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Dissertação de Mestrado

CAROLINE CRISTINA PINTO-SOUZA

ESTUDO DOS POLIMORFISMOS GENÉTICOS DA ARGINASE NA PRÉ-ECLÂMPSIA

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia,
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para obtenção do título de Mestre em Biotecnologia,
Área de concentração: Genética Humana e Médica/Farmacogenética.

Orientadora: Profa. Dra. Valéria Cristina Sandrim
Programa de Pós-Graduação em Biotecnologia, IBB-UNESP

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Palavras-chave: Arginase; Nitrito; Óxido nítrico; Polimorfismos genéticos; Pré-eclâmpsia.

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(Salmos 37:5-7)

PRÓLOGO

Esta dissertação será apresentada na forma de uma breve introdução acerca da desordem hipertensiva gestacional denominada pré-eclâmpsia, bem como, os aspectos envolvidos na fisiopatologia desta doença, como a disfunção endotelial, a relação entre a biodisponibilidade de óxido nítrico com a arginase e com os polimorfismos genéticos das duas isoformas (1 e 2) desta enzima. E na sequência, dois manuscritos relativos ao projeto de dissertação consoante as normas da revista *Nitric Oxide* à qual foram submetidos para publicação.

Lista de abreviaturas e siglas

ARG1 **-** gene que codifica a arginase 1

ARG2 - gene que codifica a arginase 2

ADMA - dimetilarginina assimétrica

BEC - S-(2-bromoetil)-L-cisteína

DE - disfunção endotelial

ELISAs - Enzyme linked immunoassays

eNOS - óxido nítrico sintase endotelial

FC - frequência cardíaca

GMPc - monofosfato de guanosina cíclica

GS - gestação saudável

hCG - gonadotrofina coriônica humana

HMOX-1 - gene que codifica a heme oxygenase-1

HP - healthy pregnant

IL-6 - interleucina 6

iNOS - óxido nítrico sintase induzída

MMP-9 - gene que codifica a matrix metaloproteinase-9

NADPH - fosfato de dinucleótido de nicotinamida e adenina

NO - óxido nítrico

NOS - óxido nítrico sintase

NOS3 - Óxido Nítrico Sintase 3, gene que codifica a eNOS

nNOS - óxido nítrico sintase neuronal

PE - pré-eclâmpsia

PlGF - fator de crescimento placentário

rs - referential snp

sEng - endoglina solúvel

SHR - spontaneously hypertensive rats

sFlt-1 - tirosina quinase fms solúvel

SNP - single nucleotide polymorphism

TGF - fator de crescimento transformador

TNF - fator de necrose tumoral

VEGF - fator de crescimento endotelial vascular

VS - volume sanguíneo

WKY - Wistar–Kyoto

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INTRODUÇÃO

CONTEXTO E PROBLEMA

Alterações vasculares observadas na gestação normal e na pré-eclâmpsia

Durante a gestação normal ocorrem diversas adaptações cardiovasculares na gestante que permitem o suprimento constante de nutrientes e metabólitos ao feto, sem comprometer as necessidades maternas. Estas alterações resultam de interações complexas entre hormônios, substâncias vasoativas e outros fatores, levando ao aumento de todos os fluidos corporais, havendo crescimento do volume sanguíneo (VS) em aproximadamente 40-50% e da frequência cardíaca (FC) em 26%. Desta maneira, o débito cardíaco, que é produto da FC x VS, se eleva em aproximadamente 30-50%. Esta ampliação permite fluxo constante e flexível conforme as necessidades do feto, além de suprir as maternas (ROVINSKY; JAFFIN, 1965; ROVINSKY; JAFFIN, 1966a; ROVINSKY; JAFFIN, 1966b)

A partir destas modificações hemodinâmicas deveria ocorrer um crescimento da pressão sanguínea durante a gravidez. No entanto, se observa o contrário, ou seja, a gravidez normal é caracterizada por diminuição da pressão sanguínea sistólica e diastólica até a 20ª semana de gestação (MACGILLIVRAY; ROSE; ROWE, 1969; PAGE; CHRISTIANSON, 1976). Este contrabalanço se deve à redução significativa da resistência vascular periférica devido, predominantemente, à vasodilatação, à diminuição da resposta aos vasoconstritores e à angiogênese (CHESLEY et al., 1965; ZUSPAN et al., 1971).

Entretanto, em algumas mulheres a gravidez não é acompanhada por tais alterações hemodinâmicas, nas quais se diagnostica, após a 20º semana de gestação, a associação de hipertensão arterial, proteinúria, disfunção hepática, comprometimento da coagulação, entre outros sinais e sintomas, caracterizando a pré-eclâmpsia (PE) (BROWN et al., 2018). A elevação da pressão sanguínea em gestantes tem efeitos deletérios sobre os sistemas vascular, hepático, renal e cerebral. Todas as complicações observadas nestes sistemas explicam a alta incidência de morbidade materno-fetal entre as mulheres que apresentam PE, tornando esta patologia uma das principais causas de morte materna no Brasil (37% das causas de morte obstétricas diretas) (LAURENTI; JORGE; GOTLIEB, 2009) e em vários outros países (LO; MISSION; CAUGHEY, 2013).

Fisiopatologia da PE

Apesar da fisiopatologia da PE ainda não estar totalmente esclarecida, atualmente é amplamente aceito que a isquemia da placenta é um fator primordial.

Durante o início do segundo trimestre da gestação (18ª–20ª semana) se instala um processo referido como *pseudovasculogenesis,* o qual é caracterizado pela migração dos citotrofoblastos em direção às arteríolas uterinas espiraladas, onde sofrem diferenciação em células com fenótipo endotelial. Neste processo ocorre remodelamento gradual da camada endotelial destes vasos e destruição do tecido elástico-muscular, tornando-os mais dilatados e ausentes de resposta às substâncias vasoativas (BROWN et al., 2018; PHIPPS et al., 2019).

Esta migração/diferenciação dos citotrofoblastos se deve às modificações nos perfis de expressão de certas citocinas, moléculas de adesão, constituintes da matriz extracelular, metaloproteinases e o antígeno de histocompatibilidade (FISHER; DAMSKY, 1993; DAMSKY; FISHER, 1998). O remodelamento das artérias uterinas espiraladas resulta na formação de um sistema local de baixa resistência arteriolar que é essencial ao aumento do suprimento sanguíneo para o desenvolvimento e crescimento do feto. Na PE, a invasão das artérias espiraladas do útero se encontra limitada, sendo que apenas 30-50% delas sofrem a invasão do trofoblasto (VON DADELSZEN; MAGEE; ROBERTS, 2003).

A média do diâmetro das artérias espiraladas de gestantes pré-eclâmpticas corresponde à metade daquela observada na gravidez normal. Esta falência do remodelamento vascular impede uma resposta adequada à alta demanda do fluxo sanguíneo que ocorre durante a gestação, diminuindo assim, a perfusão útero-placentária. Deste modo, provocando isquemia da placenta, resultando na hipóxia do tecido (falta ou ausência de oxigenação) (Figura 1) (RANA; BURKE; KARUMANCHI, 2020).

Entretanto, qual seria a origem da PE? Por que em algumas mulheres a migração e diferenciação dos citotrofoblastos estão comprometidas? Esta pergunta permanece sem resposta. Propõe-se que fatores maternos relacionados à predisposição genética, má adaptação imunológica à gravidez e doenças vasculares pré-existentes possam estar envolvidas neste distúrbio hipertensivo. Logo, é provável que haja diversas etiologias ou predisposições desta doença com efeitos que resultam num grupo comum de sinais e sintomas que caracterizam a PE (VON DADELSZEN; MAGEE; ROBERTS, 2003; MOL et al., 2016).

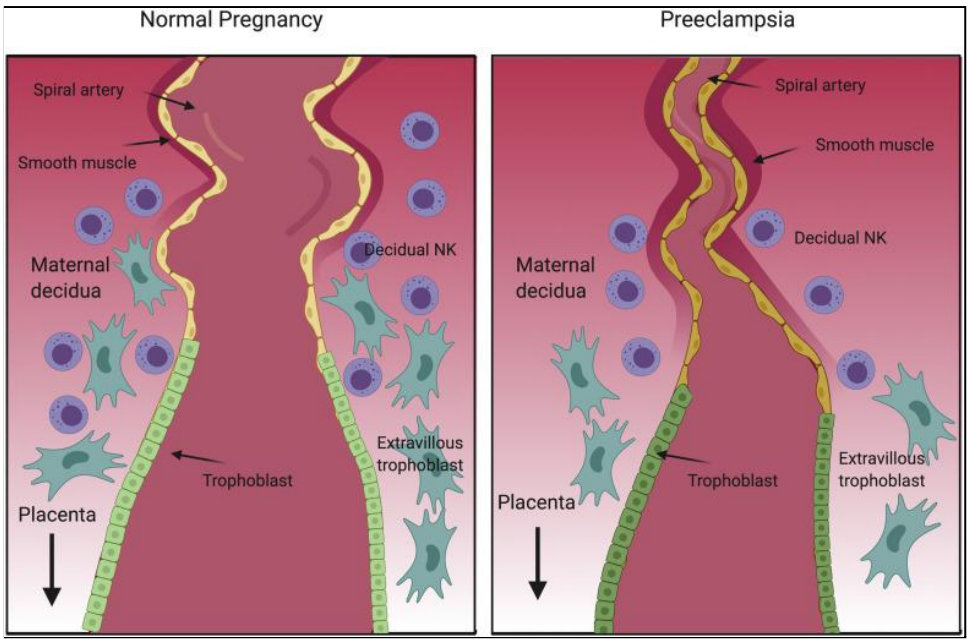

Figura 1. Representação do remodelamento das artérias espiraladas na gestação normal e na pré-eclâmpsia. (Imagem reproduzida de RANA; BURKE; KARUMANCHI, 2020).

Como citado acima, existem indicações de que a isquemia placentária seja o gatilho que leva às circunstâncias clínicas notadas na PE. Sugere-se que a isquemia desse tecido acarrete a liberação na circulação materna de fatores que modificam a função endotelial da grávida alterando o balanço entre as substâncias vasodilatadoras, como o óxido nítrico (do inglês, *nitric oxide*, NO), a prostaciclina, e induzindo aumento de responsividade aos potentes vasoconstritores angiotensina II e endotelina (ROBERTS, 1998; IVES et al., 2020). Dados obtidos em vários estudos (ROBERTS, 1998; BRENNAN; MORTON; DAVIDGE, 2014; GOULOPOULOU; DAVIDGE, 2015; IVES et al., 2020) pressupõem que a disfunção endotelial (DE) generalizada observada na PE é a principal causa das anormalidades clínicas percebidas nesta doença.

Especificamente, a perda do controle do tônus vascular provoca hipertensão, eleva a permeabilidade vascular glomerular, desencadeando a proteinúria (excreção acima de 300 mg na urina de 24 horas). Também, a expressão endotelial alterada de fatores da coagulação resulta na coagulopatia (PINHEIRO; GOMES; DUSSE, 2013). O soro de mulheres com PE apresenta concentrações elevadas de marcadores de DE, como o fator de necrose tumoral (TNF), interleucina (IL-6), produtos de lipídeos oxidados, estresse oxidativo e dimetilarginina assimétrica (ADMA) (POWE; LEVINE; KARUMANCHI, 2011; BROWN et al., 2018; PHIPPS et al., 2019).

Por conta da vasodilatação ser essencial para acomodar o aumento do volume sanguíneo verificado na gravidez, é esperado que os mediadores que provocam vasodilatação possuem função essencial neste processo. E como na PE se observa a DE e a vasoconstrição generalizada, é possível que as ações destes mediadores estejam comprometidas.

Papel do NO na gestação normal e na PE

O principal regulador do tônus vascular é o NO. Este gás, no endotélio (camada mais interna dos vasos sanguíneos), é sintetizado pela sintase endotelial do óxido nítrico (eNOS) através da conversão do aminoácido L-arginina e oxigênio em L-citrulina e NO (KRÓL, M.; KEPINSKA, M., 2020), sendo também produzido pelas isoformas neuronal (nNOS) e induzível (iNOS) (DUSSE et al., 2003). Pelo intermédio do segundo mensageiro monofosfato de guanosina cíclica (GMPc), o NO provoca o relaxamento do músculo liso vascular, levando, por conseguinte, à queda da liberação de cálcio dentro da célula (DUSSE et al., 2003; FÖRSTERMANN; SESSA, 2012).

Vários trabalhos vêm demonstrando que a modulação da via arginina/NO/GMPc desempenha um importante papel na gravidez (KHALIL, HARDMAN, 0'BRIEN, 2015; HSU; TAIN, 2019; SUTTON; GEMMEL; POWERS, 2020). De maneira geral, durante uma gestação normal, é constatada uma elevação dos níveis de NO pela atividade da eNOS (KHALIL; HARDMAN; 0'BRIEN, 2015; SUTTON; GEMMEL; POWERS, 2020). A causa específica deste aumento é desconhecida, porém, é sugerido que o aumento da tensão de cisalhamento (KHANKIN et al., 2021) e de certos hormônios, tais como, o 17-β-estradiol (WIECZOREK; BREWER; MYATT, 1995) e gonadotrofina coriônica humana (hCG) (VAN VOORHIS et al., 1995) estimulem a atividade desta enzima. Especificamente na placenta, a atividade da eNOS é imprescindível, no sentido que o NO sintetizado mantém localmente a resistência vascular baixa além de atenuar a ação dos vasoconstritores (BAYLIS, 2006). Tem sido proposto que a diminuição na produção de NO na placenta poderia acarretar perfusão anormal tecidual, o que é evidenciado nos estados da PE (Figura 2) (KHALIL; HARDMAN; 0'BRIEN, 2015).

Com relação à produção sistêmica de NO, foi demonstrado que a vasodilatação da artéria braquial mediada pelo fluxo dependente deste gás era aproximadamente três vezes menor em grávidas com PE comparadas às grávidas normais (COCKELL; POSTON, 1997).

Artérias de mulheres pré-eclâmpticas também apresentam déficit da vasodilatação mediada por NO em estudos de tecido isolado em banho de órgão (ANUMBA et al., 1999).

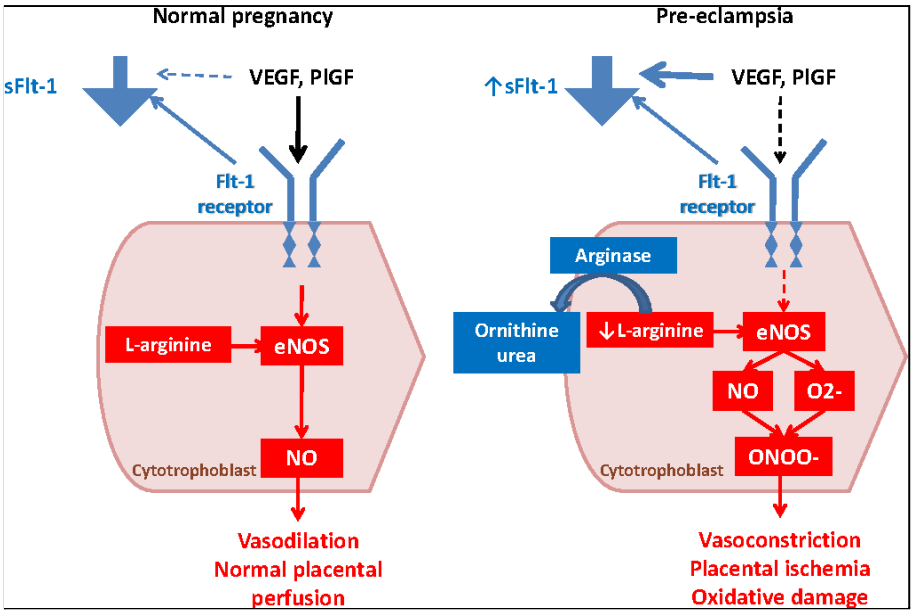

Figura 2. A interação entre L-arginina e Óxido nítrico na patogênese da pré-eclâmpsia (Imagem reproduzida de KHALIL; HARDMAN; 0'BRIEN, 2015).

O grupo de pesquisa de Sandrim et al. (2008) foi pioneiro em quantificar os níveis plasmáticos de nitrito (um biomarcador mais fidedigno usado para avaliar a biodisponibilidade de NO) em gestantes com PE, hipertensas gestacionais comparando-as com gestantes saudáveis. No artigo publicado no *Journal of Hypertension,* os autores reportaram de modo inédito na literatura, que os dois primeiros grupos têm níveis diminuídos de nitrito total e plasmático (SANDRIM et al., 2008). A partir deste dado, analisaram os fatores, importantes na síndrome hipertensiva, que poderiam estar regulando os níveis circulantes de NO. Assim verificaram:

1) Fatores anti-angiogênicos [tirosina quinase fms solúvel (sFlt-1) e endoglina solúvel (sEng)] altamente expressos e relacionados com a PE (MAYNARD; KARUMANCHI, 2011). A sEng é um co-receptor da superfície celular para os membros da família do fator de crescimento transformador (TGF), como TGF-β1 e TGF-β3, os quais atuam como potentes inibidores da diferenciação e migração do trofoblasto (JONES et al., 2006). Além disso, a sEng prejudica a ligação de TGF-β1 a seus receptores e sinalização, dificultando a ativação da eNOS e vasodilatação (VENKATESHA et al., 2006).

Durante a gravidez normal, a homeostase dos vasos é mantida por níveis fisiológicos de sinalização do fator de crescimento endotelial vascular (VEGF) e fator de crescimento placentário (PLGF). Na PE, o excesso de secreção placentária de sFlt-1 inibe a sinalização de VEGF e PlGF na vasculatura. Isto acarreta em disfunção da célula endotelial, incluindo a diminuição de prostaciclinas, da produção de NO e liberação de proteínas pró-coagulantes, levando às manifestações clínicas observadas na PE (KHALIL; HARDMAN; 0'BRIEN, 2015). Sandrim et al. (2008) encontraram correlações negativas entre os níveis circulantes destes dois marcadores anti-angiogênicos e nitrito. Logo, propuseram que na PE, parte da diminuição nos níveis de NO poderia se dar pelo aumento de sFlt-1 e sEng;

2) ADMA - inibidor endógeno da NOS.

Várias evidências apontam o efeito do inibidor endógeno da NOS, ADMA que compete com a NOS pelo substrato, a L-arginina. Altos níveis de ADMA são encontrados em doenças cardiovasculares, incluindo a PE (SAVVIDOU et al., 2003). Porém, até o momento da publicação de Sandrim et al., (2010c), faltava na literatura, trabalhos que mostrassem se o alto nível de ADMA em PE se correlacionaria com a biodisponibilidade de NO. Desta maneira, os autores demonstraram que os níveis destes dois biomarcadores se correlacionaram negativamente, indicando que uma causa da redução de nitrito na PE poderia ser devido à alta concentração de ADMA nestas gestantes (SANDRIM et al., 2010c).

3) Consumo de NO.

Nenhuma pesquisa prévia havia explorado se a baixa biodisponibilidade de NO se associaria ao consumo de moléculas presentes no plasma, com por exemplo, a hemoglobina livre. Sabia-se que o aumento dos níveis intravasculares de hemoglobina conseguiria reduzir a biodisponibilidade de NO, e também, a difusão para musculatura lisa vascular, diminuindo a vasodilatação (REITER et al., 2002). Assim, através de um ensaio que avalia o consumo de NO, Sandrim et al. (2010a) observaram que o plasma de gestantes com PE consumia 63% a mais desta molécula gasosa em comparação ao ensaio com plasma de gestante saudável. Além disso, viram que os níveis de hemoglobina livre nestas mulheres eram 53% maiores. Na subsequência, correlacionaram estes dois fatores e observaram uma correlação positiva entre hemoglobina plasmática e consumo de NO ($r = 0,61$, $P < 0,0001$) (SANDRIM et al., 2010a).

4) Polimorfismos localizados no gene que codifica a eNOS (*NOS3*)

Caracterizados pela alteração na sequência de nucleotídeos, na disposição do DNA, os polimorfismos genéticos estão presentes em uma determinada população com frequência alélica superior a 1%, todavia, sem causar danos letais. (NUSSBAUM; MCINNES; HUNTINGTON, 2008). À vista disso, outra condição com possibilidade de modular os efeitos de NO nas gestantes pré-eclâmpticas é a presença de variantes localizadas no gene que codifica a eNOS, o Óxido Nítrico Sintase 3 (*NOS3*). Sandrim et al. (2010d) verificaram que haplótipos - combinação de alelos, da *NOS3,* se associam aos níveis plasmáticos de nitrito em gestantes. E que diferentes haplótipos deste gene se encontram vinculados à resposta clínica às drogas anti-hipertensivas, segundo critério definido por Sandrim et al., (2010b) de respondedor e não-respondedor.

Estudos da arginase em PE

Outro fator relacionado aos níveis de NO e pouco explorado na PE é a arginase (BERKOWITZ et al., 2003), uma enzima presente em diversos organismos (DZIK, 2014) e encontrada no endotélio vascular e nas células do músculo liso (CHOI et al., 2012; PERNOW; JUNG, 2013). Uma de suas ações primárias é a catálise da conversão da L-arginina em L-ornitina e uréia (ASH, 2004). A expressão da arginase é estimulada por uma variedade de fatores como: TNF, interferon-ɣ, interleucinas (MORRIS, 2005; DURANTE; JOHNSON; JOHNSON, 2007), angiotensina II (TOQUE et al., 2010), espécies reativas de oxigênio e nitrogênio (THENGCHAISRI et al., 2006), entre outras. A arginase apresenta-se em duas formas, a arginase I (citosólica) e expressa principalmente no fígado; a arginase 2 (mitocondrial) é altamente expressa nos rins. Sendo que ambas são localizadas no endotélio da vasculatura, possuem mecanismos semelhantes, metabólicos idênticos e requerem o manganês como cofator (WU; MORRIS, 1998; CALDWELL et al., 2015).

Estas enzimas podem ser expressas em vários tipos de células diferentes e induzidas por uma ampla variedade de agentes e condições, dependendo do tecido e da espécie. A atividade da arginase tem dois objetivos homeostáticos principais: primeiro, livrar o corpo da amônia por meio da síntese de ureia e, segundo, produzir ornitina, o precursor das poliaminas e prolinas (Figura 3) (WU; MORRIS, 1998; CALDWELL et al., 2015).

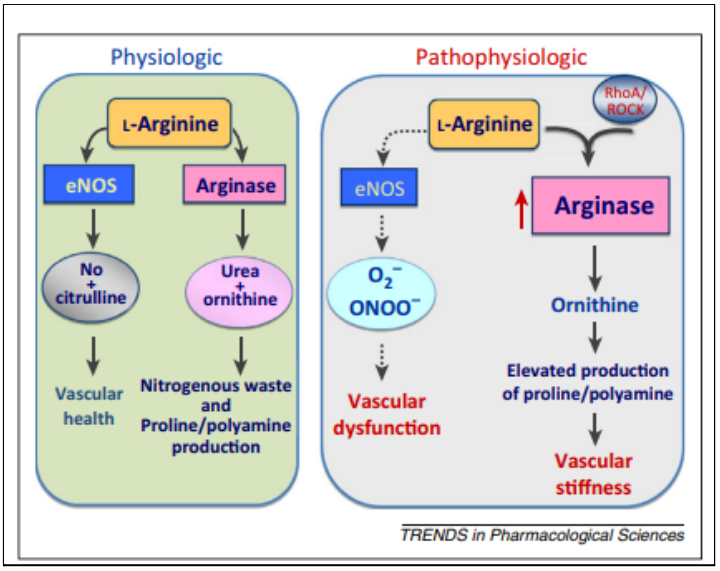

Figura 3. Atividades fisiológicas e fisiopatológicas da eNOS e arginase. (Imagem reproduzida de CALDWELL et al., 2015).

Estas enzimas estão co-localizadas com a eNOS no endotélio (CAMA et al., 2003) e competem com a NOS pelo mesmo substrato (PERNOW; JUNG, 2013; DURANTE; JOHNSON; JOHNSON, 2007). Assim, altos níveis de atividade da arginase poderiam levar à diminuição da biodisponibilidade do substrato da NOS (L-arginina) decrescendo, portanto, a síntese de NO (BERKOWITZ et al., 2003; DURANTE; JOHNSON; JOHNSON, 2007; PERNOW; JUNG, 2013; QUITTER et al., 2013). Esta competição pelo substrato vem sendo demonstrada em algumas condições referentes à disfunção cardiovascular (PERNOW; JUNG, 2013; QUITTER et al., 2013; JOHNSON et al., 2015).

Poucas pesquisas na literatura avaliaram algum aspecto da arginase em PE (NORIS et al., 2004; BERNARDI et al., 2008; SANKARALINGAM et al., 2009; SANKARALINGAM; XU; DAVIDGE, 2010; GONZÁLEZ-GARRIDO CHEM et al., 2013; TANAKA et al., 2018). McCann Haworth et al. (2021) demonstraram que hemácias de gestantes portadoras de pré-eclâmpsia induzem DE por mecanismos dependentes de arginase e estresse oxidativo. A expressão e atividade da arginase está aumentada nas hemácias dessas gestantes e está associada à gravidade da pré-eclâmpsia. Bernardi et al. (2008) avaliaram níveis plasmáticos da arginase e verificaram um aumento significativo.

Trabalho recente, realizado por nosso grupo, mostrou associação dos níveis circulantes de arginase 2 com gravidade na pré-eclâmpsia e não-responsividade ao tratamento anti-hipertensivo (BERTOZZI-MATHEUS et al., 2021).

Entretanto, dosagens independentes, relacionadas à quantificação das duas isoformas da arginase (1 e 2) são escassas na literatura e ainda se desconhece qual delas contribuiria para a atividade plasmática, ou se estaria vinculada à gravidade da pré-eclâmpsia e à resposta à terapia anti-hipertensiva, ou ainda associada aos diferentes órgãos-alvo. Um outro estudo (TANAKA et al., 2018) reportou uma correlação entre sFlt-1 e arginase, porém, um número amostral muito baixo de pacientes foi utilizado (11 grávidas saudáveis e 10 com PE), o que reduz o poder da pesquisa, dificultando conclusões que relacionam estes dois biomarcadores.

Ainda que a maioria das investigações sobre arginase e PE sejam realizadas com placenta, um grupo de cientistas (SANKARALINGAM; XU; DAVIDGE, 2010), procedeu aos ensaios *in vitro* de PE para exploração da enzima. Sugere-se que a fisiopatologia desta doença hipertensiva envolve dois estágios: o primeiro se relaciona à isquemia da placenta (decorrente da falência do remodelamento das artérias uterinas), que libera na circulação sistêmica, fatores que atuam sobre o endotélio vascular. Assim promove a DE generalizada (segundo estágio), a qual caracteriza as consequências clínicas da síndrome. Por conseguinte, com base nesta teoria proposta, alguns grupos desenvolveram um modelo designado pela incubação do plasma/soro de gestantes com PE em cultura de células endoteliais.

Conforme descrito em uma revisão (LUIZON; SANDRIM, 2013), este modelo tem potencial de auxiliar em muito no desenvolvimento de novas terapias ao tratamento da PE. Já se verificou que o plasma de gestantes com PE modulava a expressão gênica (avaliada por microarranjos de DNA) das células endoteliais comparado às gestantes saudáveis (MACKENZIE et al., 2012). Posteriormente, tais autores passaram a conectar nos seus trabalhos, ensaios clínicos aos *in vitro*. Alguns artigos já foram publicados utilizando esta metodologia e vários resultados bem interessantes foram reportados (LUIZON et al., 2016; SANDRIM et al., 2016; ROCHA-PENHA et al., 2017; CALDEIRA-DIAS et al., 2018; CALDEIRA-DIAS et al., 2019). Incluindo uma pesquisa que analisou mudanças na expressão de redes gênicas relacionadas à célula endotelial, entre pacientes pré-eclâmpticas responsivas e não responsivas à terapia antihipertensiva (LUIZON et al., 2016) e à modulação da expressão de endotelina-1 por microRNAs (CALDEIRA-DIAS et al., 2018).

Com relação à arginase, somente um estudo empregou tal procedimento *in vitro* para investigar os efeitos em células endoteliais incubadas com plasma de gestantes com PE (SANKARALINGAM; XU; DAVIDGE, 2010). Os autores concluíram que o aumento da expressão da arginase nas células incubadas com plasma de PE consegue induzir o desacoplamento da NOS por depleção da L-arginina pela arginase, favorecendo a produção do superóxido (O_2-).

Na inibição da arginase através da S-(2-boronoetil)-L-cisteína (BEC) houve redução de formação de O_2-. Entretanto, de modo interessante, ocorreu elevação dos níveis de nitrotirosina (marcador de dano celular oxidativo). Uma possível explicação a este evento é a reação entre o excesso de formação de NO (consequente do aumento da biodisponibilidade do substrato L-arginina pela inibição da arginase) que reage com o O_2- gerado via NADPH oxidase (NOS e NADPH oxidase são as maiores fontes de produção de O_2- no endotélio). E isto pode ter resultados deletérios neste último. Os autores mostraram ainda que a suplementação de L-arginina na presença do inibidor da arginase nas culturas *in vitro* levou a um aumento adicional de peroxinitrito (SANKARALINGAM; XU; DAVIDGE, 2010). Portanto, os autores indicam que outros trabalhos poderiam ser direcionados avaliando a inibição simultânea da arginase e da NADPH oxidase, propondo que esta dupla ação seria benéfica ao endotélio frente ao plasma de gestantes com PE.

Polimorfismos genéticos da arginase

Outro ponto ainda não explorado sobre arginase na pré-eclâmpsia é a análise de polimorfismos de base única (*SNP,* do inglês, *single nucleotide polymorphism*) localizados nos genes que codificam a arginase 1 (*ARG1*) e a arginase 2 (*ARG2*). SNPs consistem na variação na sequência de DNA que afeta somente uma base nitrogenada na ordem do genoma, entre indivíduos de uma espécie ou entre pares de cromossomos de um indivíduo (Figura 4) (LONETTI et al., 2016). Encontram-se por toda região do genoma: íntrons, éxons, regiões intergênicas, promotores ou *enhancers* (acentuadores) (Figura 5) (LOURENÇO; CHOUPINA, 2019), sendo considerados excelentes marcadores genéticos por rastrearem a herança do segmento genômico correspondente em famílias e populações (NUSSBAUM; MCINNES; HUNTINGTON, 2008).

Os SNPs podem ser usados para diagnóstico pré-natal de doenças genéticas; em aplicações forenses como o teste de paternidade e inclusive, na medicina personalizada baseada em genômica que verifica se determinadas variantes influenciam na eficácia ou segurança de medicamentos específicos (NUSSBAUM; MCINNES; HUNTINGTON, 2008).

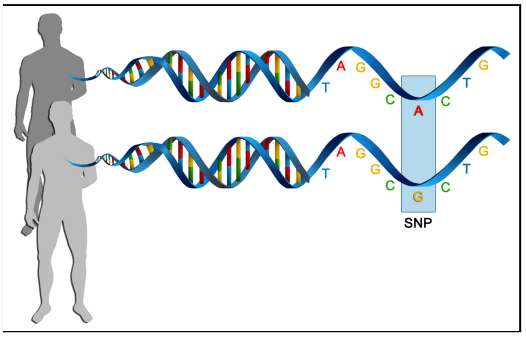

Figura 4. Esquematização da ocorrência de SNP. (Imagem reproduzida de LONETTI et al., 2016).

Em relação à identificação e detalhamento dos polimorfismos, eles se iniciam pelo prefixo "rs" (do inglês, *referential snp*), o qual é seguido de uma sequência numérica única e arbitrária. E para sinalizar a troca de uma base nitrogenada por outra (representadas pela primeira letra, em maiúsculo), se utiliza o símbolo ">". Logo, A>C significa a mudança da adenina pela citosina (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2005).

Na literatura, os SNPs com maiores informações funcionais no *ARG1* são o rs2781659, onde foi demonstrado que o alelo G reduz a expressão da região promotora da *ARG1* (DUAN et al., 2011), e o rs2791665 que também modula o efeito da expressão gênica (DUAN et al., 2011). Interessantemente, polimorfismos presentes neste gene estão associados a condições clínicas/processos biológicos em que o NO participa como pressão sanguínea (MEROUFEL et al., 2009) e infarto do miocárdio (DUMONT et al., 2007). Quanto ao gene *ARG2,* não há resultados funcionais relacionados aos polimorfismos, apesar dos SNPs rs3742879 e o rs10483901 afetarem a concentração de NO exalado (SALAM et al., 2011).

E neste contexto, portanto, se encaixam os campos da Farmacogenômica e da Farmacogenética, os quais, através da leitura de perfis genéticos, viabilizam uma prescrição particularizada, auxiliando nas escolhas mais adequadas de drogas e de suas doses, proporcionando mais benefícios ao indivíduo e reduzindo a incidência de danos ao organismo (WEINSHILBOUM; WANG, 2006).

Além disso, contribuem às percepções inerentes sobre alvos a serem estudados na elaboração de fármacos, que complementariam a terapia fornecida por antihipertensivos, prevenindo doenças e complicações cardiovasculares (FONTANA; LUIZON; SANDRIM, 2015; LUIZON et al., 2017).

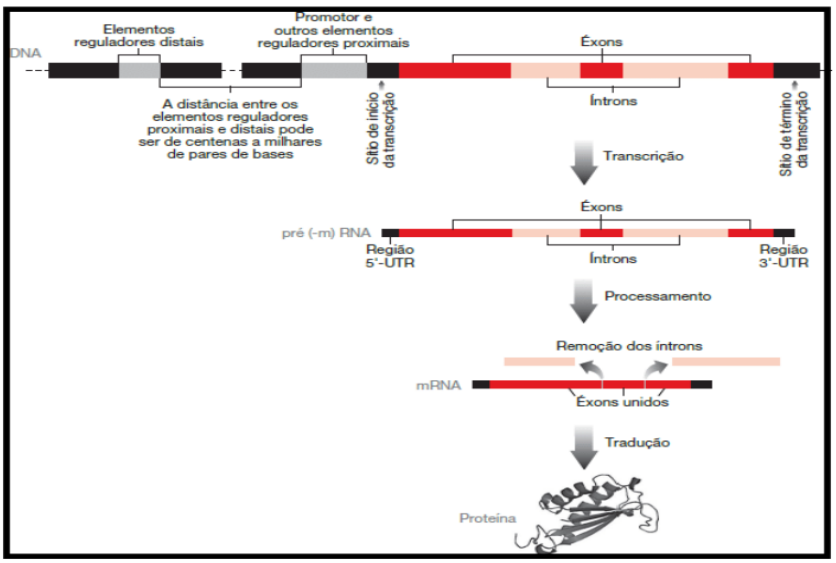

Figura 5. Estrutura do gene eucarioto para elucidação das diferentes localizações dos SNPs no genoma. (Imagem reproduzida de LOURENÇO; CHOUPINA, 2019).

Por meio do banco de dados *ClinVar*, é possível acessar um arquivo público que relata as relações entre variações e fenótipos humanos, com evidências de apoio, agregando dados de vários grupos, como laboratórios, UniProt, painéis de especialistas e diretrizes práticas, para determinar se há consenso acerca da interpretação dos materiais submetidos. Por isso, os genótipos identificados se encontram em constante atualização (LANDRUM et al., 2013; LANDRUM et al., 2016).

Atualmente, as informações disponíveis ao se realizar uma pesquisa no *ClinVar* são: a variante (localização), o gene, a proteína alterada, a condição clínica, o significado clínico (patogênico, benigno, significado incerto). Além do status de revisão (quantidade de submissões e verificação da convergência ou divergência dos dados) e o número de acesso (LANDRUM et al., 2013; LANDRUM et al., 2016).

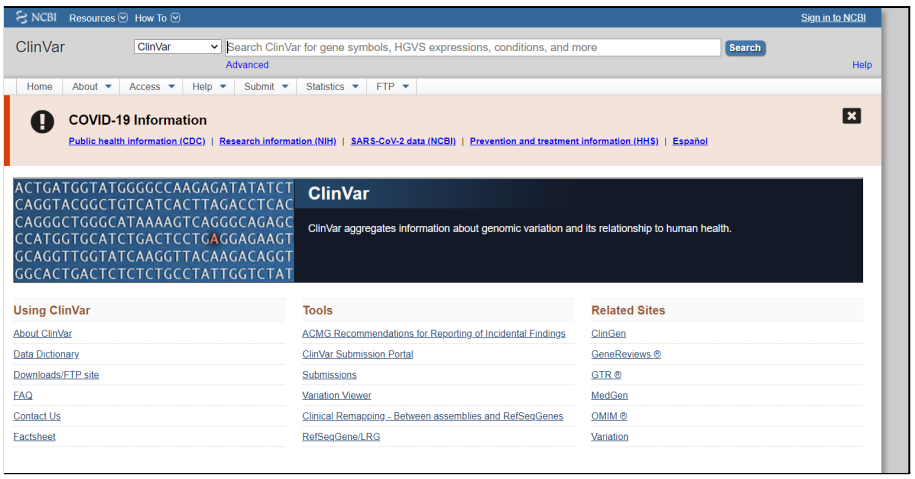

Figura 6. Website da *ClinVar*. Disponível em: <https://www.ncbi.nlm.nih.gov/clinvar/>. (Acesso em 23 de set. de 2021).

Portanto, elaboramos uma síntese para facilitar a compreensão das principais características de cada SNP do nosso estudo, incluindo a mudança das bases nitrogenadas, localização no gene, consequências funcionais, a frequência em determinadas populações étnicas e a sua exploração em trabalhos científicos.

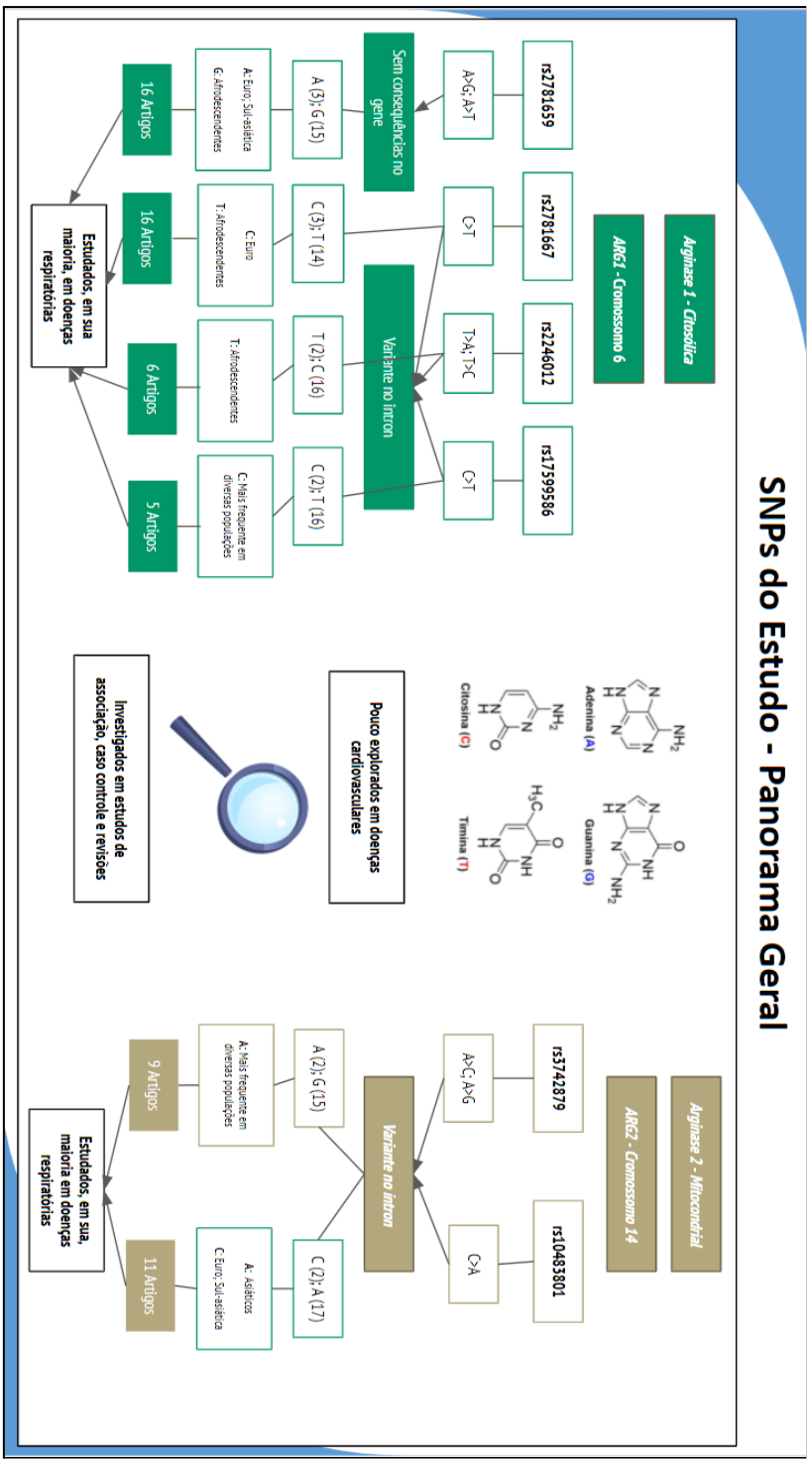

Figura 7. Breve síntese dos SNPs deste estudo.

JUSTIFICATIVA

Apesar da grave consequência da PE na saúde global, poucas frentes de tratamento se encontram disponíveis. Conhecer os mecanismos pelos quais a DE é promovida nesta síndrome é essencial para indicar intervenções mais efetivas. Neste contexto, a via do NO vem sendo explorada em nosso laboratório em diversas frentes, e no atual projeto, propomos o estudo da arginase. Esta enzima compete com a eNOS pelo substrato (L-arginina), diminuindo potencialmente a biodisponibilidade de NO, o que poderia comprometer a função endotelial.

Embora estudada em diferentes patologias de modo profundo, em PE, poucos trabalhos e com baixo número de participantes, trouxeram dados relacionados à arginase. Neste sentido, entender os mecanismos de ação da arginase nesta condição clínica se faz necessário. Neste projeto, será explorado o envolvimento da arginase na frente genética, verificando como polimorfismos genéticos que estão associados às modulações funcionais da arginase 1 e 2 podem estar vinculados à predisposição da PE, bem como na modulação dos níveis plasmáticos destas enzimas e na resposta aos antihipertensivos.

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MANUSCRITO REFERENTE AO PROJETO DE MESTRADO

RESUMO

PINTO-SOUZA, C. C. **Estudo dos polimorfismos genéticos da arginase na pré-eclâmpsia**. 2021. Dissertação (Mestrado), Instituto de Biociências de Botucatu - Universidade Estadual Paulista, Botucatu, 2021.

A pré-eclâmpsia (PE) se encontra associada à redução da biodisponibilidade do óxido nítrico (NO). A arginase se relaciona à síntese de NO, porém, é relativamente inexplorada na PE. No entanto, nenhum estudo anterior examinou se as variações nos genes *ARG1* e *ARG2,* que codificam a arginase*,* afetam a biodisponibilidade do NO e o risco de desenvolver PE. Neste trabalho, comparamos a frequência dos alelos e genótipos de polimorfismos de nucleotídeo único (SNPs) em *ARG1* (rs2781659; rs2781667; rs2246012; rs17599586) e *ARG2* (rs3742879; rs10483801) em mulheres grávidas saudáveis (GS) e PE, e examinamos se estes SNPs afetam as concentrações plasmáticas de nitrito (um marcador da formação de NO) nestes grupos. Também verificamos se haveria modulação dos níveis plasmáticos da arginase e nitrito pelos polimorfismos em mulheres com PE responsivas ou não à terapia anti-hipertensiva. Os genótipos para os SNPs de *ARG1* e *ARG2* foram determinados pela sonda *Taqman*, o nitrito do plasma, por um ensaio de quimioluminescência baseado em ozônio. As concentrações das isoformas de arginase foram medidas em amostras de plasma usando kits ELISA disponíveis comercialmente. Em relação aos SNPs de *ARG1*, as frequências dos portadores de G para rs2781659, e as frequências do alelo C para rs2246012 foram maiores na PE em comparação com mulheres GS. Além disso, o genótipo GG de rs2781659 e o genótipo TT de rs2781667 foram associados ao nitrito plasmático mais elevado em GS. Nossos resultados sugerem que os SNPs de *ARG1* aumentam a susceptibilidade à PE e modulam o nitrito plasmático a níveis elevados em mulheres GS. Além disso, o SNP rs3742879 de *ARG2* está associado à não responsividade e modula os níveis de arginase 2 e nitrito. Enquanto os polimorfismos de *ARG1* não têm esse efeito.

Palavras-chave: arginase, óxido nítrico, nitrito, pré-eclâmpsia, polimorfismos genéticos, gravidez; responsividade

TITLE PAGE (1)

Effects of arginase genetic polymorphisms on nitric oxide formation in healthy pregnancy and in preeclampsia

Caroline C. Pinto-Souza[1], Fernanda Coeli-Lacchini[2] , Marcelo R. Luizon[3], Ricardo C. Cavalli[4], Riccardo Lacchini[5], Valeria C. Sandrim[1]*

[1]Department of Biophysics and Pharmacology, Institute of Biosciences of Botucatu, Universidade Estadual Paulista (UNESP), Distrito Rubiao Junior, Botucatu, Sao Paulo, 18618-689, Brazil

[2]Department of Clinical Analyses, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto, Sao Paulo, 14040-903, Brazil

[3]Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, 31270-901, MG, Brazil

[4]Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Sao Paulo, 14049-900, Brazil

[5]Department of Psychiatric Nursing and Human Sciences, Ribeirao Preto School of Nursing, University of Sao Paulo (USP), Ribeirao Preto, Sao Paulo, 14049-900, Brazil

Corresponding author.

***Author for correspondence:**

Valeria C. Sandrim, PhD - Department of Pharmacology Institute of Biosciences of Botucatu São Paulo State University (UNESP) Distrito de Rubiao Junior S/N, Zip code: 18618-000 Botucatu, SP, Brazil

Phone: +55 14 3880 0228. E-mail address: valeria.sandrim@unesp.br (V.C. Sandrim).

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Paper presentation

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Caroline C. Pinto-Souza[1] , Fernanda Coeli-Lacchini[2] , Marcelo R. Luizon[3] , Ricardo C. Cavalli[4], Riccardo Lacchini[5], Valeria C. Sandrim[1]; Arginase in pre-eclampsia: study of genetic polymorphisms and circulating factors. 5th Symposium of Vascular Biology. February 24th-26th, 2021. Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. (Video Poster presentation - online edition).

Ethical approval and consent of participants

Human research approval was obtained from the Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (FMRP-USP). All participants provided written informed consent.

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A. For which reasons the study was conducted?

- There are few treatment fronts for hypertensive disorders of pregnancy.
- Arginase is related to NO synthesis and less explored in preeclampsia studies.
- Verification of how genetic polymorphisms that are associated with the functional modulations of arginase 1 and 2 can be linked to the predisposition of PE.
- No previous study has examined whether genetic variations in the *ARG1* and *ARG2* genes encoding arginase affect NO formation.

B. Which are the main findings?

- The *ARG1* SNPs rs2781659 and rs2246012 were associated with the risk of developing preeclampsia.
- We found novel effects of *ARG1* SNPs on plasma nitrite levels in healthy pregnant women.
- The *ARG1* SNPs rs2781659 and rs2781667 affected plasma nitrite levels in healthy pregnant women.

C. What does this study brings as a novelty about what is already known?

- First association study of *ARG1* and *ARG2* genetic polymorphisms and preeclampsia.
- *ARG1* SNPs may increase preeclampsia risk and affect nitrite levels in healthy pregnant women.

Keywords: Arginase polymorphisms, Preeclampsia, NO bioavailability

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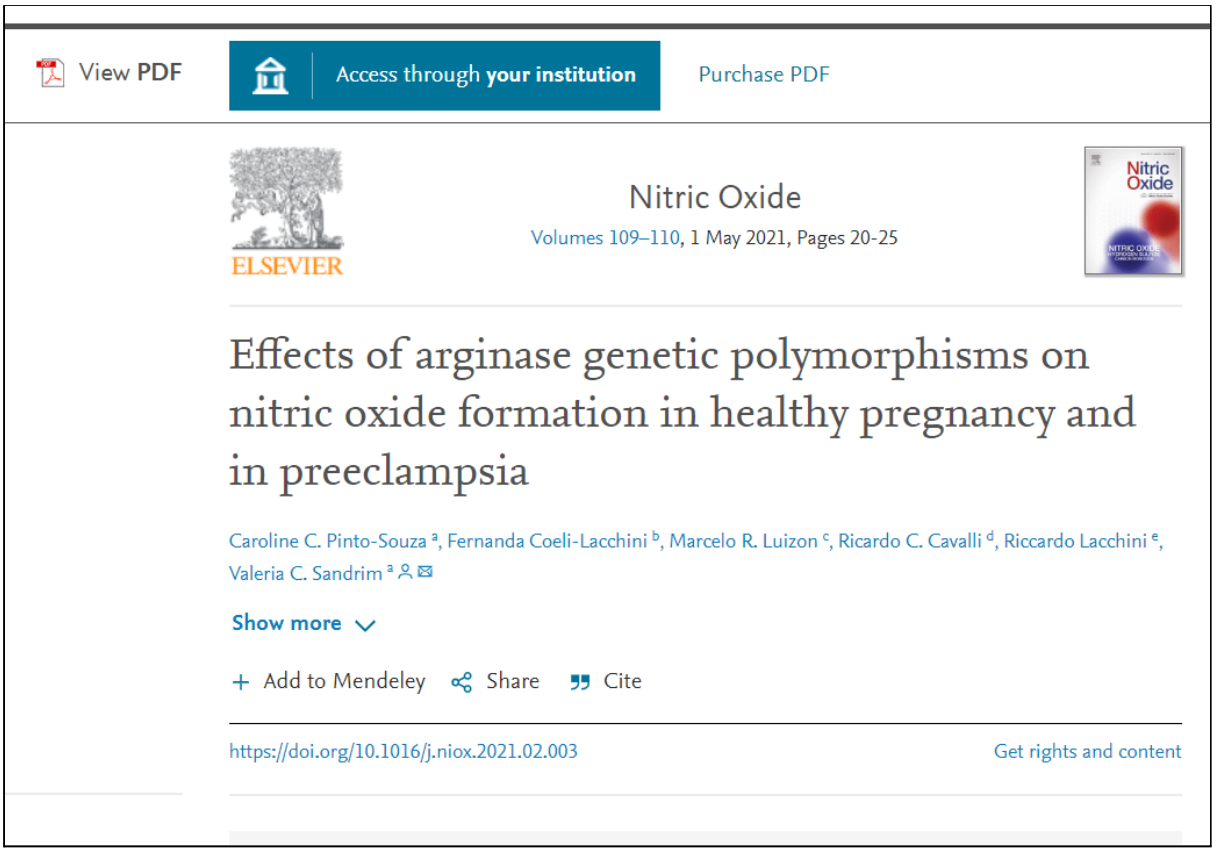

Figura 8. Artigo publicado na Revista *Nitric Oxide*.

ABSTRACT

Background and aims: Preeclampsia is associated with reduced nitric oxide (NO) bioavailability. Arginase is related to NO synthesis, but relatively unexplored in preeclampsia. However, no previous study has examined whether variations in *ARG1* and *ARG2* genes affect NO bioavailability and the risk of preeclampsia. Here, we compared the alleles and genotypes of single nucleotide polymorphisms (SNPs) in *ARG1* (rs2781659; rs2781667; rs2246012; rs17599586) and *ARG2* (rs3742879; rs10483801) in healthy pregnant women and preeclampsia, and examined whether these SNPs affect plasma nitrite concentrations (a marker of NO formation) in these groups. **Methods:** Genotypes for the *ARG1* and *ARG2* SNPs were determined by Taqman probe and plasma nitrite by an ozone-based chemiluminescence assay. **Results:** Regarding *ARG1* SNPs, the GG genotype and G allele frequencies for rs2781659, and the C allele frequencies for rs2246012 were higher in preeclampsia compared to healthy pregnant women. Moreover, the GG genotype for rs2781659 and the TT genotype for rs2781667 were associated with higher plasma nitrite in healthy pregnant women. We found no association of *ARG2* polymorphisms with preeclampsia or nitrite levels in the study groups. **Conclusions:** Our results suggest that SNPs of *ARG1* increase the risk of preeclampsia and modulate plasma nitrite levels in healthy pregnant women.

Keywords: arginase, nitric oxide, nitrite, pre-eclampsia, genetic polymorphisms, pregnancy

INTRODUCTION

Preeclampsia (PE) is a hypertensive disorder of pregnancy that occurs after 20 weeks of gestation, which may include proteinuria and lesions of target organs such as the brain, liver, and kidneys [1]. PE is linked to a higher risk for cardiovascular outcomes and can lead to seizure and death [2–5]. PE affects 2–8% of all pregnancies, and is a major contributor to maternal and fetal morbidity and mortality [2,3].

Although the pathophysiology of PE is not completely known, endothelial dysfunction is a hallmark, as it is characterized by decreased nitric oxide (NO) bioavailability [6]. Therefore, the peripheral vascular resistance is induced, promoting hypertension and other major symptoms such as edema, proteinuria, and inappropriate platelet aggregation [7]. In the endothelium, NO is synthesized by the conversion of its precursor L-arginine by the enzyme eNOS (endothelial nitric oxide synthase) [8].

NO promotes relaxation of smooth muscle cells by activating soluble guanylate cyclase (sGC) and resulting in higher cyclic guanosine monophosphate (cGMP) release [9], thereby, contributing to the vascular tone regulation and decrease of platelet aggregation. The modulation of the arginine/NO/cGMP pathway has been shown to play an important role in pregnancy [10–12]. Moreover, nitrite concentrations, a stable NO metabolite and a marker of NO formation, was shown to be reduced in the plasma of preeclamptic women [13,14].

Arginase is related to NO synthesis, although not very well explored in PE. The isoforms arginase 1 and arginase 2 compete for the same substrate, the L-arginine [15–17]. Therefore, arginase depletes the substrate of eNOS contributing to the reduction of NO bioavailability [15–18]. Arginase is present in several organisms [19] and its isoforms are expressed in several tissues/cells, including the endothelial cells, vascular endothelium and smooth muscle cells [16,20]. Arginase was shown to be associated with cardiovascular dysfunction [16,18,21]. Notably, higher arginase activity was found in PE compared to healthy pregnant [22]. Moreover, arginase was related to oxidative stress [23–25]; and to sFlt-1 (soluble fms-like tyrosine kinase-1), the antiangiogenic factor highly expressed in PE [26]. Given the relevance of arginase for cardiovascular diseases and implications to NO bioavailability, it is possible that genetic polymorphisms located in the genes that codify arginase 1 (*ARG1*) and arginase 2 (*ARG2*) may modulate NO synthesis and consequently affect the risk of PE.

Single nucleotide polymorphisms (SNPs) of *ARG1* and *ARG2* were evaluated in respiratory diseases [27–29] and erectile dysfunction [30, 31], but there is fewer data regarding cardiovascular studies [32–35]. Notably, the G allele for the rs2781659 SNP in the promoter region was shown to reduce *ARG1* expression [36], and the rs2791665 SNP also modulates the effect of *ARG1* expression in vitro [36]. As for the *ARG2*, the rs3742879 SNP was shown to affect the concentration of exhaled NO [29]. In this study, we compared the allele and genotype frequencies of SNPs located in *ARG1* (rs2781659; rs2781667; rs2246012; rs17599586) and *ARG2* (rs3742879; rs10483801) between healthy pregnant women and in PE. Moreover, we examined whether *ARG1* and *ARG2* SNPs modulate circulating levels of nitrite.

MATERIAL AND METHODS

Subjects

Human research approval was obtained from the Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (FMRP-USP). All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital at the FMRP-USP. We studied 109 healthy women with uncomplicated pregnancies, and 144 pregnant with PE. According to the ACOG 2013 [1], PE was defined as pregnancy-induced hypertension (≥140 mmHg systolic and ≥90 mmHg diastolic on two or more measurements with an interval of at least 6 h between one take and the next) in a woman after 20 weeks of gestation. And returning to normal by 12 weeks postpartum, plus significant proteinuria (>0.3 g/L in 24-h urine).

In the absence of proteinuria, PE is diagnosed as hypertension in association with thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, cerebral or visual symptoms [1]. Only a subset of pregnant women had plasma available to measure the nitrite levels (healthy pregnant, n = 100; PE, n = 83). In the clinical attendance, written informed consent was provided by volunteers who agreed to participate in the study, maternal venous blood samples were collected into tubes containing EDTA (DNA extraction to genotyping) and containing heparin (to measure nitrite). Plasma samples were obtained from whole blood after centrifugation at 1000g for 10 min and stored at − 70 ◦C until assayed.

Genomic DNA was extracted from the cellular fraction of 1 mL of whole blood by a salting-out method and stored at − 20 ◦C until analyzed.

Measurement of nitrite concentrations

Nitrite concentrations were measured using an ozone-based chemiluminescence assay, as previously described [13]. Briefly, 200 μL of plasma aliquots analyzed in triplicate were injected into a solution of acidified triiodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, General Electric Company, Boulder, CO, USA). Approximately 8 mL of triiodide solution (2.0 g of potassium iodide and 1.3 g of iodine dissolved in 40 mL of water with 140 mL of acetic acid) were placed in the purge vessel into which plasma samples were injected. The triiodide solution reduced nitrite to NO gas, which was detected by the NO analyzer.

Genotyping

Genotypes were determined by Taqman Allele Discrimination assays using probes and primers designed by Applied Biosystems (Foster City, CA, USA). Assay IDs for SNPs were: C___3063957_10 (rs2781659), C__15933286_10 (rs2781667), C__15933284_10 (rs2246012), C__25596209_10 (rs17599586), C__25960528_10 (rs3742879) and C___2778311_10 (rs10483801). All experiments were performed with JumpStart Taq ReadyMix for Quantitative PCR 1× (Sigma Aldrich, St Louis, MO, USA), Taqman assays 1× and 5 ng of template in 10 μL reaction volume. Thermal cycling was performed under standard conditions and the StepOnePlus Real-Time PCR equipment (Applied Biosystems, Foster City, CA, USA) recorded fluorescence. The results were analyzed with manufacturer's software.

Statistical analysis

The clinical characteristics of healthy pregnant women and PE were compared by Student's unpaired t-test, Mann-Whitney U test, or $\chi 2$ as appropriate.

The effects of the different genotypes for the *ARG1* and *ARG2* SNPs on nitrite concentrations in healthy pregnant women and in PE were compared by Student's unpaired t-test and one-way ANOVA with post hoc tests. Distributions of genotypes were assessed for deviation from the Hardy-Weinberg equilibrium. Statistical analysis was performed with GraphPad Prism 5.0. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Table 1 summarizes the characteristics of the 253 pregnant women enrolled in this study. Healthy pregnant women and PE groups present similar age, ethnicity, current smokers; primigravida, heart rate, hemoglobin, and hematocrit. Healthy pregnant and PE women were sampled at 37.0 (36.0–38.0) and 35.0 (32.0–38.0) weeks of gestation, respectively. As expected, higher systolic and diastolic blood pressures were found in the PE compared with healthy pregnant women. Higher fasting glucose and body mass index were found in the PE compared with the healthy pregnant group. Lower newborn weights and gestational ages at delivery were found in the PE compared with the healthy pregnant group. Lower plasma nitrite levels and higher sFlt-1 concentration were found in PE than in healthy pregnant group.

Table 2 shows the results of the single-locus analysis. The distribution of genotypes for each SNP showed no deviation from HardyWeinberg equilibrium. Genotypes and allele distributions from the *ARG1* SNPs rs2781659 and rs2246012 were different when the PE group was compared with the healthy pregnant (Table 2). The GG genotype and G allele of rs2781659, and the C allele carriers of rs2246012 were more frequent in the PE group compared to healthy pregnant women (Table 2). Conversely, we found no significant differences for *ARG2* SNPs in PE (Table 2). Next, we examined whether the SNPs may modulate nitrite levels in the groups healthy pregnant and PE. Clinical characteristics of these women are shown in Supplementary Table 1S, which were similar to the data reported in Table 1.

Fig. 1 shows plasma nitrite concentrations in healthy pregnant women grouped by *ARG1* genotypes. For healthy pregnant women, higher plasma nitrite levels were found in carriers of the GG genotype compared to AG genotype for the rs2781659 SNP (Fig. 1A).

Similarly, higher plasma nitrite levels were found in carriers of the TT genotype compared to CT genotype (Fig. 1B). We found no significant differences regarding the rs2246012 and rs17599586 SNPs (Fig. 1). Regarding the PE group, we found no *ARG1* genotypes associated with nitrite levels (Fig. 2). Fig. 3 shows plasma nitrite concentrations in healthy pregnant women and PE grouped by *ARG2* genotypes. We found no effect of *ARG2* SNPs on nitrite levels in both groups (Fig. 3).

DISCUSSION

This study was the first to analyze *ARG1* and *ARG2* polymorphisms in PE and in healthy pregnant women, and verify whether they modulate plasma nitrite levels in these groups. The main novel findings reported here were that: (1) the GG genotype and G allele frequencies for the *ARG1* rs2781659 were more frequent in PE group, and the GG genotype was associated with higher plasma nitrite levels in healthy pregnant women; that (2) the TC and CC genotypes and the C alleles for the *ARG1* rs2246012 SNP were also more frequent in PE group, and that, (3) the TT genotype for the rs2781667 was associated with higher plasma nitrite levels in healthy pregnant group.

To our knowledge, no previous study has examined whether *ARG1* and *ARG2* polymorphisms are associated with PE. For the first time, we showed that the GG genotype and the G allele for the *ARG1* rs2781659 SNP may increase the risk of PE. The G allele for the rs2781659 promoter SNP was shown to reduce the *ARG1* expression [36]. Interestingly, this functional data supports our finding that the healthy pregnant women carrying the GG genotype had higher nitrite levels. Conversely, this is not in agreement with our initial hypothesis that lower arginase would increase L-arginine levels and enhance the substrate availability to eNOS. However, one study reported that L-arginine supplementation concomitant with arginase inhibition in endothelial cell incubated with plasma from PE (in vitro model) increase peroxynitrite levels since a higher production of superoxide is observed and it reacts with NO synthesized by eNOS [38].

Few studies have measured the expression/activity of arginase in PE [22–26,37]. An increased arginase activity was observed in the plasma, but not placenta, in patients with PE [37]. Moreover, regarding isoforms, only tissue expressions were reported, but not circulating levels [23,24,38].

Interestingly, although arginase 2 was more expressed in the vasculature of PE compared to healthy pregnant women, similar levels of arginase 1 were found in these groups [38]. Regarding blood pressure, the T allele for the rs2781667 SNP was significantly associated with decreased systolic blood pressure, an effect observed in both heterozygote and homozygote subjects [32]. Accordingly, we found that healthy pregnant women carrying the TT genotype had higher nitrite levels. Therefore, the T allele for the rs2781667 SNP could be related to plasma nitrite modulation, because eNOS competes for L-arginine with *ARG1* and its downregulation promotes an increase in NO bioavailability and decreased systolic blood pressure. Moreover, the T allele or TT genotype for the rs2781667 SNP were associated with an increased risk of cardiovascular disease, higher arginase activity, and reduced levels of nitric oxide metabolites [33–35].

Besides, subjects carrying the T allele for the rs2781666 and rs2781667 SNPs had a 1.5-fold increased risk of developing essential hypertension phenotypes [33]. Finally, subjects carrying the CT and TT genotypes for the rs2781667 SNP exhibited lower nitrite concentrations [35]. Although no search has examined the effects of arginase polymorphisms on NO formation, a previous study found that the AA genotypes for the *ARG1* rs2781659 SNP and the TT genotype for the rs2781667 SNP were associated with higher severity in patients with clinical erectile dysfunction [30]. The authors also found that genotypes of these two SNPs and rs17599586 were associated with reduced plasma arginase activity in patients with clinical erectile dysfunction [30,31]. Our study was the first that examined whether *ARG1* and *ARG2* polymorphisms affect plasma nitrite levels in healthy pregnant women and in PE. Although the TC and TT genotypes and the C allele frequencies for the *ARG1* rs2246012 SNP were less frequent in healthy pregnant women than in PE, we found no differences in plasma nitrite levels according to SNP genotypes in both groups.

Moreover, there are little data for rs2246012, concerning respiratory diseases [28,39], cancer [40], and erectile dysfunction [30] with no significant results. Therefore, further research of these SNPs is needed to discover individual genotype effects of *ARG1* on nitrite levels, and to confirm their functional consequences. Arginase 2 is a mitochondrial enzyme widely expressed in the kidney, prostate, gastrointestinal tract, and vasculature [16,20]. Moreover, in endothelial cell culture incubated with plasma from pregnant women, vasculature and placenta tissue, arginine 2 is elevated in PE compared to healthy pregnant women [22–26,38].

However, we found no association of *ARG2* genotype/alleles or the genotype effects on nitrite levels, both in healthy pregnant women and in PE. Although it is possible that arginase 2 could actively participate in PE physiopathology, probably genetic polymorphisms of *ARG2* may have small effects in PE. The *ARG2* rs10483801 SNP was associated with different sickle cell disease phenotypes [41,42]. Moreover, arginase 2 concentrations were found to be associated with increased risk for clinical erectile dysfunction [30].

We were not able to find an effect of the six *ARG1* and *ARG2* polymorphisms examined on plasma nitrite levels presumably, because these levels are greatly reduced in PE, which makes it difficult to observe genetic effects. In agreement with the present findings, we previously found an effect of haplotypes of NOS3 gene on nitrite levels only in healthy pregnant women, but not in PE [43]. Moreover, nitrite is a surrogate indicator of NO bioavailability and not of its production. In PE, a higher level of superoxide radical is present, and this radical reacts rapidly with NO reducing its availability. We suppose that we can only observe the effect of SNPs of *ARG1* on plasma nitrite in healthy pregnant women because in these women the pathways related to nitrite formation are more direct and do not suffer the influence of pathophysiological process, such as exacerbated oxidative stress.

Recently, one interesting study found that the *ARG2* SNP rs3759757 was associated with blood L-arginine concentration in adult males. Nevertheless, SNPs located in *ARG1* (rs2246012 and rs2781667) were not correlated with these levels [44]. Therefore, based on these results it is unlikely that SNPs located in *ARG1* and associated with PE may be related with circulating L-arginine levels and contributing to the arginase pathway in PE.

CONCLUSION

We conclude that polymorphism in *ARG1* increases the risk of PE and may affect circulating nitrite levels in healthy pregnant women. Although this study does not provide the molecular mechanisms, it highlights relevant differences for *ARG1* polymorphisms between healthy pregnancy and PE groups. Since arginase is scarcely studied in pregnancy, our novel findings provide perspectives to further the role of arginase in healthy pregnancy and the pathophysiology of PE.

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TABLES

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

Parameters	Healthy Pregnant (n = 109)	Preeclampsia (n = 144)	*P value*
Age (years)	25.2 ± 5.9	26.8 ± 6.7	0.0750
Ethnicity (% White)	62.9	67.3	0.5532
Current Smokers (%)	15.4	9.3	0.1917
Primigravida (%)	44.0	44.7	0.8869
BMI, kg/m²	27.0 (24.6 - 30.5)	31.8 (27.5 - 37.0)	< 0.0001
SBP (mmHg)	110.0 (100.0 - 120.0)	140.0 (130.0 - 150.0)	< 0.0001
DBP (mmHg)	70.0 (70.0 - 80.0)	90.0 (80.0 - 96.0)	< 0.0001
HR (beats per min)	82.5 (80.0 - 88.0)	80.0 (80.0 - 88.0)	0.0807
Fasting Glucose (mg dl^{-1})	75.0 (67.0 - 85.0)	80.0 (73.0 - 100.8)	0.0018
Hemoglobin (g dl^{-1})	12.0 (10.9 - 12.7)	12.0 (11.0 - 12.7)	0.9645
Hematocrit (%)	35.9 (32.6 - 38.1)	36.0 (33.3 - 38.2)	0.5724
Creatinine (μmol l^{-1})	NA	0.7 (0.6 - 0.8)	NA
Proteinuria, mg/24h	NA	886.0 (390.2 - 2043.0)	NA
GA at blood collection, w	37.0 (36.0 - 38.0)	35.0 (32.0 - 38.0)	0.0001
GA at delivery, w	40.0 (39.0 - 41.0)	37.0 (34.0 - 39.0)	< 0.0001
Newborn weight (g)	3390.0 (3085.0 - 3745.0)	2705.0 (1846.0 - 3275.0)	< 0.0001
Plasma Nitrite (nM)	139.1 (81.1 - 214.6)	90.5 (58.3 - 138.7)	0.0007
Plasma sFlt-1 (ng/ml)	3.9 (2.7 - 5.2)	11.2 (3.7 - 16.4)	< 0.0001

- Data are expressed as media, median (25th – 75th percentile) or percentage. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; HR, heart rate; NA, not applicable.

Table 2. Genotypes and alleles frequencies for the *ARG1 and ARG2* polymorphisms in healthy pregnant women and preeclampsia.

Gene, SNP	Healthy pregnant (%)	Preeclampsia (%)	OR (95% CI)	*P value*
ARG1				
rs2781659, A>G				
AA	41 (37.6)	36 (25.0)	1.000 (Reference)	–
AG	51 (46.8)	76 (52.7)	1.697 (0.958-3.005)	0.063
GG	17 (15.6)	32 (22.3)	2.144 (1.023-4.490)	**0.033***
A allele	134 (61.5)	150 (52.1)	1.000 (Reference)	–
G allele	84 (38.5)	138 (47.9)	1.468 (1.026-2.099)	**0.035***
rs2781667, C>T				
CC	35 (32.1)	33 (22.8)	1.000 (Reference)	–
CT	54 (49.5)	76 (53.0)	1.493 (0.827-2.693)	0.182
TT	20 (18.3)	35 (24.2)	1.856 (0.927-3.839)	0.093
C allele	125 (57.3)	144 (50.0)	1.000 (Reference)	–
T allele	93 (42.7)	144 (50.0)	1.344 (0.943-1.915)	0.101
rs2246012, T>C				
TT	85 (77.5)	94 (65.6)	1.000 (Reference)	–
TC+CC	24 (22.5)	50 (34.4)	1.884 (1.067-3.326)	**0.027***
T allele	192 (88.1)	233 (80.9)	1.000 (Reference)	–
C allele	26 (11.9)	55 (19.1)	1.743 (1.053-2.886)	**0.037***
rs17599586, C>T				
CC	82 (75.2)	117 (81.2)	1.000 (Reference)	
CT	24 (22.0)	24 (16.7)	0.701 (0.372-1.319)	0.329
TT	3 (2.8)	3 (2.1)	0.700 (0.137-3.561)	0.693
C allele	188 (86.2)	258 (89.6)	1.000 (Reference)	
T allele	30 (13.8)	30 (10.4)	0.728 (0.424-1.250)	0.268
ARG2				
rs3742879, A>G				
AA	67 (61.5)	92 (63.9)	1.000 (Reference)	0.872
AG	38 (34.9)	47 (32.6)	0.901 (0.529-1.532)	0.786

GG	4 (3.6)	5 (3.5)	1.000 (Reference)	1.000
A allele	172 (78.9)	231 (80.2)	0.922 (0.596-1.427)	0.739
G allele	46 (21.1)	57 (19.8)		
rs10483801, A>C				
AA	11 (10.1)	14 (9.7)	1.000 (Reference)	0.486
AC	39 (35.8)	63 (43.8)	1.269 (0.524-3.076)	0.651
CC	59 (54.1)	67 (46.5)	0.892 (0.376-2.117)	0.829
A allele	61 (28.0)	91 (31.6)	1.000 (Reference)	0.433
C allele	157 (72.0)	197 (68.4)	0.841 (0.571-1.238)	

- Abbreviations: *ARG*, Arginase gene; CI, confidence interval; OR, odds ratio.

FIGURE LEGENDS

Figure 1. Plasma nitrite levels in healthy pregnant women grouped by genotypes for the *ARG1* polymorphisms. (A) rs2781659 (GG [205.6 (154.2–421.8)] versus AG genotype [110.9 (62.4–164.6)] ($P < 0.05$). (B) rs2781667 (TT [186.1 (93.4–313.5)] versus CT genotype [106.7 (67.5–168.1)]. (C) rs2246012 (TT [127.2 (80.6–199.0)] versus TC + CC genotypes [141.5 (93.2–313.5)]. (D) rs17599586 (CC [127.8 (80.6–195.0)] versus CT + TT genotypes [145.5 (85.7–211.6)]. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P < 0.05$ as compared to heterozygotes.

Figure 2. Plasma nitrite concentrations in preeclampsia grouped by genotypes for the *ARG1* polymorphisms. (A) rs2781659 (AA [86.9 (55.1–139.2)]; AG [94.9 (60.0–150.1)] and GG genotypes [103.6 (66.0–190.0)]). (B) rs2781667 (CC [86.9 (55.1–139.2)]; CT [95.7 (59.6–163.5)] and TT genotypes [94.0 (66.0–189.4)]). (C) rs2246012 (TT [85.2 (57.9–147.4)] versus TC + CC genotypes [108.6 (76.9–176.4)]). (D) rs17599586 (CC [88.7 (59.6–155.9)] versus CT + TT genotypes [95.7 (71.0–175.2)]). The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

Figure 3. Plasma nitrite concentrations in healthy pregnant women (A and B) and in preeclampsia (C and D) grouped by genotypes for the *ARG2* polymorphisms. (A) rs3742879 (AA [123.1 (64.1–195.7)] versus AG + GG genotypes [141.5 (93.4–239.3)]). (B) rs104833801 (CC [124.4 (81.3–195.7)] versus AA + CC genotypes [135.1 (80.9–207.2)]). (C) rs3742879 (AA [88.0 (59.4–166.5)] versus AG + GG genotypes [95.6 (64.2–137.2)]). (D) rs104833801 (CC [87.9 (59.8–134.8)] versus AA + CC genotypes [97.6 (66.2–175.2)]). The maximum value for plasma nitrite concentrations is different between healthy pregnant and preeclampsia. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

FIGURE 1

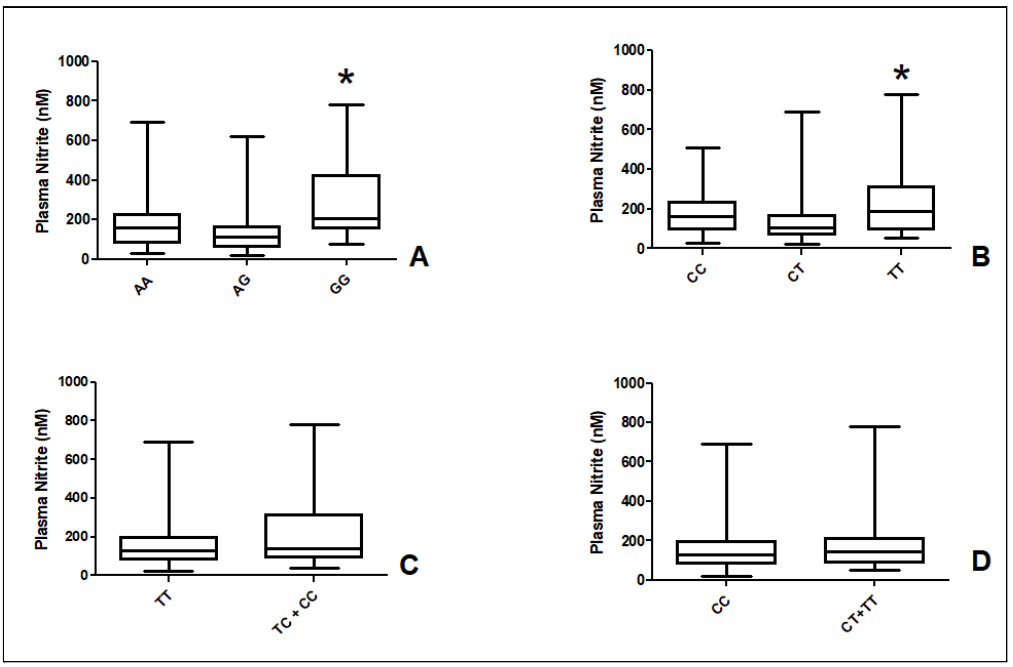


FIGURE 2

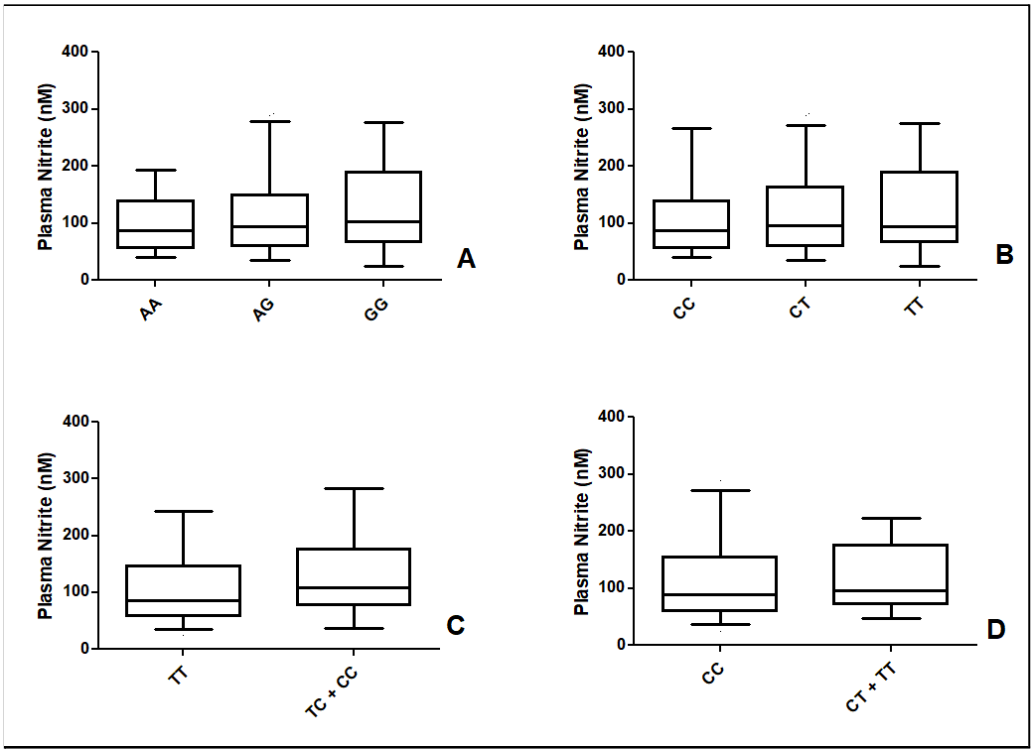
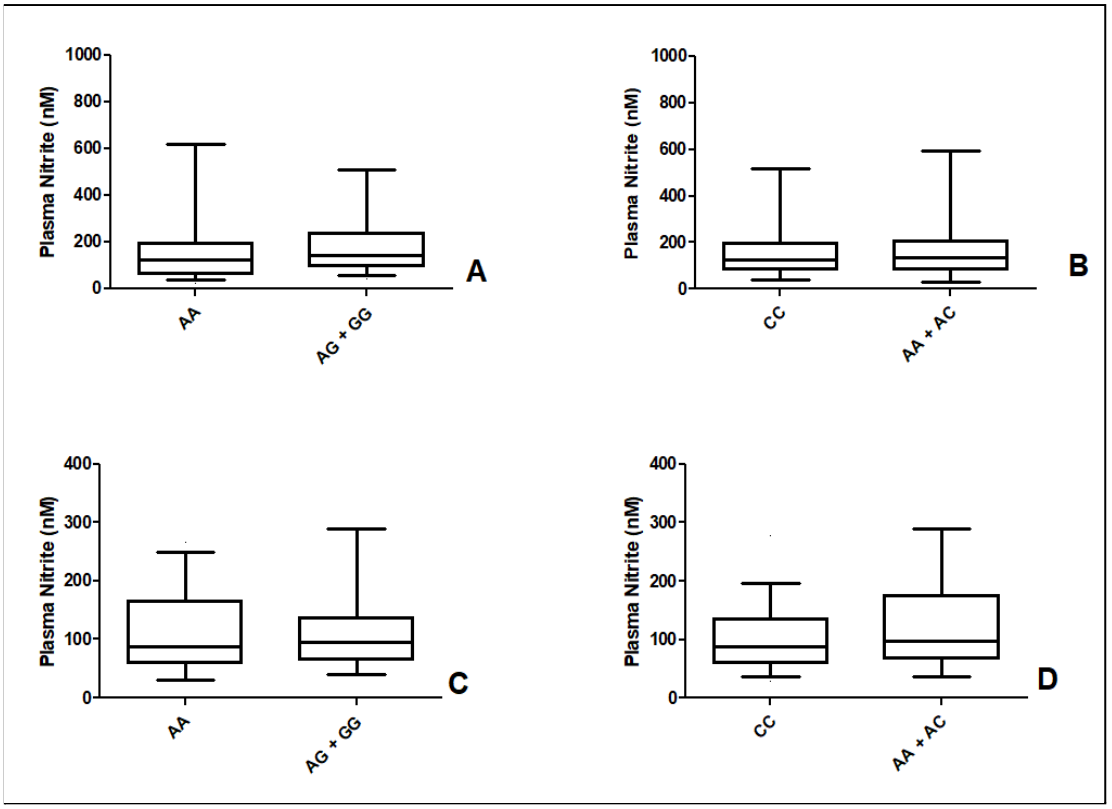


FIGURE 3


Plasma Nitrite (nM)
1000
800
600
400
200
0
AA
AG + GG
A
Plasma Nitrite (nM)
1000
800
600
400
200
0
CC
AA + AC
B
Plasma Nitrite (nM)
400
300
200
100
0
AA
AG + GG
C
Plasma Nitrite (nM)
400
300
200
100
0
CC
AA + AC
D

SUPPLEMENTARY INFORMATION

Title of the manuscript:

Effects of arginase genetic polymorphisms on nitric oxide formation in healthy pregnancy and in preeclampsia

Authors list:

Caroline C. Pinto-Souza[1], Fernanda Coeli-Lacchini[2], Marcelo R. Luizon[3], Ricardo C. Cavalli[4], Riccardo Lacchini[5], Valeria C. Sandrim[1]*

Supplementary Table 1S. Clinical characteristics of the participants with plasma nitrite data.

Parameters	Healthy pregnancy (n = 100)	Preeclampsia (n = 83)	*P value*
Age (years)	25.3 ± 6.0	26.8 ± 6.7	0.0841
Ethnicity (% White)	53.6	66.3	0.0611
Current Smokers (%)	16.4	10.8	0.2071
Primigravida (%)	44.0	44.7	0.5678
BMI, kg/m²	26.6 (24.6 - 30.6)	32.0 (26.7 - 38.4)	< 0.0001
SBP (mmHg)	110.0 (101.5 - 120.0)	140.0 (130.0 - 150.0)	< 0.0001
DBP (mmHg)	70.0 (70.0 - 80.0)	84.0 (80.0 - 95.5)	< 0.0001
HR (beats per min)	82.0 (80.0 - 88.0)	80.0 (80.0 - 86.0)	0.1196
Fasting Glucose (mg dl^{-1})	75.0 (67.0 - 84.7)	79.0 (72.6 - 99.9)	0.0115
Hemoglobin (g dl^{-1})	12.0 (10.9 - 12.7)	12.0 (11.0 - 12.8)	0.8807
Hematocrit (%)	35.9 (33.2 - 38.0)	36.0 (33.3 - 38.8)	0.5998
Creatinine (μmol l^{-1})	NA	0.6 (0.6 - 0.7)	-
Proteinuria, mg/24h	NA	760.8 (371.0 - 1436.0)	-
GA at blood collection, w	37.0 (36.0 - 38.0)	35.0 (32.0 - 37.0)	< 0.0001
GA at delivery, w	40.0 (39.0 - 41.0)	37.0 (35.3 - 39.0)	< 0.0001
Newborn weight, g	3395.0 (3106.0 - 3749.0)	2900.0 (2040.0 - 3350.0)	< 0.0001
Plasma nitrite (nM)	140.1 (81.4 - 216.7)	87.2 (55.7 - 126.5)	< 0.0001
Plasma sFlt-1 (ng/ml)	3.9 (2.6 - 5.2)	11.4 (3.7 - 16.6)	< 0.0001

- Data are expressed as media, median (25th – 75th percentile) or percentage. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; NA, not applicable.

TITLE PAGE (2)

ARG2 single nucleotide polymorphism rs3742879 modulates arginase 2 and nitrite levels and is associated with nonresponsiveness to antihypertensive therapy in preeclampsia

Caroline C. Pinto-Souza[1], Marcelo R. Luizon[2], Fernanda Coeli-Lacchini[3], Riccardo Lacchini[4], Ricardo C. Cavalli[5], Valeria C. Sandrim[1]*

[1]Department of Biophysics and Pharmacology, Institute of Biosciences of Botucatu, Universidade Estadual Paulista (UNESP), Distrito Rubiao Junior, Botucatu, Sao Paulo, 18618-689, Brazil

[2]Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, 31270-901, Brazil

[3]Department of Clinical Analyses, Toxicology and Food Science, School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto, Sao Paulo, 14040-903, Brazil

[4]Department of Psychiatric Nursing and Human Sciences, Ribeirao Preto School of Nursing, University of Sao Paulo (USP), Ribeirao Preto, Sao Paulo, 14049-900, Brazil

[5]Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo (USP), Ribeirao Preto, Sao Paulo, 14049-900, Brazil

Corresponding author.

***Author for correspondence:**

Valeria C. Sandrim, PhD - Department of Pharmacology Institute of Biosciences of Botucatu São Paulo State University (UNESP) Distrito de Rubiao Junior S/N, Zip code: 18618-000 Botucatu, SP, Brazil

Phone: +55 14 3880 0228. E-mail address: valeria.sandrim@unesp.br (V.C. Sandrim).

Competing financial interests: The authors declare no competing financial interests

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Paper presentation

Caroline C. Pinto-Souza[1] , Marcelo R. Luizon[2] , Fernanda Coeli-Lacchini[3], Riccardo Lacchini[4], Ricardo C. Cavalli[5], Valeria C. Sandrim[1]; Studies of arginase polymorphisms of nitric oxide formation and plasma arginase levels in women with pre-eclampsia responsive or not to antihypertensive therapy, subclassified as to ethnic. 6th Workshop of Biotechnology – Workbiotech, August 10th-12th, 2021, São Paulo State University, Botucatu, São Paulo, Brazil. (Video Poster presentation - online edition).

Caroline C. Pinto-Souza[1] , Fernanda Coeli-Lacchini[2] , Marcelo R. Luizon[3] , Ricardo C. Cavalli[4], Riccardo Lacchini[5], Valeria C. Sandrim[1]; The rs3742879 *ARG2* polymorphism is associated with preeclampsia nonresponsiveness and modulates arginase 2 and nitrite levels. 24th National Biomedic Meeting, October 21th-24th, 2021, São Paulo State University, Botucatu, São Paulo, Brazil. (Submitted).

Ethical approval and consent of participants

Human research approval was obtained from the Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (FMRP-USP). All participants provided written informed consent.

Abstract word count: 267 words

Main text word count: 2722 words

A. For which reasons the study was conducted?

- Arginase is related to NO synthesis and less explored in preeclampsia studies.
- No previous study has examined whether genetic variations in the *ARG1* and *ARG2* genes encoding arginase affect NO bioavailability and plasma arginase levels in responsive and nonresponsive PE women.
- It is known that pharmacological treatments do not inhibit the pathophysiological changes observed in PE, nevertheless, the use of antihypertensive drugs may avoid cardiovascular and acute cerebrovascular events related with rapid increases in blood pressure levels.
- Currently, no antihypertensive medication used in the treatment of hypertension during pregnancy involves a mechanism of action targeting the arginase biology.
- This work may help to understand the relevance of *ARG1* and *ARG2* and their genetic polymorphisms to the pathophysiology and therapeutic responses of PE.

B. Which are the main findings?

- G allele frequency of rs3742879 is lower in nonresponsive preeclampsia.
- G-carriers (rs3742879) are associated with increased plasma nitrite levels in nonresponsive preeclampsia.
- G-carriers (rs3742879) are associated with decreased plasma arginase 2 levels in nonresponsive preeclampsia.

C. What does this study brings as a novelty about what is already known?

- This study was the first to analyze arginase polymorphisms in preeclampsia responsiveness to the antihypertensive therapy.
- Our findings indicate that *ARG2* rs3742879 polymorphisms may be involved in the NO bioavailability, arginase 2 levels and in the nonresponsiveness of PE women

Keywords: Arginase polymorphisms, Preeclampsia, Responsiveness, NO bioavailability, Plasma arginase

ABSTRACT

Background and aims: Preeclampsia (PE) is associated with reduced bioavailability of nitric oxide (NO). Another factor related to NO synthesis is arginase; since this enzyme competes for the same substrate as the NO synthase precursor, the L-arginine. This study is the first to analyze whether genetic variations in the *ARG1* and *ARG2* genes encoding arginase affect NO bioavailability and plasma arginase levels in PE responsiveness to the antihypertensive therapy. Thus, in this work, we aimed to examine if *ARG1* and *ARG2* polymorphisms modulates NO bioavailability and plasma arginase levels in responsive and nonresponsive PE. Therefore, comparing in the groups, the alleles and genotypes frequencies of six arginase SNPs located in the genes *ARG1* (rs2781659; rs2781667; rs2246012; rs17599586) and *ARG2* (rs3742879; rs10483801) and if these polymorphisms affect the concentrations of plasma nitrite, a stable NO metabolite and the plasma arginase levels. **Methods:** Real Time PCR and Taqman allelic discrimination assays determined the genotypes. Plasma nitrite (nM) concentrations [mean ± standard deviation] were determined using an ozone-based chemiluminescence assay. Both arginase isoforms concentrations (ng/ml) [mean ± standard deviation] were measured in plasma samples using commercially available ELISA kits. **Results:** The *ARG2* SNP rs3742879 G allele was less frequent in non-responsive patients. In this same group, we found higher levels of arginase 2 when the AA genotype [19.1 ± 17.3] was compared to G carriers [9.2 ± 7.5]. Accordingly, in plasma nitrite, the AA genotype [78.5 ± 37.9] showed lower levels than G carriers [110.2 ± 52.8]. However, we did not find this modulation in arginase and nitrite levels in *ARG1* polymorphisms. **Conclusions:** Thus, the results of this work may help to understand the relevance of *ARG1* and *ARG2* and their genetic polymorphisms for the pathophysiology and therapeutic responses of PE.

Keywords: preeclampsia; nitric oxide; arginase; genetic polymorphisms; responsiveness; pregnancy

INTRODUCTION

Preeclampsia (PE) is a hypertensive syndrome and has been the main cause of mortality and morbidity among pregnant women in several countries [1–3]. This syndrome is also characterized by target organ damage, including impaired liver function, cerebral edema, renal insufficiency and visual disturbances [1–3]. In addition, PE is associated with fetal complications such as intrauterine growth restriction, preterm birth and perinatal death [1–3].

The current treatment options for PE are limited. And in the context of the disease, drug administration must be applied, as the antihypertensive therapies help to decrease the blood pressure and prevent adverse maternal and fetal outcomes, such as cardiovascular and acute cerebrovascular events [4,5]. The guidelines for antihypertensive treatment of pregnant disorders (NHBPEP, National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy) include therapeutic recommendations based on specific diagnosis (mild-to-moderate hypertension, severe hypertension and PE) and on the targeted blood pressure level. Typically, the therapy includes methyldopa - (centrally acting adrenergic inhibitor), nifedipine (calcium channel blocker) and hydralazine (peripheral vasodilator) [1,6]. However, about 40% of PE pregnant women do not respond to the antihypertensive therapy, still, this subgroup of patients develops the worst clinical outcomes [7–10].

The pathophysiology of PE is complex and involves several processes. One of these is related to the deficiency of the nitric oxide (NO) pathway [11–13]. A possible cause of this reduced bioavailability could be the high expression of the arginase enzyme, which competes with eNOS, the enzyme responsible for NO synthesis, mainly in the endothelium, by the substrate (L-arginine) [14–16]. Arginase is found in vascular endothelium and smooth muscle cells [16,17]. One of its primary actions is the catalysis of the conversion of L-arginine into L-ornithine and urea [14]. It is presented in two forms, arginase I (cytosolic) - codified by *ARG1*, mainly expressed in the liver and arginase 2 (mitochondrial) - codified by *ARG2*, mainly expressed in the kidneys [18].

Our group previously identified the effects of *ARG1* and *ARG2* polymorphisms on nitric oxide bioavailability in PE and healthy pregnancy (HP) [19]. Considering *ARG1* SNPs, the GG genotype and G allele frequencies for rs2781659 and the C allele frequencies for rs2246012 were higher in PE compared to the control group.

Furthermore, the GG genotype for rs2781659 and the TT genotype for rs2781667 were associated with higher plasma nitrite in HP [19]. Notwithstanding, we found no association of *ARG2* polymorphisms with PE or nitrite levels in both groups [19].

In addition, our group evidenced that lower arginase 2 plasma levels were found in PE without severe features and responsive to antihypertensive drugs when compared to HP. While in nonresponsive PE, this isoform concentration was higher [20]. However, to date, genotypic and allelic distribution related to *ARG1* and *ARG2* polymorphisms have not been evaluated for PE antihypertensive response.

Therefore, the objective of our work was to compare the allele and genotype frequencies of SNPs located in *ARG1* (rs2781659; rs2781667; rs2246012; rs17599586) and *ARG2* (rs3742879; rs10483801) between responsive and nonresponsive PE. Likewise, we sought to analyze if *ARG1* and *ARG2* SNPs could modulate circulating levels of nitrite, a stable NO metabolite, and arginase isoforms and contribute to alterations in the bioavailability of NO.

MATERIALS AND METHODS

Subjects

Human research approval was obtained from the Institutional Review Board at the Ribeirao Preto Medical School of University of Sao Paulo (FMRP-USP). All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital at the FMRP-USP. We studied 81 PE women responsive and 69 PE women nonresponsive to total antihypertensive therapy. According to the ACOG 2013 [1], PE was defined as pregnancy-induced hypertension (≥ 140 mmHg systolic and ≥ 90 mmHg diastolic on two or more measurements with an interval of at least 4 (four) hours between one take and the next) in a woman after 20 weeks of gestation associated with proteinuria and target organ damage [1]. In the present study, women with preexisting hypertension, with or without superimposed PE were not included.

Antihypertensive Treatment and Drug Response Evaluation

Responsiveness to therapy was evaluated through the clinical and laboratory parameters (see below) in response to the antihypertensive drugs treatment.

The initial antihypertensive drug was methyldopa (1,000–1,500 mg per day) followed by nifedipine (40–60 mg per day). In case of lack of significant responses to methyldopa, as a third drug we used hydralazine (5–30 mg). One of the following clinical and laboratory outcomes were considered to classify a patient as nonresponsive to antihypertensive therapy [6,21]:

(1) Clinical symptoms including blurred vision, persistent headache or scotoma, persistent right upper quadrant or epigastric pain;

(2) Systolic blood pressure > 140 mmHg and diastolic blood pressure > 90 mmHg, as assessed by the blood pressure curve;

(3) Hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome; creatinine > 1.2 mg per 100 mL; aspartate aminotransferase > 70 Ul1; thrombocytopenia below 100,000mm3, hemolysis with total bilirubin above 1.2 and or lactic dehydrogenase above 600 and

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

In the clinical attendance, written informed consent was provided by volunteers who agree to participate in the study, maternal venous blood samples were collected into tubes containing EDTA (used in arginase samples and in DNA extraction to genotyping) and containing heparin (to measure nitrite). Plasma samples were obtained from whole blood after centrifugation at 1000g for 10 min and stored at –70ºC until assayed. Genomic DNA was extracted from the cellular fraction of 1 mL of whole blood by a salting-out method and stored at –20ºC until analyzed.

Patients subsets

As a subgroup of PE women had plasma nitrite available for dosing (responsive, n = 52; nonresponsive, n = 39), Supplemental Table S1 was constructed, showing the clinical characteristics of this subset. Either, a subset of PE women had plasma arginase 1 and 2 available for dosing (responsive, n = 29; nonresponsive, n = 27), thus, Supplemental Table S2 was constructed, showing the clinical characteristics of this other subset.

Enzyme linked immunoassays (ELISAs) of Arginase 1 and Arginase 2 and Measurement of Nitrite Concentrations

Human Arginase 1 and Arginase 2 concentrations were measured in plasma samples using commercially available ELISA kits (MBS912500 and MBS2021960 respectively, purchased from MyBioSource; San Diago, CA, USA), following the manufacturer's instructions. Nitrite concentrations were measured using an ozone-based chemiluminescence assay, as previously described [22].

Genotyping

Genotypes were determined by Taqman Allele Discrimination assays using probes and primers designed by Applied Biosystems (Foster City, CA, USA). Assay IDs for SNPs were: C___3063957_10 (rs2781659), C__15933286_10 (rs2781667), C__15933284_10 (rs2246012), C__25596209_10 (rs17599586), C__25960528_10 (rs3742879) and C___2778311_10 (rs10483801). All experiments were performed with JumpStart Taq ReadyMix for Quantitative PCR 1x (Sigma Aldrich, St Louis, MO, USA), Taqman assays 1x and 5 ng of template in 10 uL reaction volume. Thermal cycling was performed under standard conditions and the StepOne Plus Real Time PCR equipment (Applied Biosystems, Foster City, CA, USA) recorded fluorescence. The results were analyzed with manufacturer's software.

Statistical analysis

The clinical characteristics of PE women responsive or not to total antihypertensive therapy were compared by Student's unpaired t-test, Mann-Whitney U-test, or $\chi 2$ as appropriate. The effects of the different genotypes for the *ARG1* and *ARG2* SNPs on nitrite and arginase concentrations of PE women responsive or not to total antihypertensive therapy were compared by Student's unpaired t-test and one-way ANOVA with post hoc tests. Distributions of genotypes were assessed for deviation from the Hardy-Weinberg equilibrium. Statistical analysis was performed with GraphPad Prism 5.0. A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Table 1 shows clinical characteristics of PE women responsive or not to total antihypertensive therapy. Nonresponsiveness was associated with higher systolic and diastolic blood pressure, higher creatinine and proteinuria levels, lower gestational age at the blood collection and at delivery, lower newborn weight and higher plasma sFlt-1, plasma arginase 1, plasma arginase 2 and urea concentrations (all $P < 0.05$).

Table 2 shows the results of the single-locus analysis. All the polymorphisms showed no deviation from Hardy-Weinberg equilibrium (all $P > 0.05$, data not shown). No differences were found in the distribution of alleles and genotypes of the *ARG1* polymorphisms. Genotype and allele distributions from the *ARG2* SNP rs3742879 were less frequent when the PE nonresponsive group was compared with the PE responsive group **(Table 2).**

Next, we examined whether the *ARG2* SNPs may modulate plasma nitrite and arginase 2 levels in the groups. In nonresponsive, we found higher plasma arginase 2 concentrations when AA genotype [19.1 ± 17.3 ng/ml] was compared with G-carriers [9.2 ± 7.5 ng/ml)]; accordingly, in plasma nitrite, AA genotype [78.5 ± 37.9 nM] showed lower levels than the G-carriers [110.2 ± 52.8 nM] (**Figure 1)**. Regarding *ARG1*, we did not find any effects of its SNPs **(Supplementary Figure 1)**.

DISCUSSION

This study was the first to compare the polymorphisms in *ARG1* and *ARG2* and to evaluate the effect of their SNPs on plasma nitrite and arginase isoforms levels in the responsive and nonresponsive subtypes of PE to drug treatment. Our results showed that 1) G carriers of rs3742879 are less frequent in nonresponsive versus responsive PE, thereby, according to 2) lower nitrite levels found in AA genotype versus G carriers in *ARG2* SNP rs3742879 in the nonresponsive group and 3) higher arginase 2 levels presented in AA genotype versus G carriers in *ARG2* SNP rs3742879 in the nonresponsive group. Thus, as major findings, we observed that rs3742879 is associated with nonresponsiveness and modulates arginase 2 and nitrite levels in this group.

Arginase is an enzyme present in several organisms [23] and found in vascular endothelium and smooth muscle cells [16,17]. It comes in two forms, arginase I (cytosolic) – codified by *ARG1* and arginase 2 (mitochondrial) codified by *ARG2*. These enzymes are co-localized with eNOS in the endothelium [18] and compete with NOS for the same substrate [15,16]. Thus, high levels of arginase activity could lead to decreased availability of the NOS substrate (L-arginine), thereby, decreasing NO synthesis [15,16,24,25]. Few studies have measured the expression/activity of this enzyme in PE [26–32].

Notwithstanding, previously, our group compared the frequency of arginase polymorphism genotypes between PE and HP and the results suggested that SNPs of *ARG1* increase the risk for the hypertensive group and modulate plasma nitrite levels in the healthy one [19].

At the same time that PE pathophysiology increases maternal fetal susceptibility of developing cardiovascular complications [1–3], effective pharmacological treatments for this disease do not exist, either, current drug administration is limited. In this respect, no study has examined whether *ARG1* and *ARG2* polymorphisms affect the responsiveness to total antihypertensive therapy and could modify arginase and nitrite concentrations. However, other studies have found differences between genotypic frequencies in PE patients responsive or not to the antihypertensive therapy in other genes as in the nitric oxide synthase *NOS3* [33].

As well, in the heme oxygenase-1 *HMOX-1* [33], it codifies the HMOX-1 enzyme which presents anti-inflammatory and antioxidant actions [34]; in the matrix metalloproteinase-9 *MMP-9* [35], which regulates pathological remodeling processes that involve inflammation and fibrosis in cardiovascular disease [36].

Substantially, there are works in animal models [RL2] considering arginase as a novel target for treating endothelial dysfunction [37–40]. It was showed that arginase inhibition with N(omega)-hydroxy-nor-L-arginine and alpha-difluoromethylornithine reduced endothelial dysfunction and blood pressure rising in spontaneously hypertensive rats (SHR) [37,38]. Experiments conducted on SHRs and Wistar–Kyoto (WKY) rats evaluated arginase activity and expression in heart, kidney, liver, lung and brain tissue extracts [39]. Only in the hearts, the prevention of hypertension by hydralazine attenuated the increase in the enzyme activity [39].

In a study done using male Wistar rats [40], searchers demonstrated that the application of arginase inhibitors (citrulline, norvaline or ornithine) to treat metabolic syndrome (induced by giving fructose 10% in drinking water *ad libitum* daily for 12 weeks) significantly reduced elevations in diastolic and systolic blood pressure. Furthermore, treatment with enzyme inhibitors administered to rats prevented the generation of impaired NO and the exacerbated formation of reactive oxygen species [40]. Thus, these findings demonstrate that exploring arginase pathways could assist to understand its evolvement in endothelial dysfunction in hypertension and be a new target to the development of antihypertensive drugs.

To our knowledge, there are few data about rs3742879 and some authors demonstrated that its G variant has been associated with lower fractional NO concentration with greater effect in children with asthma [41]; also, each variant allele of this SNP has been associated with an increased risk of this disease [42]. Which contrasts with our results, as we observed that only the G allele differs in the frequency distribution on the PE subtypes. Moreover, this G variant being less frequent in the nonresponsive group indicates that although this group of patients do not respond to the antihypertensive treatment, presents higher NO bioavailability compared with those who respond. This inverse relation could be explained by the inducible NOS generation, knowing that it is released in the face of an inflammatory stimulus [43–45] – more present in the nonresponsive, as this subset of patients develops the worst clinical outcomes, as previously explored in our group [7–10].

The expression of arginase is stimulated by a variety of factors such as: tumor necrosis factor (TNF)-α, interferon-ɣ, interleukins [15,46], angiotensin II [47], reactive oxygen and nitrogen species [48], among others. Likewise, the endothelial dysfunction present in PE would exacerbate this harmful environment [11,12].

Moreover, based on experimental evidence, associations of arginase variants with asthma might depend on the airway inflammatory state and exposures that mediate oxidant stress, both of which are strong determinants of this respiratory illness [42].

Considering NO data, the results of *Salam et al., 2010* [41] probably differ from ours as they used the fractional NO concentration to assess the oxidative and nitrosative stress in the airways. While the methodology of this work applied the ozone-based chemiluminescence assay to analyze the nitrite levels [22], a NO stable metabolite and a surrogate indicator of the presence of this gas molecule, it does not predict the exact value. Concerning circulating arginase 2 concentrations, when compared with healthy subjects, increased levels of this enzyme were found in patients with clinical erectile dysfunction [49], which pathophysiology reveals endothelial dysfunction and impaired relaxation of smooth muscle cells [50]. In addition, the isoform concentrations are also associated with increased risk for developing the disease [49]. Accordingly, the nonresponsive group exhibited higher levels in plasma arginase 2 than the responsive group, which reveals the difference between their pathophysiological mechanisms.

Nevertheless, a recent study from our group reported that PE pregnant women exhibited different profiles of arginase 2 expression when considering the severity of the disease and also their responsiveness to antihypertensive therapy [20]. Either, arginase 2 levels were positively correlated with blood pressure levels in non-severe PE and responsive PE to antihypertensive therapy [20]. Although, in this work, we did not find any modulation of the isoform levels in the responsive group.

Regarding rs10483801, as revised, it is associated with different phenotypes of sickle cell disease [51,52]. In the respect of *ARG1*-related SNPs, we did not find any effects over the biomarkers. Besides, previously we pointed out that rs2781659 G-carriers and rs2246012 C-carriers were more frequent in PE women versus HP [19], which indicates an association of these polymorphisms with the risk to develop the disease. Nevertheless, more studies on the *ARG1*, *ARG2* genes are needed to identify the individual functional effect of each of the explored polymorphisms.

LIMITATIONS

We did not have the plasma from all pregnant women genotyped, which reduced the number of samples analyzed in the groups responsive and nonresponsive PE.

Furthermore, as a subset of patients had the biomarkers plasma nitrite; plasma arginase 1 and plasma arginase 2 dosed, the statistics power is not enough to detect small differences between groups. Therefore, we could not explore the entire effects of the polymorphisms.

CONCLUSION

Our findings indicate that *ARG2* rs3742879 polymorphisms may be involved in the nitric oxide bioavailability, arginase 2 levels and in the nonresponsiveness of PE women. Currently, no antihypertensive medication used in the treatment of hypertension during pregnancy involves a mechanism of action targeting the arginase biology. Thus, the results from this work may help to understand the relevance of *ARG1* and *ARG2* and their genetic polymorphisms to the pathophysiology and therapeutic responses of PE.

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TABLES

TABLE 1. Clinical characteristics of the participants included in this study.

Parameters	Responsive	Nonresponsive	*P*
N	**81**	**69**	
Age (years)	26.8 ± 6.1	26.8 ± 7.3	0.9913
Ethnicity (% White)	69.1	66.2	0.7002
Current Smokers (%)	13.6	4.4	0.0561
Primigravida (%)	43.2	47.1	0.6381
SBP (mmHg)	130.0 (120.0 - 140.0)	150.0 (140.0 - 165.0)	< 0.0001
DBP (mmHg)	80.0 (76.0 - 90.0)	90.0 (82.0 - 100.0)	< 0.0001
HR (beats per min)	80.0 (80.0 - 85.5)	80.0 (77.5 - 88.0)	0.5488
Fasting Glucose (mg dl^{-1})	81.0 (74.5 - 98.5)	79.0 (70.5 - 104.3)	0.9138
Hemoglobin (g dl^{-1})	12.0 (11.0 - 12.7)	12.0 (11.0 - 12.7)	0.5834
Hematocrit (%)	28.8 (27.5 - 30.2)	29.0 (27.4 - 30.8)	0.7212
Creatinine (μmol l^{-1})	0.6 (0.5 - 0.7)	0.7 (0.6 - 0.8)	0.0011
Proteinuria, mg/24h	509.1 (357.6 - 1156.0)	1463.0 (743.3 - 3270.0)	0.0003
GA at blood collection, w	37.0 (34.0 - 39.0)	33.0 (31.0 - 36.0)	< 0.0001
GA at delivery, w	39.0 (38.0 - 39.0)	34.00 (31.2 - 37.0)	< 0.0001
Newborn weight, g	3185.0 (2650.0 - 3495.0)	1998.0 (1236.0 - 2605.0)	< 0.0001
Plasma nitrite (nM)	88.0 (54.3 - 120.3)	88.7 (58.7 - 142.6)	0.6153
Urea (mg dl^{-1})	16.0 (11.0 - 22.0)	23.0 (15.2 - 30.0)	0.0002
Plasma sFlt-1 (ng/ml)	6.0 (2.0 - 12.0)	16.0 (9.0 - 19.0)	0.0004
Plasma arginase 1 (ng/ml)	5.1 (3.0 - 8.0)	6.5 (5.0 - 11.5)	0.0437
Plasma arginase 2 (ng/ml)	6.3 (4.3 - 11.4)	9.3 (5.5 - 20.6)	0.0084

Data are expressed as media, median (25th – 75th percentile) or percentage. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HR, heart rate; NA, not applicable; SBP, systolic blood pressure. *P < 0.05 vs responsive PE.

TABLE 2. Genotypes and alleles for the *ARG1 and ARG2* SNPs in PE patients: responsive and nonresponsive to antihypertensive therapy.

Gene, SNP **N**	**Responsive (%)** **81**	**Nonresponsive (%)** **69**	***P value***
ARG1 **rs2781659, A>G**			
AA	23.7	27.9	0.8395
AG	53.8	51.5	
GG	22.5	20.6	
A allele	50.6	53.7	0.6005
G allele	49.4	46.3	
rs2781667, C>T			
CC	22.2	24.6	0.8894
CT	53.1	53.6	
TT	24.7	21.7	
C allele	48.8	51.4	0.6431
T allele	51.2	48.6	
rs2246012, T>C			
TT	68.4	61.8	0.4026
TC+CC	31.6	38.2	
T allele	63.9	58.5	0.2676
C allele	36.1	41.5	
rs17599586, C>T			
CC	75.0	85.5	0.1107
CT + TT	25.0	14.5	
C allele	86.9	92.0	0.1519
T allele	13.1	8.0	
ARG2 **rs3742879, A>G**			
AA	57.1	63.3	-
AG	36.4	24.7	0.193

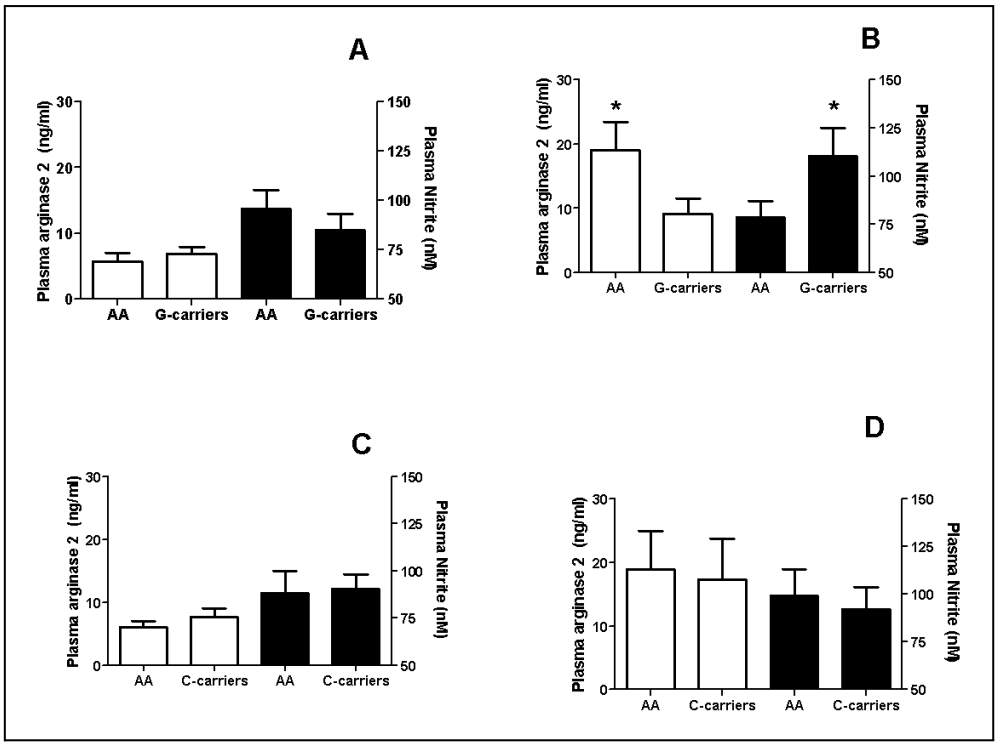
GG	6.5	11.4	0.082
A allele	75.3	84.8	–
G allele	24.7	15.2	**0.049***
rs10483801, A>C			
AA	10.1	7.3	
AC	39.2	47.8	0.5418
CC	50.6	44.9	
A allele	29.7	31.2	0.7921
C allele	70.3	68.8	

Abbreviations: *ARG*, Arginase gene; CI, confidence interval; OR, odds ratio.

FIGURE LEGENDS

Figure 1. Plasma arginase 2 and nitrite levels in PE women responsive (R) or not (NR) to antihypertensive therapy grouped by genotypes of *ARG2* polymorphisms. Only a subgroup of patients presents the biomarkers concentrations, thus, after the levels presented there is a representative number. **A**. **rs3742879** (R). We did not observe differences in plasma arginase 2 between genotypes AA [5.8 ± 4.6 ng/ml; n = 15] and G-carriers [6.8 ± 3.9 ng/ml; n = 12], neither in plasma nitrite: AA genotype [95.6 ± 46.7 nM; n = 24], G-carriers [84.8 ± 40.1 nM; n = 22]. **B**. **rs3742879** (NR). We found higher plasma arginase 2 concentrations when AA genotype [19.1 ± 17.3 ng/ml; n = 16] was compared with G-carriers [9.2 ± 7.5 ng/ml; n = 10], $p < 0.05$; while in plasma nitrite AA genotype [78.5 ± 37.9 nM; n = 20] showed lower levels than the G-carriers [110.2 ± 52.8 nM; n = 13], $p < 0.05$. **C**. **rs10483801** (R). We did not observe differences in plasma arginase 2 between AA genotype [6.0 ± 3.3 ng/ml; n = 11] and C-carriers [7.7 ± 4.5 ng/ml; n = 13] neither in plasma nitrite: AA genotype [88.2 ± 47.5 nM; n = 16], C-carriers [90.4 ± 42.0 nM; n = 31]. **D**. **rs10483801** (NR). We did not observe differences in plasma arginase 2 between AA genotype [19.0 ± 19.7 ng/ml; n = 11] and C-carriers [17.4 ± 24.0 ng/ml; n = 14], neither in plasma nitrite: AA genotype [99.1 ± 50.4 nM; n =13]; C-carriers [92.2 ± 52.2 nM; n = 21]. Data in the graph are presented as mean ± standard deviation.

FIGURE 1


A
Plasma arginase 2 (ng/ml)
Plasma Nitrite (nM)
AA
G-carriers
AA
G-carriers
B
*
*
C
C-carriers
D

SUPPLEMENTARY INFORMATION

Title of the manuscript:

***ARG2* single nucleotide polymorphism rs3742879 modulates arginase 2 and nitrite levels and is associated with nonresponsiveness to antihypertensive therapy in preeclampsia.**

Authors list:

Caroline C. Pinto-Souza[1], Marcelo R. Luizon[2], Fernanda Coeli-Lacchini[3], Riccardo Lacchini[4], Ricardo C. Cavalli[5], Valeria C. Sandrim[1]*

Supplementary Table Clinical 1. Clinical characteristics of the subset patients with plasma nitrite.

Parameters	Responsive	Nonresponsive	*P*
N	**52**	**39**	
Age (years)	26.7 ± 5.8	27.9 ± 8.0	0.4033
Ethnicity (% White)	70.4	61.5	0.3342
Current Smokers (%)	15.4	5.1	0.1216
Primigravida (%)	40.4	38.5	0.8527
BMI, kg/m²	32.9 (28.8 - 39.7)	30.8 (26.5 - 36.4)	0.1469
SBP (mmHg)	130.0 (120.0 - 140.0)	150.0 (140.0 - 160.0)	< 0.0001
DBP (mmHg)	80.0 (70.0 - 90.0)	90.0 (80.0 - 100.0)	0.0008
HR (beats per min)	80.0 (80.0 - 84.0)	80.0 (76.0 - 88.0)	0.2490
Fasting Glucose (mg dl^{-1})	80.5 (75.1 - 96.8)	78.5 (69.3 - 104.3)	0.8705
Hemoglobin (g dl^{-1})	12.0 (11.2 - 12.8)	12.2 (11.0 - 12.9)	0.8779
Hematocrit (%)	36.3 (33.9 - 38.6)	36.5 (33.3 - 39.0)	0.9686
Creatinine (μmol l^{-1})	0.6 (0.5 - 0.7)	0.7 (0.6 - 0.8)	0.0033
Proteinuria, mg/24h	443.0 (333.1 - 945.3)	1167.0 (760.8 - 2389.0)	0.0012
GA at blood collection, w	36.5 (32.8 - 38.0)	33.0 (32.0 - 36.0)	0.0032
GA at delivery, w	38.5 (38.0 - 39.0)	36.00 (32.0 - 37.0)	< 0.0001
Newborn weight, g	3203.0 (2614.0 - 3508.0)	2045.0 (1460.0 - 2865.0)	< 0.0001
Plasma nitrite (nM)	82.8 (54.3 - 120.3)	88.0 (58.1 - 133.7)	0.6153
Urea	16.0 (11.8 - 21.3)	22.0 (15.8 - 28.0)	0.0030
Plasma sFlt-1 (ng/ml)	6.1 (2.0 - 11.7)	16.0 (9.3 - 21.2)	0.0001
Plasma arginase 1 (ng/ml)	5.1 (3.0 – 8.7)	6.5 (5.0 – 11.5)	0.0510
Plasma arginase 2 (ng/ml)	5.7 (2.9 – 8.4)	9.3 (5.5 – 20.7)	0.0067

Data are expressed as media, median (25th – 75th percentile) or percentage. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HR, heart rate; NA, not applicable; SBP, systolic blood pressure. *P < 0.05 vs responsive PE.

Supplementary Table Clinical 2. Clinical characteristics of the subset patients with plasma arginase 1 and 2.

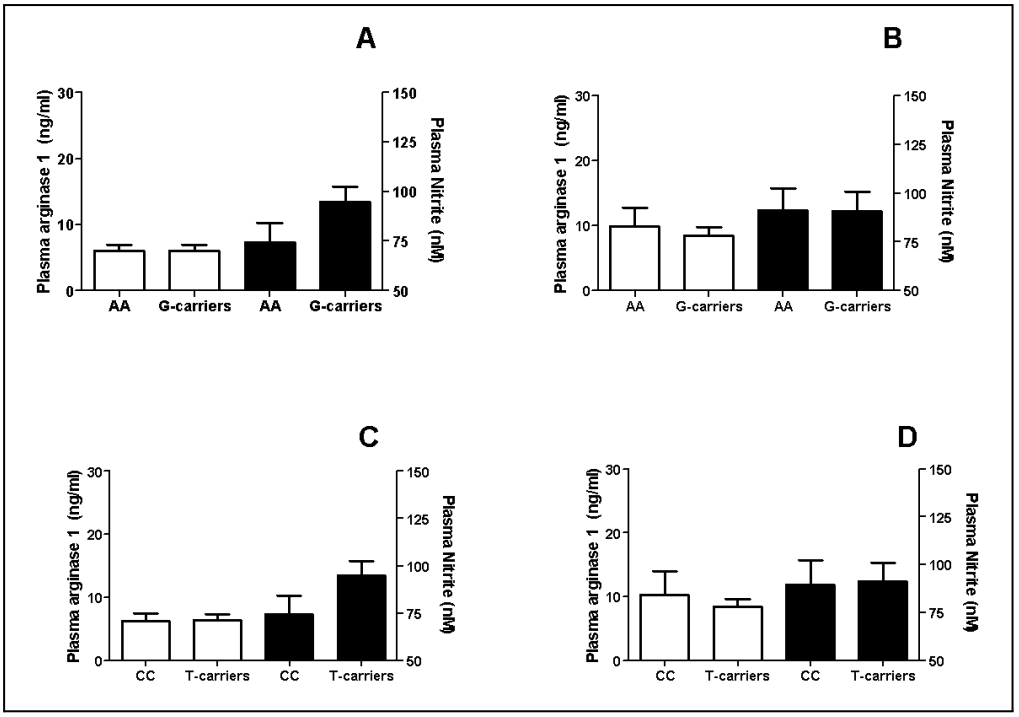
Parameters	Responsive	Nonresponsive	*P*
N	**29**	**27**	
Age (years)	26.9 ± 5.6	28.2 ± 7.9	0.4650
Ethnicity (% White)	64.3	70.4	0.6307
Current Smokers (%)	6.9	8.0	0.8733
Primigravida (%)	37.9	40.7	0.8297
BMI, kg/m²	34.3 (27.3 - 37.8)	31.6 (27.7 – 38.6)	0.4870
SBP (mmHg)	130.0 (120.0 - 140.0)	150.0 (140.0 - 160.0)	< 0.0001
DBP (mmHg)	80.0 (70.0 - 90.0)	90.0 (82.0 - 100.0)	0.0006
HR (beats per min)	80.0 (80.0 - 84.5)	80.0 (79.5 - 88.0)	0.2759
Fasting Glucose (mg dl^{-1})	80.0 (75.0 - 99.4)	77.0 (69.0 - 98.3)	0.5857
Hemoglobin (g dl^{-1})	12.0 (11.3 -- 13.0)	12.4 (11.5 - 13.2)	0.4604
Hematocrit (%)	35.0 (34.0 -- 39.6)	37.6 (34.5 - 39.0)	0.3753
Creatinine (μmol l^{-1})	0.6 (0.6 - 0.7)	0.7 (0.6 - 0.8)	0.0126
Proteinuria, mg/24h	375.5 (320.2 - 757.3)	1163.0 (509.7 - 3764.0)	0.0081
GA at blood collection, w	36.0 (29.0 - 38.0)	33.0 (31.5 - 36.0)	0.0528
GA at delivery, w	39.0 (37.0 - 40.0)	34.00 (32.0 - 37.0)	< 0.0001
Newborn weight, g	3125.0 (2750.0 - 3450)	2040.0 (1300.0 - 2865.0)	< 0.0001
Plasma nitrite (nM)	80.4 (55.8 - 110.3)	101.3 (83.5 –– 150.1)	0.0400
Urea	17.0 (13.0 - 21.0)	23.0 (18.0 - 30.0)	0.0034
Plasma sFlt-1 (ng/ml)	4.0 (1.5 - 11.5)	15.4 (9.2 - 16.5)	0.0003
Plasma arginase 1 (ng/ml)	5.1 (3.0 – 8.7)	6.5 (5.0 – 11.5)	0.0510
Plasma arginase 2 (ng/ml)	5.7 (2.9 – 8.4)	9.3 (5.5 – 20.7)	0.0067

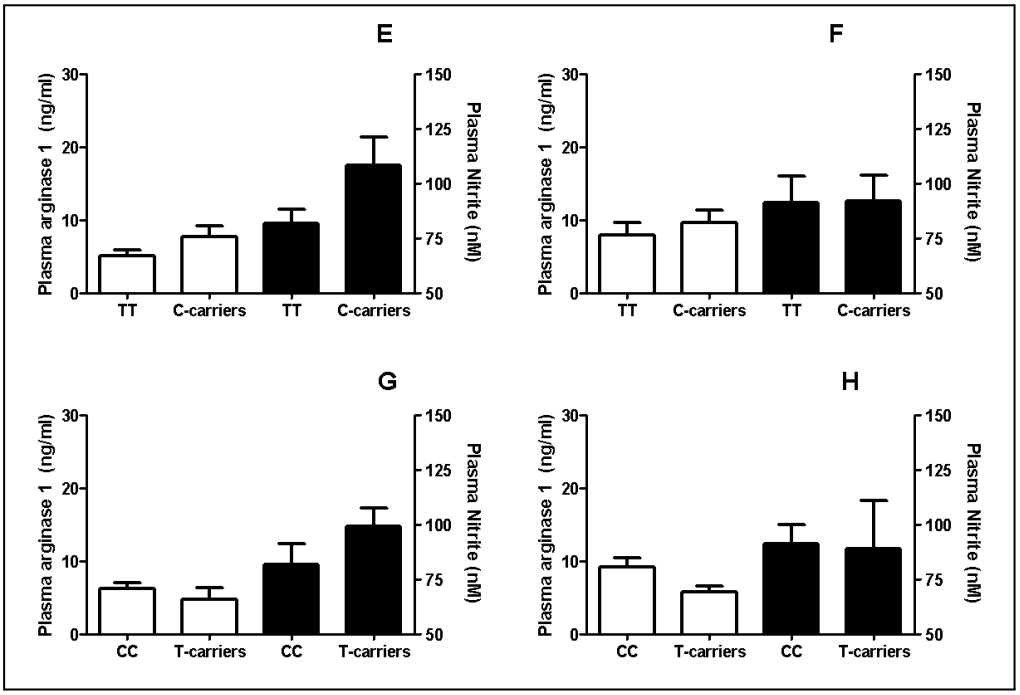
Data are expressed as media, median (25th – 75th percentile) or percentage. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GA, gestational age; HR, heart rate; NA, not applicable; SBP, systolic blood pressure. *P < 0.05 vs responsive PE.

FIGURE LEGEND

Figure 1S. Plasma arginase 1 and nitrite levels in PE women responsive (R) or not (NR) to antihypertensive therapy grouped by *ARG1* polymorphisms genotypes. Only a subgroup of patients presents the biomarkers concentrations, thus, after the levels presented there is a representative number. **A**. **rs2781659** (R). We did not observe differences in plasma arginase 1 between genotypes AA [6.0 ± 2.9 ng/ml; n = 9] and G-carriers [6.0 ± 4.0 ng/ml; n = 10]; neither in plasma nitrite: AA genotype [74.5 ± 33.6 nM; n = 12], G-carriers [94.9 ± 45.5 nM; n = 35]. **B**. **rs2781659** (NR). We did not observe differences in plasma arginase 1 between genotypes AA [9.8 ± 7.3 ng/ml; n = 6] and G-carriers [8.4 ± 5.9 ng/ml; n = 21], neither in plasma nitrite: AA [91.2 ± 29.3 nM; n = 7], G-carriers [91.0 ± 50.4 nM; n = 26]. **C**. **rs2781667** (R). We did not observe differences in plasma arginase 1 between AA genotype [6.2 ± 3.3 ng/ml; n = 7] and C-carriers [6.4 ± 3.8 ng/mL; n = 18]; neither in plasma nitrite: AA genotype [74.5 ± 33.6 nM; n = 12], C-carriers [94.9 ± 45.5 nM; n = 35. **D**. **rs2781667** (NR). We did not observe differences in plasma arginase 1 between AA genotype [10.3 ± 8.1 ng/ml; n = 5] and C-carriers [8.4 ± 5.8 ng/ml; n = 22], neither in plasma nitrite: AA genotype [89.5 ± 31.8 nM; n = 6]; C-carriers [91.3 ± 49.4 nM; n = 27]. Data in the graph are presented as mean ± standard deviation. **E**. **rs2246012** (R). We did not observe differences in plasma arginase 1 concentrations when the TT genotype [5.2 ± 3.1 ng/ml; n = 19] was compared with C-carriers [7.7 ± 4.3 ng/ml; n = 9], neither in plasma nitrite: TT genotype [81.7 ± 37.5 nM; n = 31] versus C-carriers [108.4 ± 50.60 nM; n = 15]. **F**. **rs2246012** (NR). We did not observe differences in plasma arginase 1 between TT genotype [8.0 ± 5.8 ng/ml; n = 12] and C-carriers [9.6 ± 6.7 ng/ml; n = 14], neither in plasma nitrite: TT genotype [91.3 ± 51.0 nM; n = 18], C-carriers [92.2 ± 42.8 nM; n = 14]. **G**. **rs17599586** (R). We did not observe differences in plasma arginase 1 between CC genotype [6.3 ± 3.6 ng/ml; n = 23] and T-carriers [4.7 ± 3.7 ng/ml; n = 5], neither in plasma nitrite: CC genotype [81.8 ± 47.2 nM; n = 26], T-carriers [99.3 ± 37.0 nM; n = 21]. **H**. **rs17599586** (NR). We did not observe differences in plasma arginase 1 between CC genotype [9.1 ± 6.4 ng/ml; n = 24] and T-carriers [5.7 ± 1.5 ng/ml; n = 3], neither in plasma nitrite: CC genotype [91.2 ± 47.6 nM; n = 30]; T-carriers [89.1 ± 37.9 nM; n = 3]. Data in the graph are presented as mean ± standard deviation.

Supplementary Figure 1





PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Arginase em pré-eclâmpsia: estudo de polimorfismos genéticos, fatores circulantes e ensaios in vitro

Pesquisador: Ricardo de Carvalho Cavalli

Área Temática:

Versão: 1

CAAE: 37738620.0.0000.5440

Instituição Proponente: Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da USP -

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.347.078

Apresentação do Projeto:

Os autores se propõem a estudar alguns dos mecanismos moleculares envolvidos no quadro da pré-eclâmpsia, entre os quais o papel de polimorfismo no gene da arginase. Essa enzima promove a degradação da L-arginina, o que diminui a dispobilidade dessa substância para a síntese de óxido nítrico (ON), o principal vasodilatador dos vasos. A deficiência do ON está documentada na pré-eclâmpsia, acreditando-se ser um dos seus fatores mais importantes.

Os autores pretendem estudar a presença de polimorfismos no gene da arginase em 150 gestantes com pré -eclâmpsia e em 150 gestantes normais, a fim de avaliar o seu eventual impacto na doença em foco.

Este é um estudo observacional derivado de outros estudos já apreciados e aprovados pelo sistema CEP/CONEP.

Objetivo da Pesquisa:

Objetivo Primário:

1)Comparar as freqüências alélicas, genotípicas e haplotípicas dos SNPs ARG1 (rs2781659, rs2781667, rs2246012 e rs17599586) e ARG2 (rs3742879 e rs10483801) entre gestantes saudáveis e com pré-eclâmpsia (responsiva e não responsiva a terapia anti38.

2) Comparar e correlacionar os níveis plasmáticos de arginase 1, arginase 2, atividade total da arginase com os níveis plasmáticos de nitrito e sFLT-1

em gestantes saudáveis e com pré-eclâmpsia (responsiva e não responsiva a terapia anti-hipertensiva)
3)Avaliar o efeito da suplementação com resveratrol e/ou do inibidor de arginase (BEC) nas culturas endoteliais incubadas com os plasmas de préeclâmpsia e gestante saudável sob os seguintes parâmetros:
- Viabilidade celular
- Citotoxicidade
- Expressão gênica e Atividade da arginase
- Produção de NO / nitrito / nitrotirosina
- Produção de espécies reativas de oxigênio
- Apoptose
- Expressão gênica e atividade da NADPH oxidase
- Marcadores de disfunção endotelial (VCAM, ICAM e E-Selectina)
4)Avaliar o efeito do plasma de gestantes com pré-eclâmpsia antes e após a ingestão de suco de uva sobre células endoteliais sob os seguintes parâmetros:
- Viabilidade celular
- Citotoxicidade
- Expressão gênica e Atividade da arginase
- Produção de NO / nitrito / nitrotirosina
- Produção de espécies reativas de oxigênio
- Apoptose
- Expressão gênica e atividade da NADPH oxidase
- Marcadores de disfunção endotelial (VCAM, ICAM e E-Selectina).

Avaliação dos Riscos e Benefícios:
Realizada. Os riscos à saúde são de fato inexistentes, pois pretende-se empregar apenas material biológico já colhido para outra pesquisa e armazenado em biorrepositório, segundo informação dos pesquisadores.

Comentários e Considerações sobre a Pesquisa:
Trata-se de estudo observacional com o emprego de amostras biológicas colhidas em 2006 e mantidas armazenadas desde então. Os participantes não serão convocados para nenhum procedimento adicional, segundo informam os autores.

Considerações sobre os Termos de apresentação obrigatória:

Os autores solicitam a dispensa de aplicação do TCLE, pois este projeto não prevê nenhum tipo de intervenção. Os autores pretendem usar amostras biológicas colhidas de participantes com e sem pré-eclâmpsia para projeto desenvolvido em 2006 e para as quais o pesquisador principal solicita autorização para criar biorrepositório sob sua responsabilidade.

Recomendações:

não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Diante do exposto e à luz da Resolução CNS 466/2012, o projeto de pesquisa, assim como a solicitação de dispensa de aplicação do Termo de Consentimento Livre e Esclarecido, podem ser enquadrados na categoria APROVADO.

Considerações Finais a critério do CEP:

Projeto Aprovado: Tendo em vista a legislação vigente, devem ser encaminhados ao CEP, relatórios parciais anuais referentes ao andamento da pesquisa e relatório final ao término do trabalho. Qualquer modificação do projeto original deve ser apresentada a este CEP em nova versão, de forma objetiva e com justificativas, para nova apreciação.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1573358.pdf	11/09/2020 10:04:53		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_FAPESP_Arginase_set_DrRicardo.docx	11/09/2020 10:04:30	Ricardo de Carvalho Cavalli	Aceito
Cronograma	cronograma.docx	17/08/2020 20:01:16	Ricardo de Carvalho Cavalli	Aceito
Orçamento	orcamento.docx	17/08/2020 20:00:59	Ricardo de Carvalho Cavalli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Dispensa_Termo.pdf	17/08/2020 19:57:32	Ricardo de Carvalho Cavalli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Dispensa_termo.docx	17/08/2020 19:57:25	Ricardo de Carvalho Cavalli	Aceito

Outros	Criacao_biorrepositorio.pdf	17/08/2020 19:57:18	Ricardo de Carvalho Cavalli	Aceito
Outros	Criacao_biorrepositorio.doc	17/08/2020 19:57:04	Ricardo de Carvalho Cavalli	Aceito
Parecer Anterior	CEP_FMPR_PE.jpg	17/08/2020 19:56:47	Ricardo de Carvalho Cavalli	Aceito
Outros	Aprovacao_orcamento.pdf	17/08/2020 19:56:15	Ricardo de Carvalho Cavalli	Aceito
Folha de Rosto	Folha_de_Rosto.pdf	17/08/2020 19:55:05	Ricardo de Carvalho Cavalli	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIBEIRAO PRETO, 19 de Outubro de 2020

Assinado por:
MARCIA GUIMARÃES VILLANOVA
(Coordenador(a))