DANIELA ALVES PEREIRA

Investigação do papel de polimorfismos do gene *ADIPOQ* na resposta à terapia anti-hipertensiva em Pré-eclâmpsia e identificação de novos genes relacionados à disfunção endotelial regulados pela Metformina

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Supervisor(a): Profa. Dra. Valéria Cristina Sandrim

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Resumo

A pré-eclâmpsia (PE) é a principal causa global de mortalidade e morbidade materna e perinatal. Está associada à liberação dos fatores antiangiogênicos sFLT-1 e sENG, à redução do óxido nítrico (NO), à disfunção endotelial e ao aumento do risco cardiovascular. A adiponectina estimula a liberação de NO via ativação da eNOS, e polimorfismos de nucleotídeo único (SNPs) no gene *ADIPOQ* influenciam seus níveis circulantes. Como 40% das gestantes com PE não respondem à terapia anti-hipertensiva, a metformina surge como possível tratamento, por reduzir sFLT-1, sENG e melhorar a função endotelial. **Na primeira linha do projeto** avaliamos se SNPs e haplótipos do gene ADIPOQ afetam os níveis de adiponectina em síndromes hipertensivas gestacionais (SHG). Foram analisados: 1) níveis plasmáticos de adiponectina em gestantes saudáveis (HP), com hipertensão gestacional (GH) e PE; 2) associação dos SNPs rs266729, rs2241766 e rs1501299 com SHG; 3) influência desses SNPs nos níveis de adiponectina. Os níveis foram mais altos em PE comparado a HP. O genótipo GG (rs266729) foi ligado ao risco de GH, enquanto o CG pode proteger contra PE. Portadoras de CG e GG apresentaram mais adiponectina do que CC. O haplótipo "G, T, G" elevou os níveis em PE, e o "C, T, T" pode ter efeito protetor contra GH. Nenhum estudo anterior investigou a relação entre SNPs do gene *ADIPOQ* e a resposta à terapia anti-hipertensiva em PE. Analisamos, então, em 215 pacientes com PE: os níveis de adiponectina entre responsivas e não responsivas ao tratamento, os SNPs rs17300539, rs266729, rs2241766 e rs1501299, além dos respectivos haplótipos. Pacientes não responsivas apresentaram adiponectina mais elevada. O genótipo TG (rs2241766) foi mais frequente nas responsivas. As não responsivas com os genótipos GG, GA+AA, CC, TT, GT+TT e com os haplótipos "G, C, T, G" e "G, C, T, T" mostraram níveis mais altos que as responsivas com os mesmos perfis. A metformina apresenta potencial terapêutico na PE, por reduzir disfunção endotelial e induzir angiogênese, embora seus mecanismos moleculares ainda não estejam completamente elucidados. **Na segunda linha do projeto**, investigamos genes associados à disfunção endotelial modulada pela metformina em células endoteliais de veia umbilical humana (HUVECs), incubadas com plasma de gestantes com PE e tratadas com metformina. As células foram expostas a 20% de plasma de PE precoce ou tardio (n=10) e 1 μM de metformina por 48 horas. Após extração de RNA e RT-qPCR, avaliamos 17 genes-alvo e 2 genes endógenos. Em HUVECs incubadas com plasma de PE precoce, comparado ao tardio, CASP3 e END1 foram regulados negativamente,

enquanto ALOX5 e SELE foram regulados positivamente. Em PE precoce comparada a PE precoce tratada com metformina, CASP3 foi regulado negativamente. Em PE tardio comparada a PE tardio tratada, VWF, ALOX5 e SELE foram regulados positivamente (VWF e SELE com P<0,05). Segundo o Reactome Pathways 2024, utilizando todos os genes que foram diferencialmente expressos em uma ou mais análises, a via de Organização de matriz extracelular foi a mais relevante. Concluímos então que SNPs e haplótipos do gene *ADIPOQ* afetam os níveis de adiponectina, a suscetibilidade à GH e PE, e a resposta ao tratamento. A metformina pode modular genes ligados à disfunção endotelial e à inflamação na PE.

Palavras-chave: Adiponectina; Disfunção endotelial; HUVECs; Metformina; Polimorfismos genéticos; Pré-eclâmpsia.

1. Introdução

A Pré-eclâmpsia (PE) é definida por hipertensão após a 20ª semana gestacional, que pode ser acompanhada por proteinúria, trombocitopenia, insuficiência hepática, dor de cabeça de início recente, edema pulmonar ou insuficiência renal com valores laboratoriais alterados, atingindo até 8% das gestações[1]. A PE é a principal causa de mortalidade e morbidade materna e perinatal mundial, onde mulheres com histórico de PE possuem elevado risco de desenvolver doenças cardiovasculares[2]. Entretanto, não existe tratamento definitivo tampouco marcadores efetivos de predição de risco para PE[3]. A terapia anti-hipertensiva com metildopa, nifedipina e hidralazina não é específica para tratar PE, mas ajuda a prolongar a gestação[4]. Todavia, um subgrupo de gestantes não responde à terapia anti-hipertensiva e está associado aos desfechos clínicos mais graves[5].

Embora os mecanismos responsáveis pela PE não estejam totalmente elucidados, a perfusão placentária reduzida é aceita como um mecanismo inicial[6]. Na gestação normal, os citotrofoblastos invadem a decídua e o miométrio para substituir as células da parede vascular das artérias espiraladas maternas. Esse processo é responsável pela perda da túnica muscular dos vasos, tornando-os grandes e de baixa resistência para que possam acomodar o aumento do fluxo sanguíneo necessário para suprir o feto. Entretanto, na PE ocorre falha da remodelação das artérias espiraladas e placentação anormal, onde os citotrofoblastos não infiltram a porção das artérias espiraladas que se encontra no miométrio. Desse modo, o calibre das artérias espiraladas permanece pequeno, mantendo sua capacidade de constrição, o que resulta em hipoperfusão placentária, hipóxia e danos extensivos no endotélio vascular (revisado em[7]). A disfunção endotelial proveniente desses danos causaria uma diminuição na produção e na atividade de agentes vasodilatadores, como a prostaciclina e o óxido nítrico (NO), o que levaria a uma redução do fluxo sanguíneo uteroplacentário, com trombose das artérias espiraladas, infarto placentário, e um aumento exponencial da produção de múltiplas citocinas e fatores de crescimento que culminam nas manifestações clínicas da PE[8].

Antes do início dos sintomas clínicos da PE se observa um aumento da liberação dos fatores antiangiogênicos receptor tirosina-quinase tipo fms-1 solúvel (sFLT-1) e endoglina solúvel (sEng), que contribuem para a ampla disfunção endotelial característica de PE e que pode levar à lesão em múltiplos órgãos[1, 9]. Concomitantemente, ocorre a diminuição nos níveis de fatores pró-angiogênicos, como o fator de crescimento

placentário (PlGF) e o fator de crescimento endotelial vascular (VEGF)[6]. O VEGF estimula a angiogênese e vasculogênese ligando-se aos receptores VEGFR1 e VEGFR2, sendo a ligação ao VEGFR2 a responsável por uma sinalização mais forte, com posterior ativação da via PI3K/AKT, fosforilação da sintase endotelial do óxido nítrico (eNOS) e produção de NO. O PIGF por sua vez liga-se ao VEGFR1 para aumentar a probabilidade de ligação do VEGF ao VEGFR2, assim como para promover a transfosforilação desse receptor, potencializando sua cascata de sinalização (revisado em[10]). O sFLT-1 é uma versão truncada e solúvel do receptor VEGFR1 que sequestra o PlGF e o VEGF que se ligariam ao VEGFR, inibindo assim a fosforilação da sintase endotelial do óxido nítrico (eNOS) e posterior produção de NO. Nesse contexto, um estudo prévio do grupo mostrou níveis diminuídos de NO em pacientes com PE, com as concentrações circulantes de nitrito (um marcador de formação endógena de NO) sendo associadas negativamente aos níveis circulantes de sFLT-1[11].

A PE é uma doença multifatorial[12] com fatores ambientais e genéticos, que envolve vários componentes moleculares em vias metabólicas, que são reconhecidos como potenciais alvos para intervenção terapêutica. Nesse contexto, estudos mostram que fatores metabólicos relacionados à obesidade também aumentam o risco de desenvolvimento de PE[13, 14], e a desregulação de adipocitocinas tem sido associada à disfunção endotelial na PE[15]. Por exemplo, a adipocitocina visfatina/NAMPT é um potencial biomarcador para disfunção endotelial, com impacto cardiovascular e pode desempenhar um papel na PE[16]. A visfatina/NAMPT regula positivamente a expressão do gene *NOS3,* que codifica a eNOS em células endoteliais[17], e tem efeito vasodilatador mediado pela liberação de NO[18]. No entanto, a visfatina/NAMPT também pode prejudicar o relaxamento dependente do endotélio através da estimulação da NADPH oxidase[19] e foi capaz de produzir disfunção endotelial *in vivo* em camundongos[20].

Nosso grupo encontrou níveis plasmáticos de visfatina/NAMPT relacionados positivamente com as concentrações de nitrito e inversamente com os níveis de sFLT-1 em gestantes saudáveis, mas relacionados inversamente com concentrações de nitrito e positivamente com os níveis de sFLT-1 na PE, que sugerem que a visfatina/NAMPT inibe a formação de NO e regula positivamente o sFLT-1 na PE[21]. Nesse contexto, examinamos as relações entre os níveis de visfatina/NAMPT e as concentrações de nitrito e sFLT-1 em pacientes classificados como responsivas ou não responsivas a terapia anti-hipertensiva usada para tratar PE e encontramos os níveis plasmáticos de

visfatina/NAMPT relacionados inversamente às concentrações de nitrito e positivamente aos níveis de sFLT-1 em gestantes com PE não responsivas à terapia anti-hipertensiva[22].

A adiponectina, uma adipocina produzida quase que exclusivamente pelo tecido adiposo branco[23], é outra adipocina que vem sendo estudada em relação ao seu papel na gestação[24, 25]. A adiponectina desempenha um papel na fisiologia vascular e tem efeitos antioxidantes e anti-inflamatórios, além de promover a angiogênese, como revisado em[23]. Estudos prévios descreveram os papéis da adiponectina na invasão e migração trofoblástica durante a gravidez[26-28]. Nesse contexto, nosso grupo encontrou níveis elevados de adiponectina em gestantes com PE quando comparado com gestantes saudáveis, correlação positiva entre níveis de adiponectina e nitrito plasmático em gestantes saudáveis, mas nenhuma correlação na PE[29]. Uma correlação positiva entre tais marcadores foi encontrada quando analisadas gestantes com PE com baixos níveis de ADMA, um bloqueador da eNOS[29]. Nosso grupo também encontrou associação positiva na PE entre a adiponectina com outras duas moléculas importantes para o processo de migração e invasão trofoblástica, a MMP-2 e seu inibidor, o TIMP-2[30].

Estudos prévios examinaram a associação de polimorfismos em genes candidatos à PE com subgrupos de pacientes responsivos e não responsivos à terapia anti-hipertensiva[5]. Entretanto, é importante ressaltar que existem desafios na definição dos critérios usados para definir a responsividade à terapia anti-hipertensiva na PE, e é possível que nossos critérios estejam associados à gravidade da PE[5]. Recentemente, nosso grupo melhorou a compreensão do fenótipo clínico do subgrupo de pacientes com PE não responsivos à terapia anti-hipertensiva, que apresentaram pressão arterial sistólica e diastólica, glicemia de jejum, creatinina, proteinúria e concentrações de sFLT-1 mais altas do que pacientes responsivas[22]. Além disso, os fenótipos de PE de início precoce, parto prematuro e restrição de crescimento intrauterino foram mais frequentes em pacientes não responsivos. As pacientes não responsivas também apresentaram menor índice de massa corporal, menor idade gestacional no parto e amostragem e seus recém-nascidos apresentaram menor peso[22, 31].

O principal foco da farmacogenética é elucidar a contribuição de polimorfismos genéticos na variabilidade interindividual das respostas (eficácia e reações adversas) aos fármacos. Embora estudos em farmacogenômica tenham levado à identificação de variantes associadas à essa variabilidade de resposta, grande parte da herdabilidade

genética dos fenótipos de resposta às drogas parece estar escondida em traços complexos multigênicos e multifatoriais[32], evidenciando a importância de estudos com mais de um gene[33].

O gene que codifica a adiponectina (*ADIPOQ*) possui polimorfismos de nucleotídeo único (SNPs) que podem afetar a concentração circulante dessa adipocitocina, mas a influência dos SNPs nos níveis da adiponectina em pacientes com PE e nos subgrupos de resposta à terapia anti-hipertensiva não foi elucidada. Nesse projeto, examinamos o efeito dos SNPs do gene *ADIPOQ* rs17300539 G>A, rs266729 C>G, rs2241766 T>G e rs1501299 G>T nos níveis da adiponectina em síndromes hipertensivas gestacionais (SHG), e a relação entre esses SNPs e a resposta à terapia anti-hipertensiva em PE.

É importante ressaltar que, embora os mecanismos subjacentes à disfunção endotelial em doenças hipertensivas gestacionais estejam se tornando cada vez mais compreendidos, isso não corresponde ao desenvolvimento ou uso de novos fármacos para tratar essas doenças, assim como é o caso da PE. Como dito anteriormente, a terapia anti-hipertensiva utilizada para tratar PE não é específica. Nesse contexto, a Metformina, o fármaco de primeira escolha para o tratamento de diabetes mellitus tipo 2 e que é usado de forma segura para tratar a diabetes gestacional[34], tem potencial para prevenir ou tratar a PE[35]. Nesse contexto, uma meta-análise recente de ensaios clínicos randomizados e estudos prospectivos mostrou que o uso de Metformina na diabetes gestacional pode reduzir a incidência de PE comparado com o uso da insulina durante a gestação[36, 37]. Estudos pré-clínicos em cultura de células primárias da placenta e células endoteliais da veia umbilical humana (HUVEC) também mostraram que a Metformina reduz a produção de sFLT-1, sEng e a disfunção endotelial, além de induzir a angiogênese[35]. Entretanto, os mecanismos moleculares subjacentes à ação da metformina e a esses efeitos, assim como os genes envolvidos na disfunção endotelial e regulados pela metformina não são totalmente conhecidos.

No fígado, principal órgão de ação, a metformina inibe a gliconeogênese dependente ou independentemente da ativação da via da AMPK[38]. Na PE, estudos sugerem que a metformina pode ter um papel na sua prevenção por meio de seu efeito no metabolismo celular, no estado antiangiogênico e em outros processos associados a essa desordem hipertensiva gestacional[35]. O efeito da metformina no estado antiangiogênico parece ocorrer ao nível das mitocôndrias[39]. O papel da mitocôndria na patogênese da

PE foi sugerido, pela primeira vez, em estudos que mostraram que seis em cada 10 mulheres com doença mitocondrial tiveram pelo menos uma gestação complicada por PE ou eclâmpsia[39]. A hipótese proposta pelos autores foi a de que essas mulheres apresentavam PE devido a disfunção mitocondrial e acúmulo de adenosina difosfato (ADP), uma vez que mitocôndrias disfuncionais não conseguiriam atender ao aumento de energia necessário durante a gravidez[39]. Após esse estudo, outros autores também publicaram acerca das implicações da mitocôndria na patogênese da PE (revisado em[40]). A análise proteômica comparativa de mitocôndrias provenientes da placenta de gestantes saudáveis e com PE mostrou regulação positiva de 4 proteínas e regulação negativa de 22 proteínas. Tais proteínas diferencialmente expressas foram identificadas como participantes de muitos processos críticos da PE, tais como geração de espécies reativas de oxigênio, apoptose, oxidação de ácidos graxos, função da cadeia respiratória e o ciclo do ácido tricardoxílico[41].

A metformina é capaz de inibir o complexo I da cadeia de transportes de elétrons da mitocôndria e o fator induzido por hipóxia 1-alfa (HIF1-alpha)[42, 43]. O HIF1-alpha já foi correlacionado ao aumento da secreção do sFLT-1 e a fisiopatologia da PE[44]. Nesse contexto, Brownfoot e colaboradores demonstraram que os efeitos da metformina sobre a produção do sFLT-1 e da sEng seriam regulados através do bloqueio da cadeia transportadora de elétrons da mitocôndria[35]. Interessantemente, o papel desempenhado por diferentes vias moleculares, assim como o da cadeia transportadora de elétrons e o das espécies reativas de oxigênio no desenvolvimento da PE pode ser analisado tanto farmacológica quanto geneticamente[45, 46].

A metformina é capaz de regular a expressão de milhares de genes[38], dentre os quais pode haver genes envolvidos em vias que atuam na homeostase vascular. Por exemplo, *GDF15* é um dos genes super-regulados pela metformina e proposto como um dos principais mediadores dos seus efeitos. O GDF15, uma citocina membro da superfamília do TGF-beta e induzida por estresse, também apresenta seus níveis aumentados durante a gestação, com o seu pico sendo atingido no terceiro trimestre[47]. Níveis diminuídos de GDF15 no primeiro trimestre da gestação está associado a abortos prematuros e parece afetar a invasão e migração dos trofoblastos extravilosos através da inibição da via de sinalização da JAG1/NOTCH3/HES1, sugerindo que o GDF15 é um potencial biomarcador para rastrear complicações relacionadas com a gravidez[48].

A PE é uma doença geneticamente complexa, onde está presente a interação entre fatores ambientais e múltiplos fatores genéticos[49], cenário que destaca a importância de estudos com o foco em investigar mais de um gene candidato, assim como examinar polimorfismos em genes de interesse que possam ter efeitos nos níveis plasmáticos de marcadores bioquímicos da doença.

Esse projeto contou com **duas linhas de pesquisa:** Na **primeira linha de pesquisa** de examinamos o efeito dos SNPs do gene *ADIPOQ* rs17300539 G>A, rs266729 C>G, rs2241766 T>G e rs1501299 G>T nos níveis da adiponectina em síndromes hipertensivas gestacionais (SHG), e a relação entre esses SNPs e a resposta à terapia anti-hipertensiva em PE. Importante notar que nosso grupo já publicou outros trabalhos usando abordagens metodológicas similares[21, 22, 50-52] e o objetivo desenvolvido envolve Projeto Temático "Endothelial Dysfunction in Hypertensive Disorders of Pregnancy" já em andamento no laboratório.

A identificação destes genes e de vias moleculares que possuem imbricação na fisiopatologia da PE, assim como o estudo do papel de polimorfismos de genes candidatos pode auxiliar na elucidação da mesma, na identificação de biomarcadores relacionados a tais processos fisiopatológicos[5, 31], além de gerar estratégias de detecção precoce de gestantes sob risco de PE.

Na **segunda linha de pesquisa** buscamos identificar genes e vias de sinalização envolvidas na regulação de genes diferencialmente expressos após a incubação com metformina de modelo *in vitro* de PE que utiliza células endoteliais de veia umbilical humana (HUVEC), que possuem sua cultura e manuseio já previamente padronizados no laboratório[53-57].

A metformina regula a expressão gênica e tem potencial para tratar a PE[38]. Nesse contexto, a hipótese desse objetivo do projeto é de que a metformina regula a expressão de genes que atuam na redução da disfunção endotelial e que tais genes interagem em vias associadas ao desenvolvimento da PE, onde a identificação dos mesmos pode ajudar a definir potenciais preditores de resposta ao tratamento com Metformina em PE.

2. Objetivo Geral

Examinar efeito de polimorfismos do gene da adiponectina, *ADIPOQ,* nas concentrações plasmáticas de adiponectina em pacientes com PE e nos subgrupos de resposta à terapia anti-hipertensiva e identificar genes e vias bioquímicas relacionados à disfunção endotelial regulados pela Metformina em modelo *in vitro* de PE.

2.1. Objetivos Específicos

1. Examinar os efeitos de polimorfismos do gene *ADIPOQ* e seus haplótipos, nos níveis de adiponectina em gestantes saudades, pacientes com hipertensão gestacional e PE;

2. Examinar os efeitos de polimorfismos do gene *ADIPOQ* e seus haplótipos, nos níveis de adiponectina nos subgrupos classificados como responsivos e não responsivos à terapia anti-hipertensiva em PE;

3. Identificar em modelo *in vitro* de PE incubado com metformina novos genes relacionados à disfunção endotelial regulados pela Metformina;

4. Identificar vias de sinalização envolvidas na regulação dos genes diferencialmente expressos pela metformina.

3. Materiais e Métodos

3.1. Coleta das amostras de gestantes com Pré-eclâmpsia e gestantes saudáveis

As análises dos efeitos dos polimorfismos do gene *ADIPOQ* foram examinadas em estudo de caso-controle composto por 205 gestantes com PE e 203 gestantes normotensas. As amostras biológicas das gestantes foram coletadas no Departamento de Ginecologia e Obstetrícia do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (FMRP/USP), em colaboração com o Prof. Ricardo Cavalli e durante a execução de Projetos aprovados pelo Comitê de Ética em Pesquisa do Hospital das Clínicas da FMRP/USP (Parecer número: 4.347.078, submetido em anexo conforme edital).

3.2. Avaliação da resposta à terapia anti-hipertensiva

A responsividade à terapia foi baseada na avaliação de parâmetros clínicos e laboratoriais em resposta à terapia anti-hipertensiva total, que consiste em: metildopa (1000-1500 mg por dia), o primeiro anti-hipertensivo de escolha e nifedipina (40-60 mg por dia) em casos de falta de resposta significativa à metildopa. A hidralazina (5-30 mg)

foi utilizada apenas em casos de crise hipertensiva. As pacientes incluídas neste estudo foram monitoradas com cautela quanto a sinais e sintomas de PE. A presença de pelo menos um dos critérios indicados abaixo foi utilizada para classificar as pacientes com PE como não responsivos à terapia anti-hipertensiva[22, 50, 58]:

(1) Visão turva, dor de cabeça persistente ou escotoma, dor persistente no quadrante superior direito ou dor epigástrica;
(2) Pressão arterial sistólica (PAS) acima de 140 mmHg e pressão arterial diastólica (PAD) acima de 90 mmHg, avaliada pela curva de pressão arterial;
(3) Hemólise, enzimas hepáticas elevadas e síndrome da baixa contagem de plaquetas (HELLP); ou proteinúria >2,0 g por 24 horas; creatinina >1,2 mg por 100 mL ou ureia >30 mg por 100 mL; aspartato aminotransferase >70 Ul -1 e alanina aminotransferase >60 Ul -1;
(4) Hipoatividade fetal ou feto não reativo, conforme revelado pela cardiotocografia; restrição de crescimento intrauterino, oligoâmnio, escore de perfil biofísico anormal e anormalidades na Doppler velocimetria, avaliadas por ultrassonografia.

3.3. Avaliação de biomarcadores no plasma por imunoensaios enzimáticos (ELISA); Genotipagem dos SNPs

A mensuração das concentrações plasmáticas de adiponectina em gestantes saudáveis e pacientes com PE foi determinada usando kits de ELISA comercial Human Adiponectin EZHADP-61K (Millipore, St. Charles, MO), seguindo recomendações do fabricante[26]. A genotipagem dos SNPs do gene *ADIPOQ*[59] foi previamente realizada em artigo prévio do grupo de pesquisa.

3.4. Cultivo celular

A linhagem HUVEC (*Human umbilical vein endothelial cells* - HUVEC Ea.hy96 (Banco de Células do Rio de Janeiro – BCRJ) foi cultivada a 37ºC em CO2 5% em meio DMEM/F12 (Gibco, EUA) suplementado com 20% (v/v) de soro fetal bovino (SFB), 100iu/mL de penicilina, 100 µg/mL de estreptomicina e 2 mMol/L de L-Glutamina (Sigma-Aldrich, EUA). Após o crescimento celular atingir a confluência de 80% e para realização do experimento, as HUVECs foram então expandidas e incubadas a 37ºC em CO2 5% em meio sem SFB, onde a suplementação foi com pool de plasma de gestantes com PE (modelo *in vitro* de PE), de acordo com grupo de estudo. Todas as incubações foram feitas na ausência (apenas meio de cultura) ou presença de cloridrato de metformina

(Merck – Número CAS:1115-70-4), em concentrações padronizadas no estudo. Projeto aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu (FMB) (Parecer número: 6.747.398).

3.5. Análise de expressão gênica

Para determinação do efeito da metformina na expressão de genes relacionados a disfunção endotelilal e envolvidos na fisiopatologia da PE, foi realizada a extração do RNA provenientes do modelo *in vitro* de PE incubado com metformina, transcrição reversa e PCR em tempo real.

3.6. Extração do RNA

O RNA total foi extraído a partir de um total de até 5x106 de células endoteliais incubadas com plasma de gestantes com PE precoce, tardia ou NT e/ou incubadas com metformina. As concentrações de metformina foram também analisadas e determinadas no estudo. A extração foi realizada utilizando kit Quick-RNA™ Miniprep Kit (ZYMO RESEARCH – R1055) de acordo com instruções do fabricante e o RNA eluido foi imediatamente convertido em cDNA.

3.7. Transcrição Reversa do RNA

O RNA total obtido através do processo de extração será quantificado utilizando o espectrofotômetro UV-Vis BioDrop µLite+. Após a quantificação o RNA foi submetido a transcrição reversa utilizando o LunaScript® RT SuperMix Kit (NEB #E3010**)** seguindo as recomendações do fabricante. O produto de cDNA foi diretamente usado para detecção por PCR em tempo real ou guardado no -20°C.

3.8. PCR em tempo real

O cDNA obtido após a transcrição reversa foi utilizado na reação de PCR em tempo real através do kit KiCqStart® SYBR® Green qPCR ReadyMix™ (KCQS00-250RXN) utilizando o sistema ABI Step One system (Applied Biosystems, Foster City, CA, USA) com volume final de 10 µL, onde o cDNA sintetizado a partir do RNA extraído foi diluido a 1,5 ng; 5 µL correspondeu ao KiCqStart® SYBR® ReadyMix™; 0,25 µL a cada um dos primers, *foward* e *reverse*, específicos do RNA de interesse, em concentração final de 0.25 µM; e o volume final foi completado com água. A reação foi incubada segundo recomendações do fabricante e ao final dos ciclos da reação, a curva de fusão (do inglês *Melting curve*) foi performada para validar a geração específica do

produto de PCR esperado. Em uma reação de PCR em tempo real, o CT (ciclo limite) é o número de ciclos necessários para o sinal fluorescente cruzar o limite. Os níveis de CT são inversamente proporcionais à quantidade de ácido nucleico alvo na amostra. Os conjuntos de dados foram primeiramente normalizado utilizando os genes constitutivos *HPRT1* e *RPLP0*. Valores de ΔΔCt e Fold change foram então calculados para identificação dos genes diferencialmente expressos.

3.9.Análise de vias

Após identificação dos genes diferencialmente expressos em modelo *in vitro* de PE incubado com metformina, os mesmos foram submetidos a análise de enriquecimento funcional através da plataforma Enrichr (https://maayanlab.cloud/Enrichr/). O enriquecimento funcional é um método computacional que verifica se um conjunto de genes de entrada se sobrepõe significativamente aos conjuntos de genes anotados[60]. A análise de enriquecimento de vias envolve determinar se os genes observados em uma lista são significativamente mais abundantes em certas vias do que seria esperado ao acaso[61]. As vias enriquecidas nos auxiliam a compreender os processos e mecanismos biológicos que são mais provavelmente afetados pelos grupos experimentais analisados[61].

3.10. Análises estatísticas

As análises estatísticas utilizadas foram testes paramétricos ou não paramétricos, a depender da normalidade dos dados. As distribuições dos genótipos dos polimorfismos foram avaliadas quanto à aderência ao equilíbrio de Hardy-Weinberg. Diferenças nas frequências dos genótipos e alelos dos SNPs foram avaliadas pelo teste do qui-quadrado ($\chi 2$). As frequências haplotípicas foram estimadas usando o pacote Haplo.stats versão 1.4.4 (https://cran.r-project.org/web/packages/haplo.stats/index.html). As diferenças nas frequências de haplótipos foram testadas usando testes $\chi 2$ e corrigidas para o número de haplótipos/comparações feitas usando a correção de Bonferroni. Valores de $p<0.05$ foram considerados estatisticamente significativos.

4. Resultados

Os resultados da **primeira linha de pesquisa** do projeto serão apresentados no formato de **dois artigos científicos** submetidos durante período de pós-doutoramento; os

resultados **da segunda linha de pesquisa** do projeto ainda não foram submetidos a nenhuma revista e serão apresentados em **formato de texto dissertativo**.

4.1. Artigo 1;

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Corresponding Author:	Marcelo Rizzatti Luizon, Ph.D. Universidade Federal de Minas Gerais Belo Horizonte, Minas Gerais BRAZIL
First Author:	Marcelo Rizzatti Luizon, Ph.D.
Order of Authors:	Marcelo Rizzatti Luizon, Ph.D.
	Ricardo N F de Castro
	Daniela Alves Pereira, PhD
	Juliana O. Cruz
	Ana C Palei
	Jackeline S Rangel Machado
	Ricardo C Cavalli
	Jose E Tanus-Santos
	Valéria C Sandrim
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TITLE PAGE

Effects of *ADIPOQ* polymorphisms and haplotypes on circulation adiponectin levels in gestational hypertension and preeclampsia

Ricardo N F de Castro [1,2,¥], Daniela A Pereira [1,3,¥], Juliana O. Cruz [1,4], Ana C Palei [5], Jackeline S Rangel Machado [6], Ricardo C Cavalli [6], Jose E Tanus-Santos [7], Valéria C Sandrim [3,], Marcelo R Luizon [1,3,*]*

[1] *Department of Genetics, Ecology and Evolution, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.*

[2] *Department of Biological Sciences, Federal Center for Technological Education of Minas Gerais (CEFET-MG), Belo Horizonte, Minas Gerais, Brazil.*

[3] *Department of Biophysics and Pharmacology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil.*

[4] *Department of Biological Sciences, State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil.*

[5] *Department of Surgery, University of Mississippi Medical Center, Jackson, MS, USA.*

[6] *Department of Gynecology and Obstetrics, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, São Paulo, Brazil.*

[7] *Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, São Paulo, Brazil*

[¥] *These authors have contributed equally to this work and share first authorship.*

[*] *These authors have contributed equally to this work and share senior authorship.*

***Corresponding author:** *Prof. Marcelo R Luizon, PhD*
Federal University of Minas Gerais
Institute of Biological Sciences
Department of Genetics, Ecology and Evolution
Belo Horizonte, Minas Gerais, Brazil
ZIP code: 31270-901
E-mail: luizonmr@gmail.com

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ABSTRACT

Imbalance of adipocytokines has been implicated in endothelial dysfunction in hypertensive disorders of pregnancy (HDP). Adiponectin is an adipocytokine that regulates metabolism, insulin sensitivity, and inflammation, and increased adiponectin levels have been associated with mortality in subjects with cardiovascular diseases. Adiponectin also plays a role in trophoblast invasion during placental development. The ADIPOQ gene has polymorphisms that modulates adiponectin levels and are linked to several diseases, including gestational hypertension (GH) and preeclampsia (PE). However, no previous study has examined whether ADIPOQ SNPs and haplotypes affect adiponectin levels in HDP. Therefore, we examined 1) plasma adiponectin levels in healthy pregnant (HP, n=182), GH (n=121), and PE (n=133) women; 2) whether ADIPOQ SNPs rs266729, rs2241766 and rs1501299, and their haplotypes are associated with susceptibility to HDP; and 3) whether these polymorphisms affect adiponectin levels in this population. Adiponectin was measured by ELISA and genotypes were determined by Taqman allele discrimination assays. Plasma adiponectin levels were higher in PE than in HP (P<0.05), including when patients were stratified by body mass index. Regarding rs266729 (C>G) SNP, the GG genotype was associated with risk for GH, and the CG genotype may protect against PE. PE patients carrying the CG and GG genotypes showed higher adiponectin levels than their CC counterparts. The 'G,T,G' haplotype showed higher adiponectin levels in PE than in HP, and the 'C,T,T' haplotype may protect against GH. Our novel findings indicate an effect of ADIPOQ SNPs and haplotypes on circulation adiponectin levels and susceptibility for the development of GH and PE.

KEYWORDS: *adiponectin, ADIPOQ, gestational hypertension, preeclampsia, polymorphisms.*

INTRODUCTION

Hypertensive disorders of pregnancy (HDP) are leading causes of maternal and perinatal mortality and morbidity, and the prevalence of gestational hypertension (GH) and preeclampsia (PE) in worldwide epidemiological studies are 1.8-4.4% and 0.2-9.2%, respectively [62]. PE is characterized as new-onset hypertension defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg occurring after 20 weeks of gestation, which are accompanied by proteinuria or other key organ injury [63, 64].

Abnormal development of placenta is closely linked to the pathophysiology of PE [7]. The invasion of cytotrophoblasts into the spiral arteries of the decidua is restricted to the superficial layers, leading to reduced perfusion in the placenta, which is followed by widespread dysfunction of the maternal vascular endothelium [65, 66]. While the mechanisms responsible for PE are not fully clarified, circulating antiangiogenic factors that are released in response to hypoxic/ischemic placental conditions may contribute to the maternal endothelial dysfunction, as reviewed elsewhere [67].

Adiponectin, a collagen-like adipocytokine regulates metabolism, insulin sensitivity, and inflammation [15], also plays a role in proliferation, differentiation, and invasion of trophoblasts during placental development, and enhances the migration of trophoblastic cells in the decidua by balancing the matrix metalloproteinases/tissue inhibitors of metalloproteinases axis [68]. Dysregulated adipocytokines, including adiponectin may be associated with endothelial dysfunction in PE [15, 69, 70]. Notably, increased adiponectin levels were significantly associated with elevated risk of all-cause and cardiovascular

mortality in subjects with cardiovascular diseases (CVD) [71], and as an independent predictor of cardiovascular and all-cause mortality in coronary artery disease patients [72].

Several GWAS have identified single nucleotide polymorphisms (SNPs) in the ADIPOQ gene as significant contributors to the variability in circulating adiponectin levels [73]. Specifically, SNP rs266729 was associated with cardiovascular and metabolic diseases [74, 75], and SNP rs1501299 as a low-risk factor for the development of CVD with type 2 diabetes [76]. Moreover, SNPs rs2241766 and rs1501299 were associated with important clinical manifestations of PE, being the rs1501299 associated with serum adiponectin level [29]. We have previously found a specific association between the CG genotype of the SNP rs266729 and PE. However, except for one study [29], no other previous study has examined whether ADIPOQ SNPs and haplotypes affect circulating adiponectin levels in HDP.

In this study, we aimed at comparing plasma levels of adiponectin in healthy pregnant (HP) women with those found in patients with GH and PE. We then examined whether the ADIPOQ SNPs rs266729, rs2241766 and rs1501299, as well as the haplotypes formed by combination of their alleles affect plasma adiponectin levels in the HP, GH, and PE groups, and whether they are associated with susceptibility to GH and PE.

METHODS

Study population

Approval to study human subjects was obtained from the Institutional Review Board at the Ribeirao Preto Medical School, University of Sao Paulo (FMRP-USP), during the execution of Projects approved by the Research Ethics

Committee of the Hospital das Clínicas of FMRP/USP (Opinion number: 4,347,078), with each participant giving signed informed consent. Consecutive enrollment of volunteers took place at the Department of Obstetrics and Gynecology within the University Hospital of the FMRP-USP. The study enrolled 436 pregnant women: 182 healthy pregnant, 121 with GH, and 133 with PE. HDP were identified following the diagnostic criteria set by the American College of Obstetricians and Gynecologists (ACOG) [63] and the International Society for the Study of Hypertension in Pregnancy (ISSHP) [64].

GH was defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg, confirmed by two separate readings at least 6 hours apart, after 20 weeks of gestation, which normalized within 12 weeks postpartum. PE was defined as GH along with considerable proteinuria (≥ 0.3 g per 24 h) and/or thrombocytopenia, pulmonary edema, impaired liver function, renal insufficiency with abnormal lab values, and new-onset headache unresponsive to all forms of management [63, 64]. Individuals with cardiac and renal diseases, diabetes, and those with chronic hypertension, with or without superimposed PE were excluded. Methyldopa was the initial antihypertensive treatment during pregnancy. If the pregnant women did not respond to methyldopa, nifedipine and/or hydralazine were added to achieve targeted blood pressure levels.

Maternal venous blood samples were collected at the time of clinic attendance. Genomic DNA was extracted from the cellular component of 1 mL of whole blood using the salting-out method and stored at -20 °C until analyzed. Plasma samples were obtained after centrifugation of whole blood collected into

tubes containing EDTA at 2000 g for 10 min. Those samples were stored at -70 ºC until assayed.

Enzyme immunoassays for adiponectin measurement

Plasma adiponectin concentrations were determined using the Human Adiponectin ELISA kit (Human Adiponectin EZHADP-61K, Millipore, St. Charles, MO, USA), following the manufacturer's instructions, as previously described [29].

Genotype determination

Genotypes for SNPs -11377C>G (rs266729; c_2412786_10) in the promoter region, 45T>G (rs2241766; c_26426077_10) in exon 2, and 276G>T (rs1501299; c_7497299_10) in intron 2 of the ADIPOQ gene were determined using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA). Genotyping was performed in a Real-Time System 7500 (Applied Biosystems, Foster City, CA, USA). Each PCR was carried out in a total volume of 6.74 mL (3.125 mL of 2 x TaqMan Universal Master Mix and 0.156 mL of primer probe (470 nM and 100 mM, respectively), 3.0 mL of DNA (100 ng/mL) and 0.46 mL purified water-free DNAse/RNAse) placed in 96-well plates. DNA with known genotypes was used as positive control and water as negative control in each experiment.

Statistical analysis

Demographics, clinical characteristics, and plasma adiponectin levels of HP, and patients with PE or GH were compared by Student's unpaired t-test, Mann-Whitney U test, one-way ANOVA, or $\chi 2$ as appropriate, and reported as mean ± s.e.m.

Distribution of genotypes was assessed for deviation from Hardy–Weinberg equilibrium using Hardy–Weinberg exact tests for each locus in each group using the web version of Genepop available at https://genepop.curtin.edu.au/genepop_op1.html [77].A value of P<0.05 was considered significant. Power calculation was performed using QUANTO version 1.2.4 [78]. Given the sample size of the study, considering the detectable odds ratio of 0.5 for the frequency of the minor allele of SNP rs266729, which was found to be significantly associated with PE, the calculated power was 0.929, considering an alpha of 0.05.

The effect of ADIPOQ genotypes on plasma adiponectin levels within each group were compared by analysis of normality (Shapiro-Wilk test), followed by Kruskal-Wallis test (not normally distributed variables) and post-hoc Dunn's Multiple Comparison test. Haplotype frequencies were estimated using Haplo.stats package in R (v4.3.1), which computes maximum likelihood estimates of haplotype probabilities [79]. The possible haplotypes including the alleles of the ADIPOQ SNPs rs266729 (C>G), rs2241766 (T>G) and rs1501299 (G>T) considered in the analyses were: 'C, T, G', 'C, T, T', 'G, T, G' and 'C, G, G'. We have excluded the haplotypes with frequency lower than 5% from the analysis. Differences in haplotype frequencies were tested using $\chi 2$ tests, and a value of P<0.05 was considered significant.

To further examine the effects of ADIPOQ haplotypes on adiponectin levels, we have also performed an additional analysis. We compared ADIPOQ haplotypes distributions in two groups of HP, GH and PE patients: the lower and the upper groups, which included subjects with the lower and upper values of plasma adiponectin levels distribution, respectively.

Linkage disequilibrium (LD) was assessed by calculating D' using the Haploview software (version 4.2; http://www.broad.mit.edu/mpg/haploview/) [80]. We used data retrieved from the 1000 Genomes Phase III study for Europeans (CEU, Utah Residents with Northern and Western European Ancestry), East Asians (CHB, Han Chinese in Beijing, China), and Africans (YRI, Yoruba in Ibadan, Nigeria) [80, 81].

The comparison of plasma adiponectin levels between groups was done by analysis of normality (Shapiro-Wilk), followed by identification of outliers by using the ROUT method for outlier identification [82], and then performance of Kruskal-Wallis test (non-parametric) and post-hoc Dunn's Multiple Comparison test. The same method was applied for the analysis of adiponectin levels concerning each SNP and their genotypes and haplotypes.

As an additional analysis, we compared ADIPOQ haplotypes distributions in two groups of HP, GH and PE patients: the lower and the upper groups, which were defined by the median of adiponectin levels on each group, including subjects with the lower and upper values of plasma adiponectin levels distribution, respectively.

Logistic regression analysis was performed using the RStudio integrated development environment (IDE) (https://posit.co/products/open-source/rstudio/) to assess the potential confounding influence of each covariate on the relationship between ADIPOQ genotypes in the PE and GH groups. The variables of clinical importance for GH and PE development were included in the multiple logistic regression models. PE and GH were considered as dependent variables.

Genotypes of ADIPOQ SNPs, age, ethnicity, body mass index, primiparity, and gestational age at sampling were considered as independent variables.

RESULTS

The clinical and demographic characteristics of all pregnant women enrolled in this study are shown in **Table 1**. *The HP and PE groups demonstrated a similar self-declared race, whereas there was a higher White prevalence in GH when compared with HP. Current smoking percentage and primiparity presented similar values between groups (all P>0.05). As expected, both PE and GH groups exhibited higher systolic and diastolic blood pressure compared to the HP group (both P<0.05). It is important to note that most of these patients were on antihypertensive medication. Additionally, patients with GH and PE were older than those in the HP group (P<0.05). Higher body mass index (BMI) and fasting glucose levels were observed in the GH and PE groups compared to the HP group, while hematocrit and hemoglobin were higher only in PE (all P<0.05). There was a lower gestational age at delivery (GAD) in the GH and PE groups, along with lower birth weight and gestational age at sampling in PE (all P<0.05) compared to the HP group. Significant proteinuria was detected in the PE group.*

We were not able to measure the plasma adiponectin levels for all subjects due to lack of plasma availability. The values shown in **Figure 1** *are for 130 HP women, 42 patients with GH, and 55 patients with PE. The clinical and demographical characteristics for this subset of subjects are shown in* **Supplementary Table 1**. *We found significant differences in plasma adiponectin levels between HP and PE (P<0.05;* **Figure 1A**). *We further stratified the subgroups of subjects according to BMI < 30 and BMI ≥ 30 kg/m^2, to examine the influence of body fat in circulating adiponectin levels. While we found increased*

plasma adiponectin in PE compared to HP for the subgroups with BMI < 30 kg/m^2 (P<0.05; **Figure 1B***), no differences were found among HP, GH, and PE groups in the subgroups with BMI ≥ 30 kg/m^2 (P<0.05;* **Figure 1C***).*

The frequencies of ADIPOQ genotypes and alleles are shown in **Table 2***. The distribution of genotypes for each SNP showed no deviation from Hardy-Weinberg equilibrium, except for SNP rs266729 (C>G) in the group of patients with PE (data not shown), a finding that may occur in case groups in case-control studies [83]. Considering codominant distribution, the CG genotype for SNP rs266729 was more common in HP compared to PE, while the GG genotype was more frequent in GH compared with HP (both P<0.05;* **Table 2***). Conversely, no differences in genotype and allele frequencies among groups were found for SNPs rs2241766 and rs1501299 (all P>0.05;* **Table 2***). The haplotype distributions are shown in* **Table 3***. The 'C,T,T' haplotype was more frequent in HP than in GH (P=0.0397;* **Table 3***). Conversely, no significant differences were found between HP and PE (all P>0.05;* **Table 3***).*

We next examined the effect of ADIPOQ genotypes and haplotypes on plasma adiponectin levels. We found no difference among groups and within the same group for SNP rs1501299 (all P>0.05; **Figure 2***). However, we found higher adiponectin levels in patients with PE carrying the TT genotype of SNP rs2241766 (T>G) when compared with TT carriers of the HP group (P<0.05;* **Figure 2***), but no differences between genotypes within each group (P>0.05;* **Figure 2***). Moreover, we found that patients with PE carrying the CG+GG genotypes for SNP rs266729 (C>G) showed higher adiponectin levels than those carrying the CC genotype and CG+GG carriers with HP (both P<0.05;* **Figure 2***). Regarding haplotypes, we found increased adiponectin levels in patients with PE*

carrying the 'G,T,G' haplotype compared to those with the same haplotype in the HP group (P<0.05; **Figure 3***). Conversely, no differences were found when comparing adiponectin levels between GH and PE nor among haplotypes within the same group (all P>0.05;* **Figure 3***).*

In addition, we compared the distribution of ADIPOQ haplotypes in the subgroups of lower and upper plasma adiponectin levels for each of the study groups, but found no differences in haplotype frequencies for the HP and GH groups (all P>0.05, **Supplementary Tables 2 and 3***, respectively). For PE, the 'C,T,T' haplotype was more frequent in the lower subgroup of adiponectin levels (P=0.0285; Supplementary* **Table 4***), while the 'G,T,G' haplotype was more frequent in the upper group of adiponectin levels (P=0.0378;* **Supplementary Table 4***).*

We further compared analysis of the influence of covariates performed using univariate and multivariate logistic regression models, evaluating the influence of each variable alone and groups of variables in the outcome for GH and PE. Univariate logistic regression showed age and body mass index during pregnancy independently associated with PE and GH compared to HP women (P<0.05 and OR>1) and gestational age at sampling primiparity associated with GH compared to HP women (P<0.05 and OR<1; **Supplementary Table 5 and 6***).*

*Logistic regression analysis adjusted for independent variables were done for each of the studied SNPs in GH and PE (***Supplementary Table 7 and Supplementary Table 8***, respectively). For the GH group, the GG genotype of the rs266729 (C>G) SNP influences the GH outcome in the model that considers only the maternal age as a covariate (***Supplementary Table 7***). On the other*

*hand, the same rs266729 SNP, in the PE group, showed influence in the disease outcome concerning the CG genotype for the multivariate model that has BMI during pregnancy and maternal age as covariates (Supplementary Table 8), and the same result was observed for the models considering maternal age or BMI in pregnancy as the only covariate besides the rs266729 (C>G) SNP (***Supplementary Table 4***).*

Finally, we assessed the pairwise LD among the SNPs rs266729 (C>G), rs2241766 (T>G) and rs1501299 (G>T) in all the studied groups. A short segment of higher LD between SNPs rs2241766 and rs1501299 was found in HP (D'=1 and (LOD)'≥2) when compared to PE (D'=1 and (LOD)'<2) and to GH patients (D'<1 and (LOD)'<2; **Supplementary Figure 1***). When analyzing LD of these SNPs in the 1000 Genomes Project populations of European (CEU), African (YRI), Chinese (CHB), and Japanese (JPT) subjects, higher LD blocks were found, particularly in Asian populations (CHB and JPT) (D=1 and LOD≥2) when compared to CEU and YRI populations (***Supplementary Figure 2***).*

DISCUSSION

The main findings reported in the present study are: (1) Patients with PE and a BMI <30 exhibited higher plasma adiponectin levels compared to HP women with same BMI range; (2) The CG genotype for SNP rs266729 (C>G) was more frequent in HP women compared to PE patients, while the GG genotype was more frequent in GH patients when compared to HP women; (3) among haplotypes, the 'C,T,T' was more frequent in HP than in GH; (4) PE patients carrying the CG+GG genotype for the SNP rs266729 (C>G) had elevated plasma adiponectin concentrations when compared to PE patients with CC genotype and to HP women with the CG+GG genotype, while PE patients carrying the TT

genotype for SNP rs2241766 (C>G) presented higher adiponectin levels when compared to the same genotype in HP women; and (5) the 'G,T,G' haplotype presented higher adiponectin levels in PE patients when compared to HP women with the same haplotype.

Adiponectin is a protein produced primarily by adipocytes, and has an influence upon metabolism homeostasis, inflammation resolution, and atherosclerosis prevention [84, 85]. During normal pregnancy and in patients with normal BMI, plasma adiponectin levels decrease as the pregnancy progresses, especially after the mid-term gestation [86]. Although circulating adiponectin may be expected to be lower in PE due to its anti-inflammatory effects [87, 88], its concentration trends diverge greatly among studies, with some reporting low levels [25, 89] and others high levels during PE [90]. Despite these conflicting results, these studies suggest a role for adiponectin in the pathophysiology of PE, and our findings of higher circulating adiponectin levels in PE support this concept. Differences found in the results of these studies may be attributed to characteristics of the included population, including ethnicity, lifestyle, and gestational age at sampling. In addition to these factors, we quantified total adiponectin concentration in plasma. However, adiponectin has three different isoforms, being the high molecular weight (HMW) isoform considered the active form [85]. Considering this, further studies are needed to clarify these different findings regarding circulating adiponectin levels in PE.

We found one previous association study with PE and GH that examined the SNPs rs266729, rs2241766 and rs1501299, which found rs266729 associated with PE [59] and also studies where the rs2241766 and rs1501299 were associated with PE, with rs1501299 showing association with protection

against preeclampsia and altered serum adiponectin levels [91, 92], although no causal link was found between genetically predicted levels of serum adiponectin and preeclampsia risk [93]. However, the present study considered the codominant and dominant models in the association analysis for genotypes of SNPs rs266729, rs2241766 and rs1501299 with HDP. Noteworthy, when considering the codominant model for the genotypic distribution of SNP rs266729, we found the GC genotype to be more frequent in HP, as a possible protective factor against PE, and the GG genotype as a risk factor of GH. Besides the single-locus analysis, our logistic regression models provided evidence that the association of the GC genotype with PE and the GG genotype with GH for the SNP rs266729 occurred even in the presence of BMI and maternal age as confounding variables. Beyond HDP, the G allele of the SNP rs266729 has been associated with an increased risk of type 2 diabetes mellitus and uncontrolled chronic hypertension [94, 95]. While these findings suggest that carriers of the G allele have an unfavorable outcome, in the present study we found that C and G allelic frequencies were statistically similar in PE or GH versus HP. These discordant findings highlight that further replication association studies in different populations are warranted in order to confirm our observations.

The examination of LD among the three ADIPOQ SNPs for each group revealed that SNP rs266729 is in equilibrium with the other SNPs analyzed in this study, whereas a short segment of higher LD between SNPs rs2241766 and rs1501299 was found in HP when compared to PE and GH. In contrast, when assessing LD of these same SNPs in populations from the 1000 Genomes project, we found high LD blocks, particularly in Asian populations (CHB and JPT). These findings highlight the need to evaluate these SNPs in future studies

involving cohorts from diverse ancestries for the validation of the SNP rs266729 as a genetic marker for HDP [95].

Regarding the haplotypes, the 'C,T,T' haplotype was more frequent in HP than in patients with GH, and may be a possible protective factor against GH. Previous studies focused on haplotypes formed by ADIPOQ SNPs in HDP have found an overrepresentation of the pooled G haplotypes versus the TT haplotype for the rs2241766 and rs1501299, respectively, in PE; however, when four different ADIPOQ SNPs were considered, the association was lost. Nonetheless, the 'C,T,T' haplotype has been associated with risk to metabolic syndrome in a cohort of Sudanese patients [96], highlighting these ADIPOQ SNPs as possible targets in further studies.

We observed that PE patients carrier of the G allele (GC+GG genotypes) for SNP rs266729 had higher circulating adiponectin levels when compared to PE patients with the CC genotype, suggesting that this SNP may play a potential role in regulating adiponectin levels in PE. Although previous studies have indicated that serum adiponectin levels are lower in patients with the GG genotype than those with TG+TT genotype for SNP rs1501299 in severe PE, we did not find differences in plasma adiponectin levels between carriers of these genotypes in PE. ADIPOQ SNPs have also been shown to affect circulating adiponectin levels in other disease settings. For example, in a subgroup of Chinese subjects, carriers of rs266729 variants were found at high risk of dyslipidemia, atherosclerosis, and coronary artery disease, being the G allele associated with decreased adiponectin [97]. This may be explained by intrinsic differences between studied populations, but it also could be explained by the

allelic and loci heterogeneity [98], lifestyle differences between subjects, and also the methodology used to measure circulating adiponectin levels.

*According to its 1f score in RegulomeDB and ENCODE gene regulation data (***Supplementary Figure 3***), the rs266729 may affect the binding of transcription factors in the ADIPOQ promoter region. Moreover, MethPrimer analysis revealed that the C to G change at SNP rs266729 position (CpG site) creates a potential methylation site (data not shown). Since DNA methylation in the promoter region is linked to gene inactivation or reduced expression [99], this result contrasts with our finding that carriers of the G allele showed higher adiponectin levels in PE. However, it is important to consider that methylation can be altered in pathological conditions such as HDP [100]. Thus, future studies need to confirm if this CpG site at the ADIPOQ locus is indeed methylated in PE.*

Furthermore, we noted that PE patients carriers of the 'G,T,G' haplotype had higher circulating adiponectin levels when compared to HP women with the same haplotype. While previous studies in cardiovascular and metabolic diseases have linked specific ADIPOQ haplotypes to altered circulating adiponectin levels [75], their role in HDP remains underexplored. Our findings suggest that, despite inconsistencies across populations, the rs266729 SNP may influence plasma adiponectin modulation in PE, potentially reflecting a specific effect distinct from other metabolic or cardiovascular phenotypes.

Finally, the analysis of upper and lower subgroups of plasma adiponectin levels revealed that the 'G,T,G' haplotype was more frequent in the subgroup of higher adiponectin levels in PE. However, the 'G,T,G' haplotype was not associated with PE or GH in the overall haplotype distribution. As aforementioned, the 'C,T,T' haplotype was more frequent in HP than GH.

Consistent with this finding, the 'C,T,T' haplotype was more frequent in the lower subgroup of adiponectin levels in PE. Taken together, we suggest that this haplotype may be associated with adiponectin levels and the outcome of HDP. Nonetheless, further studies are needed to explore this hypothesis. These findings are also aligned with the proposal that comparing extremes of phenotype distribution improves our abilities to find genetic contributions to particular phenotypes [101, 102].

This study has limitations. First, the number of patients included in the study was relatively small, with an even smaller subset having plasma samples available to measure adiponectin levels. Nevertheless, we identified significant associations involving genotypes and haplotypes of ADIPOQ with PE and GH, especially involving SNP rs266729. Furthermore, we did not assess adiponectin levels in placental or adipose tissues, leaving the extent of the SNPs effects at the tissue level unknown. In conclusion, we found that the GG genotype for the ADIPOQ SNP rs266729 (C>G) was associated with risk for GH, whereas the CG genotype and the 'C,T,T' haplotype may protect against PE and GH, respectively. In addition, CG and GG genotypes for the same SNP as well as the 'G,T,G' haplotype showed higher plasma adiponectin levels in PE subjects. In conclusion, in this study, we evidenced that the addressed ADIPOQ genotypes and haplotypes may affect circulating adiponectin in PE and affect the susceptibility to GH or PE. These findings suggest that ADIPOQ polymorphisms have an effect on circulating adiponectin levels and an association with susceptibility for development of GH and PE.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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FIGURES

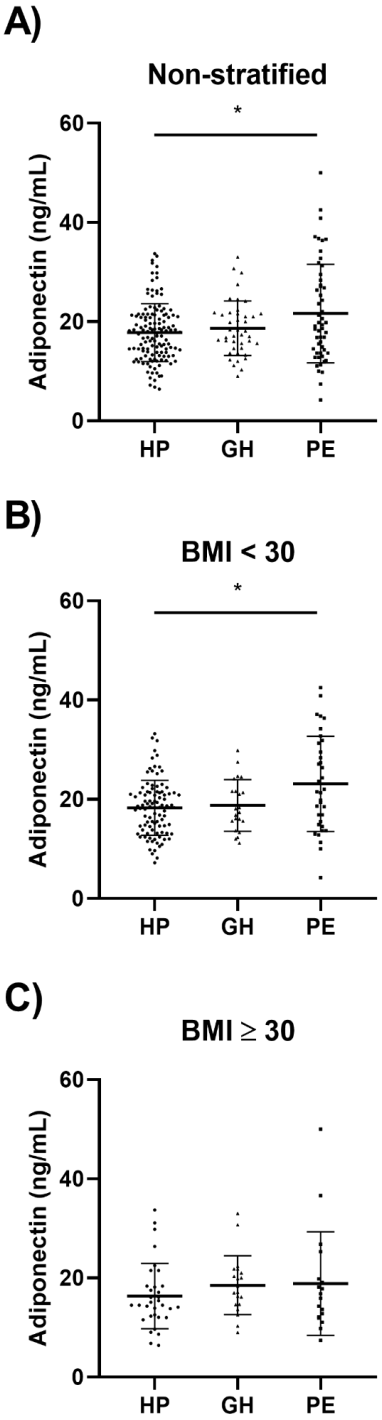


Figure 1 Scatter-plots of plasma adiponectin concentrations (Median; Minimum – Maximum). In **Figure 1A**, healthy pregnant [HP, N=130; 17.80 (6.40-33.70)], patients with gestational hypertension [GH, N=42; 17.75 (9.00-33.00)] and preeclampsia [PE, N=54; 19.25 (4.20- 50,00)]. In **Figure 1B**, [HP, N=97; 18.40 (7.20-33.20)], patients with GH [GH, N=22; 17.75 (11.20-29.90)] and PE [PE, N=35; 21.50 (4.20- 42,50)]. In **Figure 1C**, [HP, N=33; 14.50 (6.40-33.70)], patients with GH [GH, N=20; 17.75 (9.00-33.00)] and PE [PE, N=19; 16.80 (7.40- 50,00)]. In * $P < 0.05$ between HP and PE groups.

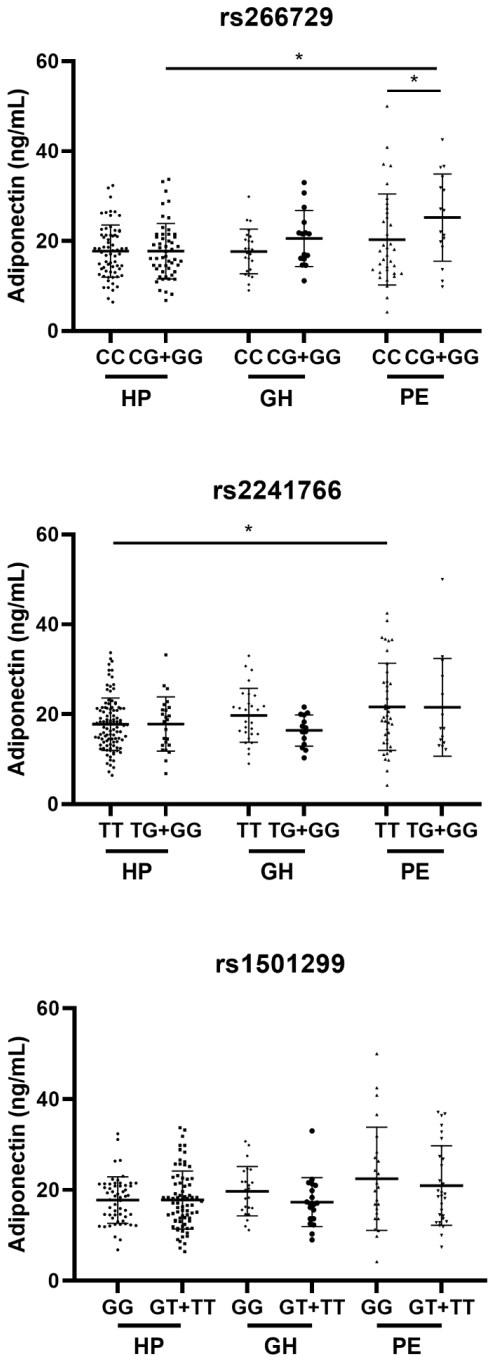


Figure 2 Scatter-plots of plasma adiponectin concentrations (Median; Minimum - Maximum) in healthy pregnant (HP) women, patients with preeclampsia (PE) and with gestational hypertension (GH) grouped according to the genotypes for three *ADIPOQ* SNPs rs266729 C>G; rs2241766 T>G; and rs1501299 G>T. *P<0.05 for rs266729 between patients with PE carrying CG+GG genotypes versus those carrying the CC genotype, and also with HP women carrying the CG+GG genotypes. *P<0.05 for rs2241766 between PE patients with the TT genotype when compared to the same genotype in the HP group.

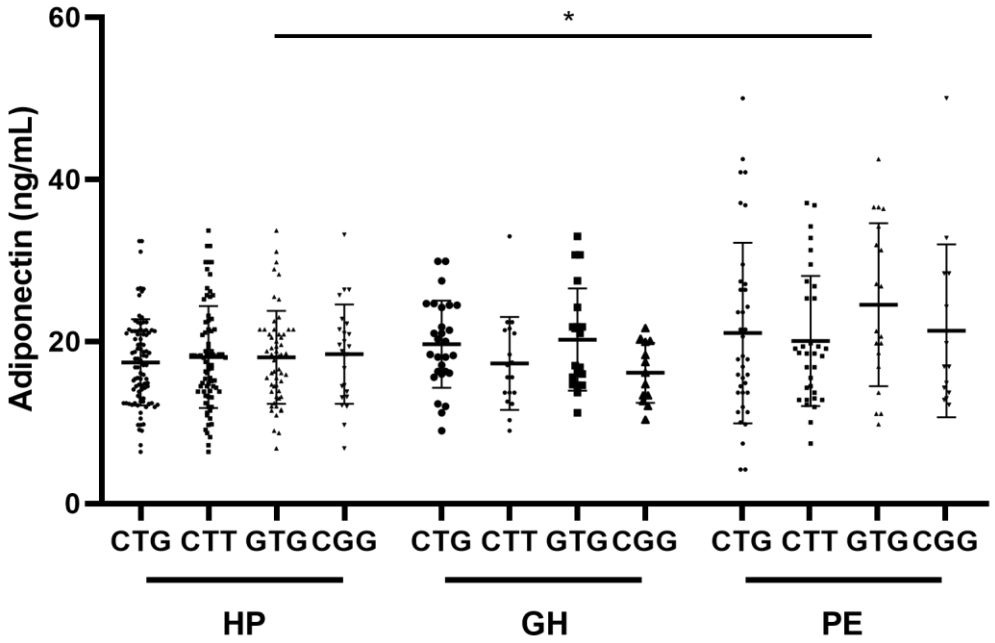


Figure 3 Plasma adiponectin concentrations for each of the most frequent *ADIPOQ* haplotypes in healthy pregnant (HP), gestational hypertension (GH), and preeclampsia (PE). *P<0.05 for comparison between PE and HP groups considering the 'G,T,G' haplotype.

TABLES

Table 1 Clinical and demographic characteristics for all subjects selected for the study.

Parameters	HP (n = 182)	GH (n = 121)	P value [a]	PE (n = 133)	P value [a]
Age (years)	24.00 (20.00 - 28.25)	26.00 (22.00 - 32.00)	**0.0088**	27.00 (22.00 - 32.00)	**0.0051**
Race (% white)	58.24	77.69	**0.0253**	72.18	0.2104
Current smoking (%)	9.89	13.22	0.4576	6.77	0.4163
Primiparity (%)	46.30	41.32	0.4680	42.11	0.4823
BMI (kg/m^2)	27.25 (19.30 - 45.70)	33.04 (19.68 - 56.22)	**<0.0001**	31.60 (21.67 - 51.85)	**<0.0001**
Pre-pregnancy BMI (kg/m^2)	21.63 (17.26 - 39.74)	29.33 (17.31 - 49.61)	**<0.0001**	25.13 (16.18 - 48.41)	**0.0014**
SBP (mm Hg)	110.00 (90.00 - 140.00)	130.00 (90.00 - 210.00)	**<0.0001**	140.00 (100.00 - 200.00)	**<0.0001**
DBP (mm Hg)	70.00 (50.00 - 90.00)	80.00 (58.00 - 130.00)	**<0.0001**	90.00 (60.00 - 130.00)	**<0.0001**
HR (beats/min)	80.00 (60.00 - 150.00)	80.00 (62.00 - 110.00)	0.2786	80.00 (60.00 - 120.00)	0.7653
Fasting glucose (mg/dL)	73.50 (54.00 - 111.00)	78.00 (60.00 - 117.00)	**0.0391**	77.00 (60.00 - 130.00)	**0.0161**

Hb (g/dL)	11.80 (2.30 - 15.00)	11.90 (7.30 - 15.70)	0.9623	12.20 (3.80 - 15.80)	**0.0103**
Hct (%)	35.25 (6.00 - 49.00)	35.70 (22.00 - 47.00)	0.9021	36.85 (12.50 - 47.00)	**0.0074**
Creatinine (mmol/L)	0.70 (0.60 - 0.80)	0.60 (0.40 - 1.00)	**0.0231**	0.70 (0.40 - 2.90)	0.4979
Proteinuria (mg/24 h)	ND	153.00 (20.00 - 291.00)	ND	665.50 (50.00 - 776.20)	ND
GAS (weeks)	37.00 (20.00 - 41.00)	37.00 (18.00 - 43.00)	0.6079	35.00 (14.00 - 42.00)	**<0.0001**
GAD (weeks)	40.00 (36.00 - 42.00)	39.00 (35.00 - 43.00)	**0.0019**	37.00 (19.00 - 42.00)	**<0.0001**
Newborn weight (g)	3265 (2970 - 4970)	3160 (1360 - 4830)	0.0458	2883 (1250 - 4235)	**<0.0001**

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GAD, gestational age at delivery; GAS, gestational age at sampling; GH, gestational hypertension; Hb, hemoglobin concentration; Hct, hematocrit; HP, healthy pregnant; HR, heart rate; ND, not determined; PE, preeclampsia; SBP, systolic blood pressure. Values are the mean ± s.e.m. [a] P < 0.05 vs healthy pregnant group. Bold values are significant.

Table 2 Genotypic and allelic frequencies for *ADIPOQ* polymorphisms in the healthy pregnant. gestational hypertension and preeclampsia groups..

ADIPOQ SNP	*HP (n = 182)*	*GH (n = 121)*	*OR (95% CI)*	*P Value[a]*	*PE (n = 133)*	*OR (95% CI)*	*P Value[a]*
rs266729							
Codominant							
CC	106 (58.24%)	64 (52.89%)	1.0000 (Reference)		84 (63.15%)	1.0000 (Reference)	
CG	62 (34.06%)	37 (30.57%)	0.9884 (0.5847 to 1.6370)	>0.9999	28 (21.05%)	0.5699 (0.3310 to 0.9658)	**0.0378**
GG	14 (7.70%)	20 (16.52%)	2.3660 (1.1540 to 4.8160)	**0.0346**	21 (15.78%)	1.8930 (0.8912 to 4.0140)	0.0986
Dominant							
CC	106 (58.24%)	64 (52.89%)	1.0000 (Reference)		84 (63.16%)	1.0000 (Reference)	
CG+GG	76 (41.76%)	57 (47.11%)	1.2420 (0.7721 to 1.9620)	0.4083	49 (36.84%)	0.8136 (0.5206 to 1.2910)	0.4152
Alleles							
C	274 (75.27%)	165 (68.18%)	1.0000 (Reference)		196 (73.68%)	1.0000 (Reference)	
G	90 (24.73%)	77 (31.82%)	1.4210 (0.9931 to 2.0250)	0.0634	70 (26.32%)	1.0870 (0.7552 to 1.5520)	0.7110
rs2241766							
Codominant							
TT	146 (80.22%)	88 (72.73%)	1.0000 (Reference)		101 (75.94%)	1.0000 (Reference)	
TG	35 (19.23%)	30 (24.79%)	0.7032 (0.4014 to 1.2210)	0.2513	27 (20.30%)	0.8968 (0.5066 to 1.5620)	0.7734
GG	1 (0.55%)	3 (2.48%)	0.2009 (0.0154 to 1.3730)	0.1575	5 (3.76%)	0.1384 (0.0117 to 1.0330)	0.0852
Dominant							

TT	146 (80.22%)	88 (72.73%)	1.0000 (Reference)		101 (75.94%)	1.0000 (Reference)	
TG+GG	36 (19.78%)	33 (27.27%)	0.6575 (0.3847 to 1.1320)	0.1616	32 (24.06%)	0.7783 (0.4576 to 1.3380)	0.4062
Alleles							
T	327 (89.84%)	206 (85.12%)	1.0000 (Reference)		229 (86.09%)	1.0000 (Reference)	
G	37 (10.16%)	36 (14.88%)	0.6475 (0.4027 to 1.0450)	0.0973	37 (13.91%)	0.7003 (0.4249 to 1.1550)	0.1685
rs1501299							
Codominant							
GG	81 (44.51%)	65 (53.72%)	1.0000 (Reference)		67 (50.37%)	1.0000 (Reference)	
GT	82 (45.05%)	47 (38.84%)	1.4000 (0.8621 to 2.3040)	0.1789	52 (39.10%)	1.3040 (0.8181 to 2.0980)	0.2801
TT	19 (10.44%)	9 (7.44%)	1.6940 (0.6990 to 4.1320)	0.2974	14 (10.53%)	1.1230 (0.5320 to 2.3120)	0.8476
Dominant							
GG	81 (44.51%)	65 (53.72%)	1.0000 (Reference)		67 (50.37%)	1.0000 (Reference)	
GT+TT	101 (55.49%)	56 (46.28%)	1.4470 (0.9055 to 2.2660)	0.1278	66 (49.63%)	1.2660 (0.8119 to 1.980)	0.3067
Alleles							
G	244 (67.03%)	177 (73.14%)	1.0000 (Reference)		186 (69.92%)	1.0000 (Reference)	
T	120 (32.97%)	65 (26.86%)	1.3390 (0.9340 to 1.9230)	0.1258	80 (30.08%)	1.1430 (0.8102 to 1.6050)	0.4883

Abbreviations: CI. Confidence Interval; OR. Odds Ratio. [a] P < 0.05 vs healthy pregnant group. Significant P values are in bold.

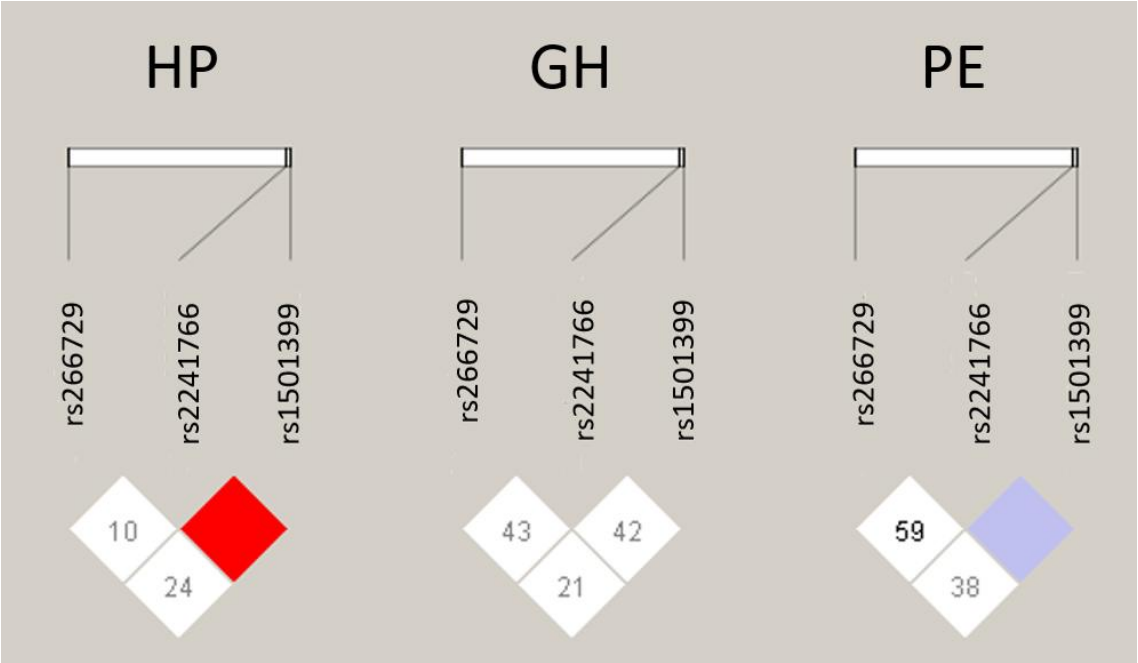
Table 3 Haplotype frequencies for ADIPOQ polymorphisms in the HP. GH and PE groups. The considered haplotypes were the ones with frequency higher than 5% among groups.

ADIPOQ Haplotypes	*HP (n = 182)*	*GH (n = 121)*	*P values[a]*	*OR (95% CI)*	*PE (n = 133)*	*P values[a]*	*OR (95% CI)*
C.T.G	0.3993 (39.93%)	0.3574 (35,74%)	0.3431	1.0000 (Reference)	0.3667 (36,67%)	0.5205	1.0000 (Reference)
C.T.T	0.2709 (27.09%)	0.1911 (19,11%)	**0.0397**	0.8132 (0.5145 – 1.2854)	0.2659 (26,59%)	0.6967	1.0463 (0.6991 – 1.5658)
G.T.G	0.1694 (16.94%)	0.2431 (24,31%)	0.0699	1.4175 (0.8836 – 2.2742)	0.1935 (19,35%)	0.5779	1.2370 (0.7776 – 1.9679)
C.G.G	0.0826 (8.26%)	0.1225 (12,25%)	0.1268	1.5546 (0.7932 – 3.0470)	0.1043 (10,43%)	0.2856	1.3732 (0.7516 – 2.5088)
		Global-stat = 8.5486. df = 5. P = 0.12848			*Global-stat = 3.7515. df = 5. P = 0.58572*		

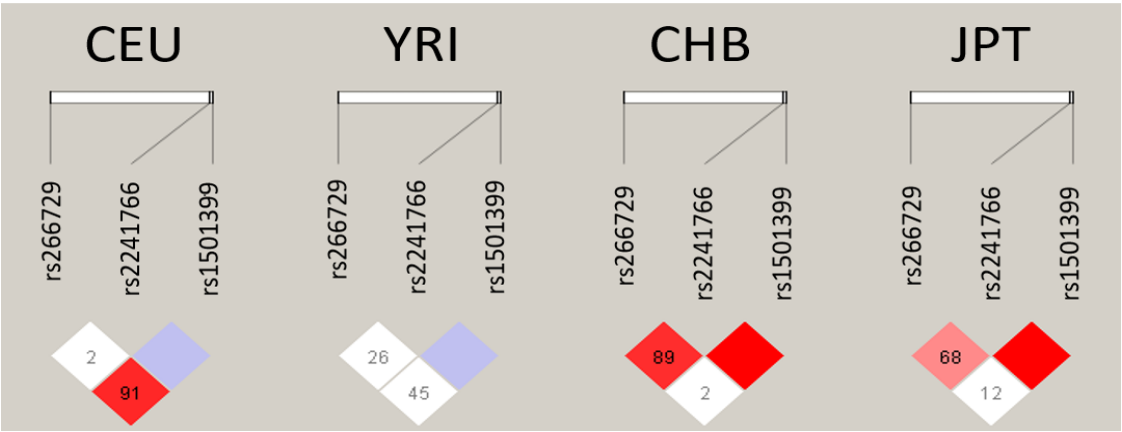
Abbreviations: CI. Confidence Interval; GH. gestational hypertension; HP. healthy pregnant; OR. Odds Ratio; PE. preeclampsia. [a] P < 0.05 vs healthy pregnant group. Significant P values are in bold.

SUPPLEMENTARY FIGURES

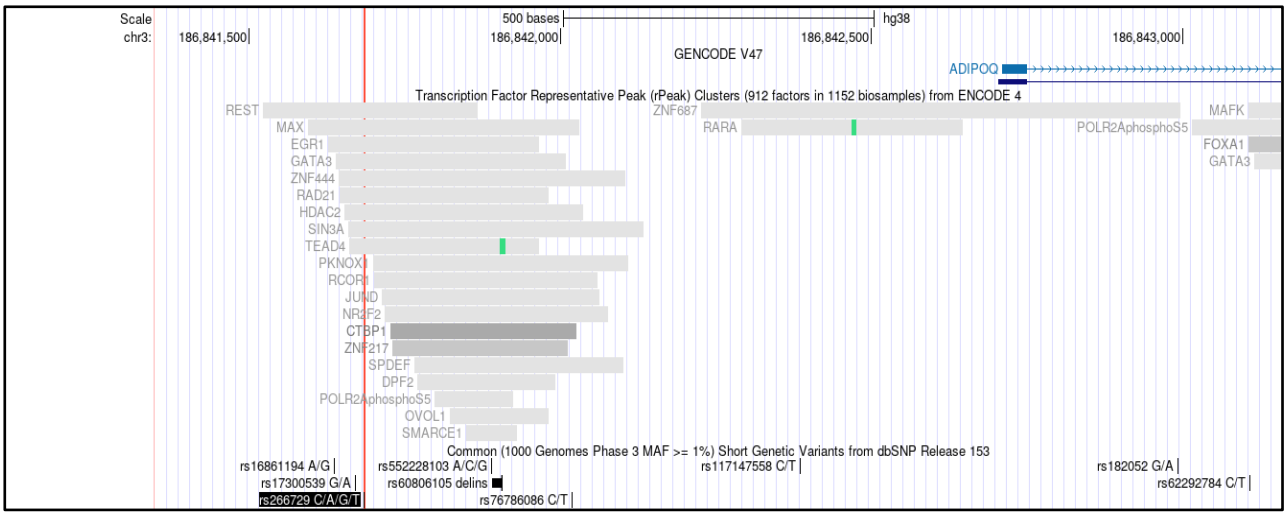
Sup. Figure 1 Linkage disequilibrium plots for the *ADIPOQ* SNPs in healthy pregnant (HP), gestational hypertension (GH), and preeclampsia (PE) groups. Values for pairwise D´ are presented in each box; those without values refer to D=1. Color scheme: bright red, D=1 and LOD≥2; blue, D=1 and LOD<2; white, D<1 and LOD<2. LOD, Logarithm of odds.



Sup. Figure 2 Linkage disequilibrium plots for the *ADIPOQ* SNPs in four different populations of the 1000 Genomes Project: European (CEU), African (YRI), Chinese (CHB), and Japanese (JPT). Values for pairwise D´ are presented in each box; those without values refer to D=1. Color scheme: bright red, D=1 and LOD≥2; blue, D=1 and LOD<2; white, D<1 and LOD<2. LOD, Logarithm of odds.



Sup. Figure 3 UCSC Genome Browser view of the *ADIPOQ* promoter region, with the location of the rs266729 SNP (red highlight) and transcription factor binding sites from the ENCODE data. The region shows an abundance transcription factor binding sites located in the SNP site and nearby.



SUPPLEMENTARY TABLES

Sup. Table 1 Clinical and demographic characteristics for subjects whose plasma samples were available.

Parameters	HP (n = 130)	GH (n = 42)	P value[a]	PE (n = 55)	P value[a]
Age (years)	24.00 (20.00 - 29.00)	27.50 (21.25 - 33.00)	0.0563	26.00 (20.25 - 32.75)	0.1593
Race (% white)	63.00	71.42	0.3555	63.63	>0.9999
Current smoking (%)	10.70	16.70	0.4151	7.27	0.5924
Primiparity (%)	44.61	40.47	0.7215	43.63	>0.9999
BMI (kg/m^2)	27.05 (24.68 - 30.00)	29.83 (27.14 - 33.11)	**0.0002**	29.13 (26.11 - 32.01)	**0.0029**
Pre-pregnancy BMI (kg/m^2)	22.20 (17.50 - 26.60)	30.06 (17.31 - 49.61)	**<0.0001**	25.15 (16.18 - 48.41)	**0.0010**
SBP (mm Hg)	110.00 (101.50 - 120.00)	130.00 (123.50 - 140.00)	**<0.0001**	140.00 (131.00 - 155.00)	**<0.0001**
DBP (mm Hg)	70.00 (70.00 - 80.00)	84.00 (73.00 - 90.00)	**<0.0001**	90.00 (80.00 - 100.00)	**<0.0001**
HR (beats/min)	80.00 (80.00 - 86.00)	82.00 (80.00 - 87.00)	0.9197	80.00 (80.00 - 87.50)	0.7698
Fasting glucose (mg/dL)	73.00 (66.00 - 82.00)	76.00 (69.00 - 87.50)	0.3965	80.00 (72.63 - 97.50)	**0.0029**
Hb (g/dL)	12.00 (11.00 - 12.75)	11.90 (11.20 - 12.70)	0.5335	12.40 (11.70 - 13.05)	0.0646
Hct (%)	35.85 (33.00 - 38.15)	35.95 (33.48 - 37.90)	0.8163	37.00 (34.50 - 39.50)	**0.0287**
Creatinine (mmol/L)	0.60 (0.60 - 0.80)	0.60 (0.50 - 0.70)	0.1574	0.70 (0.60 - 0.80)	0.8952
24 h Pr (mg/24 h)	ND	146.00 (110.00 - 166.50)	ND	726.20 (302.80 - 1777)	ND

GAS (weeks)	37.00 (36.00 - 38.00)	37.00 (36.00 - 38.00)	0.9397	35.00 (32.00 - 37.00)	**<0.0001**
GAD (weeks)	40.00 (39.00 - 41.00)	39.00 (38.00 - 39.50)	**0.0133**	36.50 (34.00-38.25)	**<0.0001**
Newborn weight (g)	3263 (2974 - 3681)	3040 (2833 - 3295)	**0.0258**	2563 (1665 - 3156)	**<0.0001**

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GAD, gestational age at delivery; GAS, gestational age at sampling; GH, gestational hypertension; Hb, hemoglobin concentration; Hct, hematocrit; HP, healthy pregnant; HR, heart rate; ND, not determined; PE, preeclampsia; SBP, systolic blood pressure. Values are the mean ± s.e.m. [a] P < 0.05 vs healthy pregnant group. Bold values are significant.

Sup. Table 2 Haplotype frequencies for *ADIPOQ* polymorphisms in the healthy pregnant group segregated according to lower and upper plasma levels of adiponectin.

Haplotypes	*Hap-Score*	*Lower (n = 66)*	*Upper (n = 64)*	*P value*	*OR (95% CI)*
C,T,G	0.9312	0.4669 (46,69%)	0.3497 (34,97%)	0.3517	1.0000 (*Reference*)
C,T,T	-0.4257	0.2359 (23,59%)	0.2926 (29,26%)	0.6703	0.6653 (0.3353 - 1.3200)
G,T,G	-0.8186	0.1164 (11,64%)	0.2128 (21,28%)	0.4130	0.4264 (0.1580 - 1.1513)
C,G,G	-1.0109	0.0472 (4,72%)	0.1077 (10,77%)	0.3121	0.4403 (0.1320 - 1.4693)

Global-stat = 5.4738. df = 5; P = 0.3608

Abbreviations: CI, Confidence Interval; OR, Odds Ratio.

Sup. Table 3 Haplotype frequencies for *ADIPOQ* polymorphisms in the gestational hypertension group segregated according to lower and upper plasma levels of adiponectin.

Haplotypes	*Hap-Score*	*Lower (n = 21)*	*Upper (n = 21)*	*P value*	*OR (95% CI)*
C,T,G	-1.7110	0.2403 (24,03%)	0.4454 (44,54%)	0.0871	1.0000 (*Reference*)
C,T,T	0.9394	0.2597 (25,97%)	0.1499 (14,99%)	0.3475	2.8999 (0.7914 - 10.6260)
G,T,G	0.0460	0.2359 (23,59%)	0.2213 (22,13%)	0.9633	1.6734 (0.4762 - 5.8810)
C,G,G	0.7716	0.1905 (19,05%)	0.1190 (11,90%)	0.4403	3.8587 (0.8139 - 18.2900)

Global-stat = 6.3497. df = 6. P = 0.3852

Abbreviations: CI, Confidence Interval; OR, Odds Ratio.

Sup. Table 4 Haplotypes frequencies for *ADIPOQ* polymorphisms in the preeclampsia group segregated according to lower and upper levels of plasma adiponectin.

Haplotypes	*Hap-Score*	*Lower (n = 28)*	*Upper (n = 27)*	*P value*	*OR (95% CI)*
C,T,G	0.4035	0.3571 (35,71%)	0.3380 (33,80%)	0.6865	1.0000 (*Reference*)
C,T,T	2.1905	0.3929 (39,29%)	0.1805 (18,05%)	**0.0285**	1.8035 (0.6678 - 4.871)
C,G,G	0.4148	0.1428 (14,28%)	0.1111 (11,11%)	0.6783	1.0252 (0.2678 - 3.925)
G,T,G	-2.0770	0.1071 (10,71%)	0.2546 (25,46%)	**0.0378**	0.5651 (0.1491 - 2.1420)

Global-stat = 8.8209. df = 5. P = 0.1164

Abbreviations: CI, Confidence Interval; OR, Odds Ratio. Significant P values are in bold.

Sup. Table 5: Gestational Hypertension univariate logistic regression.

Logistic Model GH	*Estimate*	*Std. Error z*	*z value*	*Pr(>|z|)*	*OR (95% CI)*
(Intercept)	-6.2472	0.8994	-6.9459	**< 0.0001**	0.0019 (0.0003 - 0.0104)
BMI (Kg/m²) during pregnancy	0.1939	0.0295	6.5662	**< 0.0001**	1.2140 (1.1491 - 1.2905)
(Intercept)	-1.7537	0.5207	-3.3682	**0.0008**	0.1731 (0.0611 - 0.4732)
Age (Years)	0.053	0.0194	2.737	**0.0062**	1.0544 (1.0156 - 1.096)
(Intercept)	1.1034	1.2454	0.886	0.3756	3.0144 (0.2681 - 37.7634)
GAS (weeks)	-0.0325	0.034	-0.9555	0.3393	0.9680 (0.9036 - 1.0341)
(Intercept)	-0.1201	0.1417	-0.848	0.3964	0.8868 (0.6710 - 1.1702)
Ethnicity (brown)	-0.642	0.3533	-1.817	0.0692	0.5262 (0.2567 - 1.035)
Ethnicity (black)	-0.2593	0.3868	-0.6705	0.5026	0.7716 (0.3544 - 1.6345)
(Intercept)	-0.2032	0.1599	-1.2707	0.2038	0.8161 (0.5951 - 1.1154)
Primiparity	-0.2022	0.2427	-0.8332	0.4047	0.8169 (0.5065 - 1.3131)
(Intercept)	-0.5046	0.1583	-3.1873	**0.0014**	0.6038 (0.4407 - 0.8207)
rs266729CG	-0.0117	0.2612	-0.0446	0.9644	0.9884 (0.5901 - 1.6458)

rs266729GG	0.8612	0.3827	2.2502	**0.0244**	2.3661 (1.1254 - 5.0979)
(Intercept)	1.0986	1.1547	0.9514	0.3414	3.0000 (0.3841 - 60.6487)
rs2241766TG	-1.2528	1.1812	-1.0606	0.2889	0.2857 (0.0137 - 2.3639)
rs2241766TT	-1.6049	1.1626	-1.3805	0.1674	0.2009 (0.0099 - 1.5963)
(Intercept)	-0.2201	0.1665	-1.3215	0.1863	0.8025 (0.5775 - 1.1108)
rs1501299GT	-0.3365	0.2474	-1.3602	0.1738	0.7143 (0.4385 - 1.1581)
rs1501299TT	-0.5272	0.4376	-1.2047	0.2283	0.5903 (0.2402 - 1.3595)

Sup. Table 6: Preeclampsia univariate logistic regression.

Logistic Model PE	***Estimate***	***Std. Error z***	***z value***	***Pr(>|z|)***	***OR (95% CI)***
(Intercept)	-4.9106	0.8052	-6.0983	**< 0.0001**	0.0074 (0.0014 - 0.0335)
BMI (Kg/m²) during pregnancy	0.1587	0.0268	5.9095	**< 0.0001**	1.1719 (1.1145 - 1.2386)
(Intercept)	-1.6228	0.5144	-3.155	**0.0016**	0.1973 (0.0706 - 0.5331)
Age (Years)	0.0554	0.0192	2.8798	**0.0040**	1.057 (1.0184 - 1.0984)
(Intercept)	5.0668	1.3634	3.7163	**0.0002**	158.6716 (12.9145 - 2764.8681)
GAS (weeks)	-0.1406	0.0376	-3.7348	**0.0002**	0.8688 (0.8031 - 0.9314)
(Intercept)	0	0.1443	0	1.0000	1.0000 (0.7532 - 1.3276)
Ethnicity (brown)	-0.3514	0.3324	-1.0571	0.2905	0.7037 (0.3626 - 1.3433)
Ethnicity (black)	-0.1112	0.3637	-0.3058	0.7598	0.8947 (0.4351 - 1.8273)
(Intercept)	-0.0892	0.1598	-0.5585	0.5765	0.9146 (0.6678 - 1.2509)
Primiparity	-0.1049	0.241	-0.4353	0.6633	0.9004 (0.5606 - 1.4437)
(Intercept)	-0.1231	0.1498	-0.8217	0.4113	0.8842 (0.6583 - 1.1855)
rs266729CG	-0.6223	0.274	-2.2708	**0.0232**	0.5367 (0.3107 - 0.9121)
rs266729GG	0.5538	0.3865	1.4331	0.1518	1.7399 (0.8233 - 3.7893)
(Intercept)	1.6094	1.0954	1.4692	0.1418	5.0000 (0.8064 - 95.7954)
rs2241766TG	-1.7476	1.1266	-1.5512	0.1209	0.1742 (0.0088 - 1.1681)
rs2241766TT	-1.9095	1.1034	-1.7307	0.0835	0.1481 (0.0077 - 0.9372)

(Intercept)	-0.0858	0.1692	-0.5069	0.6122	0.9178 (0.6578 - 1.2787)
rs1501299GT	-0.2999	0.2481	-1.2087	0.2268	0.7409 (0.4545 - 1.2035)
rs1501299TT	-0.2196	0.3907	-0.562	0.5741	0.8028 (0.3677 - 1.7189)

Sup. Table 7: Gestational Hypertension multivariate logistic regression.

Logistic Model GH	***Estimate***	***Std. Error z***	***z value***	***Pr(>|z|)***	***OR (95% CI)***
(Intercept)	-6.7643	1.0407	-6.4996	**< 0.0001**	0.0012 (0.0001 - 0.0081)
rs266729CG	-0.1070	0.3112	-0.3437	0.7311	0.8986 (0.4848 - 1.6478)
rs266729GG	0.4082	0.4625	0.8824	0.3775	1.5040 (0.6070 - 3.7693)
Age (years)	0.0368	0.0222	1.6581	0.0973	1.0374 (0.9934 - 1.0840)
BMI (Kg/m²) during pregnancy	0.1776	0.0295	6.0136	**< 0.0001**	1.1944 (1.1304 - 1.2697)
(Intercept)	-6.1319	0.9006	-6.8085	**< 0.0001**	0.0022 (0.0003 - 0.0116)
rs266729CG	-0.1237	0.3085	-0.401	0.6884	0.8836 (0.4792 - 1.612)
rs266729GG	0.3884	0.4555	0.8526	0.3939	1.4746 (0.6039 - 3.6473)
BMI (Kg/m²) during pregnancy	0.1899	0.0296	6.4094	**< 0.0001**	1.2092 (1.1443 - 1.2857)
(Intercept)	-1.8839	0.5330	-3.5343	**0.0004**	0.1520 (0.0522 - 0.4247)
rs266729CG	0.0216	0.2737	0.0789	0.9371	1.0218 (0.5951 - 1.7436)
rs266729GG	0.9140	0.4144	2.2056	**0.0274**	2.4943 (1.1173 - 5.7440)
Age (years)	0.0539	0.0197	2.7380	**0.0062**	1.0553 (1.0159 - 1.0977)
(Intercept)	-5.5939	1.5834	-3.5329	**0.0004**	0.0037 (0.0002 - 0.1247)
rs2241766TG	-0.9645	1.2544	-0.7688	0.4420	0.3812 (0.0170 - 3.8723)
rs2241766TT	-1.3663	1.2228	-1.1173	0.2639	0.2551 (0.0118 - 2.4217)
Age (years)	0.0352	0.0224	1.572	0.116	1.0358 (0.9914 - 1.0828)
BMI (Kg/m²) during pregnancy	0.1822	0.0298	6.1181	**< 0.0001**	1.1999 (1.1350 - 1.2761)

(Intercept)	-5.2258	1.5266	-3.4232	**0.0006**	0.0054 (0.0003 - 0.1670)
rs2241766TG	-0.7064	1.2478	-0.5661	0.5713	0.4934 (0.0222 - 5.0167)
rs2241766TT	-1.1699	1.2215	-0.9578	0.3382	0.3104 (0.0144 - 2.9859)
BMI (Kg/m²) during pregnancy	0.1950	0.0298	6.5463	**< 0.0001**	1.2153 (1.1497 - 1.2924)
(Intercept)	-0.0132	1.2279	-0.0107	0.9914	0.9869 (0.1068 - 21.6036)
rs2241766TG	-1.6379	1.2009	-1.3639	0.1726	0.1944 (0.0091 - 1.6741)
rs2241766TT	-1.8497	1.1753	-1.5738	0.1155	0.1573 (0.0076 - 1.2828)
Age (years)	0.0544	0.0198	2.7480	**0.0060**	1.0559 (1.0162 - 1.0985)
(Intercept)	-6.7060	1.0469	-6.4059	**< 0.0001**	0.0012 (0.0001 - 0.0087)
rs1501299GT	-0.4331	0.2983	-1.4521	0.1465	0.6485 (0.3589 - 1.1589)
rs1501299TT	-0.4977	0.5449	-0.9133	0.3611	0.6080 (0.1975 - 1.7112)
Age (years)	0.0371	0.0220	1.6843	0.0921	1.0378 (0.9940 - 1.0840)
BMI (Kg/m²) during pregnancy	0.1831	0.0297	6.1670	**< 0.0001**	1.2009 (1.1363 - 1.2770)
(Intercept)	-6.0642	0.9052	-6.6990	**< 0.0001**	0.0023 (0.0004 - 0.0126)
rs1501299GT	-0.4709	0.2958	-1.5921	0.1114	0.6244 (0.3472 - 1.1104)
rs1501299TT	-0.3893	0.5227	-0.7447	0.4565	0.6776 (0.2321 - 1.8386)
BMI (Kg/m²) during pregnancy	0.1953	0.0297	6.5772	**< 0.0001**	1.2157 (1.1504 - 1.2928)
(Intercept)	-1.6181	0.5348	-3.0257	**0.0025**	0.1983 (0.0682 - 0.5578)
rs1501299GT	-0.2407	0.2588	-0.9301	0.3523	0.7861 (0.4721 - 1.3040)
rs1501299TT	-0.4729	0.4707	-1.0045	0.3151	0.6232 (0.2366 - 1.5310)
Age (years)	0.0532	0.0194	2.7387	**0.0062**	1.0547 (1.0158 - 1.0965)

Abbreviations: BMI, Body mass index; CI, confidence intervals; OR, odds ratio; GH, gestational hypertension. Significant P values are in bold.

Sup. Table 8: Preeclampsia multivariate logistic regression**.**

Logistic Model PE	*Estimate*	*Std. Error z*	*z value*	*Pr(>|z|)*	*OR (95% CI)*
(Intercept)	-5.6026	0.9455	-5.9255	**< 0.0001**	0.0037 (0.0005 - 0.0220)
rs266729CG	-0.6548	0.3105	-2.1090	**0.0349**	0.5195 (0.2794 - 0.9471)
rs266729GG	0.2618	0.4591	0.5702	0.5686	1.2992 (0.5296 - 3.2518)
Age (years)	0.0449	0.0213	2.1073	**0.0351**	1.0459 (1.0036 - 1.0912)
BMI (Kg/m²) during pregnancy	0.1465	0.0275	5.3197	**< 0.0001**	1.1578 (1.0995 - 1.2253)
(Intercept)	-4.6682	0.8164	-5.7184	**< 0.0001**	0.0094 (0.0018 - 0.0437)
rs266729CG	-0.6063	0.3027	-2.0032	**0.0452**	0.5454 (0.2982 - 0.9801)
rs266729GG	0.2705	0.4459	0.6066	0.5441	1.3106 (0.5494 - 3.2028)
BMI (Kg/m²) during pregnancy	0.1554	0.0272	5.7063	**< 0.0001**	1.1681 (1.1100 - 1.2355)
(Intercept)	-1.5974	0.5284	-3.0233	**0.0025**	0.2024 (0.0704 - 0.5620)
rs266729CG	-0.6797	0.2881	-2.3589	**0.0183**	0.5068 (0.2850 - 0.8845)
rs266729GG	0.6093	0.4158	1.4654	0.1428	1.8392 (0.8252 - 4.2718)
Age (years)	0.0592	0.0197	3.0002	**0.0027**	1.0610 (1.0213 - 1.1038)
(Intercept)	-3.8556	1.4515	-2.6563	**0.0079**	0.0212 (0.0013 - 0.5975)
rs2241766TG	-1.9015	1.2587	-1.5107	0.1309	0.1493 (0.0066 - 1.4532)
rs2241766TT	-2.1229	1.2339	-1.7205	0.0853	0.1197 (0.0054 - 1.1006)

Age (years)	0.0435	0.0210	2.0699	**0.0385**	1.0444 (1.0027 - 1.0890)
BMI (Kg/m²) during pregnancy	0.1524	0.0275	5.5401	**< 0.0001**	1.1646 (1.1062 - 1.2326)
(Intercept)	-3.1721	1.4093	-2.2508	**0.0244**	0.0419 (0.0029 - 1.1213)
rs2241766TG	-1.7039	1.2578	-1.3547	0.1755	0.1820 (0.0080 - 1.7502)
rs2241766TT	-1.8470	1.2315	-1.4998	0.1337	0.1577 (0.0072 - 1.4282)
BMI (Kg/m²) during pregnancy	0.1605	0.0272	5.9042	**< 0.0001**	1.1741 (1.1159 - 1.2418)
(Intercept)	0.3505	1.1710	0.2993	0.7647	1.4198 (0.1876 - 29.4092)
rs2241766TG	-1.9896	1.1424	-1.7416	0.0816	0.1368 (0.0068 - 0.9507)
rs2241766TT	-2.2134	1.1197	-1.9768	**0.0481**	0.1093 (0.0056 - 0.7181)
Age (years)	0.0613	0.0196	3.1277	**0.0018**	1.0632 (1.0237 - 1.1057)
(Intercept)	-5.6971	0.9471	-6.0151	**< 0.0001**	0.0034 (0.0005 - 0.0200)
rs1501299GT	-0.0674	0.2825	-0.2384	0.8116	0.9349 (0.5368 - 1.6286)
rs1501299TT	-0.1232	0.4569	-0.2695	0.7875	0.8841 (0.3553 - 2.1563)
Age (years)	0.0394	0.0208	1.8912	**0.0586**	1.0401 (0.9989 - 1.0841)
BMI (Kg/m²) during pregnancy	0.1505	0.0272	5.5366	**< 0.0001**	1.1624 (1.1047 - 1.2293)
(Intercept)	-4.8163	0.8210	-5.8662	**< 0.0001**	0.0081 (0.0015 - 0.0380)
rs1501299GT	-0.1557	0.2776	-0.5609	0.5749	0.8558 (0.4957 - 1.4753)

rs1501299TT	-0.0889	0.4439	-0.2002	0.8413	0.9150 (0.3780 - 2.1794)
BMI (Kg/m²) during pregnancy	0.1580	0.0269	5.8786	**< 0.0001**	1.1712 (1.1137 - 1.2378)
(Intercept)	-1.5266	0.5317	-2.8710	**0.0041**	0.2173 (0.0752 - 0.6079)
rs1501299GT	-0.1763	0.2584	-0.6824	0.4950	0.8384 (0.5044 - 1.3907)
rs1501299TT	-0.1547	0.4174	-0.3708	0.7108	0.8566 (0.3727 - 1.9378)
Age (years)	0.0552	0.0193	2.8622	**0.0042**	1.0568 (1.0181 - 1.0983)

Abbreviations: BMI, Body mass index; CI, confidence intervals; OR, odds ratio; PE, preeclampsia. Significant *P* values are in bold.

4.2. Artigo 2;

Título: “ADIPOQ genotypes and haplotypes, circulating adiponectin levels and responsiveness to antihypertensive therapy in preeclampsia” – Submetido a revista Pharmacogenomics, fator de impacto 1.9

Pharmacogenomics

ADIPOQ genotypes and haplotypes, adiponectin levels and responsiveness to antihypertensive therapy in preeclampsia

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ABSTRACT

Aims: We compared plasma adiponectin levels between patients with preeclampsia (PE) classified as responsive and nonresponsive to antihypertensive therapy; Moreover, we examined whether *ADIPOQ* SNPs rs17300539, rs266729, rs2241766, and rs1501299 and their haplotypes are associated with responsiveness to antihypertensive therapy, and whether they affect plasma adiponectin levels in 215 pregnant women with PE.

Patientes & Methods: Genotypes for *ADIPOQ* SNPs were determined using TaqMan allelic discrimination assays, and circulating adiponectin concentrations were measured by ELISA.

Results: Patients with PE classified as nonresponsive to antihypertensive therapy showed higher plasma adiponectin levels. The TG genotype of the rs2241766 SNP was more frequent in responsive PE patients. Nonresponsive PE patients carrying the GG or GA+AA genotypes of the rs17300539 SNP, and CC, TT, and GT+TT genotypes of the rs266729, rs2241766, and rs1501299 SNPs, respectively. as well as the 'G,C,T,G' and 'G,C,T,T' haplotypes showed higher plasma adiponectin levels than responsive PE patients carrying the same genotypes/haplotypes.

Conclusions: Nonresponsive PE patients showed higher plasma adiponectin levels, the TG genotype of the rs2241766 SNP was more frequent in the responsive PE patients. Genotypes of rs17300539, rs266729, rs2241766, and rs1501299 SNPs and haplotypes may affect adiponectin levels in patients with PE classified as nonresponsive to antihypertensive therapy.

Keywords: adiponectin; ADIPOQ; antihypertensive agents; genetic polymorphisms; pharmacogenetics; preeclampsia.

Tweetable Abstract

Patients with preeclampsia (PE) classified as nonresponsive to antihypertensive therapy showed higher adiponectin levels. Genotypes of *ADIPOQ* SNPs rs17300539, rs266729, rs2241766 and rs1501299, and their haplotypes, may affect adiponectin levels in nonresponsive patients with PE.

INTRODUCTION

Preeclampsia (PE) is characterized as new-onset hypertension defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg occurring after 20 weeks of gestation on two or more occasions at least 4 hours apart and in a woman who was at baseline normotensive, which can be accompanied by proteinuria and/or thrombocytopenia, impaired liver function, pulmonary edema, new-onset headache unresponsive to all forms of management, and renal insufficiency with abnormal lab values [64, 103, 104]. PE is classified, based on the onset of the disease and diagnose, as early and late-onset preeclampsia, whether manifesting < 34 or ≥ 34 weeks of gestation, respectively [105]. PE is marked by endothelial dysfunction and can lead to multiple maternal and fetal health complications [9], such as eclampsia (seizures), HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome [106], and death in the most severe cases. In this context, antihypertensive treatment is crucial during PE management in an attempt to prolong gestation and provide adequate time for fetal development [107]. Nevertheless, almost 40% of the women with PE do not respond to the currently recommended antihypertensive therapy, being classified as a group of nonresponsive patients, which is associated with the worst clinical outcomes [5].

Although the etiology of PE is not yet fully elucidated [13], hypoxia and oxidative stress lead the placenta to release circulating antiangiogenic factors, including soluble endoglin and soluble fms-like tyrosine kinase 1 (sFlt-1), which have been proposed to cause a systemic inflammatory response and generalized maternal endothelial dysfunction [67, 108]. Notably, there is clinical evidence of decreased nitric oxide (NO) formation in PE [11, 109], and sFlt-1 is inversely related to NO bioavailability in PE [11]. Furthermore, obesity is a major risk factor for PE [14, 110], and dysregulated adipocytokine release by adipocytes may be associated with endothelial dysfunction in PE [15].

Adiponectin is an adipocyte-derived hormone expressed by the *ADIPOQ* locus that emerged as a potential cardiovascular biomarker due to its role in modulating vascular function. It has also been shown to influence insulin sensitivity and exhibit anti-inflammatory properties [111]. Furthermore, a deficiency in adiponectin may be linked to the endothelial dysfunction [112], which is a hallmark of PE [113]. Indeed, altered circulating levels of adiponectin have been observed in cardiovascular diseases [114]. Adiponectin can activate endothelial nitric oxide synthase, stimulating the production of NO in endothelial cells, and inhibit superoxide formation [111]. We had previously evaluated whether the single nucleotide polymorphisms (SNPs) rs17300539, rs266729, rs2241766, and rs1501299 of the *ADIPOQ* gene were associated

with PE [59]. However, no previous study has examined whether *ADIPOQ* SNPs are associated with responsiveness to antihypertensive therapy in PE.

In the present study, we aimed (1) to compare plasma levels of adiponectin in patients with PE classified as responsive and nonresponsive to antihypertensive therapy; (2) to examine whether *ADIPOQ* SNPs rs17300539, rs266729, rs2241766, and rs1501299, and their haplotypes, are associated with antihypertensive therapy responsiveness in PE; (3) to examine whether the genotypes and haplotypes affect plasma adiponectin levels in patients with PE classified as responsive or nonresponsive to the antihypertensive therapy. We further assessed the pairwise linkage disequilibrium among the *ADIPOQ* SNPs in each one of the groups.

PATIENTS & METHODS

Study population

The study involving human subjects was approved by the Institutional Review Board of the Ribeirao Preto Medical School of University of Sao Paulo (RPMS-USP), and each participant providing signed informed consent. Volunteers were enrolled at the Department of Obstetrics and Gynecology within the University Hospital of the RPMS-USP. The study included 215 pregnant women diagnosed with PE, which was defined as pregnancy-induced hypertension (≥140 mmHg systolic and ≥90 mmHg diastolic on two or more measurements, at least 4 h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks post-partum, and accompanied by one or more of the following criteria: proteinuria (Pr; ≥0.3 g/24 h), thrombocytopenia, impaired liver function, new-onset headache unresponsive to all forms of management, pulmonary edema, or renal insufficiency with abnormal laboratory values [64, 103]. For this study we did not include women with cardiac conditions, renal diseases, diabetes, and pre-existing hypertension, with or without superimposed PE. The 215 patients with PE were classified as responsive (n=115) and nonresponsive (n=100) to the antihypertensive therapy. The responsiveness to the antihypertensive therapy was evaluated as described below.

Maternal venous blood samples were collected. Plasma was obtained by centrifugation of tubes containing whole blood with anticoagulant and stored at -70°C until assayed. Genomic DNA was extracted from the cellular component of 1 mL of whole blood using the salting-out method, and stored at -20°C until analyzed.

Antihypertensive treatment and drug response evaluation

All the patients was under treatment and responsiveness to antihypertensive therapy was based on evaluation of clinical and laboratory parameters in response to the use of these drugs: methyldopa (1000–1500 mg per day) as the first antihypertensive drug of choice, nifedipine (40–60 mg per day) in cases of lack of significant response to methyldopa, and hydralazine (5–30 mg) in cases of hypertensive crisis. Patients were classified as non-responsive only if they did not respond to any of the therapy available. The patients were monitored with caution for signs and symptoms of PE, with fetal surveillance and laboratory tests at least once a week. The presence of at least one of the following clinical and laboratory outcomes was considered to classify the patients with PE as nonresponsive to antihypertensive therapy [50, 58, 115, 116].

- Clinical symptoms including blurred vision, persistent headache or scotomata, persistent right upper quadrant or epigastric pain;
- Systolic blood pressure (SBP) above 140 mmHg and diastolic blood pressure (DBP) above 90 mmHg, as assessed by the blood pressure curve;
- HELLP syndrome; or Pr >2.0 g per 24 h; creatinine >1.2 mg per 100 mL or blood urea nitrogen >30 mg per 100 mL; aspartate aminotransferase >70 Ul^{-1} and alanine aminotransferase >60 Ul^{-1};
- Fetal hypoactivity or nonreactive fetus, as revealed by cardiotocography; intrauterine growth restriction (IUGR), oligoamnio, abnormal biophysical profile score and Doppler velocimetry abnormalities, as evaluated by ultrasound.

Genotype determination

Genotypes for ADIPOQ SNPs were determined using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA): -11391G>A (rs17300539; c_33187774_10) and -11377C>G (rs266729; c_2412786_10) in the promoter region; 45T>G (rs2241766; c_26426077_10) in exon 2; and 276G>T (rs1501299; c_7497299_10) in intron 2. Genotyping was performed using a Real-Time System 7500 from Applied Biosystems, Foster City, CA, USA, as previously described [59].

Enzyme immunoassay for adiponectin measurement

Plasma adiponectin concentrations were determined with the Human Adiponectin ELISA kit (Human Adiponectin EZHADP-61K – Millipore, St. Charles, MO), following the instructions provided by the manufacturer, as previously described [26].

Statistical analysis

Statistical analysis was carried out using GraphPrism 5.0. The clinical characteristics and adiponectin levels of responsive and nonresponsive women were compared by Student's unpaired t-test, Mann-Whitney U test, or $\chi 2$ as appropriate and reported as mean ± s.e.m. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium (HWE), and differences in genotype and allele frequencies between groups were assessed using $\chi 2$ test or Fisher's exact test. A value of $P<0.05$ was considered significant. The effects of genotypes and haplotypes on circulating adiponectin levels were compared by analysis of variance followed by student's unpaired *t*-test, Mann-Whitney U test and also Tukey test (normally distributed variables) or Kruskall–Walli's test followed by Dunn's Multiple Comparison test (not normally distributed variables).

Haplotype frequencies were estimated by using Haplo.stats package version 1.9.3 (http://cran.r-project.org/web/packages/haplo.stats/index.html)[79]. Only haplotypes combining the alleles of the four *ADIPOQ* polymorphisms rs17300539 (G>A), rs266729 (C>G), rs2241766 (T>G), and rs1501299 (G>T) that showed frequencies higher than 5% were considered in the haplotype analysis, namely: 'GCTG', 'GCTT', 'GGTG', and 'GCGG'. Differences in haplotype frequencies were tested using function haplo.cc in the Haplo.stats, which computes haplotype specific P-values, Odds Ratios, and 95% Confidence intervals. Linkage disequilibrium (LD) was assessed by calculating D′ and logarithm of odds (LOD') using Haploview software (version 4.2; http://www.broad.mit.edu/mpg/haploview/)[80].

RESULTS

The clinical and demographic characteristics of PE patients classified as responsive or nonresponsive to antihypertensive therapy are shown **Table 1**. Age, ethnicity (% white), % of current smokers, heart rate, fasting glucose, hemoglobin, hematocrit, and % of primiparity were similar between the groups (all $P>0.05$). Nonresponsive PE patients showed higher SBP and DBP, creatinine, and 24 h proteinuria than responsive PE patients (all $P>0.05$). Noteworthy, early-onset PE, preterm birth, and IUGR were more frequent in nonresponsive PE patients (all $P<0.05$). Moreover, nonresponsive PE patients showed lower body mass index (BMI) before and during pregnancy, newborn weight, and gestational age at delivery and at blood sampling (all $P>0.05$).

Due to the nonavailability of plasma samples, we could not determine adiponectin levels for all PE subjects enrolled in this study. Nonetheless, we were able to measure plasma adiponectin concentrations in 27 responsive patients and 26 nonresponsive patients (**Figure 1**).

We found higher adiponectin levels in nonresponsive PE patients when compared to responsive patients (P<0.05; **Figure 1**).

Although we were not able to access BMI values before pregnancy for all of our patients, since adiponectin is an adipocyte-derived cytokine, we examined plasma adiponectin concentration in responsive (n = 22) and nonresponsive (n = 21) patients with pre-pregnancy BMI lower than 30 kg/m^2, and also found higher adiponectin levels in nonresponsive PE patients compared to the responsive counterpart (P<0.05; **Supplementary Figure 1**). In this context, we also performed a correlation analysis between systolic/diastolic blood pressure and adiponectin levels in the responsive and nonresponsive patient's group, however we found no association (**Supplementary Figure 2).**

The distribution of genotypes for each *ADIPOQ* SNP studied in healthy pregnant and in PE patients showed no deviation from Hardy-Weinberg equilibrium, except for the rs266729 SNP in the group of PE patients (data not shown; P<0.05). However, this finding may be expected in cases of case-control association studies [117]. Noteworthy, we have not found deviation for this rs266729 SNP in the healthy pregnant (control) group, as previously reported [59]. Genotype, allele, and haplotype frequencies in the responsive and non-responsive PE groups are shown in **Tables 2 and 3**. We found that the TG genotype of the SNP rs2241766 was more frequent in the responsive group when compared to the nonresponsive group of patients with PE (P<0.05; **Table 2**), when considering a codominant distribution of the T and G alleles. However, we found no significant differences in the distribution of *ADIPOQ* haplotypes between the groups (P>0.05; **Table 3**).

Next, we examined whether *ADIPOQ* genotypes modulate circulating adiponectin concentrations in responsive and nonresponsive PE patients. We found significant effects of *ADIPOQ* genotypes on adiponectin concentrations for all SNPs, as shown in **Figure 2.** Nonresponsive PE patients carrying both GG and GA+AA genotypes of the rs17300539 G>A SNP showed higher adiponectin levels than responsive PE patients carrying the same genotypes (both P<0.05; **Figure 2A**). Moreover, nonresponsive PE patients carrying CC, TT or GT+TT genotypes of the rs266729 C>G, rs2241766 T>G, and rs1501299 G>T polymorphisms, respectively, showed higher adiponectin levels when compared with responsive PE patients carrying the same genotypes (all P<0.05; **Figure 2B, 2C, and 2D,** respectively). Further, we examined the effects of *ADIPOQ* haplotypes on plasma adiponectin concentrations. Nonresponsive PE patients carrying the 'G,C,T,G' and 'G,C,T,T' haplotypes showed higher

plasma adiponectin levels than responsive PE patients with the same haplotypes (both $P<0.05$; **Figure 3**).

Finally, we assessed the pairwise LD among the rs17300539 (G>A), rs266729 (C>G), rs2241766 (T>G), and rs1501299 (G>T) SNPs in nonresponsive and responsive PE patients (**Figure 4A and 4B**). A short segment of higher LD between SNPs rs2241766 and rs1501299 was found in nonresponsive PE patients (D' = 1 and LOD' ≥2) when compared to responsive PE patients (D' = 1 and LOD' <2; **Figure 4A-B**). In addition, a lower LD was found between rs17300539 and rs266729 in responsive PE patients (D' <1 and LOD' <2) when compared to nonresponsive counterparts (D' = 1 and LOD' <2; **Figure 4A-B)**. The other SNPs showed similar LD among each other when compared between these two groups.

DISCUSSION

The main novel findings of this study were: (1) nonresponsive PE patients presented higher plasma adiponectin levels than responsive PE patients; (2) the TG genotype of the rs2241766 *ADIPOQ* SNP was found to be more frequent in responsive patients when compared to nonresponsive patients, however, the distribution of haplotypes formed by the SNPs rs17300539, rs266729, rs2241766 and rs1501299 was not associated with responsiveness in PE; and (3) nonresponsive PE patients carrying the GG or GA+AA genotypes of the rs17300539 polymorphism, and CC, TT, and GT+TT genotypes of the rs266729, rs2241766, and rs1501299 SNPs, respectively, as well as those carrying the 'G,C,T,G' and 'G,C,T,T' haplotypes showed higher plasma adiponectin levels than responsive PE patients carrying the same genotypes/haplotypes.

Adiponectin is an adipocyte-derived collagen-like protein with important metabolic and anti-inflammatory effects that circulates in human plasma at high concentrations [84, 85]. In normal pregnancy, circulating adiponectin levels decrease over time and, due to its insulin-sensitizing effect, it would be expected to find decreased levels in PE patients [87]. However, discordant results have been reported regarding maternal circulating adiponectin levels in patients with PE. Some previous studies evaluating plasma samples of healthy pregnant women and patients with PE, with similar BMI, showed increased adiponectin levels in PE [26, 118-120], but others have found lower levels in PE when compared to healthy pregnant women [121, 122]. Studies evaluating serum samples are also controversial, including findings of increased levels in severe PE patients when compared to mild PE patients, with no significant difference for BMI among the groups (all >25) [123], and higher levels in PE patients than in

healthy pregnant women [123, 124], but a significant decrease of this adiponectin levels in overweight PE patients compared with normal weight PE patients [123, 124]; however, others have also shown decreased levels in PE [90, 125, 126], and a study reported no differences in adiponectin levels between PE and healthy pregnant women [85]. Nevertheless, no previous study has examined adiponectin levels in PE patients classified as responsive or nonresponsive to the antihypertensive therapy. In this study, we observed that nonresponsive PE patients presented higher adiponectin levels than responsive PE patients.

Different races, lifestyle, and behaviors of included population as well as distinct diagnostic criteria of PE may explain conflicting results among studies. Moreover, there are three isoforms of adiponectin, low-, medium-, and high-molecular weight, which are active [85]. As such, differences in adiponectin levels might be confined in one or more of these isoforms. Furthermore, adiponectin has an important regulatory function on the integrity of the vasculature system, with both anti-atherogenic and angiogenic effects [88]. Total and HMW adiponectin have been reported as the most biologically active isoform, and high concentrations of these isoforms of adiponectin are associated with cardiovascular death and heart failure in general population and diabetics, while low concentrations have been associated with obesity, metabolic syndrome, insulin sensitivity and type 2 diabetes, as revised elsewhere [127]. In this context, overweight/obese normal pregnant women were previously associated with decreased circulating adiponectin multimers, but not preeclamptic women, which suggests altered regulation of adiponectin in PE [88, 128].

A prior study demonstrated a positive correlation between circulating adiponectin and soluble endoglin in PE, but not in HP [129]. Additionally, circulating adiponectin has been correlated positively with sFlt-1 and negatively with placental growth factor (PlGF) in PE [130]. Curiously, overweight PE patients exhibited decreased circulating adiponectin levels compared to normal weight PE patients [44]. Our earlier study ratified these findings by reporting a positive correlation of adiponectin with sFlt-1 and soluble endoglin only in PE, while a negative correlation between adiponectin and BMI was observed exclusively in HP [26]. Moreover, although higher plasma adiponectin and lower plasma nitrite (a marker of NO formation) levels were found in PE when compared to HP, we observed a positive correlation between these markers only in HP [29]. Taken together, these studies suggest that increased levels of adiponectin in PE may be due to a compensatory effect in response to defects in angiogenesis and vascular injury [131]. However, further studies are needed in order to clarify these different findings.

Antihypertensive drug therapy reduces risk of severe hypertension during PE [116], and its major goal is to prevent cardio- and cerebrovascular consequences caused by severe hypertension, as well as prolong gestation improving both maternal and fetal outcomes [132]. However, a large number of patients do not respond to the antihypertensive therapy in PE [50, 115], presenting higher SPB and DBP even after receiving intense antihypertensive therapy. In this context, pharmacogenomics research may prove to be an important tool to improve antihypertensive therapy for these patients with PE classified as nonresponsive [5, 133]. Interesting, PE patients classified as nonresponsive to antihypertensive therapy were associated to worse clinical parameters when compared to responsive patients. Indeed, many clinical findings used to define antihypertensive responsiveness are shared with the definition of severe PE, like elevated levels of creatinine and liver enzymes, thrombocytopenia, persistent right upper quadrant, epigastric pain, and new-onset visual and cerebral disturbances [103]. Consequently, antihypertensive drug responsiveness could possibly be associated to the disease severity [5]. Nonetheless, findings from the present study suggest a role of adiponectin in the pathophysiology of PE, especially in nonresponsive PE patients and perhaps more so in patients with severe PE.

To our knowledge, no previous study has examined whether *ADIPOQ* SNP (rs17300539, rs266729, rs2241766, rs1501299) genotypes or the haplotypes formed by them are associated with responsiveness to antihypertensive treatment in PE. Although we did not find association between *ADIPOQ* SNP haplotypes and the responsiveness to antihypertensive therapy, our analyses showed that the TG genotype of the rs2241766 SNP was more frequent in responsive PE patients when compared to nonresponsive PE patients. In this context, the association between the SNPs rs2241766 and rs1501229 and some clinical parameters observed was previously evaluated in PE [91] SBP and proteinuria were lower in patients with severe PE carrying the TT genotype of the SNP rs2241766 when compared to those carrying the TG+GG genotype. In addition, blood pressure and proteinuria were lower in severe PE patients carrying the GG genotype of the rs1501299 SNP when compared to those carrying the TG+TT genotype [91]. The T allele of the rs2241766 T>G polymorphism has also been associated with low birth weight in patients with PE [134]. Moreover, the T allele of rs1501299 G>T polymorphism was more frequent in HP when compared to PE, and the TT genotype was associated to protection against PE in comparison to the GT and GG genotypes. About the haplotypes formed by those two SNPs, the ‘T, T’ haplotype was more frequent in HP than in PE [92].

We also investigated the effects of these *ADIPOQ* SNP genotypes and haplotypes on plasma adiponectin levels in the responsive and nonresponsive PE groups. Regarding the effects of the genotypes, nonresponsive patients carrying GG or GA+AA, CC, and TT and GT+TT genotypes of rs17300539, rs266729, rs2241766, and rs1501299 polymorphisms, respectively, showed higher adiponectin levels than responsive PE patients carrying same genotypes. Along these same lines, serum adiponectin levels were increased in severe PE patients carrying the GT+TT genotype of the rs1501299 SNP when compared to those carrying the GG genotype [49]. With regard to the effects of the haplotypes, nonresponsive PE patients carrying 'G, C, T, G' or 'G, C, T, T' haplotypes showed increased adiponectin levels when compared to responsive PE patients carrying the same haplotypes. Although we have not found differences within the same group, one reason that could explain the observed differences between the groups may be the fact that nonresponsive PE patients showed higher adiponectin levels overall.

Concerning to the fact that PE patients nonresponsive to the antihypertensive therapy could possibly be associated to the disease severity [5], previous studies have suggested that adiponectin has a role in the pathophysiology of resistant hypertension, a multifactorial disease defined as uncontrolled blood pressure despite the use of ≥3 antihypertensive drugs or controlled blood pressure after use of ≥4 drugs [135]. Moreover, resistant hypertension patients carrying the CC genotype of the rs266729 C>G SNP showed higher adiponectin concentration when compared to patients carrying the G allele [135]. Regarding the rs1501299 G>T SNP, patients carrying the GG genotype presented lower adiponectin levels compared to T allele carries [135]. Furthermore, the GG and CG+GG genotypes of rs266729 C>G SNP were associated with uncontrolled hypertension, and the CG genotype was associated with higher SBP [136]. The haplotypes 'G, T' and 'G, G' formed by those two SNPs were also associated to uncontrolled hypertension, with the 'G, T' being additionally associated with higher DBP [136]. Taken together, these findings demonstrate that *ADIPOQ* polymorphisms may modulate circulating adiponectin levels and have been associated with cardiovascular disease, supporting the relevance of examining the proposed SNPs in genetic association studies in PE.

Regarding the pairwise LD among the rs17300539, rs266729, rs2241766 and rs1501299 SNPs in our study groups, we found a short segment with higher pairwise LD between the exonic rs2241766 and intronic rs1501299 when compared to the other *ADIPOQ* SNPs. This evidence suggests the existence of a possible positive selective pressure for this intronic region on the *ADIPOQ* gene [137]. Although we did not examine other potential functional *ADIPOQ* SNPs that could affect circulating adiponectin levels, these SNPs could be interesting

candidates for future studies validating the role of *ADIPOQ* polymorphisms in the modulation of *ADIPOQ* expression and the susceptibility for nonresponse to antihypertensive therapy in different populations of pregnant women with PE.

The present study has limitations. First, we could only measure adiponectin levels in a small number of patients. Despite this, we found a significant difference in plasma adiponectin levels between the responsive and nonresponsive PE groups, and we also found differences in adiponectin concentrations when comparing the groups according to the different genotypes and haplotypes for the *ADIPOQ* SNPs. Second, we have not measured placental or tissue adiponectin levels, neither determined the molecular weight of adiponectin (i.e. low, medium, and high), rather we considered the total plasma adiponectin concentration. Finally, we have not measured when adiponectin levels start to increase in nonresponsive versus responsive PE patients, and future longitudinal studies are needed in order to investigate whether the increase in adiponectin levels could either be compensatory or contribute to the development of PE as well as to the lack of response to antihypertensive therapy in PE.

CONCLUSIONS

In conclusion, patients with PE classified as nonresponsive to antihypertensive therapy showed higher plasma adiponectin levels when compared to the responsive group of PE patients. Nonresponsive PE patients carrying the GG or GA+AA genotypes of the rs17300539 SNP, and the CC, TT, and GT+TT genotypes of the rs266729, rs2241766, and rs1501299 SNPs, respectively. as well as the ‘G,C,T,G’ and ‘G,C,T,T’ haplotypes showed higher plasma adiponectin levels than responsive PE patients carrying the same genotypes/haplotypes. Moreover, we found that the TG genotype of the rs2241766 SNP was more frequent in the responsive group when compared to the nonresponsive group. Our novel findings suggest that *ADIPOQ* genotypes and haplotypes affect circulating adiponectin levels in patients with PE classified as nonresponsive to the antihypertensive therapy.

ARTICLE HIGHLIGHTS

- First study that examined whether *ADIPOQ* SNPs and haplotypes are associated to responsiveness to antihypertensive therapy in preeclampsia (PE).
- Patients with PE classified as nonresponsive to antihypertensive therapy showed higher adiponectin plasma levels when compared to the responsive PE patients.

- Patients with PE classified as nonresponsive to antihypertensive therapy showed higher plasma adiponectin levels when compared to the responsive group of PE patients.
- The TG genotype of the *ADIPOQ* SNP rs2241766 was more frequent in PE patients classified as responsive to antihypertensive therapy.
- Nonresponsive PE patients carrying the GG or GA+AA genotypes of the rs17300539 SNP, and the CC, TT, and GT+TT genotypes of the rs266729, rs2241766, and rs1501299 SNPs, respectively. as well as the 'G,C,T,G' and 'G,C,T,T' haplotypes showed higher plasma adiponectin levels than responsive PE patients carrying the same genotypes/haplotypes.
- Our novel findings suggest that *ADIPOQ* genotypes and haplotypes affect circulating adiponectin levels in patients with PE classified as nonresponsive to the antihypertensive therapy.

AUTHOR CONTRIBUTIONS

DAP, RNFC, ACP, RCC, VCS, and MRL have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; DAP, RNFC, ACP, RCC, VCS, and MRL have drafted the work or revised it critically for important intellectual content, and all authors have also approved the final version of the work to be published; All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FIGURES AND TABLES

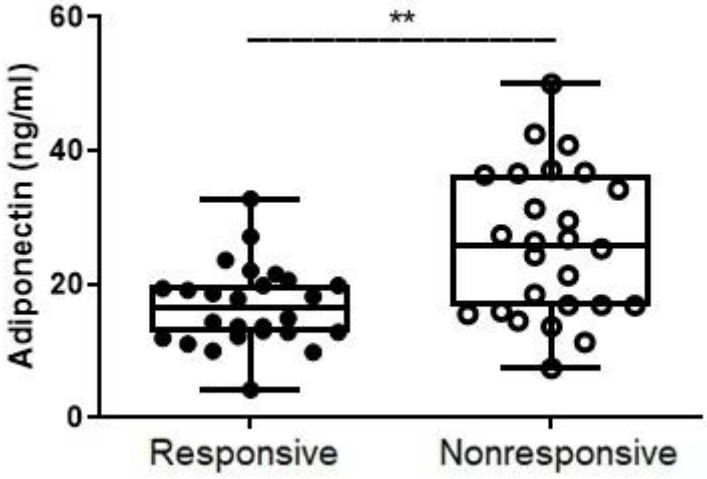


Figure 1. Adiponectin concentrations in plasma from patients with preeclampsia classified as responsive (N=27) and nonresponsive (N=26) to the antihypertensive therapy. Unpaired t-test presented as a Box plot showing the median and min to max values.

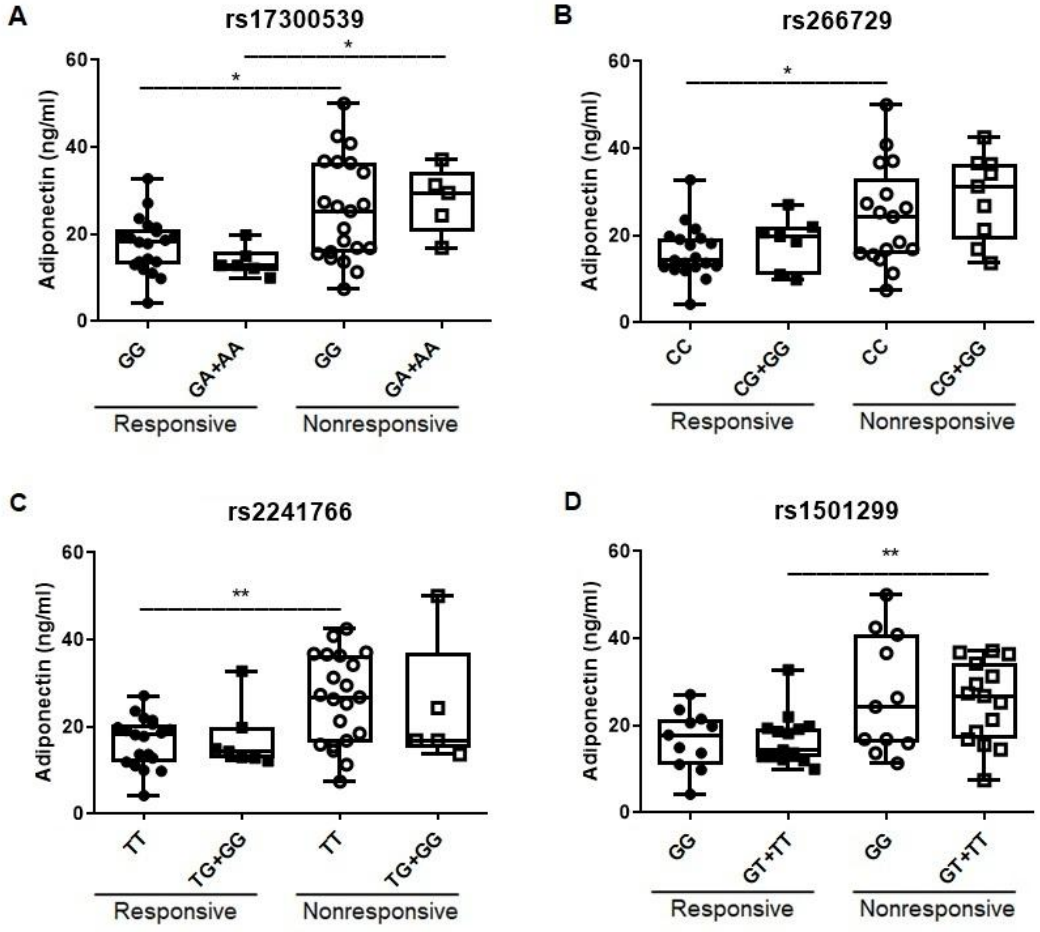


Figure 2. Adiponectin concentrations in plasma from patients with preeclampsia classified as responsive (N=27) and nonresponsive (N=26) to antihypertensive therapy grouped according to their genotypes for the SNPs *of ADIPOQ* gene **(A)** rs17300539 (G>A), **(B)** rs266729 (C>G), **(C)** rs2241766 (T>G), and **(D)** rs1501299 (G>T). Mann-Whitney U test presented as a Box plot showing the median and min to max values.

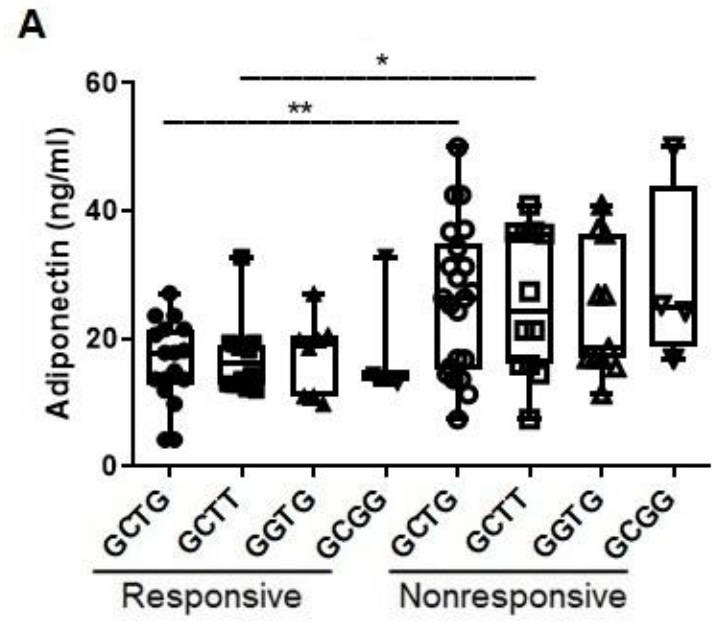


Figure 3. Adiponectin concentrations in plasma from patients with preeclampsia classified as responsive (N=27) and nonresponsive (N=26) to antihypertensive therapy grouped according to their haplotypes formed by the combination of alleles for the *ADIPOQ* SNPs rs17300539 (G>A), rs266729 (C>G), rs2241766 (T>G) and rs1501299 (G>T). One-way ANOVA and Mann-Whitney U test presented as a Box plot showing the median and min to max values.

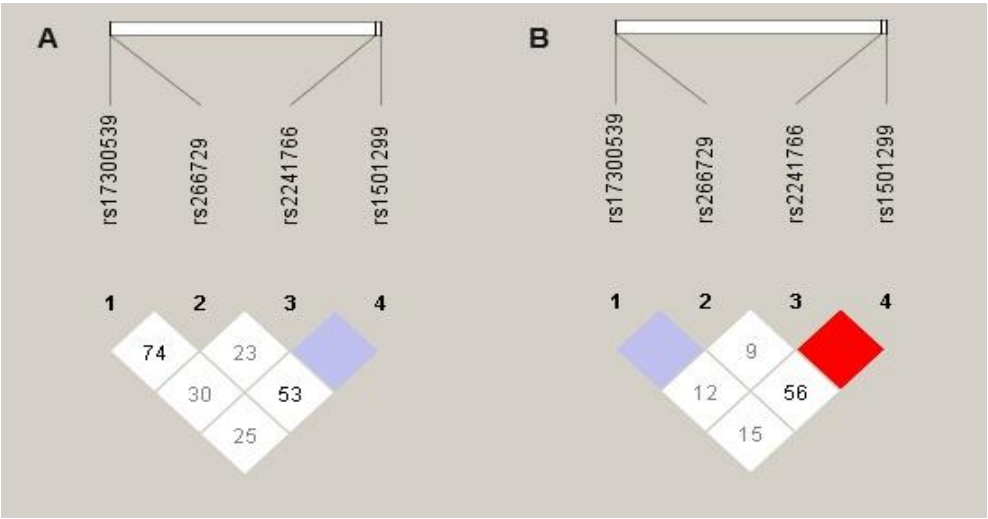


Figure 4. Pairwise linkage disequilibrium (LD) between the SNPs rs17300539, rs266729, rs2241766 and rs1501299 in *ADIPOQ* gene in patients with preeclampsia classified as (A)

responsive, and (B) nonresponsive to the antihypertensive therapy. Intensity of LD is reflected in each box by color and numeric value. The number is disequilibrium value (D′) (for example, 74 means D′ = 0.74). Color scheme: bright red, D' = 1 and (LOD)' ≥2; shades of pink/red, D' <1 and LOD' ≥2; blue, D' = 1 and LOD' <2; and white, D' <1 and LOD' <2. LOD': Logarithm of Odds.

Table 1. Clinical and demographic characteristics of patients with preeclampsia classified as responsive and nonresponsive to the antihypertensive therapy.

Parameters	Responsive n = 115	Nonresponsive n = 100	*P*
Patient-related factors			
Age (years)	26.5 ± 0.6	26.7 ± 0.7	0.989
Ethnicity (% White)	71.3	69.0	0.915
Current smokers (%)	12.2	5.0	0.099
BMI (Kg m–2) during pregnancy	33.9 ± 1	30.5 ± 1	**0.000**
BMI (Kg m–2) before pregnancy	29.5 ± 0.9	24.8 ± 0.8	**0.000**
SBP (mmHg)	132.2 ± 1.7	149.6 ± 1.9	**0.000**
DBP (mmHg)	83.2 ± 1.0	93.7 ± 1.2	**0.000**
HR (beats per min)	82.8 ± 0.7	82.7 ± 1.1	0.558
Fasting glucose (mg/dL)	82.6 ± 2.2	88.9 ± 3.3	0.441
Hb (mg/dL)	11.9 ± 0.1	11.9 ± 0.2	0.641
Hct (%)	35.9 ± 0.3	36.0 ± 0.5	0.645
Creatinine (mg/dL)	0.68 ± 0.0	0.75 ± 0.0	**0.000**
24h Pr (mg per 24h)	1106 ± 219.8	1758 ± 234.6	**0.015**
Pregnancy-related factors			
Primiparity (%)	42.1	48.0	0.624
GAD (weeks)	38.1 ± 0.2	33.7 ± 0.5	**0.000**
Newborn weight (g)	2998 ± 67.8	2007 ± 90.12	**0.000**

	Responsive	Nonresponsive	*P*
GAS (weeks)	35.70 ± 0.4	32.60 ± 0.5	**0.000**
Early-onset PE (%)	6.96	47.47	**0.000**
Preterm birth (%)	12.17	62.63	**0.000**
IUGR (%)	14.78	50.50	**0.000**

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GAD, gestational age at delivery; GAS, gestational age at sampling; Hb, hemoglobin concentration; Hct, hematocrit; HR, heart rate; IUGR, intrauterine growth restriction; PE, preeclampsia; SBP, systolic blood pressure; 24-h Pr, 24-h proteinuria. Values are the mean±s.e.m.

P responsive vs. nonresponsive. Significant *p* values are in bold.

Table 2. *ADIPOQ* genotype and allele frequencies in patients with preeclampsia responsive and nonresponsive to the antihypertensive therapy

	Genotype or allele	Responsive	Nonresponsive	OR (95% CI)	*P*
rs17300539 G>A		**(n = 78)**	**(n = 74)**		
Codominant	GG	66 (84.6%)	65 (87.8%)	1.000 (Reference)	-
	GA	9 (11.5%)	9 (12.2%)	1.015 (0.379-2.721)	1.000
	AA	3 (3.9%)	0 (0%)	0.145 (0.007-2.866)	0.245
Dominant	GG	66 (84.6%)	65 (87.8%)	1.000 (Reference)	-
	GA+AA	12 (15.4%)	9 (12.2%)	0.762 (0.301-1.930)	0.642
Alleles	G	141 (90.4%)	139 (93.9%)	1.000 (Reference)	-
	A	15 (9.6%)	9 (6.1%)	0.609 (0.258-1.437)	0.292
rs266729 C>G		**(n = 77)**	**(n = 70)**		
Codominant	CC	46 (59.7%)	47 (67.1%)	1.000 (Reference)	-
	CG	18 (23.4%)	17 (24.3%)	0.924 (0.425-2.012)	1.000
	GG	13 (16.9%)	6 (8.6%)	0.452 (0.158-1.290)	0.207
Dominant	CC	46 (59.7%)	47 (67.1%)	1.000 (Reference)	-
	CG+GG	31 (40.3%)	23 (32.9%)	0.726 (0.3700-1.427)	0.394
Alleles	C	110 (71.4%)	111 (79.3%)	1.000 (Reference)	-
	G	44 (28.6%)	29 (20.7%)	0.653 (0.381-1.119)	0.138

rs2241766 T>G		**(n = 73)**	**(n = 70)**		
Codominant	TT	54 (74%)	59 (84.3%)	1.000 (Reference)	-
	TG	18 (24.7%)	7 (10%)	0.356 (0.138-0.919)	**0.045**
	GG	1 (1.3%)	4 (5.7%)	3.661 (0.397-33.80)	0.370
Dominant	TT	54 (74%)	59 (84.3%)	1.000 (Reference)	-
	TG+GG	19 (26%)	11 (15.7%)	0.530 (0.231-1.215)	0.153
Alleles	T	126 (86.3%)	125 (89.3%)	1.000 (Reference)	-
	G	20 (13.7%)	15 (10.7%)	0.756 (0.370-1.544)	0.475
rs1501299 G>T		**(n = 77)**	**(n = 70)**		
Codominant	GG	40 (51.9%)	34 (48.6%)	1.000 (Reference)	-
	GT	29 (37.7%)	29 (41.4%)	1.176 (0.591-2.342)	0.726
	TT	8 (10.4%)	7 (10%)	1.029 (0.338-3.133)	1.000
Dominant	GG	40 (51.9%)	34 (48.6%)	1.000 (Reference)	-
	GT+TT	37 (48.1%)	36 (51.4%)	1.145 (0.599-2.188)	0.742
Alleles	G	109 (70.8%)	97 (69.3%)	1.000 (Reference)	-
	T	45 (29.2%)	43 (30.7%)	1.074 (0.656-1.770)	0.800

Abbreviations: CI, confidence interval; *ADIPOQ*, Adiponectin; OR, odds ratio.

P responsive vs. nonresponsive. Significant *p* values are in bold.

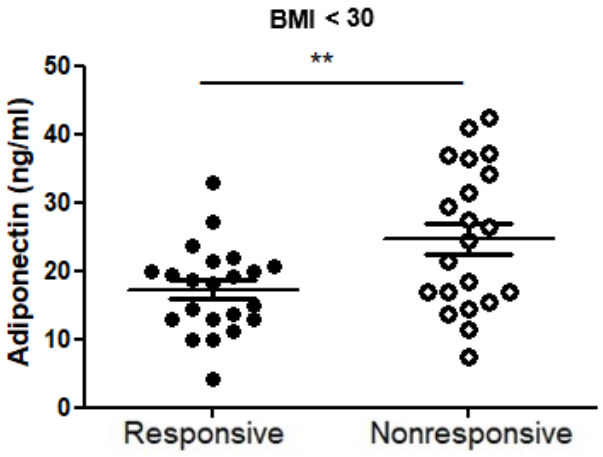
Table 3. *ADIPOQ* haplotype frequencies in patients with preeclampsia responsive and nonresponsive to the antihypertensive therapy

Haplotype	**Responsive** (n = 65)	**Nonresponsive** (n = 62)	***P*-values**	**OR (95% CI)**
GCTG	0.305	0.426	0.074	1.000 (Reference)
GCTT	0.243	0.237	0.958	0.743 (0.390-1.415)
GGTG	0.207	0.164	0.490	0.639 (0.319-1.282)
GCGG	0.083	0.066	0.622	0.597 (0.219- 1.624)
		global-stat = 4.456, df = 8, p = 0.814		

Abbreviations: CI, confidence intervals; *ADIPOQ*, Adiponectin; OR, odds ratio; RF, relative frequency; Global-stat, global score statistics.

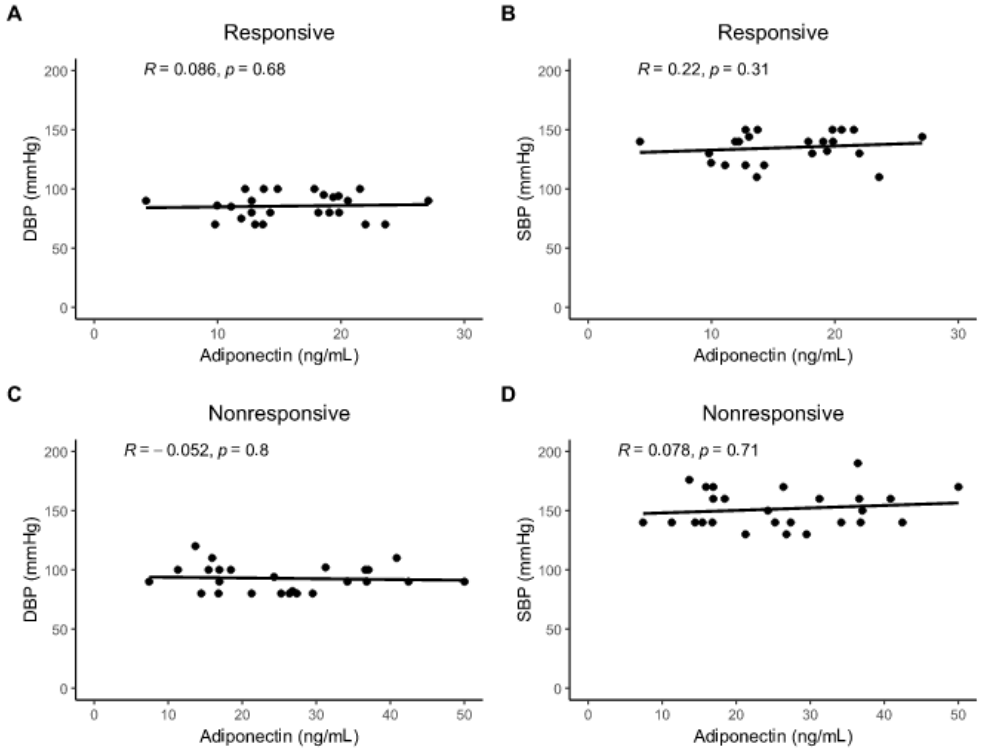
P responsive vs. nonresponsive.

SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Adiponectin concentrations in plasma from patients with preeclampsia classified as responsive (N=22) and nonresponsive (N=21) to the antihypertensive therapy categorized according to pre-pregnancy BMI lower than 30 kg/m^2. The bars show the mean ± s.e.m. ***P*<0.01.

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. *Correlation analysis between Diastolic Blood Pressure (DBP) (A & C) or Systolic Blood Pressure (SBP) (B & D) and plasma adiponectin levels in patients with preeclampsia classified as responsive and nonresponsive to antihypertensive therapy.*

The regression lines are plotted. The R- and p-values are reported. R: Spearman's coefficient.

4.3. Segunda linha de pesquisa do projeto;

Título proposto: “Differently Expressed Genes Regulated By Metformin In An *In Vitro* Model Of Preeclampsia”

Neste estudo, nosso objetivo foi identificar genes relacionados à disfunção endotelial regulados pela metformina em células endoteliais da veia umbilical humana (HUVECs) incubadas com plasma de gestantes com PE e tratadas com metformina. Assim como identificar vias de sinalização envolvidas na regulação desses genes diferencialmente expressos. A partir dessas análises, identificamos que a metformina é capaz de regular genes relacionados à disfunção endotelial e também à inflamação na PE. Com isso, nossa pesquisa pode auxiliar na compreensão dos mecanismos moleculares relacionados aos efeitos da metformina na PE e na identificação de potenciais novos biomarcadores do uso e da resposta à metformina.

4.3.1. Resultados

Concentrações do cloridrato de metformina foram avaliadas quanto a citotoxicidade antes do início dos experimentos. Foram realizados ensaios de viabilidade e citotoxicidade, conforme descritos a seguir.

O ensaio de viabilidade celular foi realizado utilizando o PrestoBlue® Cell Viability Reagent (Invitrogen) segundo as instruções do fabricante. Os procedimentos foram realizados em placas de 96 poços incluindo 5 réplicas de cada um dos grupos, que eram, células endoteliais (controle sem tratamento), células endoteliais incubadas com cloridrato de metformina a 0,05µm, 0,5µM, 1µM, 500µM, 2500µM ou 10000 µM, células endoteliais incubadas com pool de plasma de gestantes com PE, modelo *in vitro* de PE (controle) e modelo *in vitro* de PE incubada com cada uma das concentrações de cloridrato de metformina descritas anteriormente **(Figura 1)**. As concentrações escolhidas para esses testes foram previamente selecionadas com base na literatura[35, 38]. O PrestoBlue® consiste em um composto baseado em resazurina, que na presença de células metabolicamente ativas, é convertido na forma reduzida à resorufina por enzimas mitocondriais, resultando em modificações na fluorescência. Para realizar o ensaio, foram descartados 110 µl do sobrenadante e em seguida adicionado 10 µL do reagente PrestoBlue®, seguido de incubação por 1 hora na estufa. A viabilidade celular foi quantificada por fluorescência em leitor.

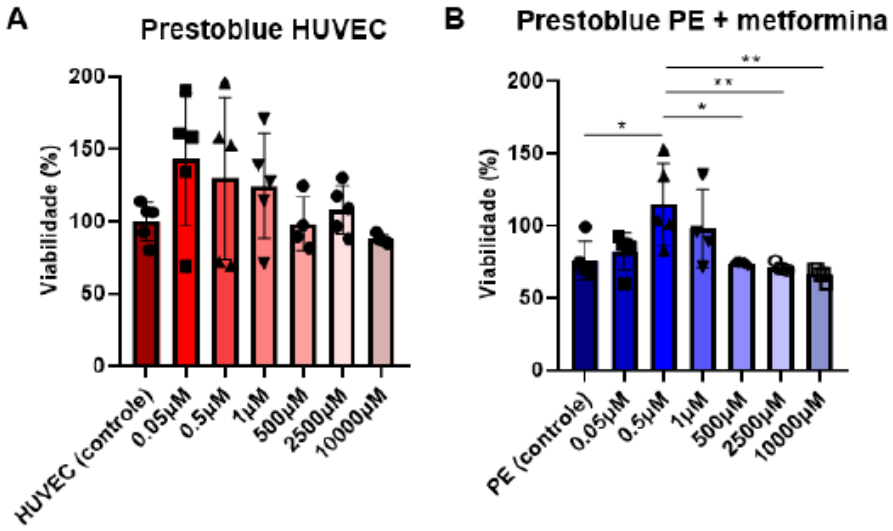


Figura 1: Ensaio de Prestoblue para determinação da viabilidade das células endoteliais. **(A)** Células endoteliais incubadas com as diferentes concentrações de metformina. **(B)** Modelo *in vitro* de PE incubado com metformina. * Indicam diferenças entre os grupos PE vs 0.05µM e 0.05µM vs 500µM, 2500µM e 10000µM. Os resultados estão apresentados como média + desvio padrão da média. (*p≤0,05; **p≤0,01 - One-Way ANOVA seguido do pós-teste de Bonferroni).

A citotoxicidade foi determinada medindo a atividade da lactato desidrogenase (LDH) no meio de cultura (Sigma-Aldrich), também seguindo as instruções do fabricante. A LDH é uma enzima intracitoplasmática que é somente liberada se a membrana plasmática estiver danificada. Uma vez o LDH no meio extracelular, este pode ser quantificado em uma reação de dois passos através da sua atividade enzimática. Primeiramente, o LDH catalisa a redução da nicotinamida adenosina dinucleotídeo (NAD+) a fosfato de dinucleótido de nicotinamida e adenina (NADPH) juntamente com a oxidação do lactato a piruvato. Em seguida, a diaforese, como uma enzima de acoplamento, usa o NADPH para catalisar a redução do sal tetrazolim (INT) a um produto de tonalidade avermelhada, o formazam, que pode ser detectado a 490 nm. Então a quantidade de formazan produzido é proporcional a quantidade de LDH liberado no meio, que é indicativo de citotoxicidade, como resultado apresentado na **Figura 2**. Foi coletado 30 µL dos sobrenadantes de cultura de cada uma das réplicas dos grupos de pesquisa conforme descrito no tópico de viabilidade e adicionado em uma nova placa de 96 poços. Logo após, foi adicionado à placa 30 µL da mistura de reação do kit PierceTM LDH Cytotoxicity Assay Kit (Thermo Fisher, Massachusetts, EUA) e incubado a temperatura ambiente, protegido da luz, durante 30 minutos. No fim, a reação foi parada com a adição de 30 µL da solução stop, e a leitura foi realizada imediatamente entre 490 nm e 680nm no leitor de placas Synergy 4 (Biotek/Agilent, Santa Clara, EUA).

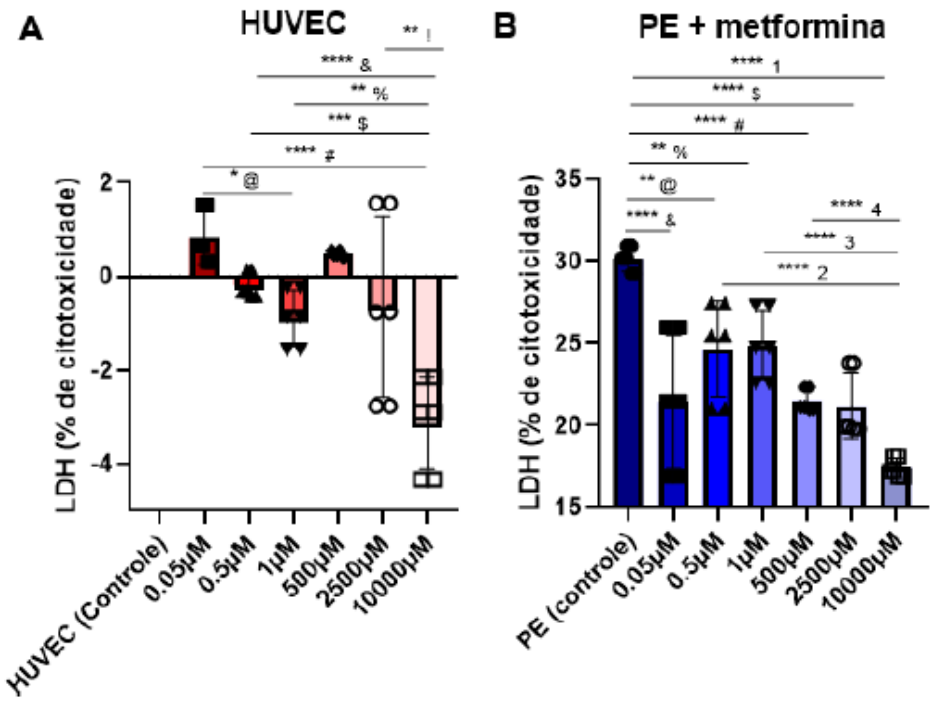


Figura 2: Ensaio de Lactato desidrogenase (LDH) para determinação da citotoxicidade das células endoteliais. (A) Células endoteliais incubadas com as diferentes concentrações de metformina. * Indicam diferenças entre os grupos. @ 0.05µM vs 1µM; # 0.05µM vs 10000µM; $ 0.5µM vs 10000µM; % 1µM vs 10000µM; & 0.5µM vs 10000µM e ! 2500µM vs 10000µM.

(B) Modelo *in vitro* de PE incubado com metformina. * Indicam diferenças entre os grupos. & Controle vs 0.05μM; @ Controle vs 0.5μM; % Controle vs 1μM; # Controle vs 500μM; $ Controle vs 2500μM; *1* Controle vs 10000μM; *2* 0.5μM vs 1000 μM; *3* 1μM vs 10000μM e *4* 500μM vs 10000μM. Os resultados estão apresentados como média + desvio padrão da média. (*p≤0,05; **p≤0,01; ***p≤0.001; p≤0.0001**** - One-Way ANOVA seguido do pós-teste de Bonferroni).

Os resultados, apesar de mostrarem diferença estatisticamente significativa entre os grupos, também mostraram que nenhuma das concentrações de metformina foi significativamente citotóxica para as células e poderiam ser utilizadas em experimentos futuros. O ensaio de prestoblue mostrou que células incubadas com metformina na concentração de 0.5μM apresentou uma maior porcentagem de células viáveis quando comparado ao controle.

Após os ensaios de viabilidade e citotocixidade foram realizados os ensaios de PCR em tempo real. Os genes apresentados na **Tabela 1** representam os 19 genes analisados, 2 genes de referência e 17 genes-alvo relacionados a fisiopatologia da PE.

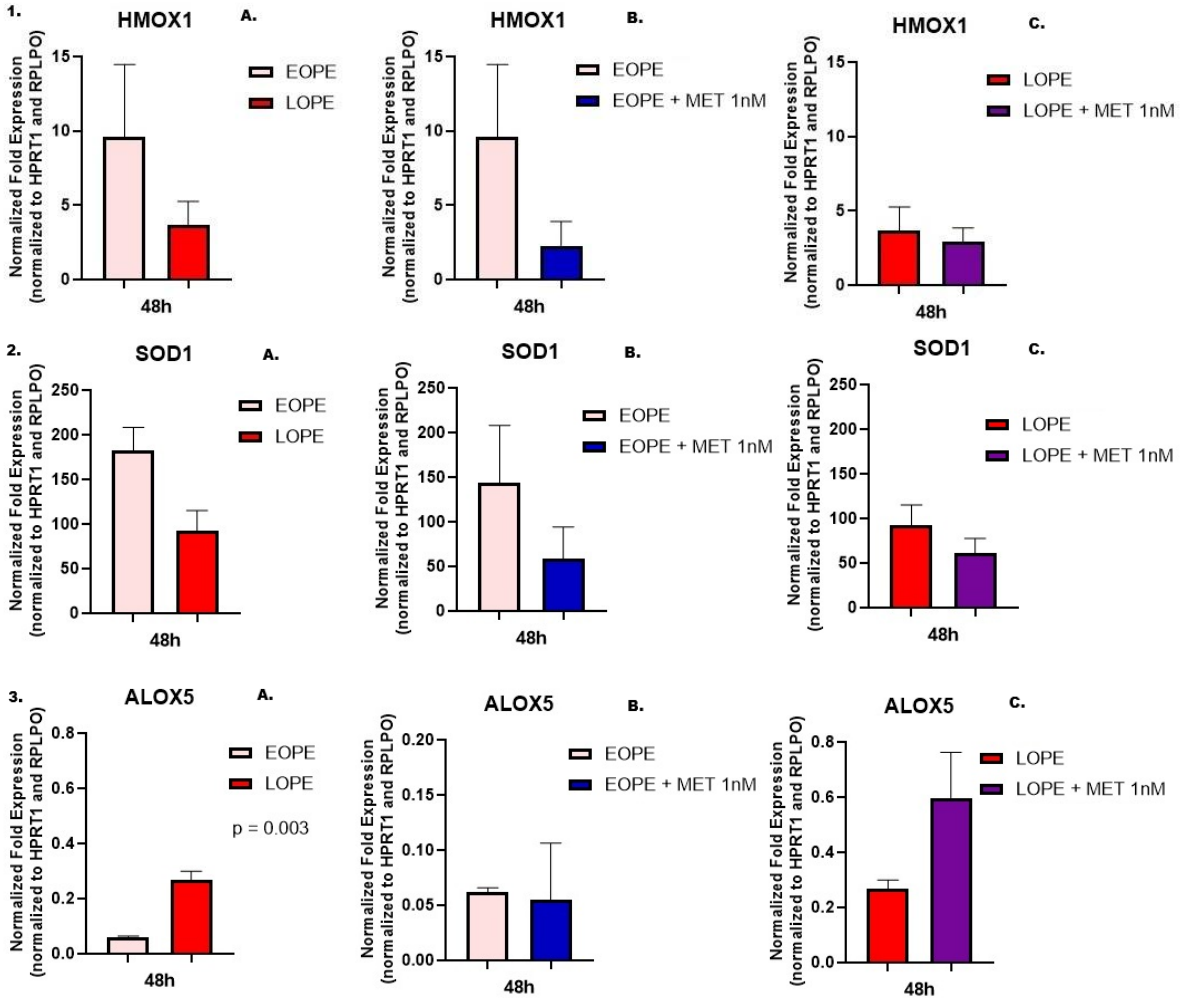
Tabela 1: Genes analisados e correlacionados a fisiopatologia da PE e primers utilizados durante PCR em tempo real.

Gene	Primer Foward	Primer Reverse
HPRT1	ATAAGCCAGACTTTGTTGG	ATAGGACTCCAGATGTTTCC
RPLP0	CGGTTTCTGATTGGCTAC	ACGATGTCACTTCCACG
HMOX1	CAACAAAGTGCAAGATTCTG	TGCATTCACATGGCATAAAG
SOD1	GAGCAGAAGGAAAGTAATGG	GATTAAAGTGAGGACCTGC
ALOX5	AAATGCCACAAGGATTTACC	ATCGCTTTGGAGTAATTCAG
ICAM	ACCATCTACAGCTTTCCG	TCACACTTCACTGTCACC
KDR	GTACATAGTTGTCGTTGTAGG	TCAATCCCCACATTTAGTTC
SIRT1	AAGGAAAACTACTTCGCAAC	GGAACCATGACACTGAATTATC
EDN1	CAAGCAGGAAAAGAACTCAG	CTGGTTTGTCTTAGGTGTTC
NOS3	CATCACCTATGACACCCTC	AGCCGCTCCTCTTAATG
MTOR	CGATGGCCAGGGATCTCTTC	GGTCTGTGTGACTTCAGCGA
VEGFA	AATGTGAATGCAGACCAAAG	GACTTATACCGGGATTTCTTG
MMP2	ATGAATACTGGATCTACTCAGC	GTATCTCCAGAATTTGTCTCC
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCAGGTCGTCCTTAT
CCL2	AGACTAACCCAGAAACATCC	ATTGATTGCATCTGGCTG
CASP1	CAACTACAGAAGAGTTTGAGG	AACATTATCTGGTGTGGAAG
CASP3	AAAGCACTGGAATGACATC	CGCATCAATTCCACAATTTC

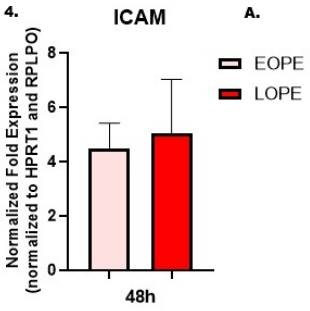
VWF	TGTATCTAGAAACTGAGGCTG	CCTTCTTGGGTCATAAAGTC
SELE	GAGAATTCACCTACAAGTCC	AGGCTTGAACATTTTACCAC

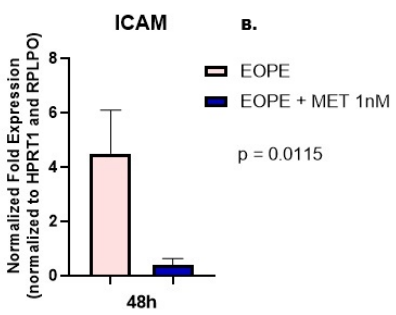
A linhagem HUVEC foi cultivada a 37ºC em CO2 5% em meio DMEM/F12 (Gibco, EUA) suplementado com 20% (v/v) de soro fetal bovino (SFB), 100iu/mL de penicilina, 100 µg/mL de estreptomicina e 2 mMol/L de L-Glutamina (Sigma-Aldrich, EUA). Após o crescimento celular atingir a confluência de 80% e para realização do experimento, as HUVECs foram então expandidas em placas de 24 poços e incubadas a 37ºC em CO2 5% por 48 horas em meio sem SFB, onde a suplementação foi com pool de plasma de gestantes com PE precoce ou tardia de acordo com grupo de estudo. Todas as incubações foram feitas na ausência (apenas meio de cultura) ou presença de cloridrato de metformina (Merck – Número CAS:1115-70-4).

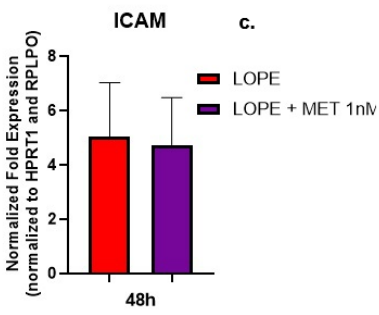
A extração do RNA foi realizada conforme descrito anteriormente e o PCR em tempo real utilizou dos primers descritos na **tabela 1**. A expressão gênica de todos os genes analizados foi normalizada utilizando média geométrica dos genes de referência *HPRT1* e *RPLP0* e os gráficos da expressão relativa normalizada são apresentados na **Figura 4**.



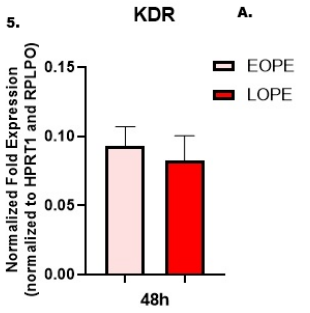
4.

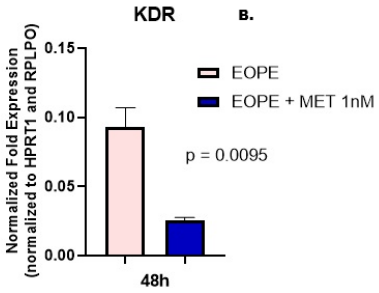

ICAM
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h

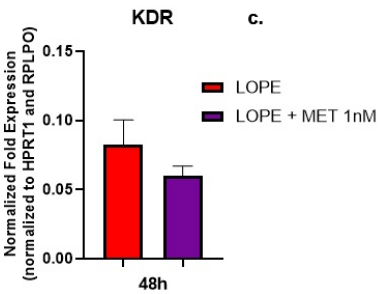

ICAM
B.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
EOPE + MET 1nM
p = 0.0115
48h


ICAM
C.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
LOPE
LOPE + MET 1nM
48h

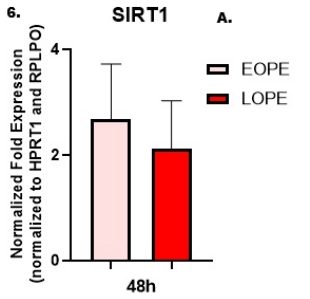
5.

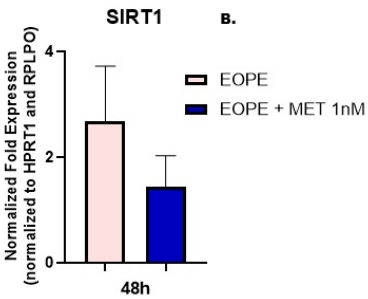

KDR
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h

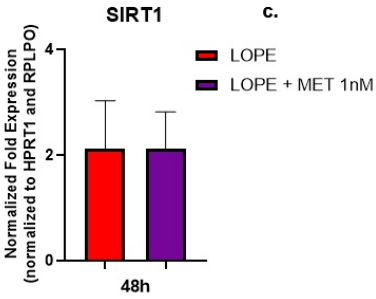

KDR
B.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
EOPE + MET 1nM
p = 0.0095
48h


KDR
C.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
LOPE
LOPE + MET 1nM
48h

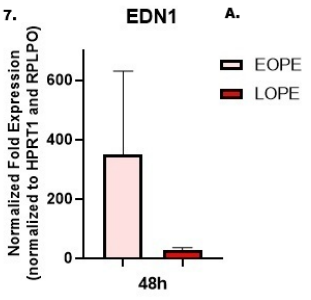
6.

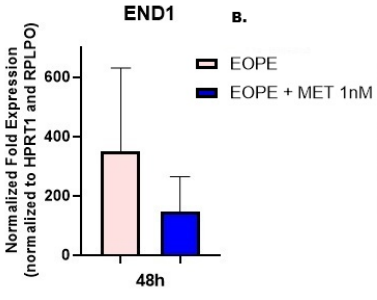

SIRT1
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h

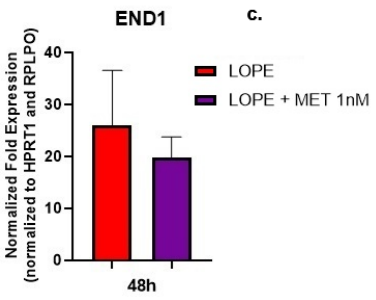

SIRT1
B.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
EOPE + MET 1nM
48h


SIRT1
C.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
LOPE
LOPE + MET 1nM
48h

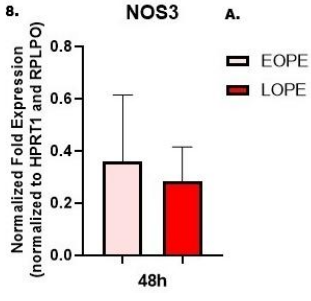
7.

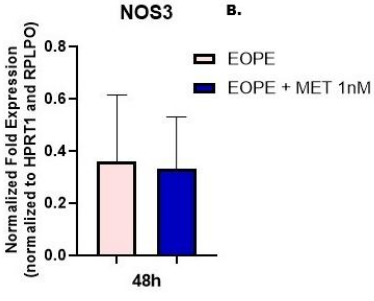

EDN1
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h

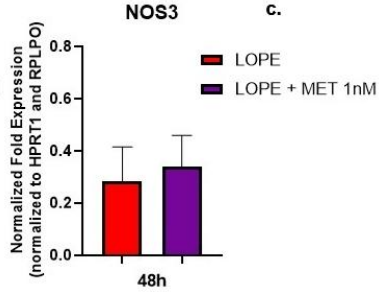

END1
B.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
EOPE + MET 1nM
48h


END1
C.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
LOPE
LOPE + MET 1nM
48h

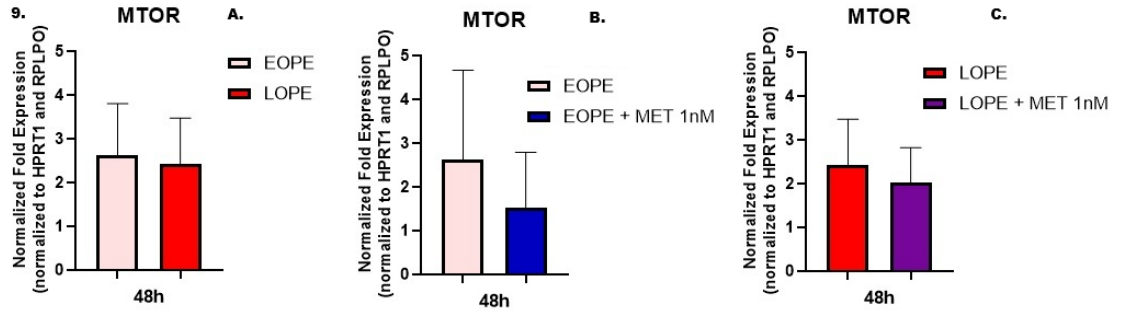
8.


NOS3
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h

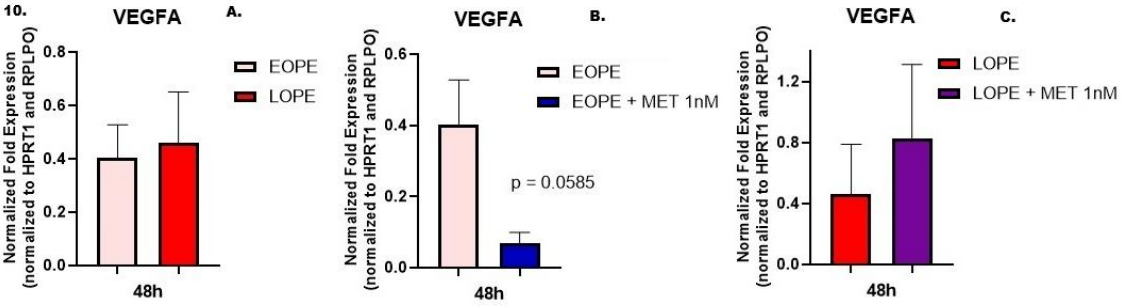

NOS3
B.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
EOPE + MET 1nM
48h


NOS3
C.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
LOPE
LOPE + MET 1nM
48h

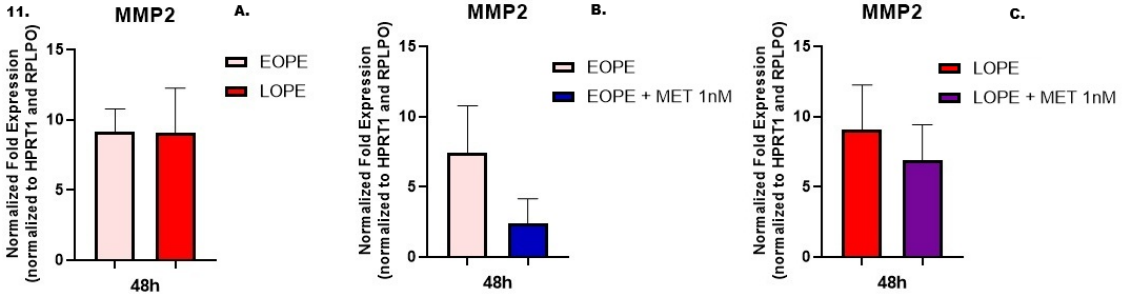
9.


MTOR
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h
MTOR
B.
EOPE
EOPE + MET 1nM
48h
MTOR
C.
LOPE
LOPE + MET 1nM
48h

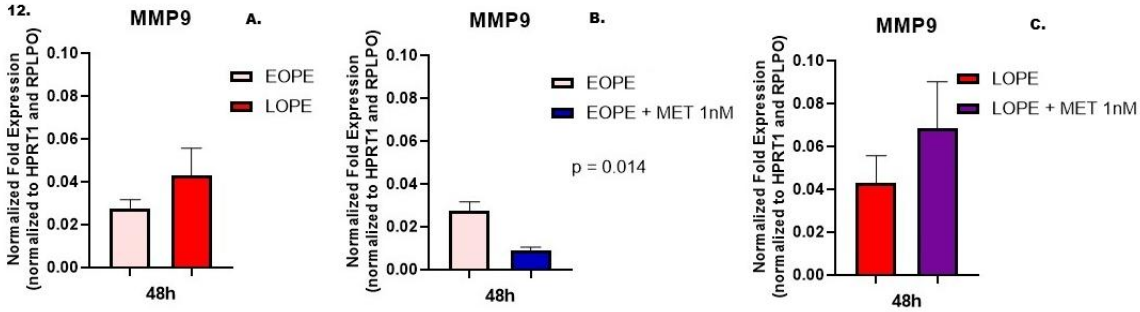
10.


VEGFA
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h
VEGFA
B.
EOPE
EOPE + MET 1nM
p = 0.0585
48h
VEGFA
C.
LOPE
LOPE + MET 1nM
48h

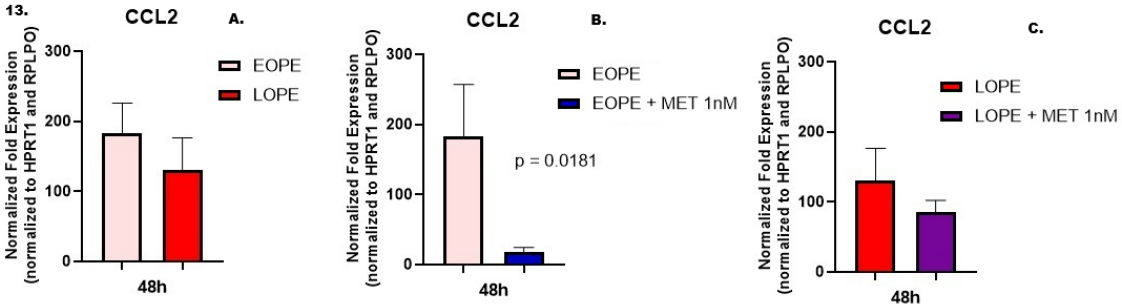
11.


MMP2
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h
MMP2
B.
EOPE
EOPE + MET 1nM
48h
MMP2
C.
LOPE
LOPE + MET 1nM
48h

12.


MMP9
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h
MMP9
B.
EOPE
EOPE + MET 1nM
p = 0.014
48h
MMP9
C.
LOPE
LOPE + MET 1nM
48h

13.


CCL2
A.
Normalized Fold Expression (normalized to HPRT1 and RPLPO)
EOPE
LOPE
48h
CCL2
B.
EOPE
EOPE + MET 1nM
p = 0.0181
48h
CCL2
C.
LOPE
LOPE + MET 1nM
48h

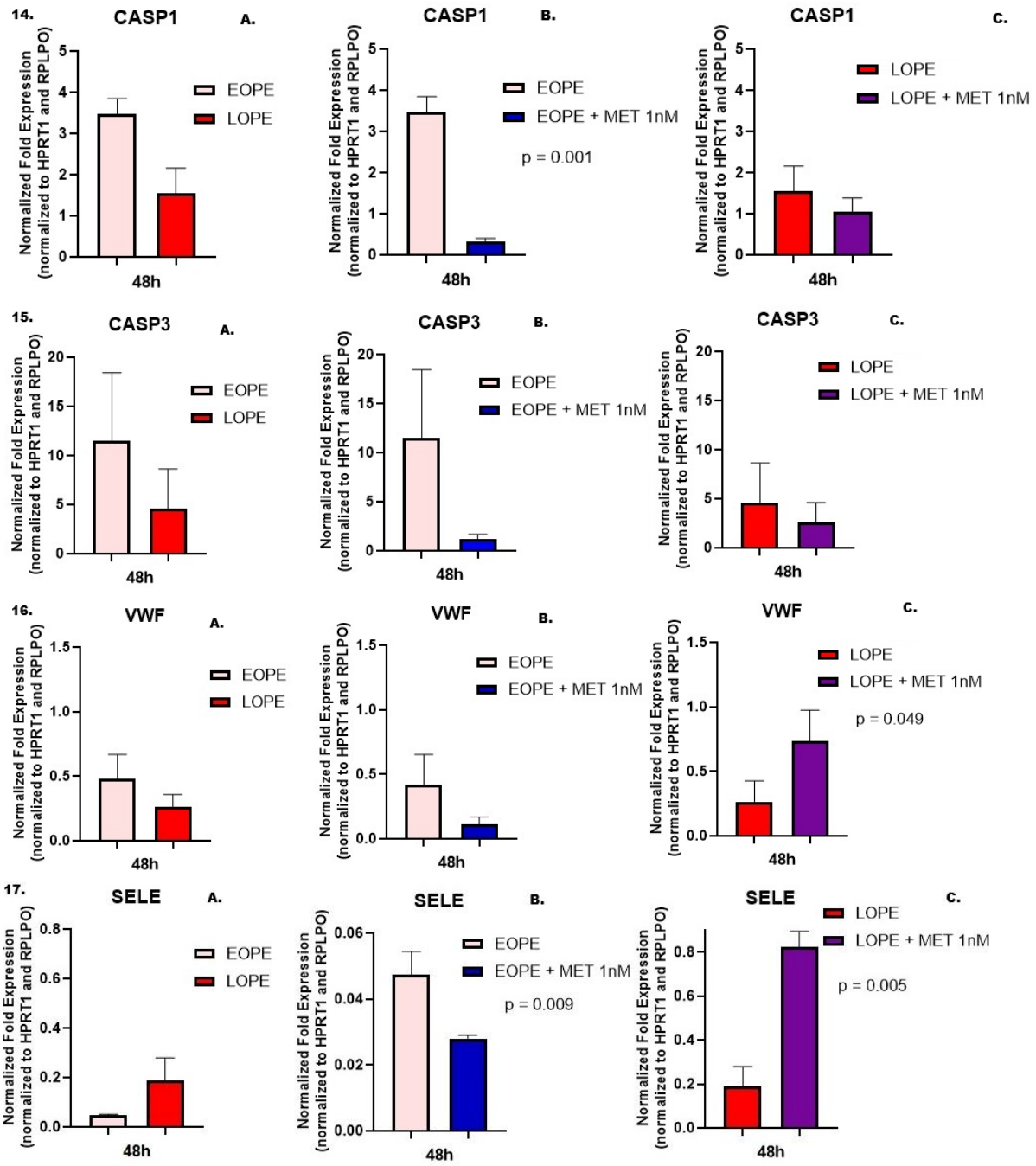


Figura 4: Expressão gênica dos 17 genes de interesse. A. Comparação entre os grupos de células incubadas com plasma de gestantes com PE preconce e PE tardia; **B.** Entre os grupos PE preconce e metformina 1nM; **C.** Entre os grupos PE tardia e metformina 1nM. A expressão gênica foi avaliada por qPCR em triplicatas biológicas, com duplicatas técnicas para cada amostra. Os valores apresentados correspondem à expressão relativa normalizada, calculada com base na média geométrica dos genes de referência *HPRT1* e *RPLP0*. Para cada amostra, a média dos valores dos genes-alvo e dos genes de referência foi utilizada no cálculo da expressão relativa. As comparações entre os grupos foram realizadas por teste t de Student, considerando *p < 0,05 como estatisticamente significativo.

Quando analizamos a expressão relativa comparando os grupos de células incubadas com plasma de gestantes com PE precoce e tardia, o único gene diferencialmente expresso foi

o *ALOX5*, onde o mesmo foi mais expressos após incubação com pool de plasma de gestantes com PE tardia. Quando comparamos os grupos PE precoce e PE precoce tratada com metformina, sete genes apresentaram expressão diferencial, sendo eles *ICAM*, *KDR*, *CCL2*, *VEGF*, *MMP9*, *CASP1* e *SELE*, onde todos os genes diferencialmente expressos apresentaram esses valores com pool de plasma de gestantes com PE precoce. Já quando comparado o grupo de PE tardia e PE tardia tratada, os único genes diferencialmente expressos foram *SELE* e *VWF,* onde ambos foram superregulados no grupo de células expostas ao pool de plasma de PE tardia mais tratamento com metformina (**Figura 4**).

Finalmente analisamos esses mesmos dados utilizando também a abordagem do $\Delta\Delta C_T$ e do $_2$-$\Delta\Delta C_T$ (mudança de expressão) para determinar a mudança na expressão gênica. O $_2$-$\Delta\Delta C_T$ é a expressão gênica normalizada ($_2$-ΔC_T) na amostra de teste, dividida pela expressão gênica normalizada ($_2$-ΔC_T) na amostra controle. Quando comparamos PE precoce com PE tardia, o grupo PE precoce foi denominado como controle; Quando comparamos PE precoce e PE precoce tratada com metformina, o grupo sem tratamento foi escolhido como controle; Já quando comparado PE tardia e PE tardia tratada com metformina, o grupo PE tardia foi o grupo controle. A mudança de expressão ou ($_2$-$\Delta\Delta C_T$) representa a mudança na expressão gênica de forma biologicamente significativa, onde valores maiores que um indicam uma regulação positiva ou superregulação, e valores menores que um indicam uma regulação negativa.

A análise do grupo PE precoce e PE tardia pode ser observada na **Tabela 2**. Os genes *CASP3* e *END1* tiveram regulação negativa, ou seja apresentaram maior expressão do grupo de PE precoce; já os genes *ALOX,* com $P<0.05$, e *SELE* apresentaram regulação positiva, com maior expressão no grupo PE tardia.

Tabela 2: Mudança de expressão genética ($_2$-ΔΔC$_T$) dos grupos PE precoce x PE tardia.

Position	Gene Symbol	Fold Change (comparing to control group)		
		Group 1		
		Fold Change	p-Value	Comments
1	HPRT1	1.13	0.693687	
2	RPLPO	0.89	0.884273	B
3	CASP3	0.20	0.487104	
4	EDN1	0.10	0.308983	
5	HMOX1	0.50	0.422122	
6	NOS3	0.88	0.597618	B
7	VEGFA	1.42	0.486351	B
8	CASP1	0.75	0.586216	
9	MMP2	1.70	0.826165	
10	SOD1	0.88	0.589498	
11	VWF	0.81	0.615062	A
12	MMP9	1.35	0.656533	B
13	ALOX5	3.80	0.022888	
14	CCL2	1.01	0.835685	
15	ICAM	1.53	0.728396	
16	SELE	2.80	0.273688	B
17	MTOR	1.02	0.902545	
18	KDR	0.77	0.476859	B
19	SIRT1	0.78	0.715019	

Legenda: $_2$-ΔΔC$_T$ é a expressão gênica normalizada ($_2$-ΔC$_T$) na amostra de teste, dividida pela expressão gênica normalizada ($_2$-ΔC$_T$) na amostra controle. Os valores de mudança de expressão maiores que 2 são indicados em vermelho; os valores de mudança de expressão menores que 0,5 e os valores de mudança de expressão menores que -2 são indicados em azul.

Quando analisamos o grupo PE precoce comparado com PE precoce tratado com metformina, o gene *CASP3* teve regulação negativa, ou seja apresentou maior expressão do grupo de PE precoce, conforme mostrado na **Tabela 3**.

Tabela 3: Mudança de expressão genética ($_2$-ΔΔC$_T$) dos grupos PE precoce x PE precoce tratada com metformina.

Position	Gene Symbol	Fold Change (comparing to control group)		
		Group 1		
		Fold Change	p-Value	Comments
1	HPRT1	1.60	0.436720	
2	RPLPO	0.62	0.435818	B
3	CASP3	0.38	0.223007	
4	EDN1	0.56	0.542366	
5	HMOX1	0.58	0.413011	
6	NOS3	1.97	0.983824	B
7	VEGFA	0.57	0.315430	B
8	CASP1	1.12	0.773497	
9	MMP2	0.64	0.515748	
10	SOD1	1.00	0.683999	
11	VWF	0.68	0.589785	A
12	MMP9	0.85	0.682543	B
13	ALOX5	1.31	0.530154	B
14	CCL2	0.67	0.468846	
15	ICAM	0.53	0.461177	
16	SELE	1.32	0.857095	B
17	MTOR	1.52	0.588604	
18	KDR	0.61	0.408199	B
19	SIRT1	1.26	0.642210	

Legenda: $_2$-ΔΔC$_T$ é a expressão gênica normalizada ($_2$-ΔC$_T$) na amostra de teste, dividida pela expressão gênica normalizada ($_2$-ΔC$_T$) na amostra controle. Os valores de mudança de expressão maiores que 2 são indicados em vermelho; os valores de mudança de expressão menores que 0,5 e os valores de mudança de expressão menores que -2 são indicados em azul.

Os últimos grupos a serem analisados foram PE tardia comparado a PE tardia tratada com metformina. Os genes *VWF*, *ALOX5* e *SELE,* onde todos foram superregulados no grupo PE tardia tratada com metformina conforme mostra a **Tabela 4**.

Tabela 4: Mudança de expressão genética ($_2$-ΔΔC$_T$) dos grupos PE tardia x PE tardia tratada com metformina.

Position	Gene Symbol	Fold Change (comparing to control group)		
		Group 1		
		Fold Change	p-Value	Comments
1	HPRT1	0.85	0.417172	
2	RPLPO	1.18	0.762773	B
3	CASP3	1.08	0.778939	
4	EDN1	0.99	0.530102	
5	HMOX1	0.99	0.547302	
6	NOS3	1.44	0.923897	B
7	VEGFA	1.95	0.535085	B
8	CASP1	0.76	0.450626	A
9	MMP2	0.76	0.498503	
10	SOD1	0.64	0.337382	
11	VWF	2.92	0.009496	
12	MMP9	1.58	0.553794	B
13	ALOX5	2.01	0.180003	B
14	CCL2	0.71	0.415118	
15	ICAM	1.02	0.673800	
16	SELE	5.46	0.014841	
17	MTOR	0.96	0.583143	
18	KDR	0.75	0.438773	B
19	SIRT1	1.09	0.739896	

Legenda: $_2$-ΔΔC$_T$ é a expressão gênica normalizada ($_2$-ΔC$_T$) na amostra de teste, dividida pela expressão gênica normalizada ($_2$-ΔC$_T$) na amostra controle. Os valores de mudança de expressão maiores que 2 são indicados em vermelho; os valores de mudança de expressão menores que 0,5 e os valores de mudança de expressão menores que -2 são indicados em azul.

Como a PE é uma doença multifatorial de origem genética complexa[138], estudar não só os transcritos diferencialmente expressos, mas também as vias biológicas no qual eles podem estar atuando, é relevante para compreender melhor a doença e buscar por novas estratégias terapêuticas[139].

Usando o banco de dados do Enrichr (https://maayanlab.cloud/Enrichr/) e os genes diferencialmente expressos em uma ou mais análises, os quais foram *ICAM*, *KDR*, *CCL2*, *VEGF*, *MMP9*, *CASP1*, *SELE*, *VWF*, *ALOX*, *CASP3* e *END1*, buscamos idenficar possíveis vias biológicas enriquecidas por esse conjunto de genes. A via de *Organização de matriz*

extracelular foi a mais relevante segundo cruzamento de dados com o do banco "Reactome Pathways 2024" (**Figura 5**).

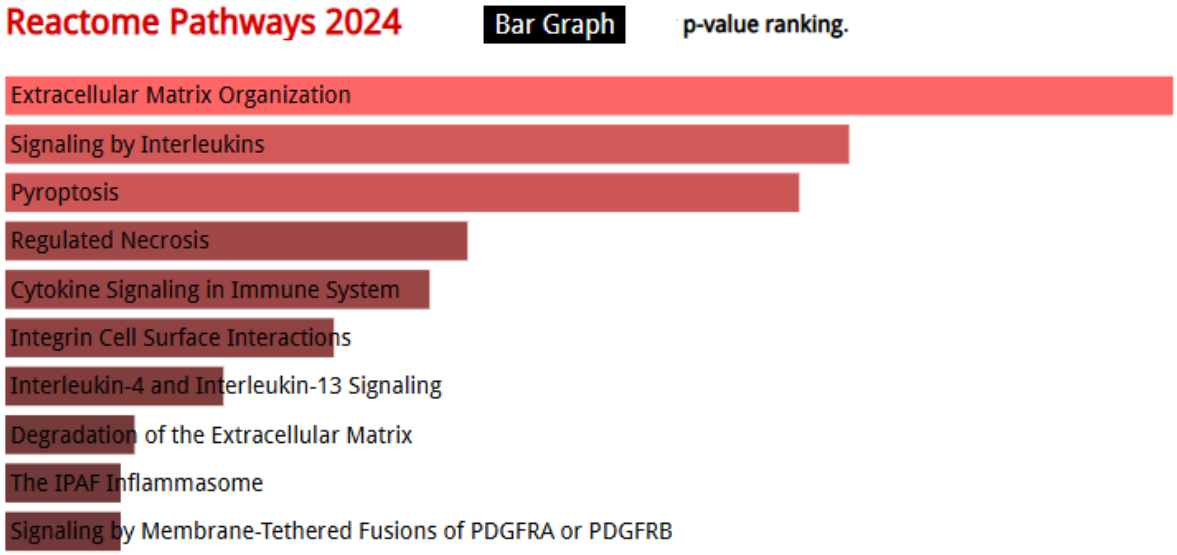


Figura 5: Análise de vias pelo Reactome Pathways 2024. As 10 principais vias biológicas com maior significância estatística. A classificação baseada no valor de *p* é derivada da execução do teste exato de Fisher para enúmeros conjuntos de genes aleatórios, a fim de calcular uma classificação média e um desvio padrão da classificação esperada para cada termo na biblioteca de conjuntos de genes e, finalmente, calcular um escore z para avaliar o desvio da classificação esperada.

Finalmente, usando o mesmo conjunto de genes, buscamos correlacionar a função dos genes selecionados a determinados processos biológicos. Para isso analisamos utilizando o banco de dados do "Gene Ontology Biological Process 2025", também performado através do Enrichr. O processo biológico mais bem rankeado baseado no valor de *p* foi de *Resposta a Lipopolissacarídeos (LPS)*, conforme apresentado na **Figura 6**.

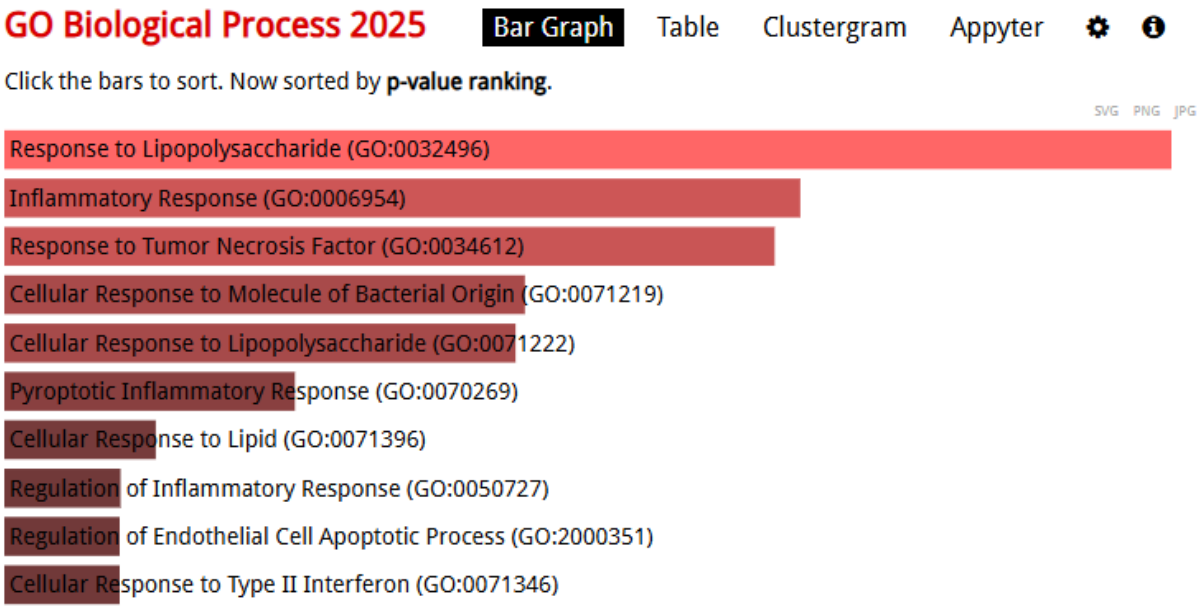


Figura 6: Análise de Ontologia Genética pelo GO Biological Process 2025. Os 10 principais processos biológicos com maior significância estatística. O valor de *p* é calculado a partir do teste exato de Fisher, que é um teste de proporção que assume uma distribuição binomial e de independência para a probabilidade de qualquer gene pertencer a qualquer conjunto.

5. Discussão

Os genes diferencialmente expressos que encontramos em nossas análises de RT-qPCR foram capazes de nos trazer informações relevantes sobre a expressão gênica diferencial na fisiopatologia da PE e também na resposta à terapia com metformina.

O gene *ALOX5*, por exemplo, que encontramos super expresso após incubação com plasma de gestantes com PE tardia, codifica a ALOX-5, uma dioxigenase que catalisa a peroxidação de ácidos graxos poliinsaturados, e também atua na mediação de diferentes tipos de morte celular, como a ferroptose, por promover um ambiente inflamatório e a peroxidação lípidica[140]. Nesse contexto, estudos já mostraram peroxidação lipídica e ferroptose em sinciciotrofoblastos de mulheres com PE tardia[141], assim como mostram super expressão de *ALOX5* associada a PE[142] e a PE tardia[143]. O gene *SELE* codifica a selectina-E, que tem sua expressão induzida por diversos agentes inflamatórios e na gestação desempenha papel importante na implantação e aceitação imunológica, assim como na migração trofoblástica para as artérias espiraladas do endométrio[144]. Entretanto, níveis elevados de selectina-E refletem disfunção endotelial e uma vez que a PE está associada à extensa ativação plaquetária e disfunção endotelial, a expressão diferencial do gene *SELE* pode desempenhar um papel na fisiopatologia da doença[145].

Em contratapartida, os genes da *CASP3* e *END1* foram super expressos em PE precoce quando comparado à PE tardia. A caspase 3, codificada pelo gene *CASP3*, desempenha um papel crucial na apoptose e na morte celular programada[146]. Durante a gestação a apoptose visa auxiliar na diferenciação, fusão e degeneração dos trofoblastos[147], porém a apoptose excessiva pode causar disfunção endotelial[148] e a apoptose parece aumentada na PE[148, 149]. Nesse contexto, outros trabalhos mostraram aumento significativo da caspase 3 em placenta de mulheres com PE e em trofoblastos induzidos com soro de mulheres com PE[148, 150]. Já o *END1* codifica a endotelina-1, que é sintetizada e secretada por um vasto número de células, incluindo as endoteliais e os sinciciotrofoblastos da placenta[151]. Nesse contexto, nosso achado corrobora com diversos estudos que observaram um aumento de duas a três vezes nos níveis circulantes de endotelina-1 em gestantes com PE, com alguns indicando inclusive uma correlação com a gravidade da doença, onde pacientes com a síndrome HELLP apresentaram níveis mais elevados do que pacientes com PE (revisado em[152]).

A PE segue sendo uma das principais causas de morbidade e mortalidade materna e fetal[153], além de ser considerada uma doença crônica com potencial de gerar insuficiência progressiva de múltiplos órgãos[153]. Contudo, não existe um tratamento definitivo e mais de 40% das gestantes não respondem a terapia antihipertensiva[5], sendo esse grupo associado aos desfechos clínicos mais graves[63]. A metformina altera a expressão de milhares de genes e demostrou ter potencial para o tratamento da pré-eclâmpsia[35]. Contudo a maneira com o qual a metformina exerce esse papel não está completamente elucidade e estudos de expressão gênica podem auxiliar na compreensão do mecanismo de ação da metformina, assim como na resposta à terapia.

Nesse contexto, quando comparamos a expressão gênica das células incubadas com plasma de gestantes com PE precoce com o grupo que recebeu plasma e metformina, diversos genes tiveram sua expressão alterada. Quando comparamos o grupo PE precoce e PE precoce tratado com metformina, o gene que apresentou expressão diferencial através da análise de $_2$-$\Delta\Delta C_T$ foi o *CASP3*, que apresentou maior expressão no grupo PE precoce não tratado, ou seja, o tratamento com metformina diminiu a expressão desse gene. Por sua vez, quando analisamos o grupo que recebeu plasma de gestantes com PE tardia comparado ao grupo PE tardia tratada com metformina os genes *VWF*, *ALOX5* e *SELE* foram todos superregulados no grupo PE tardia tratada com metformina.

A metformina além de reduzir os níveis de sFlt-1 e sEng, aumenta a biodisponibilidade de óxido nítrico e inibi a secreção de moléculas de adesão pelas células endoteliais, bloqueando a síntese de radicais livres derivados do oxigênio e proteases, reduzindo a lesão do endotélio[35, 154]. Corroborando com nosso trabalho, estudos mostraram que a metformina foi capaz de diminuir os níves de caspase 3 em plasma e placenta de ratas do modelo de PE[155]. Esses estudos mostram que a metformina pode atuar na PE para além da diminuição dos marcadores antiangiogênicos sFlt-1 sEng[156].

O gene *VWF* é responsável por codificar o fator de von Willebrand (vWF), uma glicoproteína produzida por células endoteliais e megacariócitos e que desempenha papel fundamental da hemostasia, auxiliando na adesão e agregação plaquetária em locais de lesão vascular e atuando como carreador e estabilizador plasmático do fator VIII da coagulação[157]. A PE está associada a extensa ativação endotelial e aumento da resposta inflamatória, onda essa ativação das células endoteliais causa níveis crescentes do vWF. Estudos anteriores demonstraram níveis aumentados do antígeno vWF em pacientes com PE, caracterizados pela ativação das células endoteliais[158]. Embora nossos achados não tenham mostrado expressão gênica diferencial do *VWF* entre o grupo induzido com plasma de PE precoce e PE tardia, podemos hipotetizar que algum fator relacionado a PE tardia possa ter levado ao aumento da expressão desse gene pós tratamento com a metformina.

Assim como o *VWF*, o *ALOX5* e o *SELE* também foram superregulados após tratamento com a metformina. O aumento da expressão desses genes já foi observada em PE[141-143, 145, 158] e esse aspécto nos induz a pensar que a metformina, como possível tratamento, poderia atuar diminuindo a expressão dos mesmos. Entretanto, a metformina pode apresentar efeitos tanto antiangiogênicos, por meio da modulação de diferentes mediadores que regem o processo de angiogênese (revisado em[159]), como pró-angiogênico, onde foram relatados regulação positiva do fator de crescimento endotelial vascular (VEGF) no câncer de mama[160] e melanoma[161]. Nesse contexto, efeitos endoteliais e cardioprotetores foram relatados para a metformina no contexto de diabetes e obesidade[162, 163]. Porém, os possiveis efeitos da modulação gênica causados pela metformina no contexto da PE e as possívis implicações dessas alterações ainda são pouco estudadas e mais investigações são necessárias.

Por último realizamos a análise de enriquecimento funcional utilizando como entrada todos os genes diferencialmente expressos encontrados quando comparados todos os grupos, afim de encontrar quais as possíveis vias e processos biológicos poderiam estar correlacionados

a esses genes. A via de *Organização de matriz extracelular* foi a mais relevante segundo cruzamento banco de dados do "Reactome Pathways 2024" e o processo biológico mais bem rankeado foi de *Resposta a LPS*.

Durante a gestação a remodelação da matriz extracelular facilita a morfogênese drástica dos tecidos maternos e placentários, necessária para sustentar o desenvolvimento fetal. Além de fornecer suporte tecidual, a matriz extracelular influencia os resultados da gravidez, uma vez que facilita a comunicação entre as células e seu microambiente para regular a adesão, migração e invasão celular[164]. Nesse contexto, a invasão placentária insuficiente contribui para o desenvolvimento da PE[165].

Embora a etiologia da PE ainda não esteja totalmente esclarecida[13], a hipóxia e o estresse oxidativo levam a placenta a liberar fatores antiangiogênicos circulantes, incluindo a endoglina solúvel e o sFlt-1, que foram propostos como causadores de uma resposta inflamatória sistêmica e disfunção endotelial materna generalizada[67, 108]. Corroborando com nossos achados, onde *Resposta a LPS* foi o processo biológico mais enriquecido pelo nosso conjunto de genes, um estudo demonstrou que o LPS, uma endotoxina das paredes celulares de bactérias gram-negativas, media processos inflamatórios associados à reprodução feminina[166]. Em estudo prévio, Faas e colaboradores desenvolveram um modelo clássico para PE, injetando LPS em ratas prenhas no 14º dia de gestação, onde o modelo apresentou todos os sintomas e as alterações patológicas placentárias que ocorrem na PE humana[167]. Esses estudos mostram que genes que participam da via de resposta a LPS também contribuem para a fisiopatologia da PE.

Em conclusão, nesse estudo evidenciamos que a metformina pode regular genes e vias envolvidos na fisiopatologia da PE e a identificação dos mesmos pode contribuir para elucidar sobre mecanismos envolvidos na redução da disfunção endotelial em PE e potenciais preditores de resposta ao tratamento com Metformina.

6. Orientações

A aluna foi co-orientadora de estudante de mestrado:

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

DECLARAÇÃO

Certifico, para os devidos fins, que a **Dra. Daniela Alves Pereira** atuou como **COORIENTADORA** do discente de Mestrado **Ricardo Nodari Fróes de Castro,** de Fevereiro/2024 a Fevereiro/2025.

Declaro, ainda, que a referida orientação finalizou-se quando o discente defendeu a dissertação intitulada "**Efeito de polimorfismos do gene ADIPOQ nas concentrações plasmáticas de adiponectina em Hipertensão Gestacional e Pré-eclâmpsia**" em 27 de Fevereiro de 2025, sob orientação do Dr. Marcelo Rizzatti Luizon, no Programa de Pós-graduação em Genética do Instituto de Ciências Biológicas da UFMG.

Belo Horizonte, 26 de março de 2025.

Prof. Dr. Fabrício Rodrigues dos Santos
Vice-coordenador do Programa de Pós-graduação em Genética
ICB/UFMG

Documento assinado eletronicamente por **Fabricio Rodrigues dos Santos**, **Subcoordenador(a)**, em 26/03/2025, às 09:11, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Referência: Processo nº 23072.200820/2025-18 SEI nº 4077166

A aluna atualmente é co-orientadora de estudante de iniciação científica:

FUNDAÇÃO vunesp

BOLSA DE ESTUDOS-Convênio UNESP/VUNESP/SEDUC
PLANO DE ATIVIDADES DO BOLSISTA
período de 01 julho/2025 a 30 junho/2027

INFORMAÇÕES GERAIS:

Nome do Bolsista: Otávio Ricardo David de Almeida Morato dos Reis
e-mail: ordam.reis@unesp.br RG: 63249986-2 CPF: 526246058-51
Curso: Ciências Biológicas Modalidade: Bacharelado Ano: 1° ano

Nome do Orientador: Valéria Cristina Sandrim
e-mail: valeria.sandrim@unesp.br RG: 27370186-1 CPF: 27709198813
Departamento: Departamento de Biofísica e Farmacologia
Unidade: Instituto de Biociências – UNESP/Botucatu

Nome do Co-orientador: Daniela Alves Pereira
e-mail: da.pereira@unesp.br RG: MG-17.048.227 CPF: 120.805.326-42
Departamento: Departamento de Biofísica e Farmacologia
Unidade: Instituto de Biociências – UNESP/Botucatu

Modalidade:

( ) I – complementação de estudos , visando à ampliação de atividades curriculares, de modo a abranger modalidades e hábitos de estudos e orientação para a leitura no interior das diversas disciplinas;
(x) II - iniciação científica, visando à introdução do aluno na metodologia e desenvolvimento de projetos de investigação;
( ) III - participação em Programas e Projetos de Extensão da Unidade Universitária ou da Universidade;
( ) IV - programas e Projetos de caráter técnico acadêmico, tais como o desenvolvimento de Monitorias, apoio na área de informática e outras atividades acadêmicas de interesse das Unidades.

TÍTULO (ou tema das atividades, com base nas modalidades possíveis):

Identificação de genes regulados pela metformina em modelo *in vitro* de pré-eclâmpsia

I - OBJETIVOS:

- Investigar genes regulados pela metformina em modelo *in vitro* de pré-eclâmpsia precoce, tardia e gestantes saudáveis incubados com metformina e/ou inibidor seletivo da AMPK;
- Identificar vias de sinalização envolvidas na regulação de genes relacionados a ação da Metformina.

FUNDAÇÃO vunesp

II – ATIVIDADES PROPOSTAS* :

- Extração de ácidos nucleicos (RNA) de células incubadas com metformina e plasma de gestantes saudáveis, com PE precoce e tardia, assim como de células incubadas com metformina e o inibidor seletivo da AMPK;
- Quantificação de ácidos nucleicos (RNA);
- Síntese de cDNA a partir do RNA extraído;
- PCR em tempo real (qPCR);
- Análises de vias de sinalização envolvidas na regulação dos genes diferencialmente expressos.

* **Observação:** Anexar resumo do projeto de pesquisa do qual o aluno participará, quando for o caso, e especificando as atividades que desenvolverá.

III – JUSTIFICATIVA:

A pré-eclâmpsia é a principal causa mundial de mortalidade e morbidade materna e perinatal. A metformina regula a expressão gênica de diversos genes e tem potencial para tratar e/ou prevenir a pré-eclâmpsia, condição onde cerca de 40% das gestantes não respondem a terapia. Nesse contexto, a identificação de novos genes relacionados à fisiopatologia da doença e regulados pela Metformina pode contribuir para elucidar sobre mecanismos envolvidos na redução da disfunção endotelial na pré-eclâmpsia e potenciais preditores de resposta ao tratamento com Metformina.

IV – CRONOGRAMA: 12 meses (por trimestre)

Atividades	1°	2°	3°	4°
Extração de RNA	x	x		
Síntese de cDNA	x	x		
PCR em tempo real		x	x	
Análises de vias			x	x
Participação em eventos científicos				x

V - DO CANCELAMENTO DA BOLSA DE ESTUDOS:

1.1. A Bolsa de Estudos poderá ser cancelada nos seguintes casos:

1.1.1. abandono do curso ou reprovação em 30% (trinta por cento) das disciplinas e/ou créditos cursados no respectivo ano letivo;

1.1.2. cancelamento de matrícula;

1.1.3. trancamento de matrícula em mais de 1/3 (um terço) das disciplinas;

1.1.4. conclusão do curso ou transferência de Instituição;

1.1.5. não comparecimento durante 15 (quinze) dias consecutivos ou 30 (trinta) intercalados às atividades programadas, sem justificativa aceita pelo professor orientador;

1.1.6. parecer desfavorável do orientador do aluno quanto às atividades desenvolvidas e/ou quanto à aprovação do Relatório de Atividades;

1.1.7. passar a receber bolsa de estudos/iniciação científica/extensão outorgadas pela UNESP ou outras instituições;

1.1.8. deixar de apresentar, nos prazos definidos, o Relatório de Atividades.

1.1.9. deixar de observar quaisquer das cláusulas aplicáveis ao caso pelo Convênio Secretaria da Educação, Universidade Estadual Paulista – UNESP e Fundação VUNESP.

1.2. A Bolsa de Estudos poderá ser temporariamente suspensa nos seguintes casos:

1.2.1. O aluno vir a fazer parte de programa de Intercâmbio Internacional, ou programa de estudos para aproveitamento curricular em instituição nacional ou internacional conveniada à UNESP, e para o qual venha a receber outra modalidade de bolsa. Nesses casos, a Bolsa de Estudos outorgada pela Fundação VUNESP poderá ser retomada quando findo o programa, vigendo por período igual ao número de meses faltantes para que se completem os dois anos de outorga da bolsa Vunesp.

1.2.2. Suspensão de matrícula, respeitadas as Resoluções UNESP sobre o tema.

1.3. Na ocorrência de qualquer um dos casos que implicam o cancelamento ou suspensão temporária da bolsa, o bolsista e orientador deverão comunicá-lo de imediato à Fundação Vunesp, sob pena de o bolsista ter de ressarcir a esta Fundação os valores indevidamente recebidos.

Botucatu, 11 de junho de 2025.

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Assinatura do Bolsista

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Assinatura do Orientador

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_____
Assinatura do Co-orientador

Aprovado no Conselho de Departamento
_____, ____/____/____
_____

* As linhas do Relatório são editáveis, use-as conforme a sua necessidade.

7. Disciplinas ministradas

A aluna participou das disciplinas conforme certificados a seguir:

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Botucatu

INSTITUTO DE BIOCIÊNCIAS UNESP BOTUCATU

ATESTADO

ATESTO, para os devidos fins, que a pós-doutoranda. Daniela Alves Pereira, RG nº MG-17.048.227, atuou em atividades de formação docente, no Instituto de Biociências - UNESP - Câmpus de Botucatu, nas turmas das disciplinas abaixo relacionadas:

SEM	CÓDIGO	DISCIPLINA	C.H. DISCIPLINA	C.H. EFETIVA	Nº DE ALUNOS
		Ano letivo: 2025			
1	IB/1202/UN-UNICA	Biomarcadores em pesquisa básica e clínica: fundamentos e novas tecnologias - complementar	60.0	20.0	35
		Ano letivo: 2024			
2	IB/1125/UN-UNICA	Fundamentos de Farmacogenética e Toxicogenética - OPT	30.0	8.0	14

* Disciplina Em Curso
** Disciplina ministrada em regime especial de recuperação (RER)

Botucatu, 16 de Julho de 2025

Juliana Ramos
Diretor Técnico de Divisão/Divisão Técnica Acadêmica

Disciplina: Biomarcadores em Pesquisa básica: fundamentos e novas tecnologias

Botucatu, São Paulo,
4 de Fevereiro de 2025

DECLARAÇÃO DE PARTICIPAÇÃO EM DISCIPLINA

Eu, Profa. Dra. Valéria Cristina Sandrim, docente responsável pela disciplina "Biomarcadores em Pesquisa básica: fundamentos e novas tecnologias", ofertada no semestre 2024/2 pelo Programa de Farmacologia do Instituto de Biociências, Universidade Estadual Paulista (UNESP), declaro que a Dra. Daniela Alves Pereira ministrou aula intitulada "Identificação/detecção de mutações e polimorfismos de nucleotídeo único (SNPs)" perfazendo a carga horária total de 2 horas.

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VALERIA CRISTINA SANDRIM
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Profa. Dr. Valéria Cristina Sandrim
Docente responsável pela Disciplina
Departamento de Farmacologia
Instituto de Biociências
Universidade Estadual Paulista

8. Comentários do Supervisor

9. Referências

1. *Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy.* Obstet Gynecol, 2013. **122**(5): p. 1122-1131.
2. Melchiorre, K., V. Giorgione, and B. Thilaganathan, *The placenta and preeclampsia: villain or victim?* Am J Obstet Gynecol, 2022. **226**(2S): p. S954-S962.
3. MacDonald, T.M., et al., *Clinical tools and biomarkers to predict preeclampsia.* EBioMedicine, 2022. **75**: p. 103780.
4. *Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.* Am J Obstet Gynecol, 2000. **183**(1): p. S1-S22.
5. Luizon, M.R., et al., *Pharmacogenetics in the treatment of pre-eclampsia: current findings, challenges and perspectives.* Pharmacogenomics, 2017. **18**(6): p. 571-583.
6. Hariharan, N., A. Shoemaker, and S. Wagner, *Pathophysiology of hypertension in preeclampsia.* Microvasc Res, 2017. **109**: p. 34-37.
7. Folk, D.M., *Hypertensive Disorders of Pregnancy: Overview and Current Recommendations.* J Midwifery Womens Health, 2018. **63**(3): p. 289-300.
8. Roberts, J.M. and C.A. Hubel, *The two stage model of preeclampsia: variations on the theme.* Placenta, 2009. **30 Suppl A**(Suppl A): p. S32-7.
9. Phipps, E.A., et al., *Pre-eclampsia: pathogenesis, novel diagnostics and therapies.* Nat Rev Nephrol, 2019. **15**(5): p. 275-289.
10. Opichka, M.A., et al., *Vascular Dysfunction in Preeclampsia.* Cells, 2021. **10**(11).
11. Sandrim, V.C., et al., *Nitric oxide formation is inversely related to serum levels of antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endogline in preeclampsia.* Hypertension, 2008. **52**(2): p. 402-7.
12. Pennington, K.A., et al., *Preeclampsia: multiple approaches for a multifactorial disease.* Dis Model Mech, 2012. **5**(1): p. 9-18.
13. Palei, A.C., et al., *Pathophysiology of hypertension in pre-eclampsia: a lesson in integrative physiology.* Acta Physiol (Oxf), 2013. **208**(3): p. 224-33.
14. Spradley, F.T., A.C. Palei, and J.P. Granger, *Increased risk for the development of preeclampsia in obese pregnancies: weighing in on the mechanisms.* Am J Physiol Regul Integr Comp Physiol, 2015. **309**(11): p. R1326-43.
15. Mori, T., et al., *Adipocytokines and endothelial function in preeclamptic women.* Hypertens Res, 2010. **33**(3): p. 250-4.
16. Nunes, P.R., et al., *The interplay between extracellular NAMPT and inflammatory cytokines in preeclampsia.* J Reprod Immunol, 2024. **163**: p. 104248.
17. Lovren, F., et al., *Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis.* Am J Physiol Endocrinol Metab, 2009. **296**(6): p. E1440-9.
18. Yamawaki, H., et al., *Visfatin causes endothelium-dependent relaxation in isolated blood vessels.* Biochem Biophys Res Commun, 2009. **383**(4): p. 503-8.
19. Vallejo, S., et al., *Visfatin impairs endothelium-dependent relaxation in rat and human mesenteric microvessels through nicotinamide phosphoribosyltransferase activity.* PLoS One, 2011. **6**(11): p. e27299.
20. Romacho, T., et al., *Visfatin/eNampt induces endothelial dysfunction in vivo: a role for Toll-Like Receptor 4 and NLRP3 inflammasome.* Sci Rep, 2020. **10**(1): p. 5386.
21. Pereira, D.A., et al., *NAMPT levels are inversely related to nitric oxide formation and positively related to soluble fms-like tyrosine kinase-1 levels in preeclampsia.* Pregnancy Hypertens, 2019. **18**: p. 137-140.
22. Pereira, D.A., et al., *NAMPT single-nucleotide polymorphism rs1319501 and visfatin/NAMPT affect nitric oxide formation, sFlt-1 and antihypertensive therapy response in preeclampsia.* Pharmacogenomics, 2021. **22**(8): p. 451-464.

23. Fang, H. and R.L. Judd, *Adiponectin Regulation and Function.* Compr Physiol, 2018. **8**(3): p. 1031-1063.
24. Briana, D.D. and A. Malamitsi-Puchner, *Reviews: adipocytokines in normal and complicated pregnancies.* Reprod Sci, 2009. **16**(10): p. 921-37.
25. Miehle, K., H. Stepan, and M. Fasshauer, *Leptin, adiponectin and other adipokines in gestational diabetes mellitus and pre-eclampsia.* Clin Endocrinol (Oxf), 2012. **76**(1): p. 2-11.
26. Eleuterio, N.M., et al., *Correlations between circulating levels of adipokines and anti-angiogenic factors in women with BMI <30 and a late-onset preeclampsia.* Hypertens Pregnancy, 2014. **33**(1): p. 72-80.
27. Ouchi, N., et al., *Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells.* J Biol Chem, 2004. **279**(2): p. 1304-9.
28. Shibata, R., et al., *Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of amp-activated protein kinase signaling.* J Biol Chem, 2004. **279**(27): p. 28670-4.
29. Eleuterio, N.M., et al., *Relationship between adiponectin and nitrite in healthy and preeclampsia pregnancies.* Clin Chim Acta, 2013. **423**: p. 112-5.
30. Eleuterio, N.M., et al., *Positive correlations between circulating adiponectin and MMP2 in preeclampsia pregnant.* Pregnancy Hypertens, 2015. **5**(2): p. 205-8.
31. Luizon, M.R., et al., *Antihypertensive therapy responsiveness and adverse outcomes in preeclampsia: insights into molecular mechanisms underlying cardiovascular and renal complications.* Front Pharmacol, 2023. **14**: p. 1281382.
32. Zanger, U.M., *Pharmacogenetics - challenges and opportunities ahead.* Front Pharmacol, 2010. **1**: p. 112.
33. Luizon, M.R., D.A. Pereira, and V.C. Sandrim, *Pharmacogenomics of Hypertension and Preeclampsia: Focus on Gene-Gene Interactions.* Front Pharmacol, 2018. **9**: p. 168.
34. Picon-Cesar, M.J., et al., *Metformin for gestational diabetes study: metformin vs insulin in gestational diabetes: glycemic control and obstetrical and perinatal outcomes: randomized prospective trial.* Am J Obstet Gynecol, 2021. **225**(5): p. 517 e1-517 e17.
35. Brownfoot, F.C., et al., *Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction.* Am J Obstet Gynecol, 2016. **214**(3): p. 356 e1-356 e15.
36. Alqudah, A., et al., *Risk of pre-eclampsia in women taking metformin: a systematic review and meta-analysis.* Diabet Med, 2018. **35**(2): p. 160-172.
37. Feig, D., *Meta-analysis suggests that metformin may reduce pre-eclampsia compared with insulin use during pregnancy.* BMJ Evid Based Med, 2019. **24**(2): p. 72-73.
38. Luizon, M.R., et al., *Genomic Characterization of Metformin Hepatic Response.* PLoS Genet, 2016. **12**(11): p. e1006449.
39. Torbergsen, T., et al., *Pre-eclampsia--a mitochondrial disease?* Acta Obstet Gynecol Scand, 1989. **68**(2): p. 145-8.
40. Romero, R., et al., *Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity.* Am J Obstet Gynecol, 2017. **217**(3): p. 282-302.
41. Shi, Z., et al., *Comparative proteomics analysis suggests that placental mitochondria are involved in the development of pre-eclampsia.* PLoS One, 2013. **8**(5): p. e64351.
42. Hur, K.Y. and M.S. Lee, *New mechanisms of metformin action: Focusing on mitochondria and the gut.* J Diabetes Investig, 2015. **6**(6): p. 600-9.
43. Madiraju, A.K., et al., *Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase.* Nature, 2014. **510**(7506): p. 542-6.
44. Tal, R., *The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis.* Biol Reprod, 2012. **87**(6): p. 134.
45. Miller, R.A., et al., *Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP.* Nature, 2013. **494**(7436): p. 256-60.

46. Portela, L.V., et al., *Intracerebroventricular metformin decreases body weight but has pro-oxidant effects and decreases survival.* Neurochem Res, 2015. **40**(3): p. 514-23.
47. Wischhusen, J., I. Melero, and W.H. Fridman, *Growth/Differentiation Factor-15 (GDF-15): From Biomarker to Novel Targetable Immune Checkpoint.* Front Immunol, 2020. **11**: p. 951.
48. Chen, Q., et al., *Serum levels of GDF15 are reduced in preeclampsia and the reduction is more profound in late-onset than early-onset cases.* Cytokine, 2016. **83**: p. 226-230.
49. Staines-Urias, E., et al., *Genetic association studies in pre-eclampsia: systematic meta-analyses and field synopsis.* Int J Epidemiol, 2012. **41**(6): p. 1764-75.
50. Luizon, M.R., et al., *Gene-gene interactions in the NAMPT pathway, plasma visfatin/NAMPT levels, and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy.* Pharmacogenomics J, 2017. **17**(5): p. 427-434.
51. Oliveira-Paula, G.H., et al., *Gene-gene interactions in the protein kinase C/endothelial nitric oxide synthase axis impact the hypotensive effects of propofol.* Basic Clin Pharmacol Toxicol, 2022. **130**(2): p. 277-287.
52. Pereira, D.A., et al., *Functional polymorphisms of NOS3 and GUCY1A3 affect both nitric oxide formation and association with hypertensive disorders of pregnancy.* Front Genet, 2024. **15**: p. 1293082.
53. Bueno-Pereira, T.O., et al., *Markers of Endothelial Dysfunction Are Attenuated by Resveratrol in Preeclampsia.* Antioxidants (Basel), 2022. **11**(11).
54. Bueno-Pereira, T.O., et al., *Nebivolol Increases Nitric Oxide Synthase via beta(3) Adrenergic Receptor in Endothelial Cells Following Exposure to Plasma from Preeclamptic Patients.* Cells, 2022. **11**(5).
55. Galvao, V.E., R.C. Cavalli, and V.C. Sandrim, *Preeclampsia and Gestational Hypertension: Biochemical and Antioxidant Features in Vitro Might Help Understand Different Outcomes.* Rev Bras Ginecol Obstet, 2021. **43**(12): p. 894-903.
56. Gomes, V.J., et al., *Monocytes from preeclamptic women previously treated with silibinin attenuate oxidative stress in human endothelial cells.* Hypertens Pregnancy, 2021. **40**(2): p. 124-132.
57. Nunes, P.R., et al., *Relevance of real-time analyzers to determine mitochondrial quality in endothelial cells and oxidative stress in preeclampsia.* Vascul Pharmacol, 2024. **155**: p. 107372.
58. Sandrim, V.C., et al., *eNOS haplotypes affect the responsiveness to antihypertensive therapy in preeclampsia but not in gestational hypertension.* Pharmacogenomics J, 2010. **10**(1): p. 40-5.
59. Machado, J.S., et al., *Polymorphisms of the adiponectin gene in gestational hypertension and pre-eclampsia.* J Hum Hypertens, 2014. **28**(2): p. 128-32.
60. Kuleshov, M.V., et al., *Enrichr: a comprehensive gene set enrichment analysis web server 2016 update.* Nucleic Acids Res, 2016. **44**(W1): p. W90-7.
61. Garcia-Campos, M.A., J. Espinal-Enriquez, and E. Hernandez-Lemus, *Pathway Analysis: State of the Art.* Front Physiol, 2015. **6**: p. 383.
62. Umesawa, M. and G. Kobashi, *Epidemiology of hypertensive disorders in pregnancy: prevalence, risk factors, predictors and prognosis.* Hypertens Res, 2017. **40**(3): p. 213-220.
63. ACOG, *Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222.* Obstet Gynecol, 2020. **135**(6): p. e237-e260.
64. Magee, L.A., et al., *The 2021 International Society for the Study of Hypertension in Pregnancy classification, diagnosis & management recommendations for international practice.* Pregnancy Hypertens, 2022. **27**: p. 148-169.
65. Maynard, S.E., et al., *Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia.* Pediatr Res, 2005. **57**(5 Pt 2): p. 1R-7R.
66. Meekins, J.W., et al., *A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies.* Br J Obstet Gynaecol, 1994. **101**(8): p. 669-74.
67. Tomimatsu, T., et al., *Pathophysiology of preeclampsia: an angiogenic imbalance and long-lasting systemic vascular dysfunction.* Hypertens Res, 2017. **40**(4): p. 305-310.

68. McDonald, E.A. and M.W. Wolfe, *Adiponectin attenuation of endocrine function within human term trophoblast cells.* Endocrinology, 2009. **150**(9): p. 4358-65.
69. Ceron, C.S., M.R. Luizon, and A.C. Palei, *The Potential Role of Visfatin in Mediating Vascular Dysfunction and Hypertension.* J Cardiovasc Pharmacol, 2023. **82**(5): p. 347-349.
70. Luizon, M.R., et al., *Effects of NAMPT polymorphisms and haplotypes on circulating visfatin/NAMPT levels in hypertensive disorders of pregnancy.* Hypertens Res, 2015. **38**(5): p. 361-6.
71. Wu, Z.J., et al., *Adiponectin is associated with increased mortality in patients with already established cardiovascular disease: a systematic review and meta-analysis.* Metabolism, 2014. **63**(9): p. 1157-66.
72. Yang, L., et al., *Prognostic value of adiponectin level in patients with coronary artery disease: a systematic review and meta-analysis.* Lipids Health Dis, 2019. **18**(1): p. 227.
73. Sarsani, V., et al., *A cross-ancestry genome-wide meta-analysis, fine-mapping, and gene prioritization approach to characterize the genetic architecture of adiponectin.* HGG Adv, 2024. **5**(1): p. 100252.
74. Pileggi, S., et al., *Association of ADIPOQ variants and heart failure in an Italian population.* Ther Adv Cardiovasc Dis, 2014. **8**(3): p. 89-96.
75. Smetnev, S., et al., *Associations of SNPs of the ADIPOQ Gene with Serum Adiponectin Levels, Unstable Angina, and Coronary Artery Disease.* Biomolecules, 2019. **9**(10).
76. Sun, K., et al., *Recessive protective effect of ADIPOQ rs1501299 on cardiovascular diseases with type 2 diabetes: a meta-analysis.* Mol Cell Endocrinol, 2012. **349**(2): p. 162-9.
77. Rousset, F., *genepop'007: a complete re-implementation of the genepop software for Windows and Linux.* Mol Ecol Resour, 2008. **8**(1): p. 103-6.
78. Gauderman, W.J., *Sample size requirements for matched case-control studies of gene-environment interaction.* Stat Med, 2002. **21**(1): p. 35-50.
79. Schaid, D.J., et al., *Score tests for association between traits and haplotypes when linkage phase is ambiguous.* Am J Hum Genet, 2002. **70**(2): p. 425-34.
80. Barrett, J.C., et al., *Haploview: analysis and visualization of LD and haplotype maps.* Bioinformatics, 2005. **21**(2): p. 263-5.
81. 1000 Genomes Project, C., et al., *A map of human genome variation from population-scale sequencing.* Nature, 2010. **467**(7319): p. 1061-73.
82. Motulsky, H.J. and R.E. Brown, *Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate.* BMC Bioinformatics, 2006. **7**: p. 123.
83. Wittke-Thompson, J.K., A. Pluzhnikov, and N.J. Cox, *Rational inferences about departures from Hardy-Weinberg equilibrium.* Am J Hum Genet, 2005. **76**(6): p. 967-86.
84. Scherer, P.E., et al., *A novel serum protein similar to C1q, produced exclusively in adipocytes.* J Biol Chem, 1995. **270**(45): p. 26746-9.
85. Song, Y., et al., *Serum levels of leptin, adiponectin and resistin in relation to clinical characteristics in normal pregnancy and preeclampsia.* Clin Chim Acta, 2016. **458**: p. 133-7.
86. Fuglsang, J., et al., *A longitudinal study of serum adiponectin during normal pregnancy.* BJOG, 2006. **113**(1): p. 110-3.
87. Catalano, P.M., et al., *Adiponectin in human pregnancy: implications for regulation of glucose and lipid metabolism.* Diabetologia, 2006. **49**(7): p. 1677-85.
88. Sprague, A.H. and R.A. Khalil, *Inflammatory cytokines in vascular dysfunction and vascular disease.* Biochem Pharmacol, 2009. **78**(6): p. 539-52.
89. Hendler, I., et al., *The levels of leptin, adiponectin, and resistin in normal weight, overweight, and obese pregnant women with and without preeclampsia.* Am J Obstet Gynecol, 2005. **193**(3 Pt 2): p. 979-83.
90. Bawah, A.T., et al., *Serum adipocytokines and adiposity as predictive indices of preeclampsia.* Clin Hypertens, 2020. **26**: p. 19.

91. Youpeng, B., et al., *Relationships among adiponectin gene polymorphisms, proteinuria and increased blood pressure in the context of placental diseases.* Hypertens Res, 2010. **33**(10): p. 1066-70.
92. Saarela, T., et al., *Adiponectin gene haplotype is associated with preeclampsia.* Genet Test, 2006. **10**(1): p. 35-9.
93. Chen, X., et al., *Circulating adipokine levels and preeclampsia: A bidirectional Mendelian randomization study.* Front Genet, 2022. **13**: p. 935757.
94. Bai, Y., et al., *The roles of ADIPOQ rs266729 and MTNR1B rs10830963 polymorphisms in patients with gestational diabetes mellitus: A meta-analysis.* Gene, 2020. **730**: p. 144302.
95. Ng, K.W., et al., *Biomarkers and point of care screening approaches for the management of preeclampsia.* Commun Med (Lond), 2024. **4**(1): p. 208.
96. Mosad, A.S., et al., *Adiponectin Gene Polymorphisms and Possible Susceptibility to Metabolic Syndrome among the Sudanese Population: A Case-Control Study.* Int J Endocrinol, 2023. **2023**: p. 5527963.
97. Wang, G., Y. Wang, and Z. Luo, *Effect of Adiponectin Variant on Lipid Profile and Plasma Adiponectin Levels: A Multicenter Systematic Review and Meta-Analysis.* Cardiovasc Ther, 2022. **2022**: p. 4395266.
98. Spracklen, C.N., et al., *Adiponectin GWAS loci harboring extensive allelic heterogeneity exhibit distinct molecular consequences.* PLoS Genet, 2020. **16**(9): p. e1009019.
99. Greenberg, M.V.C. and D. Bourc'his, *The diverse roles of DNA methylation in mammalian development and disease.* Nat Rev Mol Cell Biol, 2019. **20**(10): p. 590-607.
100. Deng, F., et al., *DNA methylation landscape in pregnancy-induced hypertension: progress and challenges.* Reprod Biol Endocrinol, 2024. **22**(1): p. 77.
101. Metzger, I.F., et al., *Effects of endothelial nitric oxide synthase tagSNPs haplotypes on nitrite levels in black subjects.* Nitric Oxide, 2013. **28**: p. 33-8.
102. Nebert, D.W., *Extreme discordant phenotype methodology: an intuitive approach to clinical pharmacogenetics.* Eur J Pharmacol, 2000. **410**(2-3): p. 107-120.
103. *Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222.* Obstet Gynecol, 2020. **135**(6): p. e237-e260.
104. Peracoli, J.C., et al., *Pre-eclampsia/Eclampsia.* Rev Bras Ginecol Obstet, 2019. **41**(5): p. e1-e2.
105. Wojtowicz, A., et al., *Early- and Late-Onset Preeclampsia: A Comprehensive Cohort Study of Laboratory and Clinical Findings according to the New ISHHP Criteria.* Int J Hypertens, 2019. **2019**: p. 4108271.
106. Weinstein, L., *Syndrome of hemolysis, elevated liver enzymes, and low platelet count: a severe consequence of hypertension in pregnancy.* Am J Obstet Gynecol, 1982. **142**(2): p. 159-67.
107. Townsend, R., P. O'Brien, and A. Khalil, *Current best practice in the management of hypertensive disorders in pregnancy.* Integr Blood Press Control, 2016. **9**: p. 79-94.
108. Redman, C.W. and I.L. Sargent, *Latest advances in understanding preeclampsia.* Science, 2005. **308**(5728): p. 1592-4.
109. Choi, J.W., M.W. Im, and S.H. Pai, *Nitric oxide production increases during normal pregnancy and decreases in preeclampsia.* Ann Clin Lab Sci, 2002. **32**(3): p. 257-63.
110. Roberts, J.M., et al., *The Role of Obesity in Preeclampsia.* Pregnancy Hypertens, 2011. **1**(1): p. 6-16.
111. Zhu, W., et al., *Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention.* Clin Sci (Lond), 2008. **114**(5): p. 361-74.
112. Ouchi, N., et al., *Association of hypoadiponectinemia with impaired vasoreactivity.* Hypertension, 2003. **42**(3): p. 231-4.
113. Powe, C.E., R.J. Levine, and S.A. Karumanchi, *Preeclampsia, a disease of the maternal endothelium: the role of antiangiogenic factors and implications for later cardiovascular disease.* Circulation, 2011. **123**(24): p. 2856-69.
114. Hafiane, A., *Adiponectin-mediated regulation of the adiponectin cascade in cardiovascular disease: Updates.* Biochem Biophys Res Commun, 2024. **694**: p. 149406.

115. Palei, A.C., et al., *Matrix metalloproteinase-9 polymorphisms affect plasma MMP-9 levels and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy.* Pharmacogenomics J, 2012. **12**(6): p. 489-98.
116. Abalos, E., et al., *Antihypertensive drug therapy for mild to moderate hypertension during pregnancy.* Cochrane Database Syst Rev, 2018. **10**(10): p. CD002252.
117. Wittke-Thompson, J.K., A. Pluzhnikov, and N.J. Cox, *Rational inferences about departures from Hardy-Weinberg equilibrium.* American Journal of Human Genetics, 2005. **76**(6): p. 967-986.
118. Naruse, K., et al., *Peripheral blood concentrations of adiponectin, an adipocyte-specific plasma protein, in normal pregnancy and preeclampsia.* J Reprod Immunol, 2005. **65**(1): p. 65-75.
119. Haugen, F., et al., *Increased plasma levels of adipokines in preeclampsia: relationship to placenta and adipose tissue gene expression.* Am J Physiol Endocrinol Metab, 2006. **290**(2): p. E326-33.
120. Ramsay, J.E., et al., *Paradoxical elevation in adiponectin concentrations in women with preeclampsia.* Hypertension, 2003. **42**(5): p. 891-4.
121. Ouyang, Y., H. Chen, and H. Chen, *Reduced plasma adiponectin and elevated leptin in pre-eclampsia.* Int J Gynaecol Obstet, 2007. **98**(2): p. 110-4.
122. Lomakova, Y.D., et al., *Decreased Adiponectin Levels in Early Pregnancy Are Associated with High Risk of Prematurity for African American Women.* J Clin Med, 2022. **11**(11).
123. Abd-Alaleem, D.I., et al., *Adiponectin levels in serum of women with preeclampsia.* East Mediterr Health J, 2011. **17**(7): p. 575-81.
124. Khosrowbeygi, A. and H. Ahmadvand, *Maternal serum levels of adiponectin in preeclampsia.* J Ayub Med Coll Abbottabad, 2009. **21**(3): p. 79-82.
125. Cortelazzi, D., et al., *Maternal and foetal resistin and adiponectin concentrations in normal and complicated pregnancies.* Clin Endocrinol (Oxf), 2007. **66**(3): p. 447-53.
126. Herse, F., et al., *Circulating and uteroplacental adipocytokine concentrations in preeclampsia.* Reprod Sci, 2009. **16**(6): p. 584-90.
127. van Andel, M., et al., *A method comparison of total and HMW adiponectin: HMW/total adiponectin ratio varies versus total adiponectin, independent of clinical condition.* Clin Chim Acta, 2017. **465**: p. 30-33.
128. Mazaki-Tovi, S., et al., *Maternal serum adiponectin multimers in preeclampsia.* J Perinat Med, 2009. **37**(4): p. 349-63.
129. Masuyama, H., et al., *Correlation between soluble endoglin, vascular endothelial growth factor receptor-1, and adipocytokines in preeclampsia.* J Clin Endocrinol Metab, 2007. **92**(7): p. 2672-9.
130. Suwaki, N., et al., *Hypoadiponectinemia and circulating angiogenic factors in overweight patients complicated with pre-eclampsia.* Am J Obstet Gynecol, 2006. **195**(6): p. 1687-92.
131. Retnakaran, A. and R. Retnakaran, *Adiponectin in pregnancy: implications for health and disease.* Curr Med Chem, 2012. **19**(32): p. 5444-50.
132. Podymow, T. and P. August, *Update on the use of antihypertensive drugs in pregnancy.* Hypertension, 2008. **51**(4): p. 960-9.
133. Williams, P.J. and L. Morgan, *The role of genetics in pre-eclampsia and potential pharmacogenomic interventions.* Pharmgenomics Pers Med, 2012. **5**: p. 37-51.
134. Bienertova-Vasku, J., et al., *Is there any link between severe pre-eclampsia and defined polymorphisms in leptin and adiponectin genes?* J Obstet Gynaecol Res, 2008. **34**(5): p. 858-64.
135. de Faria, A.P., et al., *Adiponectin -11377C/G and +276G/T polymorphisms affect adiponectin levels but do not modify responsiveness to therapy in resistant hypertension.* Basic Clin Pharmacol Toxicol, 2015. **117**(1): p. 65-72.
136. Cunha, W.R., et al., *Effects of ADIPOQ and NOS3 SNPs/haplotypes on blood pressure control in patients with adherence to antihypertensive therapy.* Pharmacogenomics, 2023. **24**(5): p. 269-281.
137. Kato, M., et al., *Linkage disequilibrium of evolutionarily conserved regions in the human genome.* BMC Genomics, 2006. **7**: p. 326.

138. Tessema, G.A., A. Tekeste, and T.A. Ayele, *Preeclampsia and associated factors among pregnant women attending antenatal care in Dessie referral hospital, Northeast Ethiopia: a hospital-based study.* BMC Pregnancy Childbirth, 2015. **15**: p. 73.
139. Berzan, E., R. Doyle, and C.M. Brown, *Treatment of preeclampsia: current approach and future perspectives.* Curr Hypertens Rep, 2014. **16**(9): p. 473.
140. Sun, Q.Y., H.H. Zhou, and X.Y. Mao, *Emerging Roles of 5-Lipoxygenase Phosphorylation in Inflammation and Cell Death.* Oxid Med Cell Longev, 2019. **2019**: p. 2749173.
141. Marin, R., et al., *Oxidative stress and mitochondrial dysfunction in early-onset and late-onset preeclampsia.* Biochim Biophys Acta Mol Basis Dis, 2020. **1866**(12): p. 165961.
142. Beharier, O., K. Kajiwara, and Y. Sadovsky, *Ferroptosis, trophoblast lipotoxic damage, and adverse pregnancy outcome.* Placenta, 2021. **108**: p. 32-38.
143. Ortega, M.A., et al., *Oxidative Stress, Lipid Peroxidation and Ferroptosis Are Major Pathophysiological Signatures in the Placental Tissue of Women with Late-Onset Preeclampsia.* Antioxidants (Basel), 2024. **13**(5).
144. Feng, Y., et al., *Role of selectins and their ligands in human implantation stage.* Glycobiology, 2017. **27**(5): p. 385-391.
145. Holthe, M.R., et al., *Different levels of platelet activation in preeclamptic, normotensive pregnant, and nonpregnant women.* Am J Obstet Gynecol, 2004. **190**(4): p. 1128-34.
146. Lakhani, S.A., et al., *Caspases 3 and 7: key mediators of mitochondrial events of apoptosis.* Science, 2006. **311**(5762): p. 847-51.
147. Chen, H., Y. Chen, and Q. Zheng, *The regulated cell death at the maternal-fetal interface: beneficial or detrimental?* Cell Death Discov, 2024. **10**(1): p. 100.
148. Cali, U., et al., *Placental apoptosis in preeclampsia, intrauterine growth retardation, and HELLP syndrome: an immunohistochemical study with caspase-3 and bcl-2.* Clin Exp Obstet Gynecol, 2013. **40**(1): p. 45-8.
149. Hung, T.H. and G.J. Burton, *Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia.* Taiwan J Obstet Gynecol, 2006. **45**(3): p. 189-200.
150. Teimoori, B., et al., *The association of the placental CASPASE-3 gene polymorphisms and preeclampsia susceptibility and in-silico analysis.* J Cell Biochem, 2018. **119**(8): p. 6756-6764.
151. Rubanyi, G.M. and M.A. Polokoff, *Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology.* Pharmacol Rev, 1994. **46**(3): p. 325-415.
152. Saleh, L., et al., *The emerging role of endothelin-1 in the pathogenesis of pre-eclampsia.* Ther Adv Cardiovasc Dis, 2016. **10**(5): p. 282-93.
153. Rana, S., et al., *Preeclampsia: Pathophysiology, Challenges, and Perspectives.* Circ Res, 2019. **124**(7): p. 1094-1112.
154. Kinaan, M., H. Ding, and C.R. Triggle, *Metformin: An Old Drug for the Treatment of Diabetes but a New Drug for the Protection of the Endothelium.* Med Princ Pract, 2015. **24**(5): p. 401-15.
155. Hao, H., et al., *Protective effect of metformin on the NG-nitro-l-arginine methyl ester (l-NAME)-induced rat models of preeclampsia.* Biochem Biophys Res Commun, 2024. **739**: p. 150996.
156. Wang, W., et al., *Epidemiological trends of maternal hypertensive disorders of pregnancy at the global, regional, and national levels: a population-based study.* BMC Pregnancy Childbirth, 2021. **21**(1): p. 364.
157. Seidizadeh, O., F. Peyvandi, and P.M. Mannucci, *Von Willebrand disease type 2N: An update.* J Thromb Haemost, 2021. **19**(4): p. 909-916.
158. Aref, S. and H. Goda, *Increased VWF antigen levels and decreased ADAMTS13 activity in preeclampsia.* Hematology, 2013. **18**(4): p. 237-41.
159. Dallaglio, K., et al., *Paradoxic effects of metformin on endothelial cells and angiogenesis.* Carcinogenesis, 2014. **35**(5): p. 1055-66.
160. Phoenix, K.N., F. Vumbaca, and K.P. Claffey, *Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model.* Breast Cancer Res Treat, 2009. **113**(1): p. 101-11.

161. Martin, M.J., et al., *Metformin accelerates the growth of BRAF V600E-driven melanoma by upregulating VEGF-A.* Cancer Discov, 2012. **2**(4): p. 344-55.
162. Cittadini, A., et al., *Metformin prevents the development of chronic heart failure in the SHHF rat model.* Diabetes, 2012. **61**(4): p. 944-53.
163. Sena, C.M., et al., *Metformin restores endothelial function in aorta of diabetic rats.* Br J Pharmacol, 2011. **163**(2): p. 424-37.
164. O'Connor, B.B., et al., *The role of extracellular matrix in normal and pathological pregnancy: Future applications of microphysiological systems in reproductive medicine.* Exp Biol Med (Maywood), 2020. **245**(13): p. 1163-1174.
165. Martinez-Fierro, M.L., et al., *Current model systems for the study of preeclampsia.* Exp Biol Med (Maywood), 2018. **243**(6): p. 576-585.
166. Dowling, O., et al., *Magnesium sulfate reduces bacterial LPS-induced inflammation at the maternal-fetal interface.* Placenta, 2012. **33**(5): p. 392-8.
167. Faas, M.M., et al., *A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats.* Am J Obstet Gynecol, 1994. **171**(1): p. 158-64.