declare that I participated in the genetic analysis, study design, and I was involved in the statistical analyses and in the manuscript writing.

Eliane Pilan: Participates of genetic analysis, manuscript writing and revision

Marcelo R Luizon: Participates of genetic analysis, manuscript writing and revision

Georgia Kors: Participates of genotype analysis and manuscript revision.

Iuly Berndt - Participates of genotype analysis and manuscript revision.

Ricardo Cavalli: Participates in the study with recruitment of the patients, samples collection and manuscript revision.

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Interactions between *NOS3* and *HMOX1* on methyldopa responsiveness in preeclampsia

INTRODUCTION

Preeclampsia (PE) is a syndrome characterized by hypertension associated with proteinuria or other systemic signs, and is consider one of the major causal factor for maternal and fetal morbidity worldwide [1]. Several studies have explored the role of an unbalance between antioxidant and oxidant agents in the pathophysiology of PE, as reviewed elsewhere [2]. However, despite these efforts, few clinical studies have analyzed the heme oxygenase-1 (HO-1), a pivotal enzyme that protects cells against oxidative stress [3–8]. HO-1cleaves heme producing bilirubin and carbon monoxide (CO) promoting cell protection, through antioxidant, antiapoptotic and anti-inflammatory properties [9] and in a rat model of PE was found a potential effect of HO-1 in regulating blood pressure levels[10].

Interestingly, a previous study found the association of the long allele of GT_n polymorphism on *HMOX1* gene that code for HO-1, and it is related to lower HO-1 expression [11], with non-severe late-onset PE [12]. However, other *HMOX1* polymorphisms were not examined in PE, with particular focus on single nucleotide polymorphisms (SNPs) located at promoter of *HMOX1*, such as the rs2071746 (A/T). This SNP was associated with protective factor for patients with stroke carriers of the A allele, and associated with higher expression of *HMOX* [13,14].

The nuclear factor-erythroid-derived 2-related factor-2 (Nrf2) regulate the expression of several antioxidant proteins, including HO-1 [15]. A SNP located at the promoter of *NFE2L2* gene

(rs35652124) was found to modulate the forearm vasodilator response in humans [16], and the C allele was associated with higher diastolic blood pressure levels in Japanese women and high risk of cardiovascular mortality in hemodialysis patients [17,18]. Notably, Nrf2 binds to the ARE at *HMOX1* promoter, and can regulated HO-1 expression[15]. Remarkably, gene-gene interactions have been also taken into account in pharmacogenomics studies [19–21]. Therefore, it is possible that combinations of *NFE2L2* and *HMOX1* genotypes may be associated with preeclampsia development and with the responsiveness to antihypertensive therapy in patients with PE. In addition, the increased oxidative stress in PE can potentially scavenge and reduce the bioavailability of nitric oxide (NO) [22] which may impaired by some SNPs on endothelial nitric oxide synthase (*NOS3*) gene. Notably, haplotypes formed by the combination of polymorphisms of were associated with different subgroups of response to antihypertensive therapy in PE [23].

In the present study, we compared the distributions of *NFE2L2* and *HMOX1* polymorphisms in PE patients who respond to antihypertensive therapy with those found in PE patients who do not respond to methyldopa or total antihypertensive therapy. We also examined whether *NFE2L2* and *HMOX1* polymorphisms affect plasma HO-1 levels in these subgroups of PE patients. In addition, we examined whether interactions among *NFE2L2*, *HMOX1* and *NOS3* polymorphisms were associated with PE, and with the responsiveness to methyldopa and total antihypertensive therapy in PE pregnant women.

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 (FMRP), University of Sao Paulo. All pregnant women were enrolled in High Risk Ambulatory, University Hospital at the FMRP. Preeclampsia was defined in accordance to the ACOG [1], with blood pressure (≥140mmHg systolic or ≥90mmHg diastolic at two or more measurements at least 6h apart) associated with severe features in a woman after 20 weeks of gestation. No women with pre-existing hypertension, with or without superimposed PE, were included in the present 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 1mL of whole blood by a salting-out method and stored at –20ºC until analyzed. Plasma was obtained from centrifugation of whole blood in EDTA at 2000g for 10min and stored at –70ºC until assayed.

Antihypertensive treatment and drug response evaluation

The patients in this study were carefully monitored for signs and symptoms of preeclampsia, 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), which were added in case of lack of significant responses to methyldopa. One of the following clinical and laboratory outcomes were considered to classify a

patient as nonresponsive to antihypertensive therapy [23]:

(1) Clinical symptoms including blurred vision, persistent headache or scotomata, persistent right upper quadrant or epigastricpain;

(2) Systolic blood pressure above 140mmHg and diastolic blood pressure above90mmHg, as assessed by the blood pressure curve;

(3) Hemolysis, elevated liver enzymes and a low platelet count (HELLP) syndrome; or proteinuria >2.0g per 24h; creatinine>1.2mg per 100mL or blood urea nitrogen>30mg per 100mL; aspartate aminotransferase >70Ul^{-1} and alanine aminotransferase >60Ul^{-1}; and

(4) Fetal hypoactivity or nonreactive fetus, as revealed by cardio tocography; intrauterine growth restriction, oligoamnio, abnormal biophysical profile score and Dopplervelocimetry abnormalities, as evaluated by ultrasound.

Genotyping

Genotypes for the rs35652124 polymorphism of the *NFE2L2* gene were determined by PCR-RFLP. The forward and reverse primers were respectively: 5'CCTAGAGGAGGTCTCCGTTAG3' and 5'CTGGTACTATTTTGTGAGTACGTG3'. The PCR reaction generated product of 608 bp that was digested with BseRI (New England Biolabs) restriction enzyme. Three bands were visualized when heterozygote 680 bp, 401 bp and 268 bp; and two (401 bp and 268 bp when TT genotype) and 608 bp when CC.

Genotypes for the rs2071746 polymorphism of the *HMOX1* gene were determined by Taqman Allele Discrimination assays (Applied Biosystems, Foster City, CA, USA) using real-time PCR. Probes and primers used in the genotyping were designed by Applied Biosystems (Assay ID: C__15869717_10 for rs2071746). PCR reactions were performed in a total volume of

12 μl (3 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). Results were analyzed with manufacturer's software. The two polymorphisms of *NOS3* were determined using also Allele Discrimination assays as described previously [24].

Enzyme immunoassays of plasma HO-1

HO-1 concentrations were measured in EDTA-plasma using a kit (R&D System), according to manufacturer's instructions.

Statistical analysis

The clinical characteristics of women with 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 *NFE2L2* and *HMOX1* polymorphisms on plasma HO-1 concentrations in the subgroups of PE patients classified as responsive or non-responsive according to antihypertensive therapy were compared one-way ANOVA. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium. The differences in genotype and allele frequencies among subgroups were assessed using χ^2test. A value of $P<0.05$ was considered statistically significant.

Multifactor dimensionality reduction (MDR) identifies interactions of genotypes for their ability to classify them into high and low-risk cells or into responsive or non-responsive groups through cross-validation (CV) steps and permutation testing [25]. We used the robust MDR

approach to characterize these interaction models, which performs constructive induction using a Fisher's Exact Test rather than a predetermined threshold and has the advantage that only statistically significant genotype combinations are considered in the MDR analysis[26]. The best interaction model was the model that had the maximum testing score and CV consistency. Permutation testing was performed to determine the statistical significance of the best model[25,27].

RESULTS

Table 1 summarizes the characteristics of the pregnant women enrolled in this study. HP and PE women showed similar ethnicity (% white), % current smokers, hemoglobin, hematocrit, and creatinine concentrations (all $P>0.05$). PE was older than HP ($P<0.05$). Increased body mass index and fasting glucose was found in PE patients compared with HP ($P<0.05$). We found lower gestational age at delivery (GAD) in PE, lower newborn weights in PE (all $P<0.05$) compared with HP. We found similar levels of plasma HO-1 between HP and PE.

The distribution of genotypes for all the polymorphisms showed no deviation from Hardy-Weinberg equilibrium (all $P>0.05$, data not shown). The *NFE2L2* and *HMOX1* alleles and genotype frequencies distributions are similar between HP and PE ($P>0.05$, Table 2). We then examined the effects of *NFE2L2* and *HMOX1* polymorphisms on plasma HO-1 levels in the groups studied. We were able to measure the HO-1 levels only for 177 HP and 116 PE patients due to we do not have plasma samples of all volunteers. We found no significant differences in HO-1 levels among *NFE2L2* and *HMOX1* genotypes neither in HP nor in PE (Figure S1, $P>0.05$).

According to responsiveness to methyldopa and to total antihypertensive therapy are shown in Table 3, *NFE2L2* polymorphism had no effects on the responses to methyldopa neither to the antihypertensive therapy. However, we found significant association of the *HMOX1*

polymorphism and methyldopa responsiveness (TT genotype is 2.9 more frequent in nonresponsive pregnant, *P=0.02, Table 3)*. Regarding total antihypertensive therapy responsiveness we not found any association with polymorphism. Regarding levels of HO-1, we found that PE genotype as AT (*HMOX1*) and responsive to methyldopa or to the total antihypertensive therapy present lower levels of HO-1 compared to AA genotype (Figure 1, P<0.05). Genotype of *NFE2L2* are not associated with HO-1 levels (Figure S2, P>0.05).

Since we intended to perform interaction analysis among all the polymorphisms, we excluded subjects with missing genotype data for any polymorphism. Then, we examined whether interactions among *NFE2L2*, *HMOX1* and *NOS3* polymorphisms were associated with preeclampsia and responsiveness to methyldopa and total antihypertensive therapy. While we found no significant interactions associated with preeclampsia neither with responsiveness to total antihypertensive therapy (*P*>0.05, Supplementary Table S1 and S2 respectively), we found m,a significant model of interaction among *HMOX1* and *NOS3* genotypes associated with responsiveness to methyldopa in PE (*P*=0.0125, Table 4). The combinations of genotypes are shown in Figure 2. The combinations of the GG genotype for the *NOS3 rs1799983* SNP with the AA and AT+TT genotypes for the *HMOX1* rs2071746 SNP were more frequent in the nonresponsive PE patients. Conversely, the combinations of the GT+TT genotypes for the *NOS3 rs1799983* SNP with the AA and AT+TT genotypes for the *HMOX1*rs2071746 A>T SNP were more frequent in the responsive subgroup of PE patients (Figure 2).

DISCUSSION

This study was the first to examine whether interactions among genes *NFE2L2*, *HMOX1*

and *NOS3* are associated with PE, and with the responses to methyldopa and total antihypertensive therapy in PE. Moreover, this study was the first to evaluate the effect of *NFE2L2* and *HMOX1* polymorphisms on plasma HO-1 levels in both in HP and PE, as responsive and nonresponsive groups to methyldopa and antihypertensive therapy in PE. Our main novel findings are (1) the TT genotype of rs2071746 are associated of nonresponse to methyldopa in PE; (2); rs2071746 affect the plasma HO-1 levels in the methyldopa and total antihypertensive responsive group of PE patients and (3) significant interactions between genotypes of the *HMOX1* rs2071746 and *NOS3* rs1799983 SNPs were found to be associated with responsiveness to methyldopa in PE.

To our knowledge only two studies have measured circulating HO-1 in preeclampsia pregnant compared to healthy pregnant women. In one study [4] higher levels of HO-1 was found while in the other study [3], serum HO-1 levels were similar between healthy pregnant women and mild preeclampsia women, as found by us. Besides we not found difference plasma HO-1 levels among genotypes (both *NFE2L2* and *HMOX1*) in PE neither in HP. While no study has examined whether the polymorphisms studied here affect the responsiveness to methyldopa and total antihypertensive therapy in PE, we have also examined the effects of these polymorphisms on plasma HO-1 levels in PE. We found that the AT genotype for the rs2071746 (*HMOX1)* polymorphism is associated with lower plasma HO-1 levels in PE patients responsive to methyldopa and total antihypertensive therapy in PE.

We found significant interactions among *HMOX1* and *NOS3* polymorphisms associated with responsiveness to methyldopa in PE. Although the single-analysis found that the *HMOX1 SNP* was more frequent in the subgroup of PE patients who are nonresponsive to methyldopa, the combinations with the genotypes for the *NOS3* rs1799983 SNP were associated with both the

responsive and the nonresponsive subgroup of PE patients. These findings suggest that specific combinations of genotypes of the *HMOX1* and *NOS3* SNPs may affect the responses to antihypertensive therapy using methyldopa in PE.

On the interaction of gene polymorphisms until now there is no data in the literature on the interaction of *NOS3* and *HMOX-1* gene polymorphisms under the responsiveness to antihypertensive therapy in preeclamptic pregnant women. However, the interplay among genes related to NO production, oxidative stress and endothelial dysfunction in PE give support for hypotheses about the underlying molecular mechanisms, which may guide further interaction analysis among functional variants affecting the expression of these genes. NO is produced by the NOS3 enzyme in the cardiovascular system. Reduced expression of *NOS3* gene leads to diminished NO formation, which plays a major role in the endothelial dysfunction associated with PE. Moreover, PE is associated with decreased NO bioavailability, which is inversely related to serum levels of the antiangiogenic factors sFlt-1 and sEng in PE[25] . Notably, several *NOS3* polymorphisms, or the combinations of their alleles into haplotypes, were associated with PE[34,35] and related to response to cardiovascular drugs.

There is mounting evidence which suggests a relationship between NO and HO-1 pathways. It seems to be a cross-regulation in which NO can be directly involved in the modulation of HO-1 expression[36] and likewise HO-1 expression may increase NO bioavailability[37]. Studies have shown that different NO-releasing substances can significantly upregulate HO-1 mRNA and protein expression, as well as the enzyme activity in different tissues[38,39,40,41] . In addition, increased endogenous NO derived from stimulated iNOS appears to enhance HO-1 protein expression, which was suppressed in the presence of NOS inhibitors[42,43,44]. These findings show that endogenously generated NO can trigger the expression

of HO-1. Nevertheless, the exact molecular mechanisms involving both exogenous and endogenously formed NO (or NO-related species) active the HO-1 gene are still not clear. On the other hand, according to Hyun-Ock Pae et al[45], there may be three possible mechanisms for vascular NO regulation via HO-1 and its products. One is through modulation of eNOS expression and activity. The eNOS activity can be altered when the concentrations of L-arginine or BH4 are low, a situation called eNOS uncoupling, in which it can generate superoxide (O2-) can react spontaneously with NO, leading to the formation of peroxynitrite (ONOO-) which, therefore, decreases NO bioavailability[46]. A study showed that increased HO-1 expression, via pharmacological Nrf2 activation, down-regulates eNOS expression, thereby contributing to eNOS coupling by ensuring stoichiometric balance between BH4 and eNOS[47]. The second possibility for regulation via HO-1 and its products is reducing NO inactivation by inhibiting sources of O2-production, such as NADPH oxidase[48,49,50,51] or up-regulating antioxidant enzymes, such as superoxide dismutase (SOD) and catalase[52,53,54]. Finally, compensating the loss of NO by CO effects, CO and NO have similar properties, as CO has been shown to reduce vasoconstriction[55] and stimulate vascular relaxation by sGC and cGMP[56].

There is a consensus about NRF2-HO-1 pathway importance in oxidative stress and in cardiovascular diseases. However, thus far, there are few studies on the possible polymorphisms implications evaluated in our study. In 2013, Xiao-li et al evaluated rs2071746 (HMOX1) polymorphism in elderly patients with coronary artery disease, stroke and peripheral arterial occlusive disease receiving regular aspirin therapy (75-160 mg per day). Their results revealed that homozygous or heterozygous patients for T allele were significantly more likely to have aspirin resistance (AT or TT genotype) in the HO-1 gene compared to the wild-type AA genotype [28.] However, other recent work with Chinese women revealed patients with rs 2071746

polymorphism in perimenopause were more susceptible to coronary artery disease (CAD) development. They also concluded that patients with AT genotype in the premenopausal CAD group and those with TT genotype in the postmenopausal CAD group lean towards have less resistance to aspirin [29]. Both studies were based on the evidence of HO-1 being involved in antithrombotic effects of aspirin (Peng L et al, 2004). In 2014 Liping Cao et al concluded that A rs2071746 allele may be a protective factor for patients with atherosclerotic stroke history. However, authors reinforced the need to investigate this polymorphism in larger populations and different ethnic groups [30].

As for NRF2, the data published so far suggest further investigation into details of its regulation in different pathways and pathological processes. Recent work has shown that in addition to HO-1 the NRF2 is responsible for several antioxidant induction and cytoprotective genes[31]. However, NRF2 dual role is already discussed in cancer and in some cardiovascular diseases. Depending on the disease transcription factor accumulation may be therapeutic or harmful [32]. In this scenario, until now we can say that there is still much to investigate the role of HO-1 and NRF2 in preeclampsia. Its importance is agreed in oxidative stress and several studies have been published in order to elucidate the mechanism of these biomolecules in hypertensive and cardiovascular diseases among others. However, the data published are contradictory and require further investigation. These molecules are probably not exclusively cytoprotective or exclusively cytotoxic but may contribute, in a complex and still enigmatic way, to inflammatory balance and repair pathways that determine cells fate and tissues in hostile contexts. This view should moderate the urge to label some genes as anti-oxidants or anti-apoptotic. It should also encourage us to consider protection may depend on activation and/or suppression of several genes (perhaps some still unknown) by weighing other variables such as age, ethnicity, and

gender.

Although there are several findings evidencing the relationship between NO and HO-1, further studies need to be performed in order to explore more possibilities and to fully elucidate all the mechanisms underlying this cross-regulation.

In conclusion, we found evidence indicating that the rs2071746 (*HMOX1)* polymorphism affect the plasma HO-1 levels in methyldopa and total antihypertensive therapy responsive group of PE patients, and we found significant interactions between genotypes of the *HMOX-1 r*s2071746 and *NOS3* rs1799983 and responsiveness to methyldopa treatment. Taken together, our findings suggest that the *HMOX1 and NOS3* polymorphisms may affect HO-1 and NO levels mainly in responsive to methyldopa PE, and may help to understand the interaction among genes in the PE.

Disclosure of Interest

The authors declare no conflict of interest.

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FIGURE TABLES

Figure 1. Plasma HO-1 levels in patients with preeclampsia grouped according to the genotypes for the *HMOX1* polymorphism and responsiveness to methyldopa (responsive A and nonresponsive, B) or total antihypertensive therapy (responsive C and nonresponsive D). The bars show the Boxplot indicates median [min−max]. $^{*}P<0.05$ versus the AA genotype. FIGURE

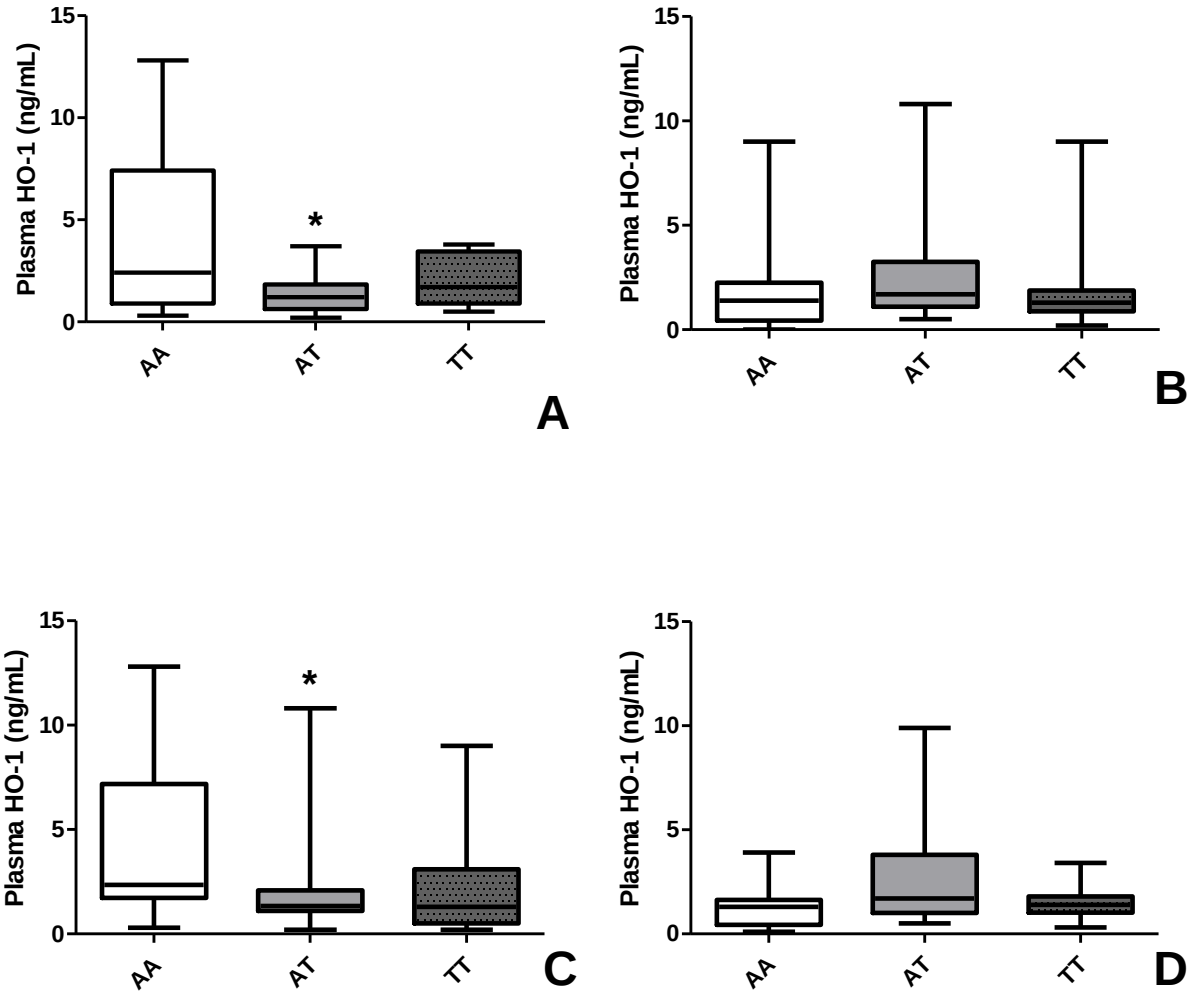

FIGURE 2

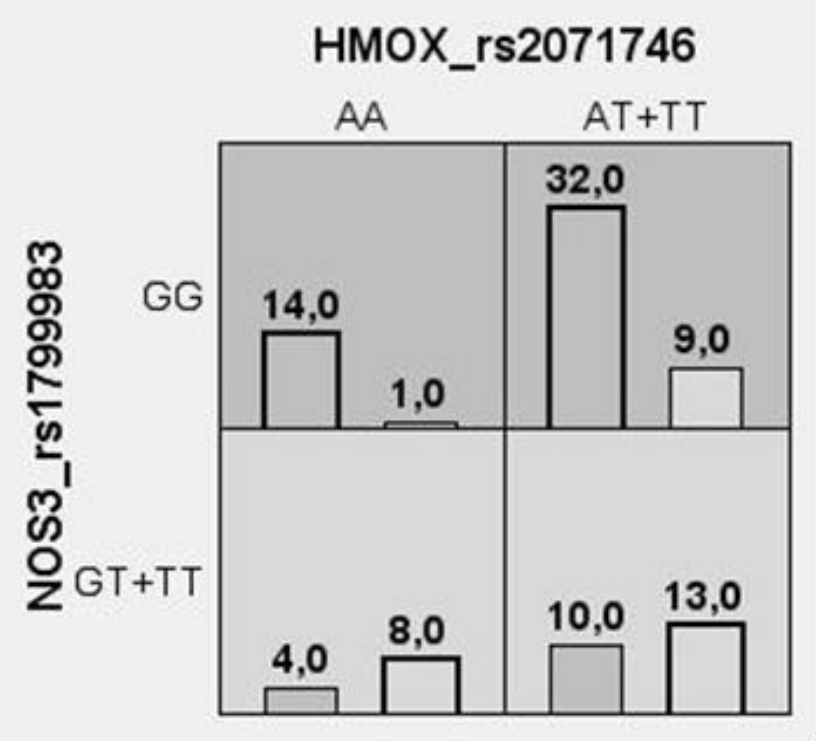

Figure 2 .The best robust MDR model of interaction between genotypes for the HMOX1 rs2071746 A>T and NOS3 rs1799983 G>T (Glu298Asp) polymorphisms when compared PE patients classified according to responsiveness to methyldopa. The distributions of nonresponsive (left bars) and responsive (right bars) patients are illustrated for each combination of multilocus genotypes. The dark grey cells are labelled as high risk or nonresponsive, light grey cells are labelled as low risk or responsive, and white cells are labelled as unknown.

Table 1. Demographic characteristics of study subjects.

Parameters	Healthy Pregnant	Preeclampsia	*P*
	(*n* = 181)	(*n* = 139)	
Age (years)	27.86±0.42	32.76±0.5*	0.0009
Ethnicity (% White)	60.8	69	0.910
CurrentSmokers (%)	11	8.6	0.974
BMI (Kg/m^2)	27.86 ± 0.3	32.76± 0.5	0.000
SBP (mmHg)	111.1±0.80	141.6±1.6*#	0.000
DPB (mmHg)	71.51±0.65	88.34±1.14*#	0.000
HR (beats/min)	82.04±0.65	82.37±0.63	0.716
Fasting Glucose (mg/dL)	75.21±1.04	80.56 ±1.76*	0.008
Hb (g/dL)	11.86 ±0.13	11.88 ±0.11	0.996
Hct (%)	35.66±0.44	35.87 ±0.301	0.686
Creatinine (□mol/L)	66.7 ±2.8	70.4 ±1.6	0.632
24-h Pr (mg/24h)	ND	892.9 ±112.2#	0.000
Primiparity (%)	45.3	42.2	0.684
GAD (weeks)	39.7±0.1	32.8±0.4*	0.000
Newbornweight (g)	3281 ±39	2591.0±69.7*	0.000
GAS (weeks)	36.5±0.3	34.0±0.3*	0.000

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; GAD, gestational age

at delivery; 24-h Pr, 24-h proteinuria; GAS, gestational age at sampling; ND: not determined (however, negative dipstick test).

Values are the mean ± S.E.M.

* $P<0.05$ *vs.* healthy pregnant group.

Table 2. Genotype and allele relative frequencies for *NFE2L2* and *HMOX1* polymorphisms in the study groups.

Genes and their Polymorphisms	Genotypes and alleles	HP (%)	PE (%)	OR (95% CI)	*P*
NFE2L2	TT	41	43	1.00 (reference)	-
rs35652124	TC	46	45	0.933 (0.515 - 1.688)	0.880
T>C	CC	13	13	0.953 (0.395 - 2.299)	1.000
	T	64	65	1.00 (reference)	-
	C	36	35	0.957 (0.536 - 1.709)	1.000
HMOX1	AA	26	27	1.00 (reference)	-
rs2071746	AT	49	49	0.963 (0.493 - 1.879)	0.963
A>T	TT	25	24	0.924 (0.425 - 2.011)	0.924
	A	52	52	1.00 (reference)	-
	T	48	49	1.041 (0.597 - 1.813)	1.000

Table 3. Genotype and allele relative frequencies for *NFE2L2* and *HMOX1* polymorphisms according to responsiveness to methyldopa or to the total antihypertensive therapy.

	Genotype or Allele	*Methyldopa responsiveness*				Antihypertensive therapy *responsiveness*			
		R (%)	NR (%)	OR (95% CI)	*P*	R (%)	NR (%)	OR (95% CI)	*P*
NFE2L2	TT	48	40	1.00 (Reference)	–	47	37	1.00 (Reference)	–
rs35652124	TC	41	47	1.376 (0.760 - 2.489)	0.365	42	48	1.452 (0.798 - 2.639)	0.229
T>C	CC	11	13	1.418 (0.573 - 3.510)	0.495	11	15	1.732 9 (0.712 - 4.216)	0.265
	T	68	64	1.00 (Reference)	–	68	61	1.00 (Reference)	–
	C	32	36	1.195 (0.665 - 2.148)	0.654	32	39	1.359 (0.759 - 2.430)	0.375
HMOX1	AA	28	26	1.00 (Reference)	–	28	24	1.00 (Reference)	–
rs2071746	AT	61	44	0.776 (0.401 - 1.503)	0.502	52	45	1.010 (0.513 -1.985)	1.000
A>T	TT	11	30	2.937 (1.226 - 7.033)	0.020	20	31	1.808 (0.826 - 3.958)	0.168
	A	58	47	1.00 (Reference)	–	54	47	1.00 (Reference)	–
	T	42	53	1.555 (0.890 - 2.722)	0.156	46	53	1.324 (0.759 - 2.308)	0.396

Abbreviations: CI, confidence interval; *NFE2L2*, Nuclear Factor, Erythroid 2 Like 2; *HMOX1*, Heme oxygenase-1; R-responsive; NR, non-responsive; OR, Odds Ratio.* P<0.05 *vs.* responsive

Table 4. Robust MDR interaction model among the *NFE2L2*, *HMOX1* and *NOS3* polymorphisms in PE patients classified as nonresponsive and responsive to methyldopa.

Interaction Models	Training score	Testing score	CVC	*P*-value
*NOS3*rs1799983	0.7220	0.7220	10/10	–
HMOX1 rs2071746; *NOS3* rs1799983	0.7186	0.6898	7/10	0.0125*
HMOX1 rs2071746; *NOS3* rs2070744*; NOS3* rs1799983	0.7183	0.6263	6/10	0.1565
NFE2L2 rs35652124; *HMOX1* rs2071746; *NOS3* rs2070744*; NOS3* rs1799983	0.5799	0.4572	10/10	0.9420

Abbreviations: CVC, cross-validation consistency; GH, gestational hypertension; HP, healthy pregnant; PE, preeclampsia; *HMOX1*, Heme oxygenase-1;MDR, Multifactor dimensionality reduction; *NFE2L2*,Nuclear Factor, Erythroid 2 Like 2*; NOS3, nitric oxide synthase 3*

* *P*-value after 1.000 permutations.

Table 5. Robust MDR interaction model among the *NFE2L2, HMOX1* and*NOS3* polymorphisms in PE patients classified as nonresponsive and responsive to total antihypertensive therapy.

Interaction Models	Training score	Testing score	CVC	*P*-value
NOS3 rs1799983	0.6383	0.6288	10/10	–
NOS3 rs2070744*;NOS3* rs1799983	0.6631	0.6321	7/10	0.1155
NFE2L2 rs35652124; *NOS3* rs2070744*; NOS3* rs1799983	0.6692	0.5870	9/10	0.3825
NFE2L2 rs35652124; *HMOX1* rs2071746; *NOS3* rs2070744*; NOS3* rs1799983	0.5412	0.4805	10/10	0.8870

Abbreviations: CVC, cross-validation consistency; GH, gestational hypertension; HP, healthy pregnant; PE, preeclampsia; *HMOX1*, Heme oxygenase-1;MDR, Multifactor dimensionality reduction; *NFE2L2*,Nuclear Factor, Erythroid 2 Like 2*; NOS3,* Endothelial nitric oxide synthase.

* *P*-value after 1.000 permutations.

Table 6. Robust MDR interaction model among the *NFE2L2, HMOX1* and *NOS3* polymorphisms when PE patients were compared with HP subjects.

Interaction Models	Training score	Testing score	CVC	*P*-value
NOS3 rs2070744	0.5652	0.5469	9/10	–
NFE2L2 rs35652124*; HMOX1* rs2071746	0.5912	0.5620	9/10	0.3620
NFE2L2 rs35652124; *HMOX1* rs2071746; *NOS3* rs207074*;*	0.6139	0.5207	6/10	0.6795
NFE2L2 rs35652124; *HMOX1* rs2071746; *NOS3* rs2070744*; NOS3* rs1799983	0.6401	0.5807	10/10	0.2235

Abbreviations: CVC, cross-validation consistency; GH, gestational hypertension; HP, healthy pregnant; PE, preeclampsia; *NFE2L2*, Nuclear Factor, Erythroid 2 Like 2*; HMOX1*, Heme oxygenase-1; *NOS3*, Endothelial nitric oxide synthase*;* MDR, Multifactor dimensionality reduction.

* *P*-value after 1.000 permutations.

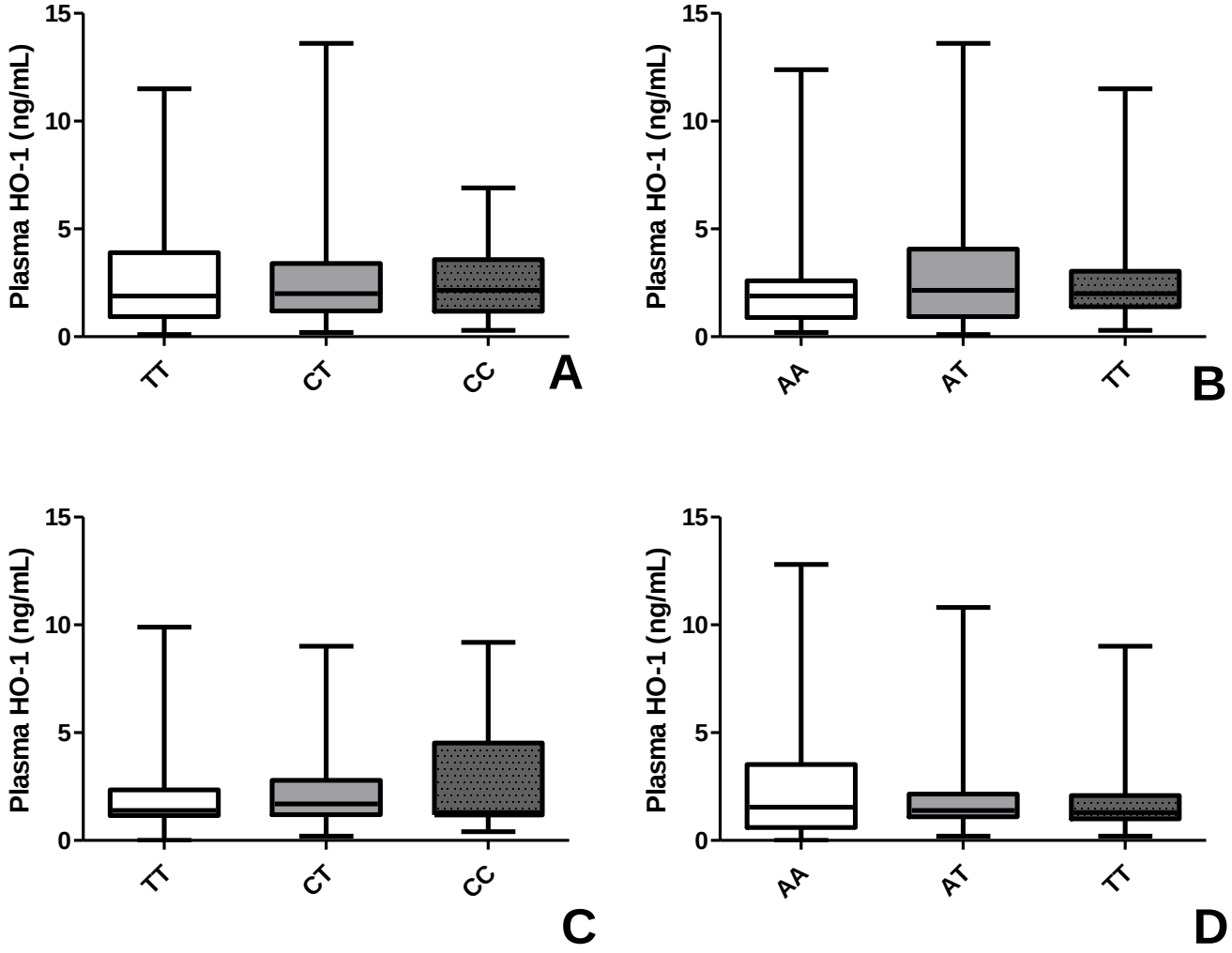

Figure S1. Relation between genotypes verses levels of HO-1. (A*) NFE2L2* in HP (A) e PE (C). *HMOX-1* in HP (B) and PE (D). There were no significant diferences.

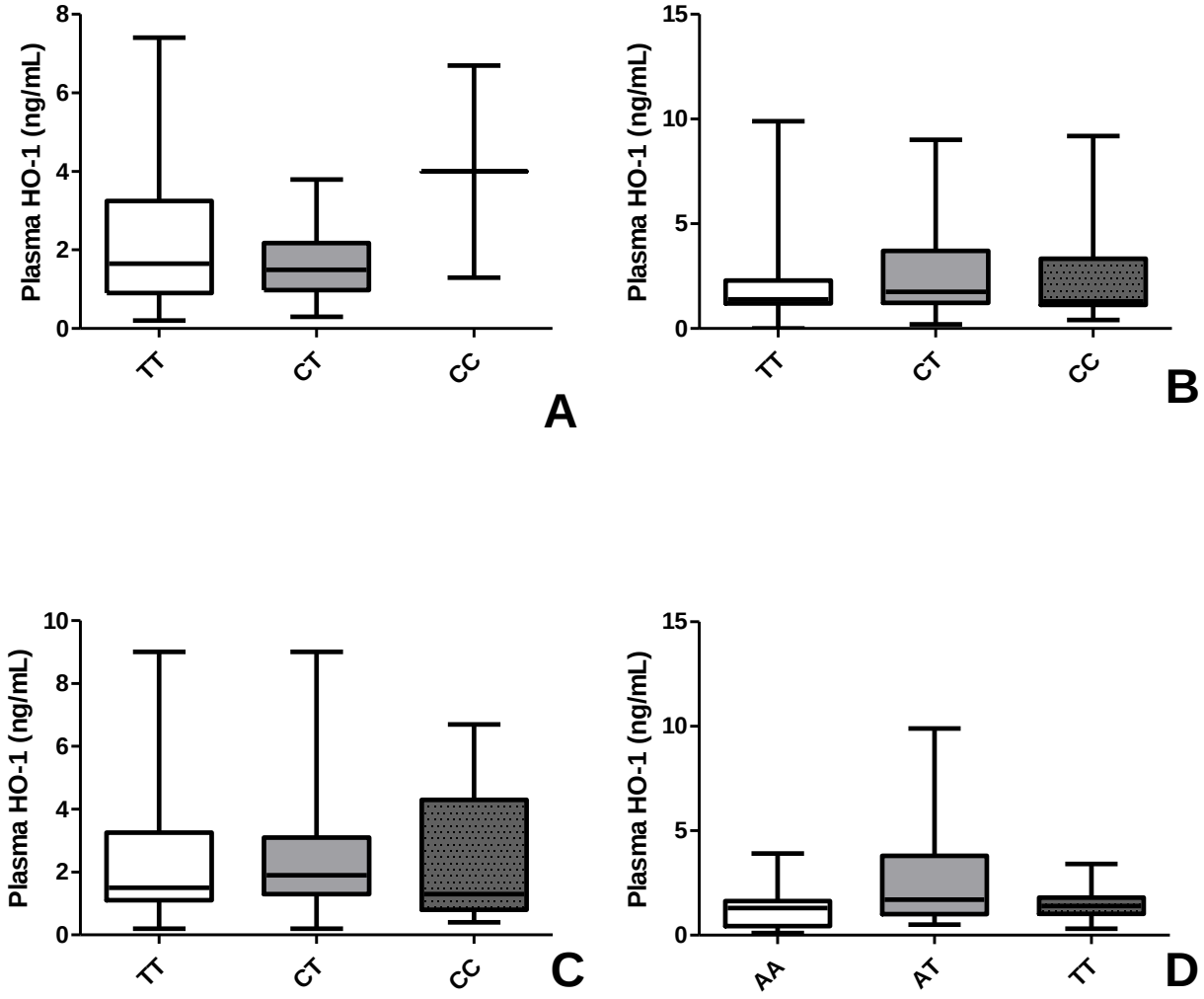

Figure S2 Effect of *NFE2L2* SNP on HO-1 in responsiveness to methyldopa (A) and nonresponsive to methyldopa (B). In C, responsive total therapy and nonresponsive D.

Conclusões:

Em conclusão, neste trabalho encontramos evidências de que o polimorfismo rs2071746 *(HMOX1*) afeta os níveis plasmáticos de HO-1 em gestantes com PE responsivas à metildopa e à terapia anti-hipertensiva total . Encontramos também interações significativas entre os genótipos do *HMOX-1* rs2071746 e *NOS3* rs1799983 sob a responsividade ao tratamento com metildopa. Em conjunto, nossos achados sugerem que os polimorfismos dos genes *HMOX1* e *NOS3* podem afetar os níveis de HO-1 e NO principalmente em resposta à metildopa em gestantes com PE, e podem ajudar a entender a interação entre esses genes na pré-eclâmpsia.

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