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Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Tocoginecologia.

Orientadora: Profª. Titular Maria Terezinha Serrão Peraçoli

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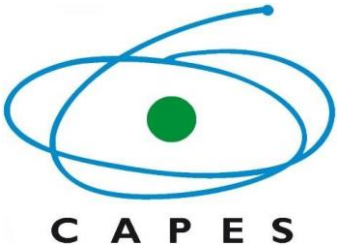

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"Eu não estou longe, apenas estou do outro lado do Caminho…"

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"A ciência é a experiência, e a experiência é um manto que se tece ao longo de vários séculos; e quanto mais o manto se estende, mais a ciência é completa e segura".

Carlo Bini

PREFÁCIO

O documento da Tese para obtenção do título de Doutora em Tocoginecologia, intitulado “Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T $CD4^+$ em gestantes portadoras de pré-eclâmpsia” encontra-se dividido em 6 partes:

Inicialmente, a Introdução aborda uma revisão a respeito da patogênese da pré-eclâmpsia (PE) e suas manifestações clínicas. Também é apresentada a literatura relacionada à resposta imune adaptativa, à diferenciação dos linfócitos T *helper* (Th)- CD4, assim como, o papel da vitamina D (VD) e da silibinina (SB) na pré-eclâmpsia.

O capítulo I contempla o manuscrito intitulado “Vitamin D modulates the transcription factors of T cell subsets to anti-inflammatory and regulatory profiles in preeclampsia”, que foi submetido à publicação ao periódico Journal of Reproductive Immunology. Os resultados desse manuscrito mostram que o tratamento in vitro com a VD sobre as células mononucleares do sangue periférico (PBMCs), diminuiu a expressão gênica dos fatores de transcrição característicos das subpopulações inflamatórias (Th1 e Th17) e aumentou os fatores de transcrição referente aos perfis anti-inflamatórios (Th2) e regulador (Treg) em gestantes com pré-eclâmpsia. Assim, sugere-se que a resposta inflamatória sistêmica observada na PE pode ser decorrente de um desequilíbrio na homeostase do sistema imunológico materno, causado pela diminuição do hormônio VD, capaz de regular a inflamação.

O capítulo II é composto pelo manuscrito “Immunomodulatory effect of vitamin D on the activators of transcription and transcription factors of $CD4^+$ T cell subsets in pregnant women with preeclampsia” que será enviado para publicação e apresenta os resultados da análise proteica dos transdutores de sinal e ativadores de transcrição (STATs), os fatores de transcrição das subpopulações de células T $CD4^+$ e as citocinas relacionadas aos perfis. Os resultados demonstraram o efeito imunomodulador in vitro da VD sobre a imunidade adaptativa, favorecendo o aumento de STATs, dos fatores de transcrição dos perfis Th2 e Treg e das citocinas relacionadas às células T em gestantes com PE. Esse manuscrito, evidenciou a capacidade da VD em modular uma importante via de sinalização JAK/STAT, restaurando o desequilíbrio das subpopulações de células T $CD4^+$ observado na PE.

O capítulo III está apresentado em forma de manuscrito que será enviado para publicação, intitulado “Silibinin downregulates the expression of the Th1 and Th17 profiles by the modulation of STATs and transcription factors in pregnant women with preeclampsia”. Foi relatado o papel do flavonoide silibinina na modulação da via JAK/STAT, com a diminuição dos ativadores de transcrição e dos fatores de transcrição de perfis inflamatórios, além da diminuição das citocinas pró-inflamatórias por linfócitos T $CD4^+$ em gestantes portadoras de PE. Sendo assim, o papel modulador da silibinina parece ter grande importância na imunidade adaptativa. Considerando que nosso grupo de pesquisa apresenta conhecimento prévio sobre o emprego in vitro de SB, com vários trabalhos associados a PE publicados em periódicos internacionais e, que não há resultados sobre o efeito desse flavonoide sobre as subpopulações de células T, decidiu-se acrescentar essa análise devido às suas propriedades anti-inflamatória, anti-oxidante e imunomoduladora já descritas em outras patologias, com processos inflamatórios intensificados. Dessa forma, são apresentados resultados obtidos de 16 gestantes pré-eclâmpticas e 16 gestantes normotensas (NT).

Finalizando a presente tese de Doutorado encontram-se as conclusões gerais baseadas nos 3 capítulos que compreenderam esse estudo, os anexos referentes aos Termos de Consentimento Livre e Esclarecido e o parecer do Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu, UNESP.

Sumário

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Alexa 647: fluorocromo alexa fluor 647

APC-H7: fluorocromo aloficocianina conjugado com o análogo de um cianina 7

BB515: fluorocromo azul brilhante 515

CBA: *cytometric bead array*

CD: *cluster* of diferenciação

CD127: *cluster* de diferenciação 127, molécula marcadora de células efetoras

CD25: *cluster* de diferenciação 25, molécula marcadora de células T reguladoras

CD3: *cluster* de diferenciação 3, molécula marcadora de células T

CD4: *cluster* de diferenciação 4, molécula marcadora de células T helper

cDNA: DNA complementar

CO_2: dióxido de carbono

EDTA: ácido etilenodiamino tetra-acético

ELISA: ensaio de imunoabsorção enzimática

FoxP3: *forkhead box P3*, fator de transcrição de células T reguladoras

GAPDH: gliceraldeído 3-fosfato-desidrogenase

GATA-3: GATA *binding protein* 3, fator de transcrição de células Th2

h: horas

H_2O_2: peróxido de hidrogênio

IFN-γ: interferon gama

IL: interleucina

IL-23R: receptor da interleucina 23

IκB: proteína inibitória da via de sinalização de NF-κB

LPS: lipopolissacarídeo

M1: monócitos classicamente ativados

M2: monócitos alternativamente ativados

mg: miligramas

MIF: mediana de intensidade de fluorescência

mL: mililitro

mm: milímetro

mmHg: milímetro de mercúrio

NF-κB: fator nuclear kappa B

NK: célula natural *killer*

NLRP: receptor *NOD-like*

nM: nanomolar

NT: normotensa

ºC: graus Celsius

p*:* nível de significância

PA: pressão arterial

PBMCs: células monucleares do sangue periférico

PBS: solução salina tamponada, *phosphate buffered saline*

PE: fluorocromo ficoeritrina

PE: pré-eclâmpsia

PE-Cy7: fluorocromo ficoeritrina conjugado com corante cianina

PerCP-Cy5.5: fluorocromo proteína piridina clorofila conjugado com corante cianina

pg: picograma

PGE_2: prostaglandina E2

qPCR: reação em cadeia da polimerase quantitativa em tempo real

RNA: ácido ribonucleico

RORγt: *retinoic-acid-receptor-related orphan nuclear receptor* gamma t

rpm: rotações por minuto

RPMI: *Roswell Park Memorial Institute*, meio sintético complexo

Runx1: *runt-related transcription factor* 1

RXR: receptor retinoide X

SB: silibinina

STAT: *Signal transducer and activator of transcription*. Transdutor de sinal e ativador da transcrição

STAT1: transdutor de sinal e ativador da transcrição 1

STAT3: transdutor de sinal e ativador da transcrição 3

STAT4: transdutor de sinal e ativador da transcrição 4

STAT5: transdutor de sinal e ativador da transcrição 5

STAT6: transdutor de sinal e ativador da transcrição 6

T-bet: *T box transcription factor*, fator de transcrição de células Th1

TGF-β1: fator transformador do crescimento beta 1

Th1: célula T *helper* 1

Th17: célula T *helper* 17

Th2: célula T *helper* 2

TNF-α: fator de necrose tumoral alfa

Treg: célula T reguladora

U/mL: unidade por mL

UVB: raios ultravioleta

VD: vitamina D

VDBP: proteína de ligação à vitamina D

VDR: receptor de vitamina D

µg: micrograma

µM: micromolar

%: percentagem

1,25 (OH)D: 1,25-dihidroxivitamina D

25(OH)D: 25-hidroxivitamina D

RESUMO

Na pré-eclâmpsia (PE) ocorre um estado de má adaptação da tolerância imunológica, caracterizado por ativação anormal do sistema imune inato e adaptativo. A ocorrência de uma resposta inflamatória sistêmica parece ser devida ao desbalanço das subpopulações de células T CD4, causado por aumento do número de células de perfis inflamatórios T helper 1 (Th1) e Th17 e diminuição das células características dos perfis anti-inflamatório e regulador, Th2 e T reg respectivamente. Portanto, o desbalanço entre as subpopulações de células T pode ser crítico para a tolerância ao feto e para prevenção da PE. O objetivo do estudo foi avaliar o efeito imunomodulador, in vitro, da vitamina D (VD) e da silibinina (SB) sobre células mononucleares do sangue periférico (PBMCs) de gestantes pré-eclâmpticas, com ênfase em subpopulações de células Th. Foram estudadas 20 gestantes portadoras de PE e 20 gestantes normotensas (NT), pareadas pela idade gestacional. Sangue periférico foi obtido das gestantes de ambos os grupos para determinação da concentração plasmática de VD [25(OH)D]. O efeito imunomodulador da VD e da SB sobre as subpopulações de células T $CD4^+$ (Th1, Th2, Th17 e Treg) foi avaliado quanto à expressão dos transdutores de sinal e ativadores de transcrição (STATs) para as células Th1 (STAT1 e STAT4), Th2 (STAT6), Th17 (STAT3) e Treg (STAT5) e dos fatores de transcrição intracelulares para células Th1 (T-bet), Th2 (GATA-3), Th17 (RORγt e Runx1) e Treg (FoxP3) por PCR quantitativo (qPCR) e/ou citometria de fluxo. As citocinas relacionadas às subpopulações de células Th, as pró-inflamatórias, IFN-γ, TNF-α, IL-6, IL-17, IL-22 e IL-23 e as anti-inflamatórias IL-10 e TGF-β, produzidas por PBMCs de gestantes pré-eclâmpticas e NT foram determinadas pelos métodos de *cytometric bead array* (CBA) e/ou Elisa. De maneira geral, os resultados mostraram que o tratamento in vitro com VD e SB é capaz de modular o desbalanço das subpopulações de células T presente na PE, diminuindo os fatores de transcrição, os ativadores de transcrição e citocinas característicos dos perfis inflamatórios e aumentando os relacionados aos perfis anti-inflamatório e regulador. Assim, o entendimento da modulação das subpopulações de células T por agentes com ações imunomoduladoras e anti-inflamatórias, como VD e SB, poderá contribuir para a melhor compreensão do envolvimento da imunidade adaptativa na fisiopatologia da PE e possivelmente propor formas alternativas para o tratamento dessa importante patologia da gestação.

Palavras-chave: PBMCs, pré-eclâmpsia, subpopulações de células T, citocinas, vitamina D e silibinina.

INTRODUÇÃO

Introdução

A pré-eclâmpsia (PE), uma síndrome específica da gravidez, acomete mulheres previamente normotensas e afeta entre 2% a 8% de todas as gestações (ACOG, 2020). Os parâmetros clínicos que identificam essa doença são fundamentados no desenvolvimento de hipertensão arterial (PA ≥ 140x90mmHg) com a presença ou não de proteinúria (≥ 300mg/24h) após a 20ª semana de gestação ou nos primeiros dias após o parto. Em algumas mulheres, pode ocorrer o aparecimento de hipertensão arterial associada a outros sinais ou sintomas da PE na ausência de proteinúria, tais como trombocitopenia, insuficiência renal, comprometimento hepático, edema pulmonar, complicações neurológicas ou hematológicas, disfunção útero-placentária e restrição de crescimento fetal (Tranquili *et al*., 2014; Mol *et al*., 2016; ACOG, 2020). Assim, os distúrbios hipertensivos da gestação constituem uma das principais causas de mortalidade materna e perinatal em todo o mundo (ACOG, 2020). Estima-se que são responsáveis por mais de 70 mil mortes maternas e 500 mil mortes fetais anualmente (Rana et *al*., 2019). Diante destas informações, ressalta-se a importância de compreender melhor essa patologia da gestação.

A PE caracteriza-se por uma intesa resposta inflamatória sistêmica, cujo distúrbio imunológico parece ter sua origem na placenta, resultante de lesão tecidual causada por isquemia/hipóxia (Huppertz, 2008). Essa inflamação sistêmica ocorre clinicamente sem sintomas de infecção microbiana e tem sido descrita como "inflamação estéril" (Nadeau-Vallée *et al*., 2016; Brien *et al*., 2019). Durante a gestação, várias moléculas e efetores imunológicos participam do microambiente imune para permitir e estabelecer a tolerância materna específica em relação ao feto semialogênico (Wang *et al*., 2020). Dentre esses componentes imunes, as células Th (helper) e suas subpopulações, Th1, Th2, Th17 e Treguladoras (Treg) desempenham importantes papéis na interface materno-fetal.

Em 1986 foi proposto o paradigma Th1/Th2 por Mosmann e colaboradores. Esse conceito foi aplicado para explicar a tolerância materna em relação ao feto e, associou-se as complicações obstétricas, como a PE ao perfil Th1 (Wegmann *et al*., 1993). O paradigma Th1/Th2 foi expandido para as células Th1/Th2/Th17/Treg, o qual permitiu compreender melhor a tolerância materna ao feto durante a gestação, com a participação das células Th17 e Treg (Saito *et al*., 2010; Jiang *et al*., 2014; Wang *et al*., 2020).

O ambiente de citocinas tem papel chave na diferenciação das células T. A geração das células Th1 ocorre através de interferon-gama (IFN-γ) e interleucina (IL)-12 que são potentes

mediadores inflamatórios e importantes na ativação da resposta imune adaptativa (Conforti-Andreoni *et al*., 2011). A presença de IL-4 é responsável por diferenciar as células Th2, enquanto as células Th17 se diferenciam na presença de IL-6 e do fator transformador do crescimento beta (TGF-β). Essas células não desempenham papéis importantes apenas na defesa contra patógenos extracelulares, mas também estão relacionadas à progressão de doenças autoimunes, condições de doenças inflamatórias crônicas e reações de rejeição de enxertos (Darmochwal-Kolarz *et al*., 2012; Hosseini *et al*., 2018). Além disso, as células Th17 induzem a ativação de células deciduais NK (dNK), prejudicando a reatividade vascular das artérias uterinas, levando à reabsorção do embrião (Peck & Mellins, 2009; Crome *et al*., 2010; Travis *et al*., 2019; Wang *et al*., 2020). Por outro lado, as células Treg, essenciais na manutenção da auto tolerância e na regulação da inflamação, desempenham importante papel na manutenção da gestação e são diferenciadas na presença de TGF-β (Saito *et al*., 2007; Saito *et al*., 2010; Figueiredo & Schumacher, 2016).

A diferenciação de células T também está relacionada com os transdutores de sinal e ativadores de transcrição, além dos fatores de transcrição específicos das subpopulações de células T, sendo a diferenciação regulada por ambos. Os ativadores de transcrição e os fatores de transcrição de acordo com cada perfil de células T são respectivamente: Th1 – STAT1/ STAT4 *(Signal transducer and activator of transcription 1/4)* e T-bet (*T box transcription factor*); Th2 - STAT6 *(Signal transducer and activator of transcription 6)* e GATA-3 *(GATA binding protein 3)*; Th17 - STAT3 (*Signal transducer and activator of transcription 3*) e RORγt (*retinoic-acid-receptor-related orphan nuclear receptor* gamma *t*); Treg - STAT5 (*Signal transducer and activator of transcription 5*) e FoxP3 *(forkhead box P3)* (Jianjun *et al*., 2010; Seif *et al*., 2017) (Figura 1).

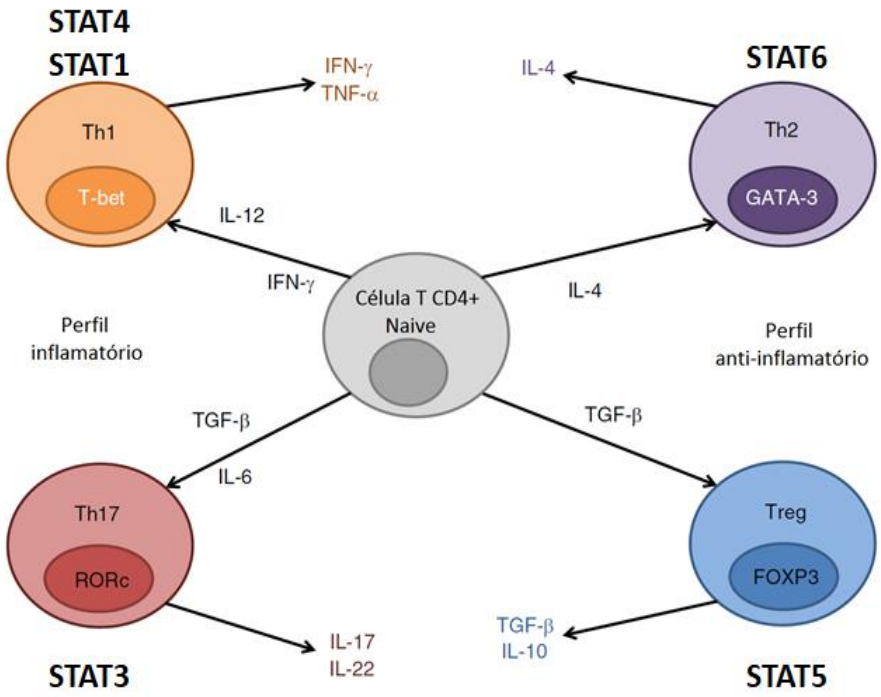

Figura 1. Diferenciação das subpopulações de células T helper (Th) em Th1, Th2, Th17 and Tregulador (Treg) a partir de uma célula T CD4$^+$ *naive*. O ambiente de citocinas durante a ativação das células T helper (Th) determina a diferenciação da célula Th efetora, por meio dos transdutores de sinais e ativadores de transcrição (STATs) seletivos, resultando na ativação dos fatores de transcrição específicos de cada subpopulação. Figura adaptada de Figueiredo A.S. & Schumacher A. Immunology, 2016.

Na PE foi relatado uma diminuição das células Treg e um aumento das células com perfil Th17 no sangue periférico de mulheres com PE quando comparadas com grávidas normotensas (Santner-Nanan *et al*., 2009). Outro estudo demonstrou que as células Th17 também estão aumentadas no sangue periférico de gestantes com PE, e que o número e a função de células Treg estão diminuídos, podendo esse desbalanço ser responsável pela ativação da resposta inflamatória nessa patologia (Darmochwal-Kolarz *et al*., 2012). Resultados obtidos por nosso grupo de pesquisa avaliou a expressão gênica e proteica dos fatores de transcrição característicos de subpopulações de células T CD4, demonstrando maior expressão de T-bet (Th1) e RORγt (Th17) associada à menor expressão de GATA-3 (Th2) e FoxP3 (Treg) na população de linfócitos T circulantes das gestantes pré-eclâmpticas em comparação às gestantes normotensas. Além disso, esses resultados foram associados à produção endógena aumentada do fator de necrose tumoral alfa (TNF-α) e IL-17 e a diminuição de IL-10, comprovando o perfil inflamatório dessas subpopulações de células T na PE (Ribeiro *et al*., 2017; Romão-Veiga *et al*., no prelo). Assim, sabe-se que as células Th17 e Treg formam um complexo para manter

a homeostase e interagem com outros tipos celulares para orquestrar a resposta imune durante a gestação (Figueiredo & Schumacher, 2016). No entanto, na PE esse complexo é falho, ocorrendo o desbalanço entre perfil inflamatório e anti-inflamatório de subpopulações de células T $CD4^+$.

Na PE pode-se notar também uma produção exacerbada de IL-2, IL-6, IL-12, IL-17, IFN-γ e TNF-α (Darmochwal-Kolarz *et al*., 2002; Peraçoli *et al*., 2007; 2011; Ribeiro *et al*., 2017; Aggarwal *et al*., 2019; Romão-Veiga *et al*., 2020), bem como a menor produção da síntese da interleucina anti-inflamatória, IL-10, importante fator capaz de regular a resposta inflamatória (Cristofalo *et al*., 2013; Raghupathy, 2013). A rede de citocinas envolvidas nos processos relacionados à gestação é complexa, uma vez que estes mediadores podem influenciar mecanismos que atuam na implantação do ovo, na evolução da gestação e no mecanismo de parto. Essa rede de citocinas quando alterada, pode estar associada com complicações como abortamento, PE e parto prematuro (Orsi & Tribe, 2008).

O desbalanço entre os perfis inflamatórios Th1 e Th17 e anti-inflamatório e regulador, Th2 e Treg respectivamente, além das citocinas pró e anti-inflamatórias na PE pode ser restaurado com fatores que merecem ser investigados quanto à sua capacidade de modular essa resposta inflamatória como, por exemplo, a vitamina D (VD) e a silibinina (SB).

A VD é um pró-hormônio multifuncional, obtido principalmente a partir da exposição à luz solar (Feldman *et al*., 2014). Esse hormônio pode ser adquirido pela dieta, mas sua principal fonte decorre da sua ativação na pele (derme e epiderme), a partir da exposição aos raios ultravioleta (UVB) (Prietl *et al*., 2013). Após a ingestão ou síntese à partir da pele, a VD na forma inativa se liga à proteína de ligação a VD (VDBP), dentro da circulação sistêmica (Streeten *et al*., 2013; Fernando *et al*., 2020). O composto inativo da VD sofre hidroxilação no fígado através da 25-hidroxilase gerando a 25-hidroxivitamina D e é então convertido por 1α-hidroxilase nos rins para a forma ativa da VD, 1,25-dihidroxivitamina D [1,25-$(OH)2$-D_3], chamada de calcitriol (Webb, 2006; Sharif *et al*., 2018). Assim, atuando como um hormônio esteróide, 1,25-dihidroxivitamina D liga-se ao receptor da vitamina D (VDR) no interior da célula e sua a ação ocorre através do heterodímero, formado por uma ligação do VDR com o receptor retinóide X (RXR), que se move para o núcleo levando à ativação de fatores nucleares que estimulam a transcrição e tradução de genes específicos (Haussler *et al*., 2011; Yang et al., 2013; Sharif *et al*., 2018). Esse caminho desacelera as células ativadas, diminuindo a produção de citocinas inflamatórias e a fosforilação de STAT (Muthian et al., 2006; Wang et al., 2013; Cantorna et al., 2014; Chen et al., 2015; Kulling et al., 2017).

Na literatura há relatos de deficiência de VD em gestantes portadoras de PE (Robinson

et al., 2013; Xu *et al*., 2014; Mohaghegh *et al*., 2015) enquanto outros mostram associação entre essa deficiência e o risco de desenvolvimento de PE (Aghajafari *et al*., 2013; Bodnar *et al*., 2014). A deficiência de VD pode desempenhar papel na disfunção endotelial, um comprometimento importante da patogênese da PE (Min, 2013). Além disso foi demonstrado que a VD inibe a ativação das células endoteliais através das citocinas e da expressão de moléculas de adesão (Reynolds *et al*., 2015; Kanikarla-Marie *et al*., 2016; Cyprian *et al*., 2019). Portanto, sugere-se que a suplementação de VD pode proteger o organismo materno, através da influência sobre a função endotelial, do controle da pressão arterial e da modulação da resposta imune em gestantes com PE (Hyppönen *et al*., 2013; Behjat Sasan *et al*., 2017).

Durante a gestação normal a VD é produzida por células trofoblásticas placentárias e da decídua humana, sendo responsável por um papel anti-inflamatório em vários órgãos, incluindo a placenta. Esse hormônio possui atividades estruturais e fisiológicas semelhantes à progesterona nos estágios iniciais da gestação, desempenhando papel importante na implantação, placentação e manutenção da gestação saudável (Monastra *et al*., 2018). Sendo assim, sugere-se que a deficiência de VD pode contribuir para o desenvolvimento da PE devido à falta de controle da inflamação nessa patologia (Xu *et al*., 2014).

Esse hormônio exerce efeitos sobre uma variedade de células, incluindo células do sistema imune, especialmente células T $CD4^+$. A ação da VD sobre células imunes é mediada através do VDR, expresso em macrófagos, células dendríticas e células T ativadas (Zhang *et al*., 2013) (Figura 2).

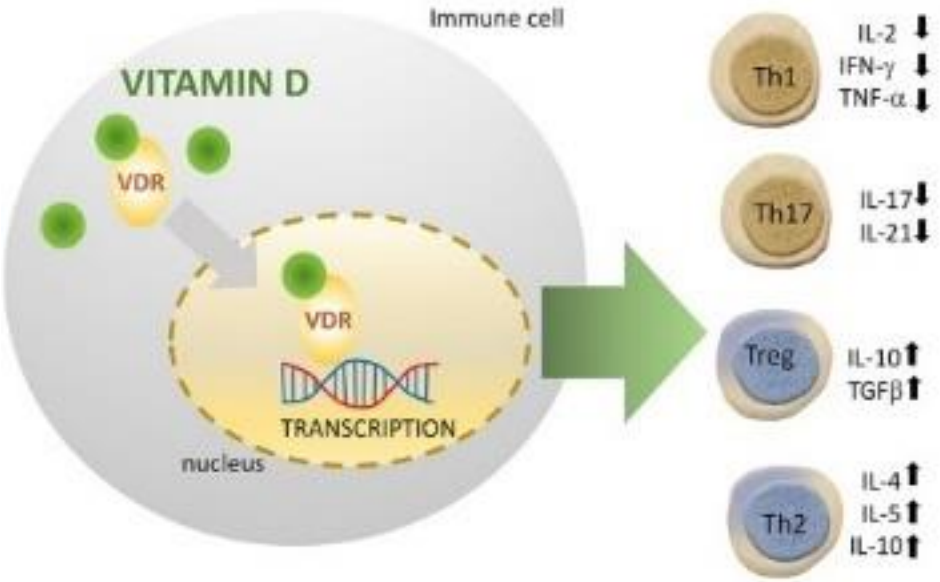

Figura 2. Efeito imunomodulador da vitamina D (VD), mediado pelo receptor de vitamina D (VDR) sobre as subpopulações de células T CD4. A VD exerce efeito imunomodulador sobre as células T, reduzindo a diferenciação e a produção de citocinas de perfis inflamatórios (Th1 e Th17) e polarizando para perfis anti-inflamatório (Th2) e regulador (Treg), além de aumentar a secreção de citocinas características desses perfis. Figura adaptada de Cyprian *et al*., 2019. Front. Immunol, 2019.

Diversos estudos investigam o papel desse hormônio sobre as subpopulações de células T. Doenças autoimunes como lúpus eritematoso sistêmico e artrite reumatoide são influenciadas por deficiência de VD. Fawaz *et al*. (2016) mostraram que a VD diminuiu a secreção de citocinas pró- inflamatórias e aumentou as anti-inflamatórias na diferenciação de células Th17. Em outro estudo, utilizando cultura de células mononucleares de pacientes com artrite reumatoide, a VD atuou como um agente modulador, suprimindo a produção de citocinas por células Th17, sendo relatado que a suplementação de VD, pode ter efeito no tratamento desses pacientes e de outras doenças autoimunes mediadas por células Th17 (Wen *et al*., 2015). Em trabalho sobre a síndrome de Behçet, mostrou-se que o uso de VD, não só inibiu a diferenciação de células Th17, como também inibiu RORc e IL-23R, moléculas importantes para a função e migração das células Th17 (Tian *et al*., 2012). A VD reduziu a diferenciação e expansão das células Th17 em crianças com asma (Hamzaoui *et al*., 2014). Na doença renal crônica, a VD aumentou a produção de citocinas por células Th2 e suprimiu a secreção de citocinas dos perfis Th1 e Th17, elucidando o importante papel da VD na comunicação entre a imunidade inata e adaptativa (Liu *et al*., 2015).

Assim, a suplementação de VD leva à modulação de perfil pró-inflamatório para um perfil imunológico tolerogênico, devido ao seu efeito sobre as diferentes subpopulações de células T (Prielt *et al*., 2013). Desta forma, o efeito imunomodulador da VD nas funções de células T incluem, supressão de proliferação, polarização do perfil Th1 para o desenvolvimento do perfil Th2, inibição das células Th17 e indução de um perfil modulador através das células Treg (Aranow, 2011).

Considerando que a adaptação materna à gestação requer uma interação rigidamente controlada entre as imunidades inata e adaptativa, para permitir o crescimento e desenvolvimento normais do semi-aloenxerto fetal (Van Rijn *et al*., 2008) uma possível forma de controlar essa interação, pode ser obtida através do uso da VD para obter o sucesso da gestação.

Outra molécula com atividades anti-inflamatória e imunomoduladora é a silibinina (SB), considerada o principal componente mais ativo e abundante da silimarina. A silimarina é um extrato obtido de frutos e sementes de *Silybum marianum* (*milk thistle*), uma das mais antigas

e tradicionais ervas medicinais, utilizada por sua ação hepatoprotetora há mais de dois mil anos (Kren & Walterová, 2005) (Figura 3). A SB apresenta atividades já descritas na literatura tais como, citoproteção, anti-inflamatórias, anti-fibróticas, anti-oxidantes e anti-carcinogênicas (Kren & Walterová, 2005; Agarwal *et al*., 2006; Deep & Agarwal, 2010).

Entre os mecanismos moleculares envolvidos nesses efeitos destaca-se a ação inibidora da SB sobre o fator de transcrição nuclear kappa B (NF-κB). De maneira geral, esse flavonóide parece interferir na cascata de transdução de sinais controlada pelo NF-κB, suprimindo a fosforilação e degradação de IκB, diminuindo assim, a translocação de NF-κB para o núcleo. (Kren & Walterova, 2005; Comelli *et al*., 2007; Esmaeil *et al*., 2017; Matias *et al*., 2019).

A SB tem sido empregada para tratamento de doenças humanas, devido a sua toxicidade extremamente baixa, tornando o tratamento seguro e não prejudicial (Hackett *et al*., 2013). Assim, este flavonoide foi efetivo no tratamento de doenças hepáticas (Tamayo & Diamond, 2007) e como terapia adjuvante no câncer (Deep & Agarwal, 2007). Em modelo experimental de artrite reumatoide, foi demonstrado que a SB inibiu a diferenciação in vitro da subpopulação Th17 (Tong *et al*., 2018). Foi observado em outro estudo, o efeito imunossupressor da SB, sobre a produção de citocinas características das subpopulações Th1/Th2/Th17 e mostrou que a SB inibiu significativamente as citocinas relacionadas ao perfil Th1 (Gharagozloo *et al*., 2013).

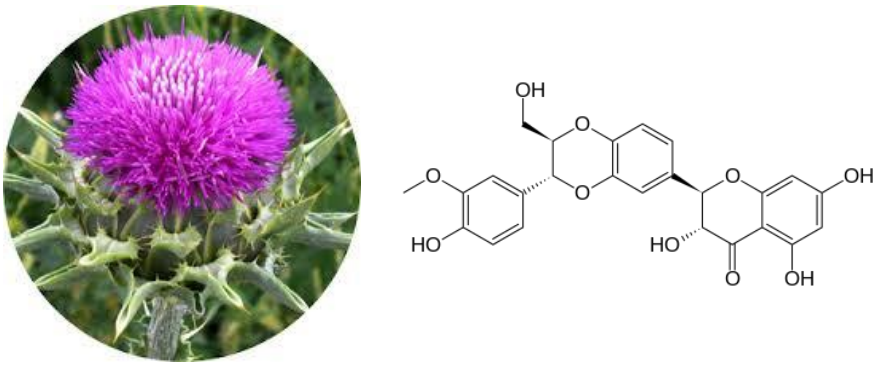

Figura 3. Imagem de *Silybum marianum* e estrutura química da silibinina. (Imagens de Bannwart et al., 2010b e do Google em Abril, 2021).

Em trabalhos anteriores do nosso grupo de pesquisa, observou-se que a SB possui propriedades anti-oxidante e anti-inflamatória, demonstradas pela inibição dose-dependente sobre a liberação do peróxido de hidrogênio (H_2O_2), da produção de TNF-α, IL-1β, TGF-β e de PGE2 por monócitos obtidos de indivíduos saudáveis estimulados com LPS e tratados com 50 μM de SB (Bannwart *et al*., 2010a, b). Além disso, a SB inibiu a ativação de NF-κB e a síntese

das citocinas pró-inflamatórias TNF-α e IL-1β, produzidas por células mononucleares de sangue periférico (PBMCs) de gestantes portadoras de PE (Giorgi *et al*., 2012; Cristofalo *et al*., 2013). Em modelo experimental de PE, induzido em ratas pela inibição da enzima óxido nítrico sintase pela inoculação de L-NAME, observou-se que o tratamento com SB melhorou o desempenho reprodutivo das ratas, normalizou os valores da pressão arterial e o número de plaquetas, além de diminuir as concentrações de proteinúria, IFN-γ, IL-1β, de TNF-α (Souza *et al*., 2012). Em outro estudo mais recente, foi demonstrado que a SB foi capaz de polarizar os monócitos de gestantes com PE, de um perfil M1 (inflamatório) para um perfil M2 (anti-inflamatório) (Gomes et al., 2020).

Frente aos resultados já obtidos pelo nosso grupo de pesquisa e a ausência de estudos que contemplem a ação da SB sobre as células T na PE, espera-se com o presente estudo avaliar se o desbalanço das subpopulações de células T, detectado na PE, poderia ser atenuado através do tratamento in vitro dessas células com a SB, um produto natural com propriedades imunomoduladoras.

Portanto, o entendimento da modulação das subpopulações de células T humanas através de agentes imunomoduladores como a VD e a SB, poderá contribuir para a melhor compreensão do envolvimento da imunidade adaptativa na fisiopatologia da PE e possivelmente, propor formas alternativas para o tratamento dessa importante síndrome da gestação.

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Capítulo I

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Vitamin D modulates the transcription factors of T cell subsets to anti-inflammatory and regulatory profiles in preeclampsia

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Short title: Modulation of T cell subsets by vitamin D in preeclampsia

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Abbreviations: CBA: cytometric bead array; **CD4:** cluster of differentiation 4; **FoxP3:** forkhead box P3; **GATA-3:** GATA binding protein 3; **IL-23R:** receptor of IL-23; **PE:** preeclampsia; **NT:** normotensive; **RORγt:** retinoic acid-related orphan receptor gamma t; **RPMI:** Roswell Park Memorial Institute; **Runx1:** runt-related transcription factor 1; **T-bet:** T box transcription factor; **Th1:** T helper cell 1; **Th2:** T helper cell 2; **Th17:** T helper cell 17; **Treg:** regulatory T cell; **VD:** vitamin D; **VDR:** receptor of vitamin D.

Abstract

Vitamin D (VD) is a multifunctional prohormone and low VD status in pregnancy may contribute to the risk of adverse perinatal outcomes, such as preeclampsia (PE). This molecule may modulate the polarization of T cell subsets during gestation. This study evaluated the in vitro immunomodulatory effect of VD on the gene expression of transcription factors and on cytokine production by T cell subsets. Twenty pregnant women with PE and twenty normotensive (NT) pregnant women were studied. Plasma concentration of VD, 25(OH)D3, was evaluated by chemiluminescence. PBMCs from preeclamptic and NT pregnant women were cultured in the absence or presence of VD to determine gene expression of T-bet (Th1), GATA-3 (Th2), RORγt, and Runx1 (Th17), FoxP3 (regulatory T cell- Treg), and the receptors of VD (VDR) and IL-23 (IL-23R) by quantitative PCR. The concentration of cytokines in the PBMC supernatant culture was determined by cytometric bead array and ELISA immunoassay. The results showed that plasmatic levels of VD were significantly lower in the PE group. The treatment of PBMCs from preeclamptic women with VD induced downregulation of genes related to inflammatory profiles (Th1 and Th17), as well as an increase of anti-inflammatory and regulatory profiles (Th2 and Treg). Thus, VD treatment decreased the release of TNF-α, IL-17, IL-6, and IL-23 while it increased the levels of IL-10 in the PE group. VD induces an immunomodulatory effect in T cell subsets from pregnant women with PE, polarizing these cells to an anti-inflammatory and regulatory profile.

Keywords: PBMCs, preeclampsia, T cell subsets, transcription factors, vitamin D.

1. Introduction

Vitamin D (VD) is a multifunctional prohormone, obtained mainly from sunlight exposition to ultraviolet radiation of energy (Feldman et al., 2014). Its deficiency has been reported around the world among pregnant women and neonates, suggesting that low VD status in pregnancy may also contribute to the risk of adverse perinatal outcomes, including hypertensive disorders such as preeclampsia (PE) (Kiely et al., 2020).

PE is a specific pregnancy syndrome that affects previously normotensive (NT) pregnant women. The clinical parameters that identify this disease are fundamental in the development of arterial hypertension associated with or without proteinuria after the 20th week of gestation or in the first days after delivery. In some pregnant women, hypertension may occur in association with other signs or symptoms in the absence of proteinuria (Tranquilli et al., 2014; Mol et al., 2016; ACOG, 2020). In this disease, there is an exacerbated systemic inflammatory response, which usually occurs without clinical symptoms of microbial infection, described as "sterile inflammation" (Nadeau-Vallée et al., 2016; Brien et al., 2019). Innate and adaptive immune cells, as well as their interactions, are responsible for this inflammation (Chen et al., 2010; Negishi et al., 2020).

During pregnancy, different molecules and immune effectors act in the immune microenvironment, allowing specific maternal tolerance toward the semi-allogeneic fetus (Wang et al., 2020). Furthermore, a balance between adaptive immunity cells is also observed according to the cytokine environment present for the maintenance and success of pregnancy (Ribeiro et al., 2017).

In recent years, the importance of VD over the immune system at the maternal-fetal interface has been increasingly recognized. A review focusing on the role of the VD in normal pregnancy suggests that this hormone may play an important role in the interaction between the maternal decidua and the fetal trophoblast, due to the modulating effect of VD on T

lymphocytes, suppressing the inflammatory response of adaptive immunity and promoting maternal tolerance (Tamblyn, 2015).

Under normal conditions, VD induces an increase in the production of Th2 cytokines, such as IL-10, and the efficiency of Treg lymphocytes. This hormone also inhibits the development of Th17 cells, which play an essential role in fighting certain pathogens and are linked to tissue damage and inflammation (Liu et al., 2015).

VD exerts effects on T cells by binding to the VD receptor (VDR), and modulating the differentiation and activation of $CD4^+$ lymphocytes. Thus, this function is considered of great importance for the adaptive immune system (Palmer et al., 2011; Cyprian et al., 2019). Naïve T cells with $CD4^+$ phenotype can differentiate into Th1, Th2, Th17, and regulatory T cells (Treg) depending on the environmental stimulus they receive (Saito et al., 2007; Saito, 2010). T cell differentiation is regulated by transcription factors T-bet (T box transcription factor), GATA-3 (GATA binding protein 3), RORγt (retinoic acid-related orphan receptor gamma t), and FoxP3 (forkhead box P3) (Zhu et al., 2010). Thus, acting as a modulator of the immune system, VD can regulate the expression of VDR in activated T cells, leading to Treg cell activation, as well as downregulation of Th17 cell generation (Vijayendra Chary et al., 2015).

Figueiredo & Schumacher (2016) reported that Th17 and Treg cells form a complex to maintain homeostasis and interact with other types of cells to orchestrate the immune response during pregnancy. However, in PE, there is an imbalance between the inflammatory and anti-inflammatory profile of T cell subsets (Ribeiro et al., 2017). VD insufficiency and Treg/Th17 imbalance in women with recurrent pregnancy loss may be restored by VD supplementation, suggesting that VD has an inhibitory effect on Th17 cells and contributes to the activation of Treg cells, and may mediate their modulatory effect by upregulating FoxP3 (Ji et al., 2019).

Considering that maternal adaptation to pregnancy requires a controlled balance between T cell subsets (Th1, Th2, Th17, and Treg) to allow normal growth and development

of the semi-allogeneic fetus, the objective of the present study was to evaluate in vitro the immunomodulatory effect of VD on gene expression of transcription factors and on cytokine production by T cell subsets obtained from pregnant women with PE.

2. Materials and methods

2.1. Study population and ethics statement

Twenty pregnant women with PE and twenty NT pregnant women were studied. These pregnant women were attended at the Obstetric Unit of Botucatu Medical School, Sao Paulo State University (UNESP), Brazil. A pregnant woman was diagnosed with PE when, without preexistent hypertension or obstetric or medical complications, arterial hypertension (≥ 140/90 mmHg) was present, with or without associated proteinuria (≥ 300 mg in urine collected over 24 h), or other serious clinical complications, in accordance with the American College of Obstetricians and Gynecologists (ACOG, 2020). The NT group was comprised of pregnant women with an uncomplicated pregnancies and was matched for gestational age with the preeclamptic group. Gestational age was determined by data about the last menstrual period and confirmed by early ultrasound examination (< 12 weeks of gestation). Proteinuria was measured in 24-hour urine by the Technicon RA-XT automation system (Miles Inc., Tarrytown, NY, USA) a colorimetric method utilized in the Clinical Laboratory of Botucatu Medical School, Botucatu, Sao Paulo, Brazil. Exclusion criteria included prior PE, multiple gestations, illicit drug use, and pre-existing medical conditions, such as diabetes, chronic hypertension, acute infectious diseases, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School (protocol number 4.418.043), and all women provided informed consent. For pregnant women aged < 18 years, written informed consent was obtained from their parents or guardians. All experiments reported in this paper were performed under standard health and safety procedures and followed relevant guidelines

and regulations from the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Treatment solution

As a treatment, 100 nM of VD (Sigma-Aldrich, St. Louis, MO, USA) was used in the cultures. This concentration was previously standardized, employing PBMCs from healthy, non-pregnant women. The viability of PE and NT PBMCs after 24h of culture with or without VD was higher than 88% in all experiments, as determined by 0.2% trypan blue exclusion test. The same reagent concentrations were used in all experiments.

2.3. Blood samples

Peripheral blood from pregnant women with PE was collected at the time of disease diagnosis, and from the NT pregnant women at the time they were matched for gestational age with the preeclamptic group. Ten milliliters of blood were collected into plastic tubes containing 10 U/mL ethylenediaminetetraacetic acid (EDTA; Becton Dickinson - BD Vacutainer; BD Biosciences, Franklin Lakes, NJ, USA) and after centrifuging for 10 min at 1500 rpm, the plasma fraction was stored in aliquots at -80 ºC until the analyses were performed.

2.4. Vitamin D plasma determination

VD, 25(OH)D, was determined by the automated chemiluminescent microparticle immunoassay (Abbott®, Santa Clara, California, USA) in the Clinical Laboratory of Botucatu Medical School, UNESP. According to the kit description, the analytical sensitivity was 1.9 ng/mL, coefficient of intra- and inter-assay variation is <10% and reference range is 0.0–160.0 ng/mL. Sufficient values were considered ≥ 30 ng/mL, insufficiency from 21–29 ng/mL and deficiency < 20 ng/mL, according to Bischoff-Ferrari et al. (2006).

2.5. PBMC Culture

After plasma separation, PBMCs were isolated by density gradient centrifugation (density [d] = 1.077) on Ficoll-Paque Premium media (GE Healthcare Bio-Sciences, Uppsala, Sweden), as previously described by Ribeiro et al. (2017). The cells were resuspended in RPMI 1640 culture medium (Sigma-Aldrich), and counting was performed using the 5% Turk dye solution (1:1) and the concentration was adjusted for 1×10^6 PBMCs/mL. PBMCs were treated in the presence or absence of VD for gene expression and cytokine determination, respectively.

2.6. Quantitative PCR

PBMCs obtained from pregnant women with PE and NT pregnant women were incubated in the presence or absence of VD for 4 h at 37 ºC in 5% CO_2. The cells were subjected to analysis of the transcriptional levels of the genes that encode proteins FoxP3, GATA-3, IL-23R, RORγt, Runx1, T-bet and VDR. Total RNA was then extracted using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to the manufacturer's protocol. One μg of RNA was incubated with DNase I Amp Grade (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) and measured by Qubit® Fluorometric Quantitation (ThermoFisher Scientific, Waltham, MA, USA). Subsequently, cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using RT GoTaq® qPCR Master Mix (Promega), according to Matias et al. (2015). A 7500 Fast Real-Time PCR System (Applied Biosystems - ThermoFisher Scientific, Waltham, MA, USA) was used for the analysis. For normalization, GAPDH was used. The primers used in the experiments are listed in Table 1. The differential expression calculation of selected genes was carried out by the data processing method compared with a standard curve. To analyze relative

gene expression, RNA expression levels in all samples were standardized of a single RNA sample, which was set to a value of 100.

Table 1: Primers for transcription factors of T cells, IL-23 and vitamin D receptors and GAPDH

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank
FOXP3	(614) CAGGAAGGACAGCACCCTTT (633)	(726) GGAAGTCCTCTGGCTCTTCG (707)	NM_014009
GATA3	(174) CTCTTCGCTACCCAGGTGAC (193)	(269) ACGACTCTGCAATTCTGCGA (250)	NM_001002295.1
IL23R	(1031) CCTGAAACAGTTCCCCAGGT (1050)	(1122) AGGTGCCCTGTAGAGATGGA (1103)	NM_144701
RORC	(363) CATGTCCCGAGATGCTGTCA (382)	(473) GGTTCCTGTTGCTGCTGTTG (454)	NM_005060.3
RUNX1	(1943) CCCGGAGGGAAACTGTGAAT(1962)	(2092) GAGTTGCATCCCTCCTCTCC (2073)	NM_001754
TBX21	(906) GGATGCGCCAGGAAGTTTCA (925)	(993) TGGAGCACAATCATCTGGGT (974)	NM_013351
VDR	(590)TGGAGACTTTGACCGGAACG(609)	(704)GCTTCGCCTGAAGAAGCCT(686)	NM_001017536.1
GAPDH	(684) CGTGGAAGGACTCATGACCA(703)	(801) GGCAGGGATGATGTTCTGGA(782)	NM_002046.4

Note: numbers indicate nucleotide positions in the corresponding transcripts

2.7. Cytokine determinations

For the quantification of the cytokines in the supernatants, the PBMCs were cultured with or without VD for 24h. After the cell treatments, IL-6, IL-10, and TNF-α levels were determined by CBA using a Th1/Th2/Th17 Cytokine Kit (Becton Dickinson - BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. The sensitivity limit was 2.4 pg/mL, 4.5 pg/mL and 3.8 pg/mL for IL-6, IL-10, and TNF-α, respectively. IL-17 and IL-23 determination were analyzed by ELISA by the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), which was used according to the manufacturer's protocol. The assay sensitivity limits of the kits were 15.6 pg/mL for IL-17 and 16.3 pg/mL for IL-23.

2.8. Statistical analysis

Data from the clinical and laboratorial characteristics of pregnant women were analyzed by non-parametric method Mann-Whitney U test. The results of gene expression were evaluated by Kruskal-Wallis test, followed by Dunn's multiple comparison test, as well as, the results of

cytokines. Correlations were performed using Spearman's correlation coefficient. The statistical program GraphPad Prism version 6.01 (GraphPad, CA, USA) was used, and the statistical significance was set at $p < 0.05$.

3. Results

3.1. Clinical and laboratorial characteristics

The analysis of clinical and laboratorial characteristics of pregnant women with PE and NT pregnant women are shown in Table 2. There was no statistical difference between the groups evaluated, regarding the parameters of age and gestational age. However, the values of systolic and diastolic blood pressure, as well as proteinuria levels, were significantly higher in the group of pregnant women with PE than in the NT pregnant women. The VD concentration was significantly lower in pregnant women with PE compared to the NT group.

Table 2. Clinical and laboratory characteristics of pregnant women with preeclampsia (PE) and normotensive (NT) pregnant women

Characteristics	Pregnant women with PE (n = 20)	NT pregnant women (n = 20)
Age, years	27 (17–45)	31 (17–39)
Gestational age, weeks	34 (24–38)	34 (24–39)
Systolic blood pressure, mmHg	150***** (140–180)	105 (95–110)
Diastolic blood pressure, mmHg	100***** (90–120)	65 (60–70)
Proteinuria, mg/24h	370***** (300–1.560)	< 300
Vitamin D [25(OH)D], ng/mL	26.3* (14.2–35.3)	30.1 (20–50.5)

Note: Results are expressed in the median with the minimum and maximum values in parentheses
* ($p < 0.05$) *vs.* NT pregnant women (Mann-Whitney U test).

3.2. Gene expression of transcription factors IL-23R and VDR in PBMCs from preeclamptic and NT pregnant women

Relative quantification for the expression of FOXP3, GATA3, IL23R, RORC, RUNX1, TBX21, and VDR genes was performed in PBMCs from 20 pregnant women with PE and 20 NT pregnant women cultivated in the presence or absence of VD.

The endogenous gene expression of T-bet (A), RORγt (B), Runx1 (C), and IL-23R (D) was significantly increased in preeclamptic women when compared to NT pregnant women (Fig. 1A–D). Treatment of PBMCs from preeclamptic women with VD led to significantly lower expression of the genes related to inflammatory transcription factors, in addition to a significant decrease in IL-23R mRNA.

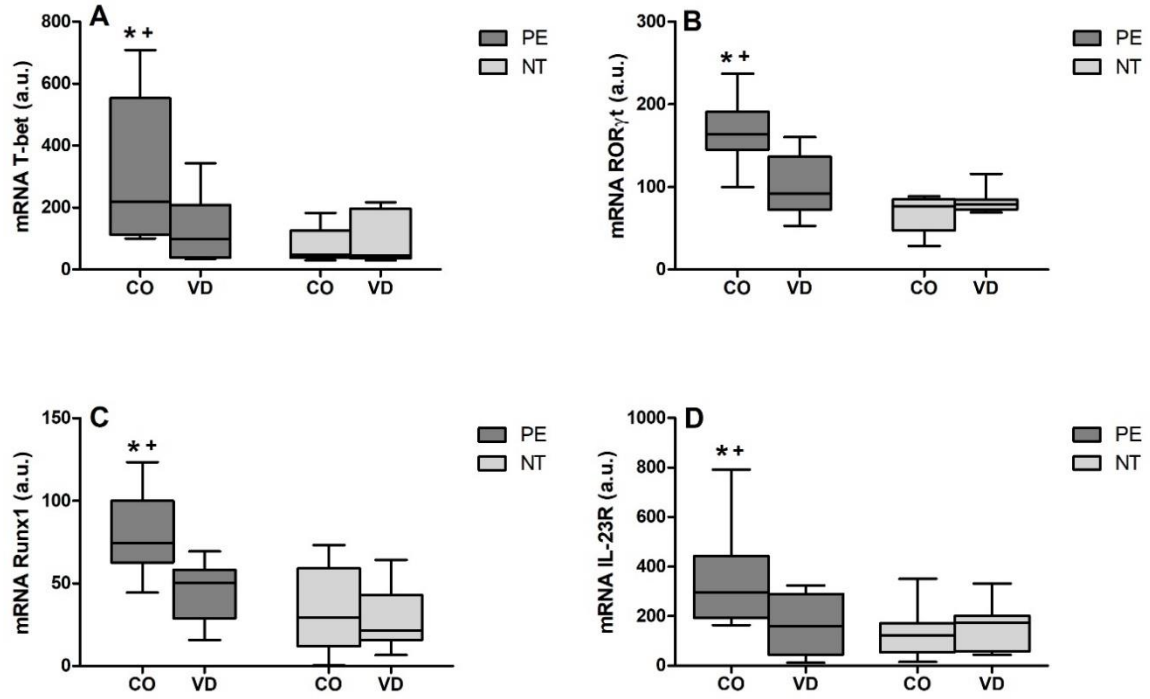

Fig. 1. Gene expression of T-bet (A), RORγt (B), Runx1 (C) and IL-23R (D) in peripheral blood mononuclear cells (PBMCs) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) by qPCR. Results are expressed as median (horizontal line), 25th and 75th percentile (box), and range (whiskers). * ($p < 0.05$) *vs* PE VD; + ($p < 0.05$) *vs* NT Co (Kruskal-Wallis test).

The anti-inflammatory and regulatory transcription factors, GATA-3 and FoxP3, as well as VDR are presented in Fig. 2. Compared with NT pregnant women, PBMCs from preeclamptic women showed a significantly lower basal expression of these genes (Fig. 2A–C). After treatment of these cells with VD, there was a significant increase in the expression of GATA-3, FoxP3 and, VDR in preeclamptic women. No significant differences were detected between the control and VD-treated cultures in the NT group.

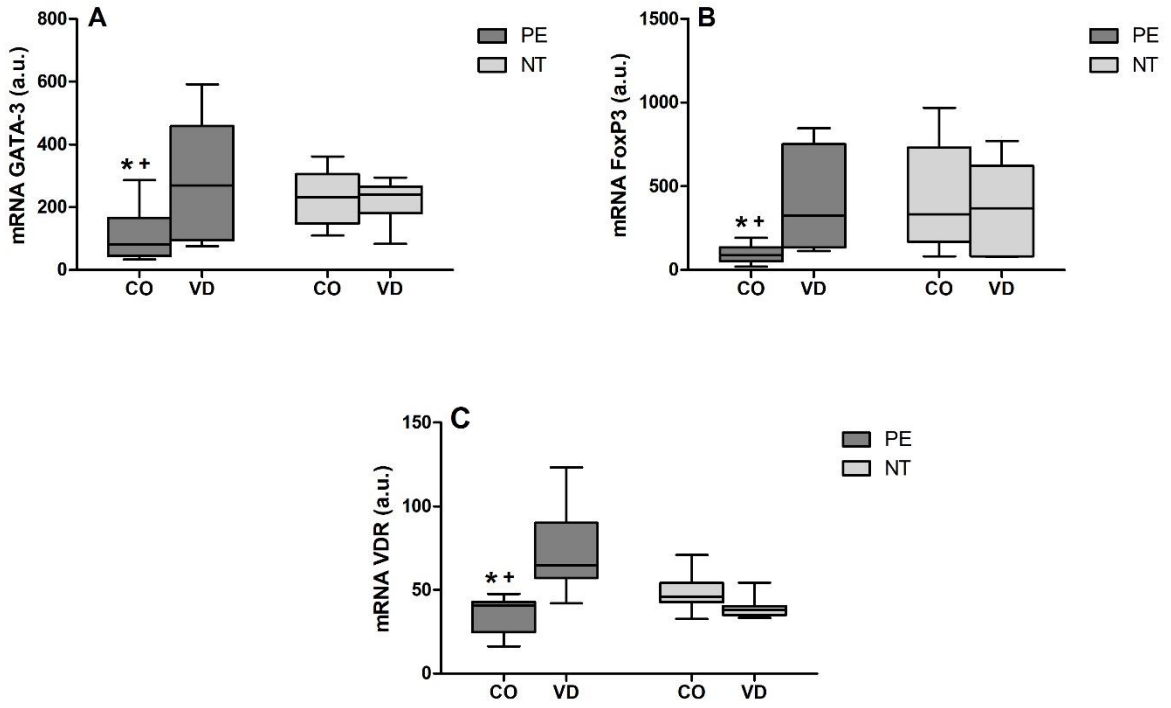

Fig. 2. Gene expression of GATA-3 (A), FoxP3 (B), and VDR (C) in peripheral blood mononuclear cells (PBMCs) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) by qPCR. Results are expressed as median (horizontal line), 25th and 75th percentile (box), and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

3.3. Correlations between the transcription factors of T cell subsets and VDR with the plasmatic levels of VD

Figure 3 shows the correlations between the inflammatory transcription factors, T-bet, RORγt and Runx1 of T cells with the plasma concentration of VD. There were negative correlations between VD x T-bet ($r = -0.59$, $p < 0.05$); VD x RORγt ($r = -0.51$, $p < 0.05$) and VD x Runx1 ($r = -0.59$, $p < 0.05$) in pregnant women with PE. Likewise, negative correlations were also observed in the NT group: VD x T-bet ($r = -0.50$, $p < 0.05$); VD vs RORγt ($r = -0.49$, $p < 0.05$) and VD x Runx1 ($r = -0.45$, $p < 0.05$) (Fig. 3A, 3B and 3C, respectively).

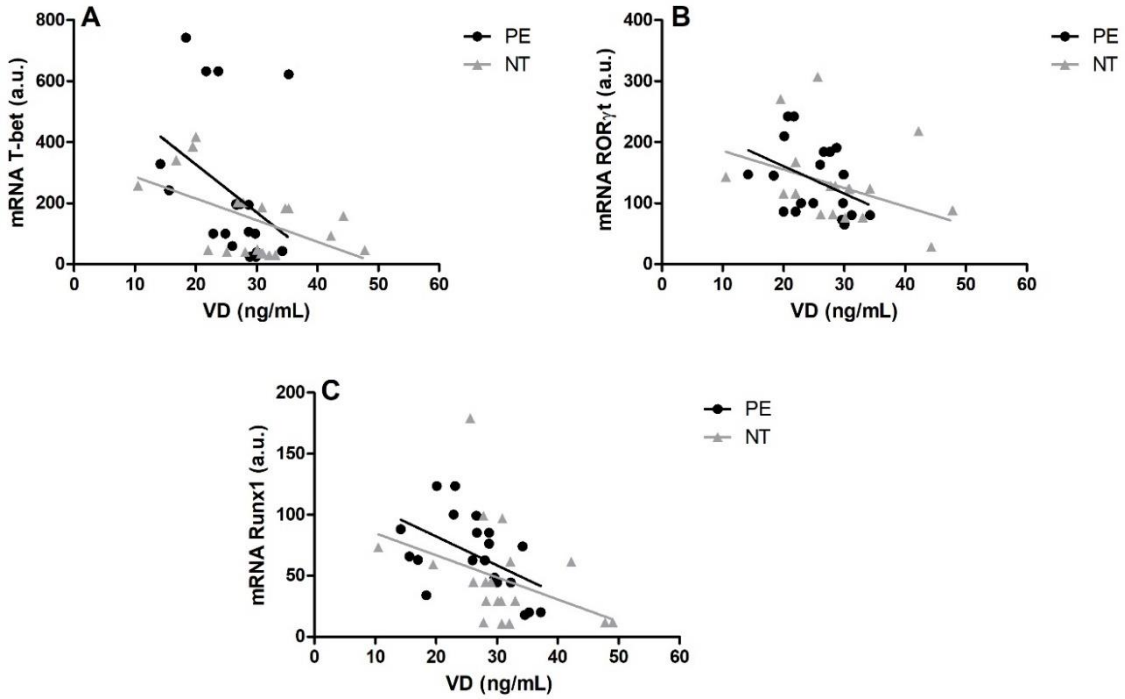

Fig. 3. Negative correlations between mRNA of T cell transcription factors of inflammatory profiles with plasma levels of vitamin D (VD) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women. (A) T-bet; (B) RORγt; (C) Runx1. The correlation was determined by Spearman's correlation coefficient. ($p < 0.05$).

Figure 4 represents the positive correlations between the T cell transcription factors of the anti-inflammatory, regulatory profiles and the VDR with the plasmatic concentration of VD in the PE and NT groups, respectively. Results were as follows: VD vs GATA-3 (Fig. 4A): ($r = 0.44$, $p < 0.05$; $r = 0.53$, $p < 0.05$), VD vs FoxP3 (Fig. 4B) ($r = 0.54$, $p < 0.05$; $r = 0.47$, $p < 0.05$) and VD vs VDR (Fig. 4C) ($r = 0.40$, $p < 0.05$; $r = 0.60$, $p < 0.05$).

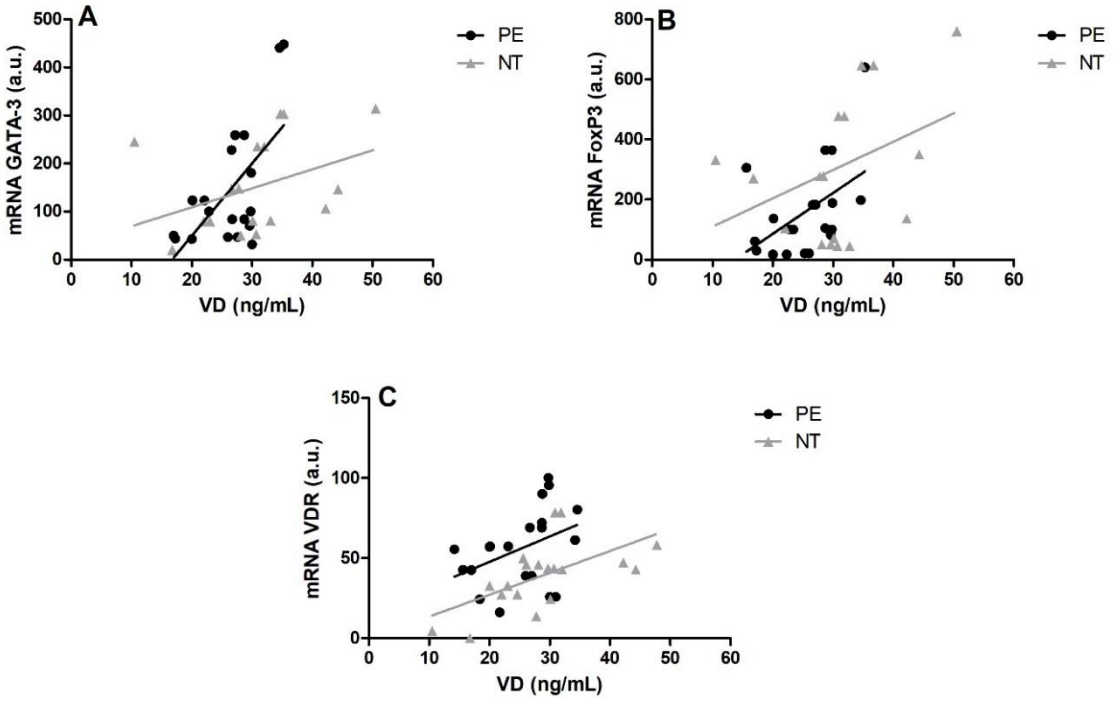

Fig. 4. Positive correlations between mRNA of T cell transcription factors of anti-inflammatory, regulatory profiles and vitamin D receptor (VDR) with plasma levels of vitamin D (VD) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women. (A) GATA-3; (B) FoxP3; (C) VDR. The correlation was determined by Spearman's correlation coefficient. ($p < 0.05$).

3.4. Cytokine determination in PBMC culture supernatants from PE and NT pregnant women

The concentrations of TNF-α, IL-17, IL-6, IL-23, and IL-10 determined in the supernatants of PBMCs obtained from 20 pregnant women with PE and 20 NT pregnant women, treated with or without VD, are shown in Fig. 5. It was observed that the basal concentrations of TNF-α, IL-17, IL-6, and IL-23 (Fig. 5A–D) produced by the PBMCs were significantly higher, while the concentration of IL-10 (Fig. 5E) was lower in pregnant women with PE, when compared to NT pregnant women. VD treatment significantly decreased the release of inflammatory cytokines (Fig. 5A–D), while the levels of anti-inflammatory cytokine IL-10 (Fig. 5E) increased significantly in the PE group.

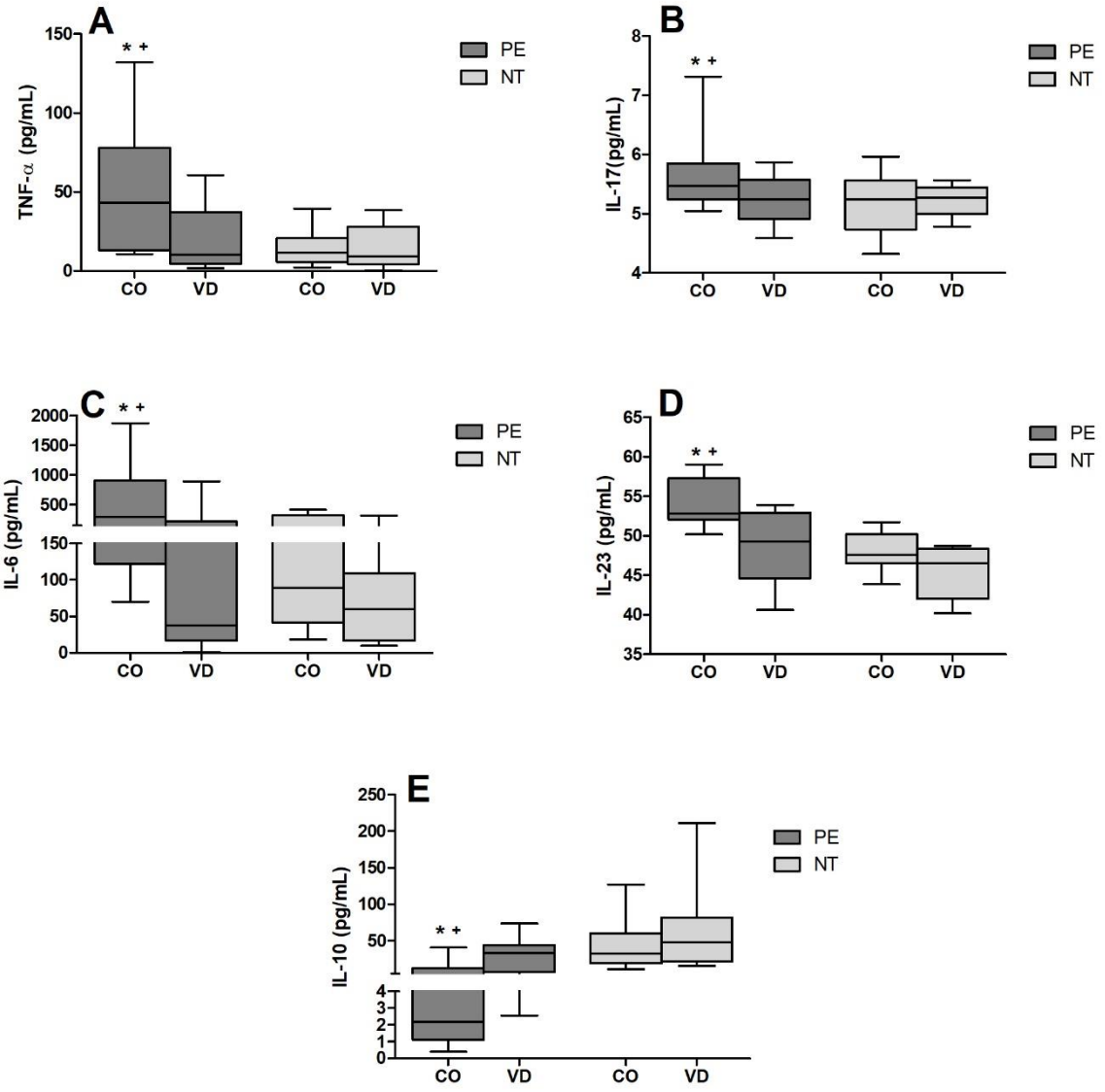

Fig. 5. The concentration of TNF-α (A), IL-17 (B), IL-6 (C), IL-23 (D) and IL-10 (E) detected in the supernatants of peripheral blood mononuclear cells (PBMCs) of 20 pregnant women with preeclampsia (PE) and 20 normotensive pregnant women (NT) cultured in vitro in the absence (Co) or presence of vitamin D (VD) for 24 hours by cytometric bead array (CBA) (A, C, and E) and ELISA (B and D) techniques. Results are expressed as median (horizontal line), 25th and 75th percentile (box), and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co. (Kruskal-Wallis test).

4. Discussion

The immunomodulatory effect of VD on the cells of adaptive immunity occurs not only by suppressing Th1 and Th17 cell activation, but also by modulating Th2 and Treg cells (Sassi et al., 2018). The present study showed that in vitro treatment of PBMCs from preeclamptic women with VD induced the downregulation of genes related to inflammatory profiles (Th1

and Th17), as well as an increase in transcription factors of anti-inflammatory and regulatory profiles (Th2 and Treg). These data were consistent with the current literature, showing that the effects of VD on adaptive immunity aroused great interest from the scientific community mainly due to its function of modulating the activation and differentiation of lymphocytes (Palmer et al., 2011; Cyprian et al., 2019). According to Prietl et al. (2013), VD exerts effects on the innate and adaptive immune systems, generating a more tolerogenic immune status, particularly due to its regulatory activities on the inflammatory response.

In the present study, we detected lower plasmatic levels of VD in preeclamptic women compared to NT pregnant women. There are reports in the literature showing that pregnant women with PE have VD deficiency (Xu et al., 2014; Mohaghegh et al., 2015, in addition to the occurrence of premature birth (Miliku et al., 2016; von Websky et al., 2018). Other studies have shown an association between this deficiency and the risk of developing PE (Aghajafari et al., 2013; Bodnar et al., 2014; Akbari et al., 2018; de Souza & Pisani, 2020, Yuan et al., 2021), suggesting that VD supplementation can protect the maternal organism and reduce the risk of developing PE (Fu et al., 2018; Aguilar-Cordero et al., 2020). Despite this evidence, further clinical studies using VD as a treatment for pregnancy syndromes should be conducted, based on the patients' clinical and personal histories, as well as ethnic and racial issues (Dovnik et al., 2018; Cyprian et al., 2019).

PBMCs from pregnant women with PE showed significantly higher basal or endogenous gene expression of T-bet and RORγt, and lower GATA-3 and FoxP3, confirming the results obtained in a previous study (Ribeiro et al., 2017). Other studies also reported that preeclamptic women have increased RORγt mRNA levels and decreased FoxP3 levels, confirming an increase in the Th17 inflammatory profile in this obstetric pathology (Cao et al., 2015; Eghbal-Fard et al., 2019). The balance between Treg and Th17 cells is important in a successful pregnancy and their imbalance can cause pregnancy diseases, such as PE (Hosseini

et al., 2018). In addition, a recent finding showed that the levels of GATA-3 mRNA, characteristic of the anti-inflammatory profile Th2, are reduced in pregnant women diagnosed with PE, suggesting that decreased levels of this mRNA seem to be associated with PE, preceding the clinical diagnosis (Whigham et al., 2019). Our results corroborate with the authors' data, showing that preeclamptic women have less endogenous gene expression of GATA-3 mRNA when compared to NT pregnant women. In agreement with the literature, this data confirm there is a deficiency of the anti-inflammatory and regulatory T cell subsets in pregnancies with PE.

Our present study brings new information, showing that VD plays an immunomodulatory role on the transcription factors of T cell subsets, demonstrated not only by a decrease of T-bet, RORγt, Runx1, and IL-23R, but also by an increase of GATA-3, FoxP3, and VDR mRNA expression. The importance of VD's role in the acquired immune response may be demonstrated by the negative correlation between plasmatic VD levels and T-bet, RORγt, and Runx1, as well as a positive correlation between this hormone levels and GATA-3, FoxP3, and VDR gene expression, both in preeclamptic and NT groups.

It is known that Runx proteins can regulate gene transcription in a context-dependent manner, binding to other transcription factors to form co-activating or co-repressor complexes. Zhang et al. (2008) described the influence of Runx1 on Th17 differentiation by inducing, binding, and acting together with RORγt during IL-17 transcription. The authors demonstrated that Runx1 binding to FoxP3 led to the inhibition of IL-17 expression, proposing that there is a complex considered a chief regulator of the proinflammatory (Th17 cells) and regulatory (Treg cells) immune responses by interactions among RORγt, Foxp3, and Runx1. To our knowledge, this is the first study reporting the effect of VD on Runx1 with PE, indicating that this hormone decreased the expression of Runx1 in an attempt to re-establish the balance of T cell subsets

and production of inflammatory cytokines in pregnant women with PE, through the Runx1, RORγt and FoxP3 complex.

In preeclamptic women, increased endogenous production of TNF-α, IL-17, and IL-6, and the decrease in IL-10 level confirmed the inflammatory cytokine characteristics of T cell subsets in PBMCs obtained from these women. Moreover, we also observed in the PE group increased basal levels of IL-23, which is a finding in accordance with Eghbal-Fard et al. (2019), who detected higher basal levels of IL-23 in supernatant obtained from PBMC cultures of preeclamptic women in relation to healthy control pregnant women. Treatment of the PBMCs with VD led to a reduction in inflammatory cytokine production, such as TNF-α, IL-6, IL-17 and IL-23, associated with higher concentration of IL-10, and showing a polarization of adaptive immune response to an anti-inflammatory or regulatory profile. The TNF-α and IL-6 decrease after treatment of PBMCs with VD is in agreement with the study by Noyola-Martínez et al. (2013) that showed downregulation of these cytokines by VD in cultured cells obtained from PE women. The authors also mentioned that the exacerbated pro-inflammatory cytokines and decreased VD production in PE pregnant women might be a consequence, rather than a cause of the disease. According to the literature, increased levels of pro-inflammatory cytokines could be related to the low levels of VD in maternal serum and placenta and might be considered as a reason for adverse pregnancy outcomes (Noyola-Martínez et al., 2013; Barrera et al., 2015; Motamed et al., 2019). Furthermore, this hormone can modulate T lymphocytes by decreasing the proliferation and production of cytokines such as IL-17, and it seems reasonable to hypothesize that VD is considered a selective/critical regulator of the immune response (Sheikh et al., 2018). Muyayalo et al. (2019) also demonstrated correlations between low plasmatic VD levels, imbalance of the Th17/Treg subsets, and increased concentrations of IL-6, and suggested that VD supplementation may protect the maternal organism through its modulating role in the immune response that is altered in PE. In addition, the mRNAs and cytokines related to each T

cell subset confirms that the maternal systemic inflammation observed in PE may be related to the imbalance of adaptive immune cells (Eghbal-Fard et al., 2019).

Our results also showed that PBMCs from preeclamptic women treated with VD enhanced IL-10 production to similar levels of endogenous production by cells of NT pregnant women, showing the immunomodulatory effect of this hormone. According to Cubro et al. (2018), dysregulation of the immune system, particularly related to IL-10 production, can lead to the development and progression of PE, since the authors observed an increase in IL-10 concentration in the groups of PE and NT pregnant women after the treatment of PBMCs with VD.

Literature states that VD is widely recognized for its immunomodulatory role in its active form 1,25-(OH)2-D3, also called calcitriol, which binds to VDRs inside cells (Yang et al., 2013). Therefore, the results obtained in this study showed that pregnant women with PE show a decrease in the gene expression of VDRs, preventing this hormone from playing its modulatory role in the inflammatory environment of PE. Another important receptor that was evaluated was IL-23R, which allows the binding of IL-23 released by dendritic and antigen-presenting cells to activate Th17 cells and contribute to the maintenance of this phenotype (Toussirot, 2012; Puig, 2017; Egeberg et al., 2020). In our study, the treatment of PBMCs with VD showed that this hormone decreased the expression of IL-23R in preeclamptic women. As a modulator of immunity, VD can inhibit the transcription of IL-23R, RORγt, and consequently, the activation of Th17 cells (Hamzaoui et al., 2014). Therefore, our hypothesis is that this hormone exerted immunomodulatory effects by downregulating the inflammatory profile due to decreased mRNA levels of IL-23R, RORγt, and Runx1 through the IL-23/IL-23R/Th17 signaling axis, and by polarizing T cells to anti-inflammatory and regulatory profiles. Another study showed that the low VD concentrations in decidual tissues were associated with recurrent

spontaneous abortion, and VD negatively correlated with IL-23, suggesting that VD might play a vital role in influencing the IL-23/IL-17 axis (Li et al., 2017).

In conclusion, the results obtained in the present study showed that in vitro treatment with VD induces an immunomodulatory effect on T cell subsets from pregnant women with PE, polarizing these cells to an anti-inflammatory and regulatory profile. The systemic inflammatory response observed in PE might be due to an imbalance in the homeostasis of the immune system, caused by decreased levels of modulatory factors, such as VD, capable of regulating inflammation. Further studies are recommended to indicate VD as a possible hormone that can be used as an alternative therapy for this important gestational syndrome.

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Author contributions

V.R.R. and M.T.P conceived the idea, designed the experiments, analyzed the data, and wrote the paper. J.C.P. selected pregnant women for the study. V.R.R., M.R.V., P.R.N. and M.L.M. performed experiments. All authors reviewed the manuscript and approved the final version for publication.

Declaration of Competing Interest

The authors declare no commercial or financial conflict of interest.

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Figure legends

Fig. 1. Gene expression of T-bet (A), RORγt (B), Runx1 (C) and IL-23R (D) in peripheral blood mononuclear cells (PBMCs) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) by qPCR. Results are expressed as median (horizontal line), 25^{th} and 75^{th} percentile (box), and range (whiskers). * ($p < 0.05$) *vs* PE VD; + ($p < 0.05$) *vs* NT Co (Kruskal-Wallis test).

Fig. 2. Gene expression of GATA-3 (A), FoxP3 (B), and VDR (C) in peripheral blood mononuclear cells (PBMCs) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) by qPCR. Results are expressed as median (horizontal line), 25th and 75th percentile (box), and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 3. Negative correlations between mRNA of T cell transcription factors of inflammatory profiles with plasma levels of vitamin D (VD) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women. (A) T-bet; (B) RORγt; (C) Runx1. The correlation was determined by Spearman's correlation coefficient. ($p < 0.05$).

Fig. 4. Positive correlations between mRNA of T cell transcription factors of anti-inflammatory, regulatory profiles and vitamin D receptor (VDR) with plasma levels of vitamin D (VD) obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women. (A) GATA-3; (B) FoxP3; (C) VDR. The correlation was determined by Spearman's correlation coefficient. ($p < 0.05$).

Fig. 5. The concentration of TNF-α (A), IL-17 (B), IL-6 (C), IL-23 (D) and IL-10 (E) detected in the supernatants of peripheral blood mononuclear cells (PBMCs) of 20 pregnant women with preeclampsia (PE) and 20 normotensive pregnant women (NT) cultured in vitro in the absence (Co) or presence of vitamin D (VD) for 24 hours by cytometric bead array (CBA) (A, C, and E) and ELISA (B and D) techniques. Results are expressed as median (horizontal line), 25th and 75th percentile (box), and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p <0.05$) vs NT Co. (Kruskal-Wallis test).

Capítulo II

Os resultados referentes à esse Capítulo serão enviados para Publicação em Periódico Internacional

Immunomodulatory effect of vitamin D on the activators of transcription and transcription factors of $CD4^+$ T cell subsets in pregnant women with preeclampsia

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Short title: Vitamin D acts on $CD4^+$ T cell subsets in preeclampsia.

Abbreviations: Alexa 647: Alexa Fluor 647**; APC-Cy7:** fluorochrome allophycocyanin conjugated to cyanine 7; **BB515:** bright blue fluorochrome 515; **CD4:** cluster of differentiation 4; **CD25:** cluster of differentiation 25; **CD127:** cluster of differentiation 127; **FoxP3:** forkhead box P3; **GATA-3:** GATA binding protein 3; **IFN-γ:** interferon gama; **IL:** interleukin; **MFI**, median fluorescence intensity**; NT:** normotensive; **PE:** fluorochrome phycoerythrin; **PerCP-Cy5.5:** fluorochrome pyridine protein chlorophyll conjugated with cyanine dye; **PE-Cy7:** fluorochrome phycoerythrin conjugated with cyanine dye; **RORγt:** retinoic acid-related orphan receptor gamma t; **RPMI:** Roswell Park Memorial Institute; **STAT:** Signal transducer and activator of transcription**; T-bet:** T box transcription factor; **Th1:** T helper cell 1; **Th2:** T helper cell 2; **Th17:** T helper cell 17; **Treg:** regulatory T cell; **VD:** vitamin D; **VDR:** receptor of vitamin D.

Abstract

An imbalance of $CD4^+$ T cell subsets present in a maternal-fetal interface is observed in preeclampsia (PE), considered one of the most common obstetric syndromes of pregnancy. A possible way to control this imbalance is with the use of vitamin D (VD). This study evaluated the in vitro modulatory effect of VD on T cells, by determination of signal transducers and activators of transcription (STATs) as well as transcription factors characteristic of each $CD4^+$ T cell subset. Twenty preeclamptic and 20 normotensive pregnant women (NT) were studied. The plasma concentration of VD was assessed by chemiluminescence. Peripheral blood mononuclear cells (PBMCs) were cultured in the absence or presence of VD to analyze the expression of activators of transcription and transcription factors by flow cytometry. Cytokines related to T cell subsets were measured in the supernatant culture by ELISA. The results showed that the plasma levels of VD were significantly lower in the PE group. Treatment of cells with VD decreased STAT1/STAT4/T-bet, and STAT3/RORγt, characteristic of the inflammatory profiles Th1 and Th17, and increased STAT6/GATA-3, and STAT5/FoxP3 of anti-inflammatory and regulatory profiles respectively. In addition, treatment with VD decreased the release of inflammatory cytokines, while increased the levels of interleukin (IL)-10 and transforming growth factor-beta (TGF-β) in the PE group. This hormone exerts immunomodulatory effects on the JAK/STAT signaling pathway, shifting the inflammatory profile by Th1/Th17 cells to an anti-inflammatory and regulatory Th2/Treg profile, and can be suggested as a promising strategy in the regulation systemic inflammatory response in PE.

Keywords: activators of transcription; preeclampsia, T cell subsets, transcription factors, vitamin D.

1. Introduction

The pregnancy has various immune effectors, molecules, and a complex network participating in the immune microenvironment to establish a specific maternal tolerance toward the semi-allogeneic fetus. An important complex network of cells is observed between T cells and their subsets [1].

Naïve T cells with $CD4^+$ phenotype can differentiate into the subsets of T cells, such as Th1, Th2, Th17, and regulatory T cells (Treg), depending on the stimulus by the microenvironment induced by cytokines [2]. Besides that, the differentiation of T cell subsets is also related to the signal transducers and transcription activators (STATs) and with the transcription factors characteristic of each subset [3, 4].

Th1 cells specific transcription factor is T-bet (transcription factor T box), and its activators of transcription are STAT1/STAT4 [5, 6]. GATA-3 (GATA 3 binding protein), and STAT6 are required for the signaling of Th2 cells [6, 7]. For Th17 cells, RORγt (retinoic acid receptor-related orphan receptor γ), and STAT3 are necessary for induction of this profile [4, 8], and FoxP3 (forkhead box P3) and STAT5 promote the differentiation of Treg cells [7, 9]. The balance between these adaptive immune cells is essential for the maintenance and success of pregnancy [2].

An imbalance of Th17/Treg cells present in a maternal-fetal interface is observed in preeclampsia (PE). This disease is considered one of the most common obstetric syndromes of gestation [10, 11], and characterized by the increase of arterial hypertension associated with or without proteinuria after the 20^{th} week of gestation or in the first days after delivery. Hypertension in association with other signs or symptoms in the absence of proteinuria may occur in some pregnant women [12-14]. Thus, Th17 inflammatory and Treg cells are considered important cells of the adaptive immunity, whose imbalance is associated with pregnancy complications [15].

Th17 lymphocytes are described for participating in a predominant Th1/Th17 over Th2/ Treg phenotype in chronic inflammatory conditions. These cells have their differentiation induced by interleukin (IL)-6 associated with transforming growth factor-beta (TGF-β) and express cytokines of the IL-17 family [16, 17].

The anti-inflammatory and immunomodulatory properties provide Treg cells with a potent ability to support maternal vascular adaptation and placental development, suppresses inflammation, and maintains the tolerance of the fetus [18]. Treg cells are differentiated in the presence of TGF-β and secrete an important anti-inflammatory cytokine, IL-10, which can suppress the production of several pro-inflammatory cytokines, and stimulate the differentiation and proliferation of Treg cells [15, 17]. $CD4^+$ $CD25^+$ $CD127^-$ cells are the phenotype of Treg cells defined in the peripheral blood, mostly are $FoxP3^{+,}$ and can effectively suppress the proliferation of $CD4^+$ $CD25^-$ T cells [19]. Furthermore, these cells are considered important players during pregnancy and PE [20].

In pregnancy with PE occurs a decrease in Treg cells, while Th17 cells, as well as the ratio of Th17/Treg cells are increased. On the other hand, in a healthy pregnancy, Treg cells are higher in the first and second trimesters, decreasing thereafter until the end of gestation, whereas Th17 cells and Th17/Treg ratio are maintained decreased or in normal levels [15, 16].

The literature suggests that more therapeutic options should be investigated to control Th cell activation, and developed for treatment of obstetrical complications [1]. A possible form to obtain this control is with the use the vitamin D (VD).

The VD is considered a pro-hormone, and its main source is obtained from exposure to the skin to ultraviolet rays (UVB) [21]. Particularly due to its regulatory activities on the inflammatory response, VD is able to generate a more tolerogenic immune status [22, 23]. The active form of VD [1,25(OH)2D] produced by monocytes/macrophages results in a change of immune status from pro-inflammatory to tolerogenic state [24].

VD has a variety of cells under its effects, including cells of the immune system, especially $CD4^+$ T cells [25]. Several studies that evaluated the effect of VD suggest that this hormone acts as an immunomodulator, not only by its ability to suppress Th1 cells and Th17 activation, but also by upregulating Th2 cells, and increasing Treg cells [26-30].

It has been suggested that VD deficiency may contribute to the development of PE due to the lack of inflammation control in this pathology [31]. Considering these observations, the present study proposed to evaluate the in vitro modulatory effect of VD on T cell subsets by analysis of the activators of transcription and transcription factors characteristic of each $CD4^+$ T cell, concomitant with related cytokines in PE and NT pregnant women. This knowledge will contribute to a better understanding of the involvement of adaptive immune response in the pathophysiology of PE, and possibly could propose a new alternative for the treatment of this pregnancy syndrome.

2. Materials and methods

2.1. Research patients and ethics statement

This study comprised forty pregnant women admitted to the Obstetric Unit of Botucatu Medical School, Sao Paulo State University, (UNESP), Brazil. Twenty pregnant women with PE were select in accordance with the American College of Obstetricians and Gynecologists [14], and it was defined, as the onset of a persistently elevated blood pressure (≥ 140/90 mmHg) associated or not with proteinuria (≥ 300 mg in urine collected over 24 h), and other serious clinical complications after 20 weeks of gestation. The group of twenty normotensive (NT) pregnant women was composed by uncomplicated pregnancy that remained non-preeclamptic, and were matched for gestational age with the preeclamptic group. Gestational age was determined by data about the last menstrual period and confirmed by early ultrasound examination (< 12 weeks of gestation). Proteinuria was measured in 24-hour urine by the

Technicon RA-XT automation system (Miles Inc., Tarrytown, NY, USA) a colorimetric method utilized in the Clinical Laboratory of Botucatu Medical School, Botucatu, Sao Paulo, Brazil. Exclusion criteria included prior PE, multiple gestations, illicit drug use, and pre-existing medical conditions, such as diabetes, chronic hypertension, acute infectious diseases, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School (protocol number 4.418.043), and all women provided informed consent. For pregnant women aged < 18 years, written informed consent was obtained from their parents or guardians. All experiments reported in this paper were performed under standard health and safety procedures and followed relevant guidelines and regulations from the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Blood collection and plasma determination

Peripheral blood samples from PE and NT pregnant women were collected by venipuncture from the antecubital vein. Ten milliliters of blood were put into plastic tubes containing 10 U/mL ethylenediaminetetraacetic acid (EDTA; Becton Dickinson - BD Vacutainer; BD Biosciences, Franklin Lakes, NJ, USA) and after centrifuging for 10 min at 1500 rpm, the plasma fraction was stored in aliquots at -80 ºC until the analysis. VD [25(OH)D] was determined by the automated chemiluminescent microparticle immunoassay (Abbott®, Santa Clara, California, USA) in the Clinical Laboratory of Botucatu Medical School, UNESP.

2.3. PBMCs Culture and treatment of VD

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (density [d] = 1.077) on Ficoll-Paque Premium media (GE Healthcare Bio-Sciences, Uppsala, Sweden), as previously described by Ribeiro *et al.* [2]. The cells were resuspended in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA), and

counting was performed using the 5% Turk dye solution (1:1 v/v) and the concentration was adjusted as needed for each of the following experiments. PBMCs were treated with or without 100 nM of VD (Sigma-Aldrich). This concentration was previously standardized, employing PBMCs from healthy, non-pregnant women. The viability of PE and NT PBMCs after 24h of culture with or without VD was higher than 88% in all experiments, as determined by 0.2% trypan blue exclusion test. The same reagent concentrations were used in all experiments. The treatment of PBMCs in the presence of VD was performed for 30 min and 24h for protein expression and cytokine determination respectively.

2.4. Cytokine production by PBMC treated with VD

The production of the cytokines were measured in the supernatants of PBMCs ($1x10^6$ cells/ mL) from preeclamptic and NT pregnant women cultured with or without VD after 24h. Then, IFN-γ, IL-6, IL-10, IL-17, IL-22, IL-23, TGF-β, and TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA), employing specific commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), which was used according to the manufacturer's instructions. Absorbance was measured using a microplate reader, and the concentration of cytokines was calculated according to the standard curve. The assay sensitivity limits of the kits were: 6.4 pg/mL for IFN-γ, 9.4 pg/mL for IL-6, 31.2 pg/mL for IL-10, 15.6 pg/mL for IL-17, 31.2 pg/mL for IL-22, 16.3 pg/mL for IL-23, 15.4 pg/mL for TGF-β, and 15.6 pg/mL for TNF-α.

2.5. Expression of activators of transcription and transcription factors in $CD4^+$ T cells

The expression of transcription activators for Th1 (STAT1, STAT4), Th2 (STAT6), Th17 (STAT3) and Treg (STAT5) cells and intracellular factors of transcription for Th1 (T-bet), Th2 (GATA-3), Th17 (RORγt) and Treg (FoxP3) was evaluated after PBMCs culture from pregnant women with PE and NT pregnant women in the presence or absence of VD (Sigma-

Aldrich) for 30 minutes. The concentration was adjusted to $2x10^5$ cells/ mL and the cells were distributed in tubes for flow cytometer (BD Biosciences). Cells were incubated with BD Biosciences antibodies with respective fluorochromes: anti-CD3 (APC-H7), anti-CD4 (PerCP-Cy5.5), anti-CD25 (BB515) and anti-CD127 (PE-Cy7) for 20 min, in the dark at 4 ° C. After centrifugation, cells were washed twice with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and centrifuged again for 5 min at 1500RPM. The cells were fixed using BD Cytofix™ Fixation Bufffer (BD Biosciences) and permeabilised using BD Phosflow™ Perm Buffer III (BD Biosciences). Then, the cells were incubated with specific Phosflow® antibodies (BD Biosciences) labelled with the fluorochromes: anti-FoxP3 (PE), anti-GATA -3 (PE), anti-RORγt (PE), anti-T-bet (PE), anti-STAT1 (Alexa 647), STAT3 (Alexa 647), STAT4 (Alexa 647), STAT5 (Alexa 647) and STAT6 (Alexa 647) for 30 min, in the dark and at room temperature. For each test, control tubes were incubated with specific isotypic antibodies for each fluorochrome. The samples were acquired in a flow cytometer of the FACSCanto™ II model (BD Biosciences) with the FACSDiva software (BD Biosciences). Cellular multiparameters were analysed using in FlowJo software, version vX.10.6 (Tree Stars Inc., Ashland, OR, USA). The acquisition of 100.000 events per sample was standardized and the population of interest was optimized to establish a gating strategy for T cell subsets. First, events were selected by the size (FSC) and granularity (SSC) expected for lymphocytes. Then, doublets were discarded using a single cell strategy (singlets gate: FSC-A x FSC-H) and fluorescence for CD3 was considered. From this gate, established on CD4 positive ($CD4^+$) lymphocytes. Additionally, Treg cell analysis was performed inside a $CD25^+CD127^-$ gate. (Suplementary Figure 1). The results were represented in median fluorescence intensities (MFIs) and in percentage of cells (%) that express activators of transcription and transcription factors.

2.6. Statistical analysis

The results were evaluated using non-parametric tests, performed on the PRISM statistical program Graph Prism version 6.01 (GraphPad, San Diego, CA, USA). The evaluation of clinical characteristics and plasma determinations of pregnant women with PE and NT pregnant women were performed using the Mann-Whitney U test. The comparison of the results of immunologic parameters between preeclamptic and NT group after the treatment with VD, was performed by the Kruskal-Wallis test, followed by multiple comparisons by the Dunn test. Statistical significance was accepted at $p<0.05$.

3. Results

3.1. Clinical and laboratorial characteristics

The analysis of clinical and laboratorial characteristics of pregnant women with PE and NT pregnant women are shown in Table 1. There was no statistical difference between the groups evaluated, in relation to age and gestational age. However, preeclamptic women presented significantly higher levels of systolic and diastolic blood pressure, as well as proteinuria, in comparison to NT pregnant women. Additionally, other information about characteristics of pregnant women is that the group of PE was composed of 40% with PE classified as mild and 60% with severe PE, classified according to ACOG, 2019[14].

Table 1. Clinical and laboratory characteristics of preeclamptic women (PE) and normotensive (NT) pregnant women.

Characteristics		Preeclamptic women (n = 20)	NT pregnant women (n = 20)
Age (years)		27 (17–45)	28 (17–39)
Gestational age (weeks)		34 (24–38)	34 (24–39)
Severity criteria	**Mild (%)**	8 (40%)	—
	Severe (%)	12 (60%)	
Systolic blood pressure (mmHg)		150***** (140–190)	105 (90–110)
Diastolic blood pressure (mmHg)		100***** (90–120)	65 (60–70)
Proteinuria (mg/24h)		480***** (300–7850)	< 300

Note: Data are expressed as the median, with the minimum and maximum values in parentheses

* ($p < 0.05$) *vs.* NT pregnant women (Mann-Whitney U test).

3.2. Determination of VD plasmatic

The concentration of VD [25(OH)D] was determined in the plasma of preeclamptic women and NT pregnant women, and results showed significantly lower levels of VD in preeclamptic group than in NT group.

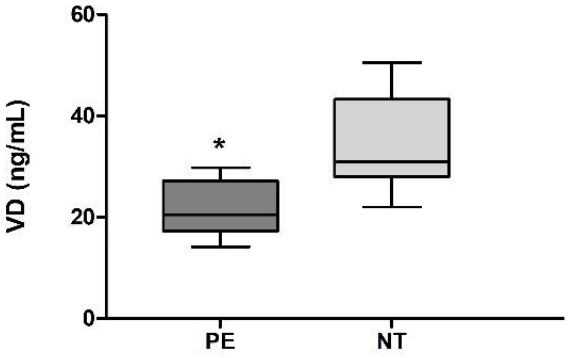

Fig. 1. Plasma concentrations of 25(OH)D obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by automated chemiluminescent technique. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs. NT (Mann-Whitney U test).

3.3. Analysis of transcription factors and activators of transcription in CD4^{+} T cell subsets from PE and NT pregnant women

Figure 2 shows two analyzes, the median fluorescence intensity (MFI) of the phosphorylated intracellular proteins and the percentage (%) of lymphocytes expressing the transcription factors of the subsets of inflammatory profiles Th1 and Th17.

The endogenous MFI of T-bet (Fig. 2A) and RORγt (Fig. 2C), as well as the percentage of cells that expressing these transcription factors (Fig. 2B and 2D, respectively) are significantly higher in pregnant women with PE when compared with NT pregnant women. The treatment with VD decreased significantly the fluorescence intensity (Fig. 2A, 2C) and the percentage (Fig. 2B, 2D) of cells in women with PE, but didn't affected the results of the NT group. Supplementary figure 2 was attached at the end of this manuscript, illustrating the results of the PE and NT groups.

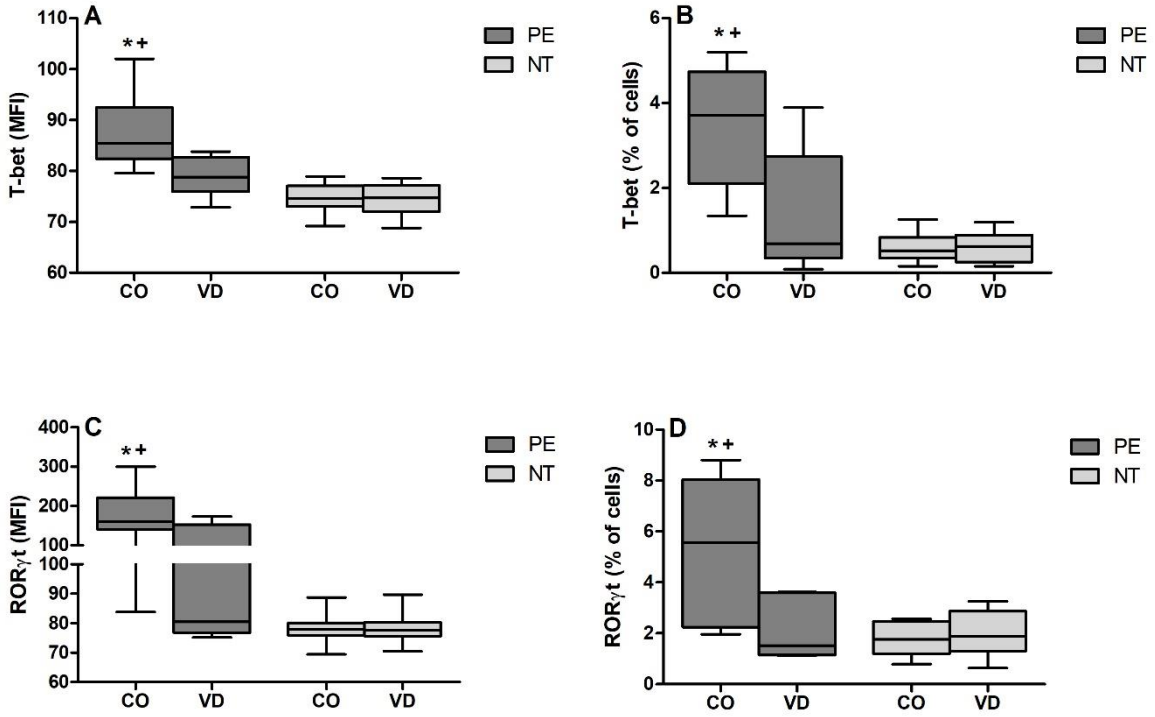

Fig. 2. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription factors in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing T-bet; (B)% of lymphocytes expressing T-bet; (C) MFI of lymphocytes expressing RORγt; (D)% of lymphocytes expressing RORγt. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

The endogenous percentage of cells that express STAT1 (Fig. 3B) is significantly higher in the PE group compared to the NT group. The treatment of lymphocytes with VD decreased the MFI (Fig. 3A) and the percentage of cells (Fig. 3B) that expressing this phosphorylated activator of transcription in preeclamptic women. In the evaluation of STAT4 and STAT3, it is noted that the MFI (Fig. 3C, 3E) and the percentage of lymphocytes (Fig. 3D, 3F) that express these activators of transcription are significantly higher in preeclamptic women when compared to NT pregnant women. After treatment with VD, there was a significant decrease in both immunologic parameters studied in the PE group (Fig. 3C-F). Supplementary figure 3 was attached at the end of this manuscript, illustrating the results of the PE and NT groups.

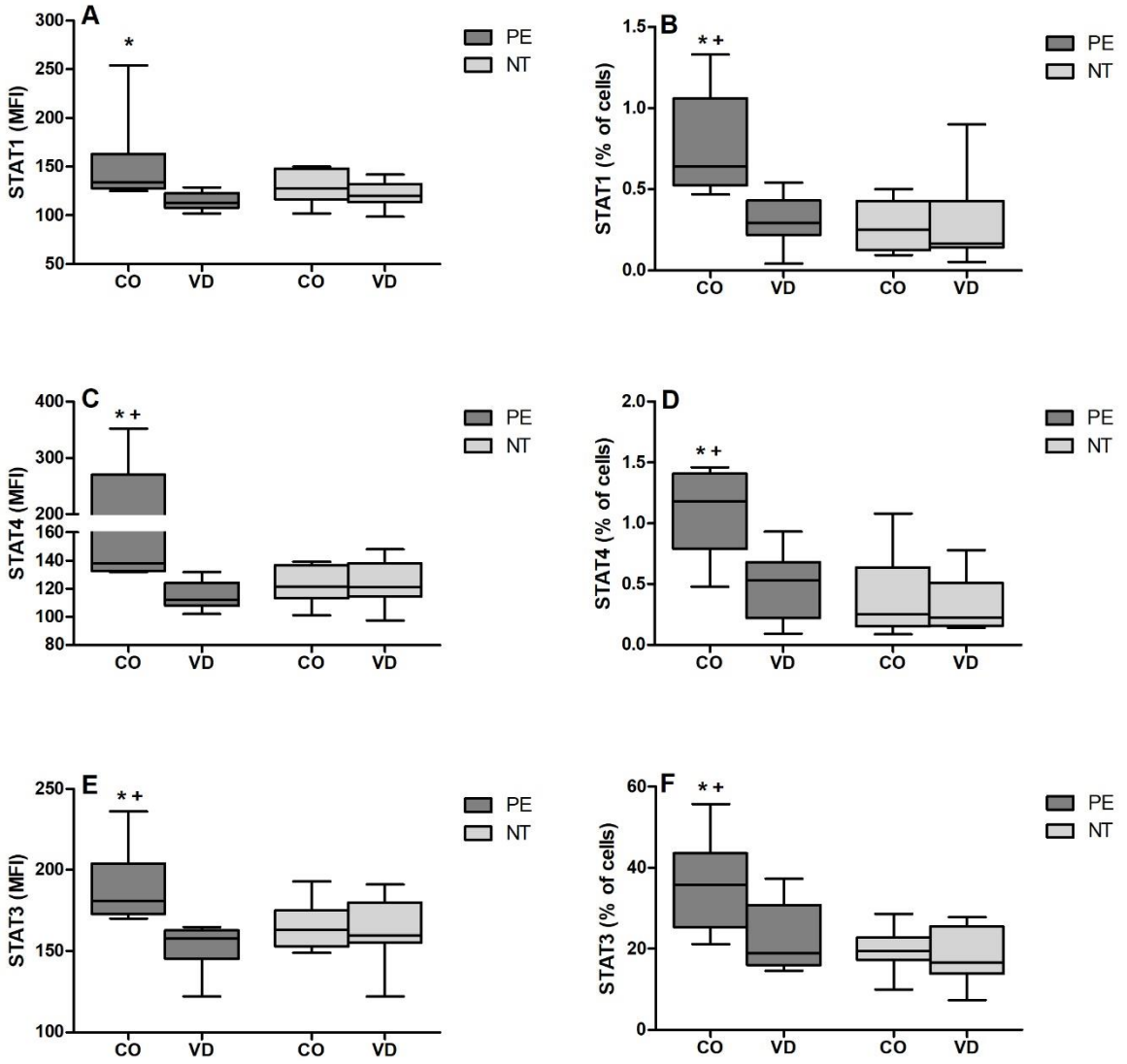

Fig. 3. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription activators in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing STAT1; (B)% of lymphocytes expressing STAT1; (C) MFI of lymphocytes expressing STAT4; (D)% of lymphocytes expressing STAT4; (E) MFI of lymphocytes expressing STAT3; (F)% of lymphocytes expressing STAT3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Figure 4 shows the analysis of the transcription factors of anti-inflammatory (Th2) and regulatory (Treg) profiles. First, it can be seen that both MFI (Fig. 4A, 4C) and the percentage of lymphocytes that endogenously express GATA-3 (Fig. 4B) and, FoxP3 (Fig. 4D) are significantly lower in pregnant women with PE in relation to the NT group. After the cell culture

in the presence of VD, a significant increase of phosphorylated transcription factors in PE group was detected by both the MFI and the percentage of cells evaluated. Treatment of cells from NT pregnant women with VD didn't interfere with the expression of these transcription factors. Supplementary figure 4 was attached at the end of this manuscript, illustrating the results of the PE and NT groups.

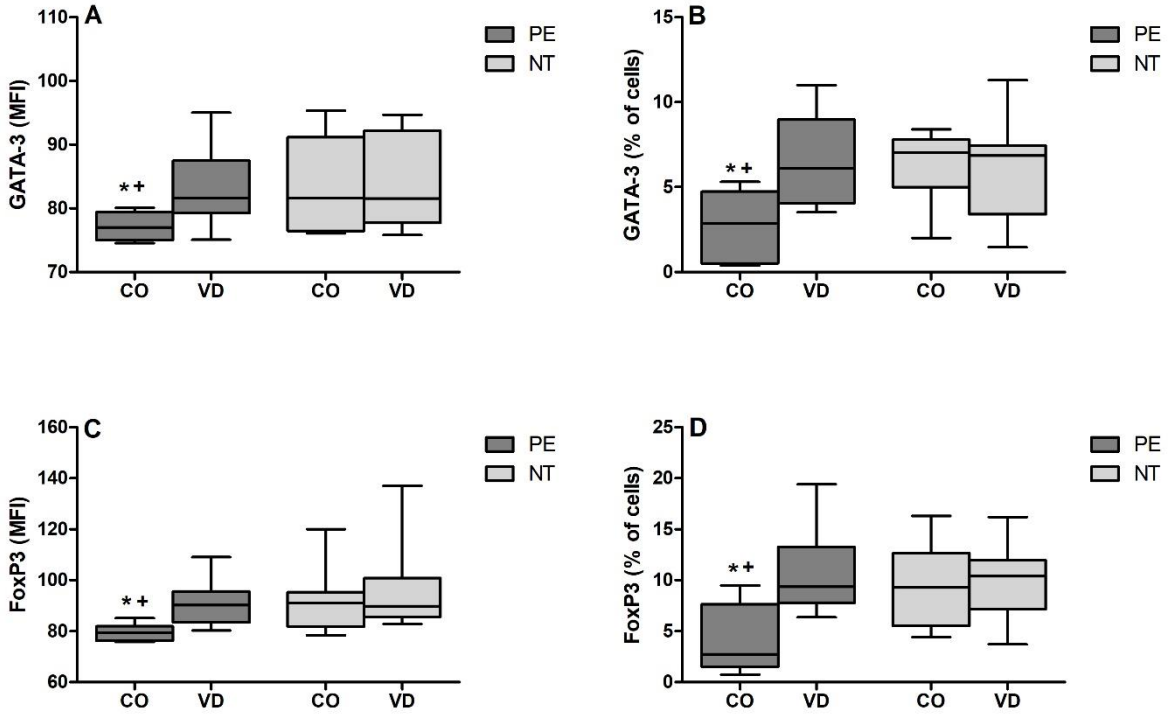

Fig. 4. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription factors in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing GATA-3; (B)% of lymphocytes expressing GATA-3; (C) MFI of lymphocytes expressing FoxP3; (D)% of lymphocytes expressing FoxP3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

$CD4^+$ T cells from pregnant women with PE have a significantly lower MFI (Fig. 5A, 5C) and percentage of cells expressing STAT6 (Fig. 5B) and STAT5 (Fig. 5D) when compared to the NT patients. In addition, the treatment with VD induced significant increase of these activators of transcription in the parameters of MFI and percentage of cells from preeclamptic women in both studied profiles. Supplementary figure 5 was attached at the end of this manuscript, illustrating the results of the PE and NT groups.

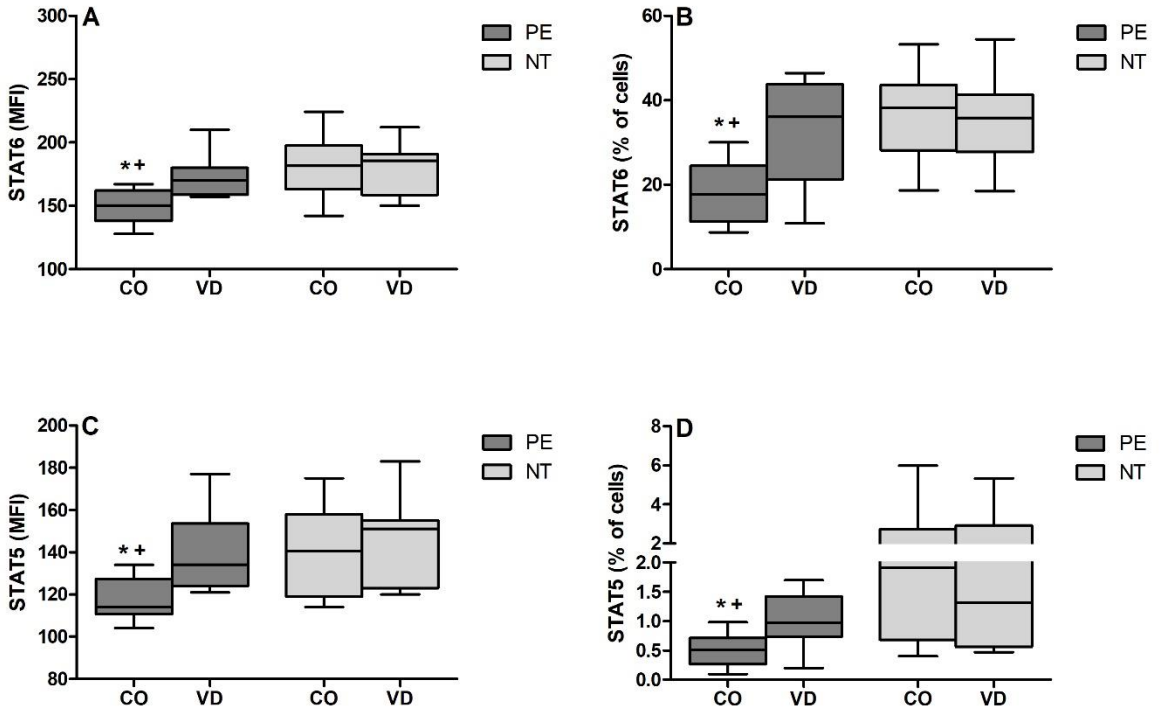

Fig. 5. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription activators in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing STAT6; (B)% of lymphocytes expressing STAT6; (C) MFI of lymphocytes expressing STAT5; (D)% of lymphocytes expressing STAT5. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Regarding $CD4^+$ lymphocytes co-expressing the transcription factors and activators of transcription double-positive, it was observed that PE patients presented a significantly higher percentage of T-bet/STAT4 (Fig. 6B) compared to NT pregnant women, while the treatment with VD decreased significantly T-bet/STAT4 (Fig.6B) and RORγt/STAT3 (Fig. 6C) co-expression in pregnant women with PE. Besides that, it was demonstrated also in PE group significantly lower percentage of lymphocytes co-expression GATA-3/STAT6 (Fig. 6D) and FoxP3/STAT5 (Fig. 6E) when compared with NT group. The culture in the presence of VD induced a significant increase of lymphocytes co-expressing transcription factors and activators of transcription characteristics of anti-inflammatory (Fig. 6D) and regulatory profiles (Fig. 6E).

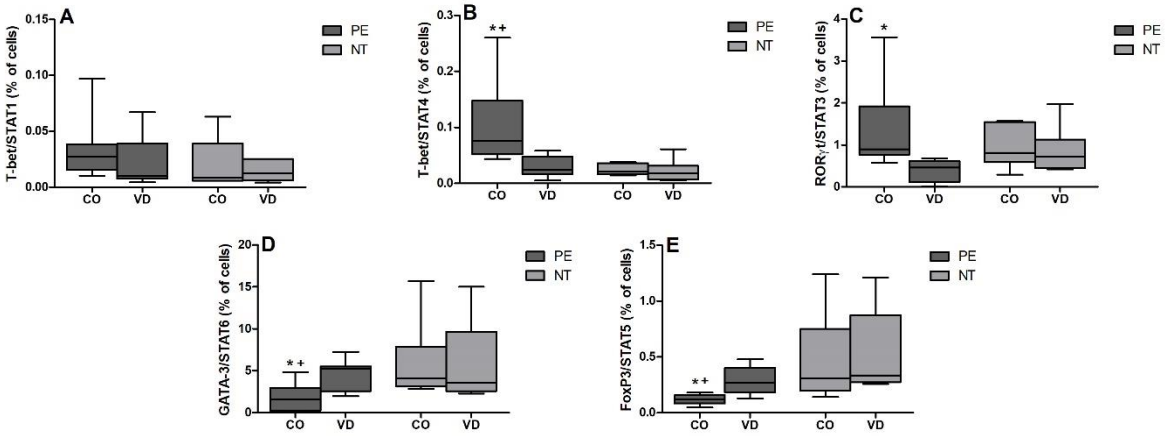

Fig. 6. Percentage of CD4$^+$ T cells co-expressing phosphorylated intracellular transcription factors and activators of transcription in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A)% of lymphocytes co-expressing T-bet and STAT1; (B)% of lymphocytes co-expressing T-bet and STAT4. (C)% of lymphocytes co-expressing RORγt and STAT3; (D)% of lymphocytes co-expressing GATA-3 and STAT6; (E)% of lymphocytes co-expressing FoxP3 and STAT5. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

3.4. Analysis of CD4$^+$ T cell subsets profile from PE and NT pregnant women

Figure 7 represents the profile of CD4$^+$ T cell subsets after the treatment with VD in preeclamptic and NT pregnant women. It was analyzed the inflammatory over anti-inflammatory profiles (Fig. 7A) and inflammatory over regulatory profiles (Fig. 7B), and the results demonstrated that women with PE show a predominance of endogenous inflammatory profiles, with an increase of Th1 and Th17 in relation to NT pregnant women. The treatment of cells with VD, induced modulation of this inflammatory response to anti-inflammatory and regulatory profiles, with the increase of Th2 and Treg cells in the PE group.

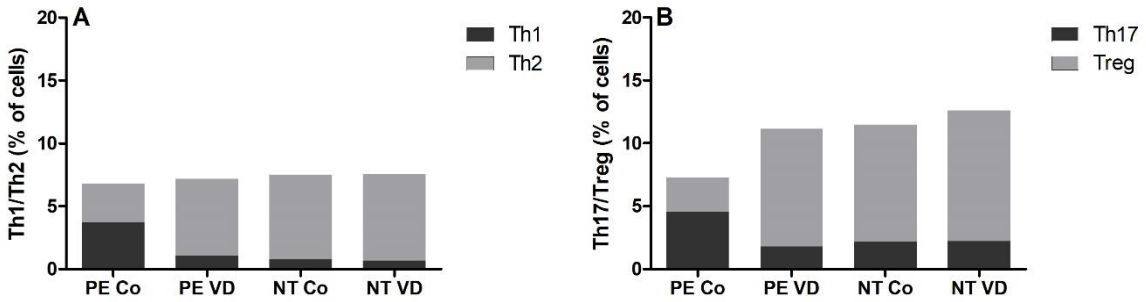

Fig. 7. Comparison between inflammatory over anti-inflammatory profiles (A), inflammatory over regulatory profiles (B) in $CD4^+$ T cell subsets obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women treated in vitro in the absence (Co) or presence of vitamin D (VD) by flow cytometry. Results are expressed in percentage (%) of $CD4^+$ T cells.

3.5. Cytokine determination in PBMC culture supernatants from PE and NT pregnant women

The production of TNF-α, IFN-γ, IL-17, IL-6, IL-22, IL-23, IL-10, and TGF-β determined in the supernatants of PBMCs obtained from pregnant women with PE and NT pregnant women, treated with or without VD, are shown in Fig. 8.

Significantly higher basal levels of inflammatory cytokines, produced by PBMCs from the preeclamptic group compared to the NT group were observed (Fig. 8A-F), whereas the concentrations of IL-10 (Fig. 8G), and TGF-β (Fig. 8H) were significantly lower in the group with PE than NT group. Cell treatment with VD led to a significant effect on cytokine production, inducing the decreased release of inflammatory cytokines (Fig. 8A-F), while the levels of anti-inflammatory cytokine IL-10 (Fig. 8G) and TGF-β (Fig. 8H) increased significantly in the pregnant women with PE. No significant effects of VD on cytokine production were seen in NT group.

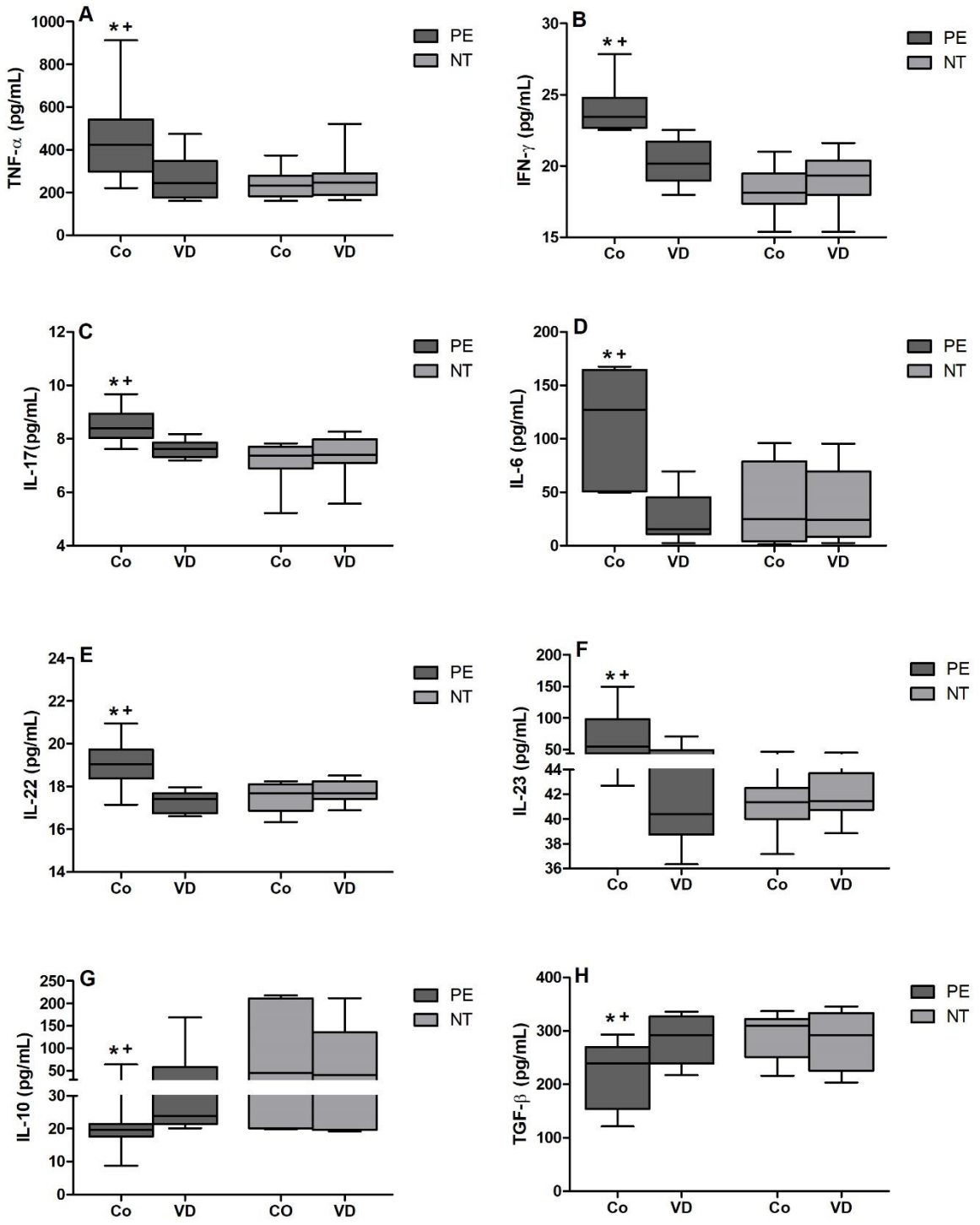

Fig. 8. TNF-α (A), IFN-γ (B), IL-17 (C), IL-6 (D), IL-22 (E), IL-23 (F), IL-10 (G), and TGF-β (H) production by peripheral blood mononuclear cells (PBMCs) detected in the supernatants of 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) for 24 hours by ELISA technique. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

4. Discussion

The regulation of immune response through components that promote maternal tolerance and the rejection of the fetus is essential to the successful outcome of pregnancy [11]. Thus, the present study evaluated in vitro, the immunomodulatory action of VD on the adaptive immune system, through determination of transcription activators and transcription factors of $CD4^+$ T cell subsets in PE and NT pregnant women. The modulatory effect of VD on cells of adaptive immunity is already well-characterized by studies that show its regulatory role on several immunological pathways in pathologies with intensified inflammatory processes, by regulating the action of lymphocytes, mast cells, antigen-presenting cells and cells present, in order to mitigate excessive inflammatory responses [32-34].

Previous studies reported an increase in Th17 and a decrease of Treg cells in pregnant women with PE [2, 35]. Recently, it was confirmed by Muyayalo *et al.* [36] that patients with PE had an increased percentage of Th17 cells, whereas, Toldi *et al*. [37] reported a decrease in Treg lymphocytes compared to NT patients. Our results showing high fluorescence intensity and percentage of $CD4^+$ cells expressing RORγt, in addition to the decrease of lymphocytes that endogenously express FoxP3 in preeclamptic women corroborate the results of the literature.

It is well-known, that VD deficiency is directly associated with the development of adverse outcomes of pregnancy, among mothers and neonates [38]. Several studies also demonstrated an association between this deficiency and the risk of developing PE [39-42]. Our results showed that pregnant women with PE had lower VD levels compared to NT pregnant women, and are in line with other studies reporting that preeclamptic women show VD deficiency [31,43].

After treatment of PBMCs with VD, there was a decrease in the inflammatory profiles (Th1 and Th17) and an increase in the anti-inflammatory and regulatory profiles (Th2 and

Treg), demonstrating an immunomodulatory action of the hormone on the adaptive immune response in preeclamptic women. Abdollahi *et al*. [44] assessed the percentage of Th17 and Treg cells in women with VD deficiency, and with a history of unexplained recurrent abortions. The authors showed that treatment of PBMCs with VD was able to increase the percentage of Treg cells, suggesting that the use of VD before pregnancy can be protective against recurrent abortions. The immunomodulatory effects of VD that have been calling special attention in the literature is the ability to suppress Th17 and increase Treg cells [28-30].

In addition to transcription factors, the STATs were also evaluated in T cell subsets to better understand the adaptive immunity in PE. The Janus kinase (JAK)/STAT pathway is an universally expressed intracellular signal transduction pathway involved in many crucial biological processes, such as differentiation and proliferation, playing critical role in orchestrating of the immune system, and modulating the polarization of T helper cells [4, 45, 46]. STATs are latent transcriptional activators that, after phosphorylation, emit signals from the cell surface to the nucleus for cytokine and growth factors activation [45]. Studies reporting the involvement of VD on T cell subsets JAK/STAT pathway in inflammatory diseases are scarce. An in vivo study evaluating the role of VD in experimental autoimmune encephalomyelitis (EAE) in rats showed that VD affects multiple signaling and metabolic pathways critical for T cell activation and differentiation into Th1 and Th17 subsets by downregulation genes of JAK/STAT, ERK/MAPK and PI3K/AKT/MTOR signaling pathways [47]. In our study, the results related to STATs endogenous expression by T cell subsets in preeclamptic women, showed a deviation towards inflammatory profiles, with an increase in STAT1, STAT4, and STAT3. After cell treatment with VD occurred a polarization of these activators of transcription to anti-inflammatory and regulatory profiles by increasing respectively STAT6 and STAT5 expression. Thus, our data confirm that in preeclamptic women, $CD4^+$ lymphocytes co-express STATs and transcription factors, and shows that the

protective effect of VD may occur by downregulation of activators of transcription, influencing the transcription of specific factors, and lead to a decrease in Th1 and Th17 cells followed by an increase in Th2 and Treg cells, of anti-inflammatory and regulatory profiles. These results are innovative in the literature related to this pregnancy syndrome. To our knowledge, there are no studies reporting the involvement of VD on JAK/STAT pathway in T cell subsets of preeclamptic women. Therefore, more studies are need to evaluate if VD plays a role on the signaling pathways of T cell subsets in PE, and improve the imbalance of the Th17/Treg cells in this pathology.

Our results showing a higher basal production of cytokines of the inflammatory profiles, Th1 and Th17, and lower production of regulatory cytokines, IL-10 and TGF-β in preeclamptic women are in accordance with the literature [2, 11]. Treatment of PBMCs with VD led to downregulation on IFN-γ, TNF-α, IL-6, L-17, IL-22 and IL-23 production, associated with an increase in IL-10 and TGF-β production, showing that VD induces the modulation of T cell subsets to an anti-inflammatory or regulatory profile. According to Cyprian *et al.* [48] VD suppress T cells proliferation, by decreasing IL-2 transcription and inhibiting the production of T helper pro-inflammatory cytokines. Our study also showed that treatment of PBMCs with VD induced an increase of IL-10 in preeclamptic women. This cytokine can suppress the production of other pro-inflammatory cytokines and stimulate the differentiation and proliferation of Treg cells [15].

In conclusion, our study showed that lymphocytes from preeclamptic women responded to in vitro treatment with VD, probably through the JAK/STAT signaling pathway, decreasing the activators of transcription and factors of transcription, shifting the inflammatory profile of Th1/Th17 cells to an anti-inflammatory and regulatory Th2/Treg profile, capable of restoring immune homeostasis in PE. These findings indicate that the immunomodulatory effect of the VD on the STAT signaling pathway of T cell subsets can be a promising and innovative strategy

in the regulation of the adaptive immune system in PE. (Attached Graphical Abstract at the end of this chapter).

Acknowledgments

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Author contributions

V.R.R., M.R.V. and M.T.P conceived the idea, designed the experiments, analyzed the data, and wrote the paper. J.C.P. selected pregnant women for the study. V.R.R., M.R.V., P.R.N., M.L.M. A.C.D. performed experiments. L.C.R.O and G.G.R performed the flow cytometry analysis. All authors reviewed the manuscript and approved the final version for publication.

Declaration of Competing Interest

The authors declare no commercial or financial conflict of interest.

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Figure legends

Fig. 1. Plasma concentrations of 25(OH)D obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by automated chemiluminescent technique. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers). * ($p < 0.05$) vs. NT (Mann-Whitney U test).

Fig. 2. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription factors in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing T-bet; (B)% of lymphocytes expressing T-bet; (C) MFI of lymphocytes expressing RORγt; (D)% of lymphocytes expressing RORγt. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 3. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription activators in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing STAT1; (B)% of lymphocytes expressing STAT1; (C) MFI of lymphocytes expressing STAT4; (D)% of lymphocytes expressing STAT4; (E) MFI of lymphocytes expressing STAT3; (F)% of lymphocytes expressing STAT3. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 4. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription factors in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing GATA-3; (B)% of lymphocytes expressing GATA-3; (C) MFI of lymphocytes expressing FoxP3; (D)% of lymphocytes expressing FoxP3. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 5. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing phosphorylated intracellular transcription activators in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A) MFI of lymphocytes expressing STAT6; (B)% of lymphocytes expressing STAT6; (C) MFI of lymphocytes expressing STAT5; (D)% of lymphocytes expressing STAT5. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 6. Percentage of $CD4^+$ T cells co-expressing phosphorylated intracellular transcription factors and activators of transcription in 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were treated in the absence (Co) or in the presence of vitamin D (VD) in vitro. (A)% of lymphocytes co-expressing T-bet and STAT1; (B)% of lymphocytes co-expressing T-bet and STAT4. (C)% of lymphocytes co-expressing RORγt and STAT3; (D)% of lymphocytes co-expressing GATA-

3 and STAT6; (E)% of lymphocytes co-expressing FoxP3 and STAT5. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 7. Comparison between inflammatory over anti-inflammatory profiles (A), inflammatory over regulatory profiles (B) in $CD4^+$ T cell subsets obtained from 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women treated in vitro in the absence (Co) or presence of vitamin D (VD) by flow cytometry. Results are expressed in percentage (%) of $CD4^+$ T cell subsets.

Fig. 8. TNF-α (A), IFN-γ (B), IL-17 (C), IL-6 (D), IL-22 (E), IL-23 (F), IL-10 (G), and TGF-β (H) production by peripheral blood mononuclear cells (PBMCs) detected in the supernatants of 20 pregnant women with preeclampsia (PE) and 20 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of vitamin D (VD) for 24 hours by ELISA technique. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Supplementary Information

Immunomodulatory effect of vitamin D on the activators of transcription and transcription factors of $CD4^+$ T cell subsets in pregnant women with preeclampsia

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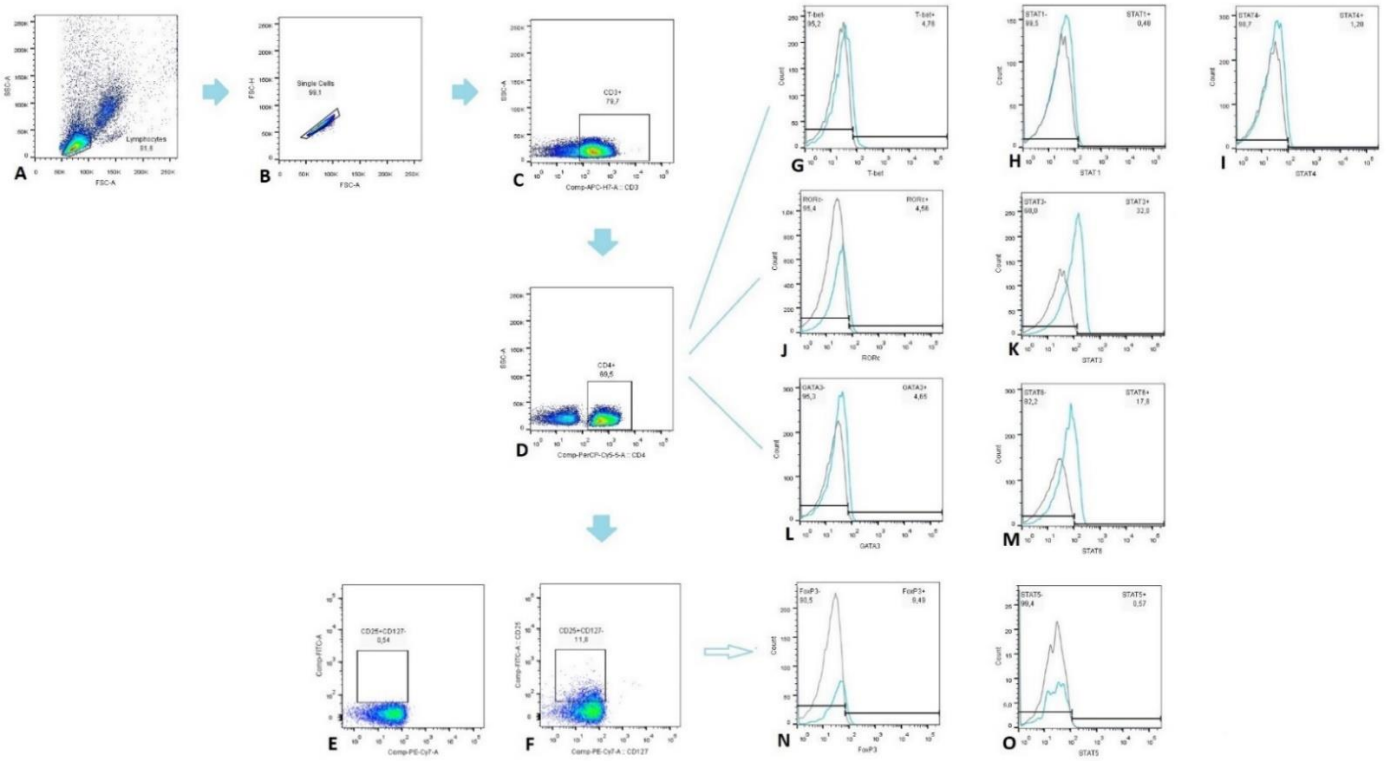
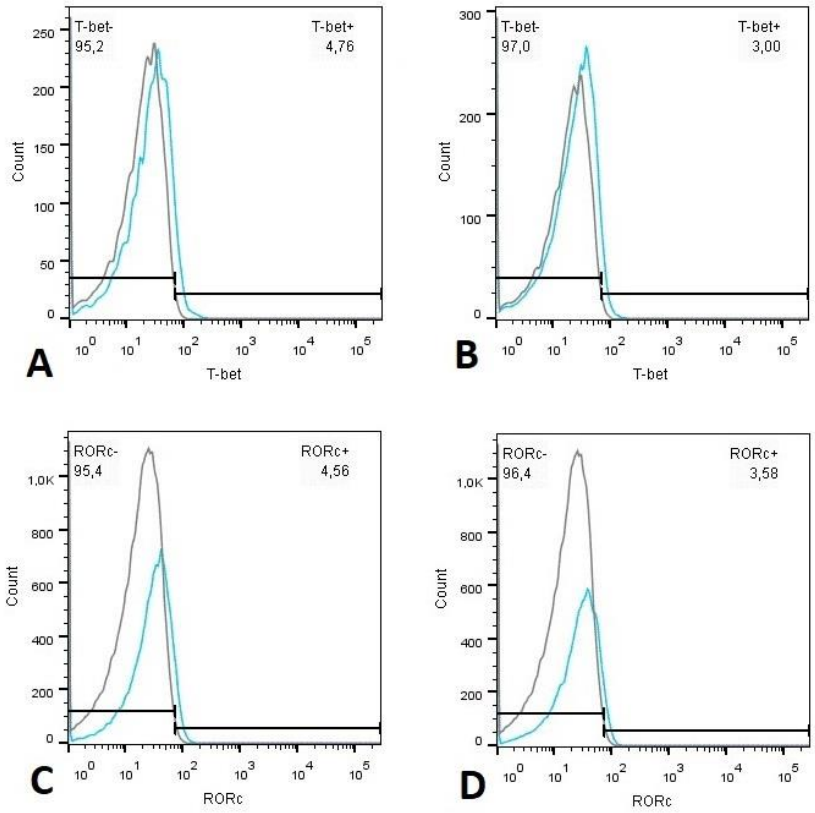
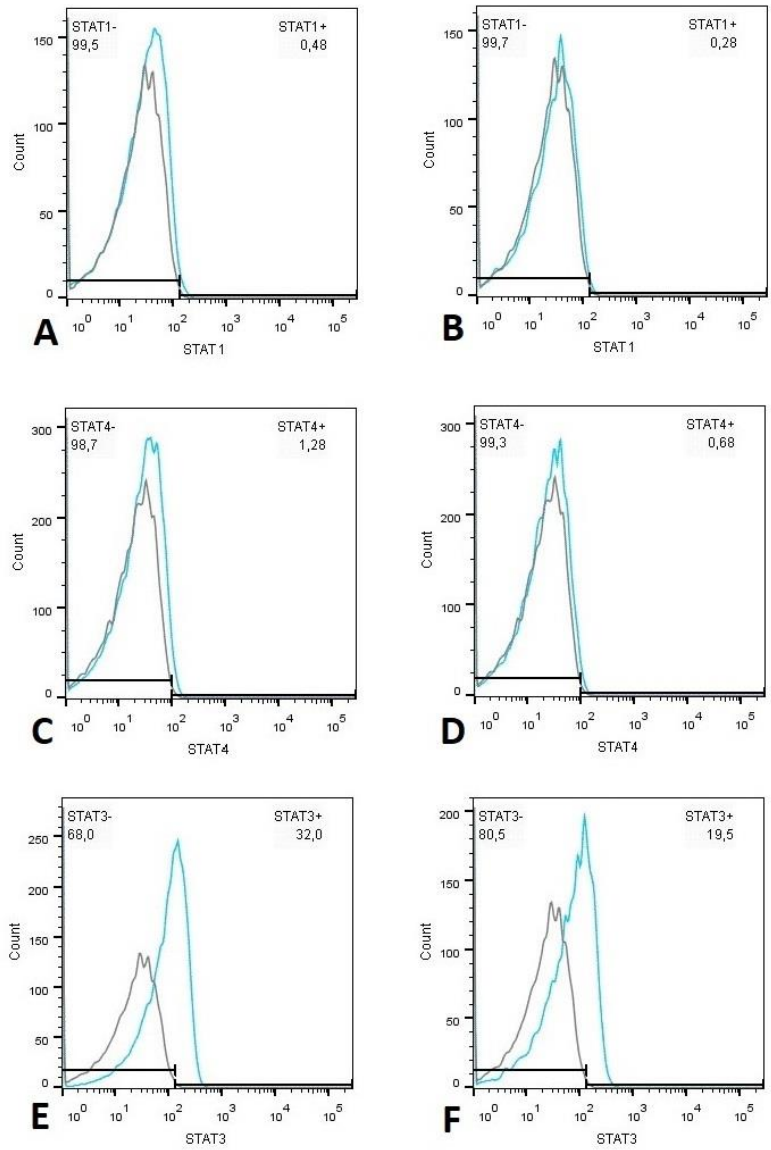


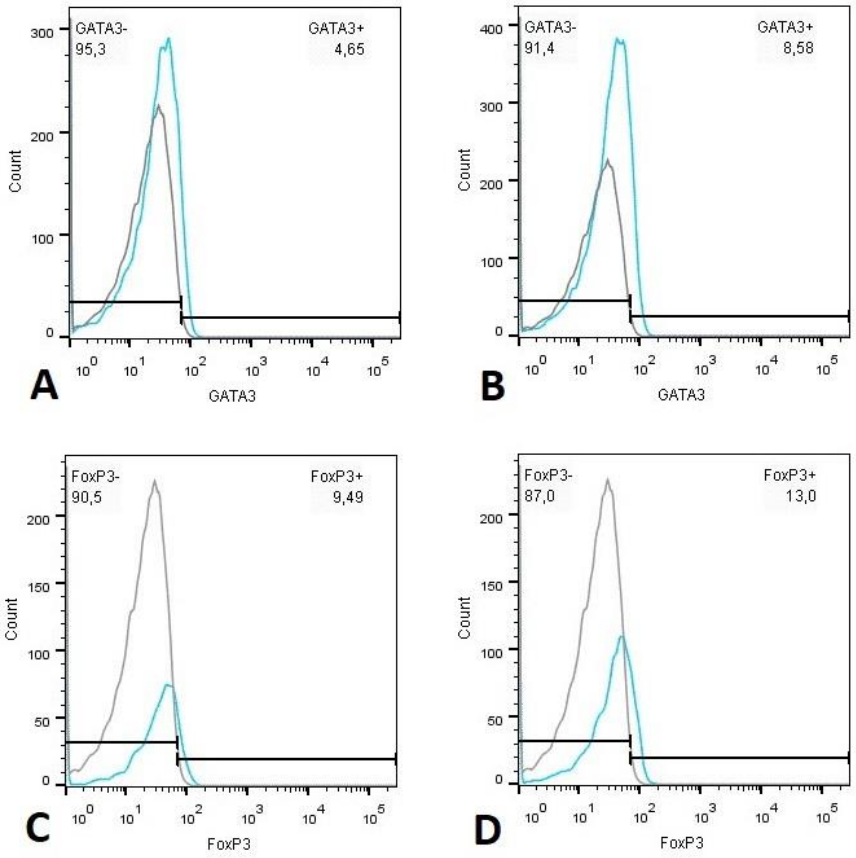
Supplementary Figure 1. Phenotypic strategy analysis of T helper lymphocytes by flow cytometry. Pseudocolor and histogram demonstrating the gates of T helper lymphocytes population analysis. (A) Gate lymphocytes were based on the size and granularity of the population. (B) Gate singlets were based on a single cell analysis. (C) The $CD3^+$ gate represents the selection of positive cells for this marker. (D) The $CD4^+$ gate represents the selection of positive cells for this marker, in which transcription activators and transcription factors were analyzed. (E) Representative pseudocolor from control non-marked lymphocytes to population $CD25^+/CD127^-$. (F) The $CD25^+/CD127^-$ gate represents the selection of cells, in which T regulatory profile transcription activator and transcription factor were analyzed. (G) Representative histogram of transcription factor T-bet (Th1). (H) Representative histogram of transcription activator STAT1 (Th1). (I) Representative histogram of transcription activator STAT4 (Th1). (J) Representative histogram of transcription factor RORγt (Th17). (K) Representative histogram of transcription activator STAT3 (Th17). (L) Representative histogram of transcription factor GATA-3 (Th2). (M) Representative histogram of transcription activator STAT6 (Th2). (N) Representative histogram of transcription factor FoxP3 (Treg). (O) Representative histogram of transcription activator STAT5 (Treg).



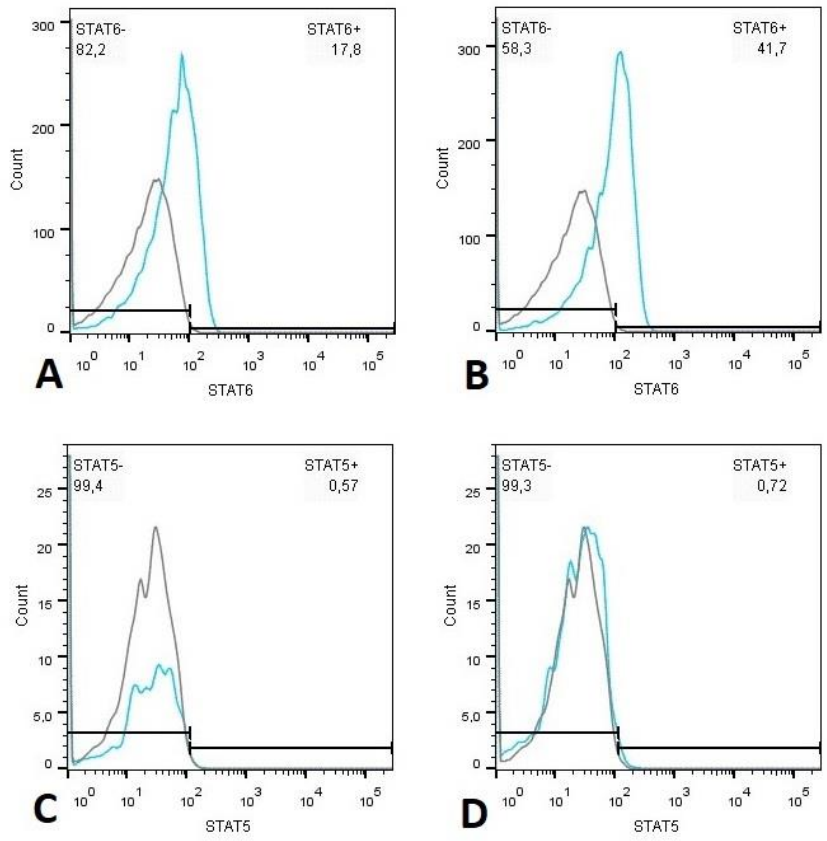
Supplementary Figure 2. Representative histogram of inflammatory transcription factors from $CD4^+$ T cell subsets T-bet (Th1) and RORγt (Th17) expressed by lymphocytes from pregnant women with preeclampsia (PE) cultured with or without Vitamin D (VD). **(A)** Endogenous expression (Co) of T-bet. **(B)** Expression of T-bet after treatment with VD. **(C)** Endogenous expression (Co) of RORγt. **(D)** Expression of RORγt after treatment with VD.



Supplementary Figure 3. Representative histogram of inflammatory transcription activators from $CD4^+$ T cell subsets T-bet (Th1) and RORγt (Th17) expressed by lymphocytes from pregnant women with preeclampsia (PE) cultured with or without Vitamin D (VD). **(A)** Endogenous expression (Co) of STAT1. **(B)** Expression of STAT1after treatment with VD. **(C)** Endogenous expression (Co) of STAT4. **(D)** Expression of STAT4 after treatment with VD. **(E)** Endogenous expression (Co) of STAT3. **(F)** Expression of STAT3 after treatment with VD.

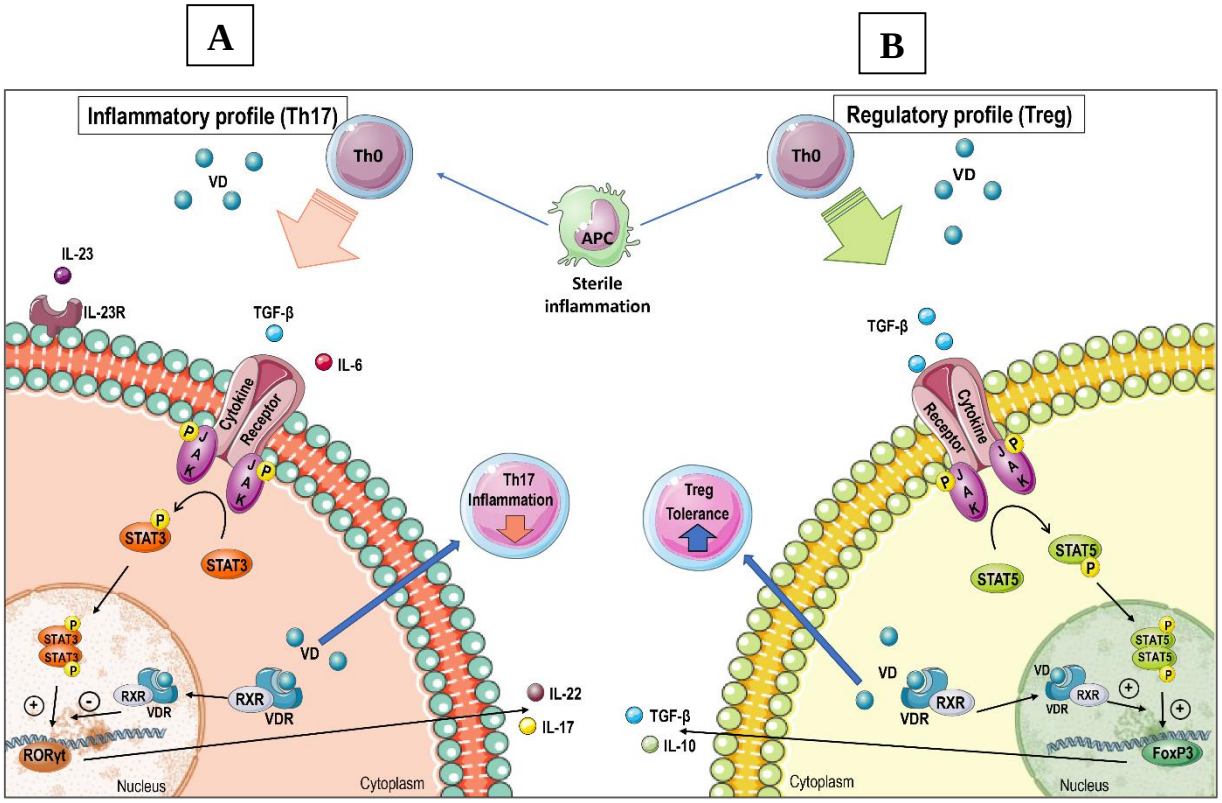


Supplementary Figure 4. Representative histogram of anti-inflammatory transcription factors from $CD4^+$ T cell subsets T-bet (Th1) and RORγt (Th17) expressed by lymphocytes from pregnant women with preeclampsia (PE) cultured with or without Vitamin D (VD). **(A)** Endogenous expression (Co) of GATA-3. **(B)** Expression of GATA-3 after treatment with VD. **(C)** Endogenous expression (Co) of FoxP3. **(D)** Expression of FoxP3 after treatment with VD.



Supplementary Figure 5. Representative histogram of anti-inflammatory transcription activators from $CD4^+$ T cell subsets STAT6 (Th2) e STAT5 (Treg) expressed by lymphocytes from pregnant women with preeclampsia (PE) cultured with or without Vitamin D (VD). **(A)** Endogenous expression (Co) of STAT6. **(B)** Expression of STAT6 after treatment with VD. **(C)** Endogenous expression (Co) of STAT5. **(D)** Expression of STAT5 after treatment with VD.

Graphical Abstract



Graphical Abstract Figure. Immunomodulatory effect of vitamin D (VD) on Th17 (A) and Treg (B) cells obtained from preeclamptic women. (A) Naïve T cells, in the presence of interleukin (IL)-6 associated with transforming growth factor beta (TGF-β) induce the inflammatory Th17 cell polarization. These cytokines bind to the receptor present in the membrane of this cell. Then, the jakus kinase (JAK) is phosphorylated and leads to the phosphorylation of the signal transducer and activator of transcription 3 (STAT3). This protein, phosphorylated by JAK, dimerizes and is transported into the nucleus to regulate the expression of the RORγt gene. In the presence of VD, this hormone binds to their nuclear receptor and downregulates the expression of RORγt, decreasing the release of IL-17 and IL-22, characteristics of this profile. Therefore, the treatment with VD decreased the inflammation. (B) Naïve T cells, in the presence of TGF-β, induce the polarization of Treg cells. This cytokine binds to the receptor on the T cell membrane, and the jakus kinase (JAK) is phosphorylated, leading to the STAT5 phosphorylation. This protein phosphorylated by JAK, dimerized, and is transported to the nucleus to regulate the expression of the FoxP3 gene. In the presence of VD, this hormone binds to its nuclear receptor and stimulates FoxP3 expression, increasing the production of IL-10 and TGF-β. The VD treatment restores the imbalance between Th17/Treg cells, modulating for the regulatory profile, by the decrease of the inflammatory lymphocytes and increasing of regulatory T cells.

Capítulo III

Os resultados referentes à esse Capítulo serão enviados para Publicação em Periódico Internacional

Silibinin downregulates the expression of the Th1 and Th17 profiles by the modulation of STATs and transcription factors in pregnant women with preeclampsia

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Short title: Silibinin acts on $CD4^+$ T cell subsets in preeclampsia.

Abbreviations: Alexa 647: *Alexa* Fluor *647;* **APC-Cy7:** fluorochrome allophycocyanin conjugated to cyanine 7; **BB515:** bright blue fluorochrome 515; **CD4:** cluster of differentiation 4; **CD25:** cluster of differentiation 25; **CD127:** cluster of differentiation 127; **CNS3:** conserved non-coding sequences; **FoxP3:** forkhead box P3; **GATA-3:** GATA binding protein 3; **IFN-γ:** interferon gama; **IkB:** inhibitory protein of the NF-κB signaling pathway; **IL:** interleukin; **MFI**, median fluorescence intensity**; NF- kB**: factor nuclear kappa B; **NLRP1:** NOD-like receptor with a pyrin domain; **NT:** normotensive; **PE:** fluorochrome phycoerythrin; **PerCP-Cy5.5:** fluorochrome pyridine protein chlorophyll conjugated with cyanine dye; **PE-Cy7:** fluorochrome phycoerythrin conjugated with cyanine dye; **RORγt:** retinoic acid-related orphan receptor gamma t; **RPMI:** Roswell Park Memorial Institute; **STAT:** *Signal transducer and activator of transcription***; T-bet:** T box transcription factor; **Th1:** T helper cell 1; **Th2:** T helper cell 2; **Th17:** T helper cell 17; **Treg:** regulatory T cell.

Abstract

Preeclampsia (PE) is a multifactorial disease and characterized by inflammation. Cells of the innate and adaptive immune system and their interactions seem to be responsible for this inflammatory state. Thus, treatment with natural products, such as silibinin (SB) can contribute to the control of this inflammation and gestational success. The present study evaluated whether the flavonoid SB, has an in vitro immunomodulatory effect on the signal transducers and activators of transcription (STAT) as well as on transcription factors of $CD4^+$ T cell subset obtained from preeclamptic and normotensive (NT) pregnant women. Peripheral blood mononuclear cells (PBMCs) from 16 preeclamptic and 16 NT pregnant women were cultured with or without SB to analyze the expression of activators of transcription and transcription factors by flow cytometry, and cytokines were measured in the supernatant culture by ELISA. The results showed that treatment with SB decreased STAT1/STAT4/T-bet, and STAT3/RORγt, characteristics of inflammatory profiles described to Th1 and Th17 T cell subsets, and increased STAT6/GATA-3, and STAT5/FoxP3 of anti-inflammatory and regulatory profiles respectively. In addition, PBMCs from preeclamptic women treated with SB released lower concentrations of inflammatory cytokines and higher levels of interleukin (IL)-10 and transforming growth factor-beta (TGF-β). Therefore, SB play an immunomodulatory role on $CD4^+$ T cell subsets in PE, led to downregulation of inflammatory profiles and upregulation on anti-inflammatory and regulatory profiles. More studies are necessary to better understand the modulation of $CD4^+$ T cell subsets by JAK/STAT pathway in this gestational pathology.

Keywords: activators of transcription; preeclampsia, NF- kB; T cell subsets, transcription factors, silibinin.

1. Introduction

Hypertensive disorders are complications that may occur during gestation, exposing the pregnant women and their fetus to the risk and life-long sequelae [1]. An example of these disorders is preeclampsia (PE), a multifactorial disease, affecting approximately 2-8% of all pregnancies [2].

The clinical parameters that identified this disease are based on the development of arterial hypertension associated or not with proteinuria after the 20th week of pregnancy or in the first days after delivery. Some cases of PE may also be related to the commitment of other organs, such as kidney and liver [3, 4]. Furthermore, PE is characterized by an inflammation that occurs for non-microbial causes and known as "sterile inflammation". Some cells responsible for this inflammation, belong to the innate and adaptive immune system and their interactions. [5, 6].

The adaptive immunity system in PE is activated by inflammatory cytokines produced by innate immune cells, which leads to increase in this inflammation [7]. Thus, the cytokine environment plays a key role in the differentiation of T cell subsets. The generation of Th1 cells were induced by interferon-gama (IFN-γ), and interleukin (IL)-12 which are potent inflammatory mediators in the activation of the adaptive immune response [8]. The presence of IL-4 is responsible for Th2 anti-inflammatory cell differentiation, while Th17 cells, are differentiated in the presence of IL-6 and transforming growth factor beta (TGF-β), and are considered the key effector T cells in the induction of the inflammatory response. On the other hand, Treg cells, essential in maintaining self-tolerance and regulating inflammation, play an important role in pregnancy maintenance and are differentiated in the presence of TGF-β [9-12].

Pregnant women with PE shows an imbalance between inflammatory profiles Th1, and Th17 and Th2 anti-inflammatory and T regulatory (Treg) profiles [11]. In a previous study, we

demonstrated in $CD4^+$ T cell subsets show higher endogenous expression of transcription factors T-bet (transcription factor T box) and RORγt (retinoic acid receptor-related orphan receptor γ) characteristics of Th1 and Th17 profiles respectively and decreased basal expression of Th2 profile, GATA-3 (GATA 3 binding protein), and FoxP3 (forkhead box P3) representing regulatory profile [7].

The signal transducers and transcription activators (STATs) present in the cytoplasm are the target of jakus kinase (JAK), which is one of the most crucial cytokine-activated transcription factors in the process of the immune response. These proteins are phosphorylated by JAK, dimerized, and transported into the nucleus through the nuclear membrane to regulate the expression of related genes, this axis is called JAK/STAT pathway [13]. According to Seif et al. [14] JAK/STAT pathway plays a critical role in the fate of Th cells.

Maternal adaptation to pregnancy requires a tightly controlled interaction between innate and adaptive immunities, to allow the normal growth and development of the semi-allogeneic fetal allograft [15]. It was hypothesized that in PE an absence of control the imbalance of T cell subsets, is due to a lack of molecules that may modulate this immune microenvironment. Thus, the use of silibinin (SB) can be a new strategy for the modulation of the JAK/STAT pathway as well as the transcription factors of $CD4^+$ T cell subsets.

SB is considered the main active component of Silymarin, an extracted from fruits and seeds of *Silybum marianum*, one of the oldest medicinal herbs, with hepatoprotective action [16]. SB presents activities already describes in the literature such as cytoprotection, anti-inflammatory, anti-fibrotic, anti-oxidant, and anti-carcinogenic [17-19].

A recent study elucidated the immunomodulatory effect of SB in monocytes from preeclamptic women, showing that SB was able to polarize these cells to an M2-like phenotype, with anti-inflammatory profile [20]. In addition, SB inhibited the activation of NF-κB and the

synthesis of pro-inflammatory cytokines, produced by peripheral blood mononuclear cells (PBMCs) of pregnant women with PE [21, 22].

Considering the properties of SB and that there is no studies on effect of SB on adaptive immunity in PE, the present study investigated whether SB has an in vitro immunomodulatory effect on the JAK/STAT signaling pathway and on transcription factors of $CD4^+$ T cell subset obtained from PE and normotensive (NT) pregnant women.

2. Materials and methods

2.1. Groups of study and ethics statement

Thirty-two pregnant women, admitted to the Obstetric Unit of Botucatu Medical School, Sao Paulo State University, (UNESP), Brazil. Sixteen pregnant women were diagnosed with PE, in accordance with the American College of Obstetricians and Gynecologists [2], defined, as the onset of a persistently elevated blood pressure (≥ 140/90 mmHg) associated or not with proteinuria (≥ 300 mg in urine collected over 24 h), and the other severe clinical complications after 20 weeks of gestation. These pregnant permanent preeclamptic until the end of pregnancy. A group of 16 pregnant normotensive (NT) women, matched for gestational age with the PE pregnant group, and composed of an uncomplicated pregnancy, that do not become preeclamptic throughout pregnancy. Gestational age was determined by data about the last menstrual period and confirmed by early ultrasound examination (< 12 weeks of gestation). Proteinuria was measured in 24-hour urine by the Technicon RA-XT automation system (Miles Inc., Tarrytown, NY, USA) a colorimetric method utilized in the Clinical Laboratory of Botucatu Medical School, Botucatu, Sao Paulo, Brazil. Exclusion criteria included prior PE, multiple gestations, illicit drug use, and pre-existing medical conditions, such as diabetes, chronic hypertension, acute infectious diseases, autoimmune, renal, and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School (protocol number 4.418.043), and all women provided informed consent. For pregnant women aged bellow 18

years old, written informed consent was obtained from their parents or guardians. All experiments reported in this paper were performed under standard health and safety procedures and followed relevant guidelines and regulations from the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Peripheral blood sample collection

For the evaluation of the signal transducer and activators of transcription, factors of transcription, and cytokine production, peripheral blood was collected at the time of disease diagnosis, and from NT pregnant women when they were matched for gestational age with preeclamptic women. Peripheral blood samples from PE and NT pregnant women were collected by venipuncture from the antecubital vein. Ten milliliters of blood were put into plastic tubes containing 10 U/mL ethylenediaminetetraacetic acid (EDTA; Becton Dickinson - BD Vacutainer; BD Biosciences, Franklin Lakes, NJ, USA).

2.3. PBMCs culture and treatment with SB

PBMCs were isolated by density gradient centrifugation method (density [d] = 1.077) on Ficoll-Paque Premium media (GE Healthcare Bio-Sciences, Uppsala, Sweden), as previously described by Ribeiro et al. [7]. The cells were resuspended in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA), and counting was performed using the 5% Turk dye solution (1:1 v/v) and the concentration was adjusted as needed for each of the following experiments. PBMCs from preeclamptic and NT pregnant women were cultured with or without 100 µM of SB (Sigma-Aldrich) for 30 min for flow cytometry analyses, and after 24h of culture for cytokine determination respectively. The SB concentration was previously standardized, employing PBMCs from healthy, non-pregnant women. The viability of PE and NT PBMCs after 24h of culture with or without SB was higher than 88% in all experiments, as determined by 0.2% trypan blue exclusion test.

2.4. Cytokine determination

The production of the cytokines were measured in the supernatants of PBMCs culture ($1x10^6$ cells/ mL) from preeclamptic and NT pregnant women cultured with or without SB for 24h. Then, IFN-γ, IL-6, IL-10, IL-17, IL-22, IL-23, TGF-β, and TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA), employing specific commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), which was used according to the manufacturer's instructions. Absorbance was measured using a microplate reader, and the concentration of cytokines was calculated according to the standard curve. The assay sensitivity limits of the kits were 6.4 pg/mL for IFN-γ , 9.4 pg/mL for IL-6, 31.2 pg/mL for IL-10, 15.6 pg/mL for IL-17, 31.2 pg/mL for IL-22, 16.3 pg/mL for IL-23, 15.4 pg/mL for TGF-β, and 15.6 pg/mL for TNF-α.

2.5. Flow cytometry analysis

The expression of transcription activators for Th1 (STAT1, STAT4), Th2 (STAT6), Th17 (STAT3) and Treg (STAT5) cells and intracellular factors of transcription for Th1 (T-bet), Th2 (GATA-3), Th17 (RORγt) and Treg (FoxP3) was evaluated after PBMCs culture from pregnant women with PE and NT pregnant women with or without 100μM of SB (Sigma-Aldrich) for 30 minutes. The concentration was adjusted to $2x10^5$ cells/ mL and the cells were distributed in tubes for flow cytometer (BD Biosciences). Cells were incubated with BD Biosciences antibodies with respective fluorochromes: anti-CD3 (APC-H7), anti-CD4 (PerCP-Cy5.5), anti-CD25 (BB515) and anti-CD127 (PE-Cy7) for 20 min, in the dark at 4 ° C. After centrifugation, cells were washed twice with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and centrifuged again for 5 min at 1500RPM. The cells were fixed using BD Cytofix™ Fixation Bufffer (BD Biosciences) and permeabilised using BD Phosflow™ Perm Buffer III (BD Biosciences). Then, the cells were incubated with specific Phosflow® antibodies (BD Biosciences) labelled with the fluorochromes: anti-FoxP3 (PE), anti-GATA -3 (PE), anti-RORγt (PE), anti-T-bet (PE), anti-STAT1 (Alexa 647), STAT3 (Alexa

647), STAT4 (Alexa 647), STAT5 (Alexa 647) and STAT6 (Alexa 647) for 30 min, in the dark and at room temperature. For each test, control tubes were incubated with specific isotypic antibodies for each fluorochrome. The samples were acquired in a flow cytometer of the FACSCanto^{TM} II model (BD Biosciences) with the FACSDiva software (BD Biosciences). Cellular multiparameters were analysed using in FlowJo software, version vX.10.6 (Tree Stars Inc., Ashland, OR, USA). The acquisition of 100.000 events per sample was standardized and the population of interest was optimized to establish a gating strategy for T cell subsets. First, events were selected by the size (FSC) and granularity (SSC) expected for lymphocytes. Then, doublets were discarded using a single cell strategy (singlets gate: FSC-A x FSC-H) and fluorescence for CD3 was considered. From this gate, established on CD4 positive ($CD4^+$) lymphocytes. Additionally, Treg cell analysis was performed inside a $CD25^+CD127^-$ gate. The results were represented in median fluorescence intensities (MFIs) and in percentage of cells (%) that express activators of transcription and transcription factors.

2.7. Statistical analysis

The results were evaluated using non-parametric tests, performed to the PRISM statistical program Graph Prism version 6.01 (GraphPad, San Diego, CA, USA). The evaluation of clinical characteristics and plasma determinations of pregnant women with PE and NT pregnant women were performed using the Mann-Whitney U test. The comparison of the results of immunologic parameters between preeclamptic and NT group after the treatment with SB, was performed by the Kruskal-Wallis test, followed by multiple comparisons by the Dunn test. Statistical significance was accepted at $p<0.05$.

3. Results

3.1. Clinical characteristics

Analysis of clinical characteristics of PE and NT pregnant women are shown in Table 1. There was no statistical difference between the groups evaluated, in relation to age and gestational age. Preeclamptic pregnant women presented significantly higher of systolic and diastolic blood pressure, as well as proteinuria, in comparison to NT pregnant women.

Table 1. Clinical characteristics of preeclamptic women (PE) and normotensive (NT) pregnant women

Characteristics	Preeclamptic women (n = 16)	NT pregnant women (n = 16)
Age (years)	27 (17–40)	28 (17–37)
Gestational age (weeks)	34 (24–37)	34 (25–39)
Systolic blood pressure (mmHg)	160***** (140–190)	100 (95–110)
Diastolic blood pressure (mmHg)	110***** (90–120)	65 (60–70)
Proteinuria (mg/24h)	460***** (300–5780)	< 300

Note: Data are expressed as the median, with the minimum and maximum values in parentheses.
* ($p < 0.05$) *vs.* NT pregnant women (Mann-Whitney U test).

3.2. Analysis of activators of transcription and transcription factors in $CD4^+$ T cell subsets from PE and NT pregnant women

$CD4^+$ T lymphocytes from 16 preeclamptic pregnant women and 16 NT pregnant women were evaluated regarding the expression of activators of transcription, and transcription factors characteristic of T cell subsets, treated or not with SB, through the analysis of the median fluorescence intensity (MFI) and the percentage (%) of cells expressing the intracellular phosphorylated proteins.

Figure 1 shows the profile Th1. Firstly, the endogenous expression of STAT1 STAT4 and T-bet determined by MFI (Fig. 1A, 1C, and 1E) and percentage of lymphocytes was significantly higher (Fig. 1B, 1D, and 1F) in the PE group than in the NT group. However, the treatment of these cells with SB decreased significantly these parameters. SB treatment didn't show the modulatory effect on lymphocytes from NT pregnant women.

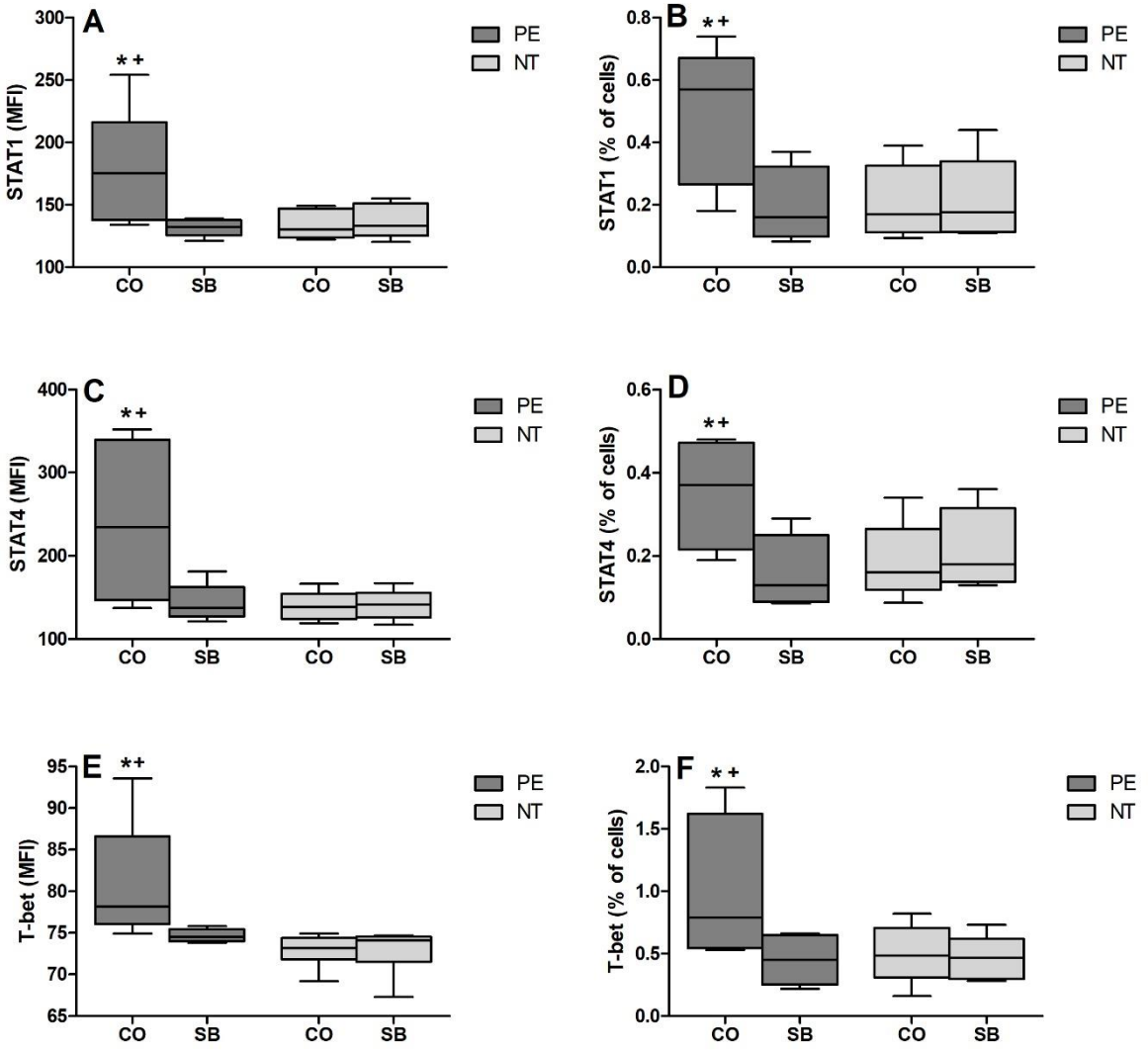

Fig. 1. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA1; (B) % of lymphocytes expressing STAT1; (C) MFI of lymphocytes expressing STAT4; (D)% of lymphocytes expressing STAT4. (E) MFI of lymphocytes expressing T-bet; (F) % of lymphocytes expressing T-bet. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers).* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Regarding, STAT3, and RORγt, characteristic of Th17 inflammatory profile, it was observed that cells from preeclamptic women have MFI (Fig. 2A, and 2C), as well as, percentage of cells (2B, and 2D) expressing both the activator and transcription factor significantly higher when compared to NT pregnant women. The addition of SB into the cultures was able to cause a significant reduction in STAT3 (Fig. 2A, and 2B) and RORγt (Fig.

2C, and 2D) in preeclamptic women, but didn't affect the expression of STAT3 and RORγt by cells of NT group.

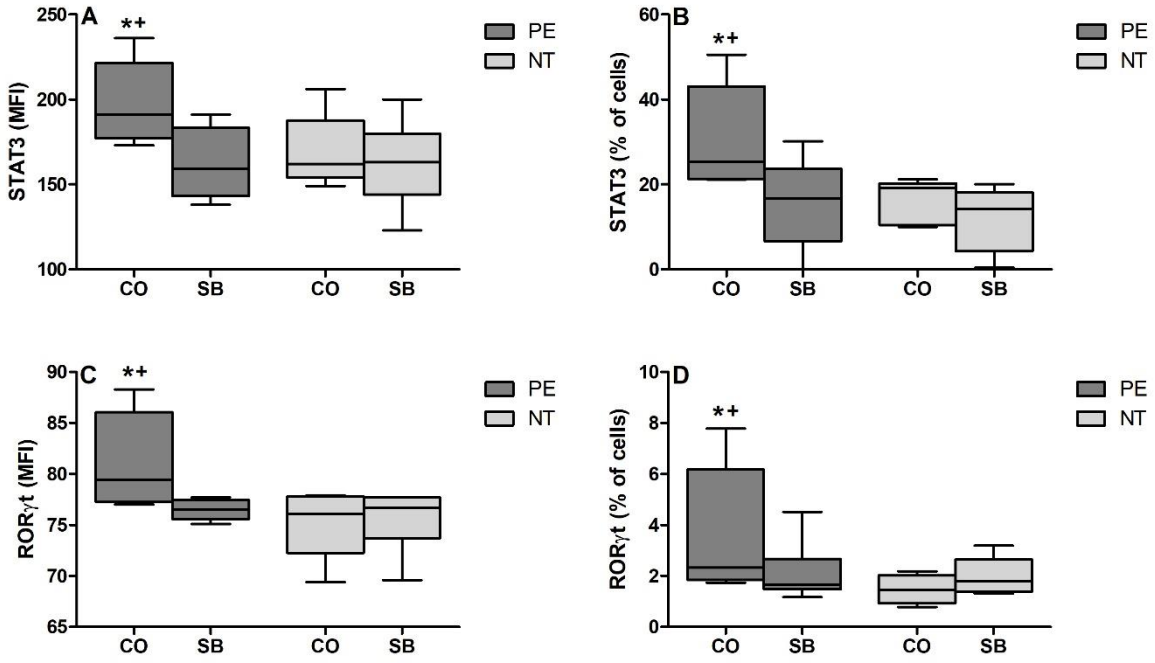

Fig. 2. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA3; (B) % of lymphocytes expressing STAT3; (C) MFI of lymphocytes expressing RORγt; (D)% of lymphocytes expressing RORγt. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Figure 3 showed the analysis of activators of transcription and the transcription factors phosphorylated of anti-inflammatory Th2 profile in $CD4^+$ T cell subsets cultured in the presence or absence of SB.

The basal expression of the STAT6 and GATA-3 were significantly lower in PE group in comparison to NT group, both analyzed by the MFI (Fig. 3A and 3C respectively) and by the percentage of lymphocytes expressed them (Fig. 3B and 3D). Treatment with SB induced a significant increase in MFI and in the percentage of cells expressing STAT6 and GATA-3 in preeclamptic pregnant women.

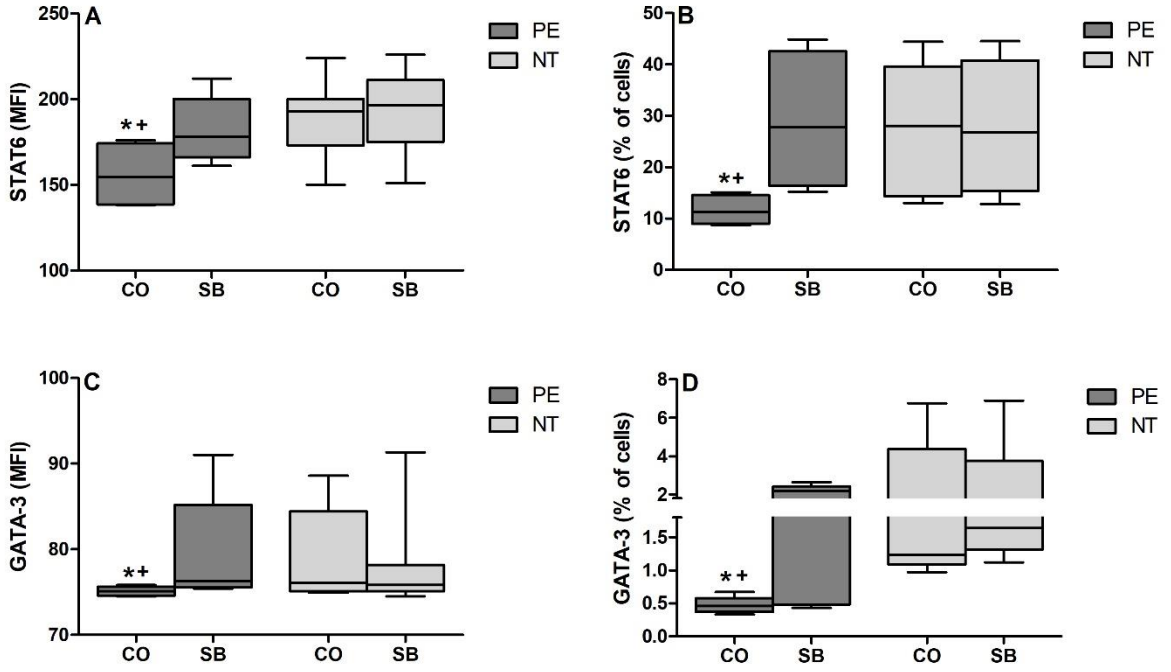

Fig. 3. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA6; (B) % of lymphocytes expressing STAT6; (C) MFI of lymphocytes expressing GATA-3; (D)% of lymphocytes expressing GATA-3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

The immunomodulatory effect of SB on the regulatory profile was represented by the expression of the STAT5 transcription activator and the FoxP3 transcription factor in the figure 4. Preeclamptic women group shows MFI (Fig. 4A and 4C) and percentage of cells that express STAT5 (Figure 4B) and FoxP3 (Figure 4D) significantly lower when compared to NT group. Lymphocyte exposure to SB shows that there is a significant increase in MFI and in the percentage of cells expressing them in the PE group.

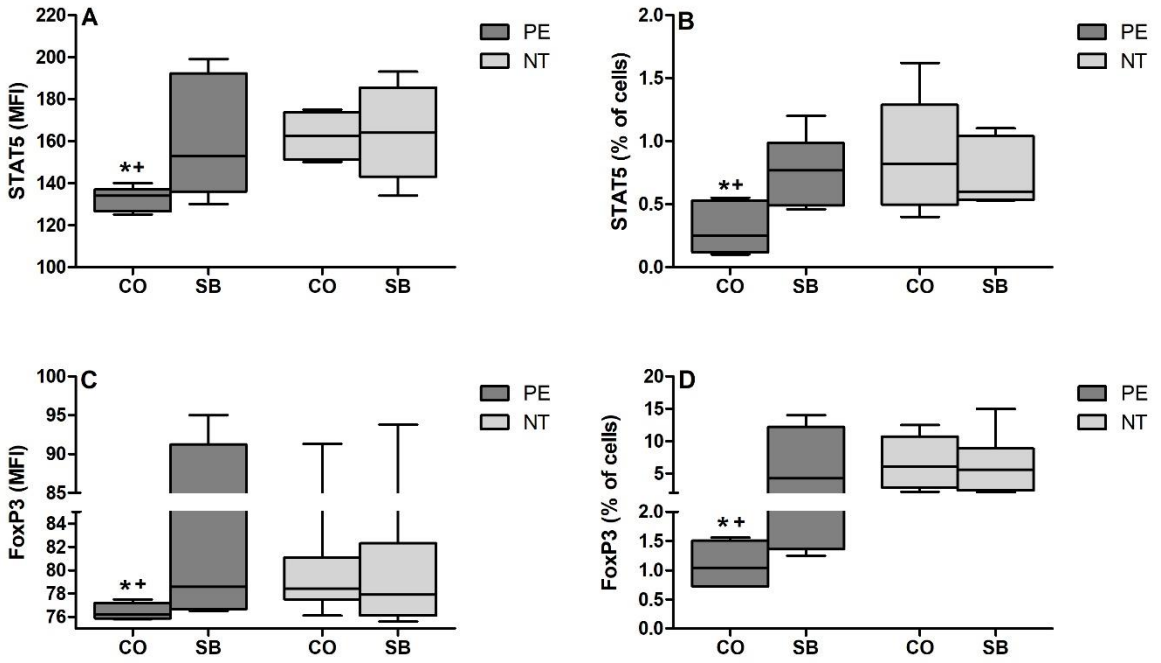

Fig. 4. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA5; (B) % of lymphocytes expressing STAT5; (C) MFI of lymphocytes expressing FoxP3; (D)% of lymphocytes expressing FoxP3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

3.3. Cytokine production in PBMC culture supernatants from PE and NT pregnant women

Figure 5 shows cytokine production in supernatants culture of PBMCs treated with or without SB from PE and NT pregnant women are demonstrated in Fig. 5. It was observed significantly higher basal levels of inflammatory cytokines (Fig. 5A-F), whereas the concentrations of IL-10 (Fig. 5G), and TGF-β (Fig. 5H) were significantly lower in the group with PE than NT group. Cell culture in the presence of SB induced a decreased of release of inflammatory cytokines (Fig. 5A-F), while the levels of anti-inflammatory cytokine IL-10 (Fig. 5G) and TGF-β (Fig. 5H) increased significantly in the pregnant women with PE.

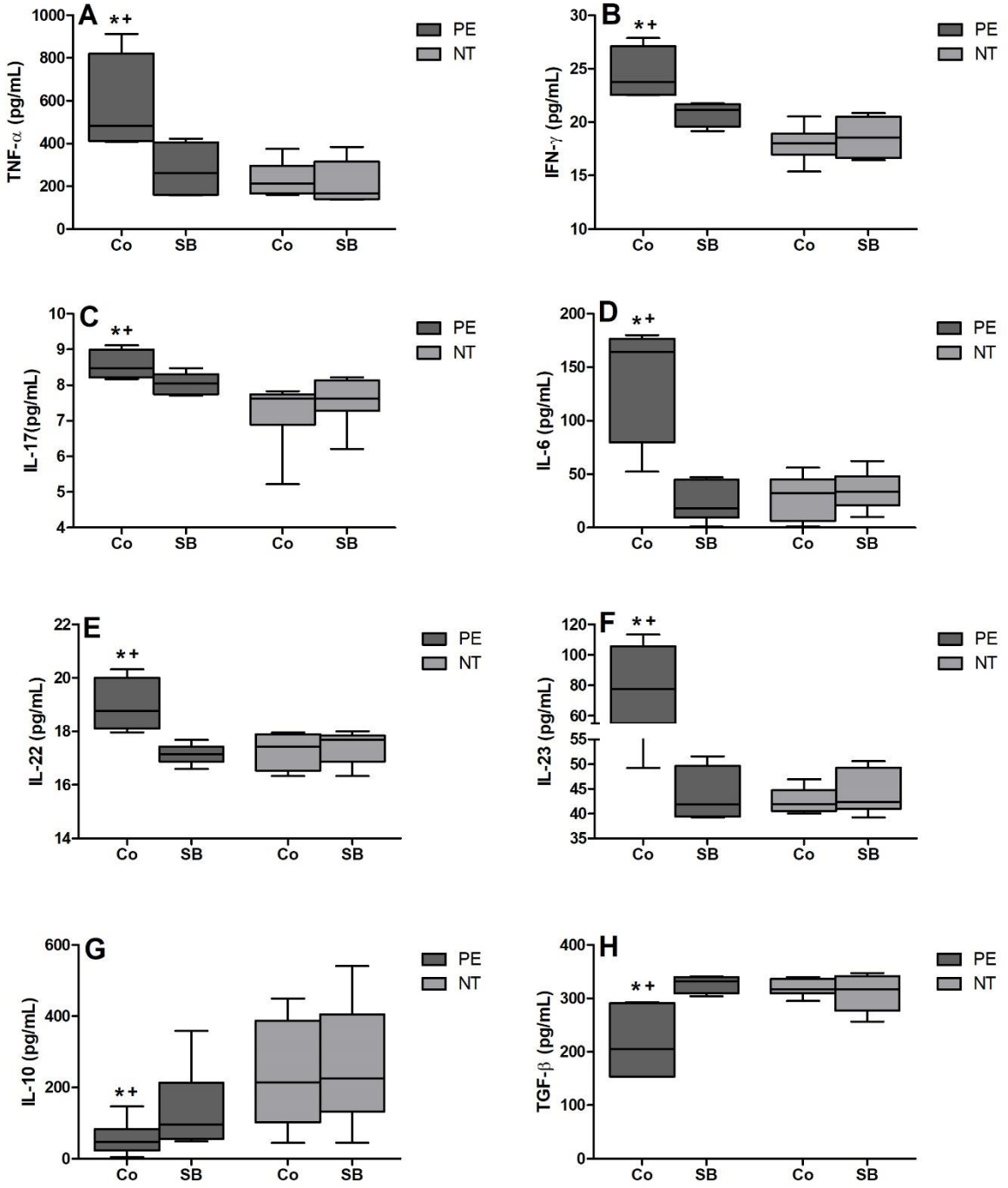

Fig. 5. Concentrations of TNF-α (A), IFN-γ (B), IL-17 (C), IL-6 (D), IL-22 (E), IL-23 (F), IL-10 (G), and TGF-β (H) detected in the supernatants of PBMCs culture of 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of silibinin (SB) for 24 hours by ELISA technique. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

4. Discussion

Several published works have demonstrated that SB, a complex flavonoid extracted from silymarin, has an important anti-inflammatory, hepatoprotective, antifibrotic, anticarcinogenic, antioxidant and immunomodulatory properties. SB has a wide spectrum of immunomodulatory effects on different conditions, and the recognition by which this agent acts on cellular and molecular pathways can be extremely important for the treatment of several pathologies [23].

The JAK/STAT is the main signaling pathway regulated by cytokines and is essential for initiating innate immunity, orchestrating the adaptive immune mechanisms, and finally suppressing the inflammatory process and immune responses [13, 24-26].

Thus, in the present study, the results demonstrated that T cells from preeclamptic women have a higher endogenous expression of STAT1, STAT4, and STAT3 and a lower expression of STAT6 and STAT5. These cells treatment with SB led to a reduction of STAT1, STAT4, and STAT3 expression. It is known that STAT1/STAT4 are key factors necessary for Th1 polarization [14, 27], while the STAT3 is required for the induction of Th17 profile [14, 28]. Besides that, associated with these reductions it was observed an increase of STAT6 and STAT5 in the presence of SB in the PE group, showing that STAT6, the important factor for Th2 profile inhibits Th1 polarization [14, 27], while STAT5 promotes Treg differentiation [29].

In addition of the activators of transcription, the results obtained by flow cytometry analysis of transcription factors involved in T cell differentiation showed that pregnant women with PE have a higher MFI and percentage of cells expressing T-bet and RORγt, and a lower of GATA-3 and FoxP3 in relation to NT pregnant women. In agreement with our findings, another study also demonstrated a higher percentage of Th17 cells, while the levels of Treg cells are lower in patients with PE than in controls patients, suggesting that a shift from Treg cells toward Th17 cells can result in PE [12]. The treatment of cells with SB decreased the

expression of T-bet and RORγt and was able to induce an increase of GATA-3 and FoxP3, specific of anti-inflammatory and regulatory profiles. In addition, culture of the cells in the presence of SB decreased the levels of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-23, and increased IL-10 and TGF-β, results that corroborate the literature [30, 31]. A study of experimental model of rheumatoid arthritis showed that SB inhibited the in vitro differentiation of the Th17 subpopulation, and the secretion of inflammatory cytokines such TNF-α, and IL-6 [32]. Thus, our results suggest that the modulatory effect of SB on T cell subsets can be a possibility to control the imbalance of Th17/Treg in PE. To our knowledge this is the first study that evaluated the immunomodulatory effect of SB on activators of transcription and transcription factors in $CD4^+$ T cell subsets obtained from preeclamptic and NT pregnant women.

The differentiation of T cell subsets is dependent on characteristic transcription factors, known for each profile. However, the literature indicates that the combination of activation of distinct signaling pathways and the concomitant mobilization of these multiple transcription factors contribute to the determination of T cell subsets [33]. Thus, the modulation of SB on activators of transcription and transcription factors in the present study may have been mediated by the transcription factor, NF-kB, on the interaction between innate and adaptive immune cells.

NF-κB is an important transcription factor that has been shown to regulate several aspects of T cells, such as development, activation, survival, and differentiation. NF-kB family is composed of five subunits: RelA (p65), RelB, c-Rel, NF-kB1, and NF-kB2 [33, 34]. A previous study from our research group reported the effect of SB on the innate immune system, specifically, on monocytes of preeclamptic women. It was demonstrated that treatment of monocytes with SB, led to reducing the basal activation of these cells, through decreasing NLRP1/NLRP3 inflammasomes and p65NF-κB activity [30].

Some studies showed the role of NF- kB in each T cell subsets. In a model using transgenic mice expressing a non-degradable form of IkB specifically in T cells, Th1 responses were significantly impaired due to diminished NF- κB activation [35]. Another report showed that mice deficient in c-Rel represented defective Th1-mediated immune responses, and inhibited the IFN-γ production by T cells [33, 36]. In relation to the Th2 profile, NF- kB was reported to be essential for the GATA-3 gene expression. It was shown also, that $CD4^+$ T cells from p-50 deficient mice (NF-kB1) did not express GATA-3 gene under Th2 differentiation over in vivo and in vitro conditions [37]. Besides that, NF-κB play a role in the differentiation and function of Th17 acting in the regulation of RORγt gene expression [38]. In the regulatory profile, it has been reported that the NF-kB, c-Rel subunit binds to conserved non-coding sequences (CNS3) playing an important role in the expression of FoxP3 and consequently, the differentiation of Treg both in the thymus and periphery [33, 39, 40].

Therefore, the immunomodulatory effect of SB observed in $CD4^+$ T cell subsets, through the STAT signaling pathway, leading to downregulation of Th inflammatory lymphocytes in pregnant women with PE, might be explained by inhibition of NF-kB activation mediated by SB. The literature broadly demonstrated that SB inhibits NF-kB activation, with suppression of IkB degradation, preventing translocation of this transcription factor into the nucleus and consequently, the transcription of genes related to the inflammatory response [23, 41].

In conclusion, the results of the present study show that SB modulates the $CD4^+$ T cell subsets, leading to an improvement in the anti-inflammatory and regulatory profiles in women with PE. Further studies are needed to understand the action of SB in the JAK/STAT pathway and transcription factors from T cell subsets in this gestational pathology.

Acknowledgments

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Author contributions

V.R.R., M.T.P. and M.R.V conceived the idea, designed the experiments, analyzed the data, and wrote the paper. J.F.A. and J.C.P. selected pregnant women for the study. V.R.R., M.R.V. and P.R.N. performed experiments. L.C.R.O and G.G.R performed the flow cytometry analysis. All authors reviewed the manuscript and approved the final version for publication.

Declaration of Competing Interest

The authors declare no commercial or financial conflict of interest.

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Figure Legends

Fig. 1. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA1; (B) % of lymphocytes expressing STAT1; (C) MFI of lymphocytes expressing STAT4; (D)% of lymphocytes expressing STAT4. (E) MFI of lymphocytes expressing T-bet; (F) % of lymphocytes expressing T-bet. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers).* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 2. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA3; (B) % of lymphocytes expressing STAT3; (C) MFI of lymphocytes expressing RORγt; (D)% of lymphocytes expressing RORγt. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 3. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA6; (B) % of lymphocytes expressing STAT6; (C) MFI of lymphocytes expressing GATA-3; (D)% of lymphocytes expressing GATA-3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 4. Median fluorescence intensity (MFI) and percentage of lymphocytes expressing activators of transcription and transcription factors intracellular phosphorylated in 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women, detected by the flow cytometry technique. The cells were cultured in the absence (Co) or presence of silibinin (SB) in vitro for 30 minutes. (A) MFI of lymphocytes expressing STA5; (B) % of lymphocytes expressing STAT5; (C) MFI of lymphocytes expressing FoxP3; (D)% of lymphocytes expressing FoxP3. The results are expressed in median (horizontal line), 25th and 75th percentile (box) and range (whiskers). * ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

Fig. 5. Concentrations of TNF-α (A), IFN-γ (B), IL-17 (C), IL-6 (D), IL-22 (E), IL-23 (F), IL-10 (G), and TGF-β (H) detected in the supernatants of PBMCs culture of 16 pregnant women with preeclampsia (PE) and 16 normotensive (NT) pregnant women cultured in vitro in the absence (Co) or presence of silibinin (SB) for 24 hours by ELISA technique. The results are expressed in median (horizontal line), 25^{th} and 75^{th} percentile (box) and range (whiskers).
* ($p < 0.05$) vs PE VD; + ($p < 0.05$) vs NT Co (Kruskal-Wallis test).

CONCLUSÕES

Conclusões Gerais

- Em conclusão, o presente estudo demonstrou que gestantes portadoras de PE apresentam níveis plasmáticos de VD, significativamente menores em relação às gestantes NT. O tratamento *in vitro* de PBMCs com VD induziu polarização das subpopulações de células T com perfis anti-inflamatório (Th2) e regulador (Treg), detectada pelo efeito imunomodulador da VD sobre a expressão gênica dos fatores de transcrição característicos dessas células no grupo PE. Assim, sugere-se que a resposta inflamatória sistêmica observada na PE pode ser decorrente de um desequilíbrio na homeostase do sistema imunológico materno, causado pela diminuição do hormônio VD, capaz de regular a inflamação.

- A análise da expressão proteica dos ativadores de transcrição (STATs) e dos fatores de transcrição das subpopulações de células T $CD4^+$, bem como das citocinas produzidas por essas células mostrou que o tratamento in vitro das células com VD modulou negativamente os perfis inflamatórios Th1/Th17, induzindo polarização das subpopulações de células T para os perfis Th2/Treg com produção de citocinas anti-inflamatórias, mediada pela via de sinalização JAK/STAT, nas gestantes com PE.

- A avaliação do tratamento in vitro das PBMCs de gestantes pré-eclâmpticas com SB mostrou que esse flavonoide parece modular a via JAK/STAT, induzindo diminuição dos ativadores de transcrição e dos fatores de transcrição de perfis inflamatórios, além da diminuição das citocinas pró-inflamatórias por linfócitos T $CD4^+$ em gestantes portadoras de PE. Sendo assim, o papel modulador da SB parece ter grande importância na imunidade adaptativa.

- Portanto, a inflamação estéril presente na PE, parece ser decorrente de um desbalanço entre as subpopulações de células T. O emprego de agentes com propriedades imunomoduladoras como a VD e SB, capazes de restabelecer o desequilíbrio do sistema imune adaptativo materno pode ser uma nova alternativa adjuvante no tratamento da resposta inflamatória sistêmica que ocorre na PE.

"É verdade que a experiência e o pensamento claro são a melhor maneira de fundamentar as convicções".

Albert Einsten

ANEXOS

Termos de consentimento livre e esclarecido (TCLE)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012
(Participante maior de 18 anos)

CONVIDO, a senhora _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.

Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.

Informamos que o material biológico colhido da Senhora, não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.
Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.

Fui informada que não terei benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informada também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de participar da pesquisa, sem prejuízo do atendimento médico que necessitar e, que meu nome não aparecerá quando os resultados forem divulgados.

Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue a Senhora devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.
Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descritos:
Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO EM PARTICIPAR de forma voluntária, estando ciente que todos os meus dados estarão resguardados através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados em revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,____/___/_____

_____ _____
Pesquisador Participante da Pesquisa

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TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO (TALE)
RESOLUÇÃO 466/2012
Para menores entre 12 a 17 anos/11meses e 29 dias

CONVIDO, você_____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.

Caso concorde em participar da pesquisa você deverá assinar este Termo de Assentimento e seu Representante Legal assinará o Termo de Consentimento Livre e Esclarecido para participantes de pesquisa entre 11 a 17 anos/11/meses e 29 dias
Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.

Informamos que o seu material biológico colhido não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.
Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.

Fui informada que não terei benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informada também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de participar da pesquisa, sem prejuízo do atendimento médico que necessitar e, que meu nome não aparecerá quando os resultados forem divulgados.
Fique ciente, que a participação neste estudo é voluntária e que mesmo após ter dado consentimento para participar da pesquisa, você poderá retirar a qualquer momento,

sem qualquer prejuízo na continuidade do tratamento, ou qualquer outra atividade em que você esteja participando.

Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue a você devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.

Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:

Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO em participar de forma voluntária, estando ciente que todos os meus dados estarão resguardos através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados e revistas científicas.

Botucatu,____/___/_____

_____ _____

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OBS: Este Assentimento Livre e Esclarecido deverá vir acompanhado do Termo de Consentimento Livre e Esclarecido de para participantes de pesquisa de 12anos a 17anos/11meses e 29 dias).

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012
(Para Responsável Legal de participantes de 12anos a 17anos/11meses e 29 dias)

CONVIDO, o Senhor (a) _____responsável pelo menor _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.

Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do sangue ou um pedaço da placenta após o parto da sua filha _____, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente

Informamos que o material biológico colhido de sua filha não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para o (a) senhor (a) assine nova autorização para utilização desse material.
Solicito também seu consentimento para consultar o prontuário médico de sua filha, para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.

Fui informada que minha filha não terá benefícios diretos com a pesquisa, mas que o resultado desse exame poderá ser enviado para mim, caso eu tenha interesse em obtê-lo. Fui informado (a) também que eu posso, a qualquer momento, esclarecer dúvidas e desistir de consentir que ela participe da pesquisa, sem prejuízo do atendimento médico que ela necessitar e, que o nome dela não aparecerá quando os resultados forem divulgados.

Informo que a participação do seu filho (a) neste estudo é voluntária e que mesmo após o senhor ter dado o consentimento para que ele participe da pesquisa, o senhor poderá retirar o consentimento a qualquer momento, sem qualquer prejuízo na continuidade do seu tratamento.

Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue ao Senhor (a) devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.

Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:

Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO na qualidade de "Representante Legal" a participação de meu (minha) filho(a) de forma voluntária, estando ciente que todos os seus dados estarão resguardados através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados em revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,_____/___/_____

_____ _____

Pesquisador Representante Legal pelo Participante da Pesquisa

Maria Terezinha S. Peraçoli – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu – UNESP
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Leandro Gustavo de Oliveira – Departamento de Ginecologia e Obstetrícia – Faculdade de Medicina Botucatu – UNESP
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Mariana Romão Veiga – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu - UNESP
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Priscila Rezeck Nunes – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu - UNESP
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Vanessa Rocha Ribeiro – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu - UNESP
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OBS: Esse Termo de Consentimento Livre e Esclarecido, deverá vir acompanhado do Termo de Assentimento Livre e Esclarecido para participantes de pesquisa de 12anos a 17anos/11meses e 29 dias).

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)
RESOLUÇÃO 466/2012 – GRUPO CONTROLE

CONVIDO, a senhora _____ para participar do Projeto de Pesquisa intitulado "Modulação da resposta inflamatória sistêmica na pré-eclâmpsia":
Projeto 1: Modulação M1/M2 em monócitos de gestantes portadoras de pré-eclâmpsia
Projeto 2: Efeito imunomodulador da vitamina D sobre a ativação de inflamassomas em tecido placentário de gestantes portadoras de pré-eclâmpsia
Projeto 3: Efeito imunomodulador da vitamina D e silibinina sobre subpopulações de células T CD4+ em gestantes portadoras de pré-eclâmpsia
Projeto 4: Efeito modulador da silibinina sobre inflamassoma NLRP3 induzido por urato monossódico em monócitos de gestantes portadoras de pré-eclâmpsia
que será desenvolvido por nós, Priscila Rezeck Nunes (bióloga), Vanessa Rocha Ribeiro (bióloga), Mariana Romão Veiga (bióloga) e Virgínia Juliani Gomes (Graduanda em Biomedicina), com orientação da Biomédica e Professora Maria Terezinha Serrão Peraçoli da Faculdade de Medicina de Botucatu –UNESP.

Sua participação nesta pesquisa se dará por ser uma gestante saudável, sem nenhum tipo de doença. Todos os dados que obtivermos com sua participação são meramente para compararmos com os dados de outros participantes que possuem a doença que estamos estudando.

Estamos estudando a pré-eclâmpsia. Para que possamos ter um resultado nesse momento precisamos coletar 10 ml do seu sangue ou um pedaço da placenta após o parto, que serão utilizados para experimentos imunológicos relacionados a doença. O risco com a coleta de sangue será a picadinha da agulha e uma manchinha roxa que desaparecerá bem rapidamente.

Informamos que o material biológico colhido da Senhora ,não será usado em sua totalidade e parte dele será armazenada na Faculdade de Medicina. Para reutilização desse material será escrito um novo projeto de pesquisa, com um novo termo de consentimento para que para a senhora assine nova autorização para utilização desse material.
Solicito também seu consentimento para levantar seu prontuário médico para coletar outras informações lá contidas, que possam fornecer subsídios para fazer comparação entre o indivíduo saudável e em tratamento de doença.

A Senhora não terá nenhum benefício em participar desta pesquisa, pois como já explicamos seus dados serão meramente para comparar com os dados de uma pessoa em tratamento de doença.

Fique ciente de que sua participação neste estudo é voluntária e que mesmo após ter dado seu consentimento para participar da pesquisa, você poderá retirá-lo a qualquer momento, sem qualquer penalidade.

Este Termo de Consentimento Livre e Esclarecido será elaborado em 2 vias de igual teor, o qual 01 via será entregue ao Senhor (a) devidamente rubricada, e a outra via será arquivada e mantida pelos pesquisadores por um período de 5 anos após o término da pesquisa.

Qualquer dúvida adicional você poderá entrar em contrato com o Comitê de Ética em Pesquisa através dos telefones (14) 3880-1608 ou 3880-1609 que funciona de 2ª a 6ª feira das 8.00 às 11.30 e das 14.00 às 17horas, na Chácara Butignolli s/nº em Rubião Júnior – Botucatu - São Paulo. Os dados de localização dos pesquisadores estão abaixo descrito:

Após terem sido sanadas todas minhas dúvidas a respeito deste estudo, CONCORDO EM PARTICIPAR de forma voluntária, estando ciente que todos os meus dados estarão resguardo através do sigilo que os pesquisadores se comprometeram. Estou ciente que os resultados desse estudo poderão ser publicados e revistas científicas, sem no entanto, que minha identidade seja revelada.

Botucatu,_____/___/_____

_____ _____

Pesquisador Participante da Pesquisa

Maria Terezinha S. Peraçoli – Departamento de Microbiologia e Imunologia – Instituto de Biociências de Botucatu – UNESP
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Parecer do Comitê de Ética em Pesquisa

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

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

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

DADOS DO PARECER

Número do Parecer: 4.418.043

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

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

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

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

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

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

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

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

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

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

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

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

Avaliação dos Riscos e Benefícios:

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

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

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

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

Comentários e Considerações sobre a Pesquisa:

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

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

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

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

Continuação do Parecer: 4.418.043

Considerações sobre os Termos de apresentação obrigatória:

Vide campo de "Conclusões ou Pendências e Lista de Inadequações".

Conclusões ou Pendências e Lista de Inadequações:

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

Considerações Finais a critério do CEP:

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

Atenciosamente,

Comitê de Ética em Pesquisa FMB/UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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

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Continuação do Parecer: 4.418.043

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

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

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Telefone: (14)3880-1609 **E-mail:** cep@fmb.unesp.br

Continuação do Parecer: 4.418.043

BOTUCATU, 24 de Novembro de 2020

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

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