UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

INVESTIGAÇÃO DA EXPRESSÃO DO GENE *IFIH1* EM MULHERES COM PRÉ-ECLAMPSIA E DO EFEITO DO BLOQUEIO DESTA HELICASE NA INDUÇÃO DE GENES RELACIONADOS À HIPERTENSÃO ARTERIAL

TESE DE DOUTORADO

ANA PAULA BOUÇAS KOCHENBORGER

Porto Alegre, Abril de 2016

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Doutor em Endocrinologia.

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Dedico essa tese ao meu companheiro Fernando, por todo amor e dedicação.
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“Quero, um dia, poder dizer às pessoas que nada foi em vão… que o amor existe que vale a pena se doar às amizades e às pessoas, que a vida é bela sim, e que eu sempre dei o melhor de mim… e que valeu a pena!”

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Esta tese de doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução geral sobre o assunto e dois manuscritos originais sobre o tema da Tese.

- **Revisão sistemática:** "Association of innate immunity genes with preeclampsia: a systematic review" (a ser submetido à revista Placenta).

- **Artigo original:** "*IFIH1* is decreased in preeclampsia, and its knockdown reduces the poly(I:C)-induced up-regulation of inflammation-, angiogenesis- and hypertension-related genes in HUVECs" (a ser submetido à revista Journal of Hypertension).

LISTA DE ABREVIATURAS PARA A INTRODUÇÃO

Ang-II	*Angiotensina-II*
DAMPs	*Danger-associated molecular patterns*
DM1	Diabetes mellitus tipo 1
DOCA	*Deoxycorticosterone acetate*
EROs	Espécies reativas de oxigênio
HA	Hipertensão arterial
IFIH1	*Interferon-induced with helicase C domain 1*
IFN	Interferon
IL-1β	Interleucina-1β
IL-8	Interleucina-8
iNOS	*Inducible nitric oxide synthase*
IRF-3	*IFN-regulatory factor*
ISGs	*Interferon-stimulated genes*
Jaks	*Janus Kinases*
LPS	Lipopolissacaridio
MAPK	*Mitogen-activated protein kinases*
MDA-5	*Melanoma differentiation-associated gene-5*
NF-kB	*Nuclear factor-kB*
NLRs	NOD–like receptors
NOD	*Nucleotide-binding oligomerization domain*
oxLDL	*Oxidized low-density lipoprotein*
PAMPs	*Pathogen-associated molecular patterns*

PE	Pré-eclampsia
PRRs	*Pattern-recognition receptors*
RIG-1	*Retinoic acid-inducible gene I*
RLHs	*RIG-I-like helicases*
RNAfd	RNA de fita dupla
TLRs	*Toll-like receptors*

LISTA DE ABREVIATURAS PARA OS ARTIGOS ORIGINAIS

AH	*Arterial hypertension*
Ang-II	*Angiotensin-II*
ANGPT-2	*Angiopoetin-2*
BMI	*Body mass index*
BP	*Blood pressure*
DAMPs	*Danger-associated molecular patterns*
DCs	*Dendritic cells*
DOCA	Acetato de deoxicorticosterona
dsRNA	*Double-stranded RNAs*
ET-1	*Endothelin-1*
HUVECs	*Human umbilical vein endothelial cells*
HWE	*Hardy–Weinberg equilibrium*
IFIH1	*Interferon-induced with helicase C domain 1*
IFN	*Interferon*
iNOS	*Inducible nitric oxide synthase*
IRF-3	*IFN-regulatory factor*
LPS	*Lipopolysaccharide*
MAPK	*Mitogen-activated protein kinases*
MDA-5	*Melanoma differentiation-associated gene-5*
MyD88	*Myeloid differentiation factor 88*
NF-kB	*Nuclear factor-kB*
NLRs	NOD–like receptors

NO	*Nitric oxide*
NOD	*Nucleotide-binding oligomerization domain*
NOS	*Newcastle-Ottawa Scale*
PAMPs	*Pathogen-associated molecular patterns*
PBMC	*Peripheral blood mononuclear cells*
PE	*Preeclampsia*
PGE-2	*Prostaglandin-2*
PIC	*Polyinosinic acid-polycitidilic* – Poly(I:C)
PRRs	*Pattern-recognition receptors*
RIG-1	*Retinoic acid-inducible gene I*
RLHs	*RIG-I-like helicases*
siRNA	*Small Interfering RNA*
STAT-1	*Signal transducer and activator of transcription-1*
T1DM	*Type 1 diabetic patients*
TLRs	*Toll-like receptors*
TNF	*Tumor necrosis factor*
TRIF	*TIR-domain containing adaptor inducing IFN-β*
VEGF	*Vascular endothelial growth factor*

SUMÁRIO

RESUMO

A hipertensão arterial (HA) é uma desordem comum com etiologia incerta. Entretanto, sabe-se que a inflamação vascular caracterizada pela infiltração de células do sistema imune é um mecanismo importante no desenvolvimento desta condição. Outro distúrbio hipertensivo conhecido é a pré-eclampsia (PE), uma síndrome específica da gravidez, caracterizada pela ativação excessiva do sistema imunológico materno, levando à inflamação e disfunção endotelial, causando hipertensão. Existem fortes evidências que receptores da imunidade inata (*pattern-recognition receptors* – PRRs) também tem um importante papel no desenvolvimento da HA e da PE.

Os PRRs reconhecem padrões moleculares associados a patógenos (*pathogen-associated molecular patterns* – PAMPs) ou a ligantes endógenos (*danger-associated molecular patterns* – DAMPs), ativando diferentes cascatas de sinalização que regulam respostas inflamatórias locais e sistêmicas. Estes receptores incluem *retinoic acid-inducible gene I* (RIG-I)-*like helicases* (RLHs), *nucleotide-binding oligomerization domain* (NOD)-*like receptors* (NLRs) e *toll-like receptors* (TLRs).

Apesar de alguns estudos já terem ligado a ativação excessiva de PRRs à PE, ainda não é claro como essa ativação leva ao desenvolvimento desta complicação. Dessa forma, visando esclarecer se diferentes PRRs estão realmente associados com PE e como isto ocorre, realizamos uma revisão sistemática da literatura sobre o assunto. Vinte e seis estudos estavam de acordo com os critérios de inclusão e foram revisados: 20 que analisaram expressões de PRRs de acordo com a presença de PE e 6 que investigaram a associação de polimorfismos nos genes para PRRs e esta complicação.

Poucos estudos analisaram expressões nos genes RIG-I, IFIH1, TLR-1, 5, 6, 7, 8 e 9 em células do sistema imune ou placentas de mulheres com PE (casos) ou mulheres grávidas saudáveis (controles). Sendo assim, ainda é inconclusivo se estes PRRs estão envolvidos no desenvolvimento da PE. Os resultados dos 10 estudos que analisaram expressões do TLR-2 em casos e controles também são contraditórios. A maioria dos estudos que investigaram expressões do TLR-3 e TLR-4 em casos e controles para PE indicam que estes receptores estão aumentados na placenta ou células imunes de mulheres com PE. Até o momento, polimorfismos nos genes *TLR-2*, *3*, e *4* e *NOD-2* não parecem estar associados com o desenvolvimento da PE.

O receptor *interferon-induced with helicase C domain 1* (IFIH1) é uma NLR que tem um papel importante na imunidade inata contra infecções virais. Interessantemente, um estudo prévio realizado pelo nosso grupo demonstrou uma expressão aumentada do gene *IFIH1* em células mononucleares de pacientes com diabetes mellitus tipo 1 (DM1) e HA comparado a pacientes normotensos. Além disso, um estudo prévio sugeriu a associação deste receptor com PE. Sendo assim, visando esclarecer o papel do IFIH1 na HA e PE, comparamos a expressão de *IFIH1* e as frequências do polimorfismo rs1990760 neste gene entre mulheres com PE (casos) e mulheres grávidas saudáveis (controles). Em um estudo experimental, também avaliamos o efeito do *knockdown* do *IFIH1* com siRNA em uma linhagem de células endoteliais humanas tratadas com ácido nucléico viral sintético (PIC) na expressão de genes relacionados à inflamação, angiogênese e hipertensão.

No estudo de caso-controle, a expressão gênica de *IFIH1* foi diminuída em placentas de mulheres com PE (n = 44) comparado ao grupo controle (n = 65) (p= 0,049). Após a exclusão das mulheres negras da amostra, esta diferença entre grupos foi mais pronunciada

(p= 0,004). As frequências do polimorfismo rs1990760 não diferiram significativamente entre mulheres com PE (n = 59) e mulheres controles (n = 134).

Para o estudo experimental, células HUVECs (*human umbilical vein endothelial cells*) foram transfectadas com um siRNA controle ou com dois diferentes siRNAs contra o *IFIH1*, por 48 h. Em seguida, as HUVECs foram transfectadas com PIC, por 24h, para mimetizar uma infecção viral. Após este período, o RNA total foi extraído para as análises de expressão gênica.

O tratamento com PIC induziu um aumento significativo nas expressões de *TNF*, *IFN-β*, *iNOS*, *VEGF*, *ANGPT-2* e *ET-1* e o bloqueio do *IFIH1* foi capaz de prevenir parcialmente o aumento nestes genes. O tratamento com PIC não induziu a expressão gênica de *PGE-2* (p >0,05). Todos os resultados de expressão gênicas foram confirmados nos experimentos usando um segundo siRNA contra *IFIH1*. O aumento nos níveis proteicos de Ang-II-induzidos por PIC pareceram diminuir após bloqueio do *IFIH1*, mas esta diferença não atingiu significância formal (p= 0,069).

Para avaliar se os genes relacionados à hipertensão e angiogênese poderiam ser induzidos em um ambiente inflamatório não viral, as HUVECs foram tratadas com a citocina pró-inflamatória IFN-β por 48 h. O tratamento com esta citocina aumentou significativamente as expressões de *STAT-1*, *PGE-2*, *ANGPT-2* e *VEGF*. Estes resultados indicam que um ambiente inflamatório não viral é suficiente para ativar a via do IFIH1 em células endoteliais. Dessa forma, estudos adicionais são necessários para definir quais PAMPs ou DAMPs podem ativar IFIH1 nessas células levando a expressão de genes relacionados à inflamação, angiogênese e hipertensão.

Em conclusão, nossos resultados sugerem que a expressão de *IFIH1* está diminuída em mulheres com PE, provavelmente contribuindo a patogênese desta complicação. Além

disso, experimentos *in vitro* nas HUVECs demonstraram que o tratamento com PIC é capaz de induzir as expressões de genes relacionadas à inflamação, angiogênese e hipertensão e que o bloqueio do *IFIH1* diminui o aumento na expressão destes genes. Este estudo contribuir com um possível novo papel do IFIH1 na patogênese da PE e HA.

ABSTRACT

Arterial hypertension (AH) is a common disorder with unknown etiology. However, it is known that vascular inflammation characterized by infiltration of immune cells is a key mechanism in the development of this condition. Other recognized hypertensive disorder is preeclampsia (PE), a specific pregnancy syndrome characterized by excessive activation of the maternal immune system, leading to inflammation and endothelial dysfunction, and then causing hypertension. There are strong evidences that innate immunity receptors (pattern-recognition receptors – PRRs) play an important role in the development of AH and PE.

PRRs recognize highly conserved pathogen-associated molecular patterns (PAMPs) as well as endogenous ligands (danger-associated molecular patterns – DAMPs), activating different signaling cascades that regulate local and systemic inflammatory responses. These receptors include retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) e toll-like receptors (TLRs).

Even though some studies have linked an excessive PRR activation to PE, it is still unclear how this activation might lead to the development of this complication. Therefore, aiming to elucidate if different PRRs are indeed associated with PE and how this occurs, we conducted a systematic review of the literature on the subject. Twenty-six studies met the inclusion criteria and were revised: 20 of them analyzed PRR expressions according to PE presence, and 6 studies investigated the association between PRR polymorphisms and this complication.

Only few studies analyzed RIG-I, IFIH1, TLR-1, 5, 6, 7, 8 and 9 expressions in immune cells or placentas from PE women (cases) and healthy pregnant women (controls); therefore, it is still inconclusive if these PRRs are involved in PE. Results from the 10 studies

that analyzed TLR-2 expressions in cases and controls are contradictory. The majority of the studies that investigated TLR-3 and TLR-4 expressions in cases and controls indicate that these PRRs are increased in placenta or immune cells from PE women. To date, polymorphisms in *TLR-2*, *3*, and *4* and *NOD-2* genes do not seem to be associated with PE development.

The interferon-induced with helicase C domain 1 (IFIH1) receptor is a NLR that plays an important role in the innate immunity against viral infections. Interestingly, a previous study published by our group demonstrated an increased *IFIH1* expression in mononuclear cells from type 1 diabetic patients with AH compared to normotensive patients. Moreover, this receptor also seems to be associated with PE. Hence, to further investigate the role of IFIH1 in AH and PE, we compared *IFIH1* expressions and frequencies of the rs1990760 in this gene between PE women (cases) and healthy pregnant women (controls). In an experimental study, we also evaluated the effect of *IFIH1* knockdown with siRNA in a human endothelial cell line treated with synthetic viral nucleic acid (PIC) in the expression of genes related to inflammation, angiogenesis and hypertension.

In the case-control study, *IFIH1* expression was decreased in placenta from PE women (n = 44) compared to the control group (n = 65) (P= 0.049). After exclusion of black women, the difference between groups was more pronounced (P= 0.004). Frequencies of the rs1990760 polymorphism did not differ significantly between PE women (n = 59) e control women (n = 134).

In the experimental study, HUVECs (human umbilical vein endothelial cells) were transfected with either a control siRNA or with two different siRNAs targeting *IFIH1* for 48h. Then, HUVECs were transfected with PIC, for 24 h, to mimic a viral infection. After this period, total RNA was extracted for gene expression analyses.

PIC treatment induced a significant increase in *TNF*, *IFN-β*, *iNOS*, *VEGF*, *ANGPT-2*, and *ET-1* expressions, and the *IFIH1* knockdown was able to partially prevent the up-regulation of these genes. PIC treatment did not influence *PGE-2* gene expression (P >0.05). All gene expression results were confirmed in experiments using a second siRNA targeting *IFIH1*. *IFIH1* knockdown seemed to prevent PIC-induced increase of Ang-II protein levels, although this difference did not reach formal statistical significance (P= 0.069).

To evaluate if hypertension- and angiogenesis-related genes could be also induced in a non-viral inflammatory environment, HUVECs were treated with the pro-inflammatory cytokine IFN-β for 48 h. Treatment with this cytokine significantly increased *STAT-1*, *PGE-2*, *ANGPT-2* e *VEGF* gene expressions. These results indicate that a non-viral inflammatory environment is sufficient to activate IFIH1 pathway. Therefore, further studies are necessary to clarify which PAMPs or DAMPs could activate IFIH1 in these cells, leading to the expression of genes related to inflammation, angiogenesis and hypertension.

In conclusion, our results suggest that *IFIH1* gene expression is decreased in placentas from PE women, probably contributing to PE pathogenesis. Furthermore, the *in vitro* experiments in HUVECs demonstrated that PIC treatment induces expressions of genes related to inflammation, angiogenesis and hypertension, which is decreased after *IFIH1* knockdown. This study adds a new possible role for IFIH1 in the pathogenesis of PE and AH.

1. INTRODUÇÃO

A hipertensão arterial (HA) é uma desordem comum com etiologia incerta [1]. Entretanto, sabe-se que a inflamação vascular caracterizada pela infiltração de células do sistema imune é um mecanismo importante no desenvolvimento desta condição [2]. A contribuição da inflamação na patofisiologia da HA é fortalecida por estudos que mostraram a expressão aumentada de moléculas de adesão e seus ligantes, extravasamento de leucócitos, produção de citocinas, aumento do estresse oxidativo e ativação das células imunes e rotas pró-inflamatórias nas artérias de pacientes hipertensivos e roedores [2, 3].

Nos últimos anos, tornou-se evidente que os componentes da imunidade inata (por exemplo, macrófagos) e adaptativa (linfócitos-T efetores e regulatórios) desempenham um papel importante na HA [1, 4]. Macrófagos e linfócitos-T se infiltram no tecido perivascular do tecido adiposo, coração e rins de pacientes hipertensos e de roedores com modelo de dano renal (revisado em [1]). Interessantemente, em ratos com ausência de macrófagos, tratamento com angiotensina-II (Ang-II) e sal de acetato de deoxicorticosterona (DOCA) foram incapazes de aumentar os níveis pressóricos ou induzir o estresse oxidativo ou a remodelação vascular que segue o estímulo hipertensivo [5, 6], fortalecendo a hipótese de que a imunidade inata tem um papel importante na HA. Embora esses estudos tenham implicado os macrófagos e outros componentes da imunidade inata no desenvolvimento da HA, os mecanismos exatos pelos quais eles agem permanecem incertos.

Harrison *et al*. [1] sugerem que tais mecanismos devem envolver produtos de macrófagos, incluindo espécies reativas de oxigênio (EROs) e citocinas pró-inflamatórias, que se difundem para as células endoteliais e vasculares, alterando as suas funções. Isto pode induzir a ativação de enzimas geradoras de EROs nestas células, estimulação da hipertrofia e

crescimento das células musculares lisas da parede vascular, produção de quimiocinas, citocinas e moléculas de adesão, alterações na produção de óxido nítrico (ON) e outras alterações da sinalização das células do endotélio vascular. Além disso, ações similares das EROs e citocinas pró-inflamatórias nas células epiteliais renais podem também promover retenção do volume de sódio, contribuindo ao desenvolvimento da HA [1].

Outro distúrbio hipertensivo conhecido é a pré-eclampsia (PE), a qual atinge 3-5% de todas as gravidezes e é uma condição rapidamente progressiva, normalmente diagnosticada por pressão arterial elevada (≥ 140/90 mmHg) e proteinúria [7, 8]. Novas definições também incluem disfunções orgânicas maternas, tais como insuficiência renal, comprometimento hepático, complicações neurológicas ou hematológicas, disfunção uteroplacentária, e restrição de crescimento fetal [8, 9].

Várias teorias têm sido propostas para explicar a etiologia da PE, incluindo invasão superficial de células trofoblásticas, levando a remodelação inadequada das artérias espirais, placentação anormal, angiogênese deficiente e disfunção do sistema imune. A placenta é a interface primária entre o feto e a mãe e desempenha um papel importante no desenvolvimento fetal. Ela facilita a transferência de substratos e participa na modulação da resposta imune materna para prevenir a rejeição imunológica do concepto [10]. De fato, a ativação do sistema imune parece ser necessária para as fases iniciais do implante. No entanto, sabe-se que a ativação excessiva do sistema imune materno pode induzir a disfunção endotelial e inflamação e pode desempenhar um papel importante no desenvolvimento desta condição [11, 12]. Estas evidências fortalecem que a imunidade inata também parece ter um papel importante no desenvolvimento da PE.

1.1. Receptores da imunidade inata do tipo *pattern-recognition receptors*

O reconhecimento de micro-organismos pelo sistema imune de mamíferos depende de componentes tanto da imunidade inata como da adaptativa. A imunidade inata é a primeira linha de defesa contra bactérias, fungos e vírus [13, 14]. A detecção de micro-organismos invasores é feita por diferentes receptores celulares do tipo *pattern-recognition receptors* (PRRs), os quais reconhecem padrões moleculares associados a patógenos (*pathogen-associated molecular patterns* - PAMPs). Estudos identificaram três grupos de PRRs: *retinoic acid-inducible gene I* (RIG-I)-*like helicases* (RLHs), *nucleotide-binding oligomerization domain* (NOD)–*like receptors* (NLRs) e *toll-like receptors* (TLRs) [15, 16].

A classe RLH compreende principalmente o RIG-I e o *interferon-induced with helicase C domain 1* (IFIH1), receptores citosólicos que reconhecem RNAs de fita dupla (RNAfd) produzidos durante a replicação e transcrição do RNA viral, desempenhando um papel importante na resposta imune desencadeada por infecção viral [13, 17]. Existem fortes evidências de que estas duas helicases utilizam vias intracelulares semelhantes para induzir uma resposta antiviral. No entanto, apesar das suas semelhanças estruturais, o IFIH1 e RIG-1 parecem estar envolvidos no reconhecimento de diferentes tipos de vírus [18].

Os NLRs estão localizados no citoplasma e no núcleo. Alguns NLRs, tais como NOD-1-2, após a detecção de fragmentos de peptidoglicanos bacterianos, ativam vias pró-inflamatórias através do *nuclear factor-kB* (NF-κB) e *mitogen-activated protein kinases* (MAPK), induzindo autofagia. Outros NLRs, tais como NLRP3, NLRP6, NLRP7, NLRP12 e NLRP4, regulam a formação de inflamassomas, levando à ativação da caspase-1 e à clivagem das citocinas pró-inflamatórias interleucina-1β (IL-1β) e interleucina-8 (IL-8) para a sua forma ativa [19].

Os PRRs mais extensivamente estudados são os TLRs, que compreendem pelo menos 10 membros [20-22]. Os TLRs de superfície celular, incluindo TLR1, TLR2, TLR4, TLR-5, TLR6 e TLR10, são essenciais para o reconhecimento de componentes da parede celular de bactérias e fungos, tais como lipopolissacarídeos (LPS) e lipopeptídeos. Por outro lado, TLR3, TLR7, TLR8 e TLR9 são expressos exclusivamente em compartimentos intracelulares, tais como os endossomos, e seus ligantes, principalmente ácidos nucleicos virais e bactérias, requerem a sua internalização antes da sinalização [20, 23, 24].

Cabe ressaltar que as NLRs, RLHs e TLRs além de detectar uma variedade de PAMPs, também reconhecem padrões moleculares endógenos associados a perigo (*danger-associated molecular patterns* - DAMPs), liberados pela morte ou dano de tecidos durante estresse ou apoptose [15].

1.2. O papel dos PRRs na resposta às infecções virais

Os PRRs específicos presentes nas células infectadas são ativados e sofrem mudanças na sua conformação, ativando cascatas sinalizadoras que culminam na produção de diversas citocinas pró-inflamatórias, quimiocinas e o interferon do tipo 1 (IFN-I) [16]. O IFN-I é uma citocina produzida pela maioria das células durante infecções virais, promovendo a expressão de diversos genes relacionados à resposta antiviral em células alvo e também modulando o sistema imune adaptativo através da ativação de células dendríticas, linfócitos-T e linfócitos-B. Estes IFNs exibem uma potente ação antiviral, protegendo as células ainda não infectadas e induzindo apoptose naquelas já infectadas [17].

Sabe-se que ilhotas pancreáticas humanas infectadas com o vírus Coxsackie B5 ou expostas a IFN-α ou IFN-γ + IL-1β aumentam a expressão do TLR-3, RIG-1 e IFIH1 [25,

26]. Tanto o RNAfd intracelular como o extracelular podem se ligar ao TLR-3 e engatilhar a produção de citocinas e quimiocinas pró-inflamatórias, levando à apoptose das células-beta através da ativação de fatores de transcrição chaves: o NF-κB e o *IFN-regulatory factor* 3 (IRF-3) [27, 28].

De fato, estudos mais recentes demonstraram que enquanto a ativação de NF-κB e IRF-3 por RNAfd extracelular é dependente de TLR-3, a ativação por RNAfd intracelular, um produto da replicação viral no citoplasma, ocorre através da ativação de RIG-1 e IFIH1 [27]. As ativações de NF-κB e IRF-3 desencadeiam a produção de IFN-α e IFN-β, os quais levam a ativação da via *janus kinases / signal transducers and activators of transcription-1* (Jak/STAT-1), e ativam a expressão de antígenos de MHC classe I e de diversas quimicionas [13, 25, 28]. Esta resposta molecular complexa leva a atração de células do sistema imune, as quais vão liberar mais citocinas pró-inflamatórias, como IFN-γ, IL-1β e TNF. A inflamação local e a ativação das defesas antivirais visam erradicar a infecção e engatilhar apoptose nas células infectadas.

1.3. O polimorfismo rs1990760 no gene *IFIH1* e sua associação com doenças autoimunes

O gene *IFIH1*, também conhecido como *Melanoma differentiation-associated gene-5* (MDA-5), codifica um receptor citoplasmático que reconhece o RNAfd e está envolvido na resposta imune desencadeada por infecção viral [18]. A ligação do RNAfd ao IFIH1 engatilha a secreção de citocinas pró-inflamatórias, particularmente IFN-I, pelas células do sistema imune, desencadeando a resposta imune (**Figura 1**) [17]. O gene *IFIH1* é expresso em baixos níveis em vários tecidos, mas é expresso em altas taxas em células do sistema imune [18, 29]. Ao nível transcricional, o IFIH1 é induzido por IFN-I, ácido retinóico e RNAfd [29].

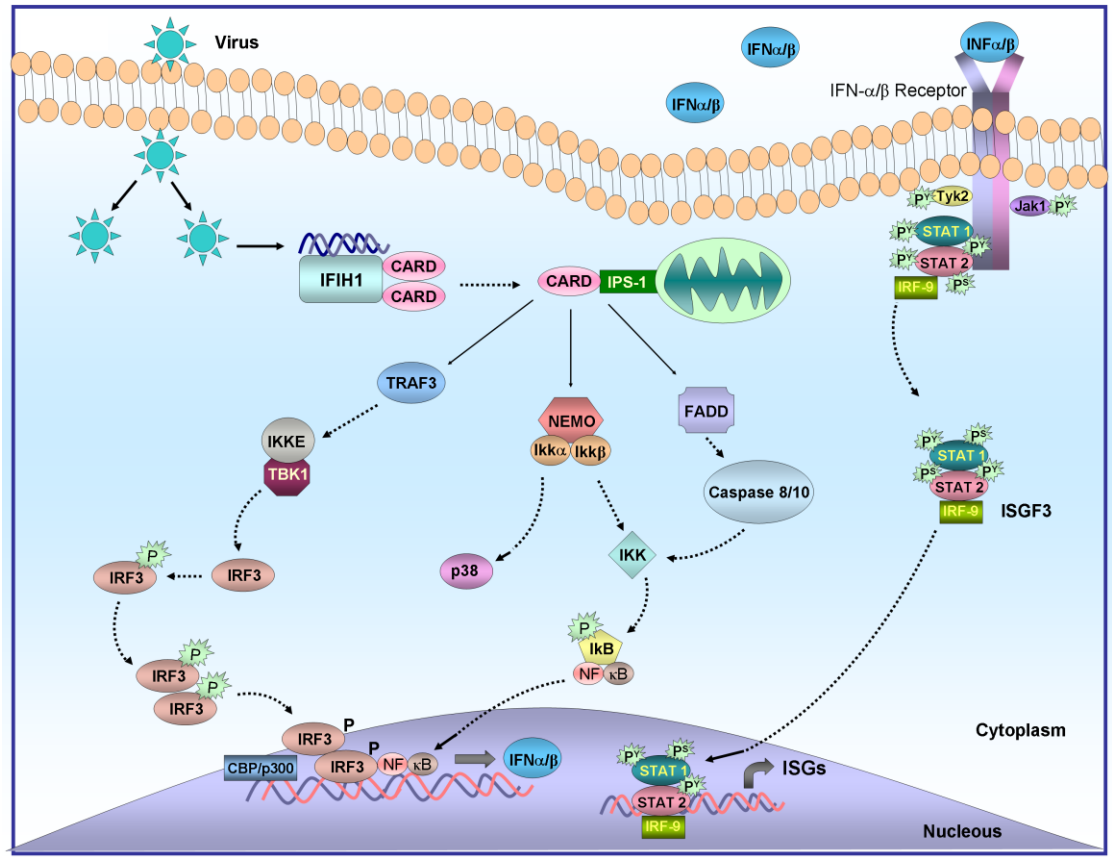

Figura 1. Sinalização antiviral através do receptor IFIH1. O RNA de fita dupla (RNAfd) produto da replicação viral é detectado pela helicase citosólica IFIH1, ativando a proteína adaptadora IPS-1 através da interação com o seu domínio CARD. A proteína IPS-1 induz via de sinalização intracelular, levando à ativação dos fatores de transcrição IRF-3 e NF-κB e consequente produção de IFNα/β pelas células infectadas. IFNα/β, por sua vez, ligam-se ao seu receptor e através da via JAK-STAT-1 induzem a expressão de genes estimulados por interferon (*interferon-stimulated genes* - ISGs) e a resposta da imunidade inata [17].

O polimorfismo rs1990760 (G/A) troca uma alanina por uma treonina no códon 946 do éxon 15, com a variante Ala946 sendo altamente conservada entre os mamíferos. Liu et al. [30] demonstrou que o genótipo G/G deste polimorfismo está associado com um aumento de 2 *fold changes* na expressão do gene *IFIH1* em células mononucleares de sangue periférico humano. Alguns estudos evidenciaram fortes associações entre o polimorfismo rs1990760 e doenças autoimunes, tais como psoríase [31], periodontite crônica [32], polimiosite [33],

esclerose múltipla [34], síndrome Aicardi-Goutières (AGS) [35] e o diabetes mellitus tipo 1 (DM1) [36-38].

Um estudo realizado pelo nosso grupo confirmou na nossa população a associação do alelo A do polimorfismo rs1990760 (G/A) com risco para o diabetes mellitus tipo 1 (DM1) (OR= 1,421; p= 0,037) [36]. Interessantemente, quando comparamos características clínicas do DM1 entre os diferentes genótipos deste polimorfismo, observamos que portadores do alelo A apresentaram níveis menores de pressão sistólica (A/A: 117,4 ± 16,0 *vs*. A/G: 121,1 ± 19,0 *vs*. G/G: 128,5 ± 18,9; p= 0,001) e diastólica (A/A: 74,4 ± 9,8 *vs*. G/A: 78,0 ± 10,8 *vs*. G/G: 82,4 ± 13,5; p <0,000001) quando comparados aos portadores do genótipo G/G. Além disso, o genótipo A/A foi associado com proteção para HA, após ajuste para idade, sexo e etnia (OR= 0,339, IC 95% = 0,14-0,84; p= 0,019) [36]. Também observamos uma expressão aumentada de *IFIH1* em células mononucleares de pacientes com DM1 e HA comparados a pacientes com DM1 normotensos (P= 0,036) [36]. Cabe ressaltar que este foi o primeiro estudo a mostrar uma associação do gene *IFIH1* com HA. Além disso, até o momento nenhum estudo avaliou polimorfismos neste gene com PE.

1.4. O papel das PRRs na hipertensão arterial e pré-eclâmpsia

O endotélio vascular não só proporciona uma barreira física controlando a permeabilidade vascular, mas também secreta uma série de substâncias vasoativas que regulam o tônus das células do músculo liso subjacentes e a remodelação da parede dos vasos sanguíneos [39]. Lesões no endotélio celular desempenham um papel chave no processo de inflamação vascular, promovendo o recrutamento de leucócitos através do aumento da produção de várias citocinas, quimiocinas e moléculas de adesão [39].

O receptor TLR-4 é expresso no endotélio e desempenha um papel fundamental no desenvolvimento da disfunção endotelial, em parte, através da ativação do inflamassoma NLRP3 [40]. Estudos *in vitro* e *in vivo* demonstraram que a administração de LPS provoca disfunção endotelial rápida nas artérias de camundongos selvagem, mas não em camundongos com o gene *TLR-4* bloqueado [40]. Da mesma forma, o receptor endossomal TLR-3 parece promover a inflamação e a disfunção em células endoteliais [41] e células vasculares do músculo liso [42].

A disfunção do sistema imune inato e adaptativo tem sido demonstrada como tendo um papel chave no desenvolvimento da PE [12, 43]. A ativação do sistema imune materno pode ser desencadeada em parte por micro-organismos invasores, assim como por agentes endógenos, os DAMPs. Neste contexto, alguns estudos experimentais e clínicos tem associado a ativação dos TLRs durante o desenvolvimento da PE [(revisto em [43])]. TLRs podem ser ativados por DAMPs liberados durante uma lesão celular, devido à má placentação, estresse oxidativo e disfunção endotelial [44, 45]. Os DAMPs que parecem ativar os TLRs levando a PE incluem, o DNA fetal, proteínas de choque térmico, fibrinogênio, ácido hialurônico, *Oxidized low-density lipoprotein* (oxLDL) e Angiotensina-II [43-46].

Chatterjee *et al.* [43, 47] demonstraram que os três receptores virais, TLR-3, RIG-I e IFIH1, são excessivamente ativados em placentas de camundongos e mulheres com PE. Os autores também mostraram que o tratamento de camundongos com RNAfd sintético intracelular (PIC) aumentou significativamente os níveis de TLR3, causando hipertensão dependente da gravidez, disfunção endotelial e inflamação placentária [43, 48]. Além disso, na PE, a necrose das células trofoblásticas resulta na liberação do DNA mitocondrial, o qual

ativa o TLR-9, induzindo a inflamação vascular excessiva e disfunção endotelial, causando hipertensão materna [49].

Resumidamente, estes estudos sugerem que uma resposta imune ativada por agentes exógenos ou endógenos pode levar à produção de citocinas pró-inflamatórias, causando um processo inflamatório tanto no endotélio vascular como na placenta, contribuindo à patogênese da HA e PE. Entretanto, estudos ainda são necessários para um melhor esclarecimento do papel do IFIH1 e outros PRRs no desenvolvimento destas doenças.

2. JUSTIFICATIVA E OBJETIVOS

Alguns estudos tem sugerido que diferentes receptores da imunidade inata (*pattern-recognition receptors* – PRRs) estão associados ao desenvolvimento da PE e HA. Entretanto, ainda não está definido quais destes receptores estão realmente associados com estas doenças e como isto ocorre.

O IFIH1 é uma NLR que tem um papel importante na imunidade inata contra infecções virais. Interessantemente, um estudo prévio realizado pelo nosso grupo demonstrou pela primeira vez uma expressão aumentada do gene *IFIH1* em células mononucleares de pacientes com DM1 e HA comparado a pacientes normotensos. Além disso, outro estudo prévio sugeriu a associação deste receptor com PE. Contudo, ainda não se sabe como o IFIH1 contribui à PE ou a HA.

Tendo em vista o exposto acima, são necessários novos estudos *in vivo* e *in vitro* que investiguem as associações entre diferentes PRRs e PE ou HA e como disfunções nestes receptores podem levar a estas complicações. O entendimento de como estes receptores da

imunidade inata podem influenciar o desenvolvimento da HA ou PE poderá levar a identificação de potenciais alvos terapêuticos.

Dessa forma, os principais objetivos desta tese foram:

- Realizar uma revisão sistemática visando sintetizar os resultados de todos os estudos que avaliaram expressão de genes para PRRs ou frequências de polimorfismos nestes genes entre mulheres com PE (casos) ou mulheres grávidas saudáveis (controles).

- Em um estudo de caso-controle, comparar a expressão do gene *IFIH1* e as frequências do polimorfismo rs1990760 neste gene entre mulheres com PE (casos) e mulheres grávidas saudáveis (controles).

- Em um estudo experimental, avaliar o efeito do bloqueio do gene *IFIH1* (knockdown com siRNAs) em uma linhagem de células endoteliais humanas tratadas com RNA dupla fita viral sintético (PIC) na expressão de genes relacionados à inflamação, angiogênese e hipertensão.

3. REFERÊNCIAS DA INTRODUÇÃO

1. Harrison, D.G., et al., *Inflammation, immunity, and hypertension.* Hypertension, 2011. **57**(2): p. 132-40.
2. Schiffrin, E.L., *T lymphocytes: a role in hypertension?* Curr Opin Nephrol Hypertens, 2010. **19**(2): p. 181-6.
3. Leibowitz, A. and E.L. Schiffrin, *Immune mechanisms in hypertension.* Curr Hypertens Rep, 2011. **13**(6): p. 465-72.
4. Verlohren, S., et al., *Immunology in hypertension, preeclampsia, and target-organ damage.* Hypertension, 2009. **54**(3): p. 439-43.
5. De Ciuceis, C., et al., *Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin II-infused macrophage colony-stimulating factor-deficient mice: evidence for a role in inflammation in angiotensin-induced vascular injury.* Arterioscler Thromb Vasc Biol, 2005. **25**(10): p. 2106-13.
6. Ko, E.A., et al., *Resistance artery remodeling in deoxycorticosterone acetate-salt hypertension is dependent on vascular inflammation: evidence from m-CSF-deficient mice.* Am J Physiol Heart Circ Physiol, 2007. **292**(4): p. H1789-95.
7. ACOG practice bulletin, *Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002.* Obstet Gynecol, 2002. **99**(1): p. 159-67.
8. Tranquilli, A.L., et al., *The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP*. Pregnancy Hypertens, 2014. **4**(2): p. 97-104.
9. Mol, B.W., et al., *Pre-eclampsia.* Lancet, 2015.
10. Zhang, S., et al., *Placental adaptations in growth restriction.* Nutrients, 2015. **7**(1): p. 360-89.
11. Borzychowski, A.M., I.L. Sargent, and C.W. Redman, *Inflammation and pre-eclampsia.* Semin Fetal Neonatal Med, 2006. **11**(5): p. 309-16.
12. Perez-Sepulveda, A., et al., *Innate immune system and preeclampsia.* Front Immunol, 2014. **5**: p. 244.
13. Wilkins, C. and M. Gale, Jr., *Recognition of viruses by cytoplasmic sensors.* Curr Opin Immunol, 2010. **22**(1): p. 41-7.
14. Pichlmair, A. and C. Reis e Sousa, *Innate recognition of viruses.* Immunity, 2007. **27**(3): p. 370-83.
15. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. **442**(7098): p. 39-44.
16. Randall, R.E. and S. Goodbourn, *Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures.* J Gen Virol, 2008. **89**(Pt 1): p. 1-47.
17. Boucas, A.P., et al., *The role of interferon induced with helicase C domain 1 (IFIH1) in the development of type 1 diabetes mellitus.* Arq Bras Endocrinol Metabol, 2013. **57**(9): p. 667-76.
18. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.* Nature, 2006. **441**(7089): p. 101-5.
19. Liu, D., A.M. Rhebergen, and S.C. Eisenbarth, *Licensing Adaptive Immunity by NOD-Like Receptors.* Front Immunol, 2013. **4**: p. 486.
20. De Nardo, D., *Toll-like receptors: Activation, signalling and transcriptional modulation.* Cytokine, 2015. **74**(2): p. 181-9.

21. Assmann, T.S., et al., *Toll-like receptor 3 (TLR3) and the development of type 1 diabetes mellitus.* Arch Endocrinol Metab, 2015. **59**(1): p. 4-12.
22. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int Immunol, 2005. **17**(1): p. 1-14.
23. Szatmary, Z., *Molecular biology of toll-like receptors.* Gen Physiol Biophys, 2012. **31**(4): p. 357-66.
24. Dunne, A. and L.A. O'Neill, *Adaptor usage and Toll-like receptor signaling specificity.* FEBS Lett, 2005. **579**(15): p. 3330-5.
25. Ylipaasto, P., et al., *Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets.* Diabetologia, 2005. **48**(8): p. 1510-22.
26. Hultcrantz, M., et al., *Interferons induce an antiviral state in human pancreatic islet cells.* Virology, 2007. **367**(1): p. 92-101.
27. Dogusan, Z., et al., *Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways.* Diabetes, 2008. **57**(5): p. 1236-45.
28. Rasschaert, J., et al., *Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells.* J Biol Chem, 2005. **280**(40): p. 33984-91.
29. Chistiakov, D.A., *Interferon induced with helicase C domain 1 (IFIH1) and virus-induced autoimmunity: a review.* Viral Immunol, 2010. **23**(1): p. 3-15.
30. Liu, S., et al., *IFIH1 polymorphisms are significantly associated with type 1 diabetes and IFIH1 gene expression in peripheral blood mononuclear cells.* Hum Mol Genet, 2009. **18**(2): p. 358-65.
31. Li, Y., et al., *Carriers of rare missense variants in IFIH1 are protected from psoriasis.* J Invest Dermatol, 2010. **130**(12): p. 2768-72.
32. Chen, G., et al., *Genetic variants in IFIH1 play opposite roles in the pathogenesis of psoriasis and chronic periodontitis.* Int J Immunogenet, 2012. **39**(2): p. 137-43.
33. Gono, T., et al., *Interferon-induced helicase (IFIH1) polymorphism with systemic lupus erythematosus and dermatomyositis/polymyositis.* Mod Rheumatol, 2010. **20**(5): p. 466-70.
34. Martinez, A., et al., *IFIH1-GCA-KCNH7 locus: influence on multiple sclerosis risk.* Eur J Hum Genet, 2008. **16**(7): p. 861-4.
35. Miner, J.J. and M.S. Diamond, *MDA5 and autoimmune disease.* Nat Genet, 2014. **46**(5): p. 418-9.
36. Boucas, A.P., et al., *The A allele of the rs1990760 polymorphism in the IFIH1 gene is associated with protection for arterial hypertension in type 1 diabetic patients and with expression of this gene in human mononuclear cells.* PLoS One, 2013. **8**(12): p. e83451.
37. Smyth, D.J., et al., *A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region.* Nat Genet, 2006. **38**(6): p. 617-9.
38. Cen, H., et al., *Association of IFIH1 rs1990760 polymorphism with susceptibility to autoimmune diseases: a meta-analysis.* Autoimmunity, 2013. **46**(7): p. 455-62.
39. Tousoulis, D., et al., *Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: therapeutic implications for cardiovascular diseases.* Atherosclerosis, 2008. **201**(2): p. 236-47.
40. Martin-Rodriguez, S., et al., *TLR4 and NALP3 inflammasome in the development of endothelial dysfunction in uraemia.* Eur J Clin Invest, 2015. **45**(2): p. 160-9.

41. Zimmer, S., et al., *Activation of endothelial toll-like receptor 3 impairs endothelial function.* Circ Res, 2011. **108**(11): p. 1358-66.
42. Yang, X., et al., *Toll-like receptor 3 signaling evokes a proinflammatory and proliferative phenotype in human vascular smooth muscle cells.* Am J Physiol Heart Circ Physiol, 2006. **291**(5): p. H2334-43.
43. Chatterjee, P., et al., *Do double-stranded RNA receptors play a role in preeclampsia?* Placenta, 2011. **32**(3): p. 201-5.
44. Riley, J.K. and D.M. Nelson, *Toll-like receptors in pregnancy disorders and placental dysfunction.* Clin Rev Allergy Immunol, 2010. **39**(3): p. 185-93.
45. Koga, K. and G. Mor, *Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy disorders.* Am J Reprod Immunol, 2010. **63**(6): p. 587-600.
46. Goulopoulou, S., C.G. McCarthy, and R.C. Webb, *Toll-like Receptors in the Vascular System: Sensing the Dangers Within.* Pharmacol Rev, 2016. **68**(1): p. 142-67.
47. Chatterjee, P., et al., *Placental toll-like receptor 3 and toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice.* PLoS ONE, 2012. **7**(7).
48. Tinsley, J.H., et al., *Toll-like receptor 3 activation during pregnancy elicits preeclampsia-like symptoms in rats.* Am J Hypertens, 2009. **22**(12): p. 1314-9.
49. Goulopoulou, S., et al., *Toll-like receptor 9 activation: a novel mechanism linking placenta-derived mitochondrial DNA and vascular dysfunction in pre-eclampsia.* Clin Sci (Lond), 2012. **123**(7): p. 429-35.

Parte I

Association of innate immunity genes with preeclampsia: a systematic review

Association of innate immunity genes with preeclampsia: a systematic review

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Abstract

Innate immune system dysfunction has been known to be a key player in PE. Activation of the maternal immunity may be triggered by invading microorganisms as well as endogenous ligands, which are detected by different pattern-recognition receptors (PRRs). PRRs include RIG-I-like helicases (RLHs), NOD-like receptors (NLRs) and toll-like receptors (TLRs). After ligand binding, PRRs activate signaling pathways that regulate local and systemic inflammatory responses. Although some studies have linked PRR activation to PE, it is still unclear how excessive PRR activation might lead to the development of this complication. Therefore, we conducted a systematic review of the literature on the subject, searching all articles that evaluated associations of PRRs with PE. Twenty-six studies met the inclusion criteria: 20 of them analyzed PRR expressions in humans or mice, and 6 studies investigated the association between PRR polymorphisms and PE. Only few studies analyzed RIG-I, IFIH1, TLR-1, 5, 6, 7, 8 and 9 expressions in immune cells or placentas from PE women and control women; therefore, it is still inconclusive if these PRRs are involved in PE. Results from the 10 studies that analyzed TLR-2 expressions in PE women and controls are contradictory. The majority of the studies that investigated TLR-3 and 4 expressions indicate that these PRRs are increased in placenta or immune cells from PE women. To date, polymorphisms in *TLR-2*, *3*, and *4* and *NOD-2* genes do not seem to be associated with PE development. In conclusion, available data in literature strongly support a role of TLR-3 and TLR-4 in the pathogenesis of PE. Further studies are needed to confirm if other PRRs are indeed involved in PE pathogenesis, and if polymorphisms in PRR genes predispose to this pregnancy complication.

Introduction

Preeclampsia (PE) affects at least 3-5% of all pregnancies and is a rapidly progressive condition usually diagnosed by new onset high blood pressure (BP) and either proteinuria or end-organ dysfunction (such as renal insufficiency, liver involvement, neurological or hematological complications, uteroplacental dysfunction or fetal growth restriction) or both after 20 weeks of gestation in a previously normotensive woman [1-3]. In women with chronic/preexisting hypertension, PE is defined by the new onset of either proteinuria or end-organ dysfunction after 20 weeks of gestation [3]. Although the origins of PE remain unclear, an activation of maternal immune system leading to inflammation and endothelial dysfunction may play an important role in the development of this condition [4, 5].

In the last few years, studies have demonstrated the involvement of specific key components of the innate immunity in the first line of defense against microorganisms. Detection of invading microorganisms is carried out by a wide range of pattern-recognition receptors (PRRs), which recognize highly conserved pathogen-associated molecular patterns (PAMPs) derived from virus, bacteria and fungi [6, 7]. Studies have identified three groups of PRRs: retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs) and toll-like receptors (TLRs) [7, 8].

The RLH class mainly comprises RIG-I and interferon-induced with helicase C domain 1 / melanoma differentiation-associated gene-5 (IFIH1/MDA-5) cytoplasmic receptors. They recognize the double-stranded RNA (dsRNA) produced during the replication and transcription of viral RNA, playing a role in the immune response triggered by viral infection [9, 10]. There are evidences that these two helicases use similar intracellular pathways to induce an antiviral response. Nevertheless, despite their structural similarities,

IFIH1 and RIG-I seem to be non-redundant and are involved in the recognition of different types of virus [11].

NLRs are located in the cytosol and nucleus, where they detect a wide variety of PAMPs as well as endogenous danger-associated molecular patterns (DAMPs). Some NLRs, such as NOD-1 and NOD-2, after sensing bacterial peptidoglycan fragments, activate pro-inflammatory NF-κB and MAPK pathways, and induce autophagy processes. Others NRLS, such as NLRP3, NLRP6, NLRP7, NLRP12 and NLRP4, regulate inflammasome formation, leading to caspase-1 activation, and consequent cleavage of the pro-inflammatory cytokines IL-1β and IL-18 into their active forms [12].

The most extensively studied PRRs are TLRs, which comprise at least 10 members with different triggers [8, 13, 14]. Cell-surface TLRs, including TLR1, TLR2, TLR4, TLR-5, TLR6 and TLR10, are essential to recognize bacterial and fungal cell wall components, such as lipopolysaccharide (LPS) and lipopeptides. On the other hand, TLR3, TLR7, TLR8 and TLR9 are exclusively expressed in intracellular compartments such as endosomes, and their ligands, mainly bacterial and viral nucleic acids, require internalization before signaling is possible [8, 15, 16].

Innate and adaptive immune system dysfunction has long been known to be a key player in PE [5, 17]. Activation of the maternal immunity may be triggered in part by invading microorganisms as well as endogenous DAMPs. In this context, some experimental and clinical studies link TLR activation to the development of PE (reviewed in [17]). TLRs have been shown to be activated by DAMPs released during cellular damage due to poor placentation, oxidative stress, and endothelial dysfunction. The DAMPs that seem to activate TLRs leading to PE include fetal DNA, heat shock proteins, hypoxia, fibrinogen, hyaluronic acid, and oxidized LDL [17-19]. RIG-I and IFIH1 also seem to be activated excessively in

placentas of mice and women with PE [17]. However, it is still unclear how excessive activation of different PRRs acting as the body's pathogen and stress responders might lead to the development of PE. Therefore, as part of the ongoing effort to confirm the association of different PRRs with PE and how this occurs, we conducted a systematic review of the literature on the subject.

Materials and Methods

Search strategy and eligibility criteria

This systematic literature search was designed and described in accordance with current guidelines [20]. PubMed and Embase repositories were searched to identify all studies that analyzed associations between PE and innate immunity genes, including: *IFIH1/MDA-5, RIG-I, TLRs,* and *NLRs*. The following medical subject headings (MeSH) were searched: "IFIH1 protein, human" OR "IFIH1 protein, mouse", "Toll-like receptor 9" OR "Toll-like receptor 6" OR "Toll-like receptor 10" OR "Toll-like receptor 8" OR "Toll-like receptor 7" OR "Toll-like receptor 5" OR "Toll-like receptor 4" OR "Toll-like receptor 3" OR "Toll-like receptor 2" OR "Toll-like receptor 1" OR "Toll-like receptors", NLRP1 protein, human" OR "NALP1 protein, mouse" OR "Nalp1 protein, rat" OR "NLRP3 protein, human" OR "CIAS1 protein mouse", "RARRES3 protein human" OR "Robo3 protein mouse", "NLRC5 protein mouse" OR "NLRP10 protein human", "Preeclampsia" OR "Hypertension, Pregnancy-Induced" OR "HELLP Syndrome" OR "eclampsia". The search was completed on March, 2016, and it was limited to English or Spanish language papers, and included both human and animal studies. All articles identified were also searched manually to identify other important citations.

We included case-control studies that evaluated the association between PE and one or more of the above mentioned genes, including studies reporting protein and gene expression data or polymorphism analyses. If data were duplicated and had been published more than once, the comprehensive study was chosen for inclusion in the systematic review. Two investigators (B.M.S. and A.P.B.) independently reviewed the titles and abstracts of all articles selected in order to evaluate whether the studies were eligible for inclusion in the systematic review. Disagreements were resolved by discussion between them and when necessary a third reviewer (D.C.) was consulted.

Data extraction and quality control assessment

Data were independently extracted by two investigators (B.M.S. and A.P.B.) using a standardized abstraction form [21, 22], and consensus was sought in all extracted items. When consensus could not be achieved, differences in data extraction were resolved by a third reviewer (D.C.) and by referencing the original publication. The following data were extracted from each individual study according the presence of PE: 1) characteristics of the studies (including name of first author, publication year, number of subjects in case and control groups, age, gestational age, body mass index (BMI), systolic BP, diastolic BP and ethnicity); 2) polymorphism frequencies (including genotype and allele distributions in case and control groups and OR (95% CI); 3) protein and gene expressions [21, 22].

Similarly, two investigators (B.M.S and A.P.B.) independently assessed the quality of each eligible study using the Newcastle-Ottawa Scale (NOS) for assessing quality of case-control studies [23]. The NOS scale contains eight items, categorized into three dimensions: selection, comparability, and exposure. For each item, a series of answer options is provided. A star score system is used to allow a semi-quantitative assessment of the study quality, such

as the highest quality studies are given a maximum of one star for each item, with exception of the item related to comparability, which allows two stars to be given. Thus, the total NOS score ranges from zero to nine stars.

Results

Literature search and characteristics of eligible studies

The strategy used to identify and select studies for inclusion in the systematic review is shown in **Figure 1**. A total of 159 possibly relevant articles were retrieved by searching the electronic databases, and 121 of them were excluded during the reading of titles and abstracts. Thirty-eight articles appeared to be eligible after this phase and, then, their full texts were evaluated. Nevertheless, after careful analysis of the full texts, another 12 studies were excluded owing to ineligible study design or because they analyzed other genes non-related to PRRs. A total of 26 articles [17, 24-46] fulfilled the eligibility criteria and were included in this systematic review.

The available characteristics of studies included in this systematic review are described in **Table S1**. The mean age of analyzed subjects was 29.5 ± 5.0 years for PE groups and 30.1 ± 8.0 years for control groups. Mean gestational age was 34.7 ± 3.0 weeks in women with PE *vs*. 37.9 ± 1.0 weeks in control women. Mean systolic BP was 156.0 ± 6.0 mmHg in cases with PE and 110.0 ± 9.0 mmHg in controls, while mean diastolic BP was 102.7 ± 6.0 mmHg in cases *vs*. 70.3 ± 5.1 in controls. Most studies were performed in European or North American populations. It is worth noting that some articles did not provide detailed information of the analyzed women (**Table S1**).

The quality of each individual study reviewed is shown in **Table S2**. The highest quality studies were awarded nine stars using NOS scale and accordingly as reported in *Material and Methods Section*. In general, most studies were considered as having moderate to good quality. None of the papers scored less than 5 stars, and 72% of them received 7 or 8 stars.

Qualitative analysis of studies that evaluated PRR gene or protein expressions in relation to PE

Twenty studies have analyzed expressions of one or more genes codifying PRRs in humans or mice with PE (cases) compared to normal pregnant females (control groups) (**Table 1**). Ten studies were done in human placenta samples and 10 studies in immune cells. Most of them reported TLR-2, -3 or -4 gene and/or protein expressions in human placenta or immune cells. No study has evaluated NLR expression.

Only one study has evaluated ***IFIH1/MDA-5*** and ***RIG-I*** expressions in humans and mice [17]. Chatterjee *et al*. [17] showed that IFIH1 and RIG-I proteins were increased in placenta from pregnant, poly I:C (PIC)-treated mice compared to normal pregnant mice. PIC is a synthetic analog of viral dsRNA. Moreover, these helicases were increased in placenta from pregnant women with PE compared to healthy pregnant women.

Three studies have investigated ***TLR-1*** expression in humans [32, 34, 47]. Two of them were not able to find any differences in the expression of this gene in dendritic cells from PE women and control women [34, 47]. In contrast, Nitsche *et al*. [32] reported a decreased *TLR-1* gene expression in neutrophils from cases compared to control women.

Ten articles have reported ***TLR-2*** expression in humans [28, 30, 33-36, 41, 43, 47, 48]. Five of these studies evaluated TLR-2 gene and/or protein expressions in placenta [28],

peripheral blood mononuclear cells (PBMCs) [30], dendritic cells [34, 47] or monocytes [36], showing similar results between PE women and control women. Four studies reported increased TLR-2 expressions in PBMCs [41], placenta (trophoblast, stroma and vascular endothelium cells, and fetal part) [35, 43] or neutrophils [48] from PE women compared to the control group. Conversely, Nitsche *et al*. [33] showed a decreased *TLR-2* gene expression in neutrophils from PE women.

Six publications have reported ***TLR-3*** expressions in humans or mice [17, 25, 34, 35, 42, 47]. Four of them showed increased TLR-3 gene or protein expressions in placentas of PE women or mice compared to controls [17, 25, 35, 42]. Panda *et al*. [47] observed increased TLR-3 protein levels in primary dendritic cells isolated from the circulation of PE women compared to cells from the control group. In contrast, the same group showed that despite differences observed at the protein level in the above-mentioned study, *TLR-3* gene expression was similar in dendritic cells isolated from PE and control groups [34].

Sixteen articles have investigated ***TLR-4*** expressions in humans [24, 28-30, 33-37, 40, 41, 43, 45-48]. Thirteen of these studies showed increased TLR4 gene or protein expressions in maternal neutrophils [48], PBMCs [30, 41], cord blood mononuclear cells [46], monocytes [36, 45], dendritic cells [47] or placenta [24, 29, 35, 37, 40, 43] from PE women compared to the control group. On the other hand, Nitsche *et al*. [33] reported decreased *TLR4* gene expression in neutrophils from PE women compared to control women, whereas two other studies were not able to find any differences in *TLR4* gene expression in dendritic cells [34] or placenta [28] from case and control groups.

While one study reported decreased *TLR-5* gene expression in neutrophils from PE women compared to the control group [32], other study showed increased expression of this gene in the placenta [43]. Only one study evaluated ***TLR-6*** gene expression in placenta,

showing increased values in cases compared to controls [43]. Similarly, one study reported increased **TLR-7** gene and protein expressions in placentas from PE women compared to the control group [25]. In the same way, only one study demonstrated increased **TLR-8** gene/protein expressions in placentas from PE women compared to healthy pregnant women [25]; although, two other studies did not find any differences in TLR-8 expressions in dendritic cells from PE and control groups [34, 47].

Three articles have described ***TLR-9*** expressions accordingly to PE occurrence. Two of them reported increased TLR-9 protein levels in placentas [35] or dendritic cells [47] from PE women, while Panda *et al*. [34] did not find any association between *TLR-9* gene expression in dendritic cells and PE occurrence.

Qualitative analysis of studies that evaluated associations between polymorphisms in genes for PRRs and PE

Only 6 studies have addressed the associations between polymorphisms in genes codifying PRRs and PE (**Table 2**). These studies focused in *TLR-2*, *TLR-3*, *TLR-4* and *NOD-2* genes.

Three articles have analyzed polymorphisms in the *TLR-2* gene [26, 27, 39]. Xie *et al*. [39] reported that the presence of Arg753Gln (G/A; rs5773708) polymorphism in this gene was associated with early-onset PE (<34 weeks' gestation; OR = 2.57, 95% CI 1.31-5.05), but not late-onset (≥34) PE in Canadian women. However, Franchim *et al*. [26] was not able to find any differences in frequencies of the *TLR-2* Arg753Gln polymorphism between Brazilian women with PE and race-matched healthy fertile women with at least 2 previous successful pregnancies (control group). Moreover, Fraser *et al*. [27] did not find any association between the *TLR-2* Arg753Gln polymorphism and PE in women from United Kingdom.

Only one study evaluated polymorphisms in the ***TLR-3*** gene regarding PE, showing similar frequencies of the Leu412Phe (A/G; 3775291) and -299698 (G/T; rs3775296) polymorphisms between case and control groups [44].

Four articles have evaluated the association between polymorphisms in the *TLR-4* gene and PE [26, 31, 38, 39]. Van Rijn *et al*. [38] analyzed *TLR-4* Asp299Gly (A/G; rs4986790) and Thr399Ile (C/T; rs4986791) polymorphisms in women with early-onset PE (cases) and women with uneventful pregnancies (controls) from Germany. These two polymorphisms were in strong linkage disequilibrium. Their results showed that after adjustment for maternal age and chronic hypertension, positivity for one or more of the minor alleles of the *TLR-4* Asp299Gly or Thr399Ile polymorphisms were more common in cases than in controls (OR = 2.9, 95% CI 1.2-6.7). Nonetheless, three other studies, performed in Brazilian [26], Hungarian [31] and Canadian [39] populations, did not find any associations between Asp299Gly and/or Thr399Ile polymorphisms and PE.

The study by Van Rijn *et al*. [38] was the only one analyzing polymorphisms in the *NOD-2* gene and development of PE. In that study, the authors showed that, individually, *NOD-2* Arg702Trp (C/T; rs2066844), Gly908Arg (G/C; rs2066845) and Leu1007fs (C/-; rs2066847) polymorphisms did not contribute to the development of PE. However, highest ORs for early-onset PE were observed for women carrying any of the five minor alleles of the *TLR-4* (Asp299Gly/Thr399Ile) or *NOD-2* (Arg702Trp/Gly908Arg/Leu1007fs) genes, within the highest tertiles for IL-6 and fibrinogen, with ORs of 6.9 (95% CI 2.1-23.2) for IL-6 and 3.8 (95% CI 1.2-11.8) to fibrinogen, respectively [38].

Discussion

Several theories have been proposed for explaining the etiology of PE, including shallow invasion of trophoblast cells leading to inadequate remodeling of the spiral arteries, abnormal placentation, deficient angiogenesis and immune system dysfunction. Indeed, immune system activation seems to be required for early stages of implantation. On the other hand, an excessive activation of the maternal immune system against the fetus may play an important role in the development of PE [17, 49, 50]. The activation of the maternal immune system might be triggered by PAMPs derived from different invading microorganisms as well as by endogenous DAMPs produced by cells and tissues during stress or apoptosis [17, 51]. Different PRRs recognize specific PAMPs or DAMPs, leading to the activation of signaling cascades and the production of proinflammatory cytokines, which coordinates local and systemic inflammatory responses [14, 52]. Although some studies have linked excessive PRR activation to the development of PE, it is still inconclusive if PRRs are indeed involved in the development of this pregnancy complication and how it occurs. Thus, we performed a systematic review of 26 studies that analyzed the potential relationship between PRR genes and PE.

As already commented, IFIH1/MDA-5 and RIG-I cytoplasmic receptors recognize different intracellular dsRNAs generated during viral replication [9]. These receptors may also recognize cell-generated small RNAs [17]. After binding with dsRNA, IFIH1 and RIG-I will activate signaling pathways leading to the activation of the transcription factors nuclear factor-kappa B (NF-κB) and interferon-regulatory factor 3 (IRF-3), ultimately driving the production of proinflammatory cytokines, chemokines, and type I interferons (IFN-I). Then, IFN-I binds to its receptor and activates the Jak-STAT pathway to drive the expression of

IFN-regulated genes and the innate immune response [9-11]. While IFIH1 and RIG-I have been implicated in a number of diseases, including type 1 diabetes mellitus (T1DM) and other autoimmune diseases [9], only one study has investigated these receptors regarding PE. Preliminary results reported by Chatterjee *et al*. [17] indicate that both receptors are activated excessively in placentas from PE women as well as placentas from PIC-treated mice. Until the present, no study has evaluated the association between polymorphisms in *IFIH1* or *RIG*-I genes and PE. Therefore, further studies are necessary to determine the role of IFIH1 and RIG-I in the development of this disease. It is worth mentioning that a previous study from our group showed increased *IFIH1* gene expression in mononuclear cells from T1DM patients with arterial hypertension compared to normotensive T1DM patients [6], also suggesting a role for this receptor in hypertension.

Among the most important families of PRRs are the TLRs, which selectively recognize several PAMPS and DAMPs [8]. For more details regarding which specific PAMP is recognized by each TLR please refer to Assmann *et al*. [14]. TLR signaling proceeds through two pathways: the myeloid differentiation factor 88 (MyD88)-mediated pathway, and the TIR-domain containing adaptor inducing IFN-β (TRIF)-mediated pathway. The MyD88 pathway leads to the activation of NF-κB and several mitogen-activated protein kinases (MAPK) that activate different genes related to inflammatory reactions. The TRIF pathway culminates in IFN-I production similarly as reported for IFIH1. TLR-3 only activates the TRIF pathway; TLR4 activates both pathways, while all other TLRs activate exclusively the MyD88 pathway [14, 53, 54].

Only few studies have analyzed TLR-1, TLR-5, TLR-6, TLR-7, TLR-8 and TLR-9 expressions in immune cells or placentas from PE women and healthy pregnant women (**Table 1**). Thus, it is still inconclusive if these TLRs are involved in the etiology of PE in

humans. Of note, Chatterjee *et al*. [25] reported that treatment of human trophoblasts with specific agonists for TLR-7 (R-837) and TLR-7/8 (CLO97) significantly increased their protein levels, leading to inflammation and immune cell activation via well-known TLR signaling pathways. Moreover, treatment of mice with R-837 or CLO97 agonists caused pregnancy-dependent hypertension, endothelial dysfunction, splenomegaly, and placental inflammation. The authors also showed that both TLR-7 and TLR-8 gene and protein expressions were increased in women with PE compared to healthy pregnant women [25]. Until this date, no study has evaluated the association between polymorphisms in *TLR-1*, *TLR-5*, *TLR-6*, *TLR-7*, *TLR-8* and *TLR-9* genes and PE.

TLR-2 recognizes PAMPs derived from bacteria, fungi or parasites [14]. Although ten studies have evaluated TLR-2 expressions in humans, the results are contradictory: while 5 studies reported similar TLR-2 levels in PE women and control pregnant women [28, 30, 34, 36, 47], 4 studies showed increased expressions in immune cells or placentas from cases compared to controls [35, 41, 43, 48], and one study observed decreased gene expression in neutrophils from PE women compared to the control group [33]. Three studies have analyzed the association between the Arg753Gln (G/A; rs5773708) polymorphism in the *TLR-2* gene and PE [26, 27, 39], also with inconclusive results. The study by Xie *et al*. [39] was the only one to report an association of this polymorphism with increased risk for PE, but only when comparing frequencies between women with early-onset PE and the control group. The authors concluded that the analyzed polymorphism appear to lower thresholds for early-onset and severe PE, but not late-onset or mild disease. The other two studies did not analyze *TLR-2* Arg753Gln frequencies according to PE severity [26, 27].

TLR-3 endoplasmic receptor recognizes viral or endogenous dsRNA [14], and it is the most abundant TLR in placenta [55]. Six studies have reported TLR-3 expressions in humans

or mice [17, 25, 34, 35, 42, 47], showing that protein levels of this PRR are increased in placenta or immune cells of PE women or mice compared to the respective control groups. Even though Panda *et al*. [47] observed increased TLR-3 protein levels in circulating dendritic cells from PE women compared to the control group; they did not observe any differences at mRNA level [34], suggesting a post-transcriptional regulation for this gene. Abrahams *et al*. [56] reported that first-trimester trophoblast cells are able to modulate the maternal immune system by their ability to secrete cytokines and chemokines following TLR-3 activation caused by PIC treatment; thus, suggesting that trophoblast cells are able to recognize and specifically respond to viral products in a regulated manner [56]. In addition, other studies in rodent models indicate a role of TLR-3 activation in detrimental pregnancy outcomes [57-59]. The only study that evaluated *TLR-3* polymorphisms was not able to find any association of these polymorphisms with PE [44].

In the same way as TLR-2, TLR-4 plasma membrane receptor recognizes PAMPs derived from bacteria, fungi or parasites [14]. Among the 16 studies that evaluated TLR-4 expressions in immune cells or placentas [24, 28-30, 33-37, 40, 41, 43, 45-48], the majority have demonstrated an increased expression of this gene in PE women compared to healthy pregnant women. An important role of TLR-4 in the development of PE is further supported by the study of Chaiworapongsa *et al*. [60] showing that TLR-4 activation by LPS inhibits the migratory capacity of the trophoblast, which might explain the impaired extravillous trophoblast invasion and remodeling of spiral arteries in the decidua from PE patients. Moreover, TLR-3 and TLR-4 activation in pregnant rats led to the development of pathological changes similar to those observed in PE women [57, 61].

In contrast, Nitsche *et al*. [33] reported decreased TLR-2 and TLR-4 expressions in neutrophils from women with mild PE compared to the control group, which goes in the

opposite way than expected considering that TLRs are possibly involved in an up-regulation of the innate immune system. The authors suggested that the decrease in TLR-2 and TLR-4 expressions might represent a compensatory reaction to the inflammatory signals present in PE women. The decrease in these TLRs would render the maternal neutrophils less able to answer to specific PAMPs or DAMPs, decelerating the inflammatory cascade seen in PE [33]. Interestingly, Panda *et al*. [47] showed that although basal expressions of TLR-3, TLR-4 and TLR-9 were increased in dendritic cells from PE women, these cells demonstrated a less robust response to stimulation with TLR ligands compared to cells from healthy pregnant controls. They hypothesized that a dysregulated patter of TLR expression and cytokine production in dendritic cells from PE patients may limit further activation by TLR engagement. Additional research is needed to clarify these contradictory results.

Four studies have analyzed the association between *TLR-4* polymorphisms and PE [26, 31, 38, 39]. Of them, only one study was able to find an association between the minor alleles of the *TLR-4* Asp299Gly and Thre399Ile polymorphism and early-onset PE [38]. The same group found no association for three attenuating polymorphisms (Arg702Trp, Gly908Arg and Leu1007fs) of the *NOD-2* gene and PE [38]. Nevertheless, the three *NOD-2* polymorphisms increased the risk for early-onset PE when in presence of the minor alleles of the *TLR-4* polymorphisms, especially within the highest tertiles for IL-6 and fibrinogen [38]. Unfortunately, this study did not analyze women with late-onset PE. Negative results regarding associations of *TLR2/4* and *NOD-2* polymorphisms with PE might be explained by the low frequency of the analyzed polymorphisms in these genes, lack of statistical power of the studies, genetic background of each population as well as differences in severity of PE. The effect of polymorphisms in PRR genes might be different between early- or late-onset PE

[39] and it should be considered when analyzing the association between polymorphisms in these genes and the disease.

In conclusion, this systematic review shows that available data in literature strongly support a role of TLR-3 and TLR-4 in the pathogenesis of PE. However, additional research is still needed for a better understanding on how an excessive activation of these TLRs in response to exogenous or endogenous danger signals can lead to PE development. Moreover, further studies are needed to confirm if other TLRs, IFIH1/MDA-5, RIG-I and NOD receptors are indeed involved in PE pathogenesis, and if polymorphisms in PRR genes predispose to this pregnancy complication. Through additional investigation of the PRR involvement in PE, it might be possible to identify potential targets for PE intervention.

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References

1. ACOG practice bulletin, *Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002.* Obstet Gynecol, 2002. **99**(1): p. 159-67.
2. Tranquilli, A.L., et al., *The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP.* Pregnancy Hypertens, 2014. **4**(2): p. 97-104.
3. American College of Obstetricians and Gynecologists, and P. Task Force on Hypertension in, *Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy.* Obstet Gynecol, 2013. **122**(5): p. 1122-31.
4. Borzychowski, A.M., I.L. Sargent, and C.W. Redman, *Inflammation and pre-eclampsia.* Semin Fetal Neonatal Med, 2006. **11**(5): p. 309-16.
5. Perez-Sepulveda, A., et al., *Innate immune system and preeclampsia.* Front Immunol, 2014. **5**: p. 244.
6. Boucas, A.P., et al., *The A allele of the rs1990760 polymorphism in the IFIH1 gene is associated with protection for arterial hypertension in type 1 diabetic patients and with expression of this gene in human mononuclear cells.* PLoS One, 2013. **8**(12): p. e83451.
7. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. **442**(7098): p. 39-44.
8. De Nardo, D., *Toll-like receptors: Activation, signalling and transcriptional modulation.* Cytokine, 2015. **74**(2): p. 181-9.
9. Boucas, A.P., et al., *The role of interferon induced with helicase C domain 1 (IFIH1) in the development of type 1 diabetes mellitus.* Arq Bras Endocrinol Metabol, 2013. **57**(9): p. 667-76.
10. Wilkins, C. and M. Gale, Jr., *Recognition of viruses by cytoplasmic sensors.* Curr Opin Immunol, 2010. **22**(1): p. 41-7.
11. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.* Nature, 2006. **441**(7089): p. 101-5.
12. Liu, D., A.M. Rhebergen, and S.C. Eisenbarth, *Licensing Adaptive Immunity by NOD-Like Receptors.* Front Immunol, 2013. **4**: p. 486.
13. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int Immunol, 2005. **17**(1): p. 1-14.
14. Assmann, T.S., et al., *Toll-like receptor 3 (TLR3) and the development of type 1 diabetes mellitus.* Arch Endocrinol Metab, 2015. **59**(1): p. 4-12.
15. Szatmary, Z., *Molecular biology of toll-like receptors.* Gen Physiol Biophys, 2012. **31**(4): p. 357-66.
16. Dunne, A. and L.A. O'Neill, *Adaptor usage and Toll-like receptor signaling specificity.* FEBS Lett, 2005. **579**(15): p. 3330-5.
17. Chatterjee, P., et al., *Do double-stranded RNA receptors play a role in preeclampsia?* Placenta, 2011. **32**(3): p. 201-5.
18. Koga, K. and G. Mor, *Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy disorders.* Am J Reprod Immunol, 2010. **63**(6): p. 587-600.
19. Riley, J.K. and D.M. Nelson, *Toll-like receptors in pregnancy disorders and placental dysfunction.* Clin Rev Allergy Immunol, 2010. **39**(3): p. 185-93.

20. Moher, D., et al., *Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement.* BMJ, 2009. **339**: p. b2535.
21. Brondani, L.A., et al., *Meta-analysis reveals the association of common variants in the uncoupling protein (UCP) 1-3 genes with body mass index variability.* PLoS One, 2014. **9**(5): p. e96411.
22. de Souza, B.M., et al., *Associations between UCP1 -3826A/G, UCP2 -866G/A, Ala55Val and Ins/Del, and UCP3 -55C/T polymorphisms and susceptibility to type 2 diabetes mellitus: case-control study and meta-analysis.* PLoS One, 2013. **8**(1): p. e54259.
23. Wells GA, S.B., O'Connell D, Peterson J, Welch V, , *The Newcastle-Ottawa scale (NOS) for assessing the quality of nonrandomized studies in metaanalysis.* p. Available: http://www.medicine.mcgill.ca/rtamblyn/Readings.
24. Bernardi, F.C., et al., *Oxidative damage, inflammation, and Toll-like receptor 4 pathway are increased in preeclamptic patients: a case-control study.* Oxid Med Cell Longev, 2012. **2012**: p. 636419.
25. Chatterjee, P., et al., *Placental toll-like receptor 3 and toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice.* PLoS ONE, 2012. **7**(7).
26. Franchim, C.S., et al., *Inflammatory mediators gene polymorphisms in preeclampsia.* Hypertension in Pregnancy, 2011. **30**(3): p. 338-346.
27. Fraser, R., et al., *Interleukin-4 -590 (C>T), toll-like receptor-2 +2258 (G>A) and matrix metalloproteinase-9 -1562 (C>T) polymorphisms in pre-eclampsia.* Bjog, 2008. **115**(8): p. 1052-6; discussion 1056.
28. Holmlund, U., et al., *The novel inflammatory cytokine high mobility group box protein 1 (HMGB1) is expressed by human term placenta.* Immunology, 2007. **122**(3): p. 430-437.
29. Kayisli, U.A., et al., *Preeclampsia-related toll receptor-4 expression in decidual cells across pregnancy.* Reproductive Sciences, 2013. **20**(3): p. 264A.
30. Medeiros, L.T.L., et al., *Monocytes from Pregnant Women with Pre-Eclampsia are Polarized to a M1 Phenotype.* American Journal of Reproductive Immunology, 2014. **72**(1): p. 5-13.
31. Molvarec, A., et al., *Toll-like receptor 4 gene polymorphisms and preeclampsia: lack of association in a Caucasian population.* Hypertens Res, 2008. **31**(5): p. 859-64.
32. Nitsche, J., et al., *Toll-like receptor 1 and Toll-like receptor 5 expression is down-regulated on maternal neutrophils in preeclampsia.* American Journal of Obstetrics and Gynecology, 2011. **204**(1): p. S274.
33. Nitsche, J.F., S.W. Jiang, and B.C. Brost, *Maternal neutrophil toll-like receptor mRNA expression is down-regulated in preeclampsia.* American journal of reproductive immunology (New York, N.Y. : 1989), 2011. **66**(3): p. 242-248.
34. Panda, B., et al., *Increase in TLR protein in preeclamptic patients does not correlate with a corresponding increase in TLR gene expression.* American Journal of Obstetrics and Gynecology, 2012. **206**(1): p. S330.
35. Pineda, A., et al., *Expression of Toll-like Receptor TLR-2, TLR-3, TLR-4 and TLR-9 Is Increased in Placentas from Patients with Preeclampsia.* Archives of Medical Research, 2011. **42**(5): p. 382-391.
36. Romao, M., et al., *TLR-4 expression and pro-inflammatory cytokine production by peripheral blood monocytes from preeclamptic women.* Pregnancy Hypertension, 2012. **2**(3): p. 276.

37. Semerci, N., et al., *Excess soluble Fms-like tyrosine kinase 1 production by endogenous activation of decidual cell toll-like receptor-4 triggers preeclampsia-related vascular dysfunctions.* Reproductive Sciences, 2014. **21**(3): p. 398A.
38. van Rijn, B.B., et al., *Maternal TLR4 and NOD2 gene variants, pro-inflammatory phenotype and susceptibility to early-onset preeclampsia and HELLP syndrome.* PLoS ONE, 2008. **3**(4).
39. Xie, F., et al., *Toll-like receptor gene polymorphisms and preeclampsia risk: A case-control study and data synthesis.* Hypertension in Pregnancy, 2010. **29**(4): p. 390-398.
40. Zhang, L. and H. Yang, *Expression and localization of TLR4 and its negative regulator Tollip in the placenta of early-onset and late-onset preeclampsia.* Hypertens Pregnancy, 2012. **31**(2): p. 218-27.
41. Al-ofi, E., S.B. Coffelt, and D.O. Anumba, *Monocyte subpopulations from pre-eclamptic patients are abnormally skewed and exhibit exaggerated responses to Toll-like receptor ligands.* PLoS One, 2012. **7**(7): p. e42217.
42. Chatterjee, P., et al., *Cotreatment with interleukin 4 and interleukin 10 modulates immune cells and prevents hypertension in pregnant mice.* American Journal of Hypertension, 2015. **28**(1): p. 135-142.
43. Dabagh-Gorjani, F., et al., *Differences in the expression of TLRs and inflammatory cytokines in pre-eclamptic compared with healthy pregnant women.* Iran J Immunol, 2014. **11**(4): p. 233-45.
44. Chen, A., et al., *Role of Toll-Like Receptor 3 Gene Polymorphisms in Preeclampsia.* Cellular Physiology and Biochemistry, 2015. **37**(5): p. 1927-1933.
45. Chen, W., et al., *Significance of toll-like receptor 4 signaling in peripheral blood monocytes of pre-eclamptic patients.* Hypertension in Pregnancy, 2015. **34**(4): p. 486-494.
46. Xia, G., et al., *Expression of Toll-like receptor 4 in neonatal cord blood mononuclear cells in patients with preeclampsia.* J Huazhong Univ Sci Technolog Med Sci, 2010. **30**(5): p. 615-9.
47. Panda, B., et al., *Dendritic cells in the circulation of women with preeclampsia demonstrate a pro-inflammatory bias secondary to dysregulation of TLR receptors.* J Reprod Immunol, 2012. **94**(2): p. 210-5.
48. Xie, F., et al., *Toll-like receptors 2 and 4 and the cryopyrin inflammasome in normal pregnancy and pre-eclampsia.* BJOG, 2010. **117**(1): p. 99-108.
49. Redman, C.W., G.P. Sacks, and I.L. Sargent, *Preeclampsia: an excessive maternal inflammatory response to pregnancy.* Am J Obstet Gynecol, 1999. **180**(2 Pt 1): p. 499-506.
50. Saito, S., et al., *The role of the immune system in preeclampsia.* Mol Aspects Med, 2007. **28**(2): p. 192-209.
51. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
52. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. **449**(7164): p. 819-26.
53. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
54. Kawai, T. and S. Akira, *TLR signaling.* Cell Death Differ, 2006. **13**(5): p. 816-25.
55. Patni, S., et al., *Expression and activity of Toll-like receptors 1-9 in the human term placenta and changes associated with labor at term.* Biol Reprod, 2009. **80**(2): p. 243-8.

56. Abrahams, V.M., et al., *Expression and secretion of antiviral factors by trophoblast cells following stimulation by the TLR-3 agonist, Poly(I : C).* Hum Reprod, 2006. **21**(9): p. 2432-9.
57. Tinsley, J.H., et al., *Toll-like receptor 3 activation during pregnancy elicits preeclampsia-like symptoms in rats.* Am J Hypertens, 2009. **22**(12): p. 1314-9.
58. Shimada, S., et al., *Expression of allograft inflammatory factor-1 in mouse uterus and poly(I:C)-induced fetal resorption.* Am J Reprod Immunol, 2003. **50**(1): p. 104-12.
59. Ilievski, V., S.J. Lu, and E. Hirsch, *Activation of toll-like receptors 2 or 3 and preterm delivery in the mouse.* Reprod Sci, 2007. **14**(4): p. 315-20.
60. Chaiworapongsa, T., et al., *Macrophage migration inhibitory factor in patients with preterm parturition and microbial invasion of the amniotic cavity.* J Matern Fetal Neonatal Med, 2005. **18**(6): p. 405-16.
61. Faas, M.M., et al., *Altered monocyte function in experimental preeclampsia in the rat.* Am J Obstet Gynecol, 2004. **191**(4): p. 1192-8.

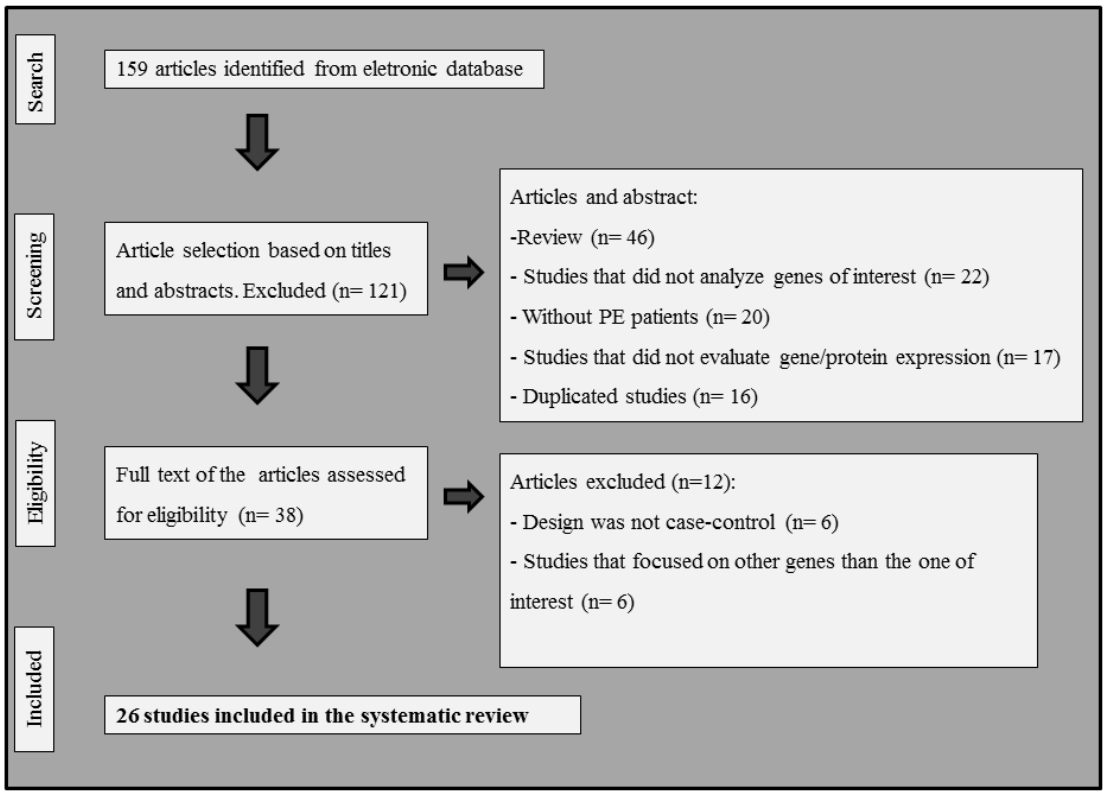

Figure 1. Flowchart illustrating the search strategy used to identify association studies of pattern-recognition receptors (PRRs) genes and preeclampsia for the systematic review.

Table 1. Relationship between gene or protein expressions of pattern recognition receptors and preeclampsia.

1st author, year [ref.]	Gene	Human or animal	Tissue /cells	Results	Method of analysis
Al-ofi *et al*., 2012 [41]	TLR-2 and TLR-4	Human	PBMCs	Both proteins were increased in cases (n=17) compared to controls (n=11).	FC
Bernardi *et al*., 2012 [24]	TLR-4	Human	Placenta	TLR-4 protein was higher in cases (n=33) than controls (n=33).	WB
Chatterjee *et al*., 2011 [17]	RIG-1, IFIH1 and TLR-3	Human and mouse	Placenta	Proteins of these 3 PRRs were increased in women with PE and PIC-treated mice compared to the respective control groups	WB and IMF
Chatterjee *et al*., 2012 [25]	TLR-3, TLR-7 and TLR-8	Human	Placenta	Expressions of these 3 genes were increased in cases (n=17) compared to controls (n=13).	GE and IHC
Chatterjee *et al*., 2015 [42]	TLR-3	Mouse	Placenta	Tlr-3 protein was increased in placenta from PIC-treated pregnant mice compared to the control group (n=3).	WB
Chen *et al*., 2015 [45]	TLR-4	Human	Monocytes	TLR-4 protein was increased in cases (n=22) compared to controls (n=23).	FC
Gorjani *et al*., 2014 [43]	TLR-2, TLR-4, TLR-5 and TLR-6	Human	Placenta	TLR-4, TLR-5 and TLR-6 were increased in cases (n=15) compared to controls (n= 15) in both maternal and fetal portions of placenta. TLR-2 was increased only in the fetal part of placenta.	GE
Holmlund *et al*., 2007 [28]	TLR-2 and TLR-4	Human	Placenta	No differences in protein levels were observed between cases (n=13) and controls (n=12).	IHC

Kayisli *et al.*, 2013 [29]*	TLR-4	Human	Placenta (DC cells)	TLR-4 protein was increased in cases (271 ± 26; n=7) compared to controls (202 ± 25; n=8).	IHC
Medeiros *et al.*, 2014 [30]	TLR-2 and TLR-4	Human	Monocytes	TLR-2 protein was similar between cases (n=85) and controls (n=52); TLR-4 was increased in cases.	FC
Nitsche *et al.*, 2011 [33]	TLR-2 and TLR-4	Human	Neutrophils	*TLR-2* and TLR-4 expressions were lower in cases than controls.	GE
Nitsche *et al.*, 2011 [32]*	TLR-1 and TLR-5	Human	Neutrophils	*TLR-1* and TLR-5 expressions were lower in cases than controls.	GE
Panda *et al.*, 2012 [47]	TLR-1 a 4, TLR-8 and TLR-9	Human	Dendritic cells	TLR-1, TLR-2 and TLR-8 proteins were similar between cases (n=30) and controls (n=30); TLR-3, TLR-4 and TLR-9 were increased in cases.	FC
Panda *et al.*, 2012 [34]*	TLR-1 a 4, TLR-8 and TLR-9	Human	Dendritic cells	Gene expressions of these genes were similar between cases (n=30) and controls (n=30).	GE
Pineda *et al.*, 2011 [35]	TLR-2, TLR-3, TLR-4 and TLR-9	Human	Placenta	Proteins concentrations of these 4 PRRs were higher in cases (n=5) than controls (n=5) in different cell types of placenta.	IMF
Romao *et al.*, 2012 [36] *	TLR-2 and TLR-4	Human	Monocytes	TLR-2 protein was similar between cases (n=30) and controls (n=20); TLR-4 was increased in cases.	FC
Semerci *et al.*, 2014 [37]*	TLR-4	Human	Placenta (DC and trophoblast cells)	TLR-4 protein was increased in cases (n=7) compared to controls (n=8).	IHC

Xia *et al*., 2010 [46]	TLR-4	Human	CBMCs	*TLR-4* gene and protein expressions were higher in cases than controls.	GE and WB
Xie et al., 2010 [48]	TLR-2 and TLR-4	Human	Neutrophils	*TLR-2* and *TLR-4* gene and protein expressions were higher in cases (n=50) than controls (n=75).	GE and FC
Zhang *et al*., 2012 [40]	TLR-4	Human	Placenta	TLR-4 protein was increased in cases with early-onset PE (n=8) compared to controls (n=8). No differences were observed between late-onset PE and the control group.	WB

Data are shown as mean ± SD or median (interquartile range). CBMCs = cord-blood mononuclear cells; DCs = decidua cells; FC = flow cytometry; GE = gene expression; IHC = immunohistochemistry; IMF = immunofluorescence; PBMCs = peripheral blood mononuclear cells; PRRs = pattern recognition receptors. * Data retrieved from abstracts from studies presented in International Congress.

Table 2. Association between polymorphisms in genes for pattern recognition receptors and preeclampsia.

1st author, year [ref.]	Gene - polymorphism	Cases	Controls	P-value / OR (95% CI)
Chen *et al.*, 2015 [44]	*TLR-3* – Leu412Phe (A/G)	N = 989	N = 1227	
	GG / AG / AA	57.0 / 34.5 / 8.5	57.0 / 36.1 / 6.9	0.346 / NA
	A allele / G allele	74.2 / 25.8	75.0 / 25.0	0.541 / 1.043 (0.91–1.19)
	TLR-3 – 299698 (5'-UTR) (G/T)	N = 989	N = 1227	
	GG / GT / TT /	58.5 / 35.5 / 6.0	55.9 / 37.1 / 7.0	0.329 / NA
	G allele / T allele	76.2 / 23.7	74.3 / 25.6	0.133 / 0.9 (0.78–1.03)
Franchim *et al*., 2011 [26]	*TLR-2* – Arg753Gln (G/A)	N = 91	N = 138	
	GG / GA / A/A	84.6 / 15.4 / 0	84.8 / 15.2 / 0	0.970 / NA
	G allele /A allele	92.3 / 7.7	92.4 / 7.6	> 0.999 / NA
	TLR-4 – Asp299Gly (G/A)	N = 109	N = 153	
	AA / AG / GG	93.6 / 6.4 / 0	87.6 / 11.7 / 0.7	0.230 / NA
	A allele / G allele	96.8 / 3.2	93.4 / 6.6	0.110 / NA
Fraser *et al*., 2008 [27]	*TLR-2* – (+2258)	N = 117	N = 146	
	GG / GA / AA	92.3 / 6.8 / 0.9	95.6 / 3.4 / 0	0.200 / NA
	G allele / A allele	95.7 / 4.3	98.3 / 1.7	NS
Molvarec *et al*., 2008 [31]	*TLR-4* – Asp299Gly (G/A)	N = 180	N = 172	
	AA / AG / GG	91.7 / 8.3 / 0	87.2 / 12.2 / 0.6	> 0.050 / NA

	A allele / G allele	95.8 / 4.2	93.3 / 6.7	> 0.050 / NA
	TLR-4 – Thr399Ile (C/T)	N = 180	N = 172	
	CC / CT / TT	91.7 / 8.3 / 0	86.6 / 12.8 / 0.6	> 0.050 / NA
	C allele / T allele	95.8 / 4.2	93.0 / 7.0	> 0.050 / NA
Van Rijn *et al*., 2008 [38]	*TLR-4* – (Asp299Gly / Thr399Ile)	N = 340	N = 113	
	Positivity for the 1 or + minor alleles	NA	NA	NA / 2.9 (1.2–6.7)
	of the two polymorphisms			
	299Asp (G allele) = 7.4%	NA	NA	NA
	399Ile (T allele) = 7.2%			
	NOD-2 – Arg702Trp (C/T) /	N = 340	N = 113	
	Gly908Arg (G/C) / Leu1007Pro (-/C)			
	702Trp allele = 4.4%, 908Arg allele	NA	NA	NA
	= 1.0%, and 1007Pro = 1.8%			
Xie *et al*., 2010 [39]	*TLR-2* – Arg753Gln (G/A)	N = 94 (42 early-onset and	N = 176	
		52 late-onset PE)		
	GG / AG / AA	91.5 / 8.5 / 0	95.5 / 4.5 / 0	0.094 / 1.95 (0.71–5.38)
	G allele / A allele	95.7 / 4.3	97.7 / 2.3	0.098 / 1.91 (0.71–5.18)
	TLR-4 – Asp299Gly (A/G)	N = 94 (42 early-onset and	N = 176	
		52 late-onset PE)		
	AA / AG / GG	86.2 / 12.8 / 1.0	89.8 / 1.2 / 0	0.310 / NA

A allele / G allele	92.6/ 7.4	94.8 / 5.2	0.140 / NA
TLR-4 – Thr399Ile (C/T)	N = 94 (42 early-onset and 52 late-onset PE)	N = 176	
CC / CT / TT	91.5 / 8.5 / 0	92.0 / 8.0 / 0	0.440 / NA
C allele / T allele	95.7 / 4.3	96.0 / 4.0	0.440 / NA

Data are expressed as percentage. N/A = not available; N/S = non-significant. Cases: women with preeclampsia. Controls: healthy pregnant women.

Table S1. Characteristics of patients with preeclampsia (cases) and healthy women with noneventful pregnancies (controls) for the studies performed in humans.

Author, year	N case	N control	Age	Gestational age (Weeks)	Ethnicity or Population	BMI (kg/m2)	SBP (mm/Hg)	DBP (mm/Hg)
Al-ofi, 2012	17	11	Case: 31.4±5.6 Control: 29.3±4.9 P = 0.26	Case: 32.9±3.7 Control: 32.0±3.7 P = 0.38	Caucasian and Black (United Kingdom)	NS	Case: 150.4±8.6 Control: 114.0±11.0 P = 0.0001	Case: 98.3±4.2 Control: 70.0±10.0 P = 0.0001
Bernardi, 2012	33	33	Case: 25.0±6.0 Control: 27.7±7.0 P = 0.35	Case: 34.0±8.5 Control: 36.1±8.4 P = 0.42	Brazilian	*Case: 23.0±6.0 *Control: 22.0±6.0 P = 0.40	Case: 150.0±12.0 Control: 115.0±9.0 P = 0.03	Case: 98.5±5.0 Control: 73.0±4.0 P = 0.002
Chaterjee, 2011	NS	NS	NS	NS	North American	NS	NS	NS
Chaterjee, 2012	17	13	Case: 26.2±1.1 Control: 24.6±1.2 P = 0.26	Case: 30.7±1.1 Control: 37.8±0.7 P < 0.05	North American	Case: 33.0±2.0 Control: 34.0±4.0 P = NS	Case: 162.0±5.0 Control: 132.0±7.0 P < 0,05	Case: 94.0±2.0 Control: 71.0±4.0 P < 0.05
Chen, 2105	987	1227	Case: 30.0±5.8 Control: 30.0±5.2 P = 0.918	Case: 35.4±3.6 Control: 39.0±1.4 P < 0.001	Asian	NS	Case: 154.1±23.2 Control: 114.0±9.9 P < 0.001	Case: 100.6±16.2 Control: 73.3±7.7

								P < 0.001
Chen, 2105	22	23	Case: 32.5±11.2 Control: 33.0±10.2 P < 0.05	Case: 36.3±0.8 Control: 36.4±0.9 P < 0.05	Asian	NS	Case: 150.4±8.9 Control: 112.0±10.0 Not associated	Case: 98.6±4.4 Control: 71.5±8.4 Not associated
Franchim, 2011	109	174	Case: 25.6±6.9 Control: 30.7±9.3 P < 0.0001	Case: 37.0±2.1 Control: 39.0±1.7 P = NS	Causasian (case)= 65 Causasian (control)= 113 Non-caucasian (case)= 44 Non-caucasian (control)= 61 (Brazilian)	Case: 23.5±1.3 Control: 23.2±1.4 P = 0.19	Case: 160.0±2.0 Control: 110.6±11.6 P < 0.00001	Case: 102.0±14.7 Control: 72.6±9.6 P < 0.0001
Fraser, 2008	117	146	Case: 29 (16-42) Control: 30 (16-40) P > 0.05	NS	Caucasian (United Kingdom)	NS	NS	NS
Gorjani, 2014	15	15	Case: 27,9 Control: 29.1 Not associated	Case: 37.6 Control: 38.1 Not associated	Iranian	NS	NS	NS

Holmlund, 2007	13	12	Case: 32 (24-37) Control: 33 (24-38) P >0.05	Case: 36 (30-42) Control: 40 (36-42) P > 0.009	Swedish	NS	NS	NS
Kayisli, 2013	7	8	NS	NS	North American	NS	NS	NS
Medeiros, 2014	85	52	**Case:** <34 weeks: 23 (14-43) ≥34 weeks: 25 (15-43) **Control:** <34 weeks: 22 (17-40) ≥34 weeks: 23 (15-40)	**Case:** <34 weeks: 31 (24–33) ≥34 weeks: 37 (34-42) **Control:** <34 weeks: 29 (24–33) ≥34 weeks: 37 (34-40)	Brazilian	NS	**Case:** <34 weeks: 160 (140-210) ≥34 weeks: 150 (140-210) P < 0,05 **Control:** <34 weeks: 105 (95-110) ≥34 weeks: 100 (95-110)	**Case:** <34 weeks: 110 (90-140) ≥34 weeks: 100 (90-130) P < 0,05 **Control:** <34 weeks: 65 (60-70) ≥34 weeks: 60 (60-70)
Molvarec, 2008	180	172	Case: 28.5±5.6 Control: 29.0±4.8 P > 0.05	Case: 34.5±2.1 Control: 39.4±1.4 P <0.001	Causasian (Hungarians)	Case: 24.2±4.5 Control: 22.1±3.9 P <0.001	Case: 168.0±1.8 Control: 112.0±12.0 P < 0.001	Case: 105.0±11.0 Control:

								71.0±9.0 P < 0.001
Nitsche, 2011	12	18	Case: 25.7±1.4 Control: 30.1±1.6 P > 0.06	Case: 38.8±0.4 Control: 38.5±0.3 P = 0.76	Causasian case (90%) Caucasian control (83%) (North American)	NS	NS	NS
Nitsche, 2011	10	12	Case: 26.6±1.6 Control: 30.6±1.3 P = 0.06	Case: 38.7±0.5 Control: 38.6±0.4 P > 0.05	North American	NS	NS	NS
Panda, 2012	30	30	Case: 33.4±6.1 Control: 26.2±4.6 P < 0.01	Case: 38.0±1.8 Control: 39.2±1.8 Not associated	Causasian case (70%) Caucasian control (73%) (North American)	Case: 34.5±3.2 Control: 27.7±3.9 P < 0.01	NS	NS
Panda, 2012	30	30	NS	Case: 38.0±1.8 Control: 39.2±1.8 Not associated (37-41)	North American	NS	NS	NS
Pineda, 2011	5	5	Case and control: (24-38)	Case: (36-38) Control: (38-40)	Mexican	NS	NS	NS
Romao, 2012	32	20	NS	NS	Brazilian	NS	NS	NS
Semerci, 2014	7	8	NS	NS	North American	NS	NS	NS
Van, 2008	340	113	Case: 30.6±4.4 Control: 32.8±4.3 P < 0.001	NS	Caucasian (Dutch)	Case: 25.7±5.3 Control: 22.7±4.0 P < 0.001	NS	NS
Xia, 2010	27	21	Case: 28.0±3.8	Case: 37.1±4.1	Chinese	NS	Case: 153.4±16.4	Case:

			Control: 27.9±3.1 Not associated	Control: 38.2±0.8 Not associated			Control: 121.6±7.0 P < 0,05	114.1±14.8 Control: 80.0±4.8 P < 0,05
Xie, 2010	25	25	Case: <34 weeks: 35 (19-43) ≥34 weeks: 34 (23-41) Control: <34 weeks: 33 (24-41) ≥34 weeks: 33 (24-39)	Case: <34 weeks: 32.1 ≥34 weeks: 37.1 Control: <34 weeks: 30.3 ≥34 weeks: 36.7	Canadian	NS	NS	NS
Xie, 2010	94	176	Case: 33 (19, 43) Control:32 (24, 42) P > 0.05	Case: 35.2 (26.3, 40.1) Control: 38.7 (26.4, 40.9) P < 0.05	Canadian	NS	NS	NS
Zhang, 2012	8	8	**Early onset:** Case: 32.6 Control: 34.7 **Late onset:** Case: 39.3 Control: 39.1	NS	Chinese	NS	NS	NS

Data are shown as mean ± SD or median (interquartile range). BMI = body mass Index; BP = blood pressure.

Table S2. Quality scores of studies included in the systematic review.

Author	Year	Selection	Comparability	Exposition	Total
Al-ofi	2012	***	***	**	********
Bernardi	2012	***	***	**	********
Chaterjee	2011	***	***	**	********
Chaterjee	2012	***	***	**	********
Chaterjee	2015	***	***	**	********
Chen	2015	***	**	**	*******
Chen	2015	***	**	**	*******
Franchim	2011	***	***	**	********
Fraser	2008	***	**	**	*******
Gorjani	2014	**	**	*	*****
Holmlund	2007	***	***	*	*******
Kayisli	2013	**	**	*	*****
Medeiros	2014	***	***	**	********
Molvarc	2008	***	**	**	*******
Nitshe (abstract)	2011	***	**	*	*****
Nitshe	2011	***	***	*	*******
Panda	2011	***	***	**	********
Panda	2012	***	**	*	******
Pineda	2011	***	**	**	*******
Romao	2012	***	**	**	*******
Semerci	2014	**	**	**	******
Van	2008	***	**	**	*******
Xia	2010	***	**	**	*******

Xie	2010	***	**	**	*******
Xie	2010	***	**	**	*******
Zhang	2012	**	**	**	******

Parte II

IFIH1 is decreased in preeclampsia, and its knockdown reduces the poly(I:C)-induced up-regulation of inflammation-, angiogenesis- and hypertension-related genes in HUVECs

IFIH1 is decreased in preeclampsia, and its knockdown reduces the poly(I:C)-induced up-regulation of inflammation-, angiogenesis- and hypertension-related genes in HUVECs

Short title: IFIH1 role on preeclampsia and expression of hypertension-related genes

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ABSTRACT

Objectives: The interferon-induced with helicase C domain 1 (IFIH1) receptor plays a role in the innate immunity against viral infections. Interestingly, we have previously reported increased *IFIH1* expressions in mononuclear cells from type 1 diabetic patients with arterial hypertension (AH) compared to normotensive patients. This receptor also seems to be associated with preeclampsia (PE). Therefore, to further investigate the role of IFIH1 in AH and PE, this study was aimed to compare *IFIH1* expressions between PE women and control women, and also to evaluate the effect of *IFIH1* knockdown in an endothelial cell line treated with synthetic viral nucleic acid (PIC) in the expression of genes related to inflammation, angiogenesis and hypertension.

Methods: *IFIH1* expression was evaluated in 44 PE women and 65 controls by RT-qPCR. Human umbilical vein endothelial cells (HUVECs) were transfected with either a control siRNA or with two different siRNAs targeting *IFIH1* for 48h. Then, HUVECs were transfected with PIC to stimulate the innate immune response for additional 24h. After this period, RNA was extracted for gene expression analyses.

Results: *IFIH1* was decreased in placenta from PE women compared to the control group (P= 0.049). After exclusion of black women, this difference between groups was more pronounced (P= 0.004). PIC treatment induced an increase in *TNF*, *IFN-β*, *iNOS*, *VEGF*, *ANGPT-2*, and *ET-1* expressions, and the *IFIH1* knockdown was able to partially prevent the up-regulation of these genes.

Conclusions: Taken together, these data suggest that IFIH1 plays a role in PE, and in the regulation inflammation-, angiogenesis- and AH-related genes in HUVECs.

Keywords: Preeclampsia, hypertension, IFIH1 receptor, innate immunity, HUVECs, siRNAs.

INTRODUCTION

In the last few years, several studies have shown the involvement of specific key components of the innate immunity in the first line of defense against microorganisms. Detection of invading microorganisms is carried out by a wide range of cell receptors of the pattern-recognition receptors (PRRs) family, which recognize highly conserved pathogen-associated molecular patterns (PAMPs) derived from virus, bacteria and fungi as well as endogenous danger-associated molecular patterns (DAMPs) (1, 2). PRRs are evolutionary conserved proteins and include retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and toll-like receptors (TLRs) (1, 3). After ligand binding, these PRRs activate signaling pathways with consequent production of pro-inflammatory cytokines, which coordinates local and systemic inflammatory responses (4).

The interferon-induced with helicase C domain 1 (IFIH1) cytoplasmic receptor (also known as melanoma differentiation-associated gene 5 – MDA-5) belongs to the RLH family, and recognizes double-stranded RNAs (dsRNAs) produced during the life cycle of most viruses, playing a major role in the immune response triggered by viral infection (4, 5). This receptor and polymorphisms in the gene codifying it have been involved in the pathogenesis of type 1 diabetes mellitus (T1DM) and other autoimmune diseases (2, 6, 7). Interestingly, in a recent study from our group, we reported an increased *IFIH1* gene expression in mononuclear cells from T1DM patients with arterial hypertension (AH) compared with normotensive T1DM patients (8). Moreover, we also observed an association between the A/A genotype of the *IFIH1* rs1990760 polymorphism and AH in the same Brazilian population (8).

Although no other study has evaluated the association between IFIH1 levels and AH, it has become evident in the last few years that components of both innate and adaptive immunity play an important role in this disease (9, 10). In mice lacking vascular macrophages, treatment with angiotensin-II (Ang-II) or deoxycorticosterone acetate (DOCA)-salt was unable to increase blood pressure (BP), probably because these animals developed less endothelial dysfunction, vascular remodeling, and oxidative stress induced by a hypertensive stimulus than their normal littermates (11, 12). These data suggest a critical role of innate immunity and low grade inflammation in hypertension.

Of note, Chatterjee *et al*. (13, 14) demonstrated that all three dsRNA sensors (IFIH1, TLR-3 and RIG-I) are activated in placentas from mice and women with preeclampsia (PE), a pregnancy-specific disease characterized by excessive activation of the maternal immune system, inflammation, and endothelial dysfunction, causing new onset hypertension and proteinuria (15). Although other studies also have linked excessive TLR activation to the development of PE (16-18), it is still inconclusive if PRRs are indeed involved in the development of this pregnancy complication as well as essential hypertension and how this occurs.

Therefore, to further investigate the potential role of IFIH1 in hypertension and PE, the present study was aimed to: 1) compare *IFIH1* gene expression and frequencies of the rs1990760 polymorphism between women with PE (cases) and healthy pregnant women (controls) from a Southern Brazilian population; and 2) evaluate the effect of *IFIH1* knockdown in an endothelial cell line treated with synthetic dsRNA [poly (I:C) – PIC) in the expression of genes related to inflammation, angiogenesis and hypertension.

METHODS

Comparisons of *IFIH1* gene expression and frequencies of the rs1990760 polymorphism between women with preeclampsia and healthy pregnant women (case-control study)

Subjects and phenotype measurements

The study population consisted of 59 women with PE (cases) and 134 normotensive pregnant women (controls) who delivered between February 2013 and July 2015 by the Gynecology and Obstetrics Service at Hospital de Clínicas de Porto Alegre (Rio Grande do Sul – Brazil). PE was diagnosed according to International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria as: 1) BP values ≥140/90 mmHg, measured in a sitting position or left lateral decubitus, on the left arm, after 5-25 min rest by a trained nurse, with a mercury sphygmomanometer. The mean of two measurements taken at least 6 h apart was used to calculate systolic and diastolic BP, and both measurements were done after 20 weeks of gestation; 2) Urinary protein/creatinine ratio ≥ 0.3 mg/mg. If this ratio was between 0.3 and 0.5 mg/mg, it was also measured 24 h-urinary protein levels to confirm the diagnosis if this value was ≥ 300 mg/24-h (19).

The control group comprised women who had to be normotensive in the index pregnancy, had a history of at least two previous uncomplicated pregnancies, without any maternal or fetal disorder. Exclusion criteria for both groups included preexisting essential hypertension, diabetes mellitus or gestational diabetes mellitus, infectious diseases, autoimmune disorders, fetal disorders or inflammatory conditions. The ethnic group was defined based on self-classification, and the ethnic proportion was similar between case and control groups: 25.0% of black women in the PE group and 20.8% of

black women in the control group (P= 0.297).

A standard questionnaire was used to collect information on age, weight, height, family history of diseases, drug treatment, gestational age, number of pregnancies, current diseases, infant birthweight, and physical activity. All case and control patients underwent physical and laboratory evaluations. Body mass index (BMI) was calculated as weight (kg)/height square (meters). Upon admission, serum and plasma samples were taken from each patient after 8 h of fasting for laboratory analyses. Plasma glucose levels were determined using the glucose oxidase method. Total proteinuria was determined by colorimetric method using the pyrogallol red and urinary creatinine levels were determined using the Jaffe reaction.

The study protocol was approved by the Ethic Committee in Research from Hospital de Clínicas de Porto Alegre and all patients gave their informed consent in writing.

Genotyping of the rs1990760 (G/A) polymorphism in the IFIH1 gene

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The *IFIH1* rs1990760 (G/A) polymorphism was genotyped using primers and probes contained in the Human Custom TaqMan Genotyping Assay 20X (Life Technologies – Thermo Fisher Scientific Inc., Waltham, MA, USA). Primer and probe sequences can be found elsewhere (8)**.** All reactions were performed in 96-well plates and ran on the 7500 Fast Real-Time PCR System (Life Technologies), as previously described (8). Power calculations (PEPI program, version 4.0) showed that this sample has a power of approximately 80% at a significance level of 0.05 to detect an odds ratio (OR) of 2.0 or higher (for the presence of the A allele).

Placenta collection and RNA extraction

To investigate *IFIH1* gene expression, approximately 2 g of placentas were obtained from a subsample of 109 patients (44 cases with PE and 65 control women). Immediately after collection, samples were preserved in RNAlater (Life Technologies) and stored at -80°C until RNA extraction and *IFIH1* expression analysis. Placenta biopsies (80 mg) were homogenized in phenol-guanidine isothiocyanate (Invitrogen – Thermo Fisher Scientific Inc.). Total RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol and resuspended in 10-50 μl of diethylpyrocarbonate treated water. Concentration and quality of total RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Only RNA samples with adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses. In addition, RNA integrity and purity were also checked on agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

Quantification of IFIH1 gene expression by Real-time qPCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 1 μg of RNA into cDNA was carried out using the SuperScript VILO Master Mix for RT-PCR (Invitrogen), following the manufacturer's protocol for the random primer method.

RT-qPCR experiments were performed in a Vii^{TM} 7 Real-Time PCR System (Life Technologies). Experiments were performed by monitoring in real-time the increase in fluorescence of SYBER Green dye. Primers for *IFIH1* and *GAPDH* genes were designed using published human gene sequences and the Primer Express 3.0

Software (Life Technologies). Primers sequences are shown in **Table 1**. PCR reactions were performed using 5 μl of 2x Fast SYBER Green Master Mix (Life Technologies), 0.5 μl (0.5 ng/μl) of forward and reverse primers for *IFIH1* or *GAPDH*, and 1.0 μl of cDNA template (1 μg/μl), in a total volume of 10 μl and following PCR conditions described elsewhere (8). Each sample was assayed in triplicate and a negative control was included in each experiment. RT-qPCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses.

Quantification of the *IFIH1* mRNA was performed using the relative standard curve method (21, 22), and the *GAPDH* gene as the reference gene. Relative standard curves were generated for both target and reference genes by preparing serial dilutions of the same cDNA sample with a known relative quantity. Then, relative amounts of each *IFIH1* cDNA sample were obtained by normalizing their signals by those of *GAPDH* gene, and are shown as arbitrary units (AU).

Experimental study of *IFIH1* knockdown in human umbilical vein endothelial cells (HUVECs)

Cell culture

Human umbilical vein endothelial cells (HUVECs; Gibco - Thermo Fisher Scientific Inc.) were cultured in Cascade Biologics 200 medium (Gibco) containing 10% low serum growth supplement (LSGS; Gibco) and penicillin (100 U/ml) / streptomycin (0.1 mg/ml) antibiotic mix (Gibco). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, and were used between passages 5-7, at 80% of confluence.

IFIH1 knockdown using RNA interference

HUVECs (1.4 x 10^5 cells/per well) were seeded into 6-well plates and cultured for 24 h prior to transfection. To evaluate the effect of *IFIH1* knockdown in HUVECS treated with synthetic dsRNA, cells were transfected either with an inactive siRNA (siCTRL; AllStars Negative Control siRNA; Qiagen, Venlo, Netherlands) or with two independent siRNAs targeting *IFIH1* (Silencer® Select Pre-designed; Ambion, Life Technologies). The sequences of the siRNAs used for *IFIH1* knockdown are as follows: siIFIH1#1 – 5'-GGUGUAAGAGAGCUACUAtt-3' (forward) and 5'-UUAGUAGCUCUCUUACACCtg-3' (reverse); and siIFIH1#2 – 5'-GUUCAGGAGUUAUCGAACAtt-3' (forward) and 5'-UGUUCGAUAACUCCUGAACca-3' (reverse).

The optimal conditions for siRNA transfection in HUVECs were first established by dose-response studies using a FITC-coupled siRNA (siGLO Green Transfection Indicator, Thermo Fisher Scientific Inc.) and viability tests with propidium iodide (5 μg/ml; Sigma-Aldrich, Poole, UK) and Hoechst 33342 (5 μg/ml; Sigma-Aldrich) dyes (23). The concentration of 50 nM of siRNAs was selected after these dose-response experiments (data not shown). The transfection procedure was performed using Lipofectamine RNAi-MAX™ (Invitrogen), according to the manufacturer's instructions. Afterwards, HUVECs were cultured for a 48 h recovery period, and then transfected with a synthetic dsRNA (PIC) to stimulate the innate immune response, as described in the following section.

Synthetic dsRNA transfection

The synthetic dsRNA, PIC (InvivoGen, San Diego, CA, USA) was used at the final concentration of 0.5 μg/ml. This concentration was also determined based on dose-

response experiments using cell survival as endpoint (data not shown). All experiments were performed with intracellular PIC, obtained through cell transfection using Lipofectamine™ LTX (Invitrogen), according to the manufacturer's instructions. HUVECs exposed to the transfectant alone were used as the control condition. Taking into account that cytoplasmic RLHs recognize dsRNAs based on their length (24), a PIC preparation with > 2000 pb was used for the experiments. After 24 h of transfection with PIC, RNA and protection extractions were performed, and samples were stored at -80°C for further analyses.

HUVECs treated with IFN-β

After binding with dsRNAs or DAMPs, IFIH1 activates signaling pathways leading to the production of type I IFNs, especially IFN-β, which drives the expression of IFN-regulated genes and the innate immune response (2). Therefore, to test if the incubation of cells with IFN-β would activate the expression of angiogenesis- and hypertension-related genes, we treated them with this cytokine. HUVECs were plated as mentioned before, and after 24 h- recovery period, they were left untreated (control condition) or treated with 500U/ml recombinant human IFN-β (Verikine™; PBL Assay Science, Piscataway, NJ, USA) for additional 48 h. Then, RNA extraction was performed, and samples were stored at -80°C for further analyses.

RNA extraction from HUVECS and quantification of gene expression by Real-time PCR

Total RNA was extracted from HUVECs using the RNeasy Mini kit (Qiagen), according to the manufacturer's recommendation. Quantification of RNA, cDNA synthesis, and RT-qPCR conditions and reagents were similar as already described for the case-control study, but the reference gene used for HUVECs was *cyclophilin*. The

following target genes were evaluated in HUVECs transfected with siRNA/PIC or from the control conditions: *IFIH1*, vascular endothelial growth factor (*VEGF*), tumor necrosis factor (*TNF*), *IFN-β*, angiopoetin-2 (*ANGPT-2*), prostaglandin-2 (*PGE-2*), endothelin-1 (*ET-1*) and inducible nitric oxide synthase (*iNOS*). For HUVECs treated with IFN-β and the respective control condition, we evaluated *VEGF*, *ANGPT-2*, *PGE-2*, and the signal transducer and activator of transcription 1 (*STAT-1*) genes. Primer sequences for all analyzed genes are shown in **Table 1**.

Immunoblotting

To confirm *IFIH1* knockdown at protein level, HUVECs were washed with cold PBS and lysed in Laemmli buffer (60 mmol/l Tris pH 6.8, 10% Glycerol, 1% SDS, 0.001% blue Bromophenol and 5% β-mercaptoethanol). Total protein extracts were resolved in 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon®-P^{SQ} membranes (Millipore, Billerica, MA, USA), and incubated with monoclonal antibody to IFIH1 (Cell Signaling Technology, Beverly, MA, USA) or GAPDH (Sigma-Aldrich). Secondary antibodies consisted of horseradish peroxidase-conjugated anti-rabbit or anti-mouse (Millipore) antibodies. Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Images were acquired in an ImageQuant LAS 500 digital imaging system (GE Healthcare, Piscataway, NJ, USA) and band densitometry analysis was performed using ImageJ 1.47v (National Institutes of Health, USA). Intensity values for IFIH1 were corrected by the values of the housekeeping protein GADPH.

ELISA for angiotensin-II in supernatants of HUVECS after IFIH1 knockdown

The supernatants of HUVECs transfected with siRNA/PIC or from the control conditions were collected and stored at -80°C until quantification of Ang-II levels using a competitive ELISA human kit (Phoenix Pharmaceuticals, CA, USA). Ang-II levels are expressed in ng/ml. All samples and standards were measured in duplicate, and the coefficient of variation was less than 5%.

Statistical analyses

Allelic frequencies were determined by gene counting and deviations from the Hardy–Weinberg equilibrium (HWE) were verified using the χ^2-test. Allele and genotype frequencies were compared between groups using χ^2-tests. The magnitude of the association between the *IFIH1* rs1990760 polymorphism and PE was estimated using OR with 95% CI. Logistic regression analyses were performed to assess the independent association of the rs1990760 polymorphism with PE, adjusting for ethnicity.

Clinical and laboratory characteristics, mRNA concentrations and protein levels were compared between groups by using χ^2 test, unpaired Student's t-test or One-way ANOVA with post-hoc tests (Bonferroni), as appropriate. Data are presented as mean ± SD or percentage. Variables with skewed distribution were log-transformed before analyses. A P value <0.05 was considered as statistically significant, and all statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL).

RESULTS

Comparisons of *IFIH1* gene expression and frequencies of the rs1990760 polymorphism between women with preeclampsia and healthy pregnant women

Sample Description

The main clinical characteristics of PE women (cases) and healthy pregnant women (controls) included in the present study are shown in **Table S1**. PE women had lower gestational age than controls (35.4 ± 3.7 *vs*. 38.9 ± 2.4; P <0.0001). As expected, gestational BMI, systolic and diastolic BP were increased in PE women compared to the control group (all P values <0.0001). Moreover, PE women had more caesarean than women from the control group (55.1% *vs*. 35.7%; P <0.0001). Age, number of pregnancies, fasting glucose levels and ethnicity did not differ significantly between groups (**Table S1**).

IFIH1 rs1990760 (G/A) distributions in women with PE and control women

Genotype and allele frequencies of the *IFIH1* rs1990760 (G/A) polymorphism in PE women and healthy pregnant women are described in **Table 2**. The frequency of the A allele of the rs1990760 polymorphism was 25.8% in white women and 15.8% in black women (P= 0.002). Neither genotype nor allele frequencies of the rs1990760 polymorphism differed statistically between case and control subjects (P= 0.938 and P= 0.435, respectively), and all genotypes were in agreement with those predicted by the HWE in the two samples (P >0.05). Frequencies of A allele carriers (dominant model) were also similar between groups, and the adjustment for ethnicity did not change this result (P= 0.590; **Table 2**). It is noteworthy that this polymorphism remained not

associated with PE when taking into account recessive or additive inheritance models (data not shown). Moreover, after the exclusion of black women from the samples, the presence of the A allele was still not associated with PE (OR= 1.408, 95% CI 0.556 – 3.575; P= 0.472 for the dominant model).

Clinical and laboratory characteristics of PE women broken down by the different genotypes of the rs1990760 polymorphism are shown in **Table S2.** Gestational age, gestational BMI, systolic and diastolic BP, infant birthweight and mode of delivery did not differ significantly among the three genotypes. However, PE women carrying the A/A genotype were older than PE women carrying the G allele (A/A 30.0 ± 6.4 *vs.* A/G 24.7 ± 5.6 *vs.* G/G 24.8 ± 5.4 years; P= 0.021).

IFIH1 gene expression in the placenta from PE women and control women

IFIH1 gene expression was analyzed in 44 PE women and 65 control women. This subsample had similar characteristics to those of the total sample (data not shown). *IFIH1* expression was increased in placentas from black women compared to white women (0.93 ± 0.33 *vs.* 0.74 ± 0.41 AU in logarithm-scale; P= 0.018). In general, placental *IFIH1* concentrations were decreased in PE cases compared to control women (0.69 ± 0.58 *vs.* 0.86 ± 0.31 AU, respectively; P= 0.049). Of note, after removal of black women from the samples, this difference between groups was more pronounced [cases (n = 33): 0.57 ± 0.58, controls (n = 53): 0.84 ± 0.29 AU; P= 0.004; **Fig. 1**]. *IFIH1* gene expression did not correlate with age (r= -0.008, P= 0.924), systolic BP (r= -0.010, P= 0.902) and diastolic BP (r= -0.085, P= 0.309) in the whole sample.

IFIH1 gene expression did not differ significantly among the three genotypes of the rs1990760 polymorphism for the total sample [G/G (n = 36): 0.8 ± 0.38, G/A (n = 69): 0.76 ± 0.43, A/A (n = 31): 0.83 ± 0.38 AU; P= 0.774]. Moreover, the rs1990760

polymorphism did not influence *IFIH1* concentrations when analyzing separately case and control women (P >0.05; data not shown).

Experimental study of *IFIH1* knockdown in human umbilical vein endothelial cells (HUVECs)

IFIH1 knockdown influences the PIC-induced activation of genes related to inflammation, angiogenesis and hypertension in HUVECs

To evaluate the role of IFIH1 in human endothelial cells treated with synthetic dsRNA (PIC), HUVECs were transfected either with an inactive siRNA (siCTRL) or with two independent siRNAs targeting *IFIH1* (siIFIH1 #1 and siIFIH1#2). *IFIH1* knockdown after 24 h and 48 h of siRNA transfection was higher than 80% at both mRNA (**Fig. 2C**) and protein levels (**Fig. 2A** and **2B**). It is noteworthy that both siRNA targeting *IFIH1* and the siCTRL did not decrease significantly cell viability compared to cells treated with the transfectant reagent only (data not shown). Since *IFIH1* knockdown was higher than 90% at the 48 h time-point, this incubation time was used for the following experiments.

After *IFIH1* knockdown for 48 h, HUVECs were transfected with PIC for additional 24 h to mimic an infectious environment. Then, we evaluated the effect of PIC treatment after *IFIH1* knockdown on mRNA expressions of inflammation-related genes (*iNOS*, *TNF*, *PGE-2*, and *IFN-β*, **Fig. 3**). As expected, PIC treatment induced a pronounced increase in both *TNF* and *IFN-β* gene expressions (PIC-treated siCTRL *vs*. untreated siCTRL; P <0.05), and *IFIH1* inhibition prevented this increase (siIFIH1#2+PIC *vs*. siCTRL+PIC; P <0.05; **Fig. 3A** and **3B**). Moreover, PIC treatment induced a 2.5 fold increase in *iNOS* expression (PIC-treated siCTRL *vs*. untreated

siCTRL; P <0.05), and the *IFIH1* knockdown reduced by 20% the PIC-induced iNOS up-regulation (siIFIH1#2+PIC *vs*. siCTRL+PIC; P <0.05; **Fig. 3C**). Although PIC treatment was not able to induce the anti-inflammatory *PGE-2* gene (PIC-treated siCTRL *vs*. untreated siCTRL; P >0.05), in the *IFIH1* knockdown condition, PIC was able to trigger an up-regulation of this gene (P <0.05; **Fig. 3D**).

Next, we investigated the effect of PIC treatment following *IFIH1* knockdown on expressions of hypertension- and angiogenesis-related genes (*ANGPT-2*, *VEGF* and *ET-1*, **Fig. 4**). Interestingly, PIC treatment significantly induced *ANGPT-2*, *VEGF* and *ET-1* gene expressions (PIC-treated siCTRL *vs*. untreated siCTRL; P >0.05), while *IFIH1* inhibition partially prevented the up-regulation of these genes (siIFIH1#2+PIC *vs*. siCTRL+PIC; P <0.05). All gene expression results obtained for siIFIH1#2 were confirmed after transfection with siIFIH1#1 (**Table 3**); supporting our data.

Ang-II mRNA showed an almost undetectable expression in HUVECs (data not shown); therefore, we analyzed Ang-II protein levels measured by ELISA of the supernatant of cells from the different experimental conditions. In accordance with expression results obtained for the other analyzed hypertension-related genes, *IFIH1* knockdown seemed to prevent PIC-induced increase of Ang-II protein levels (siCTRL+PIC: 0.14 ± 0.09 *vs*. siIFIH1#2+PIC: 0.02 ± 0.03 pg/ml), although this difference did not reach formal statistical significance (P= 0.069). This result was not confirmed for siIFIH1#1 (P= 0.834).

An inflammatory environment induces expression of angiogenesis- and hypertension-related genes in HUVECs

After binding with dsRNAs or DAMPs, IFIH1 activates signaling pathways leading to the production of type I IFN, which will bind to its receptor and activate the Jak-STAT

pathway to drive the expression of IFN-regulated genes and the innate immune response (2). Therefore, to evaluate if hypertension- and angiogenesis-related genes could be also induced in a non-viral inflammatory environment, HUVECs were treated with the pro-inflammatory cytokine IFN-β for 48 h (**Fig. 5**). To this, first we checked if IFN-β treatment was able to induce the innate immune response in HUVECs by evaluating *STAT-1* gene expression. As expected, IFN-β stimulated a 3 fold increase in *STAT-1* expression compared to the untreated condition (**Fig. 5A**, $P < 0.05$). The anti-inflammatory gene *PGE-2* also was significantly induced by IFN-β (**Fig. 5B**, $P < 0.05$). In agreement, with the PIC treatment results, IFN-β treatment was also able to induce an up-regulation of *ANGPT-2* and *VEGF* genes (**Fig. 5C and 5D**; $P < 0.05$).

DISCUSSION

As a part of the attempt to clarify the role of IFIH1 in PE, we compared *IFIH1* gene expression and frequencies of the rs1990760 polymorphism between women with PE and healthy pregnant women. *IFIH1* gene expression was significantly decreased in placentas from PE women compared to the control group. Only one previous study has investigated this PRR regarding PE. Results reported by Chatterjee *et al*. (13) showed that IFIH1 was activated in placentas from pregnant, PIC-treated mice or women with PE compared to the respective control groups, conflicting with the present data. One possible explanation for this discrepancy is that we analyzed *IFIH1* mRNA levels, while Chatterjee *et al*. (13) evaluated IFIH1 protein levels. Therefore, mRNA levels and protein concentrations of this receptor may not correlate. Of note, Chatterjee *et al*. (13) only showed qualitative preliminary results, without quantification of IFIH1 protein levels between groups, and with no description of sample numbers and characteristics.

Even though some studies have shown excessive TLRs activation in immune cells or placenta from women with PE (14, 16-18), Nitsche *et al.* (25) observed decreased *TLR-2* and *TLR-4* expressions in neutrophils from PE women compared to controls. Considering that TLRs as well as IFIH1 are possibly involved in an up-regulation of innate immunity, it would be expected an increase in their gene expressions not the decrease described by Nitsche *et al.* (25). The authors suggested that the decrease in *TLR-2*/*TLR-4* expressions might represent a compensatory reaction to inflammatory signals present in PE. The reduction in these TLRs would render the maternal neutrophils less responsible to specific PAMPs or DAMPs, decelerating the inflammatory cascade seen in PE (25). This hypothesis could also explain our present data; however, further studies are needed to confirm it.

The *IFIH1* rs1990760 polymorphism has been associated with T1DM and other autoimmune diseases (2, 26, 27). Moreover, in a previous study from our group, this polymorphism was associated with protection for AH in T1DM patients (8). However, in the present study, no significant association between the rs1990760 polymorphism and PE was observed. Until this date, no other study has evaluated the association between *IFIH1* polymorphisms and PE. Interestingly, Xie *et al*. (28) reported that the rs5773708 polymorphism in the *TLR-2* gene was associated with PE, but only when comparing frequencies between women with early-onset/severe PE and the control group. Since the majority of our case group was constituted by late-onset PE, we were not able to compare frequencies of the *IFIH1* rs1990760 polymorphism according to PE severity. Hence, we cannot exclude the possibility that this polymorphism might be associated with early-onset/severe PE. In addition, *IFIH1* gene expression seems to be not influenced by the different rs1990760 genotypes.

Although some studies have suggested that innate immunity has a critical role in AH and PE (9, 11-13), it is still unclear how dysregulation of the innate immunity receptors might lead to the development of these diseases. Therefore, aiming to contribute to this subject, we next evaluate the effect of *IFIH1* knockdown in HUVECs treated with PIC in the expression of genes related to inflammation, angiogenesis and hypertension. Our results showed that *IFIH1* knockdown prevented the PIC-induced activation of *IFN-β* and *TNF* genes, which was expected since it is well known that, after ligand binding, IFIH1 initiates a pro-inflammatory response either through activation of nuclear factor-kappa B (NF-κB) or through production of IFN-β. Then, NF-κB activation leads to production of pro-inflammatory cytokines, including TNF (2, 29). Although little is known about the effect of IFN-β on hypertension, IFN-β therapy was associated with AH in patients with multiple sclerosis, probably due to stimulation of thromboxane cascade and TNF production (30).

TNF is a pleiotropic cytokine that is elevated in chronic inflammatory states such as AH (31). In general, high levels of TNF seem to decrease BP, while chronic moderately elevated TNF levels have been associated with increased NaCl retention and hypertension (31). In hypertensive rats infused with Ang-II and fed with a high-salt diet, etanercept (an anti-TNF treatment) delayed progression of hypertension (32). Moreover, infusion of Ang-II for 2 weeks was unable to increase BP in *TNF*-knockout mice (33), indicating that TNF mediates Ang-II-dependent AH. TNF also seems to play a role in PE, since TNF infusion for 5 days increased BP in pregnant rats but not in non-pregnant female rats, possibly due to increased ET-1 levels (34).

Nitric oxide (NO) is an important vasodilator produced by vascular endothelium, and its endogenous formation is essential to maintain normal BP (35). It is well known that high NO production, catalyzed by iNOS activity, plays an important role in AH

(35). Pro-inflammatory cytokines, such as TNF and IL-1β, as well as bacterial or viral pathogens activate iNOS and generate high concentrations of NO mainly via NF-κB activation (36). In humans, increased iNOS expression was observed in cutaneous microvasculature of hypertensive patients compared to the control group (37). Although the stimulus that increased iNOS in these patients remains unclear, possible candidates include elevated TNF, Ang-II, IL-6 or cyclooxygenase (37). Heo *et al*. (38) reported that lipopolysaccharide (LPS) induced inflammatory responses in human aortic vascular smooth muscle cells via increased *TLR-4 expression*, iNOS-induced NO production, and *VEGF* expression. Accordingly, in the present study, PIC treatment induced an increase in *iNOS* expression, which was partially prevented by *IFIH1* knockdown.

PGE-2 is a lipid mediator that induces the activation of many signaling pathways to exert both pro- and anti-inflammatory responses (39). In general, PGE-2 is activated at sites of inflammation, where it acts as a potent vasodilator (40). Moreover, PGE-2 has different effects on BP regulation depending on the site of action. In this context, rodent studies showed that centrally administered PGE-2 elevates BP, while systemically administered PGE-2 produces a hypotensive effect (40). PIC or LPS treatments seem to induce PGE-2 in different cell types (41-44). In this context, several lines of evidence suggest that the production of PGE-2 during inflammation constitutes a negative feedback mechanism which limits the production of pro-inflammatory mediators, including TNF and IFN-β (42, 45-47). Our study showed that IFN-β treatment increases *PGE-2* expression in HUVECs, which is in agreement with an immunosuppressant effect of PGE-2 aiming to decrease IFN-β levels. However, we also showed that PIC treatment was not able to induce *PGE-2* gene, while after *IFIH1* knockdown, PIC triggered an up-regulation of this gene. Although this last result was observed for the two siRNAs targeting *IFIH1*, it is an intriguing result that needs further elucidation.

In this study, PIC treatment also induced *ANGPT-2*, *VEGF* and *ET-1* expressions, and the *IFIH1* knockdown partially prevented the up-regulation of these genes. In addition, PIC-induced Ang-II up-regulation seems to decrease after *IFIH1* blockade. VEGF is a potent angiogenic factor in endothelial cells and enhances vascular permeability (48). In accordance with our data, PIC also induced VEGF in mesothelial cells, and the induction was partially prevented by siRNAs targeting *TLR-3*, *RIG-I* or *IFIH1* viral sensors (49). Activation of different TLRs, including TLR-3, also produced increased VEGF synthesis in human bronchial epithelial cells (50). Furthermore, LPS induced VEGF production in human aortic vascular smooth cells (38).

ANGPT-2 is another angiogenic factor secreted by endothelial cells, and functions as an Ang-1 antagonist, promoting vascular destabilization and permeability, thereby facilitating VEGF-dependent vessel growth (51). Of note, VEGF, TNF, and Ang-II up-regulate ANGPT-2 expressions (52-54). To date, only one study related ANGPT-2 with innate immunity. Kranidioti *et al.* (55) reported that ANGPT-2 was increased in serum from patients with septic shock compared to control subjects. In addition, release of ANGPT-2 from healthy mononuclear cells was stimulated by serum of patients with septic shock but not by serum of non-shocked patients, which was modulated by TLR-4 and TNF (55).

ET-1 is a potent vasoconstrictor but has also been shown to exert pleiotropic effects in different biological processes such as cancer, ischemia/reperfusion injury, cardiovascular diseases, diabetes mellitus, and adaptive immune response (56). ET-1 expression is up-regulated by TNF and Ang-II (57). Recently, a role for ET-1 in innate immunity has been suggested in different cells types (56, 58-60). Farina *et al*. (58) demonstrated that PIC, but not other TLR ligands, highly stimulated ET-1 in endothelial cells and dermal fibroblasts. Also, human pulmonary artery smooth muscle cells

expressed high levels of TLR-3 upon PIC treatment, and responded to its activation by releasing ET-1 (59). Spirig *et al*. (56) showed that stimulation of human monocyte-derived dendritic cells with exogenous or endogenous TLR-4 and TLR-2 agonists induced ET-1 production in a dose- and time-dependent manner. Moreover, vascular endothelium infected with Herpes simplex virus type 1 can active ET-1 production, suggesting that this protein is an important player in innate immunity (60). Thus, the above-mentioned studies are in line with data from the present study.

Ang-II is a vasoconstrictive peptide that plays a central role in BP regulation (61). Importantly, Ang-II has been classified as a DAMP for TLR-4 activation (62). Hence, Ang-II treatment in renal tubular epithelial cells caused an activation of TLR-4 pathway with subsequent TNF and IL-1β up-regulation (61). Accordingly, PIC also induced Ang-II in the present study, and the *IFIH1* knockdown seems to prevent this increase. It remains to be confirmed if Ang-II also acts as a DAMP for IFIH1.

Taking into account the present results and the aforementioned literature, it seems that PIC-induced TNF up-regulation is activating *ANGPT-2*, *ET-1* and *iNOS* gene expressions. Then, ANGPT-2 is further activating *ET-1* and *VEGF* expressions. VEGF may also contribute to maintain increased levels of ANGPT-2. *IFIH1* knockdown partially prevented the PIC-induced up-regulation of these genes, suggesting that this PRR may have a key role in hypertension or PE after activation by PAMPS or DAMPS. Our data showing that INF-β treatment also induces an increase in *ANGPT-2*, *PGE-2* and *VEGF* expressions in HUVECs indicates that a non-viral inflammatory environment is sufficient to activate this response. Therefore, further *in vivo* studies are necessary to clarify which PAMPs or DAMPS could activate IFIH1, leading to the expression of genes related to inflammation, angiogenesis and hypertension.

In conclusion, our results suggest that *IFIH1* gene expression is decreased in placentas from PE women, probably contributing to PE pathogenesis. Furthermore, the *in vitro* experiments in HUVECs demonstrated that PIC treatment induces *TNF*, *IFN-β*, *iNOS*, *ANGPT-2*, *VEGF*, *ET-1* and AngII expressions, which is decreased after *IFIH1* knockdown. This study adds a new possible role for IFIH1 in the pathogenesis of PE and AH.

Declaration of Interest

The authors declare no potential conflicts of interest relevant to this article.

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Author contribution statement

A.P.B designed the study, researched data, collected samples, performed the experiments and wrote the manuscript. A.P.R collected samples and researched data. B.M.S, D.A.S and P.S.N researched data and contributed to discussion. A.C.B and S.M.C contributed to discussion and reviewed the manuscript. D.C. design of study, contributed to the discussion, and reviewed the manuscript.

References

1. Assmann TS, Brondani Lde A, Boucas AP, Canani LH, Crispim D. Toll-like receptor 3 (TLR3) and the development of type 1 diabetes mellitus. Arch Endocrinol Metab. 2015;59(1):4-12.
2. Boucas AP, Oliveira Fdos S, Canani LH, Crispim D. The role of interferon induced with helicase C domain 1 (IFIH1) in the development of type 1 diabetes mellitus. Arq Bras Endocrinol Metabol. 2013;57(9):667-76.
3. De Nardo D. Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine. 2015;74(2):181-9.
4. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol. 2008;89(Pt 1):1-47.
5. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009;420(1):1-16.
6. Kato H, Fujita T. Autoimmunity caused by constitutive activation of cytoplasmic viral RNA sensors. Cytokine Growth Factor Rev. 2014;25(6):739-43.
7. Cen H, Wang W, Leng RX, Wang TY, Pan HF, Fan YG, et al. Association of IFIH1 rs1990760 polymorphism with susceptibility to autoimmune diseases: a meta-analysis. Autoimmunity. 2013;46(7):455-62.
8. Boucas AP, Brondani LA, Souza BM, Lemos NE, de Oliveira FS, Canani LH, et al. The A allele of the rs1990760 polymorphism in the IFIH1 gene is associated with protection for arterial hypertension in type 1 diabetic patients and with expression of this gene in human mononuclear cells. PLoS One. 2013;8(12):e83451.
9. Verlohren S, Muller DN, Luft FC, Dechend R. Immunology in hypertension, preeclampsia, and target-organ damage. Hypertension. 2009;54(3):439-43.
10. Schiffrin EL. T lymphocytes: a role in hypertension? Curr Opin Nephrol Hypertens. 2010;19(2):181-6.
11. Ko EA, Amiri F, Pandey NR, Javeshghani D, Leibovitz E, Touyz RM, et al. Resistance artery remodeling in deoxycorticosterone acetate-salt hypertension is dependent on vascular inflammation: evidence from m-CSF-deficient mice. American journal of physiology Heart and circulatory physiology. 2007;292(4):H1789-95.
12. De Ciuceis C, Amiri F, Brassard P, Endemann DH, Touyz RM, Schiffrin EL. Reduced vascular remodeling, endothelial dysfunction, and oxidative stress in resistance arteries of angiotensin II-infused macrophage colony-stimulating factor-deficient mice: evidence for a role in inflammation in angiotensin-induced vascular injury. Arterioscler Thromb Vasc Biol. 2005;25(10):2106-13.
13. Chatterjee P, Weaver LE, Chiasson VL, Young KJ, Mitchell BM. Do double-stranded RNA receptors play a role in preeclampsia? Placenta. 2011;32(3):201-5.
14. Chatterjee P, Weaver LE, Doersch KM, Kopriva SE, Chiasson VL, Allen SJ, et al. Placental toll-like receptor 3 and toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice. PLoS ONE. 2012;7(7).
15. Mol BW, Roberts CT, Thangaratinam S, Magee LA, de Groot CJ, Hofmeyr GJ. Pre-eclampsia. Lancet. 2015.
16. Xie F, Hu Y, Turvey SE, Magee LA, Brunham RM, Choi KC, et al. Toll-like receptors 2 and 4 and the cryopyrin inflammasome in normal pregnancy and pre-eclampsia. BJOG. 2010;117(1):99-108.

17. Bernardi FC, Felisberto F, Vuolo F, Petronilho F, Souza DR, Luciano TF, et al. Oxidative damage, inflammation, and Toll-like receptor 4 pathway are increased in preeclamptic patients: a case-control study. Oxid Med Cell Longev. 2012;2012:636419.
18. Pineda A, Verdin-Teran SL, Camacho A, Moreno-Fierros L. Expression of Toll-like Receptor TLR-2, TLR-3, TLR-4 and TLR-9 Is Increased in Placentas from Patients with Preeclampsia. Archives of Medical Research. 2011;42(5):382-91.
19. Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, et al. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. Pregnancy Hypertens. 2014;4(2):97-104.
20. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y). 1993;11(9):1026-30.
21. Biosystems A. Relative Quantitation of Gene Expression Experimental Design and Analysis: Relative Standard Curve Method and Comparative ct Method (DeltaDelta-ct). Guide to Performing Relative Quantitation of Gene Expression Using Real-time Quantitative PCR. 2004.
22. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22.
23. Moore F, Colli ML, Cnop M, Esteve MI, Cardozo AK, Cunha DA, et al. PTPN2, a candidate gene for type 1 diabetes, modulates interferon-gamma-induced pancreatic beta-cell apoptosis. Diabetes. 2009;58(6):1283-91.
24. Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med. 2008;205(7):1601-10.
25. Nitsche JF, Jiang SW, Brost BC. Maternal neutrophil toll-like receptor mRNA expression is down-regulated in preeclampsia. American journal of reproductive immunology (New York, NY : 1989). 2011;66(3):242-8.
26. Chen G, Zhou D, Zhang Z, Kan M, Zhang D, Hu X, et al. Genetic variants in IFIH1 play opposite roles in the pathogenesis of psoriasis and chronic periodontitis. Int J Immunogenet. 2012;39(2):137-43.
27. Martinez A, Santiago JL, Cenit MC, de Las Heras V, de la Calle H, Fernandez-Arquero M, et al. IFIH1-GCA-KCNH7 locus: influence on multiple sclerosis risk. Eur J Hum Genet. 2008;16(7):861-4.
28. Xie F, Hu Y, Speert DP, Turvey SE, Peng G, Money DM, et al. Toll-like receptor gene polymorphisms and preeclampsia risk: A case-control study and data synthesis. Hypertension in Pregnancy. 2010;29(4):390-8.
29. Oliveira L, Sinicato NA, Postal M, Appenzeller S, Niewold TB. Dysregulation of antiviral helicase pathways in systemic lupus erythematosus. Front Genet. 2014;5:418.
30. Modrego PJ, Gazulla J. Arterial hypertension induced by interferon beta 1b in a patient with multiple sclerosis. Mult Scler. 2012;18(11):1655-6.
31. Ramseyer VD, Garvin JL. Tumor necrosis factor-alpha: regulation of renal function and blood pressure. Am J Physiol Renal Physiol. 2013;304(10):F1231-42.
32. Elmarakby AA, Quigley JE, Pollock DM, Imig JD. Tumor necrosis factor alpha blockade increases renal Cyp2c23 expression and slows the progression of renal damage in salt-sensitive hypertension. Hypertension. 2006;47(3):557-62.
33. Sriramula S, Haque M, Majid DS, Francis J. Involvement of tumor necrosis factor-alpha in angiotensin II-mediated effects on salt appetite, hypertension, and cardiac hypertrophy. Hypertension. 2008;51(5):1345-51.

34. LaMarca BB, Bennett WA, Alexander BT, Cockrell K, Granger JP. Hypertension produced by reductions in uterine perfusion in the pregnant rat: role of tumor necrosis factor-alpha. Hypertension. 2005;46(4):1022-5.
35. Oliveira-Paula GH, Lacchini R, Tanus-Santos JE. Inducible nitric oxide synthase as a possible target in hypertension. Curr Drug Targets. 2014;15(2):164-74.
36. Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. Biol Chem. 2003;384(10-11):1343-64.
37. Smith CJ, Santhanam L, Bruning RS, Stanhewicz A, Berkowitz DE, Holowatz LA. Upregulation of inducible nitric oxide synthase contributes to attenuated cutaneous vasodilation in essential hypertensive humans. Hypertension. 2011;58(5):935-42.
38. Heo SK, Yun HJ, Noh EK, Park WH, Park SD. LPS induces inflammatory responses in human aortic vascular smooth muscle cells via Toll-like receptor 4 expression and nitric oxide production. Immunol Lett. 2008;120(1-2):57-64.
39. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 2011;31(5):986-1000.
40. Yang T, Du Y. Distinct roles of central and peripheral prostaglandin E2 and EP subtypes in blood pressure regulation. Am J Hypertens. 2012;25(10):1042-9.
41. de Oliveira AC, Yousif NM, Bhatia HS, Hermanek J, Huell M, Fiebich BL. Poly(I:C) increases the expression of mPGES-1 and COX-2 in rat primary microglia. J Neuroinflammation. 2016;13(1):11.
42. Tanigawa T, Odkhuu E, Morikawa A, Hayashi K, Sato T, Shibata R, et al. Immunological role of prostaglandin E2 production in mouse auditory cells in response to LPS. Innate Immun. 2014;20(6):639-46.
43. Pindado J, Balsinde J, Balboa MA. TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase A2/cyclooxygenase-2 pathway. J Immunol. 2007;179(7):4821-8.
44. Endo Y, Blinova K, Romantseva T, Golding H, Zaitseva M. Differences in PGE2 production between primary human monocytes and differentiated macrophages: role of IL-1beta and TRIF/IRF3. PLoS One. 2014;9(5):e98517.
45. Xu XJ, Reichner JS, Mastrofrancesco B, Henry WL, Jr., Albina JE. Prostaglandin E2 suppresses lipopolysaccharide-stimulated IFN-beta production. J Immunol. 2008;180(4):2125-31.
46. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 2009;30(10):475-87.
47. Full F, Gack MU. Prostaglandin E2: the villain in the host response to influenza virus. Immunity. 2014;40(4):453-4.
48. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989;246(4935):1306-9.
49. Wornle M, Sauter M, Kastenmuller K, Ribeiro A, Roeder M, Mussack T, et al. Role of viral induced vascular endothelial growth factor (VEGF) production in pleural effusion and malignant mesothelioma. Cell Biol Int. 2009;33(2):180-6.
50. Koff JL, Shao MX, Ueki IF, Nadel JA. Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. Am J Physiol Lung Cell Mol Physiol. 2008;294(6):L1068-75.
51. Fagiani E, Christofori G. Angiopoietins in angiogenesis. Cancer Lett. 2013;328(1):18-26.

52. Zhang L, Yang N, Park JW, Katsaros D, Fracchioli S, Cao G, et al. Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res. 2003;63(12):3403-12.
53. Kim I, Kim JH, Ryu YS, Liu M, Koh GY. Tumor necrosis factor-alpha upregulates angiopoietin-2 in human umbilical vein endothelial cells. Biochem Biophys Res Commun. 2000;269(2):361-5.
54. Otani A, Takagi H, Oh H, Koyama S, Honda Y. Angiotensin II induces expression of the Tie2 receptor ligand, angiopoietin-2, in bovine retinal endothelial cells. Diabetes. 2001;50(4):867-75.
55. Kranidioti H, Orfanos SE, Vaki I, Kotanidou A, Raftogiannis M, Dimopoulou I, et al. Angiopoietin-2 is increased in septic shock: evidence for the existence of a circulating factor stimulating its release from human monocytes. Immunol Lett. 2009;125(1):65-71.
56. Spirig R, Potapova I, Shaw-Boden J, Tsui J, Rieben R, Shaw SG. TLR2 and TLR4 agonists induce production of the vasoactive peptide endothelin-1 by human dendritic cells. Mol Immunol. 2009;46(15):3178-82.
57. Woods M, Wood EG, Bardswell SC, Bishop-Bailey D, Barker S, Wort SJ, et al. Role for nuclear factor-kappaB and signal transducer and activator of transcription 1/interferon regulatory factor-1 in cytokine-induced endothelin-1 release in human vascular smooth muscle cells. Mol Pharmacol. 2003;64(4):923-31.
58. Farina G, York M, Collins C, Lafyatis R. dsRNA activation of endothelin-1 and markers of vascular activation in endothelial cells and fibroblasts. Ann Rheum Dis. 2011;70(3):544-50.
59. George PM, Badiger R, Shao D, Edwards MR, Wort SJ, Paul-Clark MJ, et al. Viral Toll Like Receptor activation of pulmonary vascular smooth muscle cells results in endothelin-1 generation; relevance to pathogenesis of pulmonary arterial hypertension. Biochem Biophys Res Commun. 2012;426(4):486-91.
60. Shcheglovitova ON, Skliankina NN, Boldyreva NV, Babaiants AA, Frolova IS, Kapkaeva MP. [Human interferon modulates infected vascular endothelium function]. Vestn Ross Akad Med Nauk. 2014(3-4):31-5.
61. Nair AR, Ebenezer PJ, Saini Y, Francis J. Angiotensin II-induced hypertensive renal inflammation is mediated through HMGB1-TLR4 signaling in rat tubulo-epithelial cells. Exp Cell Res. 2015;335(2):238-47.
62. Goulopoulou S, McCarthy CG, Webb RC. Toll-like Receptors in the Vascular System: Sensing the Dangers Within. Pharmacol Rev. 2016;68(1):142-67.

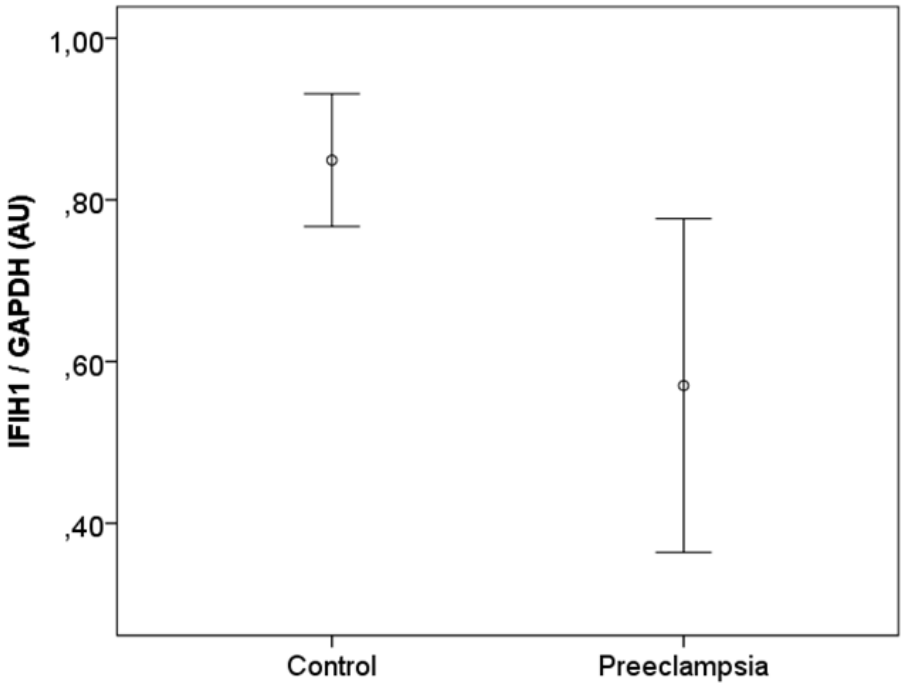

Figure 1. *IFIH1* gene expression is decreased in placenta from PE women. *IFIH1* gene expressions were assessed in placenta from women with preeclampsia (PE) (n = 33) and healthy pregnant women (controls, n = 53) (P= 0.004). Only white women were included in this analysis. P value was obtained using Student's t-test. Data are presented as median (95% CI) and *GAPDH* was used as the reference gene. AU = arbitrary units.

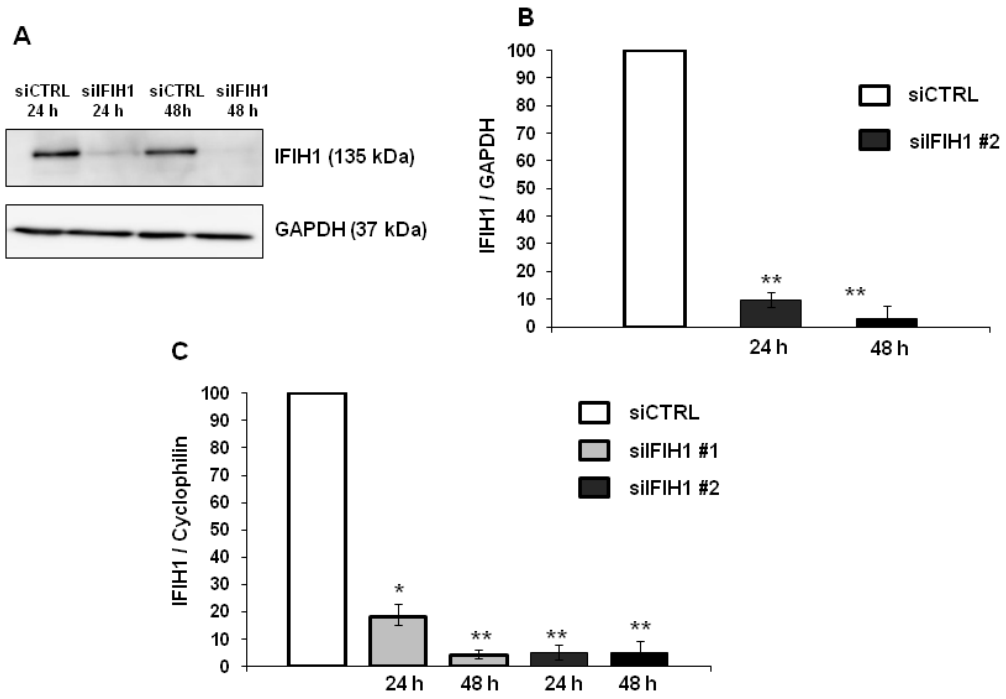

Figure 2. *IFIH1* knockdown in HUVECs. A) Representative immunoblottings for IFIH1 and GAPDH proteins after 24 h and 48 h of *IFIH1* knockdown using siIFIH1 #2 in HUVECs. B) Relative quantification of IFIH1 protein corrected by the values of GAPDH (n = 3 for each group; results for siIFIH1#2). C) Quantification of *IFIH1* gene expression corrected by *cyclophilin* reference gene (n = 3 by group). Data are presented as mean ± SD. *P < 0.001 *vs*. siCTRL; **P < 0.0001 *vs*. siCTRL (Student's *t*-test).

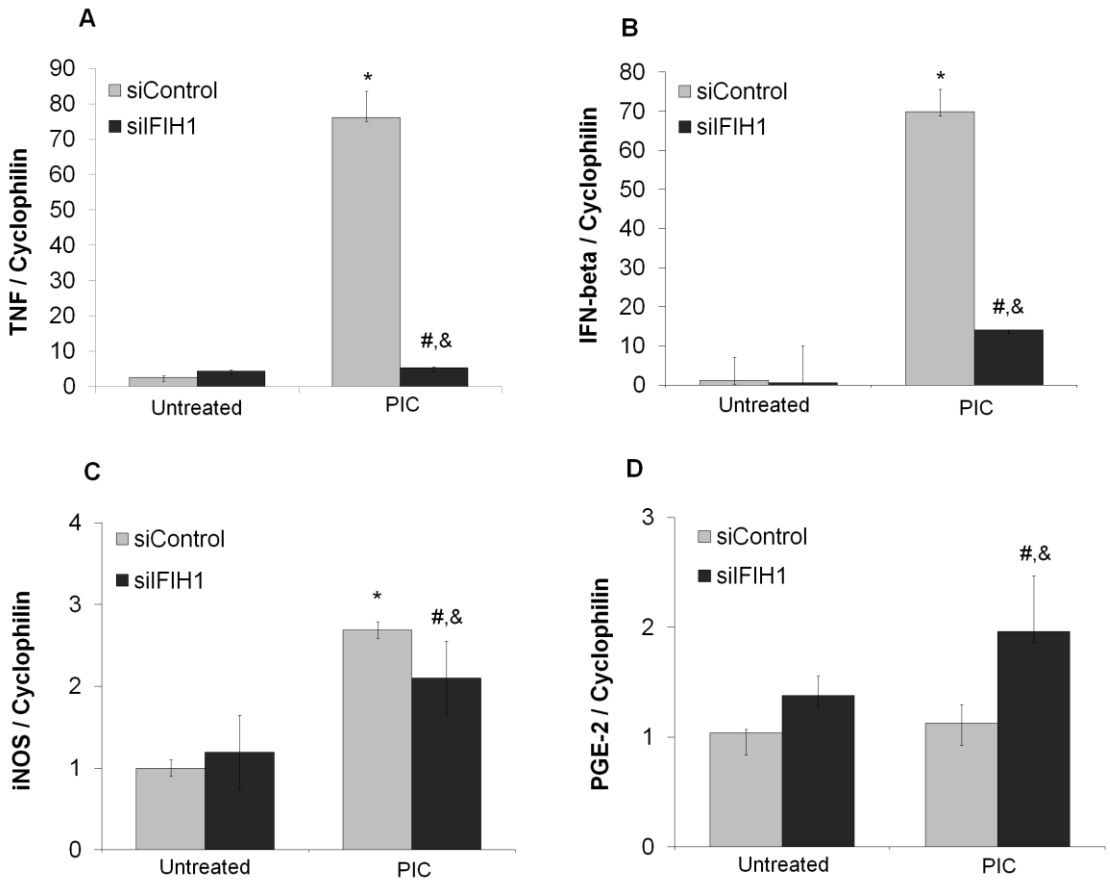

Figure 3. **Expressions of inflammation-related genes after *IFIH1* knockdown in HUVECs treated with PIC.** Following 48 h of *IFIH1* knockdown, cells were transfected with PIC for additional 24 h, and expressions of *TNF* **(A)**, *IFN-β* **(B)**, *iNOS* **(C)** and *PGE-2* **(D)** were evaluated by RT-qPCR. Values were corrected for the reference gene *cyclophilin.* Results are shown as mean ± SD (n = 3). *P <0.05 *vs.* untreated siCTRL; $^{\#}$P <0.05 *vs.* untreated siIFIH1#2, and $^{\&}$P <0.05 *vs.* siCTRL + PIC. All comparisons were done using Student *t*-tests.

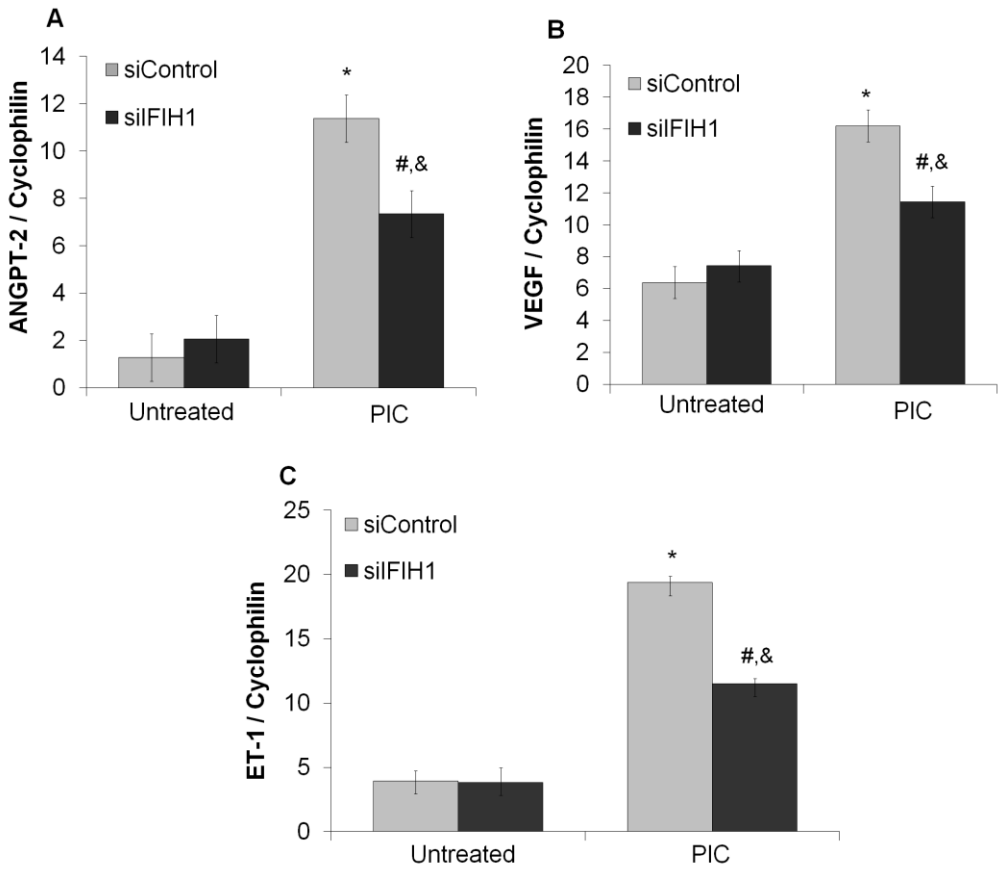

Figure 4. Expressions of hypertension- and angiogenesis-related genes after *IFIH1* knockdown in HUVECs treated with PIC. Following 48 h of *IFIH1* knockdown, cells were transfected with PIC for additional 24 h, and expressions of *ANGPT-2* **(A)**, *VEGF* **(B)** and ET-1 **(C)** were evaluated by RT-qPCR. Values were corrected for the reference gene *cyclophilin.* Results are means ± SD (n = 3). *P < 0.05 *vs*. untreated siCTRL; $^{\#}$P < 0.05 *vs*. untreated siIFIH1#2; $^{\&}$P < 0.05 *vs*. siCTRL + PIC. All comparisons were done using Student *t*-tests.

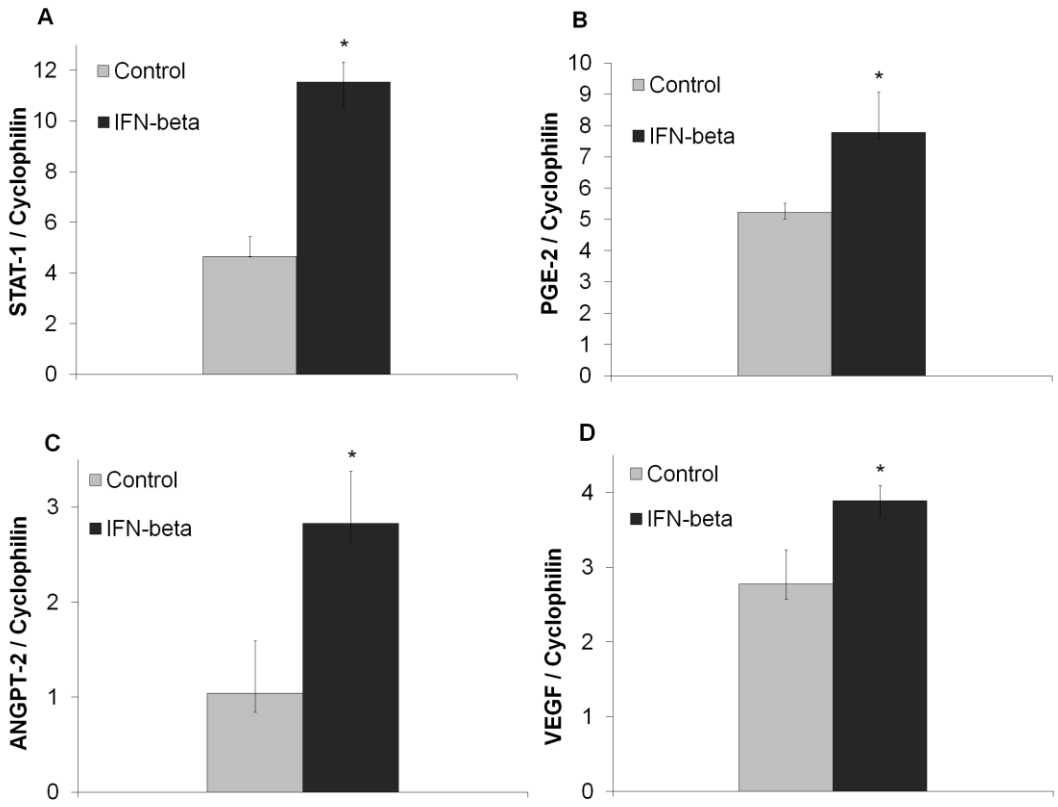

Figure 5. Expressions of genes related to inflammation, hypertension, and angiogenesis after IFN-β treatment in HUVECs. Cells were treated with IFN-β or let untreated (control condition) for 48 h in culture, and then expressions of *STAT-1* **(A)**, *PGE-2* **(B)**, *ANGPT-2* **(C)** and *VEGF* **(D)** were evaluated by RT-qPCR. Values were corrected for the reference gene *cyclophilin.* Results are means ± SD (n = 3). *P < 0.05 *vs*. control condition. All comparisons were done using Student *t*-tests.

Table 1. Primer sequences used for RT-qPCR analyses.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	ACCCACTCCTCCACCTTTG	CTCTTGTGCTCTTGCTGGG
Cyclophilin	GCCGATGACGAGCCCTTG	TGCCGCCAGTGCCATTATG
IFIH1	ATGGAAAAAAAAAGCTGCAAAAGA	GTACTTCCTCAAATGTTCTGCACAA
STAT-1	GTGGAAAGACAGCCCTGCAT	CCAACAGTCTCAACTTCACAGTGA
VEGF	GGCGAGGCAGCTTGAGTTAA	CACCGCCTCGGCTTGTC
TNF	CCCAGGGACCTCTCTCTAATCA	GGTTTGCTACAACATGGGCTACA
IFN-β	GAACTTTGACATCCCTGAGGAGATT	ATGCGGCGTCCTCCTTCT
ANGPT-2	GCCGCTCGAATACGATGACT	AGCTCATTAGCCACTGAGTGTTGT
PGE-2	ACAGAGGTGACAGCCCAACAG	CCATGGAGGCAAAGGGATT
ET-1	CCCTCCAGAGAGCGTTATGTG	CCCGAAGGTCTGTCACCAA
iNOS	CCCAAGGTCTATGTTCAGGACAT	CACATCCCCFCAAACATAGAG

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; *IFIH1*, Interferon induced with helicase C domain 1; *STAT-1*, Signal transducer and activator of transcription 1; *VEGF*, *Vascular endothelial growth factor*; *TNF*, Tumor necrosis factor; *IFN-β*, *Interferon β;* *ANGPT-2*, Angiopoetin-2; *PGE-2*, Prostaglandin-2; *ET-1*, Endothelin-1; *iNOS*, Inducible nitric oxide synthase.

Table 2. Genotype and allele frequencies of the *IFIH1* rs1990760 G/A polymorphism in women with preeclampsia (PE) and healthy pregnant women (controls).

	PE women (n = 59)	**Control women (n = 134)**	**Unadjusted P***	**Adjusted OR (95% CI) / P†**
Genotype				
G/G	25.4 (15)	27.6 (37)	0.938	1
G/A	52.5 (31)	50.0 (67)		0.826 (0.328-2.080)/0.685
A/A	22.1 (13)	22.4 (30)		1.017 (0.465-2.225)/0.966
Allele				
G	0.516	0.526	0.435	-
A	0.484	0.474		
Dominant model				
G/G	25.4 (15)	27.6 (37)	0.861	1
G/A – A/A	74.6 (44)	72.4 (97)		1.225 (0.585-2.565)/0.590

Data are presented as % (n) or proportion. * P values were computed by χ^2 tests comparing PE women and healthy pregnant women. †Adjusted OR (95% CI) and P values obtained by logistic regression analyses adjusting for ethnicity.

Table 3. Effect of synthetic dsRNA treatment (PIC) after *IFIH1* knockdown on expressions of genes related to inflammation, angiogenesis and hypertension.

Gene	**siCTRL + PIC**	**siIFIH1#1 + PIC**	**P values**
VEGF	19.2 ± 1.8	10.9 ± 1.4	0.040
TNF	18.3 ± 1.7	7.8 ± 4.0	0.031
IFN-β	17.4 ± 1.4	4.3 ± 0.8	<0.001
ANGPT-2	5.7 ± 0.8	4.2 ± 0.05	0.029
PGE-2	2.2 ± 0.3	5.3 ± 0.6	0.001
ET-1	16.4 ± 1.7	11.6 ± 0.4	0.007
iNOS	5.1 ± 0.5	4.0 ± 0.5	0.004

Mean ± SD values are derived from 3 experiments after correction by values of the reference gene (*Cyclophilin*). Data were analyzed using unpaired Student's *t*-test.

Table S1. Clinical characteristics of women with preeclampsia and control subjects.

Characteristics	**PE group (n = 59)**	**Control (n=134)**	**P value**
Age (years)	25.8 ± 5.9	25.0 ± 6.5	0.383
Ethnicity (% black women)	25.0	20.8	0.297
Gestational age (weeks)	35.4 ± 3.7	38.9 ± 2.4	<0.0001
Gestational BMI (kg/m^2)	33.4 ± 7.1	30.0 ± 4.8	<0.0001
Systolic BP (mm/Hg)	146.5 ± 17.8	122.2 ± 11.6	<0.0001
Diastolic BP (mm/Hg)	90.89 ± 14.4	72.8 ± 9.3	<0.0001
Infant birthweight (g)	2638.9 ± 894.3	3214.7 ± 602.9	<0.0001
Pregnancy number (n)	2.0 ± 1.3	2.1 ± 1.4	0.500
Fasting glucose (mg/dl)	78.6 ± 6.1	77.8 ± 8.6	0.567
Mode of delivery:			
Normal (%)	43.5	64.3	0.006
Cesarean (%)	55.1	35.7	

Results are shown as means ± SD, n or %. Data were analyzed using Student's *t*-test. PE= preeclampsia; BMI = body mass index; BP = blood pressure.

Table S2. Clinical and laboratory characteristics of women with preeclampsia broken down by the different genotypes of the rs1990760 (G/A) polymorphism in the *IFIH1* gene.

Characteristics	**Genotypes**			**P values**
	G/G (n = 15)	**G/A (n = 31)**	**A/A (n = 13)**	
Age (years)	24.8 ± 5.4	24.7 ± 5.6	30.0 ± 6.4	0.021
Gestational age (weeks)	35.5 ± 3.8	34.8 ± 3.9	36.9 ± 3.6	0.276
Gestational BMI (kg/m^2)	34.8 ± 7.7	31.9 ± 8.0	34.5 ± 5.6	0.388
Systolic BP (mm/Hg)	145.5 ± 16.2	145.4 ± 18.2	144.9 ± 16.6	0.995
Diastolic BP (mm/Hg)	87.7 ± 14.2	90.8 ±10.5	87.8 ± 13.6	0.641
Infant birthweight (g)	2793.6 ± 966.3	2440.1 ± 947.3	2967.3 ± 740.1	0.176
Mode of delivery:				
Normal % (n)	25.0 (6)	45.8 (11)	29.2 (7)	
Cesarean % (n)	26.5 (9)	55.9 (19)	17.6 (6)	0.571

Data are expressed as mean ± SD or percentage. *P-values were obtained by One-Way ANOVA or χ^2 tests, as appropriate. BMI = body mass index; BP = blood pressure.

4. CONCLUSÕES

A nossa revisão sistemática demonstrou que os dados disponíveis na literatura sugerem fortemente o papel do TLR-3 e TLR-4 na patogênese da PE. No entanto, pesquisas adicionais ainda são necessárias para compreender melhor como a ativação excessiva destes TLRs em resposta a um sinal de perigo endógeno ou exógeno leva ao desenvolvimento PE. Além disso, mais estudos são necessários para confirmar se outros receptores do tipo TLRs, IFIH1/MDA5, RIG-1 ou NOD estão realmente envolvidos na patogênese da PE e se polimorfismos nos genes dos PRRs predispõe a esta complicação da gravidez. Através de uma investigação mais aprofundada do envolvimento PRR na PE pode ser possível identificar potenciais alvos terapêuticos para esta complicação.

No nosso estudo de caso-controle, a expressão do gene *IFIH1* foi diminuída na placenta de grávidas com PE em comparação ao grupo controle, sugerindo um papel deste gene no desenvolvimento desta complicação da gravidez. Em contraste, o polimorfismo rs1990760 no gene *IFIH1* não foi associado com PE na nossa população. Dessa forma, estudos adicionais em diferentes populações, com números amostrais maiores e considerando-se a PE de diferentes graus de severidade são necessários para elucidar o papel deste polimorfismo na PE.

No estudo experimental em células HUVECs, demonstrou-se que o tratamento com PIC induz a expressão de *TNF*, *IFN-β*, *iNOS*, *ANGPT-2*, *VEGF*, *ET-1* e AngII, o que foi parcialmente prevenido após bloqueio do gene *IFIH1* nestas células. Isto sugere que o gene *IFIH1* tem um papel importante na ativação de genes relacionados à inflamação, angiogênese e hipertensão em células endoteliais humanas após ser ativado por ligantes exógenos (virais) ou endógenos. Este estudo adiciona um novo papel possível do IFIH1 na patogênese da PE e HA.

5. COLABORAÇÃO EM OUTROS ESTUDOS DURANTE O ANDAMENTO DO DOUTORADO

1. Bianca Marmontel de Souza, Marcus Michels, Denise Alves Sortica, **Ana Paula Bouças**, Jakeline Rheinheimer, Marjoriê Piuco Buffon, Andrea Carla Bauer, Luís Henrique Canani, and Daisy Crispim. Polymorphisms of the UCP2 Gene Are Associated with Glomerular Filtration Rate in Type 2 Diabetic Patients and with Decreased UCP2 Gene Expression in Human Kidney. **Plos One**, v. 28, p. 10, 2015.

2. Rheinheimer J; Bauer AC; Silveiro SP; Estivalet AAF; **Bouças AP**; Rosa AR; Souza BM; Oliveira FS; Cruz LA; Brondani LA; Azevedo MJ; Lemos NE; Carlessi R; Assmann TS; Gross JL; Leitão C; Crispim D. Human pancreatic islet transplantation: an update and description of the establishment of a pancreatic islet isolation laboratory. **Archives of Endocrinology and Metabolism**, v. 59, p. 161-170, 2015.

3. Assmann TS; Brondani LA; **Bouças AP**; Canani LH; Crispim D. Toll-like receptor (TLR3) and the development of type 1 diabetes mellitus. **Brazilian Arquives of Endocrinology and Metabolism**, v. 59, p. 4-12, 2015.

4. L.A. Brondani, T.S. Assmann, B.M. Souza, **A.P. Bouças**, L.H. Canani, D. Crispim. Meta-analysis reveals the association of common variants in the uncoupling protein (UCP) 1-3 genes with body mass index variability. **Plos One**, v. 9, p. e96411, 2014

5. L.A. Brondani, B.M. Souza, T.S. Assmann, **A.P. Bouças**, A.C. Bauer, L.H. Canani, D. Crispim. Association of the UCP polymorphisms with susceptibility to obesity: case-control study and meta-analysis. **Molecular Biology Reports**, v. 48, p. 5053-67, 2014.