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Juliana de Oliveira Cruz

ALTERAÇÕES EPIGENÉTICAS ENVOLVIDAS NA VIA DE INVASÃO TROFOBLÁSTICA NA PRÉ-ECLÂMPSIA PRECOCE E TARDIA

Belo Horizonte
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Juliana de Oliveira Cruz

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Tese apresentada ao Programa de Pós-Graduação em Genética, da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética.

Orientador: Prof. Dr. Marcelo Rizzatti Luizon

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Às oito horas do dia **31 de janeiro de 2023**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"ALTERAÇÕES EPIGENÉTICAS ENVOLVIDAS NA VIA DE INVASÃO TROFOBLÁSTICA NA PRÉ-ECLÂMPSIA PRECOCE E TARDIA"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Marcelo Rizzatti Luizon**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

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Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 31 de janeiro de 2023.

Marcelo Rizzatti Luizon - Orientador

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
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Programa de Pós-Graduação em Genética

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"ALTERAÇÕES EPIGENÉTICAS ENVOLVIDAS NA VIA DE INVASÃO TROFOBLÁSTICA NA PRÉ-ECLÂMPSIA PRECOCE E TARDIA"

Juliana de Oliveira Cruz

Tese aprovada pela banca examinadora constituída pelos Professores:

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RESUMO

A pré-eclâmpsia (PE) é definida por hipertensão após a 20ª semana gestacional, podendo ser acompanhada de proteinúria ou danos em outros órgãos alvo. A PE pode ser classificada de acordo com a idade gestacional de início dos sintomas como de início precoce (EOPE < 34 semanas de gestação) e de início tardio (LOPE ≥ 34 semanas de gestação). Entretanto, não é claro se EOPE e LOPE possuem diferentes etiologias e fisiopatologias ou resultam de um processo gradual da mesma condição. O desenvolvimento placentário anormal e pobre invasão trofoblástica são fatores primários na PE. Diversos mecanismos epigenéticos participam da regulação gênica e modulam o desenvolvimento e diferenciação celular. O objetivo deste trabalho é avaliar alterações epigenéticas em regiões regulatórias de genes envolvidos na via de invasão trofoblástica em gestantes com PE (EOPE, LOPE) e gestantes saudáveis. No **capítulo 1**, foi realizada uma revisão sistemática para identificar genes diferencialmente metilados na placenta de gestantes com PE e as vias nas quais eles estão envolvidos. Descrevemos uma diferença significativa na metilação global do DNA (DNAm) entre PE e controle, e um efeito pronunciado na DNAm de genes específicos em PE, especialmente em EOPE e PE prematuro. A partir destes achados, selecionamos genes candidatos para análise de DNAm e expressão gênica em placenta e sangue periférico de gestantes com PE (EOPE e LOPE) e em controles. O **capítulo 2**, apresenta uma análise da metilação e expressão do gene *TIMP3* em placentas de gestantes com EOPE e LOPE. Identificamos 28 sondas diferencialmente metiladas no promotor do gene *TIMP3* em PE, 38 em EOPE, 20 em LOPE e quatro em PE a termo versus controles, e oito em EOPE versus LOPE. Além disso, encontramos uma hipometilação de mais de 70% nos grupos de comparação, e uma expressão aumentada de TIMP3 em amostras de placentas correspondentes de PE, EOPE e LOPE compradas com os controles. Esses achados destacam o papel da metilação do DNA na região promotora do gene *TIMP3* como um mecanismo epigenético envolvido na fisiopatologia da PE. O **capítulo 3**, apresenta os níveis de TIMP3 circulantes em gestantes com PE e controles. No estudo primário, pacientes com PE e hipertensão gestacional exibiram concentrações aumentadas da proteína TIMP-3 em comparação com mulheres grávidas saudáveis e mulheres não grávidas. Esses achados foram confirmados no estudo de replicação. Não foi encontrada diferença nas concentrações de TIMP-3 entre EOPE e LOPE. Além disso, as concentrações de TIMP-3 foram significativamente correlacionadas com as concentrações plasmáticas de TIMP-1 e MMP-2 na

PE. No **capítulo 4**, mostramos os mecanismos epigenéticos que regulam a expressão da isoforma N-terminal Truncada da Matrix Metalloproteinase-2 (NTT-MMP-2), pela ativação de um promotor latente localizado no primeiro íntron do gene *MMP2*. Demos destaque nas descobertas recentes sobre a presença da isoforma NTT-MMP-2 na disfunção renal e nas doenças cardíacas. Diante do exposto, podemos concluir que alterações epigenéticas em regiões regulatórias de genes que participam da via de invasão trofoblástica estão relacionadas ao desenvolvimento da pré-eclâmpsia e seus subtipos EOPE e LOPE.

Palavras-chave: CpG-SNPs. Invasão Trofoblástica. Metilação do DNA. Placentação.

ABSTRACT

Preeclampsia (PE) is defined as hypertension after the 20th gestational week and may be accompanied by proteinuria or damage to other target organs. PE can be classified according to the gestational age of onset of symptoms as early-onset (EOPE < 34 weeks of gestation) and late-onset (LOPE ≥ 34 weeks of gestation). However, it is unclear whether EOPE and LOPE have different etiologies and pathophysiology or result from a gradual process of the same condition. Abnormal placental development and poor trophoblastic invasion are primary factors in PE. Several epigenetic mechanisms participate in gene regulation and modulate cell development and differentiation. The objective of this work is to evaluate epigenetic changes in regulatory regions of genes involved in the trophoblastic invasion pathway in pregnant women with PE (EOPE, LOPE) and healthy pregnant women. In chapter 1, a systematic review was performed to identify differentially methylated genes in the placenta of pregnant women with PE and the pathways in which they are involved. We describe a significant difference in global DNA methylation (mDNA) between PE and control, and a pronounced effect on gene-specific mDNA in PE, especially in EOPE and premature PE. From these findings, we selected candidate genes for analysis of mDNA and gene expression in the placenta and peripheral blood of pregnant women with PE (EOPE and LOPE) and controls. In chapter 2, we present an analysis of TIMP3 gene methylation and expression in the placentas of pregnant women with EOPE and LOPE. We identified 28 differentially methylated probes in the TIMP3 gene promoter in PE, 38 in EOPE, 20 in LOPE, four in term PE compared to controls, and eight in EOPE compared to LOPE. Furthermore, we found hypomethylation of more than 70% in the comparison groups, and an increased expression of TIMP3 in samples from corresponding placentas of PE, EOPE, and LOPE compared to controls. These findings highlight the role of DNA methylation in the promoter region of the TIMP3 gene as an epigenetic mechanism involved in the pathophysiology of PE. In chapter 3, we present circulating TIMP3 levels in pregnant women with PE and controls. In the primary study, patients with PE and gestational hypertension exhibited increased concentrations of TIMP-3 protein compared to healthy pregnant women and non-pregnant women. These findings were confirmed in the replication study. No difference was found in TIMP-3 concentrations between EOPE and LOPE. Furthermore, TIMP-3 concentrations were significantly correlated with plasma concentrations of TIMP-1 and MMP-2 in PE. In Chapter 4, we show the epigenetic mechanisms that regulate the expression of the N-Terminal Truncated isoform of Matrix Metalloproteinase-2 (NTT-MMP-2), by activating a latent promoter located in the first

intron of the MMP2 gene. We highlighted the recent findings on the presence of the NTT-MMP-2 isoform in renal dysfunction and heart disease. Given the above, we can conclude that epigenetic alterations in regulatory regions of genes that participate in the trophoblastic invasion pathway are related to the development of preeclampsia and its EOPE and LOPE subtypes.

Keywords: CpG-SNPs. Trophoblastic Invasion. DNA Methylation. Placentation.

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1. INTRODUÇÃO

1.1 PRÉ-ECLÂMPSIA

A pré-eclâmpsia (PE) é a maior causa de mortalidade e morbidade materna e fetal no mundo, afeta mais de 9% das gestações e é responsável por cerca de 70.000 e 500.000 mortes maternas e fetais, respectivamente (RANA *et al.*, 2019), principalmente em países em desenvolvimento (DULEY, 2009). No Brasil, a PE tem uma prevalência de 8,2% (GUIDA *et al.*, 2022). Entretanto, é relatado que mulheres negras não hispânicas e indígenas americanas ou nativas do Alasca são afetadas desproporcionalmente pela PE (JHONSON; LOUIS 2022).

A PE é uma doença progressiva da gestação que envolve múltiplos órgãos (CHAPPELL *et al.*, 2021). A definição clínica da PE pode variar a depender das diretrizes formuladas por diferentes organizações internacionais (MOL *et al.*, 2016). A única característica consistente é a presença de nova hipertensão, que vem acompanhada de outros sintomas que distinguem a PE de outras doenças hipertensivas gestacionais. A PE é definida por nova hipertensão (Pressão Arterial Sistólica ≥ 140 mmHg e Pressão Arterial Diastólica ≥ 90 mmHg) mensuradas mais de duas vezes com diferença de 4 horas (PHIPPS *et al.*, 2019). Uma atualização dos critérios de diagnóstico do *American College of Obstetricians and Gynecologists* (ACOG) retirou a presença da proteinúria como um critério clínico, desde que a hipertensão seja acompanhada por danos em outros órgãos alvo, tais como trombocitopenia, disfunção hepática, insuficiência renal, edema pulmonar, restrição de crescimento fetal e novos distúrbios cerebrais ou visuais (ACOG, 2019; ACOG, 2020; PHIPPS *et al.*, 2019).

A PÉ tem etiologia heterogênea e pode apresentar múltiplos processos fisiopatológicos, por vezes sobrepostos e que, portanto, ativam vias que levam a uma manifestação clínica comum, o aumento da pressão arterial (ROBERTS *et al*., 2021). Dessa forma, uma das possíveis subclassificações da PE é de acordo a idade gestacional de início dos sintomas, como: a PE de início precoce (EOPE, do inglês *early-onset preeclampsia*), que se inicia antes da 34ª semana de gestação; a PE de início tardio (LOPE, do inglês *late-onset preeclampsia*), que se inicia na 34ª ou após a 34ª semana de gestação. Embora tais subtipos apresentem características clínicas similares, a EOPE é associada com um pior desfecho materno e fetal, com complicações como a restrição de crescimento intrauterino e nascimentos prematuros (ACOG, 2019; KOVO *et al.*, 2012; VON DADELSZEN; MAGEE; ROBERTS, 2003). Achados prévios suportam que alterações da placenta são mais frequentes na EOPE (EGBOR *et al.*, 2006; NISHIZAWA *et al.*, 2007; VAN DER MERWE *et al.*, 2010),

mas não é claro se EOPE e LOPE possuem diferentes etiologias e fisiopatologias ou podem resultar de um processo gradual de uma mesma condição (OGG *et al.*, 2012; PHIPPS *et al.*, 2016).

A etiologia da PE não é completamente compreendida, mas a predisposição é multifatorial. Os fatores de risco para desenvolvimento da PE incluem fatores demográficos, tais como idade, ancestralidade, primiparidade, pequeno período de coabitação sexual, gestação de gêmeos, múltiplas gestações, histórico familiar, estresse psicológico, condições médicas pré-existentes, e fatores ambientais, tais como obesidade, estilo de vida e poluição do ar (QU; KHALIL, 2020). Adicionalmente, há a influência de fatores genéticos, angiogênicos, metabólicos, imunológicos, estresse oxidativo, infecção por SARS CoV-2, entre outros fatores biológicos (WANG; RANA; KARUMANCHI, 2009; JUNG *et al*., 2022) da mãe e do feto que estão associados ao desenvolvimento da PE (GRAY; SAXENA; KARUMANCHI, 2018). A herdabilidade genética estimada da PE é de aproximadamente 55%, sendo 35% materna e 20% fetal/paterna (CNATTINGIUS *et al.*, 2004; ESPLIN *et al.*, 2001; LIE *et al.*, 1998; NILSSON *et al.*, 2004; SERRANO *et al.*, 2020).

A patogênese da PE pode ser originada por dois estágios: a placentação anormal e pelo desenvolvimento de síndrome materna. Na placentação anormal, a PE se origina na placenta e começa com a síndrome placentária. Uma placenta pré-eclâmptica possui condições patológicas como aterose (obliteração parcial, espessamento de camadas e presença de vasos hialinizados), estreitamento esclerótico das artérias e arteríolas, disposição de fibrinas e infartos, que são característicos da hipoperfusão placentária e isquemia, e parecem se correlacionar com a gravidade da PE (HECHT *et al.*, 2017; ZHANG, 2018).

A isquemia placentária, hipóxia, estresse oxidativo e desregulação de fatores imunológicos são característicos da PE por placentação anormal. A isquemia placentária e hipóxia são demonstradas pelas mudanças fisiológicas nas artérias espiraladas que são restritas à decídua na PE, enquanto em gestações normais se estende até próximo ao miométrio (BROSENS; ROBERTSON; DIXON, 1972). Além disso, há uma invasão superficial da decídua, que resulta em segmentos estreitos e não dilatados das artérias espiraladas, que acaba levando a hipoperfusão uterina (BURTON *et al.*, 2009; ZHOU *et al.*, 1997; ZHOU; DAMSKY; FISHER, 1997). Acredita-se que o estresse oxidativo tenha um papel fundamental no desenvolvimento da placenta. É postulado que a regressão normal das vilosidades periféricas no início da gestação ocorre por um mecanismo que envolve estresse oxidativo e apoptose (HUNG; SKEPPER; BURTON, 2001). Um desbalanço entre mecanismos oxidantes e pró-oxidantes está presente na PE, que pode ser por remodelamento

defeituoso da artéria espiralada que leva a lesões por repetidos eventos de isquemia e reperfusão (BURTON *et al.*, 2009).

A PE por síndrome materna demonstra como as marcas da PE não são restritas à placenta e levam a danos generalizados na mãe. As lesões patológicas da PE e eclâmpsia são caracterizadas por danos endoteliais difundidos em vários órgãos (HECHT *et al.*, 2017). A síndrome materna engloba processos patológicos como a endoteliose glomerular, o desbalanço de fatores angiogênicos, a hipertensão, a disfunção endotelial, a obesidade, a resistência à insulina e outros fatores (PHIPPS *et al.*, 2019).

1.2 PLACENTAÇÃO

A placenta é um órgão vital com funções endócrinas, imunológicas e fisiológicas. A placenta é o primeiro órgão materno-fetal a se desenvolver e desempenha um papel fundamental na saúde da mãe e do feto, impactando até a saúde futura de ambos. O desenvolvimento placentário anormal é fator primário nas grandes síndromes gestacionais tais como a PE, restrição do crescimento fetal, aborto espontâneo recorrente e natimorto. Mesmo diante do papel fundamental da placenta na gestação, o conhecimento acerca do seu desenvolvimento ainda é limitado (TURCO; MOFFETT, 2019).

A formação da placenta ocorre após a implantação do blastocisto no endométrio entre 7-9 dias após a fertilização. A fase de blastocisto consiste essencialmente no trofectoderma e massa celular interna que posteriormente se divide em epiblasto e endoderma primitivo. Após a implantação, a invasão inicial do embrião no endométrio que se transformará na decídua é alcançada pelas células trofoblásticas, sendo uma camada externa de sinciciotrofoblastos que envolve uma camada interna de células do citotrofoblasto proliferativo. Nessa fase também ocorre o desenvolvimento da linhagem proliferativa que secretam enzimas que digerem a decídua, permitindo sua expansão para os tecidos subsequentes, isso por volta de 12-15 dias pós fertilização (HEMBERGER; HANNA; DEAN, 2020).

Por volta do 18 dia pós fertilização o saco amniótico e vitelino, e o córion já estão formados. Por volta do 35 dia pós fertilização já é estabelecida a placenta madura que é formada pela expansão localizada das vilosidades coriônicas, cada uma consistindo de um núcleo mesenquimal que contém vasos sanguíneos fetais, uma camada de células citotrofoblásticas e uma camada externa de células sinciciotrofoblásticas, que se banham diretamente no sangue materno (HEMBERGER; HANNA; DEAN, 2020). Essa é uma breve descrição do complexo processo de placentação que envolve diferenciação, proliferação e

migração de diferentes tipos celulares e tem a participação de diversos hormônios e proteínas mediando esses processos (TURCO; MOFFETT, 2019).

As células trofoblásticas desempenham as principais funções da placenta. O sinciciotrofoblasto está em contato direto com as secreções glandulares e sangue materno, sendo assim, o principal local de troca materno-fetal de gases e nutrientes necessários ao desenvolvimento fetal-placentário (TEASDALE; JEANJACQUES, 1986). É também um importante órgão endócrino que secreta hormônios e proteínas na circulação materna que leva a adaptações fisiológicas e metabólicas da gestação. Além disso, é uma barreira imunológica protetora que não expressa moléculas de antígeno leucocitário humano, assim, mesmo na presença do alogênico, as células imunes não o detectam como o não próprio (MOFFETT; LOKE, 2006; TURCO; MOFFETT, 2019).

Os citotrofoblastos têm papel no remodelamento vascular. Eles migram da decídua para o miométrio e invadem a artéria espiralada materna, transformando-as de vasos de alta resistência e pequeno calibre em vasos de baixa resistência e alto calibre capazes de fornecer perfusão placentária adequada para sustentar o feto em crescimento. Durante o processo de invasão vascular, os citotrofoblastos se diferenciam de um fenótipo epitelial para um fenótipo endotelial, citotrofoblastos endovasculares, que substituem as células endoteliais maternas (Fig. 1) (WANG; RANA; KARUMANCHI, 2009). Depois da transformação arterial, os citotrofoblastos se movem de forma retrógrada pela artéria para formar um tampão que impede a entrada de líquido no espaço interviloso até o final do primeiro trimestre, quando é estabelecida a circulação hemocorial completa (TURCO; MOFFETT, 2019). Na PE, os citotrofoblastos não conseguem adotar um fenótipo endotelial invasivo. Em vez disso, a invasão das artérias espirais é superficial e elas permanecem vasos de resistência de pequeno calibre (WANG; RANA; KARUMANCHI, 2009).

Além das células trofoblásticas, a placenta contém outras células como fibroblastos, células imunológicas e vasculares no núcleo do estroma das vilosidades. Essas células são geradas a partir do mesênquima extraembrionário, provavelmente do citotrofoblasto viloso que sofre transição epitélio mesênquima, ou se origina no epiblasto com contribuição do mesoderma embrionário após a gastrulação (BOSS; CHAMLEY; JAMES, 2018). Esses diferentes tipos celulares encontrados na placenta exibem diferentes características morfológicas, transcricionais e epigenéticas, apresentando sinais celulares específicos e que podem levar a associações espúrias em estudos genéticos e epigenômicos na placenta (ROBINSON *et al.*, 2019).

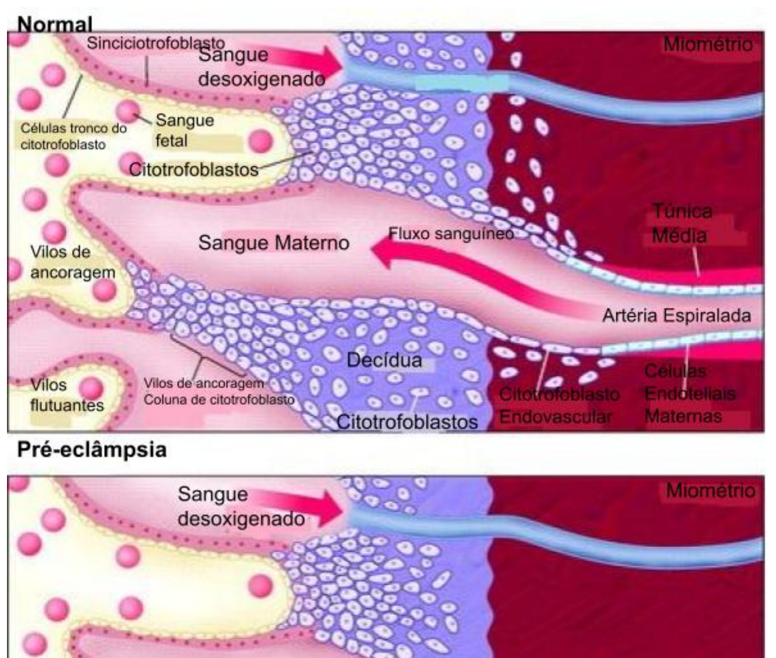

Figura 1: Placentação normal e placentação na PE. Adaptado de WANG; RANA; KARUMANCHI, 2009.

1.3 MODIFICAÇÕES EPIGENÉTICAS

A regulação da expressão gênica é promovida por mecanismos epigenéticos, tanto durante o desenvolvimento quanto em células e tecidos diferenciados (CARLBERG; MOLNÁR, 2018). Essa regulação é realizada a nível pré- e pós-transcricional, modulando a ligação de fatores de transcrição e outras proteínas regulatórias. Os mecanismos epigenéticos incluem a metilação do DNA e RNA, modificações de histonas, interação promotor-acentuador e ação de diferentes RNAs não codificadores (CHEN *et al.*, 2017). No presente trabalho, o foco será na metilação do DNA e nas modificações de proteínas histonas, mesmo sabendo que os outros tipos de modificações também têm papel na fisiopatologia da PE.

A metilação do DNA genômico de citosinas é a modificação epigenética mais bem estudada em humanos e outros mamíferos. No processo de metilação do DNA, ocorre a adição de um grupo metil no carbono 5 da citosina. Essa adição do grupo metil é mediada pelas enzimas DNA metiltransferases (Fig. 2A) (JONES, 2012). A princípio, qualquer

citosina pode ser metilada na sua 5ª posição, mas a metilação em citocinas que são seguidas por guaninas - dinucleotídeos CpGs - são as que têm mais conhecimento da sua funcionalidade, em particular se ocorrem em agrupamentos (*clusters*). Esses *clusters*, denominados de ilhas CpGs, são regiões genômicas de 500 a 2000 pb de tamanho e alta densidade (mais de 55%) de sítios CpGs (Fig. 2B). As Ilhas CpG estão geralmente associadas a regiões reguladoras como promotores de genes ou outras regiões de ligação a fatores de transcrição/proteínas, que tendem a ser hipometiladas para permitir que ocorram essas ligações (DEATON; BIRD, 2011). A metilação de ilhas CpGs localizadas em promotores gênicos é naturalmente associada à repressão gênica, mas a metilação na região do corpo gênico foi descrita associada com genes ativos, desencadeando o aumento da transcrição e/ou *splicing* alternativo (SMITH; MEISSNER, 2013).

Outro ponto de regulação da expressão genética são os polimorfismos de nucleotídeos únicos (SNPs, do inglês Single *Nucleotide Polymorphisms*) localizados em sítios CpGs (CpG-SNPs), que podem controlar os níveis de metilação do DNA de uma população por inserir ou abolir sítios CpGs e, consequentemente, interferir na ligação de proteínas regulatórias (HOFFMANN *et al.*, 2017; HU; LI, 2018) (Fig. 2C). Nesse contexto, a metilação alelo específica pode se sobrepor a *loci* de risco conhecidos e auxiliar na interpretação de variantes associadas a doenças complexas (HU; LI, 2018). Entretanto, a correlação entre SNPs e metilação alelo específica não foi examinada na PE.

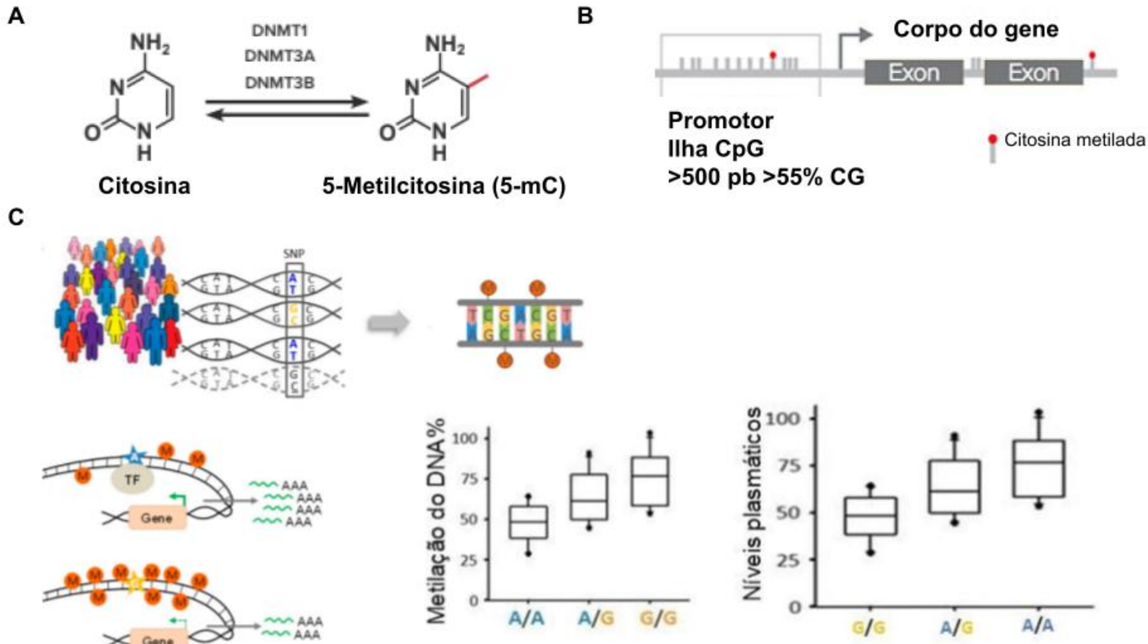

Figura 2: **A)** Representação esquemática do processo de metilação do DNA em citosinas. **B)** Representação esquemática de uma ilha CpG (>500 pb, >55% CG) localizada no promotor de um gene. **C)** Papel dos SNPs na metilação do DNA. A presença da base guanina (G, em amarelo) ao invés da adenina (A, em azul) cria um sítio de metilação na sequência, como visto à direita. Esse é um exemplo de variante genética que afeta a metilação do DNA, onde a base A em um elemento regulatório é sítio de ligação para um fator de transcrição, que se liga e realiza a transcrição. Na presença da base C, que está metilada, o fator de transcrição não se liga, reduzindo assim a transcrição. Como visto à direita os gráficos de comparação dos níveis de metilação e níveis plasmáticos, que são correlacionados negativamente, tendo aumento da metilação no genótipo G/G com redução dos níveis plasmáticos no mesmo. Adaptado de HOFFMANN *et al*., 2017.

Apesar da metilação em dinucleotídeos CpGs ser a mais importante funcionalmente, a metilação também pode ocorrer em dinucleotídeos CpH (H=A, C ou T) que parece ter um papel na memória epigenética (CARLBERG; MOLNÁR, 2018). As enzimas DNA metiltransferases (DNMTs), são uma família de enzimas canônicas e não canônicas que realizam a metilação do DNA genômico. A DNMT1 é responsável pela manutenção da metilação do DNA durante o processo de replicação, juntamente com um complexo com a enzima UHRF1 que reconhece os domínios hemi-metilados e recruta a DNMT1 para realizar a adição do grupo metil (BOSTICK *et al.*, 2007). As enzimas DNMT3A e DNMT3B realizam a metilação *de novo* nos estágios iniciais da embriogênese, mas há relatos de ação das DNMT3A e DNMT3B em estágios diferenciados na ausência da DNMT1 funcional. Essas três enzimas são as “escritoras” da metilação do DNA (Fig. 2) (CARLBERG; MOLNÁR, 2018; JIA *et al.*, 2007; LYKO, 2018).

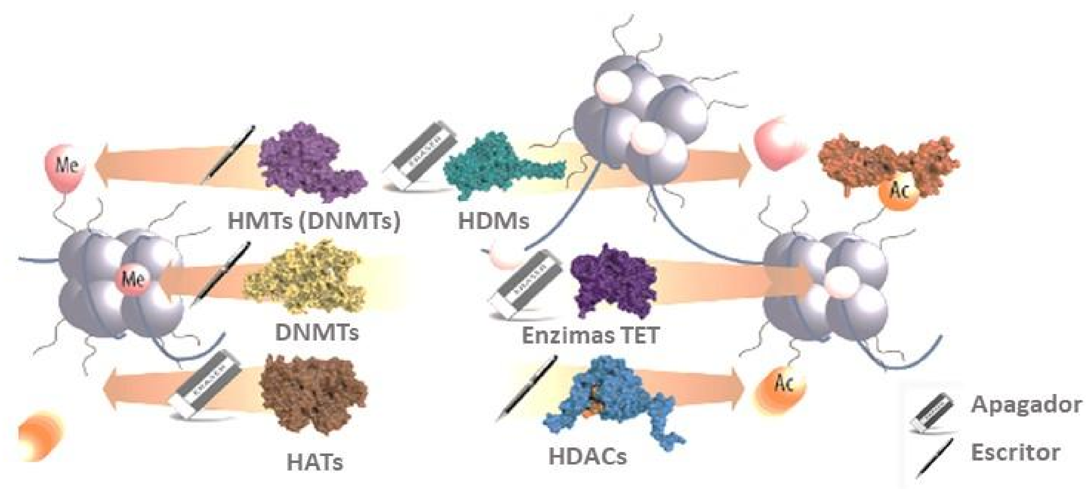

Figura 3: Enzimas escritoras e apagadoras das modificações covalentes de histonas e DNA genômico. As enzimas DNMTs realizam a metilação do DNA genômico e das proteínas histonas, as marcas de metilação do DNA são apagadas de forma ativa pelas enzimas TET e das histonas são apagadas pelas histonas demetilases (HDMs). A acetilação das histonas é realizada pelas histonas acetilases (HATs) e a desacetilação pelas histonas desacetilases (HDACs). Adaptado de CARLBERG; MOLNÁR, 2018.

Na ausência da DNMT1/UHRF1 funcional, ciclos repetidos de replicação do DNA levam a perda passiva do grupo metil com um apagamento global da 5mC do genoma materno durante a pré-implantação. A desmetilação ativa é um processo de várias etapas que envolvem as enzimas metilcitosina dioxigenases da família *ten-eleven translocation* (TET): TET1, TET2, TET3, que convertem 5mC em 5hmC. Em outras duas rodadas de oxidação, as TETs convertem 5hmC em 5-formilcitosina (5fC) e em 5-carboxilcitosina (5caC). As citosinas oxidadas são desaminadas para 5-hidroxiuracil (5hmU), que cria uma incompatibilidade de ligação com a guanina, e é removida pela glicosilase TDG. O local é então reparado pela maquinaria de reparo por excisão de base, resultando na desmetilação geral da citosina específica (Fig. 3) (CARLBERG; MOLNÁR, 2018; LYKO, 2018).

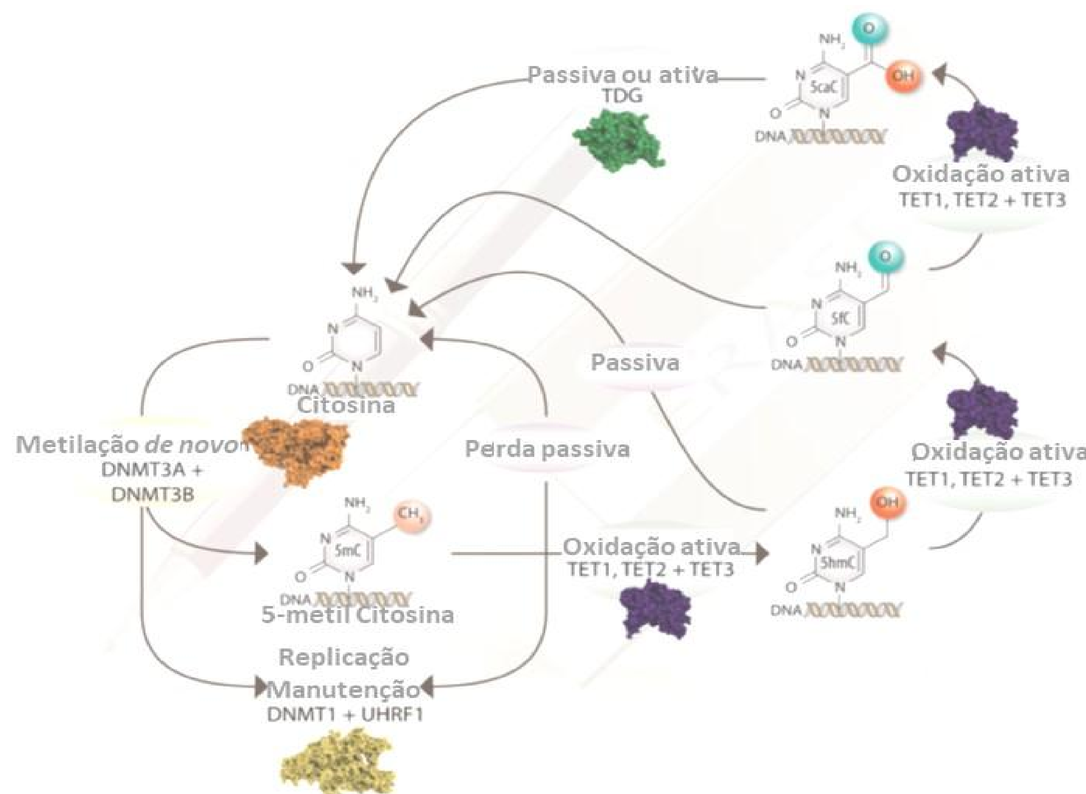

Figura 4: Escrita e apagamento da metilação do DNA. A metilação *de novo* do DNA é realizada pelas enzimas DNMT3A e DNMT3B e a metilação de manutenção e replicação é realizada pela DNMT1 em conjunto com o UHRF1. O apagamento da metilação é realizado de forma passiva na ausência das DNMTs e de forma ativa pela ação das enzimas TETs. Adaptado de CARLBERG; MOLNÁR, 2018.

Há um grande esforço da comunidade científica para entender a distribuição e funcionalidade da metilação do DNA, que levou ao surgimento de vários métodos de mapeamento da 5-mC. Embora existam muitas abordagens para estudar a metilação do DNA, a melhor técnica deve ser determinada com base nos objetivos e recursos para o experimento, sendo que cada método possui vantagens e desvantagens. Esses métodos são divididos em quantificação global da DNAm, enriquecimento da DNAm e a análise da DNAm com resolução de um único nucleotídeo. Entre esses métodos, chamo atenção para o último que engloba as tecnologias: *Whole Genome Bisulfite Sequencing* (WGBS), *Reduced Representation Bisulfite Sequencing* (RRBS), *Targeted Bisulfite Sequencing* e *Methylation Arrays*. Essas tecnologias têm variação de resolução, cobertura, quantidade de DNA necessário para o experimento, vantagens, desvantagens e custo. O *Methylation Arrays* é o mais indicado para identificação de regiões diferencialmente metiladas em amostras clínicas (SUN, CUNNINGHAM, SLAGER, KOCHER, 2015; BECK, BEN, SKINNER, 2022).

O *Methylation Arrays* permite a quantificação da metilação de citocinas específicas. O DNA é tratado com bissulfito de sódio e amplificado por meio do PCR para mostrar o padrão

de DNAm. Esse DNA é então combinado com sondas complementares que visam locais de metilação específicos. Duas sondas por alvo podem ser utilizadas, uma complementar a citosina metilada e outra a citosina não metilado. Alternativamente, uma única sonda pode ser utilizada para distinguir entre os estados de metilação com base em uma única extensão de base sobre o local de metilação. Nucleotídeos marcados de forma diferente revelam níveis de metilação de um sinal que é específico e quantificável para o estado não metilado ou metilado em cada local. A cobertura é alta (>96%) para ilhas CpG, entretanto, é perdido a maioria dos outros (>98%) sítios CpG no genoma (SUN, CUNNINGHAM, SLAGER, KOCHER, 2015; BECK, BEN, SKINNER, 2022).

As proteínas histonas são modificadas por enzimas, as chamadas modificações pós-traducionais. Essas proteínas recebem grupos químicos como metil, acetil, fosfato, entre outros em suas caudas. As modificações das histonas participam da regulação da expressão gênica modulando o grau de compactação da cromatina (CARLBERG; MOLNÁR, 2018; CHEN *et al.*, 2017). A metilação de histonas é realizada pelas histonas metiltransferases (HMTs) e ocorre frequentemente nas histonas H3 e H4 em resíduos específicos de lisina (K) e arginina (A), que podem ser mono-, di- ou tri-metiladas. A metilação da lisina da histona pode levar à ativação ou à inativação a depender da posição genômica onde está localizada. A metilação das H3K9, H3K27 e H4K20 são importantes marcas de inativação, ou seja, reprimem a transcrição por formação de heterocromatina. Já a metilação de H3K4 e H3K36 são consideradas marcas de ativação, deixando a sequência de DNA disponível para a transcrição (CARLBERG; MOLNÁR, 2018; CHEN *et al.*, 2017; JENUWEIN; ALLIS, 2001). A acetilação de histonas, que em sua maioria ocorre nos resíduos de lisina, é realizada pelas enzimas histonas acetiltransferases (HATs) e geralmente está associada a ativação e abertura da cromatina. A desacetilação é realizada pelas enzimas histonas desacetilases (HDACs) que leva a condensação da cromatina e a inativação da transcrição gênica (CARLBERG; MOLNÁR, 2018).

Uma molécula de RNA que não é traduzida em proteína é definida como RNA não codificante, cujas classes incluem RNAs de transferência e RNAs ribossomais, e os pequenos RNAs como microRNAs (miRNAs), siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs e os lncRNAs que são de grande importância na regulação gênica (APICELLA *et al.*, 2019; HOMBACH; KRETZ, 2016).

1.4 MODIFICAÇÕES EPIGENÉTICAS NA PLACENTAÇÃO

Os mecanismos epigenéticos regulam a expressão tanto na diferenciação celular, quanto no desenvolvimento dos tecidos placentários (HENIKOFF; GREALLY, 2016; ZHANG; PRADHAN, 2014) modulando a acessibilidade de fatores de transcrição e outras proteínas regulatórias pré e pós-transcricional (APICELLA *et al.*, 2019). Há descrição de metilação e acetilação de histonas, lncRNA, miRNAs e metilação no corpo do gene e em regiões regulatórias envolvidos no desenvolvimento placentário (ABELL *et al.*, 2011; APICELLA *et al.*, 2019; DOKRAS *et al.*, 2006; RAHAT *et al.*, 2016). O estudo das modificações epigenéticas na placenta e na PE é recente, mas a DNAm é o mecanismo epigenético mais bem explorado na placenta, existindo várias revisões que tratam sobre o tema (APICELLA *et al.*, 2019; CRUZ *et al.*, 2020; ROBINSON *et al.*, 2019; ROBINSON; PRICE, 2015). A maior parte do epigenoma placentário possui altos níveis de metilação do DNA (SCHROEDER *et al.*, 2013). Entretanto, domínios parcialmente metilados cobrem 37% do epigenoma da placenta e são hipometilados, essa hipometilação é constante durante a gestação e entre indivíduos (SCHROEDER *et al.*, 2013).

Recentemente, foi descrito o papel da DNAm na diferenciação do trofoblasto humano (GAMAGE *et al.*, 2018). Linhagem de células trofoblásticas lateral, o citotrofoblasto viloso e trofoblasto extraviloso todos isolados da mesma placenta de primeiro trimestre, possuem metilomas diferentes, sendo um perfil de metilação mais semelhante entre os trofoblastos da população lateral e os citotrofoblastos, enquanto o trofoblasto extraviloso apresentou a maior diferença entre eles (GAMAGE *et al.*, 2018). Trofoblastos de população lateral apresentam diferenças de metilação de genes e miRNAs envolvidos na regulação de ciclo celular e pluripotência; e os trofoblastos extravilosos apresentam metilação diferencial em genes envolvidos na transição epitelial-mesenquimal e nas vias de metástase, que podem dar a aquisição da capacidade invasiva das células. Entretanto, não foi observada uma correlação positiva entre metilação e redução da expressão desses genes, o que sinaliza para o efeito de outras marcas epigenéticas nesse processo (GAMAGE *et al.*, 2018).

A ação da família de genes homeobox (HOX) é crucial para o desenvolvimento da placenta humana (CHUI *et al.*, 2010; OUDEJANS *et al.*, 1990; QUINN *et al.*, 1997; RAJARAMAN *et al.*, 2008) e a maioria deles se encontram hipometilados no decorrer da gestação. No entanto, alguns dos genes *HOX* apresentam padrão de metilação variável, com uma tendência para aumento da metilação ao longo da gestação. *TLX1*, *HOXA10* e *DLX5* têm um aumento da metilação e redução da expressão ao longo da gestação (NOVAKOVIC *et al.*, 2017). O silenciamento desses genes por siRNA em trofoblastos primários desencadeia a perda de proliferação e um aumento de expressão de marcadores de diferenciação, como o

ERVW-1, que sugere que a redução dessas proteínas é necessária para um desenvolvimento do sinciciotrofoblasto (NOVAKOVIC *et al.*, 2017).

O processo de placentação tem características semelhantes à invasão de tumores malignos. O epigenoma de células cancerosas e células da placenta apresenta semelhanças do padrão de metilação, com destaque para uma hipometilação em todo genoma com particular hipermetilação em ilhas CpGs (NORDOR *et al.*, 2017; SCHROEDER *et al.*, 2013). Notavelmente, a hipometilação na placenta não é uniforme. Os domínios parcialmente metilados (>100 Kb) são regiões de metilação do DNA reduzida que cobrem cerca de 37% do genoma placentário (SCHROEDER *et al.*, 2013). Até o momento, esses domínios parcialmente metilados são descritos apenas para a placenta, culturas celulares e células cancerosas (NORDOR *et al.*, 2017; SCHROEDER *et al.*, 2013; SCHROEDER; LASALLE, 2013).

Essa hipometilação do genoma placentário e de células cancerosas afeta regiões do genoma que contém genes relacionados à transição epitélio mesenquimal, resposta imune e inflamação, todas essas vias são associadas ao fenótipo de câncer. Essa semelhança do início do processo de placentação e os tumores malignos também é observada no padrão de metilação dos genes supressores tumorais *RASSF1A* e *SERPINB5*. A metilação do DNA no promotor desses genes supressores tumorais regula sua expressão e afeta a capacidade de migração e invasão das células trofoblásticas (CHIU *et al.*, 2007; DOKRAS *et al.*, 2006).

Um agrupamento de genes localizados no cromossomo 19 (C19MC) e outro no cromossomo 14 (C14MC) expressa um grande número de miRNAs detectados na placenta (BENTWICH *et al.*, 2005; DONKER *et al.*, 2012; MORALES-PRIETO *et al.*, 2013). O agrupamento C19MC inclui 46 genes de miRNA intrônicos que expressam 58 espécies de miRNA regulados por uma região de *imprinting* paterno rica em dinucleotídeos CpGs (NOGUER-DANCE *et al.*, 2010; TSAI *et al.*, 2009). Esses miRNA são exclusivos de primatas e expressos quase que exclusivamente na placenta, sendo chamados de trofimiRs. O primeiro agrupamento é expresso em células embrionárias e em células tronco, mas sua expressão é reduzida quando as células já estão diferenciadas, indicando um papel na manutenção do estado celular indiferenciado (BAR *et al.*, 2008; REN *et al.*, 2009; STADLER *et al.*, 2010). Na placenta humana, os miRNA deste agrupamento são detectados na 5ª semana gestacional e aumentam gradualmente no decorrer da gestação (ZHANG; WANG; SU, 2008).

O cluster C14MC inclui os miRNAs: miR-127, miR-345, miR-370, miR-431 e miR-665 que tem expressão reduzida no decorrer da gestação e estão envolvidos na regulação da resposta imunossupressora, anti-inflamatória e resposta isquemia/hipóxia (GU *et al.*, 2013;

LIANG *et al.*, 2007). Há descrição de inúmeros miRNAs provavelmente envolvidos no desenvolvimento da placenta, regulando genes que participam da diferenciação, invasão e proliferação das células trofoblásticas (APICELLA *et al.*, 2019).

Os lncRNAs atuam na proliferação, invasão, migração e progressão do ciclo celular (MCANINCH; ROBERTS; MIOTO, 2017), mas seu papel no desenvolvimento da placenta ainda é pouco conhecido (APICELLA *et al.*, 2019). Os genes *H19, SPRY4-IT1, MIR503HG, LINC00629, MEG3, MALAT1, RPAIN* e *TUG1* são alguns dos lncRNAs descritos no desenvolvimento da placenta (BRANNAN *et al.*, 1990; CHEN *et al.*, 2015; MUYS *et al.*, 2016; SONG *et al.*, 2017; XU *et al.*, 2017; ZHANG *et al.*, 2015; ZOU *et al.*, 2013). Por fim, os achados sobre modificações de histonas na placenta são escassos e, em sua maioria, baseados em estudos em camundongos (APICELLA *et al.*, 2019).

1.5 ALTERAÇÕES GENÉTICAS NA PE

Polimorfismos genéticos e alterações epigenéticas são a base de muitas doenças complexas, incluindo a PE. Os mecanismos epigenéticos são cada vez mais considerados decisivos no início e gravidade das doenças placentárias em humanos (VAIMAN, 2017). A maior parte do conhecimento sobre o desenvolvimento inicial da placenta são oriundos de estudos em camundongos e mostram a dependência de fatores de transcrição altamente conservados em mamíferos eutérios, o que sugere que parte das descobertas podem ser aplicadas para humanos, mesmo com as possíveis diferenças nas cascatas gênicas (NIAKAN; EGGAN, 2013). Cada uma das linhagens de células da placenta depende de um subconjunto específico de genes (MALTEPE *et al.*, 2010).

A predisposição genética à PE é poligênica e os genes podem estar envolvidos na má adaptação imunológica, isquemia placentária e estresse oxidativo (MÜTZE *et al.*, 2008). De acordo com estes autores, genes candidatos podem ser agrupados de acordo com seu papel fisiológico. O grupo I inclui os genes *AGT*, *ACE*, *AT1R*, *Renin*, *PRCR*, *ET-1*, *ERα*, *ERβ*, *eNOS*, *ENG*, *ERAP1*, *ERAP2* e *VEGF*, que inclui as proteínas vasoativas e do remodelamento vascular que participam do sistema renina-angiotensina regulando a pressão arterial, o remodelamento dos vasos e o volume de fluído durante a gestação, tendo ainda uma interação com o sistema imune (SHAH, 2006; WILSON *et al.*, 2003). A trombose vilosa e infartos na placenta são característicos da PE, e genes envolvidos na trombofilia e hipofibrinólise estão

inclusos no grupo II: *FVL*, *protrombina*, *MTHRF*, *PAI-1*, *Fator V*, *Leiden*, *GPIIIa*, *β-fibrogin*, *Fator XIIIA*, *β-sintase*, *Fator VII e cistationina.*

O estresse oxidativo na placenta, resultante da placentação anormal e posterior lesão por isquemia e reperfusão tem papel central na etiologia da PE (GUPTA; AGARWAL; SHARMA, 2005). Adicionalmente, alterações em genes envolvidos na geração de espécies reativas de oxigênio podem levar ao aumento da disfunção endotelial por meio da peroxidação lipídica (GUPTA; AGARWAL; SHARMA, 2005; WILSON *et al.*, 2003). O grupo III inclui os genes envolvidos no estresse oxidativo, metabolismo de lipídios e danos endoteliais: *EPHX*, *GST*, *CYP1A1*, *SOD2*, *LPL*, *LEP*, *ApoE*, *CETP*, *LIPC*, *LIPE*, *ApoCIII*, *MPO*, *NADPH/NADH oxidase*.

A resposta imunológica de adaptação materno-fetal, que inclui componente paterno, é central na patogênese da PE (SIBAI; DEKKER; KUPFERMINC, 2005; WILSON *et al.*, 2003). Assim, o grupo IV inclui genes candidatos que codificam componentes do sistema imunológico (MÜTZE *et al.*, 2008), dentre eles *HLA*, *TNFα*, *IL-1α e β*, *IL-6*, *IL-1RA*, *IL-10*, *IFNγ*, *TGF*, *receptor CD14* e *CTLA-4*. A maioria dos estudos de associação de polimorfismos dos genes dos grupos I a IV, descritos acima, não observaram associação significativa. Embora alguns estudos apresentam resultados promissores, a maioria indica que tais genes não são determinantes, mas sim contributivos na etiologia da PE (MÜTZE *et al.*, 2008).

Genes relacionadas a placentação e *imprinting* estão inclusos no grupo V. A má placentação é vista como causa primária da PE, assim têm sido propostos alguns genes conhecidos no processo de diferenciação e manutenção das células trofoblásticas em roedores e seus homólogos em humanos, entre eles *Mash2*, *Hand1, Cdx2, Tead4* e *Gcm1* (HARAM; MORTENSEN; NAGY, 2014; VAIMAN, 2017). Outros genes nesse grupo incluem os inibidores de serina protease da placenta (SERPINs), a família de retrovírus HERV-W (ex. *sincintina I* e *II*), metaloproteinases de matriz extracelular (MMPs) e seus inibidores teciduais (TIMPs), *WWTR1* e *ACVR2* (BUIMER *et al.*, 2008; HARAM; MORTENSEN; NAGY, 2014; MÜTZE *et al.*, 2008; SOYGUR; MOORE, 2016). O *imprinting* genômico na placenta é crítico para a placentação normal e desenvolvimento do embrião e do fetal (TYCKO, 2006). Agrupamentos envolvendo genes como *H19*, *STOX1, CTNNA3* e *KCNMA1* estão em vias desreguladas na PE (VAN DIJK *et al.*, 2005).

O grupo VI inclui os fatores de crescimento *IGF-I* e *II, PIGF, IGFBP* e seu ligante *PAPPA2*, *FLT1*, que tem papel crítico na regulação do crescimento fetal e da placenta no decorrer da gestação (BERMINGHAM *et al.*, 2000; DIZON-TOWNSON *et al.*, 1996; GICQUEL; LE BOUC, 2006; GODDARD *et al.*, 2007; WILSON *et al.*, 2003).

Acima foram citados apenas alguns dos possíveis genes candidatos com papel na fisiopatologia da PE, para a qual a base genética é indiscutível. Entretanto, entender os mecanismos subjacentes à sua fisiopatologia é uma tarefa difícil por várias razões. Primeiro, pela interação gênica e de processos genéticos/epigenéticos. Segundo, pois a PE afeta cerca de metade da população, mas só manifesta na idade reprodutiva e é restrita à gravidez. Além disso, ainda tem os problemas metodológicos de definição da PE, critérios de inclusão e exclusão, diferença étnica, efeito genético materno e fetal (paterno) e tamanho amostral (COLHOUN; MCKEIGUE; SMITH, 2003; MÜTZE *et al.*, 2008; ROBERTS; GAMMILL, 2005).

1.6 ALTERAÇÕES EPIGENÉTICAS NA PE

A DNAm é o mecanismo epigenético mais bem estudado na placenta (ROBINSON *et al.*, 2019). Alterações na DNAm na PE são muito bem documentadas na placenta, linhagens celulares trofoblásticas, sangue periférico materno, células brancas do cordão umbilical e células materna endotelial (APICELLA *et al.*, 2019). A alteração de expressão em vários genes na placenta com PE é em parte explicada por alteração da DNAm (VAIMAN; CALICCHIO; MIRALLES, 2013). Tais estudos mostram um grande número de genes diferencialmente metilados envolvidos em vias decisivas no processo de placentação, e há um padrão de hipometilação no epigenoma de placentas de gestantes com PE comparadas com controle (APICELLA *et al.*, 2019; CRUZ *et al.*, 2020). A lista de genes diferencialmente metilados na placenta de gestação normal e com PE é extensa, como apresentado nas revisões (APICELLA *et al.*, 2019; CRUZ *et al.*, 2020).

O estudo pioneiro sobre miRNAs na placenta de gestantes com PE identificou 153 miRNAs, sendo miR-210, miR-155, miR-200b regulados positivamente na PE (PINELES *et al.*, 2007). A primeira análise global identificou 11 miRNAs regulados positivamente e 23 regulados negativamente. Muitos dos clusters regulados negativamente são encontrados nas regiões 13q31.3, 14q32.31, Xq26.2, Xq26.3, enquanto clusters regulados positivamente são encontrados na região 19q13.42 (ZHU *et al.*, 2009). Uma análise integrativa dos dados disponíveis identificou redes regulatórias envolvidas na resposta aos níveis de nutrientes, diferenciação celular, junção celular e componentes da membrana (BIRÓ; NAGY; RIGÓ, 2017). A análise de diferentes tipos celulares foi conduzida para identificar miRNAs diferencialmente expressos na placenta e em amostras de plasma de mulheres com PE devido

ao seu potencial como biomarcador clínico (APICELLA *et al.*, 2019). Análises transcriptômicas dos RNAs totais da placenta identificaram lncRNAs diferencialmente expressos entre PE e controle, tendo uma diferença entre EOPE e LOPE (LONG *et al.*, 2016; TONG *et al.*, 2018). A maioria desses lncRNAs já foram identificados previamente em células cancerosas, associados a proliferação, migração e invasão celular (HOSSEINI *et al.*, 2017).

Pouco se sabe sobre a participação das modificações pós-traducionais das caudas das histonas (código de histonas) na regulação gênica em PE (APICELLA *et al.*, 2019). Por exemplo, a cascata de sinalização HIF-KDM3A-MMP12 promove a invasão trofoblástica e remodelamento da artéria espiralada em ratos e na placenta humana. A condição de hipóxia leva a ativação de HIF e a expressão de KDM3A, que altera a perfil de metilação das histonas de genes que promovem o desenvolvimento das células trofoblásticas invasivas e o remodelamento do tecido com ativação do MMP12 no trofoblasto (CHAKRABORTY *et al.*, 2016). A hipóxia também atua na histona demetilase JMJD6, que tem sua atividade muito reduzida na placenta com PE comparada com controle (ALAHARI *et al.*, 2018). As HDACs já foram investigadas na placenta com PE, mas apenas a HDAC9 teve regulação negativa a nível de mRNA e proteína no sinciciotrofoblasto. Nocautes gênicos de HDAC9 em células HTR-8/SVneo inibem a invasão e migração das células trofoblásticas. Interessante notar que *TIMP3*, um inibidor de MMPs envolvido na invasão e remodelamento tecidual, é um alvo direto da HDAC9 e é regulado positivamente na ausência da HDAC9 (XIE *et al.*, 2019).

Mesmo com o aumento de estudos sobre o papel das marcas epigenéticas na regulação do desenvolvimento placentário e na PE, pouco se sabe realmente como essas modificações alteram a expressão gênica e qual a dinâmica das diferentes marcas epigenéticas no tecido placentário e desenvolvimento da PE (APICELLA *et al.*, 2019).

2. JUSTIFICATIVA

Estudos de metilação do DNA (DNAm) em diferentes tecidos sugerem a existência de alterações de metilação em larga escala no genoma em processos de desenvolvimento de doenças. Os diferentes tipos celulares encontrados na placenta exibem diferentes características morfológicas, transcricionais e epigenéticas, apresentando sinais celulares específicos e que podem levar a associações espúrias em estudos genéticos/epigenéticos na placenta.

A PE é uma doença heterogênea e multifatorial, considerada a principal complicação da gestação. Portanto, é importante entender os mecanismos fisiopatológicos que podem contribuir para o desenvolvimento da PE. Nesse contexto, estudos epigenômicos que examinem tecidos ou células específicas podem contribuir para o entendimento do processo placentário normal e anormal. Portanto, é importante avaliar mecanismos epigenéticos em regiões regulatórias de genes que participam de vias relacionadas à fisiopatologia da PE, como a via de invasão trofoblástica.

3. HIPÓTESE

Há diferenças no epigenoma de placentas de gestantes com PE e sem PE, e essas diferenças são particulares entre EOPE e LOPE.

4. OBJETIVO GERAL

Avaliar alterações epigenéticas em regiões regulatórias de genes envolvidos na via de invasão trofoblástica em gestantes com PE (EOPE, LOPE) e gestantes saudáveis.

4.1. OBJETIVOS ESPECÍFICOS

1) Identificação de genes candidatos envolvidos em vias de invasão trofoblástica;

2) Investigar o papel da metilação do DNA na regulação da expressão do gene *TIMP-3* na PE, nos subtipos EOPE e LOPE, e em gestantes sem PE.

3) Avaliar o potencial mecanismo de alterações epigenéticas na regulação da isoforma truncada do gene *MMP-2* e sua relação com doenças cardíacas e renais.

CAPÍTULO 1

INVESTIGAÇÃO DE GENES CANDIDATOS ENVOLVIDOS EM VIAS DE TROFOBLÁSTICA

O conteúdo deste objetivo foi publicado na Revisão Sistemática citada nesta referência:

CRUZ, J. O., CONCEIÇÃO, I. M. C. A., TOSATTI, J. A. G., GOMES, K. B., LUIZON, M. R. Global DNA methylation in placental tissues from pregnant with preeclampsia: A systematic review and pathway analysis. *Placenta*, *101*, 97–107, 2020.

CAPÍTULO 2 E 3

INVESTIGAR O PAPEL DA METILAÇÃO DO DNA NA REGULAÇÃO DA EXPRESSÃO DO GENE *TIMP-3* NA PE, NOS SUBTIPOS EOPE E LOPE, E EM GESTANTES SEM PE.

O conteúdo deste objetivo foi publicado nos artigos originais citados nestas referências:

CRUZ, J. O., CONCEIÇÃO, I. M. C. A., SANDRIM, V. C., LUIZON, M. R. Comprehensive analyses of DNA methylation of the TIMP3 promoter in placentas from early-onset and late-onset preeclampsia. *Placenta*, *117*, 118–121, 2022.

PALEI, A. C., CRUZ, J. O., CHAGURI, J. L., *et al*. Circulating levels of tissue inhibitor of metalloproteinase 3, a protein with inhibitory effects on angiogenesis, are increased in pre-eclampsia [published online ahead of print, Nov 5]. *Int J Gynaecol Obstet,* 2022.

CAPÍTULO 4

AVALIAR O POTENCIAL MECANISMO DE ALTERAÇÕES EPIGENÉTICAS NA REGULAÇÃO DA ISOFORMA TRUNCADA DO GENE MMP-2 E SUA RELAÇÃO COM DOENÇAS CARDÍACAS E RENAIS.

O conteúdo deste objetivo foi publicado no artigo de revisão citado nesta referência:

Cruz, J. O., Silva, A. O., Ribeiro, J. M., Luizon, M. R., Ceron, C. S. Epigenetic Regulation of the N-Terminal Truncated Isoform of Matrix Metalloproteinase-2 (NTT-MMP-2) and Its Presence in Renal and Cardiac Diseases. *Frontiers in genetics*, *12*, 637148, 2021.

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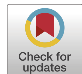

Global DNA methylation in placental tissues from pregnant with preeclampsia: A systematic review and pathway analysis

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ABSTRACT

Pre-eclampsia (PE) is the major cause of fetal and maternal mortality and can be classified according to gestational age of onset into early-onset (EOPE, <34 weeks of gestation) and late- (LOPE, ≥34 weeks of gestation). DNA methylation (DNAm) may help to understand the abnormal placentation in PE. Therefore, we performed a systematic review to assess the role of global DNAm on pathophysiology of PE, focused on fetal and maternal tissues of placenta from pregnant with PE, including EOPE and LOPE. We searched the databases EMBASE, Medline/PubMed, Cochrane Central Register of Controlled Trials, Scopus, Lilacs, Scielo and Google Scholar, and followed the MOOSE guidelines. Moreover, we performed pathway analysis with the overlapping genes from the included studies. Twelve out of 24 included studies in the qualitative analysis considered the classification into EOPE and LOPE. We did not found heterogeneity in the criteria used for diagnosis of PE, and a few studies evaluated whether confounding factors would influence placental DNAm. Fourteen out of 24 included studies showed hypomethylation in placental tissue from pregnant with PE compared to controls. The differences in DNAm are specific to genes or differentially methylated regions, and more evident in EOPE and preterm PE compared to controls, rather than LOPE and term PE. The overlapping genes from included studies revealed pathways relevant to pathophysiology of PE. Our findings highlighted the heterogeneous results of the included studies, mainly focused on North America and China. Replication studies in different populations should use the same placental tissues, techniques to assess DNAm and pipelines for bioinformatic analysis.

1. Introduction

Preeclampsia (PE) is defined as a new-onset hypertension (systolic blood pressure (SBP) ≥ 140 mmHg and diastolic blood pressure (DBP) ≥ 90 mmHg) after 20 weeks of gestation, which may be combined with proteinuria [1]. PE affects up to 9% of all pregnancies and is the major cause of fetal and maternal mortality and morbidity [2]. PE has a heterogeneous etiology and is classified according to gestational age of onset into late- (LOPE, ≥34 weeks of gestation) and early-onset (EOPE, <34 weeks of gestation), which is considered a more severe form of PE [3–5]. However, it is unclear whether EOPE and LOPE have different etiologies and pathogenesis or are the graduation of the same condition [6,7].

DNA methylation (DNAm) is primarily restricted at context of addition of a methyl group to the C5 position of the cytosine-guanine dinucleotide (CpG) [8]. Methylation of CpG sites are naturally associated with transcriptional repression when located in gene promoters, but with increased transcription when located in gene body [9,10]. Most of the human placental methylome is hypermethylated, but 37% of it is covered by partially methylated domains that are hypomethylated and constant through gestation and between individuals [11,12].

Notably, different cell types of placenta exhibit different transcriptional, epigenetic, and morphological features, which can conceal cell-specific signals and lead to spurious associations in different DNAm studies in PE [13]. In this context, epigenomic studies examining tissue or cell-specific signatures may contribute to understand both the normal and abnormal placentation processes, Therefore, it is relevant to assess the available DNAm data in placental tissues from PE pregnant.

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In this study, we aimed to perform a systematic review to assess the role of global DNAm in the pathophysiology of PE focused on the side of placental tissue evaluated and considering the classification into EOPE and LOPE. Moreover, we performed pathway analysis with the overlapping genes found in the included studies.

2. Materials and methods

This study was conducted according to The Cochrane Handbook for Systematic Reviews of Interventions guideline [14], and results will be reported in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist [15]. The protocol of current study was registered on International Prospective Register of Systematic Reviews (PROSPERO [CRD42020161780]).

2.1. Search strategy

The search question was composed by Population, Variable, Outcome (PVO) (Population = pregnant, Variable = preeclampsia, Outcome = methylation). A literature review was conducted by searching the electronic databases EMBASE, Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Scopus, Lilacs (Latin American and Caribbean Health Sciences), Scielo and Google Scholar to identify studies published until April 2020 that investigated PE, placenta, and global DNAm. The initial search included the Medical Subject Headings (MeSH) entry terms: 'Pregnancy', 'Pre-Eclampsia' and 'DNA Methylation', which were then included for a high-sensitivity search strategy in the Medline/PubMed, as described on Supplementary Material 1.

The same terms were used to search for gray literature and conference proceedings (Google Scholar). All potentially eligible studies were considered for review, regardless the language and publication date.

2.2. Inclusion and exclusion criteria

We included case-control studies including EOPE, LOPE or PE as case group, and control groups without chronic hypertension or gestational hypertension, gestational diabetes mellitus (GDM) and other well-known risk factors. The outcome was considered as the comparison of DNAm between PE and control groups.

We excluded studies that did not report the placental tissue or that evaluated only chorionic villous tissue, trophoblast cell lines, blood cells, and whole blood. We further excluded studies without matched control groups, or with control group composed of pregnancies with complications other than PE, such as GDM.

2.3. Study selection and data extraction

Initially, the studies retrieved from the databases were input on a single electronic library and duplicates were excluded using the EndNote® software. Two reviewers (J.O.C. and I.M.C.A.C.) independently analyzed the titles and abstracts of articles retrieved, reviewed the full-text articles, and used a standard data extraction protocol. Any disagreements were solved by a third reviewer (M.R.L.). The extracted data included the sample size, study design, maternal age, gestational age, tissue evaluated, applied technique, criteria for diagnosis of PE, and classification into EOPE and LOPE.

2.4. Assessment of bias across studies

The risk of bias in individual studies was independently assessed by two reviewers (J.O.C. and I.M.C.A.C.) following the Newcastle-Ottawa Quality Assessment Scale, according to The Cochrane Handbook's recommendations [14]. The tool used is structured into five domains: (1) patient selection (generalization and applicability); (2) comparability of groups in the study; (3) methods for assessing outcomes (cohort studies); (4) evidence of exposure (case-control) and (5) adequate follow-up. Any disparity was solved by a third reviewer (M.R.L.).

2.5. Pathway analysis

We manually curated the overlapping genes found in the included studies (Supplementary Table 1), and interrogated them for significant well-curated signaling pathways obtained from KEGG 2019 Human Pathway [16] sorted by p-value ranking <0.5 using Enrichr [17].

3. Results

We found 988 publications in the electronic databases (Fig. 1). After exclusion of 353 duplicates, 635 articles were selected for title and abstract analysis. Of these, 515 articles were subsequently excluded for several reasons (Fig. 1), resulting in 120 studies for complete reading. Literature reviews, studies focused on the analysis of specific genes, and studies that did not specify the tissue evaluated were also excluded. Finally, 24 full-text articles remained for the systematic review [18–41] (Table 1).

3.1. Included studies

Out of the 24 articles included, 10 (41.6%) had data from North American populations, including Canada and USA [18–22,25,31,35,36,40], eight (33.3%) from China [23,26,29,30,34,37,38,41], three (12.5%) from The Netherlands [24,32,33], and other three (12.5%) from India [28], Republic of Korea [27] and Australia [39]. Among the studies, seven (29.1%) validated their results in another independent cohort [20,25,26,29,36,40,41], and eight (33.3%) presented internal validation with the same samples but using a different technique [19,21–23,30,34,35,39]. Thirteen studies (54.2%) evaluated the fetal side of placenta [18–21,24–26,32–34,36,39,40], four (16.6%) used the maternal side [22,28,29,38], and seven (29.2%) did not specify the placental side used [23,27,30,31,35,37,41]. Twelve studies (50%) considered the classification into EOPE and LOPE [20,23–25,29,32–36,40,41], and 14 (58.3%) used the technique Infinium Human Methylation 450 Bead Chip array [18–20,22,24,25,27,31–36,39] (Table 1).

Regarding the quality assessment according to Newcastle-Ottawa scale, two studies scored nine points [36,40], 11 scored eight [19,20,23–26,28,29,32,33,35] and other 11 scored seven [18,21,22,27,30,31,34,37–39,41] (Supplementary Table 2). The bioinformatic analysis of global DNAm data greatly varied among the included studies. Therefore, a meta-analysis was not possible due to the heterogeneity of placental tissues evaluated, the applied technique used to assess global DNAm, and the different methods used for bioinformatic analysis, which hindered the quantitative comparison between the included studies.

Regarding the criteria used for PE diagnosis, 21 studies (87.5%) were based in evidence of new-onset hypertension (SBP $\geq$ 140 mmHg and DBP $\geq$ 90 mmHg) and proteinuria ($\geq$0.3 g/day or $\geq$2+ dipstick in urine sample of 24 h) after 20 weeks of gestation [18–22,24–36,39–41]. Five of these studies were based in these criteria plus others, such as maternal organ dysfunction, hematological disturbances and uteroplacental dysfunction [21,27,36,39,40]. One study (4.2%) were based in new-onset hypertension (SBP $\geq$160 mmHg and DBP $\geq$110 mmHg) and significant proteinuria (42 g or 3+ in urine sample of 24 h) after 20 weeks of gestation [23]. Two studies (8.3%) did not describe the criteria used for PE diagnosis [37,38] (Table 1).

Fourteen studies (58.3%) found a decreased DNAm level in placentas from PE pregnant compared to controls [20,22,24–27,29–35,38,40]. Although 22 studies (91.6%) used paired maternal age [18–20,22–36,38–41], 18 (75%) used gestational age as covariate for the DNAm analysis [18,20–22,24–27,29,31–36,39–41]. The ratio of male/female of infants varied among studies, and 12 (50%) used gender as covariate [20–22,24,25,29,32,33,35,36,39,40].

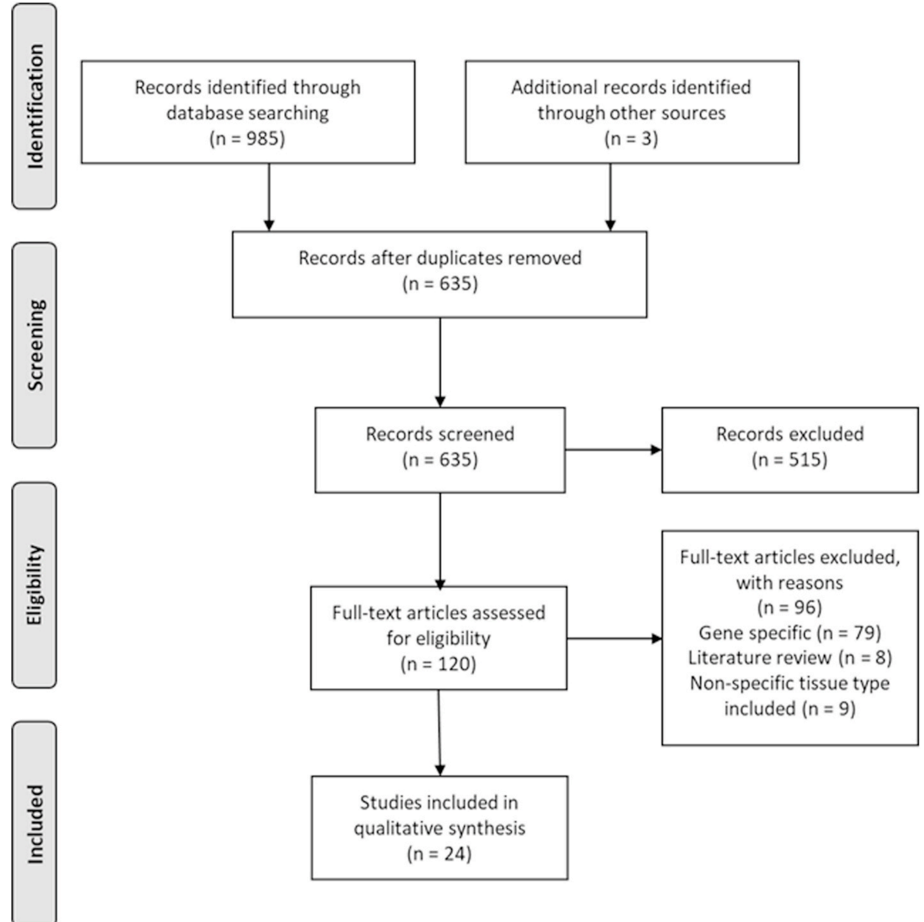

Fig. 1. Flow diagram of study selection for systematic review of published articles on the role of DNAm in placental tissue from pregnant with PE.

3.2. Global DNAm in placenta of PE pregnant compared to controls

Seven studies did not specify which side of placental tissue was analyzed [23,27,30,31,35,37,41] (Table 1). Four studies were case-control of PE pregnant compared to control, which found heterogeneous patterns of decreased DNAm levels in PE [27,30,31,37]. Remarkably, the number of differentially methylated genes (and the % of hypomethylated) reported were 3.878 (55.2%) [30], 1.664 (60.2%) [37], 617 (80.7%) [31] and 365 (89.9%) [27].

Notably, global DNAm was greatly discordant in maternal peripheral blood and placenta from PE pregnant, with 71 and 365 differentially methylated CpGs loci, respectively [27]. A total of 48 overlapping genes were found in the included studies (Fig. 2; Supplementary Table 1A), which were related to signaling pathways relevant to pathophysiology of PE (Fig. 3A).

Two studies found that global DNAm was significantly higher in EOPE compared to controls, but not statistically higher in LOPE compared to controls [23,41]. The methylation density in the Alu and LINE-1 repeats, and *H19* presented the same results for global methylation analysis [23]. Other study found 403 differentially methylated genes (68.2% hypermethylated) in placentas of LOPE [41].

PAPPA gene was exclusively hypomethylated in EOPE in other study, while the promoter and upstream enhancer regions of *INHBA* and *FN1* were hypomethylated in EOPE and LOPE + Intrauterine growth restriction groups. For these candidate genes, a positive correlation between DNAm and gene expression in placenta was found in case, but not in control group. The DNAm of *INHBA* and *FN1* was correlated with protein levels in maternal blood in the second and third trimester of gestation in PE, respectively [35]. Moreover, potential confounding factors in the assessment of DNAm showed an association of birthweight with *INHBA* and *FN1* methylation, and gestational age with *FN1* methylation [35].

3.3. Global DNAm in fetal side of placenta from PE pregnant compared to controls

Thirteen studies examined the DNAm in fetal side of placenta [18–21,24–26,32–34,36,39,40] (Table 1). Four case-control studies compared PE to controls [18,21,26,39]. One study did not find differentially methylated CpG sites between cases and controls. However, a significant correlation was found between methylation and gestational age corrected by birthweight, but no correlation with other clinical factors [21].

The methylation profiles of genes between studies were discordant in PE. For example, while one study found that 65.5% of 296 genes were hypomethylated [26], other study found that 70.6% of 303 genes were hypermethylated [39]. Notably, only *PPARG* (hypomethylated) and *ADORA2B* (hypermethylated) were commonly found in these studies [26,39]. In maternal peripheral blood, 207 CpG sites were differentially methylated (64% hypermethylated) in PE, and approximately 75% of them were concordant and hypermethylated in placenta [18].

Eight studies included the classification into EOPE and LOPE [20,24,25,32–34,36,40]. While 192 loci were hypomethylated in EOPE, none was differentially methylated in LOPE [40]. Conversely, 248 and 275 genes were differentially methylated (74.5% and 98.9% hypomethylated) in EOPE [20,34]. Multiple genes related to stress pathways and steroid production were associated with differentially methylated CpG sites in EOPE compared to controls. *NR3C1* and *CRHBP* were

Table 1
Characteristics of the studies examining global DNA methylation in placental tissues from pregnant with preeclampsia.

Author; Data; Country.	Study design; Duration (years); Study validation status	No. of patients; mean age (years mean ± standard deviation)		Mean gestational age (weeks mean ± standard deviation)		Sample size in the global analysis		Diagnosis criteria of PVO	Tissue evaluated	Applied technique
		Case	Control	Case	Control	Case	Control			
Anderson et al., 2014; USA [18]	Case-control study; NR; NR	PE: 6; 22.8 ± 1.4	6; 27.5 ± 3.65	38.4 ± 0.58	40 ± 0.49	PE: 6	6	New-onset hypertension (SBP≥ 140 mmHg or DBP≥ 90 mmHg) and proteinuria (≥+ 1 single sample or > 300 mg/24 h) after 20 weeks of gestation	Placental fetal side, white blood cells	Infinium HumanMethylation450 BeadChip array
Anton et al., 2014; USA [19]	Case-control study; 4; Internal validation	TPE: 19; 28.0 ± 8.1 PTPE: 12; 27.7 ± 7.6″	14; 27.0 ± 7.2	TPE: 38.9 ± 1.0; PTPE: 31.2 ± 4.0	39.2 ± 1.2	TPE: 19 PTPE: 12	14	New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Blair et al., 2013; Canada [20]	Case-control study; NR; Independent validation	EOPE: 20; 33.5 ± NR	20; 31.5 ± NR	31.8 ± NR	31.8 ± NR	EOPE: 20	20	New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Bourque et al., 2010; Canada [21]	Case-control study; NR; Internal validation	PE: 17; NR IUGR: 13; NR PE + IUGR: 21; NR	22; NR	PE: 35.9 ± NR IUGR: 35.4 ± NR PE + IUGR: 32.5 ± NR	39.0 ± NR	PE: 4IUGR: 5	5	(1) New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation (2) Sibai's criteria (3) British Eclampsia Survey Team criteria to define eclampsia	Placental fetal side	GoldenGate Methylation Cancer Panel 1 arrays
Chu et al., 2014; USA [22]	Case-control study; 10; Internal validation	PE; 24; 27.9 ± 7.2	24; 29.3 ± 5.4	35.9 ± 4.0	39.3 ± 1.2	PE: 24	24	New-onset hypertension (SBP ≥140 mm Hg and/or DBP ≥90 mm Hg) and proteinuria (≥300 mg of protein in 24 h or ≥2+ dipstick) after 20 weeks of gestation	Placental maternal side	Infinium HumanMethylation450 BeadChip array
Gao et al., 2011; China [23]	Case-control study; 2; Internal validation	EOPE: 10; 31.2 ± 5.1 LOPE: 10; 30.4 ± 3.7	24; 30.6 ± 4.1	EOPE: 32.3 ± 1.2 LOPE: 36.8 ± 2.1	38.3 ± 18.4	EOPE: 10 LOPE: 14	24	New-onset hypertension (SBP of ≥160 mmHg or DBP of ≥110 mmHg) and significant proteinuria (42 g per 24 h or ≥3+) after 20 weeks of gestation	Placenta	Immunohistochemistry
Herzog et al., 2017; The Netherlands [24]	Case-control study; 2; NR	EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5	Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1	EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9	Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9	EOPE: 13 LOPE: 16	Uncomp.: 36 FGR: 27 PTB: 20	New-onset hypertension (SBP≥140 and DSP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after the 20 weeks of gestation	Placental fetal side, UC-WBC, HUVEC	Infinium HumanMethylation450 BeadChip array
Hogg et al., 2013; Canada [25]	Case-control study; NR; Independent validation	EOPE: 19; 34.2 ± 6.0 LOPE: 18; 33.5 ± 5.5 nIUGR: 13; 34.7 ± 5.3	111; 33.10 ± 4.74	EOPE: 31.9 ± 3.3 LOPE: 37.5 ± 2.3 nIUGRn: 36.4 ± 2.3	35.1 ± 4.2	EOPE:19	19	New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/d or ≥2+ dipstick) after 20 weeks of gestation	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Jia et al., 2012; China [26]	Case-control study; NR; Independent validation	PE: 9; 29.0 ± 2.9	9; 28.0 ± 2.6	35.0 ± 2.6	39.4 ± 0.2	PE:3	3	New-onset hypertension (SBP >140 mmHg and DBP >90 mmHg) with proteinuria (300 mg/24 h) after 20 weeks of gestation	Placental fetal side	Methylated DNA immunoprecipitation (MeDIP)
Kim et al., 2016; Republic of Korea [27].	Case-control study; NR; NR	PE: 12; 32.3 ± 5.4	12; 31.6 ± 2.4	33.1 ± 3.3	33.1 ± 3.3	PE:12	12	New-onset hypertension (SBP ≥ 140 mmHg or DBP ≥ 90 mmHg) and proteinuria (>300 mg/day or >2+ dipstick) or other adverse conditions after 20 weeks of gestation	Placenta and peripheral blood	Infinium HumanMethylation450 BeadChip array
			30; 22.9 ± 3.2		39.2 ± 1.2		30			

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Table 1 (*continued*)

Author; Data; Country.	Study design; Duration (years); Study validation status	No. of patients; mean age (years mean ± standard deviation)		Mean gestational age (weeks mean ± standard deviation)		Sample size in the global analysis		Diagnosis criteria of PVO	Tissue evaluated	Applied technique
		Case	Control	Case	Control	Case	Control			
Kulkarni et al., 2011; India [28]	Case-control study; 2; NR	TPE: 30; 22.3 ± 3.0 PTPE: 27; 24.00 ± 3.7		TPE: 38.8 ± 0.9 PTPE: 34.0 ± 1.6		TPE: 30 PTPE: 27		New-onset hypertension (SBP >140 mmHg and DBP >90 mmHg) with proteinuria (>1þ or 300 mg/24 h) after 20 weeks of gestation	Placental maternal side	Methylamp Global DNA Methylation Quantification Kit
Li et al.,2020; China [29]	Case-control study; 1; Independent validation	EOPE: 20; 31.6 ± 4.6	TB: 20; 32.8 ± 5.1PB: 20; 31.5 ± 4.9	32.8 ± 1.4	TB: 39.1 ± 0.7; PB: 33.7 ± 1.7	EOPE: 4	TB:4; PB:4	New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks of gestation	Placental maternal side	Infinium HumanMethylation850 BeadChip array
Liu et al., 2014; China [30].	Case-control study; NR; Internal validation	PE: 27; 30.1 ± 2.7 GDM: 28; 30.8 ± 1.4	30; 29.7 ± 1.8	PE: 37.7 ± 0.9 GDM: 36.2 ± 0.8	36.4 ± 0.5	PE: 27 GDM: 28	30	New-onset hypertension (SBP ≥ 140 and DBP ≥90 mmHg) and proteinuria (≥2+ or ≥ 300 mg in 24 h) after 20 weeks of gestation	Placenta	385 K Human CpG Island plus Promoter arrays
Martin et al., 2015; USA [31]	Case-control study; NR; NR	PE: 19; 28.4 ± NR	17; 28.2 ± NR	38.6 ± NR	32.8 ± NR	PE:19	17	New onset hypertension (≥140/ 90 mmHg) and proteinuria (>300 mg of protein in a 24 h or protein/creatinine ratio of 0.3 mg/dL) after 20 weeks of gestation	Placenta	Infinium HumanMethylation450 BeadChip array
Van Den Berg et al., 2017; The Netherlands [32]	Case-control study; NR; NR	EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5	Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1	EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9	Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9	EOPE: 13 LOPE: 16	Uncomp.: 36 FGR: 27 PTB: 20	New-onset hypertension (SBP≥140 and DBP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after 20 weeks of gestation	Placental fetal side, UCL, HUVEC	Infinium HumanMethylation450 BeadChip array
Van Den Berg et al., 2020; The Netherlands [33].	Case-control study; NR; NR	EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5	Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1	EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9	Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9	EOPE: 13 LOPE: 16	Uncomp.: 36 FGR: 27 PTB: 20	New-onset hypertension (SBP≥140 and DBP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after 20 weeks of gestation	Placental fetal side, UC-WBC, HUVEC	Infinium HumanMethylation450 BeadChip array
Wang et al., 2019; China [34]	Case-control study; NR; Internal validation	EOPE: 30; 31.23 ± 5.26	30; 30.1 ± 4.0	33.7 ± 3.5	39.1 ± 2.3	EOPE:20	20	New-onset hypertension (SBP ≥140 mmHg and DBP ≥90 mmHg) and proteinuria (≥300 mg/day from 24 h) after 20 weeks of gestation	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Wilson et al., 2015; Canada [35]	Case-control study; NR; Internal validation	EOPE: 20; NR LOPE: 11; NR LOPE + IUGR: 8; NR IUGR: 10; NR	37; NR	NR	NR	EOPE: 20 LOPE: 11 LOPE + IUGR: 8 IUGR: 10	37	New-onset hypertension (SBP >140 and DBP >90 mm Hg) and proteinuria (>300 g/day) after 20 weeks gestation	Placenta	Infinium HumanMethylation450 BeadChip array
Wilson et al., 2018; Canada [36]	Case-control study; NR; Independent validation	EOPE: 22; 33.3 ± NR LOPE: 18; 34.0 ± NR IUGR: 11; 34.3 ± NR	PTC: 24; 32.5 ± NR TC: 19; 34.9 ± NR	EOPE: 32.0 ± NR LOPE: 37.4 ± NR IUGR: 36.6 ± NR	PTC: 32.6 ± NR TC: 38.4 ± NR	EOPE: 22 LOPE: 18 IUGR: 11	PTC: 24 TC: 19	New-onset hypertension (BSP >140 mmHg and >90 mmHg) and proteinuria (>300 mg/day) after 20 weeks gestation ii) HELLP syndrome without hypertension or proteinuria; or iii) eclamptic seizure without previous hypertension or proteinuria	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Xuan et al., 2016; China [37]	Case-control study; NR; NR	PE: 6; 29.8 ± NR	6; 30.2 ± NR	38.0 ± NR	39.5 ± NR	PE: 6	6	Not reported	Placenta	NimbleGen Human DNA Methylation 3 × 720 K CpG Island Plus RefSeq Promoter Microarray
Yan et al., 2013, China [38]	Case-control study; 1; NR	PE: 30; 28.5 ± 3.8	30; 27.9 ± 3.0	36.1 ± 2.3	39.2 ± 0.8	PE:5	5	Not reported	Placental maternal side	Agilent Human CpG Island Microarray
				35.0 ± 0.8	39.0 ± 0.2	PE:8	16			

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Table 1 (*continued*)

Author; Data; Country.	Study design; Duration (years); Study validation status	No. of patients; mean age (years mean ± standard deviation)		Mean gestational age (weeks mean ± standard deviation)		Sample size in the global analysis		Diagnosis criteria of PVO	Tissue evaluated	Applied technique
		Case	Control	Case	Control	Case	Control			
Yeung et al., 2016; Australia [39]	Case-control study; 13; Internal validation	PE: 8; 28.0 ± 2.0	16; 32.0 ± 1.0					New-onset hypertension (SBP ≥140 mmHg and/or DSP ≥90 mmHg) and proteinuria (2 on dipstick or 300 mg/24 h) or renal insufficiency, liver disease, neurological problems, and hematological disturbances after 20 weeks of gestation	Placental fetal side	Infinium HumanMethylation450 BeadChip array
Yuen et al., 2010; Canada [40]	Case-control study; NR; Independent validation	EOPE: 4; 33.3 ± NR LOPE: 4; 36.4 ± NR IUGR: 4; 37.3 ± NR	EOPE: 4; 36.0 ± NR LOPE: 5; 37.2 ± NR C: 5; 35.9 ± NR	EOPE: 31.0 ± NR LOPE 38.0 ± NR IUGR 33.0 ± NR	EOPE: 29.6 ± NR LOPE: 38.0 ± NR C: 37.8 ± NR	EOPE: 4 LOPE: 4 IUGR: 4	EOPE: 4 LOPE: 5 C: 5	(1) New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation (2) Sibai's criteria (3) British Eclampsia Survey Team criteria to define eclampsia	Placental fetal side	Illumina GoldenGate Methylation Cancer Panel I array
Zhu et al., 2015; China [41]	Case-control study; NR; Independent validation	LOPE: 20; 27.7 ± NR	20; 26.7 ± NR	38.2 ± NR	38.7 ± NR	LOPE: 20	20	New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks of gestation	Placenta	Methylated DNA immunoprecipitation + deep sequencing

Abbreviations: C, control; EOPE, early-onset PE; FGR, normotensive fetal growth restricted; GDM, gestational diabetes mellitus; HUVEC, human umbilical vein endothelial cells; IUGR, intrauterine growth restriction; NR, Not reported; LOPE, late-onset PE; nIUGR, normotensive Intrauterine growth restriction; PE, preeclampsia; PTB, normotensive preterm birth; PTC, preterm control; PTPE, preterm preeclampsia; TC, term control; TPE, term preeclampsia; UCL, umbilical cord leukocytes; UC-WBC, umbilical cord white blood cells; Uncomp., Uncomplicated; USA, United States of America.

hypermethylated, while regions associated with *CRH*, *CYP11A1*, *HSD3B1*, *TEAD3* and *CYP19* were hypomethylated in EOPE [25].

The comparison between EOPE and normotensive preterm births (PB) revealed 697 differentially methylated genes (67% hypomethylated) in placenta of EOPE. One differentially methylated CpG was found in EOPE compared to uncomplicated pregnancies controls and normotensive pregnancies with fetal growth restricted in placental tissue [24]. Significant differences in CpG methylation of circadian clock genes were found to be tissue-specific, in umbilical cord leukocytes (31), placenta (7), and HUVEC (1).

In placental tissue, the circadian clock genes *AKT1*, *BHLHE41*, *CSKN1E*, *PRDX1*, and *RORA* were hypomethylated in EOPE and significantly different from spontaneous PB [32]. The *CDH13*, *IGF2BP2* and *LSAMP* genes were also hypomethylated in placental tissue of EOPE and different from spontaneous PB. Notably, *CDH13* was hypermethylated in umbilical cord white blood cells of EOPE, and it was differentially methylated in EOPE compared to all study groups (uncomplicated controls, fetal growth restriction and PB) [33]. Other studies using the same set of samples found no difference in LOPE compared to all groups [24,31–33].

Noteworthy, six studies that examined the fetal side of placenta in EOPE or LOPE compared to controls had similar conclusions [20,24,25,32–34], and the number and methylation status of genes or regions are described above. In summary, five of these studies showed a pattern of hypomethylation in EOPE [20,24,32–34]. Differentially methylated CpGs sites were found in EOPE compared to controls [20,25,34]. EOPE were also different from spontaneous PB, fetal growth restriction and uncomplicated controls, and these differences were more evident when EOPE was compared to spontaneous PB controls [32,33]. Moreover, EOPE differed from spontaneous PB controls but not from fetal growth restriction or uncomplicated controls [24]. Notably, the differentially methylated sites were associated with cardiovascular system, stress pathways, steroid production and circadian clock genes. These findings suggest that EOPE have an increased placental dysregulation of DNAm, and support the hypothesis that EOPE and LOPE have different etiologies.

Most the 1.703 CpG sites were hypomethylated in EOPE compared to PB. Only five sites were differentially methylated between LOPE and term controls, which were not unique to LOPE [36]. Three studies showed that DNAm is affected by gestational age and fetus gender, which is a potential bias for DNAm analysis [20,25,40]. A total of 21

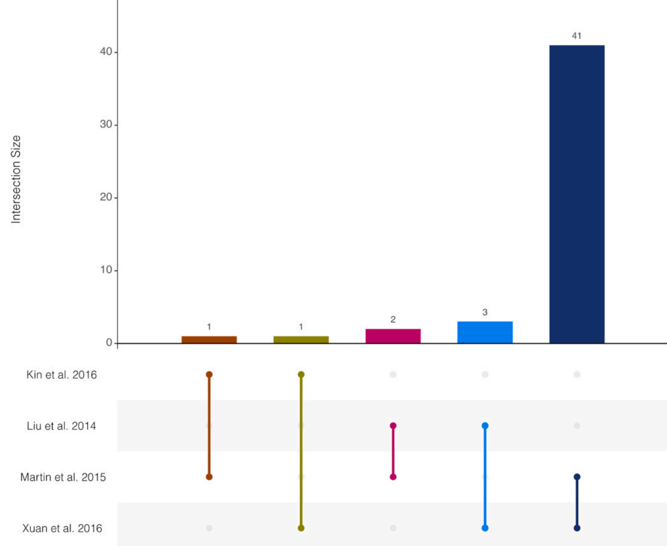

Fig. 2. Overlapping genes found in the included studies from PE pregnant compared to control groups (not specified the side of placental tissue evaluated).

overlapping genes were found among these studies (Fig. 4; Supplementary Table 1B), which were related to relevant pathways for PE (Fig. 3B).

Only one study defined preterm and term delivery for PE [19], and found 229 differentially methylated genes between controls and PE (term and preterm, 89.6% hypermethylated in PE) but none differentially methylated in term PE. Nevertheless, 1.448 differentially methylated genes were found between control and preterm PE (91.8% hypermethylated). Moreover, 118 differentially methylated genes were found between term and preterm PE (91.6% hypermethylated in term PE) [19].

3.4. Global DNAm in maternal side of placenta from PE pregnant compared to controls

Four studies considered the maternal side of placenta for DNAm analysis, and compared PE pregnant [22,29,38], term and preterm PE [28] to control, and one compared PE pregnant to PB and term birth (TB) [29].

While altered methylation levels were reported for 23 genes (52% hypermethylated) in PE [38], 10 hypomethylated CpG sites were identified in PE, and 49 differentially methylated CpG sites (78% hypomethylated) in EOPE [22]. The mean of global DNAm was higher in preterm and term PE compared to control, but the increase was significant only for term PE. Global DNAm was significant associated with SBP and DBP in term PE [28].

Global DNAm levels in placentas of PE were similar to PB, and the levels for PE and PB were higher than TB and, therefore, placental methylation levels were related to gestational age. Moreover, 2.400 differentially methylated genes were found between PE and TB (75.7% hypermethylated in PE), and 808 differentially methylated genes between PE and PB (68.8% hypomethylated in PE). Finally, 3.969

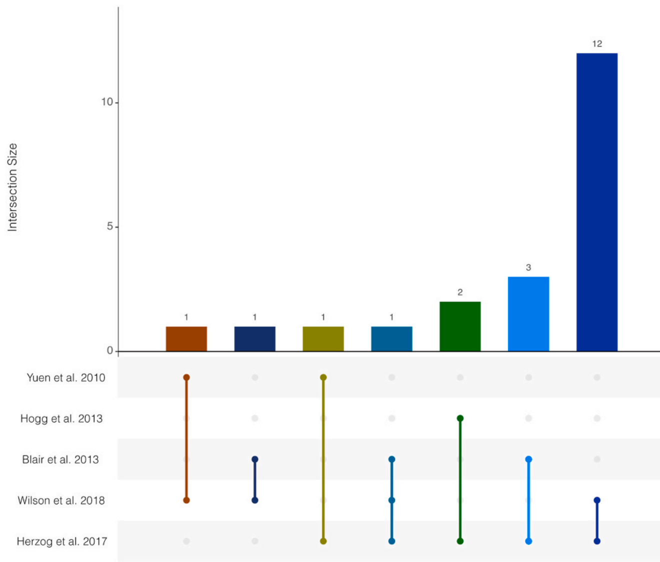

Fig. 4. Overlapping genes in the included studies in fetal side of placenta from EOPE and LOPE compared to control groups.

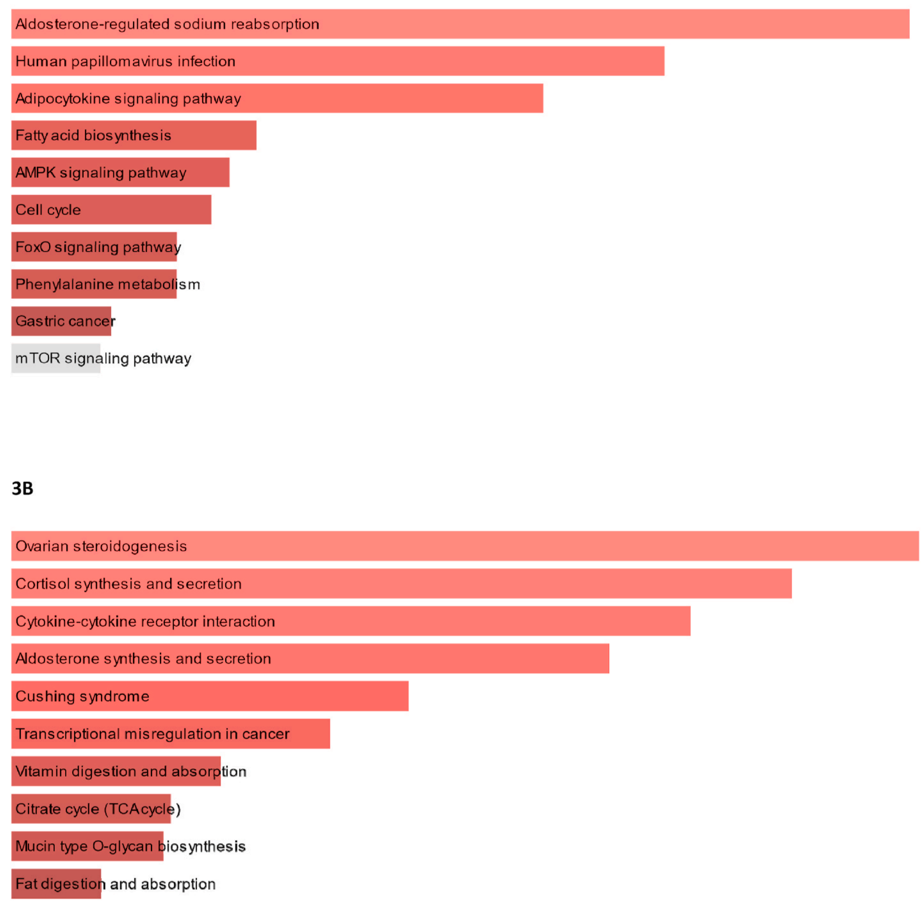

Fig. 3. Pathway analysis of the overlapping genes found among the included studies. **(A)** For studies performed with placental tissue from PE pregnant compared to controls (not specified the side of placental tissue). **(B)** For studies performed with fetal side of placenta from EOPE and LOPE compared to controls.

differentially methylated genes were found between PB and TB (80.4% hypermethylated in PB) [29].

4. Discussion

PE account for up to 26% of maternal deaths worldwide and is the main complication of pregnancy [3]. Therefore, it is important to understand the mechanisms that may lead to PE. This study is the first systematic review that assessed the role of global DNAm in the pathophysiology of PE, with focus on both maternal and fetal placental tissues. Our novel findings highlighted that the included studies show highly heterogeneous results, and that the differences between PE and controls are specific to genes or differentially methylated regions. These differences are more evident when EOPE and preterm PE were compared to controls, rather than LOPE and term PE. Most of the studies have a pattern of hypomethylation in placental tissue from PE pregnant compared to controls. Conversely, the placental methylome of normal pregnancy is hypermethylated outside the partially methylated domains [12].

It is important to highlight that tissues from the fetal or maternal side of placenta may account for the heterogeneous results of DNAm found among the studies included in this systematic review. We have also included studies that did not specify which side of placental tissue was analyzed, but their results were considered in a separate section. Therefore, the design of upcoming studies should focus on this methodological issue, which could help the understanding of the effects of different levels of DNAm on the different sides of placenta.

4.1. Confounding factors of DNA methylation in PE

The heterogeneous findings can be due to the multifactorial nature of PE, biological variation, study design and/or statistical analysis. Studies that examined whether confounding factors (age and fetal gender) would affect DNAm are discordant: some found an effect of maternal age and gestational age [20,22,31,35,39,40,42], but others did not [21]; some showed an effect of fetal gender [21,22,39,40,42], but another did not [21].

Placental DNAm showed a difference according to gestation stages [43], with a hypomethylation in early pregnancy and an increased methylation in later pregnancy [44]. A progressive increase of DNAm levels were found from the first to third trimesters, mainly in genes related to immune regulators, which reflect the placental immune modulation during pregnancy [45]. Therefore, the ideal design is to perform experiments with samples matched for both maternal and gestational age or follow-up studies to avoid and exclude this potential bias, and the exclusion of probes located in the sex chromosomes is required during the analysis, mainly in the X chromosome.

Genetic variation in different populations is another confounding factor that can alter DNAm in specific regions. For instance, probes that overlap with single nucleotide polymorphisms (SNPs) are usually excluded during bioinformatic analysis of global DNAm. However, SNPs that occur within CpG dinucleotide (CpG-SNPs) may lead to alteration of methylation in a region and thereby affect gene expression [46]. The correlation of CpG-SNPs with complex diseases is well-described [47], including type 2 diabetes [48] and cancer [49]. However, the association between CpG-SNPs and PE has not been examined. Further studies focused on potential CpG-SNPs and promoter region methylation may help to interpret findings from candidate gene association studies in PE for *NAMPT* [50,51], *NOS2* [52], *NOS3* [53] and *TIMP1* [54]. Potential CpG-SNPs of these candidate genes were also studied in subgroup of PE who were nonresponsive to antihypertensive therapy, and these follow-up epigenetic studies may further help to reveal targets for PE therapy [55,56].

4.2. Use of DNA methylation as a prognostic marker in PE

The placenta is a complex temporary organ that provides the fetal development, and composed by several number of cells and exhibiting regional variations. Placenta is divided into maternal and fetal sides, and the later carry the paternal genome [57]. A cohort analysis found that 35% of the genetic predisposition to PE is attributed to maternal characteristics and 20% to fetal effects that include the paternal genome, which suggest a genetic influence on PE development. The remaining is attributed to couple, environment and unmeasured factors [58].

DNAm is a tissue-specific epigenetic mark [59]. Notably, it is difficult to interpret DNAm data obtained from different pregnant related tissues and cell cultures. For example, only EOPE is related with levels of DNAm of circadian clock and clock-controlled genes in placental and newborn tissues. The same samples of placenta showed a decrease of differentially methylated CpG sites compared to umbilical cord leukocytes [32]. Approximately 75% of differentially methylated CpG sites overlapped between maternal white blood cell in first trimester compared to fetal side of placenta [18]. However, placental tissue showed an increase in differentially methylated CpG loci compared to peripheral blood at delivery [27].

Unfortunately, studies including the maternal side of placenta are scarce, and the DNAm data in the maternal and fetal sides of placenta showed highly discordant results. Therefore, to establish which placental side would be most suitable for studies focused on diagnostic markers for PE is unclear. Moreover, it is difficult to extrapolate the results from placenta to a description of diagnostic markers based on DNAm in the maternal blood. Further studies comparing DNAm between the maternal side of placenta and peripheral blood throughout gestation could help to establish novel diagnostic markers for PE.

4.3. Role of DNA methylation in the pathophysiology of PE

Our review highlighted a differential DNAm pattern in EOPE and preterm PE. These findings suggest that methylation have a pathophysiological role on early stages of pregnancy, and that placental epigenetic dysregulation may affect the initial steps of these early severe forms of PE. Indeed, EOPE was shown to exhibit a more severe form of PE [5], and a remarkable placental dysfunction [4].

The causes of PE are heterogeneous. Usually, there is a failure in the remodeling of the spiral uterine arteries by the trophoblastic cells, which can be triggered by an exacerbated immune response at the maternal-fetal interface [60]. These events are associated to poor placental perfusion, leading to physiological changes and gene expression in response to hypoxia and reoxygenation [61].

Transcriptional and epigenetic mechanisms control placental development and cytotrophoblast differentiation, and are activated by oxygen levels during pregnancy [62]. In primary cultures of human cytotrophoblasts and syncytiotrophoblasts, CpGs sites became hypermethylated in cytotrophoblasts exposed for 24 h to <1% oxygen. However, these same sites became hypomethylated upon differentiation of cytotrophoblast into syncytiotrophoblasts [63], and they showed hypomethylation in EOPE [20]. These findings suggest an imbalance of these cells in the expression of hypoxia-related genes in PE.

The number of overlapping genes among studies is low, ranging from 48 in PE versus controls in maternal side of placenta to 21 in EOPE, LOPE versus control in fetal side of placenta, and only *LIMCH1* is repeated in three studies. Some genes are well characterized in hypoxia and trophoblast invasion (*TERT*, *ALDH1A3*, *IRS1*), which are crucial during PE development. Other gene families frequently appear and are differentially methylated between study groups, such as *CXCL, SERPIN* and *TMEM*, which are involved in angiogenesis, inflammation, migration, cell proliferation, and invasion in types of cancer [64–66].

Altered methylation of specific genes was shown in PE (Supplementary Table 1). *LEP* [67], *PAPPA2* [68–70] and *YWHAQ* [71] showed increased expression and decreased DNAm in preeclamptic placentas.

Additionally, LEP and PAPPA2 protein was altered in maternal serum before the onset of PE symptoms [72,73], and had an increased expression in placentas from PE pregnant from the third trimester [42]. A decreased expression of *PLXNB1* in preeclamptic placentas may be responsible for the deficiency in Met signaling and in PE development [74].

The increased expression of *HSF1* in endothelial cells from term PE suggests a possible protective role as stress specific natural adaptive response against the generated stress [75,76]. *CRH*, *CYP11A1, TEAD3* showed an increased DNAm in preeclamptic placentas, suggesting a hormonal involvement in PE [25]. Moreover, the high expression of *CYP11A1* induces trophoblast autophagy, inhibits trophoblastic invasion and proliferation, as well as increases apoptosis [77,78].

Altered serum levels and placental tissue expression of CXC chemokines, including *CXCL9*, *CXCL10* and *CXCL12*, which participate in several processes triggered by PE, such as neovascularization, embryonic development and inflammatory responses, suggest their role in pathogenesis of PE [79]. Notably, these genes are related to ovarian steroidogenesis, cortisol synthesis and secretion, and cytokine-cytokine receptor interaction (Fig. 3B).

The pathway related to the overlapping genes is already described on process associated with PE, as trophoblast invasion. During pregnancy, AMP-activated protein kinase (AMPK) is necessary for the correct placental differentiation, nutrient transportation, maternal and fetal energy homeostasis, and protection of the fetal membrane. This activation is required for placental differentiation and vasodilation of uterine artery. Therefore, AMPK deficiency induces poor placentation, which results in angiogenic imbalance [80].

Many signaling pathways are involved in PE and are affected by oxidative stress, such as forkhead transcription factors of the O class (FOXO) family. Oxidative stress is responsible for the initiation or progression of pathological process in female reproduction, such as PE. The normal level of reactive oxygen species plays an important regulatory role through various signaling transduction pathways in folliculogenesis, corpus luteum oocyte maturation and feto-placental development, and FOXO is a bond of the different signaling pathway, playing an important role in signaling networks [81]. Insufficient spiral arteries remodeling in PE was associated to higher placental oxidative stress and the generation of oxidized fatty acids [82], as well as an increase of placental dimethyl acetal fatty acid [83], leptin, chemerin and fatty acid binding protein-4 in all pregnancy trimesters and forms of the disease [84].

5. Conclusion

In this systematic review, we found that there are significant differences on global DNA methylation levels between PE and controls, and a pronounced effect on DNAm of specific genes in PE, especially in EOPE and preterm PE. However, these studies should be replicated using the same placental tissues, and the same techniques and pipelines for bioinformatic analysis, in order to reduce variations between the studies. Biological variation cannot be avoided, so it is important to carry out studies in different populations, since the available results are mainly focused on samples from North America and China, and studies from literature have already shown the role of CpG-SNPs on epigenomic changes.

Authors contributions

JOC, IMCA, JAGT, KBG, and MRL have made substantial contributions to the conception or design of the work. All authors have made contributions to the analysis or interpretation of data; All authors have drafted the manuscript or revised it critically for important intellectual content; All authors have read and approved the final version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2020.09.004.

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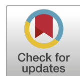

Comprehensive analyses of DNA methylation of the *TIMP3* promoter in placentas from early-onset and late-onset preeclampsia

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A R T I C L E I N F O

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A B S T R A C T

Preeclampsia (PE) is classified into late-onset (LOPE) or early-onset (EOPE) according to gestational age of onset (≥34 or <34 weeks, respectively), and into preterm and term (delivery at <37 or ≥37 weeks, respectively). An imbalanced expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) impairs proper placentation in PE, and DNA methylation (DNAm) may affect their expression. We performed comprehensive analyses of DNAm and *TIMP3* expression in placentas from PE reclassified into EOPE, LOPE, and term PE. We identified significant differentially methylated probes at the *TIMP3* promoter in PE (28), EOPE (38), LOPE (20), and term PE (4) compared to controls, and in EOPE vs. LOPE (8). Moreover, we found a hypomethylation >70% in all groups (except EOPE vs. LOPE) and an increased *TIMP3* expression in corresponding placental samples from PE, EOPE and LOPE compared to controls ($p<0.05$). Our findings highlight the role of DNAm of the *TIMP3* promoter region regarding an epigenetic mechanism in PE.

1. Introduction

Preeclampsia (PE) is classified into late-onset (LOPE) or early-onset (EOPE) according to gestational age of onset (≥34 or <34 weeks of gestation, respectively) [1–3], and into preterm and term PE (delivery at <37 or ≥37 weeks of gestation, respectively) [4]. Noteworthy, an imbalanced expression of matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs) and plasma levels were reported in PE [5–9]. Specifically, tissue inhibitor of metalloproteinase-3 (TIMP3) exhibits high affinity for proteoglycans in the extracellular matrix and an extensive range of substrates, including all MMPs, as reviewed elsewhere [10].

DNA methylation (DNAm) is the transfer a methyl group onto the C5 position of the cytosine-guanine (CpG) dinucleotide, and is associated with gene silencing when located in the promoter region [11]. Differentially methylated probes (DMPs) are sequences that interrogate CpG dinucleotides, identify and differentiate a CpG methylation signature as hypomethylated or hypermethylated.

DNAm is a well-studied epigenetic mechanism in placenta, and global DNAm was shown to have a role on pathophysiology of PE [12–14]. A recent study identified and validated consistent differences in placental DNAm across multiple PE cohorts and perceived widespread inconsistency between studies [15]. However, while TIMP3 is unique among the four TIMPs [10], no such comprehensive analyses have focused on DNAm of *TIMP3*.

Therefore, we analyzed seven independent DNAm microarray datasets to identify DMPs of *TIMP3* promoter in placentas from pregnant women with PE reclassified into EOPE, LOPE, and term PE compared to controls. Moreover, we analyzed one corresponding gene expression data to assess the effect of DNAm of *TIMP3* promoter on gene expression.

2. Methods

2.1. DNA methylation and gene expression datasets

DNAm datasets (GSEs), mostly HumanMethylation450 BeadChip (Illumina), and one corresponding gene expression dataset comparing placental samples from PE ($n = 182$) versus pregnant without PE

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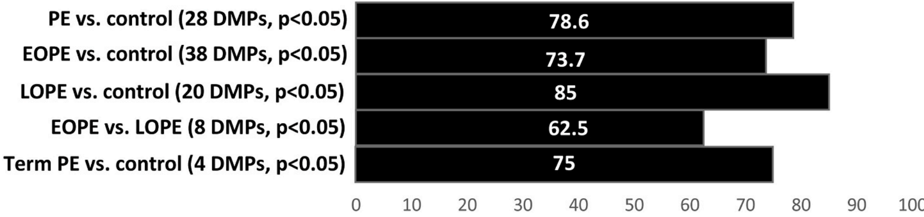

Fig. 1. Number of significant differentially methylated probes (DMPs) and their percentage of hypomethylation in each comparison between the study groups.

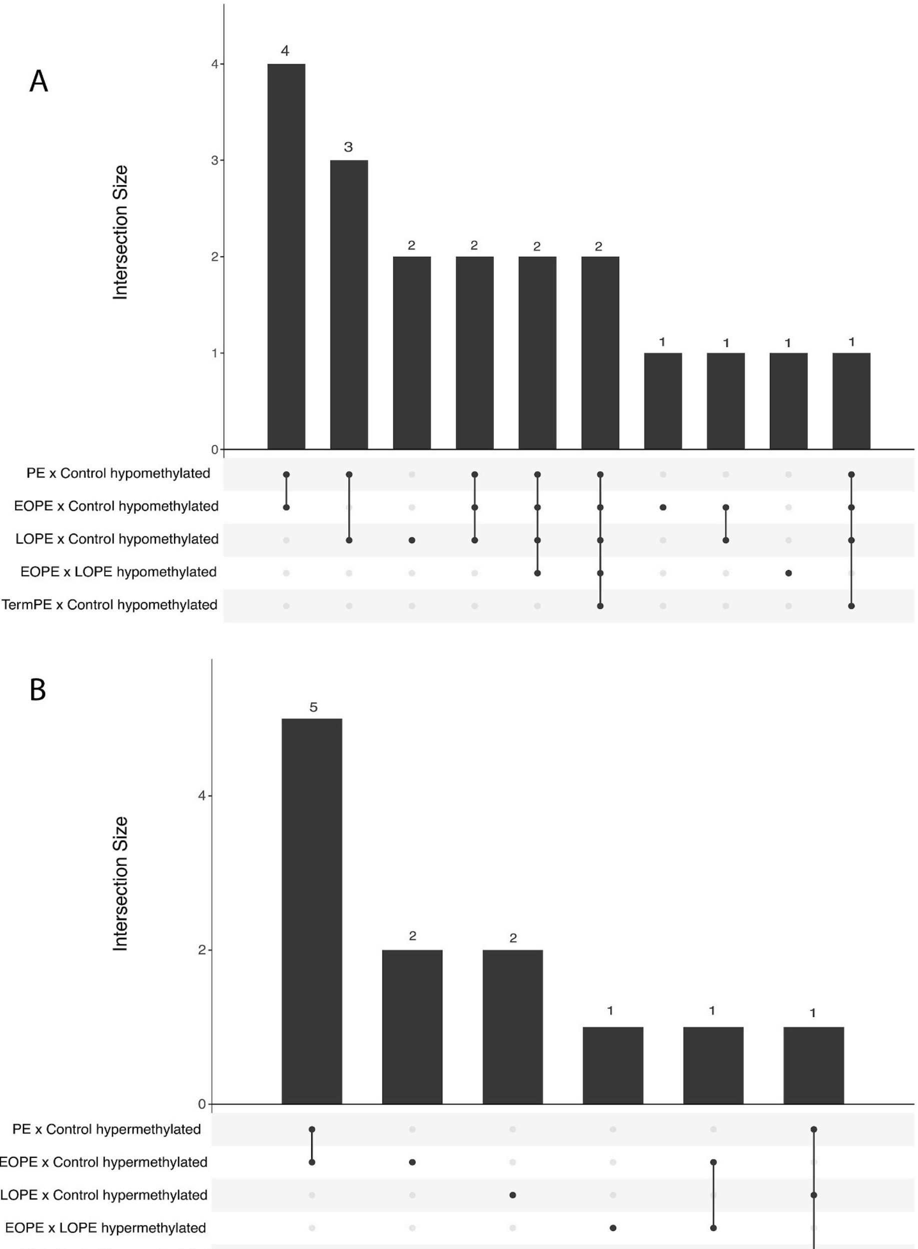

Fig. 2. Upset plots highlighting the overlap of significant DMPs found to be either (A) hypomethylated or (B) hypermethylated among the groups compared.

(controls, n = 147) were obtained from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/), which are detailed in Supplementary Table 1.

2.2. Data acquisition and processing

GEO2R online software was used to analyze the raw submitter-supplied DNAm datasets to identify DMPs of *TIMP3* locus and differences on *TIMP3* expression between the groups compared. GEO2R was executed by selecting the chosen GSEs, controls and case groups

reclassified according to both the gestational age of onset and gestational age at delivery as follows: PE (n = 146), EOPE (n = 66), LOPE (n = 49) and term PE (n = 19). Output tables were fully downloaded and further analyses were made using R packages (readr and tidyverse). Probe to gene annotation was made using data from their manufacturers available as R public packages [16–18].

Probes were filtered by location at or nearby the *TIMP3* promoter. The cut-off standards used to identify significant DMPs were t >< 2 and $p < 0.05$, and log Fold Change ≥1 and $p < 0.05$ were used to identify significant differences on *TIMP3* expression. Groups were analyzed in the comparisons: PE vs. control, EOPE vs. control, LOPE vs. control, EOPE vs. LOPE, and term PE vs. control (Supplementary Table 2). Shared DMPs between these groups were identified using upset plots. However, we could not perform correlation analysis between expression and methylation probes, due to the small number of differential expression probes available that were paired with methylation probes.

3. Results

We identified significant DMPs in PE (28), EOPE (38), LOPE (20), and term PE (4) compared to controls, and in EOPE vs. LOPE (8). We also found a hypomethylation profile >70% in all groups compared to controls (except EOPE vs. LOPE) (Fig. 1; Supplementary Table 3). Notably, we found an increased *TIMP3* expression in corresponding placental samples from pregnant with PE, EOPE and LOPE compared to controls ($p < 0.05$; Supplementary Table 2). However, we were not able to perform correlation analysis between these data.

We then assessed the overlap of significant DMPs among the groups compared to check for differences on DNAm of *TIMP3* promoter region between EOPE and LOPE. The comparisons PE *vs.* control and EOPE *vs.* control shared 11 hypomethylated (Fig. 2A) and five hypermethylated DMPs (Fig. 2B), while EOPE *vs.* control and LOPE *vs.* control share eight hypomethylated DMPs (Fig. 2A).

4. Discussion

Our main novel findings were the highest number of significant DMPs of the *TIMP3* promoter region and their overlap among the compared groups, highlighting the hypomethylation profile and increased *TIMP3* expression in placentas from PE and its subclassifications into EOPE and LOPE. In agreement with our findings, *TIMP3* promoter was found to be hypomethylated in placentas of EOPE [19], and it was also hypomethylated with increased *TIMP3* expression in placentas from PE [20].

A systematic review on global DNAm in placental tissues from pregnant with PE compared to controls showed a hypomethylation pattern, which was more evident in EOPE and preterm PE [14]. Notably, our global DNAm analyses highlighted differences in specific genes or different methylated regions [14]. However, we found only eight DMPs at *TIMP3* promoter when compared EOPE and LOPE, which are suggested to have different etiologies [14,21], and this may be due to TIMP3 role on pathophysiology of PE. While proper placentation and trophoblast invasion requires balanced expression levels of MMPs and TIMPs [8], an imbalanced expression and plasma levels of MMPs and TIMPs was found in PE [5–9]. Therefore, an increased *TIMP3* expression could inhibit MMPs activity and lead to abnormal placental in EOPE. In this regard, genetic polymorphisms affecting the expression of MMPs and TIMPs [22,23], and other epigenetic mechanisms of gene expression [24], may also affect the imbalanced expression of MMPs and TIMPs in PE. Moreover, TIMP3 acts as an antiangiogenic factor blocking VEGF by binding to VEGF receptor-2 [25], thereby leading to widespread endothelial dysfunction that trigger multisystemic damage of PE [26].

Our novel findings highlight DMPs of the *TIMP3* promoter region in EOPE and LOPE, contribute with DNAm data as an epigenetic pathophysiologic mechanism altered in PE, and with *TIMP3* as candidate gene for a biomarker for early detection of PE.

Author contributions

JOC, IMCA, VCS and MRL have contributed to the conception and to the analysis or interpretation of data of the work. All authors have drafted or critically revised the manuscript. All authors have read and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2021.12.003.

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CLINICAL ARTICLE

Obstetrics

Circulating levels of tissue inhibitor of metalloproteinase 3, a protein with inhibitory effects on angiogenesis, are increased in pre-eclampsia

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Abstract

Objective: To assess and compare circulating tissue inhibitor of metalloproteinase 3 (TIMP-3) concentrations between women with pre-eclampsia and healthy pregnant women. We also aimed to determine the relationships between circulating TIMP-3 and matrix metalloproteinase 2 (MMP-2), MMP-9, TIMP-1, and TIMP-2 concentrations in pre-eclampsia.

Methods: A primary case–control study included patients with pre-eclampsia ($n = 219$) and gestational hypertension ($n = 118$), healthy pregnant women ($n = 214$), and non-pregnant women ($n = 66$), and a replication case–control study included patients with pre-eclampsia ($n = 177$) and healthy pregnant women ($n = 124$), all from southeastern Brazil. Plasma TIMP-3, MMP-2, MMP-9, TIMP-1, and TIMP-2 concentrations were assessed using commercially available enzyme-linked immunosorbent assay kits, and the relationships between them were analyzed using Spearman's correlation.

Results: In our primary study, patients with pre-eclampsia and gestational hypertension exhibited increased TIMP-3 concentrations compared with healthy pregnant women (both $P < 0.0001$) and non-pregnant women (both $P < 0.001$). These findings were confirmed in the replication study, showing elevated TIMP-3 concentrations in women with pre-eclampsia versus healthy pregnant women ($P < 0.001$). We found no difference in TIMP-3 concentrations between early-onset and late-onset pre-eclampsia. Moreover, TIMP-3 concentrations were significantly correlated with plasma concentrations of TIMP-1 ($r = 0.2333$; $P = 0.0086$) and MMP-2 ($r = 0.2159$; $P = 0.0156$) in pre-eclampsia.

Conclusions: Circulating TIMP-3 concentration is increased in women with pre-eclampsia compared with healthy pregnant women, and it is positively correlated with plasma MMP-2 and TIMP-1 concentrations in pre-eclampsia.

Ana C. Palei, Juliana de O. Cruz, Marcelo R. Luizon and Valéria C. Sandrim contributed equally to this work.

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gestational hypertension, matrix metalloproteinase-2, pre-eclampsia, tissue inhibitor of metalloproteinase 1, tissue inhibitor of metalloproteinase 3

1 | INTRODUCTION

Hypertensive disorders, including gestational hypertension and pre-eclampsia, affect 5%–10% of pregnancies worldwide.[1,2] Pre-eclampsia is a multisystem health condition characterized by new-onset hypertension and proteinuria or damage in other organs after 20 weeks of pregnancy.[3] Considering the gestational age at onset, pre-eclampsia can be classified as early-onset (<34 weeks of pregnancy) or late-onset (≥34 weeks of pregnancy).[4,5]

Tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous inhibitors of matrix metalloproteinases (MMPs) comprising four members, TIMP-1 to TIMP-4. The *TIMP3* gene is relatively highly expressed in different tissues,[6] and it is regulated at transcriptional and post-transcriptional levels, as reviewed elsewhere.[7] TIMP-3 is unique among the four TIMPs because of its high affinity for the proteoglycans in the extracellular matrix, including all MMPs, a disintegrin and metalloproteinases (ADAMs), and ADAMTSs (ADAM with thrombospondin motifs).[7,8] Among its critical roles, TIMP-3 acts as a regulator of uterine extracellular matrix degradation during embryo implantation.[9] Importantly, the balance between TIMPs and MMPs is the key to the stability and normal function of the extracellular matrix, which is a complex process involving the ability of the MMPs to degrade proteins of the extracellular matrix, whereas the TIMPs inhibit this process.[7,10] An imbalanced in the placental expression of MMPs and TIMPs and their plasma levels has been reported in hypertensive disorders of pregnancy,[11,12] including pre-eclampsia.[13-18]

The roles of TIMP-3 in cardiovascular pathologies, namely myocardial disease, cardiac inflammation, aortic aneurysm, and atherosclerosis, are reviewed elsewhere,[7] as is its therapeutic potential and application as a biomarker for predicting cancer progression.[19] Our comprehensive analyses of DNA methylation of the *TIMP3* promoter in placentas from early-onset pre-eclampsia and late-onset pre-eclampsia[20] confirmed that its promoter is hypomethylated and *TIMP3* is overexpressed in placentas from women with pre-eclampsia.[21,22] Increased *TIMP3* mRNA levels resulting in increased TIMP-3 protein production could inhibit the activity of MMPs, thereby leading to impaired trophoblast invasion and abnormal placentation in pre-eclampsia. However, no previous study has examined TIMP-3 protein levels in pre-eclampsia or gestational hypertension.

In this study, we assessed TIMP-3 concentration in plasma from women with pre-eclampsia in two populations, including the subgroups classified into early-onset pre-eclampsia and late-onset pre-eclampsia, and plasma from women with gestational hypertension, healthy pregnant women and non-pregnant women. Moreover, we examined the relationships between circulating TIMP-3 concentrations and MMP-2, MMP-9, TIMP-1, and TIMP-2 concentrations in plasma from women with pre-eclampsia, women with gestational hypertension, and healthy pregnant women.

2 | MATERIALS AND METHODS

2.1 | Participants

The primary case–control study was approved by the Institutional Review Board (#4682/2006, June 2006) of the University Hospital of Ribeirao Preto Medical School, Brazil, and included 219 women with pre-eclampsia, 118 with gestational hypertension, 214 healthy pregnant women, and 66 non-pregnant women. The replication case–control study was approved by the Ethics Committee (#4.418.043, November 2020) of the Botucatu Medical School, Brazil, and included 177 women with pre-eclampsia and 124 healthy pregnant women. All participants from both case–control studies were consecutively enrolled in the outpatient clinics of their respective Departments of Obstetrics and Gynecology. All participants provided written informed consent.

Gestational hypertension was defined as a systolic blood pressure of 140 mm Hg or more or a diastolic blood pressure of 90 mm Hg or more, or both, on two or more measurements at least 6 h apart after 20 weeks of pregnancy, in a woman with previously normal blood pressure.[3,23] Pre-eclampsia was defined as gestational hypertension along with proteinuria or other sign of end-organ damage in a woman after 20 weeks of pregnancy, according to the American College of Obstetricians and Gynecologists criteria.[23] Women with pre-existing hypertension, with or without superimposed pre-eclampsia, were not included in the study. The exclusion criteria for all study groups were as follows: hemostatic abnormalities, chronic hypertension, cancer, multiple pregnancy, diabetes, and cardiovascular, autoimmune, renal, and hepatic diseases. We conducted our studies in compliance with the principles of the Declaration of Helsinki.

2.2 | Collection and preparation of plasma samples

Maternal venous blood samples were collected into tubes containing ethylenediamine tetraacetic acid at the time of clinic attendance. No sample was collected at delivery. Plasma was obtained from centrifugation of whole blood, and stored at −80°C until it was used to assess MMP and TIMP concentrations using the methods described below.

2.3 | Plasma TIMP-3 measurement

The concentration of TIMP-3 in plasma from women with gestational hypertension, women with pre-eclampsia, healthy pregnant women, and non-pregnant women was determined using the commercially available Duoset enzyme-linked immunosorbent assay kit (catalog number: DY973; R&D Systems), according to the manufacturer's instructions. Assay range was 62.5–4000 pg/ml.

2.4 | Plasma MMP-2, MMP-9, TIMP-1, and TIMP-2 measurements

Plasma MMP-2, MMP-9, TIMP-1, and TIMP-2 concentrations were measured using commercially available enzyme-linked immunosorbent assay kits, as previously described.[14-16]

2.5 | Statistical analyses

Student *t* test, Mann–Whitney *U* test, and χ^2 test were used as appropriate to examine the differences in clinical parameters and circulating TIMP-3 concentrations between women with pre-eclampsia, women with gestational hypertension, healthy pregnant women, and non-pregnant women; healthy pregnant women acted as the control group. Differences in circulating TIMP-3 concentrations were similarly analyzed when the pre-eclampsia group was classified into the subgroups of early-onset pre-eclampsia and late-onset pre-eclampsia, term and preterm pre-eclampsia, or pre-eclampsia with and without fetal intrauterine growth restriction.

The relationships between circulating TIMP-3 concentrations with plasma concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 were analyzed using Spearman's correlation (*r* and *P* values). GraphPad Prism 5.0 (GraphPad) was used for statistical analysis and preparation of graphs. A value of *P* less than 0.05 was considered the level of statistical significance.

3 | RESULTS

The demographic and clinical characteristics of women enrolled in the primary case–control study are shown in Table 1. Women with pre-eclampsia and those with gestational hypertension were older than healthy pregnant women ($P = 0.0017$ and $P = 0.0004$, respectively), but younger than non-pregnant women ($P = 0.0124$ and $P = 0.0272$, respectively). Body mass index (BMI; calculated as weight in kilograms divided by the square of height in meters) was not different between women with pre-eclampsia and healthy pregnant women ($P > 0.05$), it was higher in women with pre-eclampsia than in non-pregnant women ($P < 0.0001$), and in women with gestational hypertension when compared with healthy pregnant and non-pregnant women ($P = 0.0136$ and $P < 0.0001$, respectively). As expected, women with pre-eclampsia exhibited higher systolic blood pressure and diastolic blood pressure than healthy pregnant and non-pregnant women ($P < 0.0001$ and $P = 0.0013$, respectively), despite the fact that most women with pre-eclampsia were receiving antihypertensive therapy. Women with gestational hypertension exhibited higher systolic blood pressure than healthy pregnant and non-pregnant women (all $P < 0.0001$), but a higher diastolic blood pressure only when compared with non-pregnant women ($P = 0.0002$). We found lower gestational age at delivery and gestational age at sampling, as well as lower newborn weight in women with pre-eclampsia than in healthy pregnant women (all $P < 0.0001$). Gestational age at delivery and creatinine ($P = 0.0022$ and $P = 0.0113$, respectively) were lower in women with gestational hypertension than in healthy pregnant women. Heart rate, fasting glucose, hemoglobin, and hematocrit showed no significant differences between groups ($P > 0.05$).

Women with pre-eclampsia showed increased circulating TIMP-3 concentrations compared with healthy pregnant ($P < 0.0001$) and non-pregnant ($P = 0.0002$) women (Table 1, Figure 1a). Women with gestational hypertension also showed increased circulating TIMP-3 concentrations compared with healthy pregnant ($P = 0.0001$) and non-pregnant ($P = 0.0031$) women (Table 1, Figure 1a). When the pre-eclampsia group was classified according to different clinical presentations, we found no differences in circulating TIMP-3 levels between early-onset pre-eclampsia and late-onset pre-eclampsia ($P > 0.05$; Figure 1c), between term and preterm pre-eclampsia, and between pre-eclampsia with and without intrauterine fetal growth restriction (all $P > 0.05$; Figures S1-1A and S1-1B, respectively).

Next, we examined the relationships between circulating TIMP-3 concentrations with plasma concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the primary case–control study. Notably, we found significant positive correlations between TIMP-3 and TIMP-1 ($r = 0.2333$; $P = 0.0086$) and between TIMP-3 and MMP-2 ($r = 0.2159$; $P = 0.0156$) concentrations in women with pre-eclampsia (Figure 2a and Figure 2b, respectively). Moreover, we found significant positive correlations between TIMP-3 and TIMP-1 concentrations in women with gestational hypertension ($r = 0.2023$; $P = 0.0254$; Figure 2c), and between TIMP-3 and TIMP-2 concentrations in healthy pregnant women ($r = 0.1817$; $P = 0.0098$) (Figure 2d). Conversely, we found no significant correlations between TIMP-3 with plasma concentrations of MMP-9 and TIMP-2 in the pre-eclampsia, gestational hypertension, and healthy pregnancy groups (all $P > 0.05$).

To assess the relationship between circulating TIMP-3 concentrations with clinical characteristics, we performed correlation analyses with blood pressure levels, BMI, and gestational age at sampling and gestational age at delivery. However, we found no significant correlations between TIMP-3 concentrations and systolic blood pressure, diastolic blood pressure, and BMI in any of the study groups (all $P > 0.05$; Figures S2 and S3). Finally, we also found no significant correlations between TIMP-3 and gestational age at sampling or gestational age at delivery (data not shown).

The demographic and clinical characteristics of women enrolled in the replication case–control study are shown in Table 2, and they show similar trends to those described for the primary case–control study. Notably, in the replication study, women with pre-eclampsia

TABLE 1 Clinical, demographic, and biochemical characteristics of women enrolled in the primary study[a]

Parameters	Pre-eclampsia (*n* = 219)	Gestational hypertension (*n* = 118)	Healthy pregnant (*n* = 214)	Non-pregnant (*n* = 66)	*P* value
Age, y	27.1 ± 6.7	27.3 ± 6.7	24.6 ± 6.5	30.9 ± 8.7	**0.0017**[b]; **0.0124**[c]; **0.0004**[d]; **0.0272**[e]
Ethnicity, % white	68.2	70.3	71.03	77.14	
Current smokers	9.04	12.7	7.36	5.45	
BMI	33.2 ± 6.6	35.6 ± 7	33.2 ± 6.6	22.7 ± 2.1	0.9992[b]; **<0.0001**[c]; **0.0136**[d]; **<0.0001**[e]
SBP, mm Hg	138.3 ± 18.6	132.1 ± 18.1	110.7 ± 11.3	114.3 ± 9.6	all **<0.0001**[b,c,d,e]
DBP, mm Hg	86.9 ± 12.9	84.2 ± 12.5	81.8 ± 10.4	77.4 ± 6.8	**0.0013**[b]; **<0.0001**[c]; 0.1392[d]; **0.0002**[e]
HR, beats/min	81.6 ± 7.6	81.6 ± 7.3	81.2 ± 8.8	ND	0.9083[b]; 0.7850[d]
Fasting glucose, mg dl^{-1}	80.2 ± 18.1	77.7 ± 10	75.4 ± 10.4	ND	0.1489[b]; 0.0968[d]
Hemoglobin, g dl^{-1}	11.7 ± 1.5	11.9 ± 1.3	11.9 ± 1.5	ND	0.3693[b]; 0.3720[d]
Hematocrit, %	35.1 ± 4.6	36 ± 3.9	35.4 ± 4.9	ND	0.6850[b]; 0.3449[d]
Creatinine, mmol L^{-1}	0.7 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	ND	0.4455[b]; **0.0113**[d]
24-h Pr, mg/24 h	1465.1 ± 2035.1	162.9 ± 78.7	ND	ND	
GAD, wk	36.2 ± 3.6	38.9 ± 1.6	39.6 ± 1.4	ND	**<0.0001**[b]; **0.0022**[d]
Newborn weight, g	2617.3 ± 919	3210.8 ± 538.7	3271.1 ± 510	ND	<**0.0001**[b]; 0.4166[d]
GAS, wk	34.1 ± 4.1	35.4 ± 4.9	36.6 ± 3.3	ND	<**0.0001**[b]; 0.2338[d]
Early-onset PE	20.9	ND	ND	ND	
Preterm birth	16	ND	ND	ND	
IUGR	9.9	ND	ND	ND	
TIMP-3, ng/ml	6764.9 ± 2136.1	6581.4 ± 2425.7	5444.3 ± 2400.1	5478.8 ± 2556.9	**0.0001**[b]; **0.0002**[c]; **0.0001**[d]; **0.0031**[e]

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by the square of height in meters); DBP, diastolic blood pressure; GAD, gestational age at delivery; GAS, gestational age at sampling; HR, heart rate; IUGR, intrauterine growth restriction; ND, not determined (however, negative dipstick test); PE, pre-eclampsia; Pr, proteinuria; SBP, systolic blood pressure; TIMP-3, metalloproteinase inhibitor 3.

[a]Values are presented as mean ± standard error of the mean or as percentages. Bold values are significant (<0.05), according to the comparisons between groups.

[b]$P < 0.05$, pre-eclampsia versus healthy pregnant women.

[c]$P < 0.05$, pre-eclampsia versus non-pregnant women.

[d]$P < 0.05$, gestational hypertension versus healthy pregnant women.

[e]$P < 0.05$, gestational hypertension versus non-pregnant women.

also showed increased circulating TIMP-3 concentrations compared with healthy pregnant women ($P = 0.0004$) (Table 2, Figure 1b). Again, we found no differences in the circulating TIMP-3 concentrations between early-onset pre-eclampsia and late-onset pre-eclampsia in the participants of the replication study ($P > 0.05$) (Figure 1d).

4 | DISCUSSION

The main novel findings reported here are that circulating TIMP-3 concentrations are increased in women with pre-eclampsia and gestational hypertension compared with healthy pregnant women in a case–control study, and this observation of increased TIMP-3 concentrations in pre-eclampsia is replicated in a different populations of pregnant women. Moreover, we found that circulating TIMP-3 concentrations are positively correlated with plasma MMP-2 and TIMP-1 concentrations in pre-eclampsia.

A balance between TIMPs and MMPs is needed for normal function of the extracellular matrix, an essential process for the remodeling of spiral arteries and establishment of proper placentation. Therefore, we have examined the relationship of TIMP-3 with MMPs and other TIMPs. Although we have found a significant association between TIMP-3 and MMP-2 and TIMP-1 in pre-eclampsia, we have not conducted functional studies proving the interaction between these proteins in placental tissue and its impact on trophoblast invasion and vascular remodeling. Nonetheless, these interesting findings serve as a foundation for future studies.

Our findings of increased circulating TIMP-3 concentration in pre-eclampsia are in agreement with previous reports describing hypomethylation of the *TIMP3* promoter,[21,22] and increased *TIMP3* expression in placental tissue from women with pre-eclampsia.[21] Our previous systematic review showed that DNA methylation is gene-specific or specific to genomic regions.[24] Similarly, by means

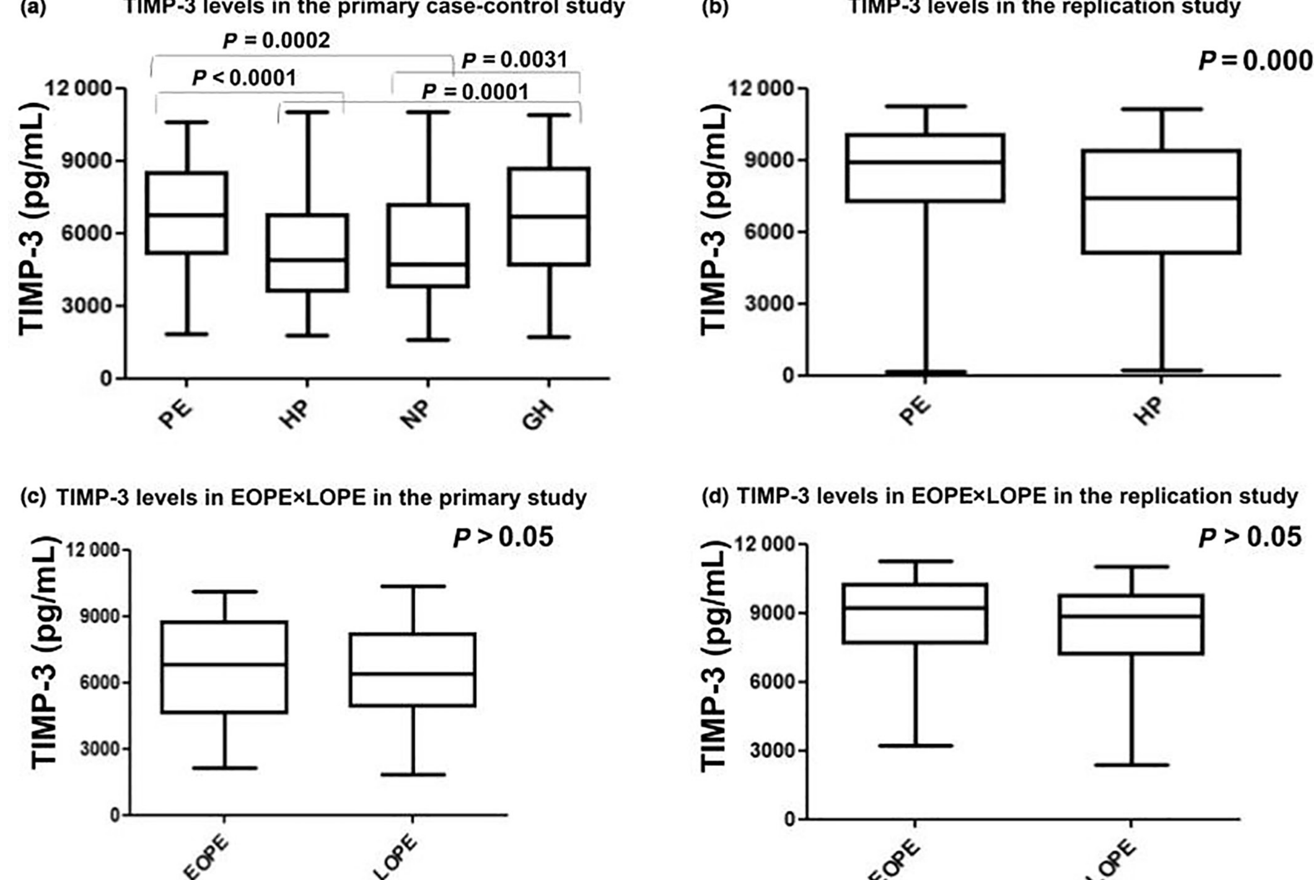

FIGURE 1 Circulating TIMP-3 concentrations considering the women with pre-eclampsia (PE), healthy pregnant women (HP), non-pregnant women (NP), and women with gestational hypertension (GH), and in the subgroups of early-onset PE (EOPE) and late-onset PE (LOPE) in the primary study (a and c) and in the replication study (b and d). The box and whisker plots show range and quartiles. The boxes extend from the 25th centile to the 75th centile, with a line at the median. The whiskers show the highest and lowest values.

of a sub-analysis of publicly available data deposited in the Gene Expression Omnibus, we have recently shown that DNA hypomethylation is specific for the promoter region of the *TIMP3* gene, leading to increased *TIMP3* mRNA levels.[20] Moreover, the lack of differences on plasma TIMP-3 levels between early-onset pre-eclampsia and late-onset pre-eclampsia reinforces our earlier results regarding DNA methylation and gene expression showing that TIMP-3 is not able to differentiate between these two pre-eclampsia phenotypes.[20]

Although the pathogenesis of pre-eclampsia is not entirely known, placental malperfusion leading to widespread maternal endothelial dysfunction is accepted as a major disease mechanism. Hence, set as two pathophysiologic stages, abnormal placental formation occurs early in the first trimester followed by a maternal syndrome in the last two trimesters characterized by an excess of anti-angiogenic and pro-inflammatory factors.[2] We have previously suggested that the MMP-TIMP system may participate in both phases of pre-eclampsia.[25] Trophoblast invasion of spiral arteries and proper placentation require balanced expression levels of MMPs and TIMPs.[17] Indeed, imbalanced placental expression of MMPs and TIMPs and their plasma levels have been reported in hypertensive disorders of pregnancy,[11,12] including pre-eclampsia.[13-18] There is robust correlative and functional evidence indicating that TIMP-3 is a key regulator of extracellular matrix remodeling during embryo implantation, as reviewed elsewhere.[9] Therefore, increased TIMP-3 levels could reduce MMP activity and lead to abnormal placental formation and function in pre-eclampsia. Meanwhile, our results showed significant positive correlations between TIMP-3 and MMP-2, which was surprising because of the inhibitory role of TIMPs on MMPs. However, the interaction between MMPs and TIMPs is complex, in that TIMPs are also involved in activation and uptake/removal of MMPs from the extracellular environment.[26] For instance, the activation of pro-MMP-2 by matrix type 3-MMP was shown to be enhanced by TIMP-3 in a dose-dependent manner.[27] The interplay between TIMP-3 and TIMP-1 has yet to be determined by future studies.

Nonetheless, TIMP-3 has functions beyond the modulation of MMP activity, such as angiogenesis and apoptosis. It has been demonstrated that TIMP-3 is a strong inhibitor of the pro-angiogenic action of vascular endothelial growth factor (VEGF), through its binding to the VEGF type 2 receptor (VEGFR2).[28] Alternatively, TIMP-3 may bind to angiotensin II type 2 receptor (ATR2), and the over-expression of both TIMP-3 and ATR2 additively inhibits VEGF-induced angiogenesis.[29] Moreover, TIMP-3 and ATR2 also have additive effects on inducing apoptosis through activation of different caspases.[29] Additionally, TIMP-3 is able to inhibit tumor necrosis factor-α converting enzyme, activating the apoptosis process in cultured rat vascular smooth muscle

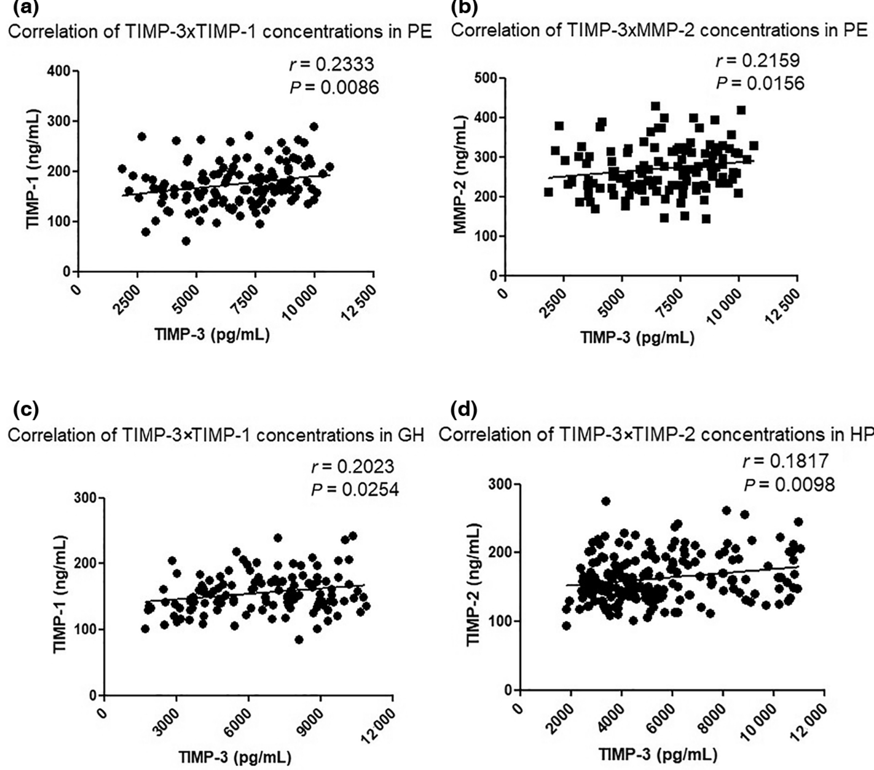

FIGURE 2 Correlations between tissue inhibitor of metalloproteinase 3 (TIMP-3) concentrations with (a) TIMP-1 and (b) matrix metalloproteinase 2 (MMP-2) concentrations in women with pre-eclampsia (PE), and with (c) TIMP-1 and (d) TIMP-2 concentrations in women with gestational hypertension (GH) and healthy pregnant women (HP) enrolled in the primary case–control study, respectively. The regression lines are plotted. The *r* and *P* values are reported, where *r* represents Spearman's correlation.

cells and tumor cell lines.[30,31] Hence, the TIMP-3 anti-angiogenic and pro-apoptotic properties may contribute to abnormal placentation associated with pre-eclampsia. Future prospective studies in humans as well as studies in animal and cell models are warranted to confirm these roles of TIMP-3 in the pathophysiology of pre-eclampsia.

Hypertension is marked by structural remodeling of the vascular extracellular matrix and the balance between MMPs and TIMPs may be altered in response to a hypertensive stimulus.[32] It has been shown that angiotensin II-induced hypertension is significantly suppressed in mice lacking the *TIMP3* gene, indicating a critical effect of TIMP-3 on preserving remodeling of the arterial extracellular matrix in response to angiotensin II.[32] Previous studies have demonstrated that MMP-2 is able to generate vasoconstrictors (e.g. endothelin-1-related peptides) and degrade vasodilators (e.g. adrenomedullin and calcitonin-gene-related peptide) and cell surface receptors (e.g. VEGFR2 and β_2-adrenergic receptor). Although unproven in the context of pre-eclampsia, MMP-2 may contribute to vasoconstriction and hypertension in pre-eclampsia through its extracellular matrix-independent actions.[25] Here we found an elevated TIMP-3 concentration in the circulation of women with pre-eclampsia and women with gestational hypertension. Similarly, higher circulating MMP-2 levels have been reported in pre-eclampsia compared with both healthy pregnant women and non-pregnant women.[14,33,34,35] Therefore, future studies should investigate the role of TIMP-3 in stimulating MMP-2 activation and their isolated and/or interactive action on promoting vascular dysfunction and hypertension in pre-eclampsia.

As previous studies have demonstrated that TIMP-3 is the primary TIMP to regulate agonist-induced vascular remodeling and hypertension,[32] we examined the relationship between TIMP-3 and blood pressure levels. Moreover, as obesity is an important risk factor for hypertension and pre-eclampsia,[36] we have also determined the relationship between TIMP-3 and BMI. However, plasma TIMP-3 concentrations were not statistically associated with either blood pressure or BMI. Furthermore, TIMP-3 levels were not different between the pre-eclampsia and gestational hypertension groups, nor between the early-onset pre-eclampsia and late-onset pre-eclampsia subgroups, suggesting that TIMP-3 is not a good blood-based biomarker to distinguish between hypertensive disorders of pregnancy or pre-eclampsia phenotypes. However, these analyses might have been biased because (1) the majority of women with pre-eclampsia and gestational hypertension were under antihypertensive therapy at the time of blood collection, and (2) patients with hypertensive disorders of pregnancy, especially pre-eclampsia, frequently develop edema, which influences BMI and hemodilution.

We were able to validate our findings of increased circulating TIMP-3 concentrations in pre-eclampsia from the primary case–control study with the results in an independent population of pregnant women of the replication study. The participants recruited for both studies were followed as outpatients in the Department of Gynecology and Obstetrics from their respective reference hospitals as soon as healthy pregnant women entered the second trimester or

TABLE 2 Clinical, demographic and biochemical characteristics of women enrolled in the replication study[a]

Parameters	Healthy pregnant (n = 124)	Pre-eclampsia (n = 177)	P value
Age, y	26.72 ± 0.710	27.32 ± 0.5828	0.5177
Ethnicity, % white	84	89	–
GAS, wk	34.33 ± 0.3538	31.87 ± 0.526	**0.0001**
Parity			
Nulliparous	64	68	–
Multiparous	36	32	–
SBP, mm Hg	115.7 ± 0.7	155.8 ± 0.2	**<0.0001**
DBP, mm Hg	73.3 ± 0.1	103.3 ± 0.2	**<0.0001**
24-h Pr, mg per 24 h	ND	1857 ± 539.4	–
Uric acid, mg/dl	ND	5.15 ± 0.2	–
Early-onset PE	ND	41	–
Severity			
Severe PE	ND	57	–
Mild PE	ND	43	–
TIMP-3, pg/ml	7296 ± 223.8	8349 ± 162.4	**0.0004**

Abbreviations: DBP, diastolic blood pressure; GAS, gestational age at sampling; ND, not determined; Pr, proteinuria; SBP, systolic blood pressure; TIMP-3, metalloproteinase inhibitor 3.

[a]Values are presented as mean ± standard error of the mean. Bold values are significant according to the comparison; $P < 0.05$, pre-eclampsia versus healthy pregnant women.

when pregnant women were diagnosed with pre-eclampsia or gestational hypertension throughout the pregnancy and postpartum. The recruitment protocols are described elsewhere.[37,38]

Although we have not enrolled patients with gestational hypertension in the replication study, the main focus of this study in particular was to determine circulating TIMP-3 levels in an independent population of patients with pre-eclampsia. In this context, we were not able to retrieve all relevant clinical data for the patients with pre-eclampsia and healthy pregnant women included in the replication study, nor to measure MMP-2, MMP-9, TIMP-1, and TIMP-2 in their plasma.

In conclusion, our novel findings provide evidence for increased circulating TIMP-3 concentrations in patients with pre-eclampsia and gestational hypertension compared with healthy pregnant women. Moreover, increased circulating TIMP-3 concentrations were positively correlated with plasma MMP-2 and TIMP-1 concentrations in patients with pre-eclampsia.

AUTHOR CONTRIBUTIONS

ACP, JdeOC, JLC, JCP, MR-V, VRR-V, RCC, PRN, MRL, and VCS contributed to the conception, design, participant recruitment, and data collection of the present study. JdeOC, PRN, MRL, and VCS contributed to data analysis. ACP, JdeOC, PRN, MRL, and VCS drafted the article. All authors were involved in interpreting the data and critically reviewing the article. All authors gave approval of the final version for publication.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The authors confirm that data supporting the findings of this study are presented. Raw data are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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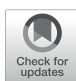

Epigenetic Regulation of the N-Terminal Truncated Isoform of Matrix Metalloproteinase-2 (NTT-MMP-2) and Its Presence in Renal and Cardiac Diseases

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Several clinical and experimental studies have documented a compelling and critical role for the full-length matrix metalloproteinase-2 (FL-MMP-2) in ischemic renal injury, progressive renal fibrosis, and diabetic nephropathy. A novel N-terminal truncated isoform of MMP-2 (NTT-MMP-2) was recently discovered, which is induced by hypoxia and oxidative stress by the activation of a latent promoter located in the first intron of the *MMP2* gene. This NTT-MMP-2 isoform is enzymatically active but remains intracellular in or near the mitochondria. In this perspective article, we first present the findings about the discovery of the NTT-MMP-2 isoform, and its functional and structural differences as compared with the FL-MMP-2 isoform. Based on publicly available epigenomics data from the Encyclopedia of DNA Elements (ENCODE) project, we provide insights into the epigenetic regulation of the latent promoter located in the first intron of the *MMP2* gene, which support the activation of the NTT-MMP-2 isoform. We then focus on its functional assessment by covering the alterations found in the kidney of transgenic mice expressing the NTT-MMP-2 isoform. Next, we highlight recent findings regarding the presence of the NTT-MMP-2 isoform in renal dysfunction, in kidney and cardiac diseases, including damage observed in aging, acute ischemia-reperfusion injury (IRI), chronic kidney disease, diabetic nephropathy, and human renal transplants with delayed graft function. Finally, we briefly discuss how our insights may guide further experimental and clinical studies that are needed to elucidate the underlying mechanisms and the role of the NTT-MMP-2 isoform in renal dysfunction, which may help to establish it as a potential therapeutic target in kidney diseases.

Keywords: acute kidney injury, alternative promoter, chronic kidney disease, histone modifications, matrix metalloproteinase-2, mitochondria, epigenetics (DNA methylation), epigenomics

INTRODUCTION

Matrix metalloproteinases (MMPs) are a large family of zinc-containing endopeptidases that participate in multiple cellular processes beyond extracellular matrix (ECM) remodeling and in kidney diseases (Tan and Liu, 2012; Parrish, 2017). Regarding the MMPs known as gelatinases, the full-length MMP-2 (FL-MMP-2) is synthesized and was originally thought to be only secreted, but later it was found to be only inefficiently targeted to the secretory pathway (Ali et al., 2012). The FL-MMP-2 can be activated by extracellular proteolysis and intracellularly by direct chemical modification by peroxynitrite/oxidative stress (Viappiani et al., 2009; Kandasamy et al., 2010; Sariahmetoglu et al., 2012).

In the renal ECM, FL-MMP-2 has a role in the regulated turnover of the tubular epithelial basement membrane (Cheng et al., 2006). However, enhanced FL-MMP-2 synthesis distorts the basement membrane architecture and results in progressive renal injury (Cheng et al., 2006), cardiac remodeling and contractile dysfunction (Jacob-Ferreira and Schulz, 2013). The regulation and role of FL-MMP-2 have been extensively studied in both kidney (Tan and Liu, 2012; Dimas et al., 2017; Mansour et al., 2017; Narula et al., 2018) and cardiac injuries (Coker et al., 1999; Wang et al., 2002; Sawicki et al., 2005; Sung et al., 2007; Ali et al., 2010). Besides ECM proteins, FL-MMP-2 also cleaves vasoactive peptides, chemokines, and intracellular sarcomere and nuclear proteins (Fernandez-Patron et al., 1999, 2000; Martinez et al., 2004; Denney et al., 2009; Jacob-Ferreira and Schulz, 2013; DeCoux et al., 2014).

A novel N-terminal truncated isoform of MMP-2 (NTT-MMP-2) was recently discovered, which is induced by hypoxia and oxidative stress by the activation of a latent promoter in the first intron of the *MMP2* gene, thereby generating a 5′-truncated mRNA transcript (Lovett et al., 2012). This NTT-MMP-2 isoform is enzymatically active but lacks the secretory sequence and the inhibitory propeptide, remains intracellular in the mitochondria, triggers mitochondrial-nuclear stress signaling *via* nuclear factor-κβ (NF-κβ) and nuclear factor of activated T cells (NFAT), and induces innate immune response genes (Lovett et al., 2012).

This perspective presents the findings about the discovery of the NTT-MMP-2 isoform, its functional and structural differences as compared with the FL-MMP-2 isoform, the alterations in the kidney of transgenic mice expressing the NTT-MMP-2 isoform, insights into the epigenetic regulation of the latent promoter located in the first intron of *MMP2* gene that support the activation of the NTT-MMP-2 isoform, and highlights the role of the NTT-MMP-2 isoform in renal and cardiac diseases.

THE FL-MMP-2 AND THE NOVEL NTT-MMP-2 ISOFORM

The structure of the 68 kDa FL-MMP-2 is a short N-terminal signal sequence for secretory vesicle processing, a propeptide domain responsible for the enzyme latency, a highly conserved zinc-binding catalytic domain and hemopexin and fibronectin domains, which binds to ECM substrates (Turck et al., 1996; Morgunova et al., 1999). The FL-MMP-2 latency is maintained by the presence of a cysteine residue of the prodomain extended along the catalytic site, the "cysteine-switch" mechanism. The mRNA transcript of FL-MMP-2 is translated, and a portion of enzymatically inactive full-length protein is secreted by vesicles to the extracellular space, where occurs the proteolytic activation of the latent MMP-2 protein by other MMPs, ending in an active 62 kDa MMP-2 after cleavage of the inhibitory prodomain. In the ECM, the active enzyme degrades ECM components, such as collagen IV, laminin, and elastin (Turck et al., 1996; Morgunova et al., 1999).

The intracellular MMP-2 was first observed distributed in a pattern consistent with sarcomeric and sarcolemmal in cardiomyocytes (Coker et al., 1999), and the cleavage of sarcomeric troponin I by the 68 kDa FL-MMP-2 following acute ischemia-reperfusion injury (IRI) of the heart was later reported (Wang et al., 2002). Next, myosin light chain-1 (Sawicki et al., 2005), α-actinin (Sung et al., 2007), and titin (Ali et al., 2010) were reported as intracellular targets of the FL-MMP-2 in cardiomyocytes, leading to impaired heart contractility. This intracellular 68 kDa FL-MMP-2 is able to escape the secretory pathway (Ali et al., 2012), and is activated by the cysteine-switch opening by reactive oxygen species and peroxynitrite (Viappiani et al., 2009), and its activity was shown to be further modulated by its phosphorylation in cardiomyocytes (Sariahmetoglu et al., 2007, 2012).

Regarding kidney diseases, most of the studies focused on the extracellular or intracellular roles of the FL-MMP-2, mainly its deleterious action in the tubular basement membrane (Cheng et al., 2006). A novel intracellular MMP-2 isoform with 65 kDa (NTT-MMP-2) was recently discovered, which is generated by the activation of an alternate promoter in the first intron of the *MMP2* gene (Lovett et al., 2012). This novel isoform was first observed in the hearts of both FL-MMP-2 transgenic mice and aging wild-type mice (Lovett et al., 2012). Cardiomyoblasts submitted to mitochondrial stress generated with transient inhibition of oxidative phosphorylation induced the NTT-MMP-2 isoform expression on mitochondria-enriched cell fractions (Lovett et al., 2012). The NTT-MMP-2 is functionally and structurally distinct from the FL-MMP-2 (**Figure 1**).

While the FL-MMP-2 is present in the cytoplasm or ECM, and in cells of control conditions, the NTT-MMP-2 is intracellular, predominantly located at or near the mitochondria, and its transcription is induced only in conditions of tissue damage (Lovett et al., 2012). Transfection of the NTT-MMP-2 cDNA in cardiomyoblasts resulted in increased luciferase reporter activity for NF-kβ, NFAT, and response elements for interferon regulatory factors (IRFs) and induced innate immune response transcription factors and chemokines/cytokines, thereby activating a proinflammatory, pro-apoptotic innate immune response (Lovett et al., 2012).

EPIGENETIC REGULATION OF THE NTT-MMP-2 ISOFORM EXPRESSION

The transcriptional start site for the FL-MMP-2 isoform is located in the 5′ flanking region of *MMP2*, and transcription from this site encodes the FL-MMP-2 beginning at M1 amino

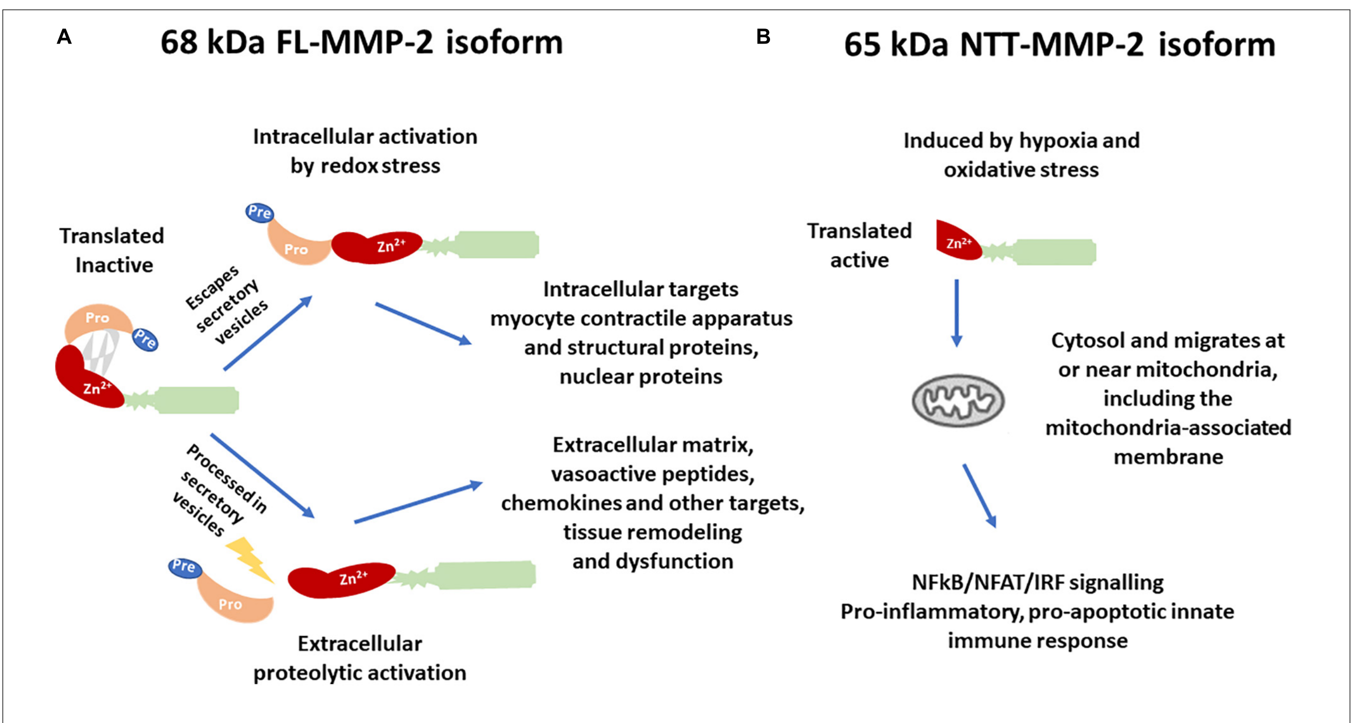

FIGURE 1 | Differences in the structure, activation, and biological targets/outcomes between **(A)** the 68 kDa full-length isoform of MMP-2 (FL-MMP-2) and **(B)** the 65 kDa N-terminal truncated isoform of MMP-2 (NTT-MMP-2).

acid in the first exon of the *MMP2* gene (Lovett et al., 2012). Transcription of NTT-MMP-2 starts with activation of a latent promoter induced by hypoxia and oxidative stress, in an alternate transcriptional start site located at the 3′ end of the first intron of the *MMP2*, which encodes the NTT-MMP-2 isoform beginning at M77 amino acid located within the second exon of *MMP2* (Lovett et al., 2012).

Figure 2 shows the promoter region of the *MMP2* with the transcriptional start site as indicated by the Eukaryotic Promoter Database (Dreos et al., 2013) and highlights the overlap of the first intron with several epigenomics data from the Encyclopedia of DNA Elements (ENCODE) (Consortium, 2012) and GENCODE consortium (Frankish et al., 2019), including ENCODE registry of candidate *cis*-regulatory elements (cCREs), DNase I hypersensitivity clusters (Thurman et al., 2012), chromatin immunoprecipitation-sequencing (ChIP-seq) data for histone marks, and transcription factor ChIP-seq clusters (Consortium, 2012). This approach using epigenomics data to identify gene regulatory regions was recently performed elsewhere (Linhares et al., 2020).

HISTONE MODIFICATIONS AND THE NTT-MMP-2 ISOFORM EXPRESSION

The trimethylation of histone H3 on lysine 4 (H3K4me3) is associated with promoters (Rosenbloom et al., 2012). The acetylation of histone H3 on lysine 27 (H3K27ac) is often found near active regulatory elements such as enhancers and distinguishes active from poised enhancers containing the monomethylation of histone H3 on lysine 4 (H3K4me1) alone (Creyghton et al., 2010).

H3K4me1 is commonly associated with distal enhancers, but it is also present at promoter regions proximal to transcriptional start site (Cheng et al., 2014; Bae and Lesch, 2020). Noteworthy, H3K4me1 peak density was recently examined around promoters in human and mouse germ cells (Bae and Lesch, 2020). H3K4me1 was found to exhibit either a bimodal pattern at active promoters, where it flanks H3K4me3, or a unimodal pattern at poised promoters, where it coincides with both H3K4me3 and trimethylation at lysine 27 of histone H3 (H3K27me3). H3K4me1 distribution was proposed as a key feature of the poised epigenetic state and poising at promoters (Bae and Lesch, 2020).

Interestingly, a region with enhancer-like signature (E1817412/enhP) identified by the ENCODE cCREs in the first intron of the *MMP2* gene overlaps with a DNase I hypersensitivity cluster, and with ENCODE ChIP-seq data for the histone modification H3K4me1, for the histone acetyltransferase EP300, and for the transcription factors FOS, JUND, and MAFK, which are known to cooperate in hypoxia-induced gene transcription (**Figure 2**). Hypoxic conditions were shown to induce the transcriptional activation of c-fos in HeLa cells (Muller et al., 1997). Notably, c-Jun was shown to functionally cooperate with hypoxia-inducible factor 1 (HIF-1) transcriptional activity in different cell types (Alfranca et al., 2002). In this context, the small Maf protein, MafG, possess a basic leucine zipper domain that is required for homodimer or heterodimer complex formation with other transcription factors. MafG was shown to interact with HIF-1α, a key factor in hypoxic response, and it was suggested to regulate the hypoxic

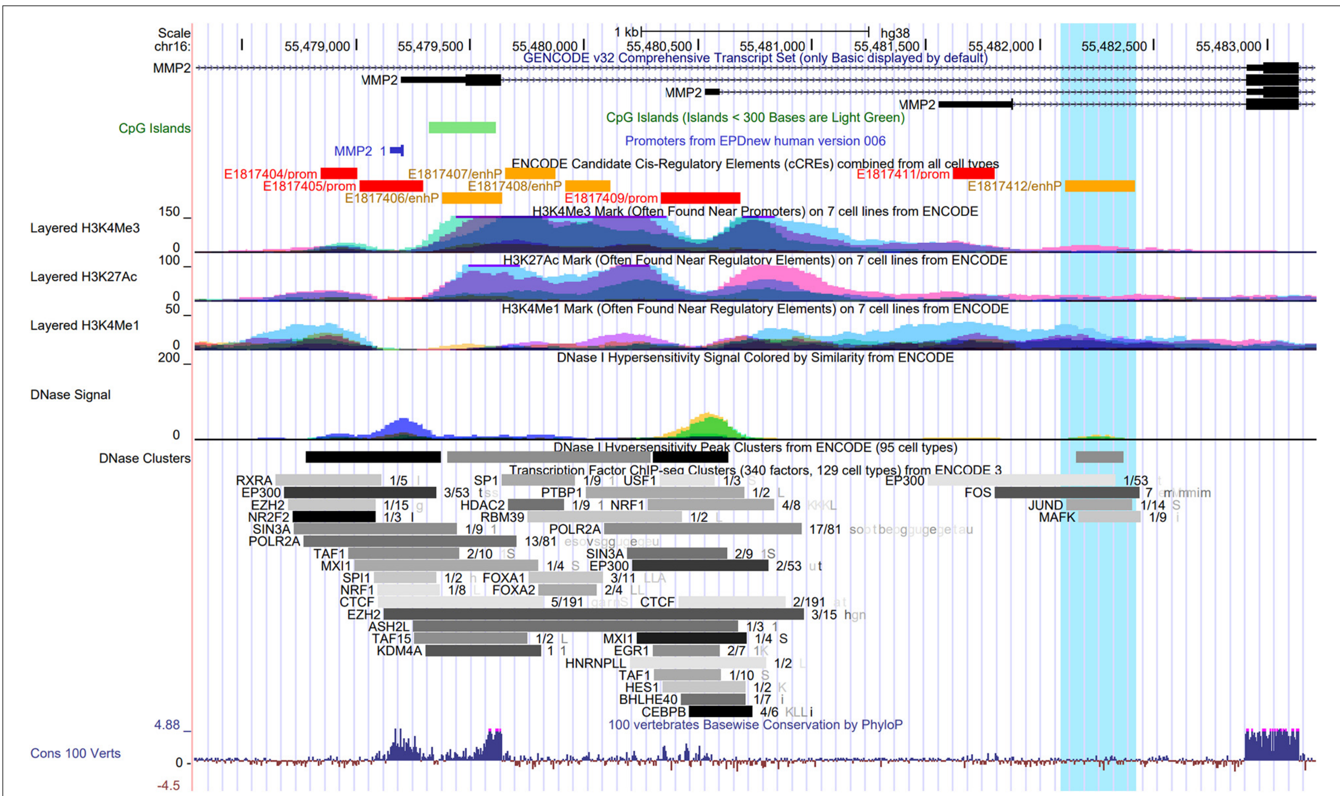

FIGURE 2 | UCSC Genome Browser view of the promoter and the first intron of the *MMP2* gene showing the overlap with several epigenomics data from the ENCODE Project, including the ENCODE registry of candidate cis-regulatory elements (cCREs), DNase I hypersensitivity clusters, ChIP-seq data for three histone marks on seven cell lines, and Transcription Factor ChIP-seq clusters. The transcriptional start site in the promoter region of *MMP2* gene is indicated by the Eukaryotic Promoter Database (EPDnew). Highlighted (in light blue) is a region with an enhancer-like signature (E1817412/enhP) identified by the ENCODE cCREs in the first intron of *MMP2*, which overlaps with a DNase I hypersensitivity cluster, and with ENCODE ChIP-seq data for the histone modification H3K4me1, for the histone acetyltransferase EP300, and for the hypoxia-induced transcription factors FOS, JUND, and MAFK.

response by detaining HIF-1α in the nucleus (Ueda et al., 2008). These data further support the presence of the poised promoter located in the first intron of the *MMP2* gene and suggest that it may be affected by a putative enhancer element activated by the binding of well-known hypoxia-induced transcription factors in the activation of the NTT-MMP-2 expression. However, this hypothesis remains to be tested.

DNA METHYLATION AND THE NTT-MMP-2 ISOFORM EXPRESSION

DNA methylation consists of the addition of the methyl group in cytosines followed by guanines, named CpG dinucleotides. CpG islands are regions enriched in CpG dinucleotides that are normally located in gene promoters and that are implicated in the regulation of gene expression (Gardiner-Garden and Frommer, 1987; Jones, 2012). ENCODE data show a CpG island located in the promoter/exon 1 of the *MMP2* gene (**Figure 2**), which are not methylated in most of the ENCODE cell lines. However, there is no CpG methylation data by Methyl 450K Bead Arrays from ENCODE, which overlap with the latent promoter region in the intron 1 of the *MMP2* gene (**Supplementary Figure 1**).

DNA methylation represses gene transcription when located in promoter regions and activates transcription when located in gene body (Jones, 2012). Methylation pattern is known to differ between promoters and alternative promoters in the same gene and among different tissues, indicating a dynamic physiological change in DNA methylation. Thus, DNA methylation may have a significant role in the differential use of alternative promoters, which may be related to the functional differentiation of promoters with or without CpG islands (Cheong et al., 2006). Moreover, intragenic DNA methylation has a major role in regulating alternative promoters in gene bodies (Maunakea et al., 2010). While there are no DNA methylation data from ENCODE for the intron 1 of the *MMP2* gene, there are other CpG islands in this region (**Supplementary Figure 1**). However, it is unknown whether the overlap of the intron 1 region with DNA methylation may affect the NTT-MMP-2 expression.

The NTT-MMP-2 isoform is induced by hypoxia (Lovett et al., 2012), which is present in cancer and other diseases (Ivan and Kaelin, 2017). The cells sense and adapt to hypoxia by activating hypoxia-inducible transcription factors, but the cells differ in their transcriptional response to hypoxia (Ivan and Kaelin, 2017). A probable explanation is that the hypoxia-inducible transcription factors do not bind to CpG dinucleotides that are

methylated. Therefore, the specific DNA methylation patterns of a cell established under normoxic conditions determine the hypoxia-inducible binding profiles for the transcription factors, and define how cell types response to hypoxia (D'Anna et al., 2020). Taken together, these processes could explain the mechanism for regulating the latent promoter located in the intron of the *MMP2* gene in the condition of hypoxia by DNA methylation. However, these hypotheses remain to be proved.

FUNCTIONAL ASSESSMENT OF THE NTT-MMP-2 ISOFORM

Cardiac-specific transgenic mice expressing the NTT-MMP-2 isoform were generated to determine its functional significance (Lovett et al., 2013). These mice developed progressive cardiomyocyte and ventricular hypertrophy associated with systolic heart failure. The NTT-MMP-2 transgenic hearts also showed more severe injury following *ex vivo* IRI. Therefore, this NTT-MMP-2 isoform induced by oxidative stress directly contributed, in the absence of superimposed injury, to cardiomyocyte hypertrophy, inflammation, systolic heart failure, and enhanced susceptibility to IRI (Lovett et al., 2013).

Further studies evaluated the effects of the presence of the NTT-MMP-2 isoform (Lovett et al., 2014). At 4 to 5-month-old transgenic mice, the NTT-MMP-2 expression was located in the mitochondria and in the Z-line of the sarcomere. When compared with wild-type mice, transgenic mice expressing the NTT-MMP-2 isoform presented impaired myocardial contraction, without decreasing myofilament force, but affecting calcium transients. However, the FL-MMP-2 impaired cardiomyocyte contractility by decreasing myofilament force. Thus, the FL-MMP-2 and NTT-MMP-2 have distinctive pathophysiological mechanisms in the cardiomyocyte by impairing different intracellular processes (Lovett et al., 2014).

Transgenic mice expressing the NTT-MMP-2 isoform specifically in the renal proximal tubule cells were generated to evaluate the effects of this isoform (Ceron et al., 2017). At 4 months of age, the NTT-MMP-2 transgenic mouse kidneys presented tubular epithelial cell necrosis, mitochondrial loss of organized cristae, and mitochondrial permeability transition, mitophagy observed at ultrastructural analysis. At 8 months old, transgenic mice expressing the NTT-MMP-2 isoform presented severe structural kidney abnormalities, as tubular atrophy, necrosis of tubular epithelial cells, and mononuclear cell infiltration and evidence of mitochondrial reactive oxygen species production. Glomerular changes were not present. At this time point, transgenic mice expressing the NTT-MMP-2 isoform also had a decrease in renal function compared with wild-type mice (Ceron et al., 2017).

NTT-MMP-2 AND RENAL DYSFUNCTION AND KIDNEY DISEASES

Delayed graft function, a clinical example of renal acute IRI, is a complication of renal transplantation, which results from tubular epithelial cell injury and has consequences as post-transplantation dialysis, increased incidence of acute rejection, and poorer long-term outcomes (Moeckli et al., 2019; Nieuwenhuijs-Moeke et al., 2020). The extent of tubular epithelial injury and the expression of both FL-MMP-2 and NTT-MMP-2 were analyzed in renal biopsies of controls and patients diagnosed with delayed graft function, and these expressions were correlated with the amount of tubular damage in patients (Wanga et al., 2015). While FL-MMP-2 expression was diffusely found in control kidney biopsies, NTT-MMP-2 was found located in a pattern characteristic of mitochondria only in biopsies of patients with delayed graft function (Wanga et al., 2015).

The mitochondrial NTT-MMP-2 isoform was also evaluated in *acute kidney injury*, a frequent complication in severely ill patients that may progress to *chronic kidney disease*. Mitochondria dysfunction increases oxidative stress and cause tubular inflammation, one of the major fibrotic processes in renal diseases (Maekawa and Inagi, 2019; Husain-Syed et al., 2020). Wild-type mice and transgenic NTT-MMP-2 mice were submitted to 40 min of unilateral renal IRI, and the contralateral non-clamped kidney was evaluated for systemic inflammatory responses (Ceron et al., 2017). At 96 h following IRI, the contralateral kidney of wild-type mice presented normal morphology, while the kidney subjected to IRI presented a mild degree of tubular dilatation, inflammation, and cast formation. However, the contralateral kidney of NTT-MMP-2 transgenic mice showed mild to moderate degrees of injury, and the kidneys subjected to IRI showed more extensive injury, with massive cast formation, tubular dilatation, and cellular inflammation than the wild-type kidneys subjected to IRI (Ceron et al., 2017).

Three weeks after IRI, the differences between wild-type and NTT-MMP-2 transgenic mice were more prominent. While IRI and contralateral of wild-type mice showed moderate injury, the kidney of NTT-MMP-2 mice subjected to IRI showed extensive mononuclear cell infiltration, fibrosis, and tubular epithelial cell dropout (Ceron et al., 2017). Moreover, the contralateral kidneys showed cellular inflammation, fibrosis, tubular dilatation, and tubular epithelial cell dropout, indicating a sustained systemic inflammatory response. These findings suggest that the NTT-MMP-2 sensitizes the kidneys to more severe IRI (Ceron et al., 2017).

The NTT-MMP-2 was also showed to induce sustained systemic inflammatory response after IRI, which was not observed in the wild-type kidneys. The kidney of the NTT-MMP-2 transgenic mice present enhanced expression of *OAS-1*, *IRF-7*, and *CXCL-10* at 96 h following IRI when compared with IRI kidneys of the wild-type mice and the contralateral kidneys, suggesting the induction of a systemic inflammatory response by NTT-MMP-2. This enhanced expression of innate immunity genes and a sustained release of danger-associated molecular patterns were persistent 3 weeks following the IRI in the kidney subjected to the injury and the contralateral kidney of NTT-MMP-2 transgenic mice (Ceron et al., 2017).

The NTT-MMP-2 also participates in chronic kidney disease. The HypoE/SR-B1 Mx1-Cre mice is a mice model of accelerated atherogenesis, which develops a diffuse atherosclerosis, chronic kidney disease and ischemic cardiomyopathy were given a

high-fat diet (Wang et al., 2014; Luk et al., 2016). After 22 days of high-fat diet, an increased expression of both FL-MMP-2 and NTT-MMP-2 was associated with tubular epithelial cell necrosis, kidney inflammation, and elevated plasma blood urea nitrogen levels when compared with normal diet-fed mice (Ceron et al., 2017).

Normal *aging* also leads to a decline in the kidney function. While the mechanisms are not fully known, oxidative stress and inflammation may participate in the aging changes in organ functions (Panickar and Jewell, 2018). NTT-MMP-2 was increased in the renal proximal tubules in aged mouse (14 months old wild-type mice), but it was absent at 4 months old wild-type mice. No differences were observed in FL-MMP-2 expression as a function of increasing age. The NTT-MMP-2 was suggested to be a link between the inflammatory state and the declined renal function that occurs on the aging process (Ceron et al., 2017).

Diabetic nephropathy is a complication of diabetes mellitus and a frequent cause of chronic kidney disease. The participation of both MMP-2 isoforms was also examined in diabetic nephropathy (Kim et al., 2017). The increased expression of the FL-MMP-2 and NTT-MMP-2 was observed in HK2 cells cultured in high glucose or 4-hydroxy-2-hexenal (an oxidative stress inductor). However, the pretreatment of HK2 cells with the antioxidant/NF-κB inhibitor pyrrolidine dithiocarbamate inhibited only the NTT-MMP-2 expression. In the murine model of type 1 diabetes mellitus induced by streptozotocin, NTT-MMP-2 was intensely expressed in the diabetic kidneys, while FL-MMP-2 was present in control and diabetic kidneys. Finally, an increase in both MMP-2 isoforms was found in renal biopsies of patients with diabetic nephropathy (Kim et al., 2017).

To explore the possible mechanisms of aging in renal function, the FL-MMP-2 and NTT-MMP-2 were examined in two mouse models of chronic kidney disease, the streptozotocin-induced model of type 1 diabetes mellitus, and the 5/6 nephrectomy model of chronic kidney disease in mice aged 8 weeks (young mice) or 14 months (old mice). The expression of both isoforms was increased independently of mice age in both mouse models. However, only the NTT-MMP-2 expression was increased in mice aged 14 months, which was associated with the tubulointerstitial fibrosis development in chronic kidney disease (Rhee et al., 2018).

The temporal and spatial locations of both MMP-2 isoforms were examined in a mouse model of type 1 diabetes mellitus induced by streptozotocin and in the db/db mouse model of type 2 diabetes mellitus. Both the FL-MMP-2 and NTT-MMP-2 were increased earlier in the kidney of streptozotocin mice than in db/db mice. However, while FL-MMP-2 was located in the cortices and outer medullae, NTT-MMP-2 was located only in the cortices. Moreover, the levels of nitrotyrosine, a marker of nitrosative stress, were increased similarly to the NTT-MMP-2 isoform (Kim et al., 2018).

NTT-MMP-2 AND HEART INJURY

Diabetic cardiomyopathy is a condition associated with enhanced reactive oxygen species production and mitochondrial dysfunction (Cieluch et al., 2020). The expression of FL-MMP-2 and NTT-MMP-2 was increased both in H9C2 cells exposed to high glucose media and in the heart of streptozotocin-induced diabetes mouse model. The FL-MMP-2 was located in the cardiomyocyte sarcomeres, and the NTT-MMP-2 mainly in the subsarcolemmal space, where mitochondria are abundant. The degree of mitochondrial damage was positively correlated to NTT-MMP-2 expression, and the decreased left ventricular systolic function observed in diabetic mice was associated with the increased expression of both MMP-2 isoforms (Lee H. W. et al., 2019).

Increased cardiac MMP-2 activity was found in hearts of mice treated with doxorubicin, in part, by upregulating NTT-MMP-2. Cardiotoxicity was attenuated by MMP inhibitors (Chan et al., 2021). Regarding MMPs inhibitors, they are shown to be protective in different models of renal and cardiac diseases, including type 1 diabetes (Yaras et al., 2008), *in vivo* renal injury (Labossiere et al., 2015; Lee T. F. et al., 2019), and other models of cardiac injury and cardiovascular dysfunction (Kandasamy et al., 2010; Rizzi et al., 2010; Guimaraes et al., 2011; Castro et al., 2012). In this context, FL-MMP-2 and NTT-MMP-2 are relevant targets of pharmacological intervention.

CONCLUSION AND PERSPECTIVES

We provide insights into the epigenetic regulation of the latent promoter located in the first intron of *MMP-2*, which support the activation of the NTT-MMP-2 isoform. Moreover, we reviewed recent evidence for the presence of NTT-MMP-2 in renal dysfunction and in kidney and cardiac diseases. Noteworthy, both the FL-MMP-2 and NTT-MMP-2 isoforms can be activated in tissue injury/oxidative stress models. However, FL-MMP-2 is directly activated by oxidative stress whereas there is transcriptional activation and expression of NTT-MMP-2. Further studies should consider that these isoforms would act in different time frames, subcellular locales, and protein targets in the development of tissue injuries and diseases. Taken together, these findings may help to understand how hypoxia and oxidative stress trigger NTT-MMP-2 expression, which are relevant pathophysiological mechanisms to several diseases. Our insights may guide further experimental and clinical studies that are needed to elucidate the underlying mechanisms and the role of NTT-MMP-2 in renal dysfunction. We expect that these future efforts may help to establish the NTT-MMP-2 as a potential therapeutic target in kidney diseases.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: UCSC Genome Browser Gateway, available at: https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&non-VirtPosition=&position=chr16%3A55478079%2D55483623&hgsid=963348143_Mp3s46YxzZ1zGnQs8Ct0oWoyZOKp.

AUTHOR CONTRIBUTIONS

JC, ML, and CC drafted the manuscript and prepared figures. JC, AS, ML, and CC edited and revised the manuscript. All authors have read and approved the final version of manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.637148/full#supplementary-material

Supplementary Figure 1 | UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. Characterization of the genomic position chr16:55,512,747-55,517,056 showing the location of CpG dinucleotides and CpG islands located in the *MMP2* gene. **(A)** The CpG methylation by Methyl 450K Bead Array ENCODE/HAIB showing the CpG dinucleotides and CpG islands in the promoter region/exon 1 and intron 1 that are not methylated in most of the ENCODE cell lines, except in the HeLa-S3 line, and the DNA methylation by reduced representation bisulfite Seq from ENCODE/HudsonAlpha showing the same pattern of methylation in this region. **(B)** The output of MethPrimer showed other CpG dinucleotides and CpG islands in the *MMP2* gene, which are not covered by the ENCODE techniques publicly available at the UCSC Genome Browser.

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Conflict of Interest: 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.

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5. DISCUSSÃO

A PE é uma doença heterogênea e multifatorial, responsável por até 26% das mortes maternas em todo o mundo, sendo considerada como a principal complicação da gestação (ACOG, 2019). Diante de sua importância populacional e no âmbito da saúde pública, é importante entender os mecanismos fisiopatológicos que podem contribuir para o desenvolvimento da PE. Com foco na metilação global do DNA, nossa revisão sistemática (**CAPÍTULO 1**) (CRUZ *et al.*, 2020) foi a primeira a avaliar o papel da metilação global do DNA na fisiopatologia da PE e tem seu foco principal nos tecidos placentários, face materna e fetal, uma vez que esse órgão pode ser a fonte primária para o entendimento da fisiopatologia e desenvolvimento da PE (CRUZ *et al.*, 2020).

Os achados destacam a heterogeneidade dos estudos incluídos na revisão sistemática e que as diferenças de metilação entre placentas de gestantes com PE e gestantes dos grupos controle são observadas em genes específicos ou em regiões diferencialmente metiladas. Essas diferenças foram mais evidentes quando gestantes com EOPE e PE prematuro foram comparadas aos grupos controles. Há um padrão de hipometilação na placenta de gestantes com PE em comparação com os controles (CRUZ *et al.*, 2020). Entretanto, fora previamente descrito que o metiloma da placenta oriunda da gravidez normal é hipermetilado fora dos domínios parcialmente metilados (SCHROEDER *et al.*, 2013).

É importante destacar que o tecido placentário que foi utilizado como amostra biológica nos estudos, pode ser responsável pelos resultados heterogêneos que foram encontrados (CRUZ *et al.*, 2020). Assim, o desenho amostral dos estudos deve levar em consideração a face da placenta que está sendo analisada, para que seja possível compreender melhor os efeitos dos diferentes níveis da metilação do DNA nos diferentes tecidos para a fisiopatologia da PE. Por exemplo, esta abordagem pode ajudar a esclarecer se uma diferença de metilação na face fetal ou materna da placenta é correlacionada com a LOPE ou EOPE respectivamente, que são subtipos da PE que apresentam desfechos clínicos diferentes e aparentam ter fisiopatologias distintas.

Conhecendo melhor os genes que estão diferencialmente metilados em cada face da placenta e as vias com as quais eles estão envolvidos, poderemos entender melhor como a ativação ou inativação dessas vias em diferentes idades gestacionais podem levar aos diferentes fenótipos que cada subtipo apresenta. Além disso, esses achados heterogêneos podem ser devido à natureza multifatorial da PE, variações biológicas e outros fatores

relacionados ao desenho do estudo que podem vir a ser fator de confusão na interpretação dos dados da metilação do DNA.

Fatores de confusão que poderiam interferir nos níveis globais de metilação, tais como sexo e idade do feto, podem levar a resultados discordantes (CRUZ *et al*., 2020). Por sua vez, a idade gestacional se mostrou significativa com diferentes níveis de metilação a depender do período gestacional (CRUZ *et al*., 2020). Isso reforça a importância de um desenho experimental bem definido, com o uso de amostras correspondentes em relação à idade materna e gestacional ou estudos longitudinais onde isso possa ser acompanhado e esses potenciais vieses possam ser excluídos.

As variações genéticas encontradas entre e intra populações também podem interferir na compreensão dos resultados da metilação do DNA, sendo assim um fator de confusão (HOFFMANN *et al*., 2017). Convencionalmente, sondas que se sobrepõem com SNPs são excluídas das análises globais de dados de metiloma. Entretanto, CpG-SNPs pode levar a alteração de metilação em uma região e, assim, afetar a expressão gênica (HOFFMANN *et al*., 2017). Nesse contexto, uma perspectiva futura do grupo de pesquisa é focar no potencial de CpG-SNPs na modulação da metilação do DNA e expressão gênica para nos ajudar na interpretação dos resultados de metilação e polimorfismos genéticos em genes candidatos para a PE.

Os subtipos EOPE e PE prematuro apresentam uma hipometilação global proeminente (CRUZ *et al*., 2020). Sugerindo que a metilação tem um papel fisiopatológico nos estágios iniciais da gravidez e que a desregulação epigenética na placenta pode afetar os estágios iniciais da gestação, levando a forma mais grave e precoce da PE. A forma precoce, de fato, exibe uma notável disfunção placentária e forma mais grave da PE (VON DADELSZEN; MAGEE; ROBERTS, 2003; CHEN *et al.*, 2012).

É consenso na literatura que uma má placentação nos primeiros estágios da gestação seja um dos mecanismos fisiopatológicos centrais envolvidos da PE. Normalmente, células trofoblásticas extravilosas do feto invadem o endométrio, realizando a decidualização, e também invadem o miométrio, substituindo células endoteliais maternas da parede da artéria espiralada por citotrofoblastos endovasculares **(Fig. 1)** (WANG *et al*., 2009). Dessa forma, transformam uma artéria de baixo calibre e alta resistência em uma artéria de alto calibre e baixa resistência. Essas mudanças fisiológicas na artéria espiralada facilitam o aumento do fluxo sanguíneo materno para o espaço interviloso (CONRAD, 2021).

Entretanto, a PE é sempre associada por uma pobre invasão das células trofoblásticas e pobre remodelamento da artéria espiralada **(Fig. 1)**, o que restringe o fluxo sanguíneo para o espaço interviloso e leva à isquemia placentária (CONRAD, 2021). Essa placentação defeituosa vem sendo associada com um grande número de doenças, que são classificadas como as grandes síndromes obstétricas. Nessas síndromes estão inclusas doenças como a PE, a restrição de crescimento intrauterino, o parto prematuro, a ruptura prematura de membranas, o aborto espontâneo tardio e o descolamento da placenta (BROSENS *et al*., 2010).

Dentre os estudos que foram incluídos na revisão sistemática (**CAPÍTULO 1)**, o número de genes em repetição é baixo, sendo 48 genes na comparação de PE versus controles quando a face da placenta não foi especificada e 21 genes na comparação EOPE, LOPE versus controle na face fetal da placenta, e apenas o gene LIM e Domínios Homólogos de Calponina 1 (*LIMCH1*) está repetido e hipometilado em três estudos (CRUZ *et al*., 2020). O gene *LINCH1* regula negativamente a propagação e a migração celular (*LIN et al*., 2017), participando da organização do citoesqueleto de actina, transcrição genica e processamento de RNA (GETTEMANS *et al*., 2005). Em um perfil de expressão gênica em placentas, que teve por objetivo identificar diferentes subclasses de PE, o *LINCH1*, juntamente com outros genes mostrou expressão diferencial capaz de distinguir quatro agrupamentos - PE materna, PE canônica, PE imunológica e parto pré-termo- sendo o *LINCH1,* o gene com maior potencial discriminatório (LEAVEY *et al*., 2016).

Mecanismos transcricionais e epigenéticos controlam o desenvolvimento placentário e a diferenciação de citotrofoblastos, e são ativados pelos níveis de oxigênio durante a gestação (SONCIN; NATALE; PARAST, 2015). Alguns genes que estão em repetição entre os estudos são bem caracterizados na hipóxia e invasão trofoblástica. Por exemplo, o gene *TERT*, que está hipermetilado na análise de gestantes com PE versus grupo controle (CRUZ *et al*., 2020), têm sua expressão aumentada em tecido placentário no primeiro trimestre da gestação, mas depois sua expressão é reduzida. Entretanto, em placentas de gestantes com PE esse aumento de expressão de *TERT* não foi observada, sugerindo seu papel nos mecanismos de diferenciação trofoblástica (AKUTAGAWA *et al*., 2007). Por sua vez, Geifman-Holtzman *et al* mostraram um aumento da expressão de *TERT* em placentas de gestantes com PE comparadas ao grupo controle. Polimorfismos maternos (não do feto) no *TERT* foram associados com parto prematuro e rompimento prematuro de membranas (MARS *et al*., 2018).

O oxigênio é necessário para o desenvolvimento e manutenção da vida, entretanto este é deletério quando os seus níveis estão desregulados. Níveis altos ou baixos de oxigênio são

prejudiciais para o desenvolvimento do embrião. Em uma gestação normal, o feto e a placenta se mantêm em um ambiente hipóxico (JAUNIAUX *et al*., 2003), essa baixa tensão do oxigênio propicia a proliferação celular e a angiogênese da placenta, enquanto no feto em desenvolvimento ajuda na vasculogênese, hematopoiese e condrogênese (GIACCIA *et al*., 2004; REAM *et al*., 2008).

Por volta da 10ª-12ª semana de gestação, a circulação intersticial se estabelece, a tensão do oxigênio aumenta e permanece nesse nível até o terceiro trimestre. Para uma função placentária normal e correto desenvolvimento do feto, a placenta se adapta às alterações dos níveis de oxigênio pela modulação da expressão do Fator Induzível por Hipóxia-1α (*HIF-1α*) e pelo aumento de defesas por meio de antioxidantes celulares (GIACCIA *et al*., 2004; REAM *et al*., 2008). Assim, a variação dos níveis de oxigênio no útero é normal e necessária. Entretanto, a hipóxia fetal excessiva, que pode ser oriunda de diversos fatores (prolapso ou oclusão do cordão umbilical, infarto placentário, hipertensão, anemia, doença pulmonar, PE e tabagismo materno), podem levar a resultados adversos, tais como morte fetal (ANDERSON, 2002), restrição de crescimento intrauterino e baixo peso ao nascimento (HUTTER *et al*., 2010).

O *HIF-1α* não faz parte da lista de genes em repetição (CRUZ *et al*., 2020), mas têm papel na regulação de genes de interesse do grupo de estudo (CRUZ *et al*., 2021; FRAGKIADAKI *et al*., 2016), uma vez que é de grande importância na resposta celular à deficiência de oxigênio, com papel preponderante durante a embriogênese (SUTTER *et al*., 2000). A região promotora de *TERT* possui dois motivos consenso de ligação de *HIF-1α*, que são essenciais para sua transativação pelo mesmo. Estudos mostraram como alterações nesses motivos podem diminuir a expressão de TERT durante a hipóxia, indicando que a regulação positiva de TERT sob condições de hipóxia é diretamente mediada por *HIF-1α*. Diante disso, esse processo de regulação da atividade do promotor do *TERT* pelo *HIF-1α* representa um mecanismo para o crescimento do trofoblasto durante a hipóxia (NISHI *et al*., 2004).

Alguns genes que apresentaram metilação diferencial entre EOPE versus LOPE no estudo global, mostraram aumento da expressão e diminuição da metilação do DNA nas placentas de PE em estudos de genes candidatos: *LEP* (hipometilado), *PAPPA2* (hipermetilado), *YWHAQ* e *PLXNB1* (hipometilados) entre PE versus controle (CRUZ *et al*., 2020; LOUWEN *et al*., 2012; KRAMER; LAMALE-SMITH; WINN, 2016; NISHIZAWA, HARUKI *et al*., 2008; WAGNER; OTOMO; CHRISTIANS, 2011; LIU *et al*., 2014; LI *et al*., 2018).

Seguindo a elucidação do papel de genes candidatos na fisiopatologia da PE, o gene *TIMP-3* se mostra relevante para estudos de associação com a PE pelo seu papel na inibição das metaloproteinases de matriz extracelular (MMPs), que participam ativamente do processo de invasão das células trofoblásticas e decidualização (WANG *et al*., 2010*)*. Além disso, o gene *TIMP-3* possui elementos regulatórios diversos. A regulação do *TIMP3* pode ser por ação de fatores de transcrição e por regulação da cromatina (LEE; YOUNG, 2013). A forma mais comum é por fatores de transcrição que envolve ELF3, SP1, SMAD2, e SMAD4 que se ligam ao promotor do *TIMP-3* e regula sua expressão (JOBLING *et al*., 2002; QURESHI *et al*., 2005; QURESHI; RICCI; ZAFARULLAH, 2008; YAN, D. *et al*., 2013; ZERROUQI *et al*., 2012). Além disso, a expressão do *TIMP3* pode ser regulada por metilação e acetilação de histonas (SU *et al*., 2019). A repressão transcricional de *TIMP-3* é associada com aumento de H3K27me3 e redução de H3K9ac no promotor do gene *TIMP-3*. A HDAC9 é uma das histonas desacetilase que tem papel descrito na repressão do *TIMP-3* por meio da hipoacetilação de histonas no promotor (XIE *et al*., 2019), KDM1A leva a repressão por desmetilação de H3K4me2 e o EZH2 com atividade de histona metiltransferase reduz a expressão de *TIMP-3* por ação nas H3K27me3 (XU *et al*., 2013).

Nossa reanálise *in silico* de dados de metilação do DNA e expressão gênica do TIMP-3 em amostras de placenta de gestantes com PE teve como objetivo entender o papel do *TIMP-3* na fisiopatologia da PE. As amostras de PE utilizadas nesse artigo foram reclassificadas de acordo com a idade gestacional de início dos sintomas em EOPE e LOPE, no intuito de compreendermos a participação do *TIMP-3* nos dois subtipos de PE **(CAPÍTULO 2)**. Nós identificamos sondas diferencialmente metiladas entre os grupos de estudo, sendo: 28 em PE, 38 em EOPE, 20 em LOPE e 4 em PE a termo comparados com controles, e 8 sondas na comparação de EOPE versus LOPE. Mais de 70% das sondas estavam hipometiladas, exceto na comparação entre EOPE versus LOPE. Nós encontramos um aumento de expressão de *TIMP-3* em PE, EOPE e LOPE comparados com o grupo controle, em amostras de placentas correspondentes às que foram utilizadas na análise de metilação do DNA (CRUZ *et al*., 2022).

A maioria das sondas diferencialmente metiladas estão em sobreposição nos grupos de estudo (CRUZ *et al*., 2022). No contexto da metilação do promotor do gene *TIMP-3,* estas sobreposições indicam que esse gene pode estar atuando na fisiopatologia de ambos os tipos de PE, EOPE e LOPE. Ainda assim, foram encontradas apenas oito sondas diferencialmente metiladas entre EOPE e LOPE (CRUZ *et al*., 2022), sendo um indício fraco para uma diferença de etiologia com participação do *TIMP-3* para esses dois subtipos de PE.

Importante notar que as sondas que foram analisadas neste estudo **(CAPÍTULO 2)** estão localizadas apenas no promotor do gene *TIMP-3* (CRUZ *et al*., 2022). Isso é uma questão de ordem técnica, uma vez que as metodologias de análise global de metilação do DNA ainda apresentam uma cobertura relativamente baixa do genoma humano e sua maioria está focada em sondas localizadas nos promotores dos genes. Na análise de genes candidatos, é interessante fazer uso de metodologias *in silico* para identificar regiões regulatórias ou ricas em conteúdo CG no gene de interesse e explorar o uso de metodologias de análise de metilação do DNA que analise essas regiões. Análises mais extensas do gene podem nos fornecer informações extras sobre o papel da metilação do DNA na expressão desses genes.

Os trofoblastos extravilosos proliferam, migram e invadem o endométrio, além de remodelar a artéria espiralada materna durante a implantação para que haja uma fixação adequada do feto em desenvolvimento com a parede do útero materno e uma troca eficiente de nutrientes entre o feto em desenvolvimento e a mãe (PIJNENBORG *et al*., 1981). Esse processo invasivo que ocorre na placentação é muito semelhante com o que ocorre durante o crescimento de células cancerosas, sendo um processo mediado pela ação de endopeptidases que degradam a matriz extracelular e ajudam na remodelação do tecido (SOUNDARAJAN; RAO 2004).

Os achados de hipometilação e aumento de expressão do *TIMP-3* na EOPE (CRUZ *et al*., 2022) pode ser um indicativo de um mecanismo fisiopatológico logo no início da gestação, onde o aumento do TIMP-3 inibe a atividade das MMPs, bloqueando o processo de degradação da matriz extracelular, que é necessário para a invasão trofoblástica, e bloqueando também o remodelamento da artéria e do tecido uterino (CRUZ *et al*., 2022). A PE é sempre associada por uma pobre invasão das células trofoblásticas e pobre remodelamento das artérias espiraladas (CONRAD, 2021), assim, esse mecanismo de inativação de MMPs pelos TIMPs, pode ser uma via que explica essa má invasão e desenvolvimento da PE. Entretanto, essa hipótese precisa ser testada experimentalmente, primeiro identificando qual das MMPs têm maior associação com a proteína TIMP-3 e como culturas celulares com diferentes níveis de TIMP-3 e MMPs se comportam em relação a invasão e métricas de remodelamento tecidual.

Além da sua função como inibidor das MMPs, o TIMP-3 age como um fator antiangiogênico e essa função parece ser independente do processo de inibição das MMPs (QI *et al*., 2003). O Fator de Crescimento Endotelial Vascular (*VEGF*) é uma subfamília que codifica proteínas sinalizadoras que promove a vasculogênese, angiogênese, migração celular, crescimento de células endoteliais e proliferação de vasos sanguíneos; além de ser um

anti-apoptótico de células endoteliais (APTE; CHEN; FERRARA 2019; PATAN 2004). A expressão do VEGF é induzida pela hipóxia e os seus receptores são FLT1/VEGFR1, KDR/VEGFR2 e FLT4/VEGRF3. A expressão desses receptores é variável em diferentes tipos de células epiteliais (MELINCOVICE *et al*., 2018). Essa família de fatores de crescimento endotelial e vascular, principalmente o VEGFA com suas isoformas, é crucial para a regulação da vasculogênese e angiogênese (TAMMELA *et al*., 2005), e o VEGFR2 tem uma das mais fortes atividades pró-angiogênicas entre os receptores (MELINCOVICE *et al*., 2018). A função do TIMP-3 como um fator antiangiogênico se dá por inibir a ligação do VEGF ao seu receptor VEGFR2, que inibe a fosforilação do VEGFR2 e assim, bloqueia uma série de eventos de sinalização a jusante (QI *et al*., 2003).

A PE é uma desordem vascular sistêmica e que tem a disfunção endotelial generalizada da mãe como uma de suas principais características (TOMIMATSU *et al*., 2019). A disfunção endotelial materna causada por fatores placentários e a teoria dos dois estágios são aceitos como uma das fisiopatologias da PE (ROBERTS; HUBEL, 2009). O estágio inicial começa com a placentação anormal com pobre invasão das células trofoblásticas e remodelamento da artéria espiralada, essa falha na remodelação resulta em perfusão placentária reduzida (HECHT *et al*., 2016), que leva ao segundo estágio, da liberação dos fatores antiangiogênicos placentários na circulação materna (TOMIMATSU *et al*., 2019).

Há evidências que o sFlt1 placentário é um dos fatores desencadeantes mais importantes para a disfunção endotelial materna (TAMIMATSU *et al*., 2017). sFlt1 é uma variante de *splicing* do VEGFR1 (Flt1) que contém apenas o domínio extracelular de ligação ao ligante de VEGFR1. O VEGFR1 é receptor ligante de VEGF e PlGF, assim o sFlt1 inibe a sinalização pró-angiogênica por se ligar a VEGF e PlGF bloqueando essa via e levando a disfunção endotelial com a diminuição de vasodilatadores como o óxido nítrico e prostaciclina e aumento de vasoconstritores como a endotelina-1 (TOMIMATSU *et al*., 2019). Os mecanismos exatos de como a sFlt1 circulante pode levar a disfunção endotelial em gestantes com PE ainda não é bem claro (TOMIMATSU *et al*., 2019). Entretanto, pelo que é conhecido sobre essa via, podemos hipotetizar um possível efeito semelhante de bloqueio da angiogênese pelo aumento dos níveis de TIMP-3 circulante, uma vez que este bloqueia a ligação do VEGF ao VEGFR2 inibindo a migração, sinalização e angiogênese a jusante (QI *et al*., 2003). Essa função antiangiogênica pode levar a uma disfunção endotelial generalizada que pode levar às condições sistêmicas que são observadas na PE, bem como pode explicar o aumento dos níveis de expressão na EOPE e LOPE.

Na sequência da investigação sobre o potencial papel do TIMP-3 na PE, comparamos os níveis circulantes do TIMP-3 entre pacientes com PE, pacientes com hipertensão gestacional, gestantes saudáveis e mulheres não gestantes, e em um estudo primário do tipo caso-controle e em uma população de replicação entre PE e gestantes saudáveis **(CAPÍTULO 3)**. No estudo primário e no estudo de replicação, os níveis plasmáticos de TIMP-3 estavam aumentados na PE em comparação com as gestantes saudáveis e não gestantes. Entretanto, não foi encontrada diferença nos níveis de TIMP-3 entre EOPE e LOPE (PALEI *et al*., 2022).

Esses resultados corroboram os dados de hipometilação e hiperexpressão do TIMP-3 que foram encontrados em amostras de placenta **(CAPÍTULO 2)** (CRUZ *et al*,. 2022). E com esses dados de proteína em pacientes com PE, podemos ver que não é possível discernir os dois tipos de PE (EOPE e LOPE) por meio dos níveis proteicos de TIMP-3. Isso reforça a hipótese da participação do TIMP-3 nos dois estágios de desenvolvimento da PE. Primeiro, por bloquear as MMPs e, portanto, levar à placentação anormal e pobre invasão trofoblástica (WANG *et al*., 2010*)*. Segundo, por bloquear a ligação do VEGF ao seu VEGFR2 que, por sua vez, leva a disfunção endotelial materna generalizada (QI *et al*., 2003).

Importante destacar que seria importante sabermos se o nível da proteína TIMP-3 varia em diferentes estágios da gestação e se teríamos, por exemplo, uma correlação de aumento no primeiro semestre com o desenvolvimento da EOPE, ou um aumento durante o terceiro trimestre e uma associação com o desenvolvimento da LOPE. Isso é uma hipótese a ser testada, possivelmente por meio de um estudo longitudinal para sabermos os níveis de TIMP-3 logo no início da implantação e invasão trofoblástica e ao longo da gestação, no intuito de identificar uma possível variação nas concentrações dos níveis de TIMP-3 de acordo com a idade gestacional de desenvolvimento dos subtipos de PE.

Interessante destacar que observamos os níveis plasmáticos de TIMP-3 como significativamente e positivamente correlacionados aos níveis plasmáticos de MMP-2 (PALEI *et al*., 2022). As MMPs são uma família de endopepitdases zinco dependentes com mais de 20 membros (LI *et al*., 2014; MONTAGNANA *et al*., 2009). Estes podem ser organizados em grupos como as gelatinases, colagenases, estromelisinas, matrilisinas e MMPs do tipo membrana (RAFFETTO; KHALIL, 2008). As MMPs são reguladas pela ativação de sua forma latente de zimogênio e a nível de expressão de mRNA, e são muitas vezes secretadas como forma inativa de pró-MMP que é clivada por outras proteínas, inclusive outras MMPs em sua forma ativa (CUI *et al*., 2017). Não apenas fatores solúveis, mas também interação célula-célula e célula-matriz são decisivos para a expressão gênica das MMPs. Essas enzimas são inibidas pela ação de inibidores teciduais endógenos de MMPs (TIMPs), e a razão

MMPs/TIMPs é o que determina a extensão da degradação da matriz extracelular e remodelação do tecido (NEGASE; WOESSNER 1999).

A inibição de cada MMP é variável em relação aos TIMPs. Por exemplo, TIMP-1 inibe fortemente a MMP-9, o TIMP-2 inibe fortemente a MMP-2 e pode inibir outras MMPs, além disso quando está em baixas concentrações, pode formar complexo TIMP-2-pro-MMP-2-MT1-MMP que resulta na ativação da pro-MMP-2 (SARKAR *et al*., 2020; BREW; NAGASE 2010; HERNANDEZ-BARRANTES *et al*., 2000). TIMP-4 pode se ligar nesse complexo TIMP-4-pro-MMP-2-MT1-MMP, não permitindo a ligação de TIMP-2 no complexo, inibindo a ativação de pró-MMP-2 via inibição de MT1-MMP (BIGG *et al*., 2001). O TIMP-3 pode formar um complexo semelhante e inibir a ativação da pró-MMP-2. Além da MMP-2, o TIMP-3 pode efetivamente inibir vários tipos de ADAMs (BREW; NAGASE, 2010).

Observando esse padrão de inibição da MMP-2 pelo TIMP-3, a correlação positiva dos seus níveis plasmáticos nas amostras de gestantes com PE seria um achado controverso (PALEI *et al*., 2022). Entretanto, a interação das MMPs e TIMPs é complexa, participando além da inibição, de ativação e endereçamento das MMPs para o ambiente extracelular (CABRAL-PACHECO *et al*., 2020; ZHAO *et al*., 2004). As atividade catalíticas das MMPs são reguladas em quatro níveis, sendo eles: expressão gênica com regulação transcricional e pós-transcricional; compartimentalização, ou seja, a localização extracelular e tecido ou célula onde a MMP é liberada; ativação da pró-enzima pela remoção do pró-domínio; e pela inibição por inibidores específicos como os TIMPs e por inibidores não específicos de proteinases, como a α 2-macrogobulina (RA; PARKS 2007; OVERALL; LÓPEZ-OTÍN 2002; LOFFEK, SCHILLING, FRANZKE 2011). Dessas quatro formas de regulação, conhecemos a ação dos TIMPs em apenas duas delas. Por isso, buscamos entender os mecanismos epigenéticos que regulam a expressão da isoforma truncada no N-terminal da MMP-2 (NTT-MMP-2) **(CAPÍTULO 4)** (CRUZ *et al*., 2021).

A MMP-2 é uma metaloproteinase do tipo gelatinase que é secretada para fora da célula, mas também pode ser encontrada no espaço intracelular e do citosol, onde age sobre vários substratos (ALI *et al*., 2012). Essa enzima, assim como as outras MMPs, foram originalmente consideradas enzimas amplas e não específicas (HANOOCKS *et al*., 2021) que estão diretamente relacionadas a praticamente todos os processos biológicos que envolvem remodelação da matriz extracelular (MEC, do inglês *extracellular Matrix*) ao longo da vida dos mamíferos, da implantação do embrião (ALEXANDER *et al*., 1996) até a morte celular e necrose (EGEBLAD, WERB 2007; CURRIE *et al*., 2007). Entretanto, estudos de proteoma

em animais modelos mostraram que além de clivar moléculas da MEC, *in vivo* essas enzimas alcançam mais substratos, incluindo citocinas, receptores e proteínas de ligação às citocinas, fatores angiogênicos e de crescimento, quimiocinas e receptores ECM, e sugerem que seu papel é de processamento, ao invés de degradação (RODRÍGUEZ *et al*., 2010).

A MMP-2 demonstrou participar da liberação proteolítica do VEGF a partir da formação de um complexo inibitório com o fator de crescimento do tecido conjuntivo (CTGF) e o regulador de afinidade do peptídeo de heparina (HARP), estimulando a neovascularização (DEAN *et al*., 2007). Essa interação, demonstra como a correlação positiva dos níveis plasmáticos de TIMP-3 e MMP-2 nas amostras de gestantes com PE (PALEI *et al*., 2022) **(CAPÍTULO 3)** não seria tão contraditório. Primeiro pelas extensas vias de ativação das MMPs, que já foram descritas, segundo que o TIMP-3 não é o principal inibidor da MMP-2. E também pois, não é porque a enzima pode realizar uma função, que irá realizar essa função (OVERALL, BLOBEL 2007). Essa interação entre inibidores e MMPs, assim como qual MMP atua sobre qual substrato é muito complexa e nem tudo que é relatado por estudos de prospecção, *in vitro* e *in vivo* em animais modelos pode ser encontrado em humanos (RODRÍGUEZ *et al*., 2010).

A isoforma NTT-MMP-2 tem sua expressão induzida por hipóxia e estresse oxidativo, que ativa o promotor latente no primeiro íntron da *MMP-2*, gerando um transcrito de mRNA truncado na extremidade 5' (LOVETT *et al*., 2012). Durante o processo de placentação e desenvolvimento do feto, este se mantém em um ambiente hipóxico, levando à proliferação celular e desenvolvimento cardiovascular (JAUNIAUX *et al*., 2003; GIACCIA *et al*., 2004; REAM *et al*., 2008).

No primeiro íntron do gene *MMP-2* temos um intensificador que se sobrepõe a um *cluster* de hipersensibilidade a DNase I, uma modificação da histona H3K4me1 e aos fatores de transcrição EP300, FOS, JUND e MAFK, que são conhecidos por participar de processos de transcrição que são ativados pela hipóxia. Adicionalmente, nessa região do promotor latente não há descrição de ilhas CpGs ou dinucleotídeos CpGs (CRUZ *et al*., 2021). A metilação do DNA reprime e ativa a transcrição gênica quando está localizada no promotor ou no corpo do gene, respectivamente (JONES, 2012). Sabe-se que o padrão de metilação difere entre promotores e promotores alternativos no mesmo gene e em diferentes tecidos, indicando uma mudança fisiológica dinâmica da metilação do DNA (CHEONG *et al*., 2006). Apesar de sabermos que a metilação intragência tem papel importante na regulação de promotores

alternativos (MAUNAKEIA *et al*., 2010), não temos evidências *in silico* da presença da metilação do DNA regulando esse promotor alternativo do gene *MMP-2* (CRUZ *et al*., 2021).

Uma das perspectivas do nosso grupo de estudo é identificar qual o papel dessa isoforma no tecido placentário, se alterações epigenéticas em suas regiões regulatórias estão associadas a alteração de sua expressão e com o desenvolvimento da PE.

6. CONCLUSÃO

Diante do exposto, é possível concluir que alterações epigenéticas em regiões regulatórias de genes que participam da via de invasão trofoblástica estão relacionadas ao desenvolvimento da pré-eclâmpsia (PE) e seus subtipos de início precoce (EOPE) e de início tardio dos sintomas (LOPE). Cabe destacar que, diferentes fatores podem influenciar nas alterações epigenéticas e isso reflete nos resultados encontrados. É importante salientar também que nem todo gene candidato que apresenta alterações epigenéticas envolvidos no desenvolvimento da PE, é capaz de distinguir entre os seus dois subtipos EOPE e LOPE. Por isso, a busca de biomarcadores de diagnóstico precoce da PE baseados em dados epigenéticos necessita de avaliações criteriosas para o seu uso na clínica.

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ANEXOS

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA
DE RIBEIRÃO PRETO DA UNIVERSIDADE DE SÃO PAULO

www.hcrp.fmrp.usp.br

Ribeirão Preto, 24 de julho de 2009

Ofício nº 2380/2009
CEP/MGV

Prezados Senhores,

O trabalho intitulado **"QUANTIFICAÇÃO DOS NÍVEIS DE ADIPONECTINA E LEPTINA EM MULHERES COM PRÉ-ECLÂMPSIA"** foi analisado pelo Comitê de Ética em Pesquisa, em sua 291ª Reunião Ordinária realizada em 20/07/2009 e enquadrado na categoria: **APROVADO, bem como o Termo de Consentimento Livre e Esclarecido,** de acordo com o Processo HCRP nº 5182/2009.

Este Comitê segue integralmente a Conferência Internacional de Harmonização de Boas Práticas Clínicas (IGH-GCP), bem como a Resolução nº 196/96 CNS/MS.

Lembramos que devem ser apresentados a este CEP, o Relatório Parcial e o Relatório Final da pesquisa.

Atenciosamente.

DRª MÁRCIA GUIMARÃES VILLANOVA
Vice-Coordenadora do Comitê de Ética em Pesquisa do HCRP e da FMRP-USP

Ilustríssimos Senhores
PROF. DR. RICARDO DE CARVALHO CAVALLI
JACKELINE DE SOUZA RANGEL MACHADO (Aluna)
Depto. de Ginecologia e Obstetrícia

Comitê de Ética em Pesquisa HCRP e FMRP-USP - Campus Universitário
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PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Modulação da resposta inflamatória sistêmica na pré-eclâmpsia
Subprojeto 1: MODULAÇÃO M1/M2 EM MONÓCITOS DE GESTANTES PORTADORAS DE PRÉ-ECLAMPSIA
Subprojeto 2:Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Subprojeto 3: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Subprojeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia

Pesquisador: Maria Terezinha Serrão Peraçoli
Área Temática:
Versão: 5
CAAE: 74067417.0.0000.5411
Instituição Proponente: Departamento de Microbiologia e Imunologia
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 4.418.043

Apresentação do Projeto:
A presente Emenda refere-se alterações nos Subprojeto 1 e Subprojeto 2, segundo o documento Carta_de_resposta_a_pendencias.pdf, postada na PB (Plataforma Brasil) em 18 de novembro de 2020.

No Subprojeto 1, não foi possível utilizar a linhagem celular THP-1, pois após várias tentativas de cultivo dessa célula em laboratório foi impossível manter a viabilidade celular. Sendo assim, foi preciso substituir as células THP-1 na metodologia por células de vinte mulheres saudáveis, que serão coletadas no ambulatório de Ginecologia e Obstetrícia. Considerando que haverá coleta de sangue de mulheres saudáveis foram incluídos TCLEs e TALE.

No Subprojeto 2, devido a resultados promissores com o uso da silibinina (outro agente

imunomodulador), decidiu-se utilizá-la também nesse projeto em andamento, uma vez que foram coletadas células suficientes para dar continuidade nos experimentos, não sendo necessário coletar maior quantidade de sangue periférico das gestantes. Dessa forma, houve apenas alteração no título e na análise laboratorial de material previamente coletado.

De acordo com retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil):

SUBPROJETO 1: A pré-eclâmpsia (PE) é uma síndrome específica da gestação que se caracteriza por um estado de má adaptação da tolerância imunológica, identificado por estresse oxidativo e ativação anormal do sistema imune inato. No plasma de gestantes portadoras de PE encontram-se níveis elevados de estruturas moleculares associadas ao estresse e morte celular, denominados padrões moleculares associados ao dano (DAMPs) tais como, proteína de choque térmico (Hsp70), high mobility group box 1 (HMGB1), Hialurona (HA) e Ácido Úrico, que parecem contribuir para a patogênese dessa doença. As DAMPs podem ativar monócitos desviando-os para perfil proinflamatório M1. O desbalanço entre citocinas pró e antiinflamatórias na PE pode ser dependente da deficiência de fatores reguladores capazes de modular essa resposta inflamatória como a vitamina D, ou ainda esse desbalanço poderia ser regulado através da administração de flavonóides com propriedades anti-inflamatórias como a silibinina. O objetivo desse trabalho é estimular e tratar monócitos com moléculas moduladoras da ativação ou regulação, para melhor compreensão das vias de ativação dos monócitos na inflamação sistêmica observada na PE e, possivelmente, propor formas alternativas para o tratamento dessa importante síndrome da gestação. Monócitos de gestantes portadoras de PE serão cultivados na presença ou ausência de hialurona de alto peso molecular (hmw-HA), vitamina D e silibinina e os monócitos de mulheres não grávidas serão cultivadas na presença ou ausência de urato monossódico (MSU), hialurona de baixo peso molecular (lmw-HA), HSP70, HMGB1, vitamina D ou silibinina, por diferentes períodos: a) por 30 min para análise da expressão de proteínas de vias de sinalização p38 MAPK, ERK1/2, IKB-, STAT1, STAT6, e NF-kBp65 por citometria de fluxo, b) por 18h para análise da expressão dos receptores TLR4, RAGE, CD64, CD44, CD163 e CD14 por citometria de fluxo e para a determinação da concentração das citocinas por IL-1, IL-6, IL-8, IL-10, IL-12p70 e TNF- por CBA e IL-23 por ELISA. Os resultados serão analisados por meio de testes paramétricos ou não-paramétricos com nível de significância de 5%.

SUBPROJETO 2: Estudos sugerem que na pré-eclâmpsia (PE) ocorre um estado de má adaptação da

tolerância imunológica, caracterizada por ativação anormal do sistema imune inato e adaptativo. As células T reguladoras (Treg) são uma população de células T responsáveis pela manutenção da tolerância e controle da inflamação, enquanto células Th17 medeiam diferentes tipos de reações inflamatórias, possuindo ações opostas. Na PE, a ocorrência de resposta inflamatória sistêmica parece ser devida ao desbalanço das subpopulações de células T CD4+ causado por aumento do número de células Th17 e diminuição das células T reguladoras em comparação às gestantes normotensas. Portanto, o balanço entre células Treg e Th17 pode ser crítico para a tolerância ao feto e para prevenção da PE. O presente estudo pretende avaliar
o efeito imunomodulador da vitamina D e da silibinina sobre as subpopulações de células T (Th17 e Treg) em gestantes préeclâmpticas. Serão estudadas 20 gestantes portadoras de PE e 20 normotensas, pareadas pela idade gestacional. Sangue periférico será obtido das gestantes de ambos os grupos para determinação da concentração plasmática de vitamina D [25(OH)D]. O efeito modulador da vitamina D e da silibinina sobre as subpopulações de células T CD4+ (Th1, Th2, Th17 e Treg) será avaliado quanto à expressão dos ativadores de transcrição para as células Th1 (STAT1 e STAT4), Th2 (STAT6), Th17 (STAT3) e Treg (STAT5) e dos fatores de transcrição intracitoplasmáticos para células Th1 (Tbet), Th2 (GATA-3), Th17 (RORt e Runx1) e Treg (FoxP3) característicos de cada subpopulação de células T, além dos receptores de IL-23 (IL-23R) e de vitamina D (VDR) por meio das técnicas de citometria de fluxo e qPCR. O perfil de citocinas pró e anti-inflamatórias produzido por essas subpopulações celulares após os tratamentos com a vitamina D e silibinina, Th1 (IFN- e TNF-), Th2 (IL-4), Th17 (IL-6, IL-17 e IL-22) e Treg (IL-10 e TGF-) no sobrenadante de cultura de células mononucleares de gestantes pré-eclâmpticas e normotensas por CBA (citometria de fluxo) e Elisa.

Objetivo da Pesquisa:

Segundo informações retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil), os objetivos do estudo são:

SUBPROJETO 1: Estimular e tratar monócitos com moléculas moduladoras da ativação ou regulação, para melhor compreensão das vias de ativação dos monócitos na inflamação sistêmica observada na PE, e possivelmente, propor formas alternativas para o tratamento dessa importante síndrome da gestação.

SUBPROJETO 2: Avaliar o efeito modulador da vitamina D e da silibinina sobre as subpopulações de células T (Th17 e T reguladoras - Treg) em gestantes pré-eclâmpticas.

A presente Emenda não se refere aos Subprojetos 3 e 4.

Avaliação dos Riscos e Benefícios:

De acordo com informações retiradas das "Informações Básicas do Projeto" na PB (Plataforma Brasil), tem-se:

RISCOS: Nos Subprojetos 1 e 2, no momento da coleta de sangue pode haver dor da picada de agulha e, raramente, formação de um pequeno hematoma no local.

BENEFÍCIOS: Os resultados do presente projeto contribuirão para melhor conhecimento dos mecanismos imunes envolvido na fisiopatologia da pré-eclâmpsia e poderão, no futuro estabelecer estratégias diferentes de prevenção e tratamento dessa patologia obstétrica.

A presente Emenda não se refere aos Subprojetos 3 e 4.

Comentários e Considerações sobre a Pesquisa:

Trata-se de Emenda ao projeto original intitulado "Modulação da resposta inflamatória sistêmica na préeclâmpsia", sob responsabilidade da Profa. Maria Terezinha Serrão Peraçoli.

Segundo o item "Justificativa da Emenda" nas "Informações básicas do projeto" da PB, houve alterações nos subprojetos 1 e 2, informações confiramdas pela pesquisadora em documento postado em 18 de novembro de 2020 (Carta_de_resposta_a_pendencias.pdf).

No SUBPROJETO 1, não foi possível utilizar a linhagem celular THP-1, pois após várias tentativas de cultivo dessa célula em laboratório foi impossível manter a viabilidade celular. Sendo assim, foi preciso substituir as células THP-1 na metodologia por células de mulheres saudáveis, que serão coletadas no ambulatório de Ginecologia e Obstetrícia.

Considerando que haverá coleta de sangue de mulheres saudáveis foram incluídos TCLEs e TALE.

No SUBPROJETO 2, devido a resultados promissores com o uso da silibinina (outro agente imunomodulador), decidiu-se utilizá-la também nesse projeto em andamento, uma vez que foram coletadas células suficientes para dar continuidade nos experimentos, não sendo necessário coletar maior quantidade de sangue periférico das gestantes. Dessa forma, houve apenas alteração no título e na análise laboratorial de material previamente coletado.

Considerações sobre os Termos de apresentação obrigatória:
Vide campo de "Conclusões ou Pendências e Lista de Inadequações".

Conclusões ou Pendências e Lista de Inadequações:

Após análise em REUNIÃO EXTRAORDINÁRIA, o Colegiado deliberou APROVAÇÃO da Emenda.

Considerações Finais a critério do CEP:

Conforme deliberação do Colegiado, em REUNIÃO EXTRAORDINÁRIA do Comitê de Ética em Pesquisa FMB/UNESP, realizada em 23/11/2020, a Emenda encontra-se APROVADA. O Pesquisador deverá enviar Relatório Final de Atividades ao final da pesquisa.

Atenciosamente,

Comitê de Ética em Pesquisa FMB/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1635771_E2.pdf	18/11/2020 14:12:48		Aceito
Outros	Carta_de_resposta_a_pendencias.pdf	18/11/2020 14:11:00	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_1_novo.docx	16/11/2020 16:28:19	Maria Terezinha Serrão Peraçoli	Aceito
Folha de Rosto	folha_de_rosto.pdf	24/09/2020 14:07:40	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_2novo.docx	23/09/2020 15:56:00	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_responsavel_novo.docx	23/09/2020 15:23:04	Maria Terezinha Serrão Peraçoli	Aceito

TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_maior_novo.docx	23/09/2020 15:22:48	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_GN_novo.docx	23/09/2020 15:22:37	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TALE_novo.docx	23/09/2020 15:22:24	Maria Terezinha Serrão Peraçoli	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_grupo_controle_NG.docx	23/09/2020 15:17:11	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_3_incluida_UNIMED.docx	02/05/2019 10:56:58	Priscila Rezeck Nunes	Aceito
Outros	Oficio_UNIMED.docx	02/05/2019 10:56:42	Priscila Rezeck Nunes	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	biorrepositorio_CEP.pdf	16/08/2017 11:44:20	Maria Terezinha Serrão Peraçoli	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_Anuencia.pdf	03/08/2017 10:32:11	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_4.docx	03/08/2017 10:26:47	Maria Terezinha Serrão Peraçoli	Aceito
Projeto Detalhado / Brochura Investigador	Subprojeto_3.docx	03/08/2017 10:26:35	Maria Terezinha Serrão Peraçoli	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

BOTUCATU, 24 de Novembro de 2020

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador(a))

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