UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

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PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO

Belo Horizonte - MG
2012

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Tese submetida ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial, para obter o grau de doutor em Ciências Farmacêuticas.

Orientadora: Profª. Drª. Luci Maria S. Dusse
Co-orientadora: Profª. Drª Karina Braga G. Borges
Co-orientador: Profº. Drº Olindo Assis M. Filho

Belo Horizonte - MG

2012

Pinheiro, Melina de Barros.
B277p Pré-eclâmpsia: inter-relação dos sistemas hemostático e inflamatório / Melina de Barros Pinheiro. – 2012.
129 f. : il.

Orientadora: Profª. Drª. Luci Maria Sant'Ana Dusse.
Coorientadores: Profª . Karina Braga G. Borges, Prof. Dr. Olindo Assis Martins Filho.
Tese (doutorado) - Universidade Federal de Minas Gerais, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas.

1. Pré-eclâmpsia – Teses. 2. Hemostasia – Teses. 3. Fibrinólise – Teses. 4. Inflamação – Teses. 5. Citocinas – Teses. I. Dusse, Luci Maria Sant'Ana. II. Gomes, Karina Braga. III. Martins Filho, Olindo Assis. IV. Universidade Federal de Minas Gerais. Faculdade de Farmácia. V. Título.

CDD 616.15

FOLHA DE APROVAÇÃO

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Dedico este trabalho

A Deus, por me abençoar e iluminar todos os dias de minha vida.
Aos meus queridos orientadores Profª Luci, Profª Karina e Dr. Olindo,
pela dedicação, ensinamentos e exemplos de vida.
Aos meus pais e irmãos, pelo amor e apoio incondicional.
Ao Luis, pelo amor, carinho e incentivo em todos os momentos.

AGRADECIMENTOS

A Deus, pela incrível tarefa da criação e pela alegria de viver que me foi concedida. Por sua luz, que me ilumina, em todos os momentos de minha vida.

A querida professora Luci Dusse, muito obrigada pela dedicação, carinho e atenção. Obrigada por sempre acreditar e confiar em mim. Você é a principal responsável por essa vitória. Agradeço pela oportunidade de convívio ao lado de uma pessoa tão iluminada como você. Obrigado por tornar todos os momentos deste trabalho um constante aprendizado. Muito obrigada por tudo!

A querida profa. Karina Braga, pela colaboração, carinho, ensinamentos e principalmente amizade. Admiro sua sabedoria e seu profissionalismo . A sua competência e humildade fazem com que você se transforme em um exemplo para todos que convivem ao seu redor. Muito obrigada!

Ao Dr. Olindo A. Martins Filho pela colaboração, dedicação e ensinamentos essenciais no desenvolvimento deste trabalho. Muito obrigada.

As minhas colegas e parceiras de coleta Lara, Patrícia, Fabiana e Letícia pela fundamental ajuda, apoio e incentivo. Sem a colaboração de vocês a realização desse trabalho não seria possível.

A equipe do laboratório Simile Imunologia Aplicada, em especial a Dra. Carla e a Gabrielle Guimarães pela calorosa acolhida e fundamental colaboração em meus experimentos.

Ao pessoal do laboratório de Biomarcadores de Diagnóstico e Monitorização – Centro de Pesquisa René Rachou – FIOCRUZ, em especial Dra. Andrea Teixeira Carvalho e Amanda Silveira, pela colaboração e grande ajuda no desenvolvimento dessa tese.

Aos amigos do Laboratório de Hematologia Clínica da Faculdade de Farmácia da UFMG pelo convivência, apoio e amizade.

Aos professores, colegas e funcionários da pós-graduação da Faculdade de Farmácia da UFMG que acompanharam e apoiaram o desenvolvimento deste projeto.

A bibliotecária da Universidade de São Paulo (USP) Riberião Preto, Maria Cristina M. Ferreira, pela ajuda fundamental em nossos trabalhos de revisão sistemática e metanálise.

Aos meus pais e meus irmãos Welbinho e Marina, que me apoiaram em todos os momentos e sempre confiaram em mim. Muito obrigada pelo amor, carinho e confiança.

Aos meus segundos pais, Zarinha e Lele, pessoas maravilhosas capazes de tornar todos os momentos mais prazerosos e suaves. Obrigada pela apoio, carinho e

dedicação.

Ao Luis, por ser tão especial e estar sempre ao meu lado me acalmando, torcendo e incentivando. Muito obrigada pelo amor e compreensão.

As minhas queridas amigas Soraya, Larissa e Pollyana, pelo incentivo, amizade e motivação nos momentos mais difíceis. Muito obrigada por estarem presentes em minha vida.

As mulheres que contribuíram voluntariamente com este estudo, sem vocês não seria possível a realização deste trabalho.

A equipe médica e de enfermagem da Maternidade Odete Valadares, Hospital Público Regional de Betim, Unidade Básica de Saúde da Família (UBSF) Guanabara / Betim, Hospital Municipal Odilon Behrens e Santa Casa de Belo Horizonte pela parceria tão fundamental para a realização desse trabalho.

A todos os professores e mestres que passaram pela minha vida, pelos ensinamentos e dedicação.

A todos que contribuíram de alguma forma para que este trabalho fosse desenvolvido.

RESUMO

A pré-eclâmpsia (PE) é uma doença multifatorial, caracterizada por hipertensão e proteinúria após a 20ª semana de gestação. A etiologia da PE ainda não é conhecida e a doença ocorre somente na presença da placenta. Clinicamente é importante diagnosticar a forma grave da doença, na qual a pressão arterial e a proteinúria estão ainda mais elevadas. A PE está associada à disfunção vascular, bem como à exacerbação da coagulação, ainda mais acentuada que aquela observada nas gestantes normotensas. O envolvimento do sistema imune na patogênese da PE é bem aceito e essa doença está associada a um estado inflamatório exagerado. Diversos polimorfismos nos genes de citocinas pró-inflamatórias parecem estar associados ao desenvolvimento da PE. Sabe-se que componentes de sistema hemostático são capazes de ativar o sistema inflamatório e vice-versa. Dessa forma, o objetivo desse estudo foi investigar a inter-relação dos sistemas hemostático e inflamatório na PE grave, por meio da determinação dos níveis plasmáticos de marcadores hemostáticos e citocinas, bem como avaliar a relação de polimorfismos nos genes das citocinas e a ocorrência de PE. Foram avaliadas 331 mulheres, sendo 108 mulheres não gestantes, 107 gestantes normotensas e 116 gestantes com PE forma grave. A PE grave foi definida por pressão arterial ≥160/110mmHg e proteinúria > 2 gL-1. Os níveis plasmáticos de PAI-1 e D-Di foram determinados por ELISA (*Kit* IMUBIND® PLASMA PAI-1 e *Kit* IMUCLONE® D-Dimer American Diagnostica® Inc., *Stamford, USA* , respectivamente). As citocinas IL-8, IL-6, IL-1β, TNF-α, IL-12, IFN-γ, IL-4, IL-5 e IL-10 foram determinadas por citometria de fluxo (Cytometric Beads Array – CBA; BD Biosciences Pharmingen, USA). A determinação dos polimorfismos nos genes das citocinas IL-6, IL-10, IFN-γ e TNF-α foi feita por PCR-SSP (Cytokine Genotyping Tray; One Lambda, Inc. Canoga Park, CA). Os dados obtidos neste estudo permitem concluir que os marcadores plasmáticos da coagulação/fibrinólise e as citocinas inflamatórias IL-6, IL-8 e IFN-γ estão elevados na PE grave e não há correlação forte entre os mesmos; a PE grave está associada a maior frequência do genótipo T/T no gene IFN-γ (+874) e esse genótipo determina o aumento desta citocina, enquanto os outros polimorfismos estudados não exercem qualquer papel nesta doença. A

revisão sistemática e metanálise investigando os níveis de D-Di na PE, revelaram que esse marcador é um candidato promissor para a monitoração da PE.

Palavras-chave: Pré-eclâmpsia; hemostasia; fibrinólise; D-Dímero; PAI-1; inflamação; citocinas;

ABSTRACT

Preeclampsia (PE) is a multifactorial disease characterized by hypertension and proteinuria after 20 weeks of gestation. The PE etiology is not known yet, and the disease occurs only in the presence of the placenta. Clinically it is important to diagnose the severe form of the disease, in which blood pressure and proteinuria are even higher. PE is associated with vascular dysfunction, as well as to exacerbation of coagulation, which is higher than those observed in normotensive pregnant women. The involvement of the immune system in the PE pathogenesis is well accepted and this disease is associated with a high inflammatory condition. Several polymorphisms in the genes of pro-inflammatory cytokines appear to be associated with PE occurrence. It is known that components of the hemostatic system are able to activate the inflammatory system and vice versa. Thus, the aim of this study was to investigate the relationship between hemostatic and inflammatory systems in severe PE, by determining plasma levels of hemostatic markers and cytokines, as well as evaluating the relationship of polymorphisms in cytokine genes and the PE occurrence. A total of 331 women were evaluated (108 non-pregnant women, 107 normotensive pregnant women, and 116 pregnant women with severe PE). Severe PE was defined as blood pressure ≥ 160/110mmHg and proteinuria > 2 g L-1. PAI-1 and D-Di Plasma levels were measured by ELISA (Kit IMUBIND® PLASMA PAI-1 and IMUCLONE® Kit D-Dimer American Diagnostica® Inc., Stamford, USA, respectively). The cytokines IL-8, IL-6, IL-1β, TNF-α, IL-12, IFN-γ, IL-4, IL-5 and IL-10 were determined by flow cytometry (Cytometric Beads Array - CBA; BD Biosciences Pharmingen, USA). The determination of polymorphisms in the IL-6, IL-10, IFN-γ and TNF-α genes was performed by PCR-SSP (Cytokine Genotyping Tray; One Lambda, Inc. Canoga Park, CA). The data obtained in this study indicate that plasma markers of coagulation/fibrinolysis and inflammatory cytokines IL-6, IL-8 and IFN-γ are elevated in severe PE and there is not a strong correlation between them. Furthermore, severe PE is associated with high frequency of T/T genotype in IFN-γ gene (+874) and this genotype determines the increase of this cytokine, while the other polymorphisms do not exert any role in this disease. The systematic review and meta-analysis investigating the D-Di levels in PE revealed that this marker is a promising candidate for monitoring of PE.

Keywords: Preeclampsia, hemostasis, fibrinolysis, D-dimer, PAI-1, inflammation, cytokines

LISTA DE ABREVIATURAS E SIGLAS

α2-AP	*α2-antiplasmin*
α2-M	*α2-macroglobulin*
ACOG	*American College of Obstetricians and Gynecologists*
ALT	*Alanine aminotransferase*
APC	*Activated protein C*
AST	*Aspartate amino transferase*
CBA	Cytometric Bead Array
CID	Coagulação intravascular disseminada
COX-2	Cyclooxygenase-2
D-Di	Dímero-D / *D-Dimer*
ELISA	*Enzyme-linked immunosorbent assay*
FVII	*Factor VII*
HELLP	*Haemolysis*, *elevated liver enzyme activity*, *low platelets*
IFN-γ	Interferon do tipo gama
IL	Interleucina
MCP-1	*Monocyte chemoattractant protein-1*
MPs	*Microparticles*
NF-κ*B*	*Transcription factor κB*
NO	*Nitric oxide*
O_2^-	*Superoxide anion*
PAI-1	Inibidor do ativador de plasminogênio do tipo 1 / *Plasminogen activator inhibitor type 1*
PAI-2	Inibidor do ativador de plasminogênio do tipo 1 / *Plasminogen activator*

	inhibitor type 2
PARs	*Protease activator receptors*
PBMC	*Peripheral blood mononuclear cells*
PCR	Reação em cadeia da polimerase
PE	Pré-eclâmpsia / *preeclampsia*
RFLP	Polimorfismo de tamanho de fragmentos de restrição
ROC	*Receiver operator characteristics*
ROS	*Reactive oxygen species*
sPE	*Severe preeclampsia*
STBM	*Syncytiotrophoblast*
TAFI	*Thrombin activatable fibrinolytic inhibitor*
TAT	Complexo trombina-antitrombina
TCLE	Termo de Consentimento Livre e Esclarecido
TF	Tissue factor
TGF-β	Fator transformador de crescimento beta
TNF-α	Fator de necrose tumoral alfa
t-PA	*Tissue plasminogen activator*
u-PA	*Plasminogen type urokinase*

SUMÁRIO

1 INTRODUÇÃO E RELEVÂNCIA

A pré-eclâmpsia (PE), na sua forma pura, caracteriza-se pelo aparecimento em grávida normotensa, após a vigésima semana de gestação de hipertensão e proteinúria. De acordo com o *Working Group on High Blood Pressure in Pregnancy* (2000) (1) e o *The American College of Obstetricians and Gynecologists* - ACOG Practice Bulletin (2002) (2), os parâmetros para diagnóstico da PE são hipertensão (pressão sanguínea sistólica ≥140 mmHg ou pressão sanguínea diastólica ≥90 mmHg, em no mínimo duas ocasiões e o intervalo entre as medições não deve ser inferior a duas horas ou superior a uma semana) e proteinúria (excreção de proteína ≥0,3 g em urina de 24 horas ou ≥30 mg/dL , ou seja, ≥+1 pelo método qualitativo de fita, em amostras isoladas).

A etiologia da PE ainda não é conhecida e a doença ocorre somente na presença da placenta. A PE constitui a principal causa de morte materna em diversos países do mundo e contribui significativamente para a prematuridade, baixo peso fetal e o aumento da mortalidade neonatal. Esta doença está associada a um elevado custo social, uma vez que frequentemente resulta na internação da gestante e do recém- nascido por vários dias. (3)

Um dos aspectos mais intrigantes da PE é o seu desfecho. Ainda não está elucidado por que algumas gestantes com PE vão até o puerpério sem maiores complicações, enquanto outras evoluem para a eclâmpsia (com surgimento de alterações neurológicas e convulsões, que podem evoluir para o coma e morte), síndrome HELLP (*Haemolysis*, *elevated liver enzyme activity*, *low platelets*) ou coagulação intravascular disseminada (CID). (3)

Segundo os critérios estabelecidos pela *American College of Obstetricians and Gynecologists (ACOG)* a PE pode ser clinicamente caracterizada nas formas leve e grave. Na forma grave da PE, os sintomas clínicos são ainda mais acentuados e os parâmetros para diagnóstico são hipertensão (pressão sanguínea sistólica ≥160mmHg ou pressão sanguínea diastólica ≥110mmHg, em no mínimo duas ocasiões e o intervalo entre as medições não deve ser inferior a seis horas ou superior a uma semana) e proteinúria (excreção de proteína ≥5g em urina de 24 horas ou ≥+3 pelo método qualitativo de fita, em amostras isoladas, coletadas em intervalo de no mínimo 4 horas). (2) Esta classificação tem sido amplamente utilizada por basear-se em critérios clínicos objetivos, refletindo seu prognóstico e

orientando a condução da gestação. Porém, de modo geral, os obstetras não esperam a obtenção de níveis tão elevados de proteinúria, pelo risco de complicações e morte da gestante, e é feita a interrupção da gestação.

Embora os sintomas da PE se manifestem após a vigésima semana de gestação, atualmente tem sido aceito que a patogênese é estabelecida muito antes e a doença ocorre em duas fases. A primeira fase se dá nas primeiras doze semanas de gestação, quando ocorre de forma defeituosa a diferenciação dos trofoblastos, invasão da decídua e remodelamento das artérias espiraladas. Isto resulta na entrada abrupta do sangue materno no espaço interviloso causando dano mecânico aos sinciciotrofoblastos, além de um suprimento irregular de sangue na placenta, com eventos de hipoperfusão e reperfusão. (4, 5) A segunda fase ocorre no segundo ou terceiro trimestres e resulta da hipoperfusão e isquemia placentária. A placenta isquêmica libera citocinas e radicais livres do oxigênio que induzem a disfunção endotelial materna sistêmica e a resposta inflamatória excessiva. (4)

O entendimento da PE como síndrome e sua diversidade de repercussões na gestante e concepto vêm sendo investigados à luz de uma nova classificação, baseada no momento do surgimento de manifestações clínicas. Dessa forma, a PE é classificada como precoce ou tardia, de acordo com a idade gestacional na qual aparecem os sintomas da doença (6). Tem sido sugerido que a PE precoce e tardia constituem entidades distintas, que refletem o mecanismo etiopatogênico que se manifestam em momentos diferentes da gestação. (7) A PE precoce, tem início antes da 34ª semana de gestação, é menos frequente, mas associa-se à forma clinicamente mais grave, refletindo lesões isquêmicas placentárias. Seu componente genético é mais acentuado (6), há maior taxa de recorrência e seu prognóstico é mais sombrio para a gestante e seu concepto. (7, 8) Nestes casos, a restrição do crescimento intrauterino é mais frequente. (9) A PE tardia, tem início a partir da 34ª semana gestacional, é a mais frequente e, em geral, é associada a uma placentação adequada ou levemente comprometida. (6) Caracteriza-se por ausência ou leve resistência ao fluxo nas artérias uterinas, menor comprometimento do crescimento fetal e resultados perinatais mais favoráveis. (10)

A gestação normal está associada a elevação dos níveis de fatores da coagulação e diminuição dos anticoagulantes naturais, o que resulta em um estado de hipercoagulabilidade. (11-13) Esse estado constitui uma adaptação fisiológica, que visa garantir um controle rápido e eficaz da hemorragia no momento do parto,

quando ocorre a separação da placenta. (13, 14) Na PE a exacerbação da coagulação é ainda maior. (15-17) Sabe-se que a PE está associada à deposição de fibrina na microcirculação placentária (18) e que a ativação e/ou dano das células endoteliais parece desempenhar um papel chave na fisiopatologia da PE e certamente contribuem para as alterações hemostáticas observadas nessa síndrome. (19, 20)

Evidências recentes sugerem que a disfunção na angiogênese (21), bem como alterações na tensão local de oxigênio (22, 23) e na resposta imunológica (24-27), constituem fatores fisiopatológicos importantes na PE. O envolvimento do sistema imune na patogênese dessa doença tem sido sugerido, principalmente pelo contexto inflamatório observado. (24, 26-28)

O modelo de regulação imunológica durante a gravidez tem por base a mudança da resposta imune materna para um estado pró-inflamatório modulado. (29-31) Este modelo baseia-se na observação de que em uma mulher saudável não gestante, a resposta imune a um antígeno dependerá, em parte, do microambiente de citocinas. Assim, um microambiente rico em interleucina (IL) 12, IL-18 e interferon do tipo gama (IFN-γ) irá favorecer o desenvolvimento de células pró-inflamatórias que secretam citocinas inflamatórias, como o fator de necrose tumoral alfa (TNF-α), IL-2 e IFN-γ. Além disso, promoverá a ativação de macrófagos e linfócitos T citotóxicos. Por outro lado, um microambiente rico em IL-10 e IL-4 irá promover a expansão de linfócitos regulatórios. (29-31)

Nas mulheres não gestantes, há um equilíbrio entre as respostas pró-inflamatória e regulatória. No entanto, durante a gestação, o equilíbrio é significativamente alterado pela presença da placenta, uma vez que progesterona e citocinas são capazes de modular as células do sistema imunológico favorecendo o estado regulatório. (32) Na PE, o desvio da resposta imune para o estado regulatório provavelmente não ocorre, ou é revertido em fases muito precoces da doença. Níveis elevados da citocina pró-inflamatória IFN-γ e reduzidos da regulatória IL-4 têm sido descritos. (33-36) Sabe-se que as citocinas pró-inflamatórias podem provocar alterações funcionais e estruturais, incluindo danos oxidativos e comprometimento dos mecanismos de vasoconstrição e relaxamento de vasos, o que resulta em alterações da integridade vascular e da hemostasia. (37) No entanto, o fator que desencadeia a resposta inflamatória excessiva na PE não é ainda totalmente conhecido. (38)

Diversos estudos têm sido realizados visando elucidar as alterações genéticas que explicariam o desenvolvimento da PE. Estes estudos têm como objetivos a análise de genes relacionados aos mecanismos de alterações fisiológicas da doença, e visam definir marcadores moleculares capazes tanto de prever o desenvolvimento da doença, como melhorar a resposta ao tratamento clínico e farmacológico. A presença de polimorfismos em um determinado gene pode ou não acarretar alterações funcionais. Polimorfismos funcionais em genes de citocinas, que podem conferir diferenças interindividuais na síntese e secreção destas proteínas, têm sido associados a doenças que têm patogênese inflamatória. (39, 40) A investigação da associação de polimorfismos nos genes de citocinas e a ocorrência de PE têm resultado em conclusões conflitantes (41-51), o que indica a necessidade de estudos em outras populações.

A principal motivação para a realização deste estudo foi o maior entendimento da inter-relação dos processos hemostático e inflamatório na PE, uma vez que poderá contribuir para a adoção de medidas importantes na sua monitoração. Sabendo que a PE é uma doença de caráter multifatorial e que os fatores genéticos podem estar associados à sua ocorrência, foi também investigado neste estudo se os polimorfismos nos genes das citocinas estariam associados à ocorrência dessa doença no nosso meio.

Considerando a complexidade da PE, bem como das lacunas existentes na literatura com relação à sua etiologia, diagnóstico e tratamento, este estudo se justifica plenamente podendo gerar conhecimentos adequados à nossa realidade.

2 OBJETIVOS

2.1 Objetivo geral

Investigar a inter-relação dos sistemas hemostático e inflamatório na pré-eclâmpsia grave, por meio da determinação dos níveis plasmáticos de marcadores hemostáticos e citocinas, bem como os polimorfismos nos genes das citocinas e a ocorrência de pré-eclâmpsia grave.

2.2 Objetivos específicos

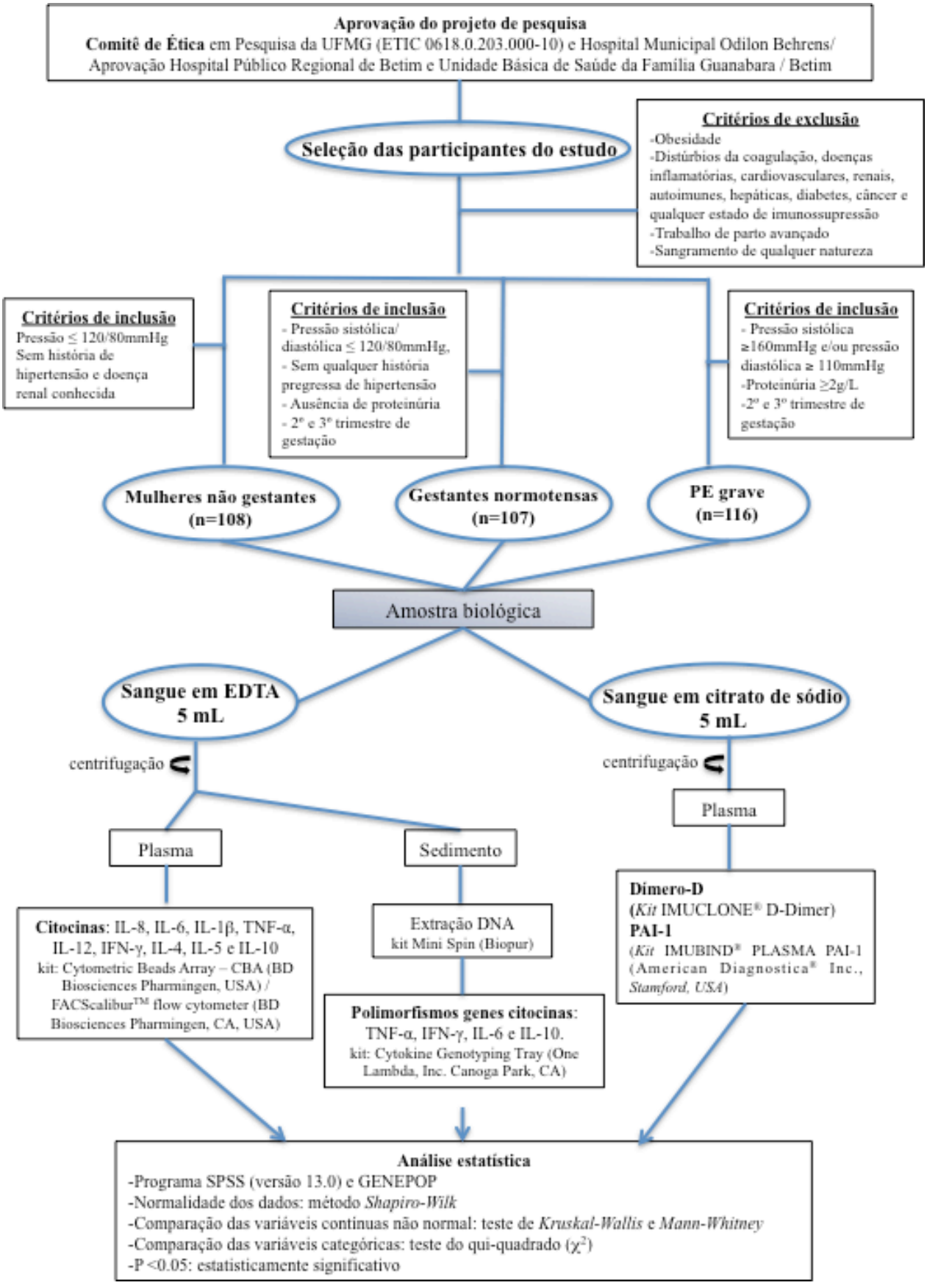
Nos três grupos avaliados, mulheres não gestantes, gestantes normotensas e gestantes com PE grave:

- Determinar os níveis plasmáticos dos marcadores da coagulação e fibrinólise, D-Di e PAI-1.
- Determinar os níveis plasmáticos das citocinas IL-8, IL-6, IL-1β, TNF-α, IL-12, IFN-γ, IL-4, IL-5 e IL-10, por citometria de fluxo.
- Correlacionar os níveis plasmáticos de D-Di e PAI-1 e de citocinas.
- Determinar a frequência dos polimorfismos nos genes das citocinas IL-6, IL-10, IFN-γ e TNF-α.
- Estabelecer a relação entre os polimorfismos dos genes das citocinas IL-6, IL-10, IFN-γ e TNF-α e os níveis plasmáticos dessas citocinas.

Além desses, também foi objetivo:

- Realizar uma revisão sistemática e metanálise sobre a associação dos níveis plasmáticos de D-Di e ocorrência de PE.

3 DELINEAMENTO EXPERIMENTAL



4 RESULTADOS

4.1 Artigos publicados

4.1.1 Pre-eclampsia: Relationship between coagulation, fibrinolysis and inflammation – *Clinica Chimica Acta*

Clinica Chimica Acta 412 (2011) 17–21

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

Pre-eclampsia: Relationship between coagulation, fibrinolysis and inflammation

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ARTICLE INFO

Article history:
Received 24 September 2010
Accepted 25 September 2010
Available online 1 October 2010

Keywords:
Pre-eclampsia
Coagulation
Fibrinolysis
Inflammation
Microparticles

ABSTRACT

Pre-eclampsia (PE) is a multi-system disorder of human pregnancy, characterised by hypertension and proteinuria. Although the pathogenesis of PE is not fully understood, predisposition to endothelial dysfunction is thought to play a crucial part. Despite intensive research there is no reliable test for screening purposes or to inform decision making towards effective treatment for PE. Understanding the link between PE, abnormal haemostatic activation and inflammation may help to elucidate some of the patho-physiology of the disease; primary preventative measures and targeted therapies at an early stage of the disease could then be considered. In the present paper we discuss potential causal links between PE, haemostasis and inflammation. The potential implications of such interaction on the pathogenesis of PE are also addressed.

Contents

1. Introduction

Pre-eclampsia (PE) is a multi-system disorder of human pregnancy, whose etiology remains poorly understood [1]. It is characterised by hypertension (diastolic blood pressure >110 mmHg on one occasion, or greater than 90 mmHg on two or more consecutive occasions at least 4 h apart) and proteinuria (either ≥300 mg protein per day or a urinary protein/creatinine ratio ≥30 mg/mmol), occurring after the 20th week of pregnancy in women who have had no previous symptoms [2]. During past decades many theories related to the etiology of PE have been proposed and challenged, while several others remain the subject of ongoing investigation. Although its pathogenesis is not fully understood, predisposition to endothelial dysfunction is thought to play a crucial part. This may trigger abnormal activation of the haemostatic and/or inflammatory systems. Indeed, maternal endothelial cell disorder can explain many of the clinical aspects associated with PE. For example, hypertension is probably due to endothelial disruption or uncontrolled vascular tone, fluid retention is a consequence of increased endothelial permeability, and clotting dysfunction results from increased blood borne pro-coagulant-microparticles [3–6]. Risk factors for PE such as chronic hypertension, renal disease and diabetes, are all conditions known to be associated with endothelial dysfunction.

Pre-eclampsia is also associated with increased inflammatory responses compared to uncomplicated pregnancy [7–9]. A history of PE increases the risk of future hypertension, ischaemic heart disease, stroke, venous thromboembolism, and the risk of PE occurring earlier in subsequent pregnancies [10–12]. Similarly, women with inherited thrombophilias are at increased risk of PE and venous thromboembolic disease [13,14].

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doi:10.1016/j.cca.2010.09.030

2. Haemostasis and pre-eclampsia

Pre-eclamptic women are known to have an increased hypercoagulable state compared to those with a normal pregnancy [4,15,16]. Activation of blood coagulation in women with PE occurs at an early stage of the disease and often antedates clinical symptoms and abnormal changes in other laboratory parameters. For example, there is a reported increase in factor VIII, von Willebrand factor, thrombin-antithrombin complex (TAT), D-dimers, soluble fibrin and thrombomodulin levels [17–22]. There is also increased resistance to the anticoagulant property of activated protein C (APC) [23]. However, antithrombin levels are described as reduced [24] and tissue factor pathway inhibitor levels unchanged [25]; platelets have a reduced half-life [26] and platelet counts are also decreased [27]. Interestingly, antithrombin, thrombomodulin and platelet counts correlate positively with the severity of the disease [22,24,26,28]. Fibrin deposition is usually found in the sub-endothelium of the glomerulus, the decidual segments of spiral arteries and occlusive lesions in placental vasculature (atherosis or atheroma-like lesions) [15,16]. Clinical manifestations of PE are considered secondary to hypoperfusion, which results from micro-thrombus formation and excess fibrin deposition affecting multiple maternal organs as well as the placenta [4].

The fibrinolytic system is also involved in PE. A significant increase in plasma plasminogen activator inhibitor type-1 (PAI-1) was reported [27,29]. Measurements of end products of fibrinolysis in both peripheral and uteroplacental circulation in normotensive and pre-eclamptic pregnancies, including soluble fibrin, TAT complex, plasmin-α_2-antiplasmin complex and D-dimers plasma, showed an abnormal haemostatic pattern occurring in women with PE compared to normal pregnancy. In the uteroplacental circulation, decreased level of soluble fibrin is consistent with increased fibrin formation as well as fibrin degradation products [21].

3. Inflammatory response and pre-eclampsia

Expatiated inflammatory reactions usually occur in women with PE compared to those with a normal pregnancy [8]. PE is associated with circulatory disturbances caused by systemic maternal endothelial cell dysfunction and/or activation; however, the causes of such dysfunction are not well understood. Pathological alterations in the endothelium have been observed in the kidney as glomerular endotheliosis [3]. The endothelium is an integral part of the inflammatory network; thus, its activation stimulates leukocytes and vice versa [30]. In PE both monocytes and granulocytes are activated and pro-inflammatory cytokines released into the circulation [31,32]. Increased cytokine concentration in PE is a potential stimulus for nicotinamide adenine dinucleotide phosphate oxidase activation, which results in increase superoxide generation [9]. Enhanced superoxide generation by placenta [33] or neutrophils [34,35] leads to an increase in oxidative stress in pre-eclamptic women. Shedding of syncytiotrophoblasts is a feature of healthy pregnancy and it has been viewed as part of syncytial renewal [36]. In PE shedding of syncytiotrophoblasts is increased. Placental ischaemia and reperfusion, as a consequence of oxidative stress, have been regarded as a major cause of syncytiotrophoblast apoptosis [36]. Oxidative stress in PE is not localised to the placenta but disseminate into the maternal circulation. Postmortem observations indicate that in some cases the lethal pathologic condition resembles that of the Shwartzman reaction, a particular form of inflammatory response to endotoxin [8].

4. Pre-eclampsia, haemostasis and inflammation

Coagulation, fibrinolysis and inflammation are integral parts of the host immune response. Activation of inflammatory and coagulation pathways is important in the pathogenesis of vascular disease and both systems interact strongly, so that coagulation and inflammatory activity mutually modulates each other (Fig. 1). Such processes appear to be intrinsically related to PE since the disease is associated with endothelial cell dysfunction, increased inflammatory responses and hypercoagulability [37].

Activation of blood coagulation produces proteases that not only interact with coagulation protein but also with specific cell receptors involved in inflammatory responses. Binding of coagulation proteases (such as thrombin and/or tissue factor) or anticoagulant proteins (e.g., APC) to protease activated receptors (PARs) may affect cytokine production or inflammatory cell apoptosis [38,39]. These receptors are localised on the vasculature on endothelial cells, mononuclear cells, platelets, fibroblasts and smooth muscle cells. Stimulation of PARs by

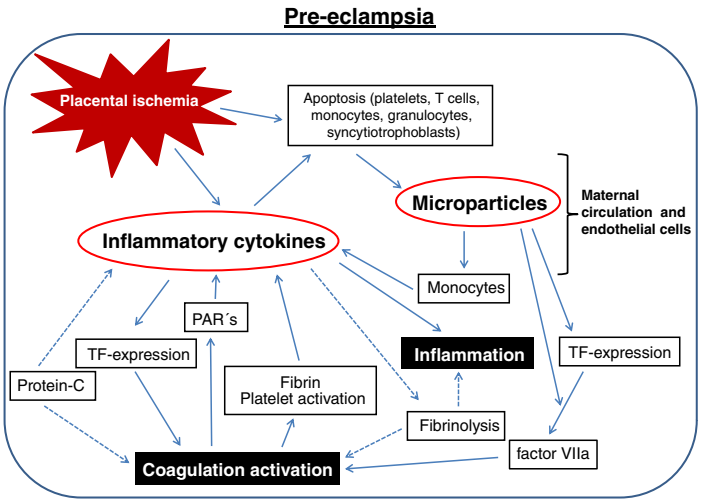

Fig. 1. A potential relationship between haemostasis, inflammation and pre-eclampsia. Pre-eclampsia is associated with placental oxidative stress and subsequent placental ischaemia and cellular apoptosis. Consequently, there is endothelial dysfunction and release of cytokines as well as microparticles that fall into the maternal circulation. Inflammatory cytokines trigger inflammation reaction which modulates coagulation. Microparticles are also able to modulate both inflammatory and coagulation pathways. Continuous arrows represent activation. Dotted arrows represent inhibition. TF, tissue factor; PARs, protease-activated receptors.

coagulation proteases leads to an induction of a number of pro-inflammatory mediators including IL-6, IL-8, tumour growth factor-β, monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor, intercellular adhesion molecule-1 and P-selectin [40,41].

In addition, the TF/Factor VII (FVII) complex induces pro-inflammatory effects in macrophages/monocyte leading to the production of reactive oxygen species (ROS) including superoxide anion (O_2^-) [42]. Superoxide causes vasoconstriction, either directly through contracting smooth muscle [43] or indirectly by inactivating nitric oxide and reducing the release of prostacyclin [44]. Vasoconstriction is associated with slow blood flux and platelet activation. In PE, superoxide generation is increased in neutrophils [34] and the placenta [33]. High concentrations of superoxide stimulate the arachidonic acid pathway in cells to produce thromboxane A_2, which is a potent stimulator of platelet activation [45]. It is well established that activated platelets secrete an array of pro-inflammatory and pro-coagulant substances stored in their alpha and dense granules. These substances induce TF synthesis in monocyte [46] and contribute to the production of interleukin IL-1, TNF-α, IL-8 and MCP-1 [47]. On the other hand, platelets can be directly activated by pro-inflammatory mediators, such as platelet activating factor [48]. Cytokines increase platelet reactivity, due to the release of large multimers of von Willebrand factor from the endothelium, which are particularly effective in promoting high shear stress rates [49]. Platelet activation and increased cytokine release are commonly seen in pre-eclamptic women [26].

Endogenous anticoagulant pathways also influence inflammatory responses [50]. Beneficial cytoprotective activities of APC include APC-mediated alteration of gene expression profiles, anti-inflammatory and anti-apoptotic activities [51,52]. Such activities require endothelial protein C receptors and PAR-1 [51,53,54]. Anti-inflammatory effects of APC on endothelial cells involve inhibition of inflammatory mediator release and expression of vascular adhesion molecules with the net result of inhibiting leukocyte adhesion and tissue infiltration. In addition, by helping to maintain endothelial barriers, APC reduces extravascular inflammatory processes through the inhibition of mediators released by leukocytes or endothelial cells [5,26,49,55]. Although there is no consensus, some groups have demonstrated a significant decrease of protein C levels in pregnancy-associated hypertensive disorders [56,57]. Thrombin–antithrombin complex can activate prothrombin activatable fibrinolysis inhibitor (pro-TAFI) to active-TAFI [58]. Activated TAFI plays a role in vascular responses to inflammation by removing the carboxyl-terminal arginine residues from C3a and C5a. It also has an important role in the regulation of inflammation by interfering in the cleavage of bradykin, osteopontin or C5a and modulating their pro-inflammatory functions [59]. Plasmin and thrombin can also activate pro-TAFI [60]. Pro-inflammatory mediators are known to up-regulate genes that stimulate α_2-macroglobulin production, which upon binding plasmin abrogates its action in degrading fibrin [61]. Thus, pro-inflammatory mediators contribute to maintaining the fibrin clot formation, as seen in PE.

Central regulators of plasminogen activators and inhibitors during inflammation are TNF-α and IL-1β [62]. The presence of these cytokines in the circulation leads to the release of plasminogen activators, particularly tissue-type plasminogen activator and urokinase-type plasminogen activator, from storage sites into vascular endothelial cells. However, this increase in plasminogen activation and subsequent plasmin generation is counteracted by a delayed but sustained increase in PAI-1 [63]. The resulting effect on fibrinolysis is complete inhibition and, as a consequence, inadequate fibrin removal, contributing to microvascular thrombosis. Inflammation is also associated with increased concentrations of plasma acute phase reactant proteins (e.g., fibrinogen and C reactive protein - CRP) [64]. High levels of fibrinogen increase blood viscosity favouring platelet activation [61,64], while CRP facilitates monocyte-endothelial cell interactions [64] and TF expression [65]. In addition, fibrin itself may act as a pro-inflammatory agent, specifically during edema accompanying acute inflammatory reactions. Fibrinogen and fibrin directly influence the production of pro-inflammatory cytokines (including TNF-α, IL-1β, and MCP-1) by mononuclear cells and endothelial cells [66]. It is known that PE is associated with decrease fibrinolysis, as shown by higher PAI-1 levels [29]. Taken together these suggest that haemostatic abnormalities are associated with abnormal inflammatory responses [67] and that the two systems (haemostasis and inflammation) are implicated in the ethio-pathogenesis of PE.

5. Pre-eclampsia and microparticles

An additional pathway through which the coagulation and inflammatory systems are generally activated is linked to microparticles (MPs) (Fig. 1). Microparticles were first described nearly 30 years ago and initially called “platelet dust.” They were described as small vesicles (>0.1 mm) and were shown to promote coagulation activation [68]. However, MPs are now considered to be membrane nano-fragments (0.05–1 μm) with pro-coagulant and pro-inflammatory properties [69]. Microparticles are generated after cell activation or apoptosis. This usually occurs following the disturbance of membrane phospholipid asymmetry and the pumps responsible for phospholipid transport. Changes in phosphatid MPs composition are not yet elucidated, but appear to differ depending on the cell origin and the stimulatory mechanisms behind their generation [70]. Microparticles are able to act on both endothelial cells [71] and smooth muscle cells [72]; as a result, they regulate vasomotor reactivity as well as angiogenesis [73]. Microparticles can provide as well as interact with TF to generate fibrin clot. In order for TF to gain its fully activity it requires the presence of PS which is exposed on apoptotic cell and MPs surfaces [74]. Moreover, MPs accelerate the interaction between TF and factor VIIa [75]. Microparticles participate in the regulation of vascular tonus, notably by decreasing the production of nitric oxide (NO). The latter is a powerful vasodilator, anti-platelet agent and a major factor for endothelial cells survival [76]. Microparticles are also able to influence smooth muscle cells directly through the activation of the transcription factor κB (NF-κB), leading to enhanced expression of inducible NOS (iNOS) and cyclooxygenase-2 (COX-2) with subsequent increase in NO and prostacyclin productions respectively, ending in a blunting of vascular contractility to agonists [72]. Microparticles also act as potent pro-inflammatory mediators, initiating an array of signal transduction pathways and gene expression profiles in endothelial cells, thereby affecting their function. They can also directly activate and stimulate monocytes to produce cytokines and ROS, resulting in an inflammatory response [75].

In PE it has been suggested that the most abundant MPs are from platelet origin [77]. Lok et al. [77] showed that the number of platelet-derived MPs decreased in PE compared to normal pregnancy, while the number of platelet-derived MPs exposing P-selectin increased. These P-selectin-exposing MPs reflect platelet activation, as is found in PE. Elevated concentrations of erythrocyte-derived MPs have been shown in PE, which are probably due to haemolysis and haemoconcentration [77]. Increased MPs from T cells, monocytes and granulocytes were reported in PE, and the number of granulocyte-derived MPs correlates with elastase, a marker of granulocyte activation and secretion [78–80]. Elevated concentrations of endothelial cell-derived MPs have been reported by some investigators but not others [81–84].

It has been reported that syncytiotrophoblasts (STBM MPs) increase during the course of pregnancy. These are surface membrane fragments shed from the outer layer of the placenta directly into the maternal blood. Higher STBM MPs during pregnancy probably result from the increasing placental volume, and reach its highest level in the third trimester [77,85]. Women with PE have increased STBM MPs compared to normal pregnancies, which is thought to directly reflect placental hypoxia and apoptosis [5,77,85–88]. Indeed, hypoxia leads to excessive ROS generation in placenta. In normal pregnancies ROS generation is

low, and antioxidative pathways are able to inactivate endogenous ROS thereby limiting placental damage. However, in PE these adaptive mechanisms are overwhelmed by enhanced production of ROS leading to an apoptotic/necrotic cascade in STBM MPs [89]. This may promote the release of syncytial products including STBM MPs. The presence of STBM MPs was specifically demonstrated to promote cell death and/or reduce proliferation of endothelial cells and to activate superoxide production in neutrophils isolated from women with PE [5,77,89–91].

In conclusion, several interfaces link coagulation and inflammation. Pro-inflammatory cytokines can affect coagulation pathways, while activated coagulation proteases and endogenous anticoagulants can modulate inflammation though specific cell receptors. Both systems have been shown to impinge on the ethio-pathogenesis of PE. However, the relationship between these and PE is complex and is far from being understood. Thus, detailed studies are required to elucidate the mechanisms governing these interactions and their relation to PE presence and/or progression.

6. Future perspectives on pre-eclampsia

- Despite intensive research, PE remains one of the leading causes of maternal death worldwide. The only definitive treatment is to deliver the baby and placenta, often prematurely, in the interest of the baby, the mother, or both. Several randomised trials have reported different means of reducing the rate or the severity of PE. These trials have some limitations (e.g., small sample size) and the results show at best minimal benefit. Thus, the classical prophylactic treatment continues i.e., control of blood pressure using antihypertensive drugs and seizure prophylaxis with magnesium sulphate (a cerebral vasodilator) [92].
- Attempts to manage inflammation and oxidative stress have not improved outcome. PE is associated with endothelial cell injury, haemostatic abnormalities and systemic inflammatory processes. Whether these events are primary mechanisms or secondary to PE needs clarification.
- PE may be linked to homeostasis involving blood coagulation, fibrinolysis and inflammation. Detailed understanding of the relationship between these three systems and PE may improve our knowledge on the patho-physiology of PE. A large-scale study correlating key markers of coagulation, fibrinolysis and inflammation and PE is required. Apart from shedding light on mechanisms, new therapeutic targets might be identified.
- The role of pro-coagulant microparticles in P-EC needs to be clarified further. Recently there have been more studies involving microparticles but the definitive role of these in PE and indeed other disease processes remains lacking.
- Finally, the causal effect of the proposed association of PE with risk of delayed cardiovascular disease and with the risk of PE occurring earlier in subsequent pregnancies should be examined further.

Acknowledgement

The authors thank CAPES, FAPEMIG and CNPq/Brazil. LMSD is grateful to CNPq Research Fellowship (PQ) and CAPES(BEX-2694.05.0).

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4.1.2 D-dimer plasma levels in preeclampsia: a systematic review and metanalysis - *Clinica Chimica Acta*

ARTICLE IN PRESS
CCA-12776; No of Pages 5

Clinica Chimica Acta xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Invited critical review

D-dimer in preeclampsia: Review and meta-analysis

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ARTICLE INFO

Article history:
Received 30 May 2012
Received in revised form 10 July 2012
Accepted 4 August 2012
Available online xxxx

Keywords:
Preeclampsia
D-dimer
Diagnosis
Systematic review
Meta-analysis

ABSTRACT

Preeclampsia is a multifactorial disease characterized by high blood pressure and proteinuria after the 20th week of pregnancy. Preeclampsia is associated with microvasculature fibrin deposition and maternal organ dysfunction. D-dimer (D-Di) has been used as a marker of production/degradation of fibrin in vivo. D-Di has emerged as a useful diagnostic tool for thrombotic conditions because its plasma concentration has a high negative predictive value for venous thromboembolism. The aim of this study was to evaluate publications that assessed plasma D-Di in preeclampsia and normotensive pregnant subjects to define its diagnostic value. A total of 194 publications were identified. Following the exclusion process, seven studies were in accordance with the pre-defined eligibility criteria. This systematic review was performed with methodologic accuracy, including a careful definition of preeclampsia and a high sensitivity literature search strategy. Quality of the included studies was assessed in accordance with widely accepted literature recommendations. Our meta-analysis indicates that increased plasma D-Di is associated with preeclampsia in the third trimester of gestation vs normotensive pregnant subjects. These preliminary findings in this select group of patients clearly highlight the need for additional comprehensive studies throughout pregnancy, including the establishment of an appropriate cut-off, in order to fully elucidate the diagnostic/prognostic role of D-Di in preeclampsia.

Contents

1. Introduction

Preeclampsia is a multifactorial disease characterized by systolic blood pressure ≥140 mm Hg or diastolic ≥90 mm Hg at bed rest on at least two occasions 6 h apart, and proteinuria ≥0.3 g/24 h, measured after the 20th week of pregnancy [1,2]. Symptoms frequently observed in preeclampsia include headache, blurred vision, and abdominal pain. The etiology of preeclampsia is unknown and the delivery of placenta

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0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2012.08.003

remains the only known treatment. This disease can progress to eclampsia (characterized by seizures as a sign of affection of the cerebral vessels), syndrome HELLP (hemolysis, elevated liver enzyme, low platelets) or disseminated intravascular coagulation [2]. Although some laboratory tests such as platelet count and liver enzymes can be used to monitor the risk of preeclampsia, the diagnosis is more effective when established by blood pressure and proteinuria measurement [2].

Preeclampsia is associated with the deposition of fibrin in microvasculature, which results in placental perfusion compromise, intrauterine fetal growth retardation [2] and dysfunction of some maternal organs [3].

In the early stages of fibrin clot formation, activated thrombin cleaves fibrinogen, a soluble plasma protein. Molecular polymerization is observed due to the formation of soluble fibrin, which is subsequently stabilized by covalent cross-linking with factor XIII—producing an insoluble fibrin matrix. Degradation is immediately initiated by plasmin, resulting in a variety of relatively stable dimeric fragments or fibrin degradation products. The smallest fragment, D-dimer (D-Di), is resistant to plasmin degradation. Therefore, D-Di specifically reflects both fibrin polymerization and breakdown [4–7].

Plasma D-Di is a well established clinical laboratory marker of this process in vivo. Additionally, D-Di is a useful diagnostic tool due to its high negative predictive value for venous thromboembolism [6,8,9].

Several studies have shown increased D-Di in preeclampsia vs normotensive pregnant subjects [10–14]. The aim of this meta-analysis was to compile and evaluate publications that assessed the D-Di by enzyme-linked immunosorbent assay (ELISA) to define its diagnostic value in preeclampsia.

2. Methods

2.1. Data sources and searches

An electronic database search was conducted for four databases (Medline, Embase, LILACS, and Web of Science) from the earliest record to August 2010. A sensitive search strategy using controlled vocabulary and free text terms was developed for each database with a combination of relevant key words such as D-Dimer, preeclampsia, eclampsia, pregnancy induced hypertension and gestational hypertension (full details of the search strategy are available on-request from the authors). Citation tracking was performed by manually screening reference lists of eligible studies. Studies included in the review were restricted to English, Spanish and Portuguese languages.

2.2. Study selection

Eligible studies included those that evaluated D-Di by ELISA, constituted by preeclamptic women and controls (normotensive pregnant). Preeclampsia was defined as systolic blood pressure ≥140 mm Hg or diastolic ≥90 mm Hg at bed rest on at least two occasions 6 h apart and proteinuria ≥0.3 g/24 h after the 20th week of pregnancy [2]. Studies with inappropriate or unclear definition of preeclampsia and those presenting insufficient results were excluded.

The retrieved papers were submitted to a rigorous selection process using a standardized protocol applied to papers by three authors independently. Disagreements were resolved by consensus.

2.3. Data extraction

For each included study, two reviewers independently extracted data such as study design, preeclampsia definition, number of preeclamptic and normotensive pregnant women in each study, gestational age at which blood collection occurred, D-Di concentration and author's conclusions. Data were adjusted to include only pregnant women in the third trimester of gestation.

Quality of the included studies was performed according to the Newcastle-Ottawa Scale recommendations [15] for nonrandomized studies in meta-analyses [16] and STROBE guidelines [17]. Five domains were considered: appropriate selection of participants, appropriate measurement of variables and outcomes, adequate follow-up rate, control for confounding via statistical adjustment and the existence of conflict of interest. This approach was designed to provide an overall quality assessment of the specific domains associated with potential source of bias in study findings and was not designed to provide a score to each individual study [18].

2.4. Data analysis

D-Di (median and standard deviation or median and ranges) from the participants (case or control group) were weighted in a meta-

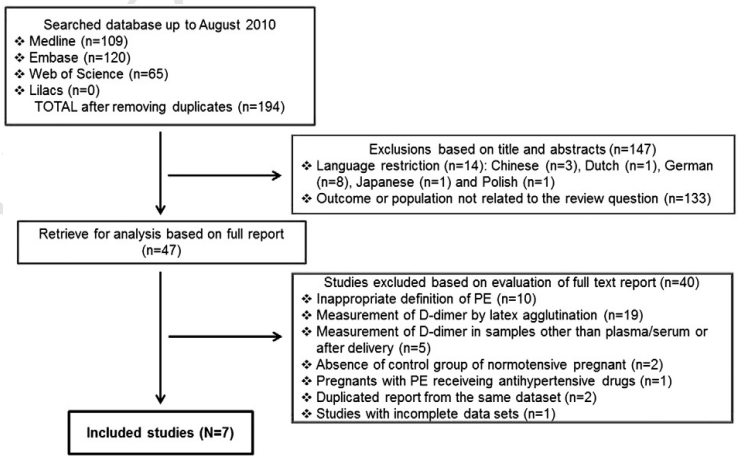

Fig. 1. Flow chart illustrating the exclusion process.

Please cite this article as: Pinheiro MB, et al, D-dimer in preeclampsia: Review and meta-analysis, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.08.003

Table 1
Methodological quality of the included studies.

Study	Selection of participants[a]		Appropriate measurement of variables and outcomes[b]		Response rate[c]	Control for confounding[d]	Funding/conflict of interest[e]
	Cases	Controls	Case	Exposure			
Catarino 2008 [20]	?	?	✓	✓	✗	✗	✓
Dusse 2003 [21]	?	?	✓	✓	✗	✗	✓
He 1997 [11]	?	?	✓	✓	✗	✗	✓
Schjtlen 1997 [13]	?	?	✓	✓	✗	✗	✓
Terao 1991 [14]	?	?	✓	✓	✗	✗	✓
Bellart 1998 [10]	?	?	✓	✓	✗	✗	✓
Heilmann 2007 [12]	?	?	✓	✓	✗	✗	✓

[a] *Selection of participants*
Case
Representative sample of the general population = ✓
Selected group of users (e.g. nurses, volunteers) = ✗
Unclear = ?

Controls
Representative sample of the general population = ✓
Selected group of users (e.g. nurses, volunteers) = ✗
Unclear = ?

[b] *Appropriate measurement of variables and outcomes*
Case definition
Secure record (e.g. surgical records) = ✓
Structured interview; written self report = ✗
Unclear = ?

Exposure definition
Objective measurement of exposure status or level = ✓
Structured interview; written self report = ✗
Unclear = ?

[c] *Response rate*
Follow-up rate ≥ 85% or non-participation detailed at each stage = ✓
Follow-up rate < 85% or no mention about non-respondents = ✗
Unclear = ?

[d] *Control for confounding*
Statistical adjustment: multivariate analysis conducted, with adjustment for potentially confounding factors
Yes = ✓; No = ✗; Unclear = ?

[e] *Funding/conflict of interest*
No = ✓; Yes = ✗; Unclear = ?

analysis using a random-effect model and were presented in a qualitative description. Statistical analyses were performed using Stata software version 12.0.

Publication bias was a matter of concern for the search strategies, but a funnel plot could not be used because the few studies included, such as the test power, would not distinguish chance from real asymmetry [19].

Table 2
Descriptive summary of the included studies.

Source	Study design and sample size	Cases characteristics	Controls characteristics	Key findings
Catarino 2008 [20]	Cross-sectional Cases: n = 44 Controls: n = 42	All preeclamptic pregnancies had blood collected before delivery (median was 37 weeks). Mean age 29.7 ± 5.3.	Normal pregnancies diagnosed on basis of clinical and ultrasound findings. They did not receive any medication to interfere with hemostasis. Mean age 30.4 ± 5.7.	There were not found differences in D-Di levels between cases (median = 488.5 ng/mL) and controls (median = 538.2 ng/mL).
Dusse 2003 [21]	Cross-sectional Cases: n = 43 Controls: n = 28	Preeclamptic women had blood samples collected on the third pregnancy semester	Health pregnant women had blood samples collected on the third pregnancy semester.	There were not found differences in D-Di levels between cases (mean = 1263.8 ng/mL) and controls (mean = 1146.6 ng/mL).
He 1997 [11]	Cross-sectional Cases: n = 30 Controls: n = 24	Preeclamptic women had blood collected between the 30th and the 35th week of gestation.	Health pregnant women had blood collected between the 30th and the 35th week of gestation.	Cases had increased values of D-Di (median = 315.0 ng/mL) when compared with controls (median = 183.0 ng/mL)
Schjetlein 1997 [13]	Cross-sectional Cases: n = 200 Controls: n = 97	Preeclamptic women had blood collected between the 27th and the 40th week of gestation. Mean age 28.0 (range 18–42)	Health pregnant women had blood collected between the 27th and the 40th week of gestation. Mean age 28.7 (range 21–40)	There was a slight increase of D-Di levels in cases (mean = 1595.0 ng/mL) when compared to controls (mean = 1390.0 ng/mL)
Terao 1991 [14]	Cross-sectional Cases: n = 13 Controls: n = 80	Preeclamptic women had blood collected on the 34th week of gestation	Health pregnant women had blood collected on the 33th week of gestation.	There was a slight increase of D-Di levels in cases (mean = 347.87 ng/mL) when compared to controls (mean = 221.52 ng/mL)
Bellart 1998 [10]	Cross-sectional Cases: n = 12 Controls: n = 65	Preeclamptic women had blood collected between the 28th and the 39th week of gestation	Health pregnant women had blood collected between the 29th and the 36th week of gestation.	There was an increase of D-Di levels in cases (median = 2090.0 ng/mL) when compared to controls (median = 545.0 ng/mL)
Heilmann 2007 [12]	Cross-sectional Cases: n = 111 Controls: n = 33	Severe preeclamptic women had blood collected after the 35th week of gestation	Health pregnant women had blood collected between the 31st and the 40th week of gestation.	There was a slight increase of D-Di levels in cases (median = 1623.60 ng/mL) when compared to controls (median = 1149.0 ng/mL)

PE: preeclampsia; D-Di: D-dimer.

Please cite this article as: Pinheiro MB, et al, D-dimer in preeclampsia: Review and meta-analysis, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.08.003

Table 3
Detailed data of D-dimer levels according to group of patient.

Study reference	Control group	Preeclamptic group
D-Di (ng/mL) (Mean ± SD)		
Dusse 2003 [21]	1146.6 (311.2)	1263.8 (411.9)
Schjtlein 1997 [13]	1390.0 (559.0)	1545.0 (849.5)
Terao 1991 [14]	221.52 (179.9)	347.87 (460.5)
D-Di (ng/mL) (Median)		
Catarino 2008 [20]	538.2 (Interquartile range 391.2; 822.8)	448.5 (Interquartile range 313.0; 1091.3)
He 1997 [11]	183.0 (Range 110.0; 340.0)	315.0 (Range 145.0; 1150.0)
Bellart 1998 [10]	545.0 (Interquartile range 225.0)	2090.0 (Interquartile range 1800.0)
Heilmann 2007 [12]	1149.0 (Interquartile range 456.0)	1623.60 (Interquartile range 932.9)

3. Results

A total of 194 unique titles were identified. Following the exclusion process (Fig. 1), nine studies were in accordance with the pre-defined eligibility criteria. Eight had detailed data sets and allowed data extraction. One study was later excluded because preeclamptic women received unusual antihypertensive drugs that could bias results. Seven studies were suitable for the systematic review.

Included studies consisted of cross-sectional analysis of D-Di in preeclamptic women and normotensive pregnant (control group). These studies were published from 1991 to 2008 in a variety of countries including Norway [13], Portugal [20], Brazil [21], Sweden [11], Japan [14], Spain [10] and Germany [12]. The methodologic quality of these studies can be considered poor (Table 1).

3.1. Participants

Participants included 453 preeclamptic women and 368 normotensive pregnant. Participants included pregnant women who had early or late, mild or severe preeclampsia. Unfortunately, detailed information regarding each group could not be accurately determined. Mean age of participants was similar among studies (28–32 years). Gestational age at the time of blood collection was also comparable (24th to 40th weeks) (Table 2).

Individually, the studies presented a relevant degree of heterogeneity concerning D-Di concentration. Mean values ranged from 222 to 1390 ng/mL and from 348 to 1545 ng/mL in the control and preeclamptic groups, respectively. Median values ranged from 183 to 1149 ng/mL and from 315 to 2090 ng/mL, respectively (Table 3).

Weighting the three studies in a meta-analysis, extracted/converted the data into median and standard deviation [13,14,21]. Under this approach, increased D-Di was observed in preeclampsia vs normal controls. Mean overall difference was 135.3 ng/mL (28.4–242.1 ng/mL, 95% CI). There was no evidence of heterogeneity among the studies (I-squared = 0.0%; P = 0.95) as presented by forest plot (Fig. 2).

4. Discussion

Despite extensive research, diagnosis of preeclampsia remains a challenge. Although supplementary tests can aid in suspected preeclampsia, diagnosis is routinely assessed by blood pressure and determination of urinary protein concentration [2]. The use of blood pressure measurement is unreliable, given the influence of body position, physical exertion and potential psychological complications, i.e., anxiety and stress [22–24]. Proteinuria is usually assessed by reagent dipsticks in a

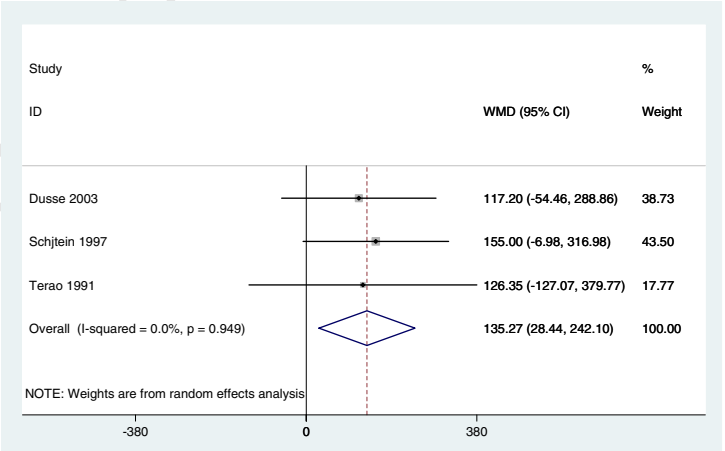

Fig. 2. Meta-analysis of the difference in means of D-dimer levels in normotensive pregnant and preeclamptic women.

Please cite this article as: Pinheiro MB, et al, D-dimer in preeclampsia: Review and meta-analysis, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.08.003

randomly collected urine sample. A 24 hour urine sample may provide more accurate results, but its collection is time consuming. Furthermore, reagent strip analysis can provide false positive results in the presence of vaginal discharge or if urine is too alkaline or contaminated, i.e., quaternary ammonium and chlorhexidine [25].

Identification of sensitive and specific biomarkers for precise diagnosis of preeclampsia is highly necessary in order to aid timely pregnancy intervention. Several laboratory markers have been proposed, but the reliability of these markers has been questioned. Although plasma D-Di has high negative predictive value for venous thromboembolism [6,8,9], its diagnostic value in preeclampsia has not been explored.

A variety of tests has been used for D-Di assessment, including ELISA, latex-based immunoassays and automated immunoturbidimetric assays [26–28]. Because ELISA is a more sensitive assay, we decided to include only studies that used this methodology. As the hypercoagulable state increases in pregnancy, we included only women in their third trimester of gestation.

A limitation of this study was the large inter-assay variation in D-Di measurement among different commercial kits. Because the same kit was used for both preeclamptic women and normotensive pregnant in each study, differences in analytic performance, i.e., precision, sensitivity, specificity, linearity were mitigated. The strength of this meta-analysis would be greatly improved if the eligible primary studies were more homogeneous regarding participants (preeclamptic women and normotensive pregnant). Our results indicate that preeclamptic women (following disease manifestation) have increased plasma D-Di, when compared to normotensive pregnant. Unfortunately, a large number of studies could not be included due to inappropriate definition of preeclampsia, regarding diagnostic procedures. The weighed overall effect showed by meta-analysis reveals the usefulness of D-Di plasma in preeclampsia. Besides, this test may also be useful for prognosis outcomes along pregnancy.

Another limitation of this review was our inability to extract data based on preeclampsia diagnosis, as early or late, mild or severe. As such, we could not exclude the possibility that specific characteristics of these subgroups could partially influence results. Selection bias was, however, avoided through use of a comprehensive search strategy in different databases. Moreover, predefined inclusion criteria were followed to avoid selection bias based on the particular characteristics stemming from the assessment of a wide range of studies. Publication bias was mitigated by searching numerous databases and performing manually citation tracking. Objective measures to assess publication bias were not effective given the few number of studies included in meta-analysis.

In conclusion, this review was conducted with methodologic accuracy that included a carefully established definition of preeclampsia and a highly sensitive literature search strategy. Methodologic quality of the included studies was assessed in accordance with widely accepted literature recommendations. Data analyses indicated a possible diagnostic role for D-Di levels in preeclampsia, especially in the third trimester of gestation. These initial findings clearly highlight the need for additional comprehensive studies throughout pregnancy, including the establishment of an appropriate cut-off, in order to fully elucidate the diagnostic/prognostic role of D-Di in preeclampsia.

Conflict of interest statement

All authors disclose no financial or personal relationship with other people or organizations that could inappropriately influence their work.

Acknowledgments

This work received grants from MCT/CT-Saúde and Decit/SCTIE/MS through CNPq-Brazil. APSMF, DRGJ, MGC and LMSD are grateful to CNPq for the received research fellowship. Authors thank Maria Cristina M. Ferreira for help in Embase data.

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Please cite this article as: Pinheiro MB, et al, D-dimer in preeclampsia: Review and meta-analysis, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.08.003

4.1.3 Fibrinolytic system in preeclampsia - *Clinica Chimica Acta*

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Contents lists available at SciVerse ScienceDirect

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Highlights

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Fibrinolytic system in preeclampsia

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► Fibrin deposition in maternal microcirculation is usually found in preeclampsia. ► There is still no consensus about the specific role of fibrinolytic system in PE.
► Blood coagulation seems to overlap the fibrinolytic regulatory mechanism in PE.

0009-8981/$ – see front matter
http://dx.doi.org/10.1016/j.cca.2012.10.060

Please cite this article as: Pinheiro MB, et al, Fibrinolytic system in preeclampsia, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.10.060

ARTICLE IN PRESS
CCA-12900; No of Pages 6
Clinica Chimica Acta xxx (2012) xxx–xxx
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Invited critical review

Fibrinolytic system in preeclampsia

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ARTICLE INFO

Article history:
Received 23 July 2012
Received in revised form 11 October 2012
Accepted 21 October 2012
Available online xxxx

Keywords:
Preeclampsia
Fibrinolysis
Coagulation
Pregnancy

ABSTRACT

Preeclampsia (PE) is a multi-system disorder of human pregnancy characterized by hypertension and proteinuria. Although its pathogenesis is not fully understood, predisposition to endothelial dysfunction is thought to play a crucial part. Normotensive pregnancy is associated with increases in coagulation factor levels and decreases in natural anticoagulation, leading to a hypercoagulable state. This state is thought to be part of a complex physiological adaptation, which ensures rapid and effective control of bleeding from the placental site at the time of placental separation. In PE, a more pronounced exacerbation of the hypercoagulable state is noticed, compared to normotensive pregnancy. Activation of coagulation in PE occurs at an early stage of the disease and often antedates the clinical symptoms. It is known that PE is associated with fibrin deposition in the kidney glomerulus, and in fatal cases, widespread fibrin deposition has been a prominent histological finding. Related to the fibrinolytic system in PE, the state of the art allows the assumption that blood coagulation overlaps the fibrinolytic regulatory mechanism, since fibrin deposition in maternal microcirculation is usually found in PE. However, there is still no consensus about its specific role. This review aims to discuss the fibrinolytic system in PE and its potential implications to the pathogenesis of this disease.

Contents

1. Preeclampsia

Preeclampsia (PE) is a multi-system disorder of human pregnancy, potentially dangerous for both mother and fetus [1]. PE is characterized by hypertension (diastolic blood pressure ≥ 110 mm Hg on one occasion, or greater than 90 mm Hg on two or more consecutive occasions at least 4 h apart) and proteinuria (either ≥ 300 mg protein per day) occurring after the 20th week of pregnancy in women who have had no previous symptoms [2].

Normotensive pregnancy is associated with increases in coagulation factor levels and decreases in natural anticoagulation, leading to a hypercoagulable state [3–5]. This state is thought to be part of a complex physiological adaptation, which ensures rapid and effective control of bleeding from the placental site at the time of placental separation. In addition, it allows the expansion of the maternal and fetal circulation at the uteroplacental interface during pregnancy [5, 6]. PE is also associated with an increased hypercoagulable state [7–9]. Fibrin deposition in the intervillous space and placental infarction has been a prominent histological finding [10]. Although PE pathogenesis is not fully understood, predisposition to endothelial dysfunction is thought to play a

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http://dx.doi.org/10.1016/j.cca.2012.10.060

crucial role and may underlie the hemostatic changes observed in this syndrome [11, 12].

2. Fibrinolytic system

Fibrinolytic system acts by breaking down the fibrin clot and ensuring hemostasis (Fig. 1). Plasmin, which is the main protease enzyme in this system, originates from plasminogen secreted by the liver. Physiologically, the activation of plasminogen into plasmin is performed by tissue plasminogen activator (t-PA) and plasminogen type urokinase (u-PA). t-PA is secreted by endothelial cells and exhibits higher proteolytic activity when bound to cells or fibrin. u-PA is likewise produced by endothelial cells, but also by monocytes and macrophages. Cell surface-bound u-PA converts plasminogen to plasmin in a much more efficient way than in solution [13]. Plasmin, in turn, cleaves and converts t-PA and u-PA into two-chain proteases, which exhibit higher proteolytic activity, implying a positive feedback for the fibrinolytic cascade [13].

Another physiological activator of plasmin is kallikrein, a serine protease [13]. Plasma contact with negatively charged surfaces, such as proteoglycans, endotoxic LPS, or different types of crystals, triggers factor XII activation, which, in turn, activates prekallikrein to kallikrein. This contact activation cascade could also be assembled at the surface of various cells, such as leukocytes, platelets, or endothelial cells, initiating the kallikrein activation, as well as the kallikrein-mediated plasminogen activation [13].

In the early stages of fibrin clot formation, activated thrombin cleaves fibrinogen, a soluble plasma protein. Molecular polymerization is observed due to the formation of soluble fibrin, which is subsequently stabilized by covalent cross-linking with factor XIII, producing an insoluble fibrin matrix. Degradation is immediately initiated by plasmin, resulting in a variety of relatively stable dimeric fragments or fibrin degradation products. The smallest fragment, D-dimer (D-Di), is resistant to plasmin degradation [14, 15].

As well as in the coagulation process, a negative feedback is essential for fibrinolytic pathway success. The main inhibitors of fibrinolysis are plasminogen activator inhibitor type 1 (PAI-1), α2-antiplasmin (α2-AP), thrombin activatable fibrinolytic inhibitor (TAFI) and, α2-macroglobulin (α2-M) [14–16].

PAI-1 is a single chain glycoprotein (52,000 kDa molecular weight), consisting of 379 amino acid residues. It lacks cysteine residues and has therefore no disulfide bridges [17, 18]. PAI-1 is secreted by endothelial cells stimulated by factors such as thrombin, endotoxin, dexamethasone, interleukin-1, tumor necrosis factor and transforming growth factor β [19]. Adipose tissue is a potential source of PAI-1 [20, 21], constituting the main inhibitor of plasminogen activation and avoiding non-fibrin bound t-PA, u-PA and plasmin [15]. The efficient fibrinolysis inhibition prevents clot premature lysis.

α2-AP, a single-chain glycoprotein, reacts with plasmin, forming a complex (plasmin–α2-AP), which is unable to break fibrin down. This complex formation in plasma is fast, with a rate constant above 10^7 M^{-1} s^{-1} [22]. In contrast, α2-AP reacts much slower with fibrin-bound plasmin [23]. Small amounts of α2-AP become cross-linked to fibrin during clotting, due to the action of factor XIIIa [24], which protects the clot from premature lysis and consequent bleeding [25]. Congenital deficiency of α2-AP is associated to a severe bleeding disorder (Miyasato disease) [14, 15, 26].

TAFI is a glycoprotein with 417 amino acids synthesized by the liver and also found in platelet granules [14, 15]. TAFI can be activated by thrombin, trypsin, kallikrein or plasmin into the active enzyme TAFIa. Its most efficient activators seem to be the thrombin and the thrombomodulin complex [27, 28]. TAFIa can potentially decrease the fibrinolytic activity by removing carboxyterminal lysine residues from partially degraded fibrin, thereby decreasing plasminogen binding to the fibrin surface. Therefore, TAFI is not an inhibitor, but an enzyme that may modulate fibrinolytic activity [2, 29, 30].

α2-M is synthesized mainly by the liver but it can also be locally synthesized by macrophages, fibroblasts, and adrenocortical cells. This molecule is a general inhibitor of both coagulation and fibrinolysis, acting as a scavenger [31]. In the fibrinolytic system, α2-M inhibits the action of plasmin and kallikrein, while in coagulation, it inhibits

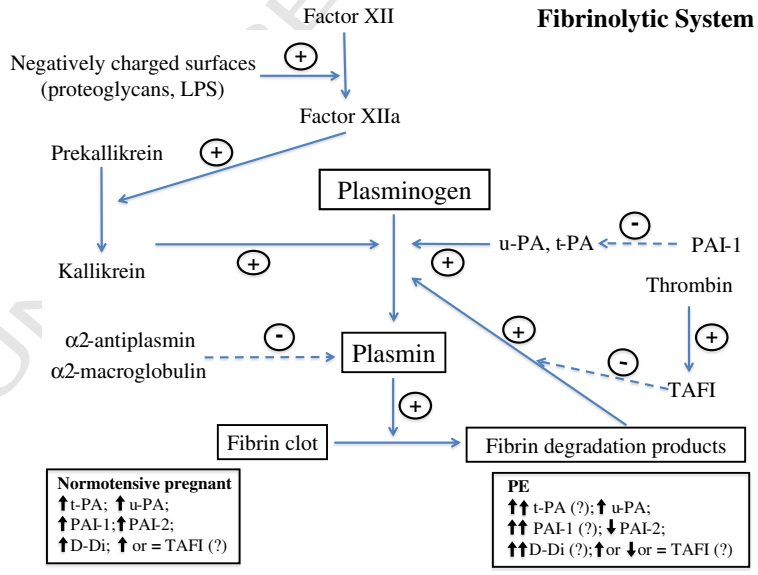

Fig. 1. Plasma contact with negatively charged surfaces (proteoglycans, LPS, or different types of crystals) triggers factor XII activation, which, in turn, activates prekallikrein to kallikrein. Generation of plasmin results from activation of plasminogen by kallikrein, tissue plasminogen activator/t-PA and plasminogen activator urokinase-type/u-PA. Plasmin acts by breaking down the fibrin clot in fibrin degradation products. t-PA and u-PA are inhibited by type 1 plasminogen activator inhibitor/PAI-1. The enzymatic activity of plasmin is inhibited by α2-antiplasmin and α2-macroglobulin. Exposed C-terminal lysine sites of plasmin-digested fibrin enhance the rate of plasmin formation, a phenomenon that is efficiently inhibited by thrombin activatable fibrinolysis inhibitor/TAFI.

Please cite this article as: Pinheiro MB, et al, Fibrinolytic system in preeclampsia, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.10.060

thrombin [32]. Besides, α2-M may act as a carrier protein because it also binds to several growth factors and cytokines, such as platelet-derived growth factor, basic fibroblast growth factor, TGF-β, insulin, and IL-1β [33].

High-efficacy cleavage of insoluble fibrin molecules by plasmin confirms its central role in fibrinolysis. Although the main targets of this enzyme are fibrinogen and fibrin, it is also able to cleave factors V and VIII, adrenocorticotropic and glucagon hormones, metalloproteinases, growth factors, and matrix proteins [13]. Besides this crucial role in the fibrinolytic system, plasmin has a number of important functions in other processes, including inflammation.

Several cells can bind plasminogen and plasmin via plasminogen-binding sites, which exposes a C-terminal lysine. Plasmin generated at the cell surface is protected from its physiological inhibitors [13] and facilitates cell migration in tissues. Moreover, plasmin is capable of triggering signaling, which depends on cellular binding via its lysine-binding sites and its proteolytic activity. Plasmin-induced signaling affects the functions of monocytes, macrophages, dendritic cells, and others [13]. *In vitro* and *in vivo* studies have demonstrated the ability of plasmin to stimulate the production of cytokines, radical oxygen species (ROS), and other mediators, thereby contributing to inflammation. Plasmin seems to be a potent chemoattractant for immune cells, since it shows monocytes and dendritic cell chemotaxis [13].

3. Fibrinolysis in normal pregnancy

A gradual decrease in fibrinolysis during pregnancy with the lowest marker values occurring in the third trimester has been reported by the first studies concerning this issue [34]. Accordingly, other investigators using clot-lysis techniques have also reported depressed fibrinolysis during normotensive pregnancy [35, 36]. However, more recent studies have shown that t-PA [37, 38] and u-PA [39] levels increase in pregnancy, suggesting an activation of the fibrinolytic system. To balance such activation, there is a several-fold increase in PAI-1 levels [37, 40–42] and placental production of another plasminogen activator inhibitor, called PAI-2 [37]. A progressive increase in D-Di levels has also been observed throughout pregnancy [38, 43, 44]. Since D-Di reflects both fibrin polymerization and breakdown [45–48], fibrinolysis has been considered active during pregnancy.

Few studies have investigated pro-TAFI/TAFIa in pregnancy and conflicting results were reported [49]. Chetaille et al. [50] did not find any difference in TAFI antigen plasma levels in a group of 12 women in the third trimester and age-matched non-pregnant controls. On the other hand, Chabloz et al. [51] reported a significant increase in TAFI antigen levels during pregnancy, which peaked in the last trimester. Mousa et al. [52] also found a gradual and significant increase in TAFI antigen and activity levels during pregnancy. The maximal level was found towards the end of pregnancy and returned to normal quite abruptly within 24 h after delivery. [53]. These studies suggest that TAFIa has a role in the thrombin generation predisposition in pregnancy [52].

4. Fibrinolysis in preeclampsia

Conflicting results have been obtained concerning the fibrinolytic system's role in PE. Several studies have shown that PAI-1 antigen [37 ,54–59] as well as t-PA levels [54–56, 60, 61] are higher in PE compared to normotensive pregnancy. Since both t-PA and PAI-1 are synthesized by the endothelial cells, their increased levels would reflect endothelial dysfunction. However, other studies have revealed a significant reduction [30, 62, 63] or no difference [64–66] in PAI-1 plasma levels comparing preeclamptic women and normotensive pregnant subjects. PAI-2 was significantly decreased in severe PE, reflecting placental insufficiency [65]. However, it is known that the binding affinity between PAI-1 and t-PA is approximately 1000-fold higher than PAI-2 [67, 68]. Thus, PAI-1 seems to play a more critical role in the regulation of fibrinolysis, while PAI-2 is thought to have a local role in the placental function during pregnancy. Chappel et al. [59] proposed that the PAI-1:PAI-2 ratio, evaluated at 20 and 24 weeks of gestation, is a promising tool for predicting PE. Accordingly, Parra et al. [69] related that pregnant women at 22 to 25 weeks who subsequently develop PE had a PAI-1:PAI-2 ratio significantly higher than control. Q4

Recently, it has been admitted that the time of clinical onset is fundamental for PE prognosis. Therefore, two different forms of the disease have been proposed: early (symptoms presented between 24 and 34 weeks' gestation) and late-onset (35–42 weeks' gestation). Wikstrom et al. [64] demonstrated decreased PAI-2 levels, increased placental oxidative stress, and increased PAI-1:PAI-2 ratio in early-onset, but not in late-onset. This finding suggests an association between early-onset PE and an abnormal placenta. Therefore, placenta seems to have a role in the development of these two forms [71]. Besides, the PAI-1:PAI-2 ratio has emerged as a useful tool for predicting early-onset PE [64].

PAI-1 anti-fibrinolytic action contributes to clot permanence, compromising throphoblast migration and invasion [72]. A low PAI-2 synthesis and an excess of PAI-1, probably due to trophoblastic and endothelial damage respectively, have been observed in Caucasian and Asian preeclamptic women and was associated with placental dysfunction [55, 70, 73]. Changes in u-PA and PAI-2 levels were also correlated with PE severity, suggesting a prognostic value for pregnancy outcome [73]. These parameters presented no changes under antihypertensive treatment [74].

Catarino et al. [75] related that after gestational age adjustment, t-PA levels remained significantly high in PE, both in maternal circulation and umbilical blood cord, suggesting that the differences observed were not significantly affected by gestational age [75]. Similarly, other studies show that PAI-1 levels remained significantly higher in preeclamptic women [76–80]. In preterm PE, there was also a related significant increase in t-PA and PAI-1 antigens and a decrease in PAI-2 levels, compared to normotensive pregnancy [55]. It has also been suggested that increased t-PA and PAI-1 antigen levels found in PE could be regarded as markers for endothelial dysfunction [38, 81], while reduced PAI-2 could reflect a decreased placental function. As PAI-1 is also an acute phase protein [82], its increased levels could indicate an abnormal condition. In this way, although t-PA levels are also increased, the increment in PAI-1 seems to be even more pronounced, contributing to the reduced fibrinolytic activity observed in PE.

It is important to highlight that Teng et al. [83] have not found t-PA increase in throphoblast cell culture under hypoxia or hypoxia-reoxygenation, although they demonstrated high PAI-1 mRNA expression and levels in the placental tissue and plasma of preeclamptic women.

A study regarding TAFI levels in pregnancies complicated by either PE or fetal growth restriction showed no significant changes compared to normal pregnancy [65]. In contrast, Wiman and Hamsten [81] found lower TAFI antigen levels in complicated pregnant women. Since the molecular mass of pro-TAFI (46 kDa) is lower than albumin (64 kDa), increased kidney loss could have contributed to this result [84]. However, another study showed no difference in TAFI antigen levels in preeclamptic women compared to normotensive pregnant subjects, but since only two severe preeclamptic women were included in this study, the researchers suggested that a compromised synthesis of pro-TAFI by the liver occurs only in the PE severe form. In this sense, they speculated that TAFI antigen levels could be affected by PE severity [85]. In fact, Martinéz-Zamora et al. [86] have found higher TAFI levels in severe preeclamptic women, associated or not with the presence of antiphospholipid antibodies.

Zhang et al. [87] found that TAFI antigen levels were significantly higher in preeclamptic women compared to normotensive pregnant

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subjects throughout the three trimesters of gestation. However, no significant difference was found between other forms of gestational hypertension compared to normotensive pregnant subjects [87]. They also verified that aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total protein plasma levels were significantly higher in the PE group. However, these values were within normal range, suggesting that hepatic function was not impaired in PE. Changes in TAFI in the three trimesters showed that when hepatic and renal functions are normal, TAFI increases throughout pregnancy in preeclamptic women compared to normotensive pregnant subjects [87]. These researchers suggested that TAFI might be used as a tool for PE diagnosis but not for other gestational hypertension. They concluded that elevated TAFI down-regulates fibrinolysis and contributes to the exacerbation of coagulation in PE.

The fibrinolytic system could be altered by microparticles (MPs). MPs are vesicles shed from the outer layer of several cells. Some studies have shown controversial results about placental platelet and endothelial MPs in PE [88]. Considering that MPs are a rich source of proteins [88, 89], they can alter fibrinolysis at the maternal–fetal interface, playing a role in microvasculature fibrin deposition in PE [90].

Plasma D-Di is a well established clinical laboratory marker of fibrin polymerization and breakdown *in vivo* [14, 15]. Several studies have shown increased D-Di in PE vs normotensive pregnant subjects [77–80, 91]. However, Catarino et al. [75] have not found any difference between women with PE and normotensive pregnant subjects. A recent meta-analysis has evaluated publications that assessed the D-Di by enzyme-linked immunosorbent assay (ELISA) to define its diagnostic value in PE. The results indicated that increased plasma D-Di is associated with PE in the third trimester of gestation vs normotensive pregnant subjects [92]. However, the authors highlighted the need for additional comprehensive studies throughout pregnancy, including the establishment of an appropriate cut-off, in order to fully elucidate the diagnostic/prognostic role of D-Di in PE.

The majority of studies evaluating hemostasis in pregnancy have assessed the markers in the peripheral circulation. However, these markers may not reflect changes in the uteroplacental circulation. This local hemostasis protects the integrity of the maternal and fetal circulations and is primed to control hemorrhage after placental expulsion. The altered hemostasis within uteroplacental circulation could lead to excessive fibrin deposition and remains poorly understood. Studies involving local hemostasis are limited, due to the difficulty in obtaining relevant samples. Bonnar et al. [84] performed a detailed sequential study of blood coagulation and fibrinolytic systems in the uteroplacental circulation. They observed a pronounced shortening of the whole blood clotting time, a significant shortening of other clotting tests and a sharp increase in factor VIII activity compared to peripheral circulation. These changes were transitory, since the levels of fibrin/fibrinogen degradation products were slightly increased in uterine blood during placental separation. The authors concluded that there is a pronounced local activation of coagulation *in vivo* [84]. Sheppard et al. [93] evaluated PAI levels in the uterine vein and the peripheral vein at the time of Caesarean section in both normotensive pregnancy and PE women. PAI-1 levels were higher in PE women in both peripheral and uterine vein blood, while PAI-2 levels were much lower in PE women in both peripheral and uterine vein blood [93].

Higgins et al. [30] simultaneously measured the end products of both coagulation (TAT complex) and fibrinolysis (PAP complex and D-Di) in samples taken from the antecubital and uterine veins. TAT complex, soluble fibrin, D-Di and PAP complex levels were all higher in the uterine vein compared to the peripheral vein, which suggests an activation of coagulation and fibrinolytic systems. However, not all the differences reached statistical significance. In normotensive pregnant subjects, only TAT and soluble fibrin levels were significantly higher in the uterine vein compared to the peripheral vein. In PE women, only TAT and D-Di levels were significantly higher in the uterine vein compared to the peripheral vein. [30].

5. Conclusion

Pregnancy is associated with significant changes in blood coagulation, natural anticoagulation and fibrinolytic system. The majority of studies revealed that both coagulation and fibrinolytic systems are activated in healthy pregnancy. Regarding PE, a more pronounced exacerbation of the hypercoagulable state is noticed, compared to normotensive pregnancy. However, related to the fibrinolytic system, there is still no consensus about the involvement of activators and inhibitors in PE, although several studies point to an increase in PAI-1 and t-PA levels and a decrease in PAI-2 levels. The real role of TAFI is also not understood. The state of the art allows the assumption that blood coagulation overlaps the fibrinolytic regulatory mechanism, since fibrin deposition in maternal microcirculation is usually found in PE. Furthermore, clinical manifestations of PE are considered secondary to hypoperfusion due to placental occlusive lesions. Better designed studies evaluating simultaneously all laboratory markers available for the fibrinolytic system assessment in both uteroplacental and peripheral circulation are needed to clarify the uncertainties and to define the role of this system in PE.

Acknowledgement

The authors thank FAPEMIG and CNPq/Brazil. LMD is grateful to CNPq Research Fellowship (PQ).

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Please cite this article as: Pinheiro MB, et al, Fibrinolytic system in preeclampsia, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.10.060

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Please cite this article as: Pinheiro MB, et al, Fibrinolytic system in preeclampsia, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.10.060

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Please cite this article as: Pinheiro MB, et al, Fibrinolytic system in preeclampsia, Clin Chim Acta (2012), http://dx.doi.org/10.1016/j.cca.2012.10.060

4.2 Artigos submetidos

4.2.1 Severe preeclampsia: association of genes polymorphisms and maternal cytokines production – *Cytokine*

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Abstract

This study aims to investigate whether the polymorphisms in TNF-α, IL-6, IFN-γ and IL-10 promoter regions are associated with preeclampsia (PE) occurrence. This study included 116 severe PE, 106 normotensive pregnant and 58 non-pregnant women. A higher frequency of the IFN-γ (+874) T/T genotype in PE comparing to the control group (PE: T/T 28% and A/A 28%; Control: T/T 7% and A/A 57%, P<0.001) was observed. TNF-α, IL-6 and IFN-γ plasma levels were higher in PE women compared to non-pregnant women (P<0.001;P<0.001;P=0.004). IL-6 and IFN-γ levels were also higher in PE women compared to normotensive pregnant (P<0.001;P=0.010). IL-10 levels were higher in normotensive pregnant compared to PE (P<0.001). Our data revealed increased IFN-γ levels in PE with “high” compared to “intermediate” and “low” phenotypes. A positive correlation between IL-6 levels and “high” phenotype in normotensive pregnant was revealed. These results suggest that IFN-γ seems to play a role in PE occurrence.

Keywords: Preeclampsia; cytokine gene polymorphism; cytokine levels

1. Introduction

Preeclampsia (PE) is a multifactorial disease characterized by systolic blood pressure ≥ 140 mmHg or diastolic ≥ 90 mmHg at bed rest on at least two occasions six hours apart, and proteinuria ≥ 0.3 g/24 h, measured after the 20th week of pregnancy [1]. Symptoms frequently observed in PE include headache, blurred vision, and abdominal pain. The etiology of PE is unknown and the delivery of placenta remains the only known treatment. Clinically, it is important to diagnosis the severe form of PE when hypertension and proteinuria are even higher. This form can progress to eclampsia (characterized by seizures as a sign of affection of the cerebral vessels), syndrome HELLP (hemolysis, elevated liver enzyme, low platelets) or disseminated intravascular coagulation [2]. PE is associated with placental disorder, endothelial cell dysfunction and systemic vasospasm. The events leading to these alterations remain unclear, but it seems like abnormal activation of the immune system plays a relevant role in PE development [2, 3].

Healthy pregnancy is associated with a controlled inflammatory process that is exacerbated in PE in response to excessive placental stimuli [4]. Previous studies suggested that cytokines might be involved in the PE pathogenesis. High levels of interleukin (IL) IL-1, IL-6 and tumor necrosis factor alpha (TNF-α), as well as IL-2 and interferon gamma (IFN-γ), have been detected in plasma and amniotic fluid of PE women. All these inflammatory cytokines seem to have deleterious effects on pregnancy development [5-7]. IL-10 has been identified as an important cytokine in successful pregnancy [8]. It has been suggested that decreased IL-10 production in PE may cause a pro-inflammatory cytokine maternal response, resulting in pregnancy complications [6, 9, 10].

It has been reported that phytohemagglutinin (PHA)-stimulated IFN-γ production in peripheral blood mononuclear cells (PBMC) in PE women is significantly higher compared to normotensive pregnant [6, 11-14]. Elevated IFN-γ levels in pregnancy can be potentially harmful to the fetus. It is known that IFN-γ inhibits the outgrowth of trophoblast cells *in vitro* [15] and synergistically stimulates the programmed death of primary villous trophoblast cells [16, 17].

Point mutations and single nucleotide substitutions (SNPs) in the regulatory regions of cytokine genes may affect cytokine transcription and influence its production level. Some of those polymorphisms have been associated with acute and

chronic rejection in organ transplantation [18], graft-versus-host disease in hematopoietic stem cell transplants [19], and several diseases predisposition [20].

Gene expression levels can affect inflammation and immune regulation. It is known that differences in cytokine allele frequencies amongst populations may contribute to difference in the incidence of many diseases. The relationship between PE and SNPs in cytokine genes has been investigated, but is still unclear [21-45]. Therefore, the aim of this study was to investigate whether the polymorphisms in TNF-α (-308 G $\rightarrow$ A), IL-6 (-174 G $\rightarrow$ C), IFN-γ intron 1 (+874 A $\rightarrow$T) and IL-10 (-1082 G $\rightarrow$ A) promoter regions are associated with PE occurrence.

2. Subjects and Methods

2.1 Ethical aspects

This study was approved by the Ethics Committee of Federal University of Minas Gerais and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

2.2 Study design

The present case-control study included 116 severe preeclamptic women, 106 normotensive pregnant and 58 non-pregnant women. These women were selected from Odete Valadares Maternity-Belo Horizonte/Brazil, Regional Public Hospital of Betim/Brazil and Healthy Center Guanabara, Betim/Brazil from 2008 to 2011.

2.3 Inclusion criteria

Severe PE was defined by systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥ 110 mmHg, presented in two consecutive occasions at bed rest at least four hours apart; and proteinuria $\geq$ 2 gL−1 or at least 2+ protein by dipstick. Normotensive pregnant had systolic/diastolic blood pressure below 120/80 mmHg and no history of hypertension or proteinuria. All pregnant women showed gestational age ≥20 weeks. Non-pregnant women had no clinical and laboratory

alterations, including hypertension.

2.4 Exclusion criteria

Exclusion criteria common for the three groups were chronic hypertension, haemostatic abnormalities, cancer, diabetes, cardiovascular, autoimmune, renal and hepatic diseases, and anticoagulant therapy.

2.5 Cytokine gene polymorphism analysis

DNA was extracted and purified from whole blood, collected in EDTA using Biopur Mini Spin Kit (Biometrix, Brazil).

Cytokine genotyping was carried out by the polymerase chain reaction (PCR) sequence-specific primer method, using the 'Cytokine Genotyping Tray' (One Lambda Inc., Canoga Park, CA, USA). The kit accuracy was checked by our laboratory using known DNA samples. The PCR products were then visualized by electrophoresis in 2% agarose gel stained with ethidium bromide and documented with a Polaroid camera. The polymorphisms analyzed in the present study were: TNF-α (-308 G→A), IL-10 (-1082 G→A), IL-6 (-174 G→C), and IFN-γ (+874 A→T).

The cytokine genotypes were grouped according to the final phenotype on gene expression. For the TNF-α gene, the genotypes were distributed as A/A and A/G (high) and G/G (low); for the IL-10 gene, the genotypes were distributed as G/G (high), G/A (intermediate) and A/A (low); for the IL-6 gene, the genotypes were distributed as G/G and G/C (high) and C/C (low); and for the IFN-γ gene, the genotypes were distributed as T/T (high), T/A (intermediate) and A/A (low) [46-49].

2.6 Determination of cytokine plasma levels

Samples collected in EDTA were centrifuged at 2,500g for 20 min at 4°C to obtain plasma and stored at -80°C until analysis. Data acquisition and analysis were performed in dual-laser FACScalibur^{TM} flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA), using the BD Bioscience CBA software. IFN-γ was determined

using the Human Th1/Th2 Cytometric Bead Array method (BD Biosciences Pharmingen, USA). IL-6, IL-10 and TNF-α were determined using Human Inflammation Kit (BD Biosciences Pharmingen, USA), according to the manufacturers' instructions. Results were expressed as mean fluorescence intensity (MFI) for each cytokine.

2.7 Statistical analysis

Statistical analysis was carried out using SPSS (version 13.0) and GENEPOP software. Hard-Weinberg equilibrium was investigated through probability test. Data normality was tested by Shapiro-Wilk test. Comparisons between two groups were made by Student t test for parametric variables and Mann-Whitney for non-parametric variables. A comparison of non-parametric variables was done by Kruskal-Wallis test amongst three groups. When differences were detected among groups, these were compared in pairs by Mann-Whitney method, followed by Bonferroni test. The comparison of categorical variables was performed using the chi-square test (χ^2). Spearman's correlations were computed to assess correlations with cytokine plasma levels and cytokine genotype. P values <0.05 were considered statistically significant.

3. Results

Table 1 summarizes the clinical characteristics of the 281 women enrolled in this study. PE women, normotensive pregnant and non-pregnant women showed similar ages (P=0.207) and body mass index (BMI) (P=0.128). Normotensive pregnant and PE women did not show differences regarding gestational age (P=0.799). As expected, systolic and diastolic blood pressures were significantly higher in PE women, comparing to the other two groups (P<0.001, in both of cases), as well as gestational weight gain, when compared to normotensive pregnant (P=0.002).

The case (PE) and control group (normotensive pregnant and non-pregnant women) were under Hardy–Weinberg equilibrium (P=0.289 and P=0.364, respectively.)

Genotyping data are presented in Table 2. It was observed a higher frequency of the IFN-γ (+874) T/T genotype in PE comparing to the control group (PE: T/T 28% and A/A 28%; Control: T/T 7% and A/A 57%, P<0.001). However, no differences between cases and controls were found in genotypes distribution for TNF-α (-308), IL-10 (-1082) and IL-6 (-174) polymorphisms.

Cytokine plasma levels were analyzed as mean fluorescent intensity (MFI) provided by the CBA immunoassay (Fig. 1). To assess whether pregnancy is able to induce different levels of cytokines, this analysis was performed separately in each group studied (PE, normotensive pregnant and non-pregnant women groups). TNF-α, IL-6 and IFN-γ plasma levels were higher in PE women compared to non-pregnant women (P<0.001; P<0.001; P=0.004, respectively). Furthermore, IL-6 and IFN-γ levels were also higher in PE women compared to normotensive pregnant (P<0.001; P=0.010, respectively). However, IL-10 levels were higher in normotensive pregnant compared to PE women (P<0.001) and non-pregnant women (P<0.001). Aiming to evaluate whether the polymorphisms in TNF-α, IL-10, IL-6 and IFN-γ genes influence the genic expression, plasma levels of these cytokines were compared to phenotypes determined by the genotypes (Table 3). Increased levels of IL-6 in “high” phenotype compared to “low” phenotype (P=0.05) were observed in normotensive pregnant. Furthermore, increased levels of IFN-γ in “high” compared to “intermediate” (P=0.012) and “low” phenotypes (P<0.001) were revealed in PE women.

In order to investigate the correlation between genotypes and cytokines plasma levels, the three groups were analyzed together. A significant positive correlation between plasma IFN-γ levels and the presence of +874T allele was observed (P<0.001, r=0.302). When the three groups were evaluated separately, a significant positive correlation between IL-6 levels and -174C allele (P=0.05, r=0,236) in normotensive pregnant was evidenced. Moreover, in the PE group, it was found a significant positive correlation between IFN-γ plasma levels and +874T allele (P=0.004, r=0.372). The other polymorphisms did not show correlation with cytokines levels.

4. Discussion

In the present study, the +874 T/T genotype in IFN-γ gene was more frequent in PE women than in the control group (normotensive pregnant and non-pregnant women). Therefore, given the decisive role of IFN-γ in pregnancy and the presence of functional polymorphisms in the first intron of the IFN-γ gene, our data suggest that this gene might plausibly be a candidate for susceptibility gene in PE. In contrast, a study involving Brazilian preeclamptic and eclamptic women showed higher frequency of IFN-γ +874 A in eclamptic women comparing to controls [26]. The authors admitted that these results were unexpected and could have occurred by chance, since they did not detect a corresponding expression in genotype frequency. However, other studies have investigated this polymorphism in preeclamptic women and did not find any association between genotypes or allele frequencies of IFN-γ gene and PE [24, 31]. These conflicting findings could have resulted from the heterogeneity in study designs, definition of phenotype, population diversity and sample size. These factors surely confound the results´ interpretation, especially in a complex disease such as PE. Moreover, few studies have been conducted to evaluate the association between IFN-γ +874 T → A gene polymorphism and PE occurrence. Although our data showed this association, further studies are necessary to confirm the relationship between this polymorphism and PE.

No association between TNF-α (-308 G→A), IL-6 (-174 G→C), or IL-10 (-1082 G→A) polymorphisms and PE was observed. These results are in line with other publications [21, 24, 26-28, 31, 35, 41-43, 45, 50-54] and in disagreement with others [24, 30-32, 36, 38, 40, 45]. A reason for discrepant results among different studies might be the selection bias and small sample size in retrospective studies, or ethnic differences among the populations studied.

There are several evidences suggesting that IL-10 has an important role in pregnancy. IL-10 has a critical function in different obstetric pathologies associated to down regulation of inflammatory responses in the placenta [55]. PE has also been associated with a deficiency of placental IL-10, which induces T lymphocytes to differentiate along the regulatory pathway and block IFN-γ production. It is known that IFN-γ is the major pro-inflammatory lymphocyte product that induces others pro-inflammatory cytokine synthesis [55]. Mirhamadian *et al*. [36] found significantly

higher C/C genotype frequency of IL-10 (-819 C → T) and (-592 C → A) in PE women. However, in agreement with other studies [25, 26, 28, 44] our data did not show any association between PE and IL-10 gene polymorphism. A recent meta-analysis showed no association between PE and IL-10 polymorphisms (-1082 G→A) [54].

IL-6 is a critical cytokine in the cascade of host response to infection. IL-6 activates the acute phase response, stimulates T lymphocytes, induces the terminal differentiation of B-lymphocytes, and induces C reactive protein production [56]. It has recently been reported that in PE, endothelial cells phagocytes kill trophoblasts shedding from placenta to maternal blood. Phagocytosis of necrotic trophoblasts cause endothelial cells activation and subsequent IL-6 release [57, 58]. Several studies have been reporting increased IL-6 levels in PE [54, 59-63].

It is known that the IL-6 production is under genetic regulation. A polymorphism in the promoter region of IL-6 (-174 G→C) gene, on chromosome 7 [64] is associated with the production of IL-6 [46]. The C/C genotype of this polymorphism is related to reduced IL-6 production, whereas homozygous G/G or heterozygous G/C displays normal production. In agreement with our results, other studies did not find an association between polymorphism in IL-6 gene promoter (-174 G→C) and PE occurrence [24, 26, 41, 43, 53], which was confirmed by a recent metanalysis [54].

TNF-α is a potent and multi-functional cytokine produced by macrophages, lymphocytes and trophoblast. It contributes to the abnormal placental invasion [65], endothelial cell damage [66] and oxidative stress [67]. An excessive inflammatory response to pregnancy seems to characterize PE, and TNF-α represents a major mediator of this reaction [51]. Our data did not show any association between polymorphism in TNF-α gene and PE occurrence, although conflicting results were previously reported [22, 28, 36, 38]. In agreement with our data, two different metanalysis [50, 54] revealed no association between polymorphism -308 G→A and PE. Nonetheless, in one of these metanalysis [54] it was found an association between high TNF-α plasma levels and PE. In accordance, our data showed high TNF-α plasma levels in PE comparing to non-pregnant women, which could suggest that this cytokine may have a role in PE.

Our data suggest that TNF-α plasma levels are not controlled by -308 G→A polymorphism. TNF-α gene cluster is located on chromosome 6 and contains many

polymorphisms. Understanding the control of its production is complex, as it depends on TNF-α allele polymorphism, which is in linkage disequilibrium with the human leukocyte antigen (HLA) genes, and also on HLA-DR polymorphism, where HLA-DR acts as an immune response modifier [24, 49].

There are several evidences supporting the hypothesis that cytokines production is associated with PE occurrence. Our data showed increased inflammatory cytokines levels, IL-6 and IFN-γ, in PE women comparing to normotensive pregnant. Supporting our findings, some studies have demonstrated an increase in IFN-γ [59-62, 68] and IL-6 in PE [54, 59-63], which was confirmed by a recent metanalysis [54]. However, we found decreased levels of the regulatory cytokine IL-10. It has been suggested that decreased IL-10 production in PE may cause a pro-inflammatory cytokine response.

In this sense, there is no consensus regarding cytokines production and PE. Several hypotheses could be proposed to explain the discrepant data. As IL-10 has a very short half-life, it is not consistently present in the circulation. Thus, a single blood sample may fail to detect a sporadic elevation or reduction in this cytokine level. Different factors, such as the effect of gestational age at the time of blood sample collection, the influence of body mass index and the assay sensitivity to measure IL-10 may also explain the divergences in results found in the studies [24].

Our data revealed higher levels of IL-6 in pregnant women with “high” phenotype compared to “intermediate” and “low” phenotypes. This finding suggests that pregnancy is able to increase IL-6 levels and this cytokine may be important to physiologic gestational development. However, it is known that IL-6 levels depend on the genotype that determines the “high” phenotype. Some cytokines such as IL-4, IL-6 and IL-10 seem to favor pregnancy success whereas others such as TNF-α and IFN-γ are harmful. In pregnancy, there is a greater increase in IL-6 production compared to the non-pregnant state [69]. IL-6 may induce prostaglandin synthesis by intrauterine tissues, suggesting its physiological role in labor. However, several studies showed that IL-6 plasma levels are higher in women presenting pregnancy complications, when compared to healthy pregnant, which suggests a role for this cytokine in these disturbances [54, 59-63, 70].

To the best of our knowledge, this is the first study investigating the relation between IFN-γ levels and gene polymorphism in PE.

In conclusion, our data revealed increased IFN-γ plasma levels in preeclamptic women with “high” phenotype compared to “intermediate” and “low” phenotypes. Besides, a positive correlation between IL-6 levels and “high” phenotype in normotensive pregnant was revealed. Moreover, this association was also observed evaluating the three groups together. These results point to the importance of IL-6 production in healthy pregnancy. On the other, IFN-γ seems to play an essential role in PE occurrence.

There are still many lacks in comprehending the complexity of PE pathogenesis. Multiple mechanisms and mediators are involved in development of PE. The severity and the time of clinical onset make us believe in the existence of different subgroups for this disease. Ignoring this fact and treating PE as a single-manifested illness may justify the conflicting results found in literature.

Understanding inflammatory response in PE is associated with another challenge, since women present distinct immunogenetics backgrounds. The inflammatory markers results will certainly reflect such differences, leading to controversial research conclusions. Moreover, maternal and fetal genes interacting with each other and a variety of environmental stimuli interfere on the PE severity and outcome. Based on these considerations, further studies are undoubtedly needed in order to clarify the association of genes polymorphisms and maternal cytokines production. In this sense, reproducing our findings in other populations will help defining the influence of genes polymorphisms and cytokine production in the pathophysiology of PE.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

The authors thank FAPEMIG and CNPq/Brazil. DUSSE, Luci M, MARTINS-FILHO Olindo Assis, TEIXEIRA-CARVALHO Andrea are grateful to CNPq Research Fellowship (PQ).

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Table 1 Clinical Characteristics of participants

Characteristics	Control Group	Preeclamptic women	P value
Age (years)	25.8 (6.22)	26.8 (7.16)	0.207
GA (weeks)	32.9 (4.68) (only normotensive pregnant)	33.0 (4.04)	0.799
GWG (Kg)	10.0 (6.75-13.55) (only normotensive pregnant)	12.7 (8.50-16.50)	0.002*
BMI (Kg/m^2)	23.25 (20.53-26.90)	23.98 (21.63-28.13)	0.128
SBP (mmHg)	110 (100.0-120.0)	170 (160.0-180.0)	<0.001*
DBP (mmHg)	70 (70.0-80.0)	110 (100.0-120.0)	<0.001*

GA: gestational age; GWG: gestational weight gain; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; * Statistic significant.
Age and GA are presented as mean (standard deviation). Student t test
GWG, BMI, SBP and DBP are presented as median (25th–75th centiles). Mann–Whitney t

Table 2 Genotype frequencies of TNF-α, IL-10, IL-6 and IFN-γ polymorphisms in women with preeclampsia (PE) and the control group

Polymorphism Genotype (phenotype[a])	Control (n = 165)	PE (n = 116)	P
TNF-α (-308 G → A)			0.483
A/A; A/G (high)	46 (0.28)	28 (0.24)	
G/G (low)	119 (0.72)	88 (0.76)	
IL-10 (-1082 G → A)			0.662
G/G (high)	16 (0.10)	11 (0.09)	
G/A (intermediate)	78 (0.47)	61 (0.53)	
A/A (low)	71 (0.43)	44 (0.38)	
IL-6 (-174 G → C)			0.130
G/G; G/C (high)	159 (0.96)	107 (0.92)	
C/C (low)	6 (0.04)	9 (0.08)	
IFN-γ (+874 A →T)			<0.001*
T/T (high)	11 (0.07)	33 (0.28)	
T/A (intermediate)	59 (0.36)	51 (0.44)	
A/A (low)	95 (0.57)	32 (0.28)	

Values in parentheses are frequency. [a]Cytokine production phenotype according to the Hoffmann et al., Pravica et al., Turner et al., and Wilson et al. * Statistically significant differenc

Table 3 Evaluation of polymorphism influence in circulating levels of cytokines

Population	Cytokines	Polymorphism Genotype (phenotype[a])			P value
		High	Intermediate	Low	
Non-pregnant	TNF-α	4.00 (3.80-4.20)	NA	3.90 (3.20-4.10)	0.812
	IL-10	4.75 (3.80-5.70)	4.00 (3.70-4.90)	4.55 (3.80-5.70)	0.515
	IL-6	7.71 (5.73-18.60)	NA	-	-
	IFN-γ	3.32 (2.00-4.91)	3.53 (2.09-4.01)	2.83 (1.33-3.96)	0.183
Normotensive pregnant	TNF-α	4.00 (3.80-4.70)	NA	3.90 (3.50-4.70)	0.585
	IL-10	8.20 (6.00-9.60)	8.65 (4.50-13.20)	8.25 (6.50-11.10)	0.456
	IL-6	8.82 (3.90-14.86)	NA	6.66 (6.21-7.10)	0.050*
	IFN-γ	4.2 (3.79-4.61)	3.54 (2.76-4.33)	3.53 (2.23-4.61)	0.390
Preeclamptic women	TNF-α	4.10 (4.20-6.00)	NA	4.10 (4.50-6.80)	0.637
	IL-10	4.55 (3.70-5.30)	5.20 (4.20-6.80)	4.15 (3.30-5.90)	0.155
	IL-6	12.92 (4.57-176.24)	NA	19.99 (9.73-75.67)	0.187
	IFN-γ	4.45 (3.18-5.73)	3.79 (2.67-4.41)	3.89 (2.62-4.83)	0.012[b]* <0.001[c]* 0.494[d]

Levels of plasma cytokine measured by median fluorescence intensities (MFI); NA, Not applicable; (-) no woman had the phenotype "low" (C / C).
[a]Cytokine production phenotype according to the Hoffmann et al., Pravica et al., Turner et al., and Wilson et al. Data were compared by the Kruskal–Wallis and Mann–Whitney test. Values are presented as median (25th–75th centiles). b. High x Intermediate; c. High x low; d. Intermediate x low.

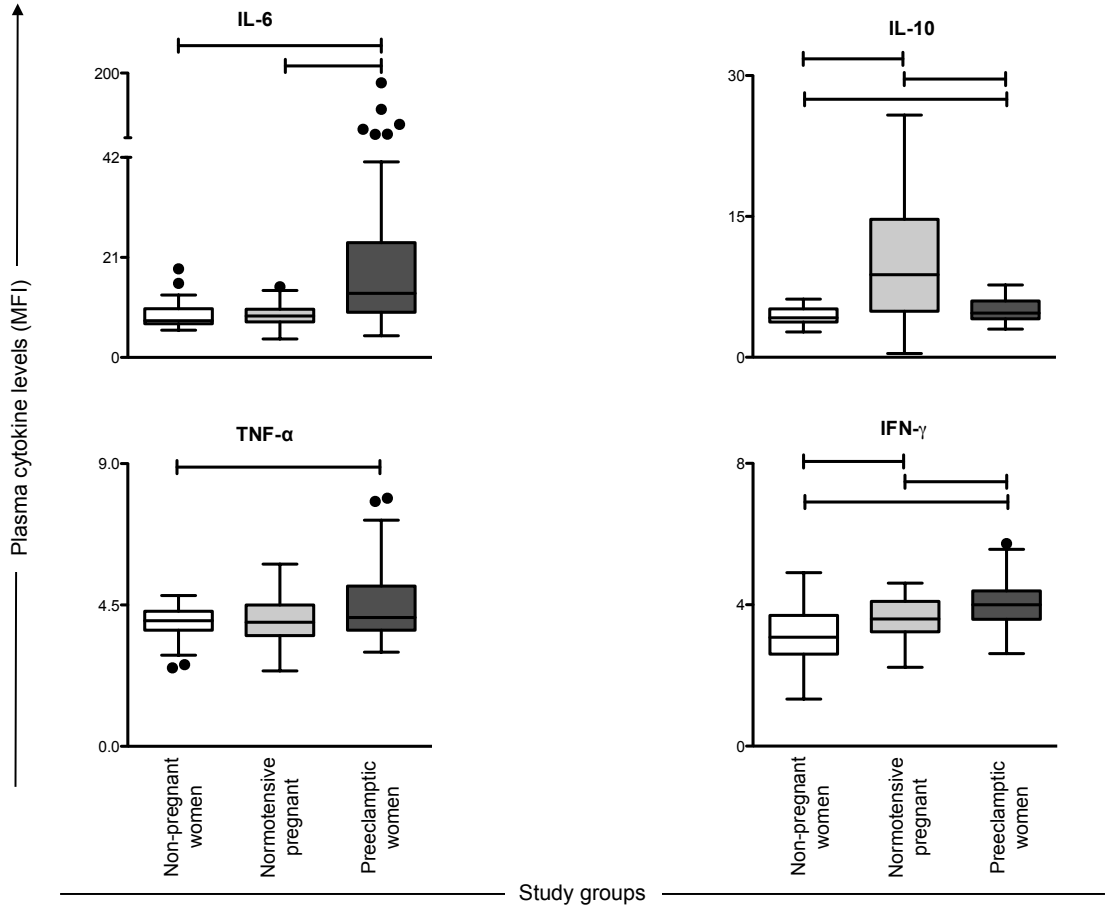

Figure 1: Cytokines plasma levels in mean fluorescence intensity (MIF) according to the groups

4.2.2 Severe Preeclampsia: Does Cytokine Network Drive To An Excessive Systemic Inflammatory State? – *Clinical Immunology*

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Severe Preeclampsia: Does Cytokine Network Drive to an Excessive Systemic Inflammatory State?

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Abstract

Recent evidence suggests that dissociation in the pro-inflammatory/regulatory immunological functions in the placental microenvironment plays a role in the preeclampsia pathogenesis. Herein, we have characterized the cytokine plasma levels in severe preeclamptic women compared to normotensive pregnant and non-pregnant women, aiming to better understand the immunological network and its clinical significance for the pathogenesis of preeclampsia. Our findings demonstrated that severe preeclamptic state is associated with high levels of pro-inflammatory cytokines IL-8, IL-6, and IFN-γ, whereas normotensive pregnancy evolves high levels of regulatory cytokine IL-10. Moreover, an outstanding pro-inflammatory "cytokine signature" could be observed in severe preeclamptic women display, while an overall regulatory state is the hallmark for normotensive pregnancy. In summary, our data showed that elevated levels of pro-inflammatory cytokines in the maternal circulation with a deviation in the "IL-8 x IL-6" axis towards IFN-γ might drive the cytokine network in severe preeclamptic women towards an excessive systemic inflammatory state.

Abbreviations: IFN-γ- interferon-gamma; IL- interleukin; TNF- tumor necrosis factor.

Keywords: Preeclampsia; cytokines; inflammation

1. Introduction

Preeclampsia is a multifactorial disease characterized by systolic blood pressure ≥ 140 mmHg or diastolic ≥ 90 mmHg at bed rest on at least two occasions six hours apart, and proteinuria ≥ 0.3 g/24 h, measured after the 20th week of pregnancy [1]. Clinically, it is important to diagnosis the severe form of preeclampsia when hypertension and proteinuria are even higher. This form can progress to eclampsia (characterized by seizures as a sign of affection of the cerebral vessels), syndrome HELLP (hemolysis, elevated liver enzyme, low platelets) or disseminated intravascular coagulation [2].

Although preeclampsia causes high maternal/fetal morbidity and mortality, the etiology of this multi-system disorder still remains to be elucidated. Recent evidence suggests that dissociation in the pro-inflammatory/regulatory immunological functions in the placental microenvironment plays a relevant role in the preeclampsia pathogenesis [3-6].

It is well established that the physiological balance between pro-inflammatory/regulatory responses presents important changes in healthy pregnancy, with a shift toward a regulatory state [7]. In preeclampsia it has been proposed that this alteration does not occur, or it is reverted in very early stages of the disease, and in consequence, it leads to a pro-inflammatory state. Previous studies showed increased levels of IFN-γ and decreased levels of IL-4 [8-11]. On the other hand, regarding to TNF-α, IL-1β, IL-6, IL-8, and IL-10 conflicting results have been found [11-17].

In the present investigation, we have characterized the cytokine

plasma levels in severe preeclamptic women compared to normotensive pregnant and non-pregnant women, aiming to better understand the immunological network and its clinical significance for the pathogenesis of preeclampsia.

2. Subjects, Material and Methods

2.1 Study Population

A total of 219 women were selected from Odete Valadares Maternity-Belo Horizonte/Brazil, Regional Public Hospital of Betim/Brazil and Healthy Center Guanabara, Betim/Brazil from 2009 to 2011. The study population was composed of three groups referred as severe preeclamptic, normotensive pregnant and non-pregnant women. The severe preeclamptic group comprises 69 women, age ranging from 14-44 years, with gestational age between 22-40 weeks. Severe preeclampsia was defined by systolic blood pressure ≥160mmHg or diastolic blood pressure ≥ 110mmHg, on more than two consecutive occasions within four hours apart and proteinuria > $2gL^{-1}$ or at least 2+ protein by dipstick. The group of normotensive pregnant was composed by 69 women, age ranging from 14-42 years, with gestational age between 20-41 weeks with systolic/diastolic blood pressure below 120/80mmHg and no history of hypertension or proteinuria. Non-pregnant women, with age ranging from 14-44 years, had no clinical and laboratory alterations. No significant differences were observed for age and gestational age. As expected, significant differences were observed for body mass index

(BMI), gestational weight gain (GWG) as well as systolic (SBP) and diastolic blood pressures (DBP). Table 1 summarizes the clinical characteristics of the study groups.

Exclusion criteria common for the three groups were chronic hypertension, haemostatic abnormalities, cancer, diabetes, cardiovascular, autoimmune, renal, and hepatic diseases, anticoagulant or corticosteroids therapy.

The Ethics Committee at Federal University of Minas Gerais-Brazil approved this study and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

2.2 Blood sampling

Five mL whole blood samples were drawn in EDTA-K_3 1.8mg/mL (Vacuette®) and centrifuged at 2,500g for 20 min at 4°C to obtain the plasma samples. One mL plasma aliquots were stored at -70°C until use for flow cytometric cytokine measurements.

2.3 Cytometric beads array for cytokine measurements

Cytokine plasma levels were determined using commercially available kits, including Human Th1/Th2 Cytometric Beads Array – CBA (BD Biosciences Pharmingen, USA) to quantify TNF-α, IFN-γ, IL-4, IL-5 and IL-10 along with the Human Inflammation kit to quantify IL-1β, IL-6, IL-8 and IL-12.

The CBA immunoassay uses 7.5μm polystyrene microbeads, assembled in distinct fluorescent sets, unique on their type four fluorescence intensity (FL-4). Each microbead is coupled to monoclonal antibody (MAb) against a given cytokine. Following incubation with the test sample, the bead-captured cytokines were detected by direct immunoassay using a “detection cocktail” of distinct MAbs labeled with type two fluorescence, phycoerythrin-PE (FL-2).

The method was carried out as recommended by the manufacturer, modified as follows: briefly, 25μL of undiluted plasma samples or standards (previously diluted) were added to 15μL of bead-mix and incubated for 90min at room temperature in the dark. The cytokine standard curves were run daily for each assay. After incubation, the samples and standards were washed with 500μL of wash buffer and centrifuged at 600g for 7min at room temperature. Subsequently, 20μL of detection cocktail were added to each tube and the bead-mix re-incubated for 90min at room temperature in the dark. Following incubation, the samples and standards were washed again with 500μL of wash buffer and centrifuged at 600g for 7min at room temperature to remove unbound detector reagent. After washing, 250μL of wash buffer was added to each tube. Data acquisition and analysis was performed in dual-laser FACScalibur™ flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA), using the BD Bioscience CBA software. Although the fluorescently labeled particles in the BD CBA immunoassay are designed to be excited by the 488nm and 532nm lasers on other BD flow cytometers, they can also be excited by the red diode laser 633nm on dual-laser BD FACSCalibur instruments. The detection of beads emission at FL-4

channel, using the excitation with 633nm laser simplifies the instrument set-up procedure and reduces the need for fluorescence compensation. Thus, a total of 1,800 beads/tube were acquired after proper set-up of a flow cytometer. Results were expressed as mean fluorescence intensity (MFI) for each cytokine.

2.4 Analysis "cytokine signatures"

The cytokine plasma levels were analyzed as the mean fluorescent intensity (MFI) provided by the CBA immunoassay. They were compared amongst preeclamptic women, normotensive pregnant and non-pregnant women (Figure 1). Additional analysis referred as "cytokine signatures were also performed as previously proposed by Luiza-Silva *et al* [18]. Briefly, the global median value for each cytokine was calculated taking the whole data universe from women (Figure 2A). The global median cut off for each cytokine were used as the cut-off edge to tag each women as they display "Low levels" (□ for all cytokines), High levels of pro-inflammatory (■ for IL-8, IL-6, IL-1β, TNF-α, IL-12, and IFN-γ) or "High levels of regulatory (▭ for IL-4, IL-5, and IL-10) cytokines. After assembling the gray-scale diagrams for each studied subgroups, the frequency (%) of women showing "High cytokine levels" was calculated (Figure 2B). This strategy allowed for computation of the percentage of patients displaying high cytokine levels. Following, the "cytokine signature" for each subgroup was then assembled as the ascendant frequency of high cytokine levels of preeclamptic women, normotensive pregnant and non-pregnant women (Figure 3A and 3B). Furthermore, these

data were also assembled was taken as the reference cytokine curve of non-pregnant women (figure 4A) and the normotensive pregnant (figure 4B) in order to identify changes in the overall cytokine patterns in preeclamptic women.

2.5 Statistical analysis

The cytokine were first evaluated comparing cytokine plasma levels, expressed as medium fluorescence intensity (MFI), amongst subgroups. This analyze were performed by Kruskal-Wallis and Dunn tests and differences considered significant at $P<0.05$ as demonstrated in Figure 1. Prior statistical analysis, the normality of data distribution was evaluated by the Kolmogorov-Smirnov test. All statistical comparisons were performed using the program GraphPad PRISM (version 5.0).

An additional strategy of data analysis were used to tag each women as they present “Low” or “High” cytokine levels, taking the global median MFI value from all data universe of a given cytokine as the cut-off as demonstrated on Figure 2A. Following data assembling of gray-scale diagrams (Figure 2B), the frequency of women with High cytokine levels were then compiled to establish the cytokine ascendant profile referred as “cytokine signatures”, illustrated in Figure 3A and B. Relevant cytokine frequency was considered when the percentage of women with high cytokine levels was above the 50^{th} percentile. Further, comparative analysis of the cytokine signatures among groups were performed by overlapping the ascendant cytokine curves of non-pregnant women (Figure 4A) or normotensive pregnant (Figure 4B). Relevant

differences in the ascendant cytokine signatures among groups were identified by comparative analysis, considering for each group only the cytokines with frequency above the 50th percentile, as illustrated in Figure 3 and 4. Spearman's rank correlations (r_S) were computed to assess correlations between inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α and IFN-γ in severe preeclamptic women, normotensive pregnant and non-pregnant women (Figure 5). The correlations that were statistically significant ($P < 0.05$) were showed.

3. Results

3.1 Severe preeclamptic state is associated with high levels of pro-inflammatory cytokines IL-8, IL-6, and IFN-γ whereas normotensive pregnancy evolves with *high levels of regulatory cytokine IL-10*

Cytokine plasma levels for the three groups are showed on Figure 1. Data analysis demonstrated that the levels of IL-8, IL-6 and IFN-γ were significantly higher in preeclamptic women as compared to non-pregnant women as well as to non-pregnant women. Moreover, the levels of TNF-α were also significantly higher in preeclamptic women in comparison with non-pregnant women. On the other hand, normotensive pregnant showed significantly higher levels of IL-10 as compared to normotensive pregnant and non-pregnant. No significant differences were observed for plasma levels of the other cytokines evaluated.

3.2 Severe preeclamptic women display an outstanding pro-inflammatory "cytokine signature" while an overall regulatory state is the hallmark of normotensive pregnancy

In order to assemble the cytokine signature of each study group, the global median plasma values for each cytokine was first calculated to establish the cut-off used to segregated women with "Low" or "High" cytokines levels, as illustrated in Figure 2A (IL-8= 2.75; IL-6= 9.31; IL-1β= 4.39; TNF-α= 4.03; IL-12= 7.77; IFN-γ= 3.79; IL-4= 1.40; IL-5= 3.13; IL-10= 4.70, all expressed in MFI). Using these values, each woman received a tag for each cytokine. Following, diagrams were used to assemble the pro-inflammatory and regulatory profiles and to calculate the frequency (%) of women showing "High cytokine levels" as showed in the Figure 2B.

The frequency of women with high cytokine levels was further compiled to establish the cytokine ascendant profile, referred as "cytokine signatures" for each study group (Figure 3A). Data analysis was carried out considering relevant only the cytokine frequencies above the 50th percentile. Using this criterion, the IL-4 was the only relevant element in the "cytokine signature" of the non-pregnant women. On the other hand, normotensive pregnant showed an outstanding frequency of regulatory cytokines IL-4, IL-5 and IL-10 along with borderline inflammatory IL-1β. Moreover, cytokine signatures of severe preeclamptic women showed a predominance of pro-inflammatory cytokines, including IL-8, IL-6, IL-1β, TNF-α, IL-12 and IFN-γ with IL-4 as the only one regulatory cytokine. These findings suggest an exacerbated inflammatory condition in severe preeclampsia and a regulated condition associated with

normotensive pregnancy. The overlay of ascendant “cytokine signatures” from the three study groups was further used to illustrate these findings (Figure 3B).

Alternatively, the ascendant “cytokine signature” from the non-pregnant group was used as a reference curve for comparative analysis with the normotensive and severe preeclamptic pregnant (Figure 4A). Considering relevant only the cytokine frequencies above the 50th percentile, data analysis demonstrated that the normotensive group displayed elevated percentage of women with high levels of IL-1β, IL-5 and IL-10 as compared to the non-pregnant group. These findings suggest that, in physiological conditions, pregnancy is characterized by a predominant regulatory cytokine profile (Figure 4A). On the other hand, severe preeclamptic group showed enhanced frequency of pro-inflammatory cytokines, including IL-8, IL-6, IL-1β, TNF-α, IL-12, and IFN-γ as compared to the non-pregnant women group (Figure 4A).

Additionally, the severe preeclamptic group showed higher frequency of pro-inflammatory cytokines, including IL-8, IL-6, TNF-α, IL-12 and IFN-γ along with lower frequency of regulatory cytokines (IL-5 and IL-10) when the ascendant “cytokine signature” from the normotensive group was used as a reference curve for comparative analysis (Figure 4B). Again, these findings suggest that severe preeclampsia evolves a high pro-inflammatory response and low participation of regulatory cytokines.

3.3 Deviation in the “IL-8 x IL-6” axis towards IFN-γ is the hallmark of the cytokine network correlation in preeclamptic women

The dynamic connections within the pro-inflammatory cytokine network were further evaluated using the correlation analysis as a tool to identify any shift in severe preeclamptic women aside from the normal pregnancy course (Figure 5). Our data pointed out to a universal axis of positive correlation between IL-8 and IL-6 in all studied groups. In non-pregnant women this axis also included an effective association with TNF-α, whereas in normotensive pregnant this common axis shifted towards a connection with IL-1β. Although the IL-1β connection is somehow preserved in severe preeclamptic women, a deviation forward IFN-γ appears as a satellite link reinforcing the pro-inflammatory cytokine network at this clinical condition.

4. Discussion

The availability of plasma panels from severe preeclamptic women, normotensive pregnant and non-pregnant women has enabled an unprecedented comparative analysis of plasma cytokines. Aiming to better understand the immunological network and its clinical significance for the pathogenesis of preeclampsia, we have performed an analysis of changes in pro-inflammatory/regulatory plasma cytokines in pregnant complicated by this intriguing disease, normotensive pregnant and non-pregnant women.

Our data reveal that severe preeclamptic state is associated with high levels of pro-inflammatory cytokines IL-8, IL-6, and IFN-γ whereas normotensive pregnancy evolves with high levels of regulatory cytokine IL-10 (Figure 1).

Previous studies showed higher IL-8 plasma levels in preeclamptic

women [10, 11, 19]. Likewise, increased IL-8 production by maternal peripheral blood mononuclear cells (PBMCs) in PE has been demonstrated [20-23]. Production of IL-8 by neutrophils that infiltrate the vasculature in women with PE [24, 25] would provide a chemotactic gradient to attract more neutrophils. These cells can adhere on the endothelium, infiltrate into the intimal space and release reactive oxygen species, myeloperoxidase, matrix metalloproteinase 8 and thromboxane, causing inflammation [26]. In this way, IL-8 seems to have a pivotal role in preeclampsia pathogenesis and severity.

Similarly, a recent metanalysis has highlighted the role of IL-6 in preeclampsia [17]. IL-6 is a multifunctional cytokine that regulates hematopoiesis, as well as the acute-phase reaction and modulates both pro- and anti-inflammatory events [27]. Chronic infusion of this cytokine to pregnant rats *in vivo* has caused hypertension and proteinuria, the two classical symptoms of preeclampsia [28, 29]. This disease is associated with endothelium activation, which justifies, at least in part, the clinical signs [30]. As it is known that IL-6 interferes in endothelial cell function [31], a role of this cytokine in preeclampsia may be admitted.

In agreement with our findings, several studies have been demonstrated high IFN-γ levels in preeclamsia [9-11, 32, 33]. However, other studies have not found an increase in this cytokine levels in preeclamptic women compared to normotensive pregnant [8, 21]. The role of IFN-γ during healthy pregnancy is still controversial. For instance, primiparous IFN-γ knockout mice experience fetal loss [34], and this cytokine can trigger spiral artery modifications [35]. However, these results were not obtained in multiparous mice.

Regarding IL-10, our results showed increased levels in normotensive pregnant compared to severe preeclamptic women and non-pregnant women (Figure 1). Previous studies reported high levels of IL-10 in healthy pregnant [13, 36-39], suggesting that successful pregnancy reflects a predominance of regulatory cytokine. Studies in mice revealed that IL-10 deficiency in early pregnancy affects trophoblast growth and differentiation, causing placental failure and abortion [40]. IL-10 also increases the resistance of trophoblasts to Fas-mediated apoptosis [41]. Inhibition of IL-10 by passive immunization (with monoclonal antibody to IL-10) during early gestation increases blood pressure in pregnant baboons [42]. Therefore, it has been suggested that decreased IL-10 production is associated with pregnancy disorders including preeclampsia [36, 43, 44].

Contrarily to our data other studies demonstrated an increase in IL-10 levels in preeclamptic women compared to normotensive pregnant [11, 17, 45, 46]. The interpretation of IL-10 results should be cautiously done. As the half-life of this cytokine is very short, it is not consistently present in circulation. Therefore, a single blood sample may fail to detect a sporadic raise or decline in this cytokine level. Besides, other factors as the effect of gestational age at the time of sample collection, the influence of body mass index and the assay sensitivity may also explain the divergences in IL-10 levels among studies [47]. In conclusion, there is no consensus regarding IL-10 production in preeclampsia.

TNF-α is a powerful pro-inflammatory cytokine and it is found in human placental and uterine cells, both early and late in gestation [48]. Several studies have reported elevated TNF-α maternal circulating levels in

preeclampsia, suggesting that TNF-α could be involved in the pathogenesis of this disease [17, 23, 49-52]. However, likewise our data, other studies have not reported significant differences in TNF-α maternal levels compared to normotensive pregnant [20, 21, 53, 54]. It is known that IL-6 can inhibit IL-1 and TNF-α [27, 55], which could be one explanation for a lack of differences for the latter two cytokines between preeclamptic women and normotensive pregnant in our study. Another justification may be due to the relatively short half-life of the cytokines, as well as possible transient and episodic release, which may result in considerable plasma levels variation not shown in a single blood sample. Although IL-1 and TNF-α were not increased in preeclamptic women, it is important to highlight that the endothelium in some patients might be more sensitive to activation by cytokines, which could lead to injuries even when the cytokines levels are normal [21].

IL-1β, IL-12, IL-4, and IL-5 were successfully detected in our studied groups but no difference was found comparing severe preeclamptic women and normotensive pregnant. Although this might represent the real condition *in vivo,* such results must be carefully interpreted. A speculative explanation could be related to the paracrine action of T-cell cytokines, which are quickly bound to receptors on neighboring cells, not being available in circulation. As a result, these cytokines plasma levels in both groups may be similar, even though an increased production has occurred in preeclampsia [21].

Complementary data analysis was applied to evaluate the plasma cytokine profile among the three groups evaluated, using the general concept of “Low” and “High” cytokine producers (Figure 2A) as proposed by Luiza-Silva et al [18]. Following data assembling on multi-cytokine diagrams, the

frequency of *High* cytokine producers was calculated for each group (Figure 2B). The comparative analysis of *High* cytokine producers among groups was performed using the 50th percentile as a limit to identify relevant differences as previously proposed by Luiza-Silva et al [18]. The comparative analysis of cytokine signatures pointed out that there is an enhanced frequency of severe preeclamptic with high levels of pro-inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α, IL-12 and IFN-γ (74%, 76%, 52%, 61%, 64% and 72%, respectively), while in normotensive group, only the frequency of women with high levels of IL-1β (53%) was obtained. On the other hand, the frequency of normotensive pregnant with high levels of regulatory cytokines IL-4, IL-5 and IL-10 (64%, 61% and 80%) were increased, while only the frequency of severe preeclamptic with high levels of IL-4 (63%) was verified (Figure 2B). These data showed that severe preeclamptic women display an outstanding pro-inflammatory “cytokine signature” while an overall regulatory state is the hallmark of normotensive pregnancy (Figure 3A and B). The major advantage of applying the cytokine signature model for data analysis was the opportunity to detect, with higher sensibility, putative minor changes in the cytokine profile not detectable by conventional statistical approaches.

In order to compare the inflammatory status between non-pregnant women *versus* normotensive pregnant or *versus* the severe preeclampsia group, the ascendant “cytokine signature” from the non-pregnant group was used as a reference curve (Figure 4A). In this way, only the cytokine frequencies above the 50th percentile were considered. Data analysis revealed that the normotensive group displayed an elevated percentage of women with high levels of IL-1β, IL-5 and IL-10, when compared to the non-

pregnant group, reinforcing that healthy pregnancy is characterized by a predominant regulatory cytokine profile (Figure 4A). Contrarily, the severe preeclamptic group showed a higher frequency of pro-inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α, IL-12, and IFN-γ, comparing to the non-pregnant group (Figure 4A). In physiological conditions, the cytokines balance is significantly altered in pregnancy by the placenta, since progesterone and cytokines modulate the immune cells by regulatory response [7]. The shift away from pro-inflammatory cytokine production during pregnancy is beneficial for this condition, since pro-inflammatory cytokines, especially IFN-γ and TNF-α, are harmful for pregnancy. Experimental studies revealed that these cytokines inhibited embryonic and fetal development [56, 57] and interrupted pregnancy when injected into pregnant mice [56]. Previous studies have shown that, particularly in the third trimester of human pregnancy, the ratio of pro-inflammatory/regulatory cytokines production by peripheral T lymphocytes is decreased, as compared to non-pregnant women [58-62]. However, there is no consensus if this decreased cytokines pro-inflammatory/regulatory ratio is due to a decreased production of pro-inflammatory cytokines [61, 62] or to an increased production of regulatory cytokines (IL-4, IL-5, IL-9, IL- 10) [58]. Our results suggest an increased production of regulatory cytokines and a normal production of inflammatory cytokines in normotensive pregnant women.

The comparison between normotensive pregnant *versus* the severe preclamptic group used the ascendant “cytokine signature” from the normotensive pregnant as a reference (Figure 4B). The severe preeclamptic group showed higher frequency of pro-inflammatory cytokines, including IL-8,

IL-6, TNF-α, IL-12 and IFN-γ, along with lower frequency of regulatory cytokines (IL-5 and IL-10). Once more, our data suggest that severe preeclampsia evolves a high pro-inflammatory response and low participation of regulatory cytokines. Accordingly to our data, Sargent et al. [63] has suggested that preeclampsia does not present a shift toward modulated response and, as a consequence, pro-inflammatory responses are not suppressed.

Correlation analysis was used as a tool to identify the dynamic connections within the pro-inflammatory cytokine network in severe preeclamptic women (Figure 5). For the three groups studied, a positive correlation between IL-6 and IL-8 was found, suggesting that these cytokines participate in the physiological mechanisms. Normotensive pregnant and preeclamptic women showed a positive correlation between IL-8 and IL-1β, suggesting that these cytokines are normally expressed in pregnancy. However, a positive correlation between IL-6 and IL-1β was observed in normotensive pregnant, but not in severe preeclamptic women. On the other hand, a positive correlation between IL-8 and IFN-γ was observed in severe preeclamptic women, but not in normotensive pregnant. It is possible to infer that this change in cytokines profile can be an important factor for the development of preeclampsia. Besides, altered cytokine levels may have a direct effect on maternal systemic vasculature. In agreement with our data, Kalinderis *et al*. [64] did not find a positive correlation between IL-6 and IL-1β in preeclamptic women.

In summary, our data showed that elevated levels of pro-inflammatory cytokines in the maternal circulation, with a deviation in the “IL-8 x IL-6” axis

towards IFN-γ, might drive the cytokine network in severe preeclamptic women towards an excessive systemic inflammatory state.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank FAPEMIG and CNPq/Brazil, and the program for technological development in tools for health-PDTIS-FIOCRUZ for the use of its facilities. OAMF, ATC and LMD are grateful to CNPq Research Fellowship (PQ).

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Legends

Figure 1 – Cytokine plasma levels in preeclamptic women (■) as compared to normotensive pregnant (■) and non-pregnant women (□). Plasma levels of pro-inflammatory (IL-8, IL-6, IL-1β, TNF-α, IL-12, and IFN-γ) and regulatory (IL-4, IL-5, and IL-10) cytokines were determined by cytometric beads array. Results are expressed in mean fluorescence intensity (MIF) data are

presented in a box plot format. The lines stretch from the 10th percentile to the upper 90th percentile, highlighting the outliers (●). The median is shown as a line across the box. Statistical analysis was performed by non-parametric Mann-Whitney test. Significant differences at $P<0.05$ are highlighted by connecting lines.

Figure 2 – Plasma cytokine cut-off and frequency of women with High levels of plasma cytokine amongst preeclamptic women, normotensive pregnant and non-pregnant women. (A) Scatter graphs employed to establish the concept of Low cytokine producers (○<global median), High pro-inflammatory cytokine producers for IL-8, IL-6, IL-1β, TNF-α, IL-12, IFN-γ (●≥global median) and High regulatory cytokine producers for IL-4, IL-5 and IL-10 (◎≥global median), all expressed in Mean fluorescence Intensity - MFI). Low (▭ for all cytokines) and High (■ for pro-inflammatory and ▬ for regulatory) cytokine producers were tagged for further frequency analysis. (B) Multi-cytokine diagrams used to quantify the frequency of women with High levels of cytokines in all studied groups. Relevant frequencies, considered for values above the 50th percentile are highlighted in **bold underline** format.

Figure 3 – "Cytokine signatures" preeclamptic women, normotensive pregnant and non-pregnant women. (A) The ascendant frequency of women with *High* levels of plasma cytokine was assembled and data expressed by bars graphs. Relevant frequencies, considered for values above the 50th percentile (cut-off dotted line) are highlighted by *. (B) The cytokine signatures were further overlaid for preeclamptic women (◍), normotensive pregnant (△)

and non-pregnant women (□) to identify relevant elements in the cytokine signature that emerge above the 50th percentile (cut-off dotted line). These elements are highlighted in the bottom “X axis” by rectangles.

Figure 4 - Comparative analysis of the cytokine signatures of preeclamptic women (■) as compared to normotensive pregnant (▭) and non-pregnant women (▭). (A) The ascendant frequency of women with high cytokine plasma levels was assembled for the non-pregnant women arm and demonstrated by bars graphs and ascendant cytokine curve (top panel). Comparative analysis with the cytokine profile of normotensive pregnant and preeclamptic women was further performed by overlaying the ascendant cytokine reference curve (middle and bottom panels). Dotted lines indicate the 50th percentiles used as the cut-off to identify relevant elements, highlighted by ↑ for increased frequencies. (B) The ascendant frequency of women with high cytokine plasma levels was also assembled for the normotensive pregnant arm and demonstrated by bars graphs and ascendant cytokine curve (top panel). Comparative analysis with the cytokine profile of preeclamptic women was further performed by overlaying the ascendant cytokine reference curve (bottom panels). Dotted lines indicate the 50th percentiles used as the cut-off to identify relevant elements, highlighted by ↑ and ↓ for increased or decreased frequencies, respectively.

Figure 5 - Correlations analysis of pro-inflammatory cytokines in preeclamptic women (■) as compared to normotensive pregnant (▭) and non-pregnant women (▭). (A) Spearman correlation indexes “r” and (B) Spearman

correlation graphs illustrated the significant connections within the pro-inflammatory cytokine network. (C) Grayscale diagram pointed out to a universal axis of positive correlation between IL-8 and IL-6 in all studied groups (□). This axis also included an effective association with TNF-α in non-pregnant women (□) and the shift towards a connection with IL-1β in normotensive pregnant (■). Deviation in the "IL-8 x IL-6" axis towards IFN-γ (■) is the hallmark of the cytokine network correlation in preeclamptic women.

Table 1 - Clinical characteristics of participants

Characteristics	Non-pregnant	Normotensive pregnant	Preeclamptic women	P value
Age (years)	25 (14-44)	24 (14-42)	26 (14-44)	0.356
GA (weeks)	-	33 (20-41)	33 (22-40)	0.799
BMI (Kg/m^2)	21.80 (19.95-	23.30 (21.00-26.70)	23.94 (21.69-	0.016*
GWG (Kg)	-	10.0 (0.1-25,4)	12.7 (2,1-76.1)	0.033*
SBP (mmHg)	120 (80-130)	110 (90-130)	170 (130-220)[a,c]	<0.001*
DBP (mmHg)	80 (50-90)	70 (50-90)	110 (90-150)[a,c]	<0.001*

GA: gestational age; GWG: gestational weight gain; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index (–): does not apply. * Statistic significant. a (non-pregnant x preeclamptic); b (non-pregnant x normotensive pregnant); c (normotensive pregnant x preeclamptic women).
Age and GA are presented as mean (standard deviation). Student t test
GWG, BMI, SBP and DBP are presented as median Mann–Whitney test and Kruskal-Wallis Test

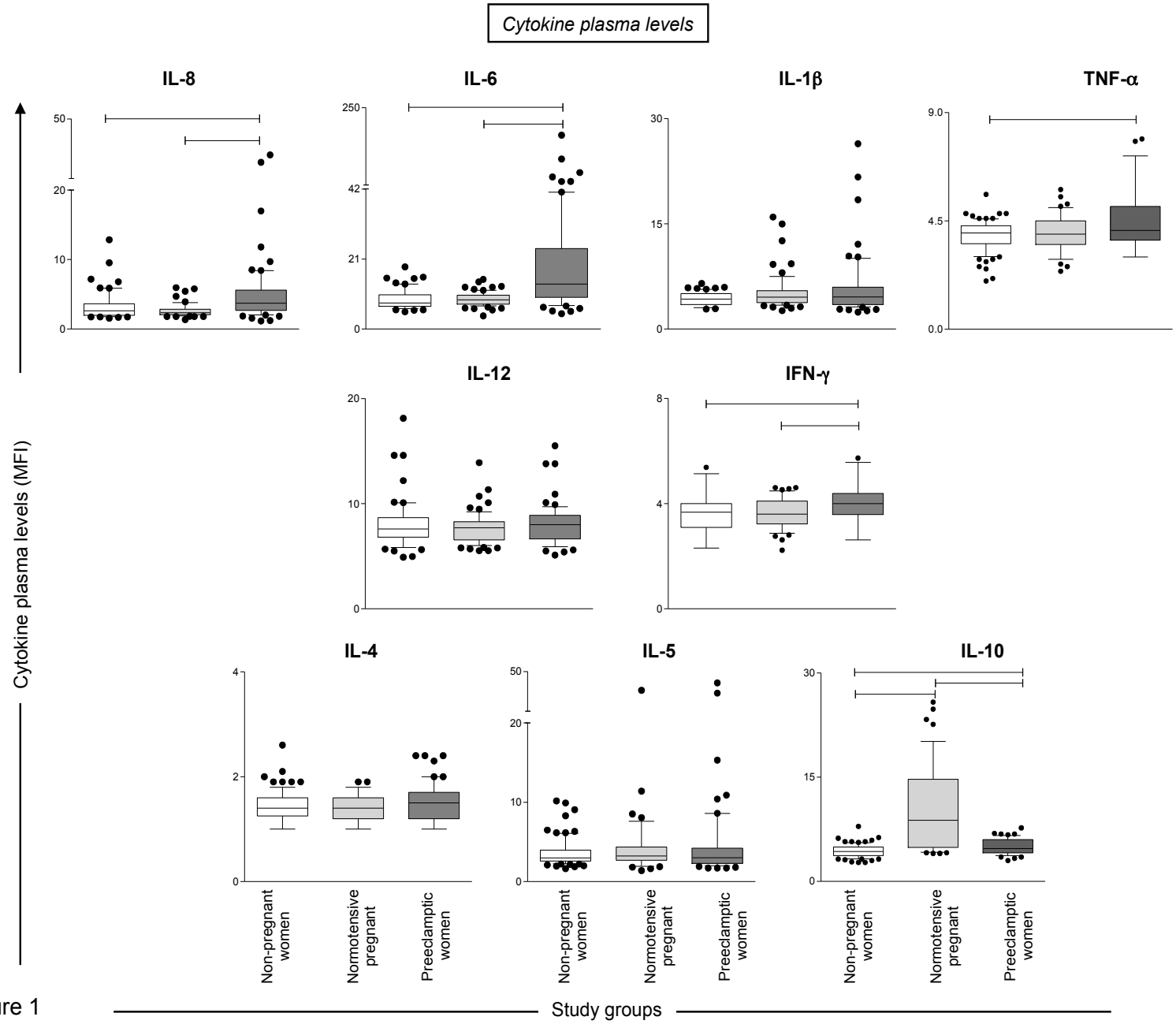

Cytokine plasma levels
IL-8
IL-6
IL-1β
TNF-α
IL-12
IFN-γ
IL-4
IL-5
IL-10
Cytokine plasma levels (MFI)
Non-pregnant women
Normotensive pregnant
Preeclamptic women
Study groups

Figure 1

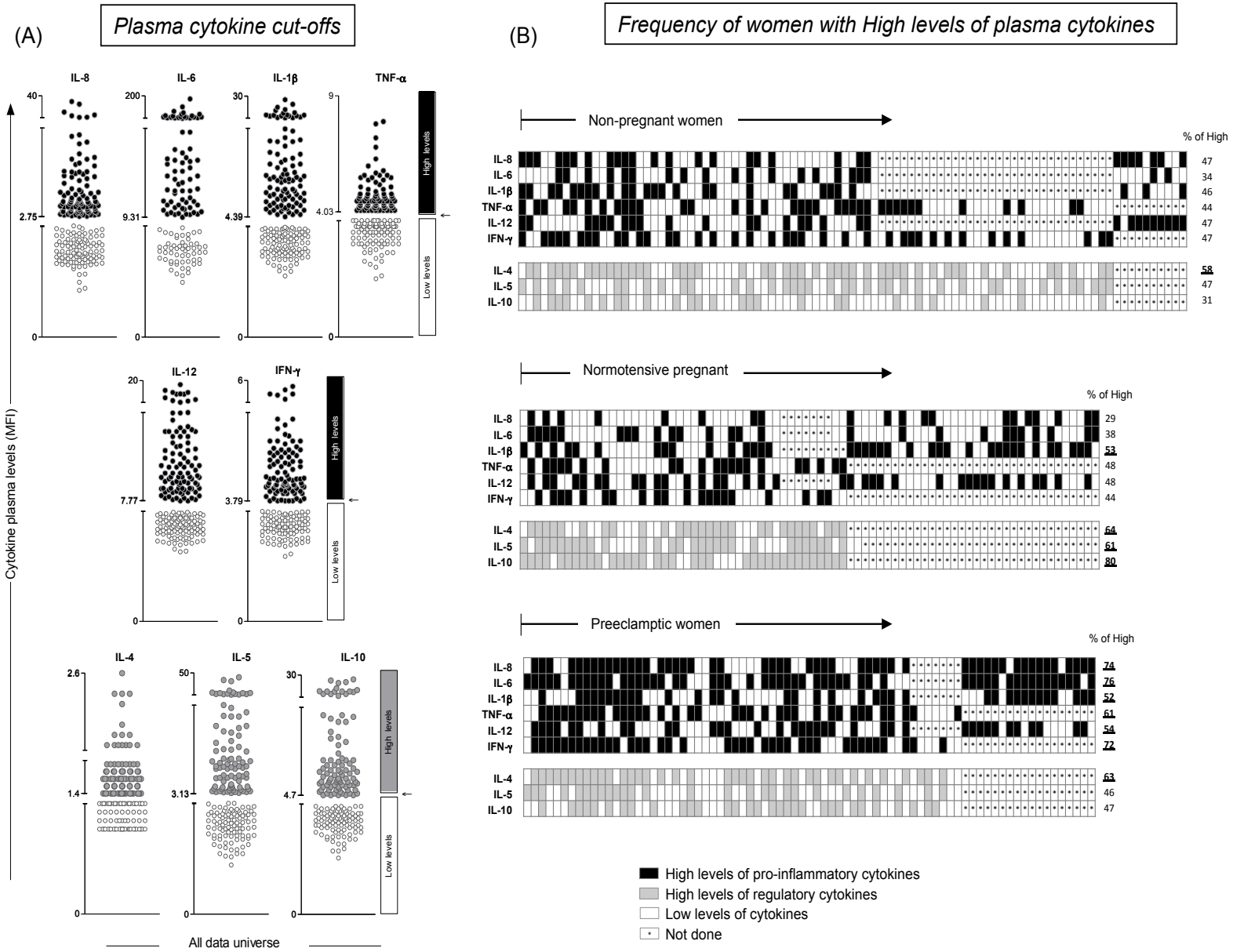

(A)
Plasma cytokine cut-offs
IL-8
IL-6
IL-1β
TNF-α
IL-12
IFN-γ
IL-4
IL-5
IL-10
High levels
Low levels
Cytokine plasma levels (MFI)
All data universe
(B)
Frequency of women with High levels of plasma cytokines
Non-pregnant women
% of High
IL-8 47
IL-6 34
IL-1β 46
TNF-α 44
IL-12 47
IFN-γ 47
IL-4 58
IL-5 47
IL-10 31
Normotensive pregnant
% of High
IL-8 29
IL-6 38
IL-1β 53
TNF-α 48
IL-12 48
IFN-γ 44
IL-4 64
IL-5 61
IL-10 80
Preeclamptic women
% of High
IL-8 74
IL-6 76
IL-1β 52
TNF-α 61
IL-12 54
IFN-γ 72
IL-4 63
IL-5 46
IL-10 47
High levels of pro-inflammatory cytokines
High levels of regulatory cytokines
Low levels of cytokines
Not done

Figure 2

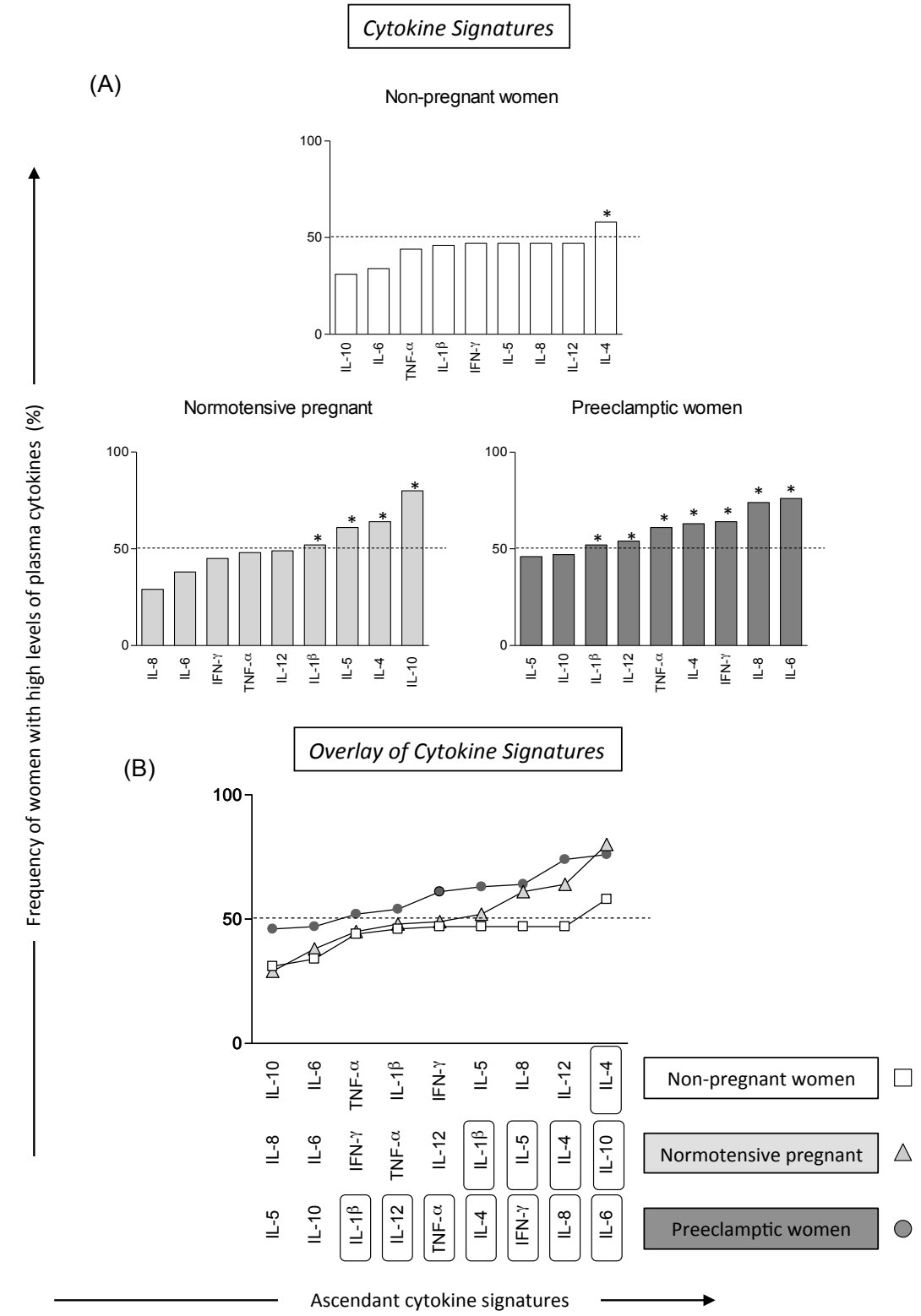
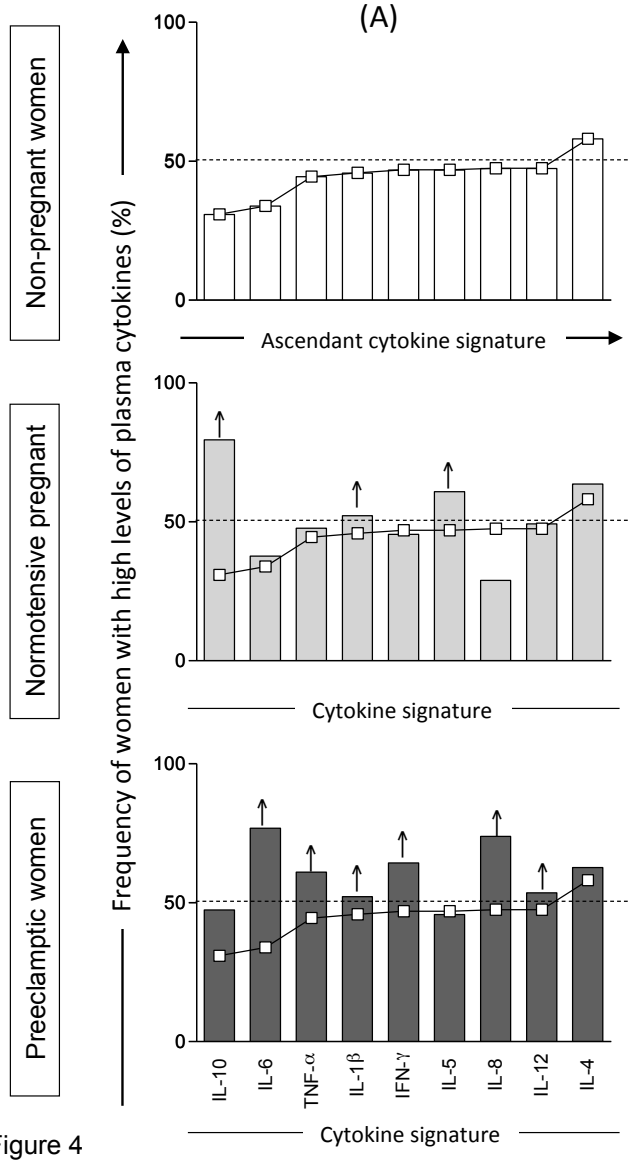

Cytokine Signatures
(A)
Non-pregnant women
100
50
0
IL-10
IL-6
TNF-α
IL-1β
IFN-γ
IL-5
IL-8
IL-12
IL-4
Normotensive pregnant
IL-8
IL-6
IFN-γ
TNF-α
IL-12
IL-1β
IL-5
IL-4
IL-10
Preeclamptic women
IL-5
IL-10
IL-1β
IL-12
TNF-α
IL-4
IFN-γ
IL-8
IL-6
Frequency of women with high levels of plasma cytokines (%)
Overlay of Cytokine Signatures
(B)
IL-10
IL-6
TNF-α
IL-1β
IFN-γ
IL-5
IL-8
IL-12
IL-4
IL-8
IL-6
IFN-γ
TNF-α
IL-12
IL-1β
IL-5
IL-4
IL-10
IL-5
IL-10
IL-1β
IL-12
TNF-α
IL-4
IFN-γ
IL-8
IL-6
Non-pregnant women
Normotensive pregnant
Preeclamptic women
Ascendant cytokine signatures

Figure 3


(A)
Non-pregnant women
Normotensive pregnant
Preeclamptic women
Frequency of women with high levels of plasma cytokines (%)
100
50
0
Ascendant cytokine signature
Cytokine signature
IL-10
IL-6
TNF-α
IL-1β
IFN-γ
IL-5
IL-8
IL-12
IL-4

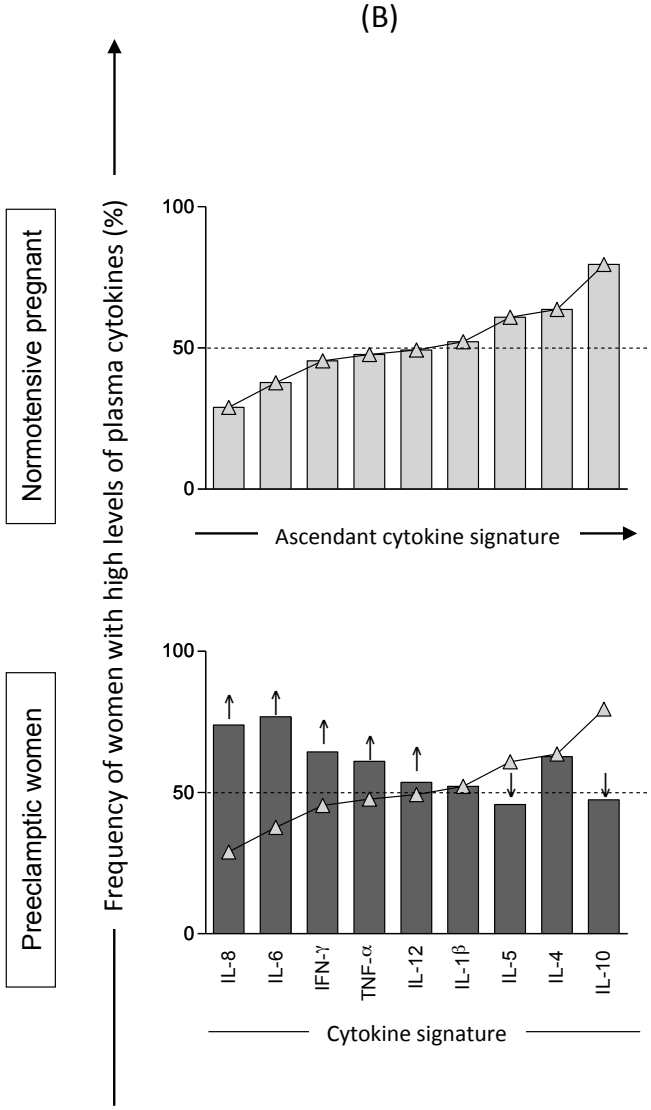
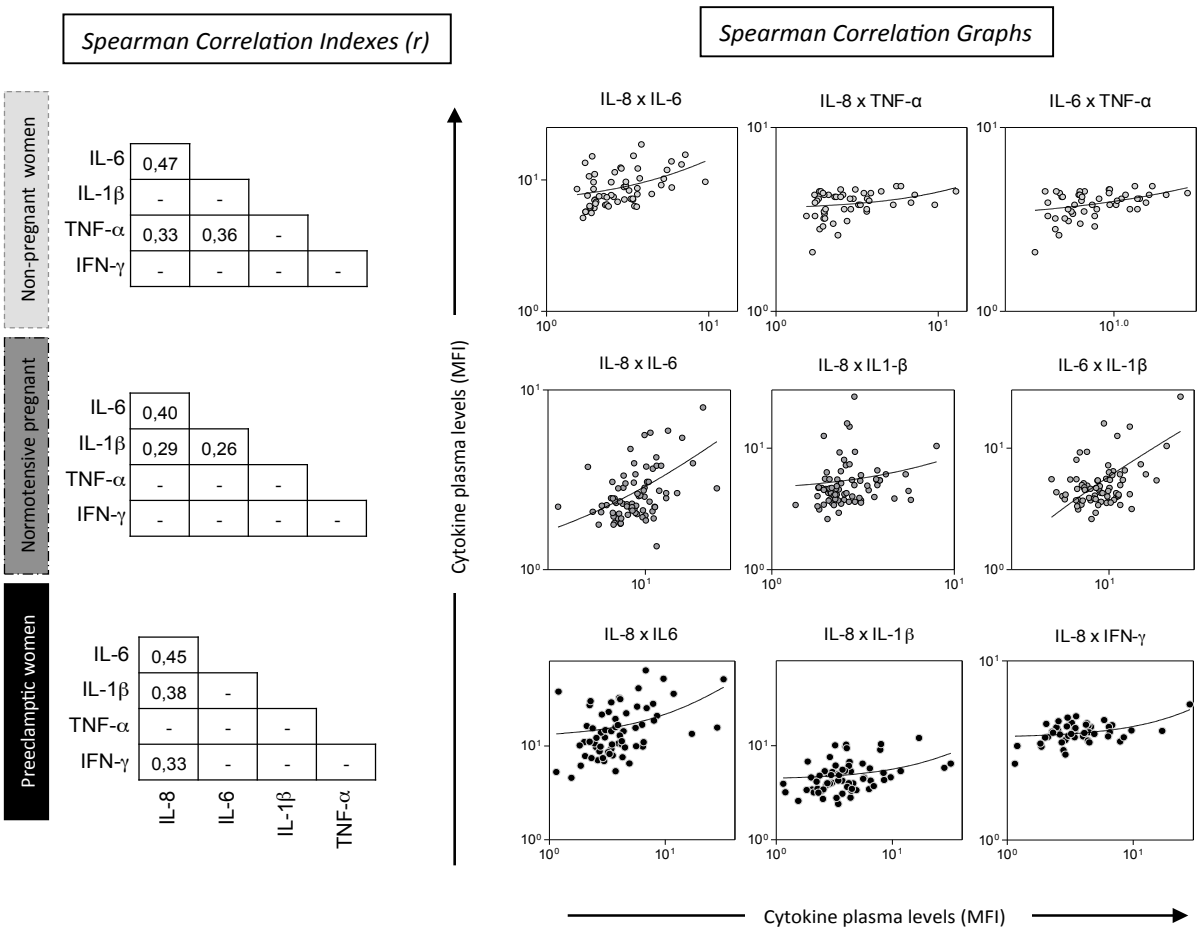

(B)
Normotensive pregnant
Preeclamptic women
Frequency of women with high levels of plasma cytokines (%)
100
50
0
Ascendant cytokine signature
Cytokine signature
IL-8
IL-6
IFN-γ
TNF-α
IL-12
IL-1β
IL-5
IL-4
IL-10

Figure 4



Cytokine Network

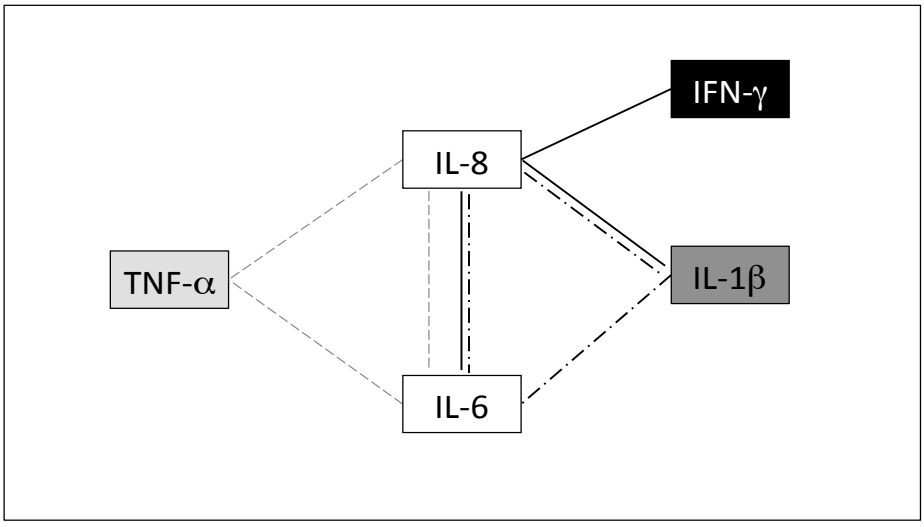


Figure 5

4.2.3 Severe Preeclampsia: How Is The Relationship Between Hemostatic And Inflammatory Parameters? - *Arteriosclerosis, Thrombosis, and Vascular Biology*

Title: Severe Preeclampsia: How is the relationship between hemostatic and inflammatory parameters?

Manuscript number: ATVB/2012/300402

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Severe Preeclampsia: How is the relationship between hemostatic and inflammatory parameters?

Hemostasis and Inflammation in Preeclampsia

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Abstract

Objective: Preeclampsia is a multi-system disorder of pregnancy characterized by hypertension and proteinuria. A predisposition to endothelial dysfunction, which may trigger abnormal activation of the hemostatic and/or inflammatory systems, is thought to play a crucial part in pathogenesis of PE. The aim of this study was to investigate the relationship between hemostatic and inflammatory parameters in women with severe PE.

Results: D-Dimer, PAI-1, IL-8, IL-6, TNF-α, and IFN-γ levels were measured in 59 pregnant with severe preeclampsia, 49 normotensive pregnant and 48 non-pregnant women. D-Dimer and PAI-1 were significantly higher in preeclamptic women comparing to normotensive pregnant and non-pregnant women. IL-8, IL-6, and IFN-γ also were significantly higher in preeclampsia comparing to normotensive pregnant. However, only IL-6 and IFN-γ were significantly higher in preeclamptic women comparing to non-pregnant. Moreover, D-Dimer and PAI-1 showed an elevated area under ROC curve (0.938 and 0.873), proving to be excellent for discriminating preeclampsia. Correlation analysis showed a weak correlation between D-Dimer and IL-8 ($r=0.597$, $P<0.001$) and between PAI-1 and IFN-γ ($r=0.397$, $P=0.045$) in preeclamptic women.

Conclusion: D-Di and PAI-1 levels showed as important tool for monitoring PE. However, no important correlation between these haemostatic markers and cytokines levels was found as expected, since hemostasis and inflammation are linked and influence each other. In conclusion, more studies are necessary to improve the knowledge of hemostasis and inflammation in PE. Apart from shedding light on pathogenesis of this intriguing disease, new therapeutic targets might be identified.

Keywords: Preeclampsia; D-Dimer; PAI-1; inflammatory cytokines

Introduction

Preeclampsia (PE) is a multi-system disorder of human pregnancy characterized by hypertension and proteinuria occurring after the 20th week of pregnancy in women who have had no previous symptoms [1, 2]. Clinically, it is important to diagnose the severe form of the disease, determined by even higher levels of hypertension and proteinuria [1]. The only definitive treatment is to deliver the baby and placenta, often prematurely, in the interest of the baby, the mother, or both[1].

PE is associated with deposition of fibrin in microvasculature, which results in placental perfusion compromised, intrauterine fetal growth retardation and dysfunction in some maternal organs [2-4]. Symptoms frequently observed in preeclamptic women include headache, blurred vision, and abdominal pain. The delivery of placenta remains the only known treatment. This disease can progress to eclampsia (characterized by seizures as a sign of affection of the cerebral vessels), syndrome HELLP (hemolysis, elevated liver enzyme, low platelets) or disseminated intravascular coagulation [5].

Although PE causes high maternal/fetal morbidity and mortality, its etiology still remains to be elucidated. A predisposition to endothelial dysfunction, which may trigger abnormal activation of the haemostatic and/or inflammatory systems, is thought to play a crucial part in pathogenesis of PE [2, 4, 6, 7].

Since hemostatic and inflammatory systems are known as important elements for the pathogenesis of vascular disease and both systems interact strongly [3], a detailed understanding of the relationship between these systems in PE may improve our knowledge on the pathophysiology of this disease. Thereby, the aim of this study was to investigate the relationship between hemostatic and inflammatory parameters in women with severe PE.

Subjects, Material and Methods

Study Population

A total of 59 pregnant with severe PE (sPE), 49 normotensive pregnant and 48 non-pregnant women were selected from Odete Valadares Maternity-Belo Horizonte/Brazil and Regional Public Hospital of Betim/Brazil and Healthy Center Guanabara, Betim/Brazil, from 2009 to 2011. Severe PE was defined by systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥ 110 mmHg, on ≥ 2 consecutive occasions ≥ 4 h apart; and proteinuria ≥ 2 gL−1 or at least 2+ protein by dipstick. The normotensive pregnant women had systolic/diastolic blood pressure below 120/80 mmHg and no history of hypertension or proteinuria. All studied women were age matched and all pregnant were gestational age matched. Non-pregnant women had no clinical and laboratory alterations.

Common exclusion criteria for the three groups were chronic hypertension, haemostatic abnormalities, cancer, diabetes, cardiovascular, autoimmune, renal and hepatic diseases, anticoagulant or corticosteroids therapy.

This study was approved by the Ethics Committee at Federal University of Minas Gerais and informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

Blood sampling

Blood samples were drawn in sodium citrate (0.129 mol/l) in 9:1 volume ratio and EDTA-K_3 1.8mg/mL (Vacuette®). Citrated blood samples were centrifuged at 2,500 g for 20 min at 4°C to obtain plasma. Samples were aliquoted and stored at 70°C until analysis of D-dimer and plasminogen activator inhibitor type-1. EDTA blood samples were centrifuged at 2,500g for 20 min at 4°C to obtain the plasma samples. One mL plasma aliquots were stored at -70°C until use for flow cytometric cytokine measurements.

Assays

D-Dimer (D-Di) and plasminogen activator inhibitor type-1(PAI-1)

Specific commercially available enzyme-linked immunosorbent assay (ELISA) *Kit* IMUCLONE® D-Dimer (American Diagnostica® Inc., *Stamford, USA*) and *Kit* IMUBIND® PLASMA PAI-1 (American Diagnostica® Inc., *Stamford, USA*), were used, according to the Manufacturer's instructions.

Cytokines

Cytokine plasma levels were determined using two commercially available kits: Human Th1/Th2 Cytometric Beads Array – CBA (BD Biosciences Pharmingen, USA) for IFN-γ, and Human Inflammation kit for IL-8, IL-6, and TNF-α. The method was carried out as recommended by the manufacturer. Data acquisition and analysis was performed in dual-laser FACScalibur™ flow cytometer (BD Biosciences Pharmingen, San Jose, CA, USA), using the BD Bioscience CBA software. Results were expressed as mean fluorescence intensity (MFI) for each cytokine.

Statistical analysis

Statistical analysis was carried out using SPSS (version 13.0). Data normality was tested by Shapiro-Wilk test. Comparisons between two groups were made by Student t test for parametric variables and Mann-Whitney for non-parametric variables. A comparison of non-parametric variables was done by Kruskal-Wallis test amongst three groups. When differences were detected, they were compared in pairs by Mann-Whitney method, followed by Bonferroni correction. Spearman's correlations were computed to assess correlations with plasma cytokine levels and hemostatic parameters. To evaluate the performance of D-Di, PAI-1, IL-8, IL-6 and IFN-γ as a tool for severe PE diagnosis, the area under the Receiver-operator characteristics (ROC) curve was calculated. P values < 0.05 were considered statistically significant.

Results

Table 1 summarizes the clinical characteristics of the 156 women enrolled in this study. Severe PE women, normotensive pregnant and non-pregnant women presented similar ages (P=0.305) and body mass index (BMI) (P=0.126). sPE women and non-pregnant did not show differences regarding gestational age (P=0.199). As expected, systolic and diastolic blood pressures were significantly higher in women with sPE (P<0.001 and P<0.001, respectively), as well as

gestational weight gain, when compared to the normotensive pregnant group (P=0.001).

Hemostatic markers and cytokine levels are summarized in Table 2. D-Di and PAI-1 were significantly higher in sPE group as compared to normotensive pregnant women (P<0.001 and P<0.001, respectively) or to non-pregnant women (P<0.001, in both cases). Furthermore, D-Di and PAI-1 were also significantly higher in pregnant women as compared to non-pregnant women (P<0.001, in both cases). Furthermore, D-Di and PAI-1 were also significantly higher in normotensive pregnant women, comparing to non-pregnant women (P<0.001, in both cases). (Figure 1).

IL-8, IL-6, and IFN-γ were significantly higher in the sPE group, comparing to normotensive pregnant women (P<0.001, P<0.001, and P=0.024, respectively), while only IL-6, and IFN-γ were higher comparing sPE and non-pregnant women (P<0.001, in both cases). IFN-γ was also significantly higher in normotensive pregnant women as compared to non-pregnant women (P=0.018). On the other hand, no difference was found for TNF-α comparing the three groups studied (Table 2).

Figure 2 presents the area under the ROC curve for D-Di, PAI-1, IL-8, IL-6 and IFN-γ and these parameters showed to be able to detect the sPE (P<0.001, P<0.001, P=0.021, and P=0.020, respectively). D-Di showed an elevated area under curve (AUC), above 0.900, proving to be excellent for detecting sPE in the population studied, as well as PAI-1 levels, which showed an AUC above 0.800. On the other hand, IL-8 and IL-6 showed to be bad for discriminating sPE (AUC=0.697 and 0.698, respectevely).

Correlation analysis showed a weak positive correlation between D-Di and IL-8 (r=0.597, P<0.001) and between PAI-1 and IFN-γ (r=0.397, P=0.045) in sPE. No statistical significant correlation was found for normotensive pregnant (Table 3).

Discussion

Hemostatic and inflammatory pathways mutually modulate and are integral parts of the host immune response [8]. Preeclamptic women are known to have an increased hypercoagulable state [9-13], as well as a higher inflammatory response [14-17]. Although some laboratory test are used to monitor pregnant in risk of PE, as platelets count and abnormal liver enzymes values, the diagnosis is established effectively by measuring blood pressure and proteinuria [5]. Therefore, to enhance our knowledge about the link between hemostasis and inflammation in PE is required.

Our present investigation showed an increase in D-Di levels in sPE women comparing to normotensive and non-pregnant women. Besides, it was observed higher D-Di levels in normotensive pregnant women comparing to non-pregnant women (Table 2). A recent metanalysis showed the ability of D-Di plasma levels to detect women with PE after the disease manifestation [18].

Regarding PAI-1, high levels were found in sPE, compared to normotensive pregnant or to non-pregnant women. Furthermore, PAI-1 plasma levels were also significantly higher in normotensive pregnant as compared to non-pregnant women. The PAI-1 AUC was 0.873, revealing that it is also a good test for detecting sPE.

Previous studies reported higher PAI-1 levels in preeclamptic women compared to normotensive pregnant [19-23]. It was also demonstrated that increased PAI-1 levels were detected preclinically in pregnant that show early evidence of placental dysfunction, as well as fetal growth restriction [24]. These findings suggest a decrease in fibrinolytic activity in PE.

Fibrinolysis *in vivo* is tightly regulated and depends on the balance between

plasminogen activators (t-PA and uPA) and plasminogen activator inhibitor (PAI-1) [25]. In the third trimester of healthy pregnancy, there is a four to five fold elevation of PAI-1 plasma levels, comparing to age matched non-pregnant women [26, 27]. Moreover, there is a major inhibition of acute endothelial t-PA release in pregnancy, attributable to excess PAI-1 [27]. It leads to a t-PA:PAI-1 ratio reduction, shifting pregnant women toward a prothrombotic state.

Taking together, our data suggest that elevated D-Di levels represent an exacerbated production of fibrin in women with sPE. D-Di levels reflect both fibrin polymerization and its breakdown *in vivo* [28-31] and the high levels found in sPE are probably due to fibrin production, since fibrinolytic system seems to be modulated by the high PAI-1 levels.

The D-Di/PAI-1 ratio in sPE, normotensive pregnant and non-pregnant women was 5.7, 4.4 and 2.8, respectively, confirming the prothrombotic state in women with sPE. A second ratio established between D-Di/PAI-1 for sPE or normotensive pregnant in relation to non-pregnant women suggests that normotensive pregnant is 57% (1.57), while sPE is 104% (2.04) more hypercoagulable than non-pregnant women. Such results were expected, since fibrin deposition is usually found in the subendothelium of the glomerulus and in decidual segments of spiral arteries in preeclamptic women [32].

Concerning cytokines, our data showed higher IL-8, IL-6 and IFN-γ levels in sPE women comparing to normotensive pregnant, which show a greater inflammation in severe preeclamptic women (Table 2). Elevated IL-6 levels in PE have also been observed in a number of studies, as demonstrated in a recent metanalysis [33].

Pro-inflammatory cytokines can induce functional and structural alterations, including oxidative damage or interference in vase constriction/relaxation, leading to alterations in vascular integrity, tone and coagulation [34]. Therefore, plasma cytokines have been suspected to be involved in the pathogenesis of PE for a long time [35, 36].

It is known that IL-8 is a potent chemotactic agent produced by activated neutrophils. Previous studies also showed high IL-8 levels in PE [37-39].

According to our data, other studies also found high IFN-γ levels in PE [38-42]. However, two studies did not find differences in IFN-γ levels comparing PE women and normotensive pregnant [43, 44]. Therefore, role of IFN-γ in the pathophysiology of PE remain to be clarified.

Our data did not show difference in TNF-α comparing the three groups (Table 2). TNF-α is a powerful pro-inflammatory cytokine and it is present in human placental and uterine cells, both early and late in gestation [45]. In agreement, other studies did not find significant difference in TNF-α levels comparing PE and normotensive pregnant [44, 46-48]. However, several studies have reported elevated TNF-α plasma levels in PE, suggesting that this cytokine is involved on the pathogenesis of this disease [33, 49-53]. The lack of consistency may be due to the relatively short half-life of the cytokine, as well as possible transient and episodic release, which may lead to a very considerable variation in its plasma levels [44].

In order to evaluate the relationship between hemostasis and inflammation in sPE, correlation analysis among the markers evaluated was performed. Only a weak positive correlation between PAI-1 and IFN-γ was found in sPE (Table 3). Similarly, regarding D-Di and cytokines correlation, only a weak positive correlation was obtained in sPE (D-Di *versus* IL-8). A previous study showed that coagulation of whole blood *in vitro* results in a detectable expression of IL-8 [54]. Fibrin can also activate endothelial cells, eliciting the synthesis of IL-6 and/or IL-8 [5, 55]. Thrombin and

fibrin can directly stimulate mononuclear cells and endothelial cells, inducing the synthesis of IL-6 or IL-8 [55].

It has been admitted that the endothelium sensibility to cytokines effects vary among subjects. As a result, normal cytokines levels could become injurious in some women, while others could tolerate high levels without endothelium lesions. This fact could explain the absent correlation between hemostatic and inflammatory markers obtained in our study [44].

To the best of our knowledge, this is the first study evaluating both coagulation and inflammatory systems in sPE. D-Di and PAI-1 levels showed to be important tool for monitoring PE. However, no important correlation between these hemostatic markers and cytokines levels was found as expected, since hemostasis and inflammation are linked and influence each other. Some speculations for the lack of the expected correlations may be done, as the multifactorial characteristics of PE, including the endothelium dysfunction, nitric oxide pathway, renin-angiotensin system and genetic factors, which represent confound factors for the disease understanding. Besides, it is possible that the hemostatic and inflammatory alterations may not be occurring simultaneously, which would prevent the joining of the cytokines and hemostatic markers' peak. Another possible explanation would be the fact that D-Di, PAI-1 and cytokines were evaluated systemically and the main alterations in PE could be occurring locally in microenvironment uterine. In conclusion, more studies are necessary to improve the knowledge of hemostasis and inflammation in PE. Apart from shedding light on pathogenesis of this intriguing disease, new therapeutic targets might be identified.

Acknowledgements

The authors thank FAPEMIG and CNPq/Brazil, and the program for technological development in tools for health-PDTIS-FIOCRUZ for the use of its facilities. OAMF, MCG, ATC and LMD are grateful to CNPq Research Fellowship (PQ).

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Legends

Table 1 - Clinical characteristics of participants
Table 2 - Hemostatic parameters and cytokines levels
Table 3 - Spearman correlation coefficients of the plasma cytokine and hemostatic Parameters

Figure 1 - Plasma Levels of Dimer-D and PAI-1
Figure 2 – Receiver-operator characteristics (ROC) curve for D-Dimer, PAI-1, IFN-γ, IL-6 and IL-8 for discriminating preeclamptic women

Table 1

Characteristics	Non-pregnant	Normotensive pregnant	Severe preeclamptic women	P value
Age (years)	25 (22-30)	23 (18-29)	26 (21-29)	0.305
GA (weeks)	-	32 (29-35)	33 (31-36)	0.199
BMI (Kg/m^2)	21.6 (20.1-25.4)	23.3 (20.9-26.9)	23.2 (21.4-28.4)	0.126
GWG (Kg)	-	8.5 (4.0-12.5)	12.3 (8.7-15.5)	0.001*
SBP (mmHg)	120 (110-120)	110 (100-110)	160 (160-180)[a,c]	<0.001*
DBP (mmHg)	80 (65-80)	70 (63-70)	110 (100-115)[a,c]	<0.001*

GA: gestational age; GWG: gestational weight gain; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index (–): does not apply. Data are expressed as median (25th–75th centiles).
Mann-Whitney test and Kruskal-Wallis Test were performed. * Statistic significant. a (non-pregnant x preeclamptic); b (non-pregnant x normotensive pregnant); c (normotensive pregnant x preeclamptic women).

Table 2

Parameters	Non-pregnant	Normotensive pregnant	Severe preeclamptic women	P value
D-Dimer (ng/mL)	116.9 (69.37-204.1) (N=48)	891.2 (712.9-1080.0) (N=49)	1641.0 (1226.0-2073.0) (N=59)	<0.001[a*] <0.001[b*] <0.001[c*]
PAI-1 (ng/mL)	41.70 (26.81-51.43) (N=31)	201.7 (172.1-250.9) (N=26)	286.8 (243.7-318.3) (N=28)	<0.001[a*] <0.001[b*] <0.001[c*]
IL-8 (MFI)	2.93 (1.97-3.85) (N=22)	2.37 (2.08-2.61) (N=30)	3.52 (2.46-4.61) (N=43)	0.193[a] <0.001[b*] 0.078[c]
IL-6 (MFI)	8.07 (6.41-10.85) (N=22)	8.43 (7.17-9.91) (N=30)	13.82 (9.65-28.13) (N=43)	<0.001[a*] <0.001[b*] 0.912[c]
TNF-α (MFI)	4.05 (3.60-4.30) (N=48)	4.00 (3.45-4.55) (N=37)	4.10 (3.78-5.15) (N=50)	0.058
IFN-γ (MFI)	3.24 (2.80-3.96) (N=48)	3.69 (3.31-4.13) (N=37)	3.98 (3.58-4.42) (N=50)	<0.001[a*] 0.024[b*] 0.018[c*]

PAI-1: Plasminogen activator inhibitor type-1; IL: Interleukin; TNF-α: Tumor necrosis facto type alpha; IFN-γ: Interferon type gamma; MFI: Mean fluorescence intensity; sPE: Severe preeclamptic women. * Statistic significant. a, sPE x non-pregnant women; b, sPE x pregnant women; c, pregnant women x non-pregnant women. Data are expressed as median (25th–75th centiles). Mann-Whitney test and Kruskal-Wallis Test were performed.

Table 3

Population	Cytokine	Hemostatic parameter	Spearman's (rho)
Normotensive pregnant	IL-8	D-Dimer	0,075
		PAI-1	0,092
	IL-6	D-Dimer	-0,006
		PAI-1	-0,054
	TNF-α	D-Dimer	-0,049
		PAI-1	-0,067
	IFN-γ	D-Dimer	-0,199
		PAI-1	0,062
Severe preeclamptic women	IL-8	D-Dimer	0,597*
		PAI-1	0,190
	IL-6	D-Dimer	0,248
		PAI-1	-0,128
	TNF-α	D-Dimer	0,099
		PAI-1	-0,221
	IFN-γ	D-Dimer	0,026
		PAI-1	0,397*

TNF, tumor necrosis factor; IL, interleukin; IFN, interferon. * Statistically significant difference (P<0,05)
Correlation analysis performed by the Spearman correlation test.

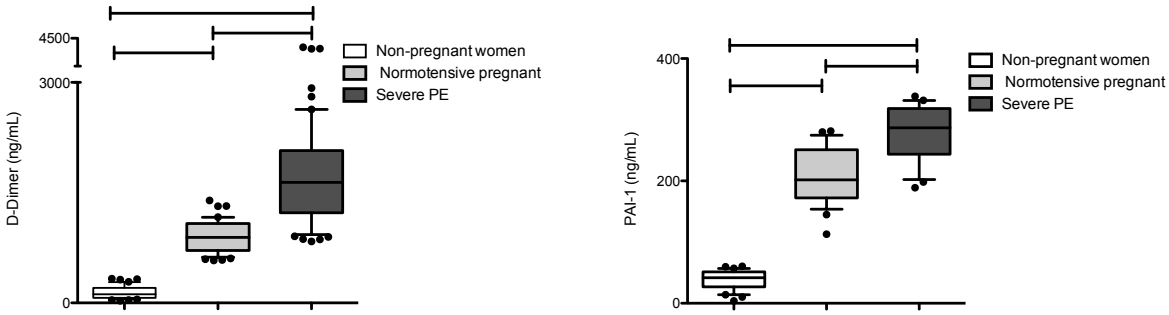


Figure 1

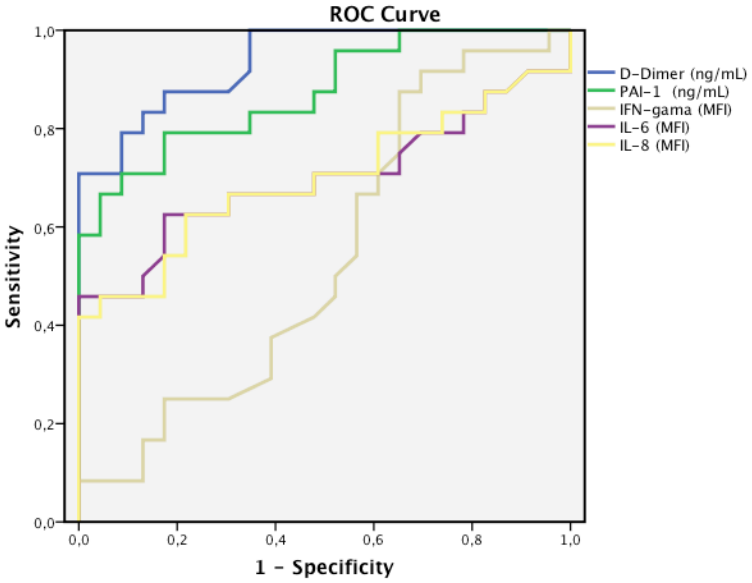
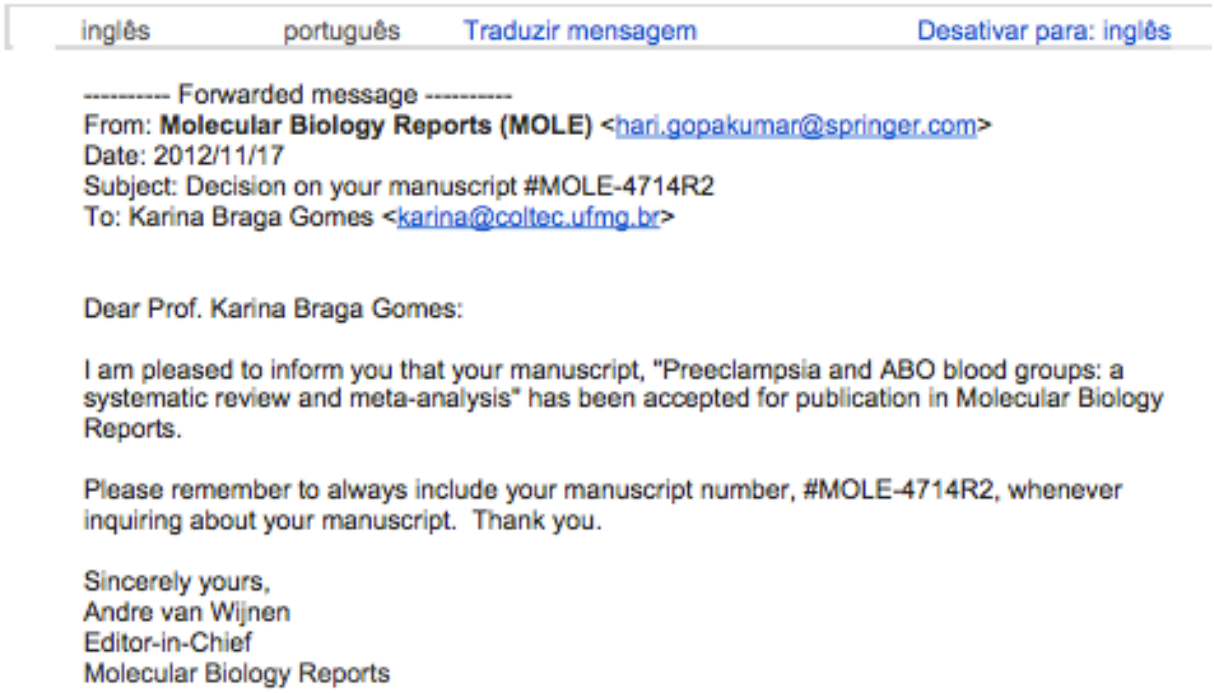


Figure 2

Area Under the Curve (AUC)		
Test Result Variable(s)	P value	Area (95% Confidence Interval)
D-Dimer (ng/mL	<0,0001	0,938 (0,875-1,000)*
PAI-1 (ng/mL)	<0,0001	0,873 (0,775-0,972)*
IFN-gama	0,647	0,539 (0,369-0,709)
IL-6	0,020	0,698 (0,540-0,856)*
IL-8	0,021	0,697 (0,540-0,853)*

4.3 Outras publicações junto ao grupo de pesquisa

4.3.1 Artigo Aceito - *Molecular Biology Reports*



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Molecular Biology Reports

Preeclampsia and ABO blood groups: a systematic review and meta-analysis

ALPOIM, P. N.; **PINHEIRO, M. B**.; FREITAS, L. G.; CARVALHO, M. G.; FERNANDES, A. P.; KOMATZUKI, F.; JUNQUEIRA, D. R. G.; GOMES, K. B.; DUSSE, L. M. S.

Abstract

Preeclampsia (PE) is a multifactorial pregnancy-specific syndrome, which represents one of the leading causes of maternal mortality worldwide. Inherited thrombophilias have been investigated as risk factor for the development of PE and it is currently known that ABO blood group may impact haemostatic balance, having the non-O blood groups (A, B or AB) subjects increased risk for thrombus formation, as compared to those of group O. We performed a systematic review of the literature for published studies investigating whether ABO blood groups could influence PE developing. A sensitive search of four databases identified 45 unique titles. Retrieved papers were assessed independently by authors and a rigorous process of selection and data extract was conduct. Methodological quality of the included studies was also evaluated. Two studies met eligibility criteria. As a main finding of our systematic review, an association between the AB blood group and the occurrence of PE was detected based on two original studies. Cconsidering the role of ABO blood groups on the hemostatic process and thrombus formation, special attention should be given to pregnant patients carrying the AB blood group in order to prevent the syndrome and improve prognosis.
Keywords: Preeclampsia, ABO Blood-Group System, Risk Factors, Systematic Review

4.3.2 Artigos em fase final de redação

4.3.2.1 Cytokines signatures in patients under hemodialysis

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Keywords: Hemodialysis, cytokines

Abstract

Objective: The aim of this study was to investigate the association between cytokines plasma levels and occurrence of vascular access thrombosis (VAT) in patients undergoing hemodialysis (HD). **Methods:** We evaluated 192 patients undergoing HD, 47 of which had VAT ("case" group) and 145 did not have this complication (control group). TNF-α, IFN-γ, IL-2, IL -4, IL-5 and IL-10 levels were performed by flow cytometry (FACScaliburTM-BD), using BAC kit (BD). **Results:** The cytokine patterns were first evaluated considering the moving average of plasma cytokine levels, expressed as medium fluorescence intensity (MFI) for the two groups. The HD patient with a higher value than the median was regarded as a "high" cytokine producer and those with lower value, as "low" cytokine producer. The "case" group showed a mixed profile of cytokine producers with an elevated percentage of "high" inflammatory cytokines IFN-γ (51%) and IL-2 (60%) and regulatory IL-4 (55%) and IL -5 (55%). For the control group it was obtained an elevated percentage of producers "high" of the regulatory cytokines IL-4 (52%) and IL-10 (52%). **Conclusions:** The elevated frequency of "high" pro-inflammatory cytokines producers and reduced percentage of regulatory cytokine IL-10 producers in "case" group compared to the control group, support the hypothesis of exacerbation of inflammation in patients who had VAT. Therefore, determination of cytokines in patients with HD can be a useful tool for the prevention of VAT.

4.3.2.2 Cytokines signatures in long-term stable renal transplantation

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Keywords: Renal transplant, cytokines, stable graft function

Abstract

Objective: In order to improve the understanding of the immune response in long-term stable renal transplantation, this study aims to investigate regulatory cytokines and pro-inflammatory plasma levels according to the time post-transplantation. **Methods:** Plasma levels of IFN-γ, IL-4 and IL-5 (Human kit Th1/Th2 cytometric Bead Array) and IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12 (Human Inflammation kit) from 120 kidney transplant patients were evaluated according to time after transplantation (in months). **Results:** The results revealed an increase in IL-4, IL-5 and IL-10 (regulatory cytokine) in patients with up to 24 months post-transplant. TNF-a levels showed to be elevated in patients with 25-60 months and up to 120 months after transplantation. The other pro-inflammatory cytokines IL-1β, IL-6, IL-8 and IL-12 levels were elevated in patients with more than 120 months after transplantation and IFN-γ has remained constant in all patients. This profile of cytokine levels after renal transplantation supported the distribution of patients into 4 groups: G1 (1-24 months), G2 (25 to 60 months), G3 (61 to 120 months) and G4 (> 120 months) after transplantation. The levels of IL-12 were significantly higher in G4 compared to G3 ($p = 0.015$). **Conclusions:** Our results allow us to infer that the loss of graft function over time is associated with an elevation of proinflammatory cytokines. Higher IL-5 levels in G1 compared to G2 suggest a modulation of the immune response in the immediate post-transplant, probably due to immunosuppressive therapy.

4.3.2.3 Cytokines signatures in long-term stable renal transplantation according to renal function

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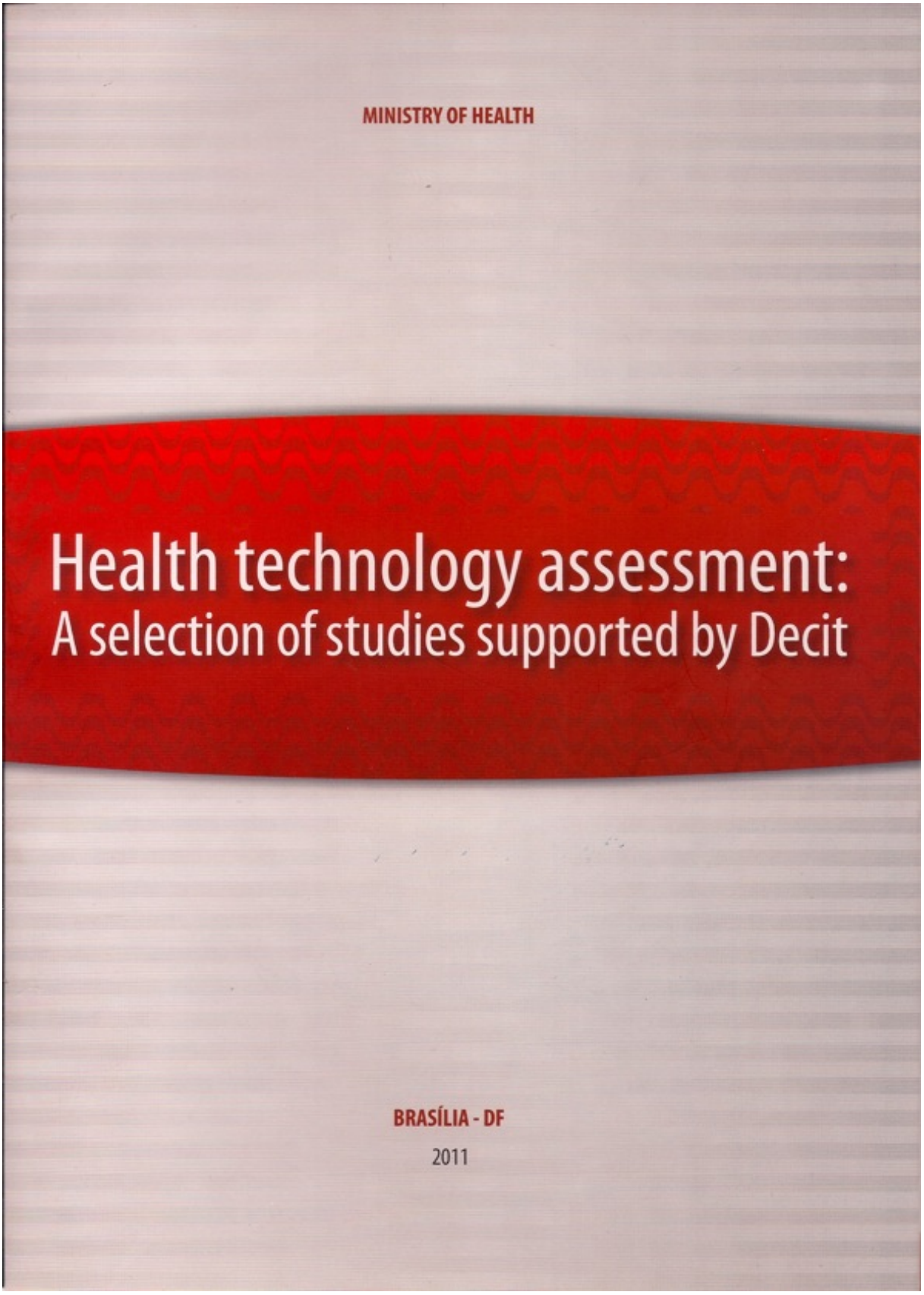
Keywords: Renal transplant, cytokines, renal function

Objective: The aim of this study was to evaluate regulatory cytokines and pro-inflammatory plasma levels in long-term stable renal transplantation, according to creatinine plasma levels. **Methods:** We evaluated 120 kidney transplant patients, with time post-transplant from 1 month to 19 years. IFN-γ, IL-4 and IL-5 (Human Th1/Th2 cytometric Bead Array ®) and IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α (Human Inflammation ®) plasma levels from 120 kidney transplanted patients were evaluated by flow cytometry ((FACScaliburTM-BD). Creatinine plasma levels were obtained from medical records. Patients were distributed into three groups: GI- creatinine <1.4 mg/dL GII- creatinine: 1.4 to 2g/dL and GIII- creatinine >2g/dL. **Results:** Our results showed a significant increase in IL-6 in GIII comparing to GI (P=0.01, Mann-Whitney test). The analysis of other cytokines according to creatinine levels revealed no significant differences comparing the three groups. **Conclusion:** It has been suggested that IL-6 is a highly sensitive marker for early detection of renal graft function loss, particularly over the years. Creatinine plasma levels increase proportionally to IL-6 increase. These results corroborate those in the literature and suggest that IL-6 may be a sensitive marker for monitoring the inflammatory process after renal transplantation and may be an important tool for diagnosis of allograft loss.

4.3.3 Resumos publicados



D-dimer plasma levels and pre-eclampsia – A systematic review

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AIM

Pre-eclampsia (PE) is associated with fibrin deposition in placental and renal microcirculation. D-dimer (D-Di) is the smallest fragment of the breaking of fibrin clot. The plasma level of this fragment has been used as a marker of production / degradation of fibrin in vivo. It is well established that plasma levels of D-Di have a negative predictive value for the diagnosis of deep vein thrombosis. Several studies show an increase of D-Di plasma levels in PE. The aim of this study was to review publications that assessed the D-Di plasma levels in pregnant women with PE and normotensive pregnant, by the most sensitive technique used (Enzyme-linked immunosorbent assay-ELISA), to define the diagnostic value of this marker for this disease.

Methods

We performed a systematic review following the methodology recommended by the Cochrane Collaboration. The electronic search included the Medline / Pubmed, EMBASE, LILACS and Web of Science (updated through August 2010) using sensitive search strategies, including a combination of free terms and controlled vocabulary from the relevant keywords. Additional studies were manually searched on the lists of references of potentially relevant articles. Only publications in English, Spanish and Portuguese were considered. We included observational studies evaluating D-Di plasma levels in pregnant women with PE and normotensive pregnant. Among the 194 titles in the literature, 47 were considered potentially eligible and 10 were selected for this review.

Conclusions and results

Among the 10 relevant studies to answer the question proposed in this systematic review, five had sufficient data and appropriately, allowing the combination of their results through a meta-analysis. These studies evaluated 347 cases of PE and 604 normotensive pregnant women. The results of the studies included in this meta-analysis showed high variability, some showed elevated D-Di plasma levels in pregnant women with PE and others in normotensive pregnant women. Analysis of the results showed no significant differences between D-Di plasma levels, measured by ELISA, in patients with PE compared to normotensive pregnant women.

Recommendations

Futher research/review required
The limitation of this systematic review was to include only articles in English, Spanish and Portuguese and should be conducted a new review including papers in other languages.

Reference

Manuscript under review.

ABO blood group and pre-eclampsia – a systematic rewiew

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AIM

Preeclampsia (PE) is associated with fibrin deposition in the placenta and kidney microcirculation. It is known that non-O blood groups (A, B or AB) subjects have increased risk for thrombus formation, as compared to those of group O. Since 1953, when Pike and Dickins related an association between O blood group and PE, several other studies were performed with the same objective, but there is no consensus until now relating to this association. In order to investigate the association between ABO blood groups and PE, a systematic review of studies published in this context was performed.

Methods

It was conducted a computerized search of the databases Medline, Embase, LILACS, Medline and Web of Science up to July 2010. Optimized search strategies were used with a combination of terms such as pre-eclampsia, eclampsia, pregnancy induced hypertension, toxaemia, HELLP syndrome and ABO(H) blood group system. Cohort, case-control and sectional studies were included if compared pre-eclamptic woman with a controlled group constituted of health pregnancy woman regarding the typed blood group, the risk factor of interest.

Conclusions and results

Overall, 46 full text articles were identified. Twenty-three studies were considered potentially relevant, of which only three were included. These studies included 507 cases of preeclampsia and severe preeclampsia among 1761 pregnant women. The studies were generally of good methodological quality although one study considered only the severe form of PE (13) and was very small (number of cases=55). Generally, no overall effect was found when all the study's findings were pooled comparing blood group A versus non-A for the risk of PE [odds ratio of 0.88 (95% CI 0.71 to 1.10) with no substantial heterogeneity (P=0.34, I2=7.0%)]. A similar result was observed when comparing group B with non-B pregnant [odds ratio of 1.08 (95% CI 0.75 to 1.56), with low heterogeneity (P=0.25, I2=28.0%)]. Once more, no overall effect was found when all the study findings were pooled as regards non-O versus O blood group [odds ratio 0.86; 95% CI 0.69 to 1.08) and no substantial heterogeneity was observed (P=0.69, I2=0.0%)]. It was observed a significant overall effect when comparing blood group AB versus non-AB pregnant [odds ratio of 2.37 (95% CI 1.62 to 3.47) with no evidence of heterogeneity (P=0.63, I2=0.0%)].

Recommendations

Futher research/review required
The limitation of this systematic review was to include only articles in English, Spanish and Portuguese and should be conducted a new review including papers in other languages.

Reference

ALPOIM, P. N. et al. ABO blood group and pre-eclampsia: a systematic review. In: INTERNATIONAL SYMPOSIUM ON WOMEN'S HEALTH ISSUES IN THROMBOSIS AND HAEMOSTASIS, 4., 2011, Berlim. **[Annal...]**. Berlim: Elsevier, 2011. (Thrombosis reserch 127: S123-S150)

5 CONSIDERAÇÕES FINAIS

A PE constitui uma das principais causas de morte materna e complicações neonatais em todo o mundo. Apesar de muito estudada, continua desafiando a comunidade científica. Uma busca no site *PubMed* revelou mais de 27 mil publicações envolvendo essa doença. No entanto, muitas lacunas ainda existem para o completo entendimento dessa doença.

A etiologia da PE é obscura e muitas teorias têm sido propostas para explicá-la. No entanto, nenhuma delas é totalmente aceita.

O único tratamento definitivo para a PE consiste na interrupção da gravidez e retirada da placenta. Em muitas das vezes esta medida é tomada prematuramente, visando garantir a vida da mãe, do bebê ou de ambos. Vários estudos randomizados têm sido desenvolvidos visando a obtenção de uma alternativa terapêutica para a PE, incluindo o uso de aspirina, heparina, concentrado de antitrombina, agentes anti-plaquetários, cálcio, L-arginina, óleo de peixe, vitaminas e outros antioxidantes. No entanto, todos estes estudos apresentam limitações, especialmente em relação ao número de gestantes avaliadas e os resultados mostram pouco ou nenhum benefício. (52)

O diagnóstico da PE constitui um grande desafio. Marcadores laboratoriais vêm sendo sistematicamente pesquisados, no entanto, nenhum exame promissor para esse diagnóstico foi ainda proposto. Rotineiramente, a propedêutica e monitorização da gestante com suspeita de PE inclui a solicitação de exames complementares como hemograma, contagem de plaquetas, enzimas hepáticas, dentre outros, mas o diagnóstico é feito efetivamente pela aferição da pressão arterial e determinação da proteinúria. Sabe-se que a medida da pressão arterial está associada a imprecisão e altera com a postura corporal e ausência de repouso prévio do paciente. A proteinúria é normalmente detectada nos laboratórios clínicos, por meio de fita reagente, uma vez que a determinação em urina de 24 horas é um método trabalhoso e demorado. Além disso, muitas vezes, o agravamento dos sintomas clínicos da gestante exige a interrupção da gestação, antes de completar a coleta de urina para esse exame. A detecção da proteinúria por meio de fita reagente está sujeita a resultados falsamente positivos quando a está alcalina, seja pela contaminação com amônia quaternária, clorhexidina, bem como pelo corrimento vaginal. (53)

Um estudo conduzido por Lindheimer, 1975 (54) revelou que a análise de material obtido por biópsia renal mostrou a presença de outras doenças renais em 20 a 40% dos casos diagnosticado como PE, o que pode resultar em assistência médica não apropriada durante a gestação, bem como no acompanhamento futuro desta mulher, além da obtenção de conclusões errôneas nas pesquisas envolvendo esta doença.

A PE manifesta-se em diferentes formas clínicas. Atualmente a doença é classificada em leve ou grave, de acordo com os níveis pressóricos e de proteinúria. Uma nova classificação da PE está sendo proposta, como precoce ou tardia, de acordo com a idade gestacional na qual surgem as manifestações clínicas. A PE precoce tem início antes da 34ª semana de gestação, é clinicamente mais grave, enquanto a PE tardia, tem início a partir da 34ª semana gestacional e é a mais frequente. (7, 8)

A PE está associada à exacerbação da coagulação. Sabe-se que na gestação normal há elevação dos níveis de fatores da coagulação e diminuição dos anticoagulantes naturais, o que resulta em um estado de hipercoagulabilidade. (11-13) Esse estado constitui uma adaptação fisiológica, que visa garantir um controle rápido e eficaz da hemorragia no momento do parto, quando ocorre a separação da placenta. (13, 14) No entanto, na PE a exacerbação da coagulação é ainda maior e ocorre deposição de fibrina na microcirculação uterina. (15-18)

A investigação da coagulação no presente estudo, por meio da determinação plasmática de D-Di, revelou níveis aumentados nas mulheres com PE grave comparadas às gestantes normotensas e mulheres não gestantes. Os níveis de D-Di refletem, tanto a polimerização, quanto a quebra de fibrina e têm sido utilizados, tanto como marcador de produção de fibrina *in vivo*, como da sua degradação. (55-58) As gestantes normotensas também mostraram níveis aumentados de D-Di quando comparadas às mulheres não gestantes. (59) Uma metanálise recente avaliando os níveis de D-Di em mulheres com PE revelou que esse marcador pode ser útil para o diagnóstico dessa doença, uma vez que seus níveis são mais elevados nas gestantes com pré-eclâmpsia no terceiro trimestre de gravidez, em comparação às gestantes normotensas. (60)

A avaliação plasmática dos níveis de PAI-1 também revelou um aumento nas gestantes com PE grave quando comparadas às normotensas e mulheres não gestantes. (59) De forma semelhante, as gestantes normotensas também

apresentaram níveis aumentados de PAI-1 em relação as mulheres não gestantes. Vários outros estudos também encontraram níveis aumentados de PAI-1 em mulheres com PE. (61-65)

A análise conjunta dos resultados de D-Di e PAI-1, neste estudo, permite concluir que os níveis elevados de D-Di nas gestantes com PE grave refletem a exacerbação da produção de fibrina, uma vez que o sistema fibrinolítico estaria modulado pelos níveis elevados de PAI-1. A proporção D-Di/PAI-1 nas gestantes com PE grave, normotensas e mulheres não gestantes foi 5,7; 4,4 e 2,8, respectivamente, confirmando o estado pro-trombótico associado à PE grave. Uma segunda razão estabelecida entre "D-Di/PAI-1" nas gestantes com PE grave ou normotensas, em relação a "D-Di/PAI-1" das mulheres não gestantes, foi 1,57 e 2,04, o que indica que a PE grave é 104% mais hipercoagulável que as mulheres não gestantes e as gestantes normotensas, 57%. (59) Este resultado era esperado, desde que pequenos coágulos de fibrina são encontrados na microcirculação de gestantes com PE grave.

A PE cursa com disfunção renal e sabendo que a antitrombina possui peso molecular reduzido (58KDa), quantidades significativas dessa podem ser perdidas na urina, o que contribuiria para o estado pró-trombótico (66). A lesão endotelial que está presente na PE, também contribui para o estado de hipercoagulabilidade, uma vez que as células lesadas expõe fator tissular, desencadeando a coagulação, além de reduzir a expressão de trombomodulina (o receptor para a trombina, importante para iniciar a ativação da via da proteína C). (20)

Uma revisão da literatura acerca da fibrinólise na PE permite concluir que a coagulação sobrepõe os mecanismos regulatórios do sistema fibrinolítico, uma vez que formação de pequenos trombos é usualmente observada na microcirculação de gestantes com PE. A oclusão e a consequente hipoperfusão tecidual justifica, em parte, os sintomas clínicos da doença. (67)

A opção de incluir no presente estudo apenas a forma grave da PE partiu da premissa de que as alterações hemostáticas e inflamatórias estariam mais acentuadas nessa forma clínica da doença. De fato, Dusse (1999) (68) em um estudo onde foram avaliados parâmetros hemostáticos de gestantes com PE grave e leve, comparando-se à gestantes normotensas, mostrou que a elevação desses foi mais acentuada naquelas com PE grave.

Diversos estudos sugerem que a PE está associada à exacerbação do processo inflamatório. (24, 26-28) A investigação dos níveis plasmáticos de citocinas pró-inflamatórias e reguladoras, no presente estudo, revelou um aumento significativo de IL-8, IL-6 e IFN-γ e diminuição de IL-10 nas gestantes com PE grave em relação às gestantes normotensas e mulheres não gestantes. O predomínio do aumento das citocinas pró-inflamatórias indica um estado de inflamação exacerbado nessa forma da doença. Nas gestantes normotensas foi obtido um aumento de IL-10, sugerindo a modulação da resposta inflamatória nessa condição clínica. Visando comparar o *status* inflamatório dos três grupos estudados de modo global, a mediana de cada citocina (considerando todas as mulheres avaliadas), foi obtida e utilizada como *cut off* para segregar como “High” ou “Low” produtoras de citocinas, aquelas que mostraram níveis maiores e menores, respectivamente. A frequência de mulheres “High” produtoras de cada citocina foi compilada e considerada como significativa quando superior a 50% e foi utilizada para obtenção da “Assinatura de citocinas”. No grupo de mulheres com PE grave, houve uma frequência maior que 50% de “High” produtoras das citocinas pró-inflamatórias IL-1β, IL-12, TNF-α, IFN-γ, IL-8 e IL-6 e da reguladora IL-4. No grupo de gestantes normotensas, as citocinas reguladoras, IL-5, IL-4 e IL-10 e apenas a pró-inflamatória IL-1β, tiveram frequência superior a 50% de “High” produtoras. Estes dados confirmam o estado pró-inflamatório na PE grave e modulado na gestação normal. (69)

Sabe-se que a hemostasia e a resposta inflamatória estão relacionadas e interagem mutuamente. (70, 71) Nos processos inflamatórios, há uma diminuição da atividade das proteínas C e S, o que concorre para o estado pró-trombótico (71-73). Além disso, as citocinas pró-inflamatórias induzem um aumento da expressão de fator tissular pelos monócitos, o iniciador do processo da coagulação. As micropartículas liberadas pelas células ativadas também expressam fator tissular desencadeando a coagulação. Por outro lado, as plaquetas ativadas, bem como o coágulo de fibrina secretam citocinas pró-inflamatórias.

Considerando a inter-relação dos sistemas hemostático e inflamatório, foi investigada, no presente estudo, a correlação entre os níveis plasmáticos de D-Di e de PAI-1 com as citocinas pró-inflamatórias IL-8, IL-6, IFN-γ e TNF-α. No entanto, foi obtida apenas uma correlação fraca entre D-Di e IL-8 ($r=0.597$) e entre PAI-1 e IFN-γ ($r=0.3975$) na PE grave. Algumas especulações podem ser feitas para justificar a

não obtenção das correlações esperadas. Dentre essas, os fatores de confusão associados à PE (caráter multifatorial da PE, disfunção endotelial, via do óxido nítrico, sistema renina-angiotensina e fatores genéticos). Além disso, é possível que as alterações dos sistemas hemostático e inflamatório não ocorram simultaneamente, o que inviabilizaria a obtenção de picos coincidentes dos marcadores avaliados. Outra possível explicação seria a avaliação sistêmica destes marcadores e a possibilidade das principais alterações hemostáticas e inflamatórias ocorrerem no microambiente uterino. (59)

Tem sido investigado se alterações genéticas poderiam explicar o desenvolvimento da PE e estudos envolvendo a análise de genes relacionados aos mecanismos de alteração fisiológica da doença vem sendo realizados. Estes estudos visam definir marcadores moleculares capazes, tanto de prever o desenvolvimento da doença, como melhorar a resposta ao tratamento clínico e farmacológico. No entanto, a investigação da associação de polimorfismos nos genes de citocinas e a ocorrência de PE têm resultado em conclusões conflitantes (41-51). Dessa forma, foi incluído no presente estudo, a investigação da associação de alguns polimorfismos nos genes da IL-6, IL-10, TNF-α e IFN-γ. Os resultados obtidos indicam que a PE grave está associada a maior frequência do genótipo +874TT no gene do IFN-γ. (74) Resultados conflitantes têm sido obtidos (42, 75) e podem ser devido a heterogeneidade no desenho do estudo, diversidade da população estudada e tamanho da amostra.

O presente estudo revelou também que o genótipo +874TT no gene IFN-γ determina o aumento nos níveis plasmáticos dessa citocina pró-inflamatória. Estes dados sugerem que a avaliação do genótipo +847TT possa ser utilizada como ferramenta adicional para avaliação da gravidade da PE. No entanto, outros estudos são necessários para confirmação da relação entre este polimorfismo e a ocorrência da PE. (74)

O avanço no entendimento da inter-relação dos sistemas hemostático e inflamatório, bem como das alterações genéticas associadas à PE alcançados neste estudo, poderá representar mais um passo na compreensão da fisiopatologia dessa doença tão complexa e abrir perspectivas para novos estudos.

5.1 Limitações do estudo

Constituem limitações deste estudo:

1. As dificuldades inerentes ao diagnóstico da PE grave.
2. A obtenção de gestantes com PE grave de quatro maternidades distintas cujo diagnóstico foi, portanto, feito por equipes obstétricas distintas.
3. A ACOG, 2002 (2) considera como critério para classificação da PE como grave, proteinúria acima de 5 g/24h. Na prática clínica, a interrupção da gestação é feita antes que a proteinúria atinja estes valores e foi utilizado como critério de inclusão, proteinúria maior que 2 g/24h.
4. A ausência de investigação laboratorial mais completa das gestantes normotensas, visando excluir qualquer alteração.
5. A ausência de investigação clínica e laboratorial das mulheres não gestantes e a inclusão mediante apenas o auto relato da sua condição clínica.

6 CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

- Houve aumento dos marcadores plasmáticos da coagulação e fibrinólise (D-Di e PAI-1) e das citocinas pró-inflamatórias IL-6, IL-8 e IFN-γ na pré-eclâmpsia grave em relação às gestantes normotensas e mulheres não gestantes
- Não houve correlação forte entre os níveis de D-Di e PAI-1 e as citocinas avaliadas
- Houve associação entre o genótipo +874TT no gene IFN-γ e a ocorrência de pré-eclâmpsia grave e esse genótipo mostrou-se associado ao aumento dessa citocina
- Não houve associação entre os outros polimorfismos estudados e a ocorrência de pré-eclâmpsia grave
- O D-Di constitui um candidato promissor para monitoração da pré-eclâmpsia

7 PERSPECTIVAS DE ESTUDOS

Avaliação dos níveis plasmáticos de D-Di ao longo da gestação e determinação do *cut off* para o diagnóstico/monitoração da pré-eclâmpsia

Avaliação dos marcadores hemostáticos e das citocinas no microambiente uterino

Investigação da associação de outros polimorfismos nos genes das citocinas e a ocorrência de pré-eclâmpsia

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ANEXO A - Parecer do comitê de ética em pesquisa da Universidade Federal de Minas Gerais

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 0618.0.203.000-10

Interessado(a): Profa. Luci Maria Sant'Ana Dusse
Departamento de Análises Clínicas e Toxicológicas
Faculdade de Farmácia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 26 de abril de 2011, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Pré-eclâmpsia: inter-relação dos sistemas hemostático e inflamatório"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2° andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

ANEXO B - Parecer do comitê de ética em pesquisa do Hospital Municipal Odilon Behrens

HOSPITAL MUNICIPAL
ODILON BEHRENS

COMITÊ DE ÉTICA EM PESQUISA

Avaliação de Projeto de Pesquisa

FR: 418196

Número do Parecer: 0681.0.000.210-11

Título do Projeto: "Pré-Eclâmpsia: inter-relação dos sistemas hemostático e inflamatório"

Pesquisador Responsável: Melina de Barros Pinheiro

PARECER DO CEP/ HOB: o projeto em apreço foi avaliado pelo CEP-HOB e aprovado.

Data da reunião: 12 de maio de 2011

Atenciosamente,

Ricardo Melo Marinho
Coordenador do CEP/HOB

ANEXO C - Declaração da Diretoria do Hospital Público Regional de Betim

Prefeitura Municipal de Betim
Secretaria Municipal de Saúde
Hospital Público Regional de Betim - HPRB
Maternidade do HPRB

SUS Sistema Único de Saúde

Declaração

Declaramos, para os devidos fins, que estamos de acordo com o desenvolvimento do projeto intitulado "PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO" da Faculdade de Farmácia da UFMG, na Maternidade do Hospital Público Regional de Betim, desde que seja aprovado por um comitê de ética em pesquisa.

Betim, 09 de novembro de 2010.

Denise da Silva Kattah
Gerente Maternidade

Clésio Gontijo do Amaral
Coordenador SEPPEM
Serviço de Educação Permanente e
Pesquisa Multiprofissional

Geraldo Marcio Viana Zanon
Diretor HPRB

ANEXO D - Declaração da Gerência da Unidade Básica de Saúde da Família (UBSF) Guanabara / Betim - MG

Prefeitura Municipal de Betim
Secretaria Municipal de Saúde
Hospital Público Regional de Betim - HPRB
Maternidade do HPRB

SUS

Declaração

Declaro, para os devidos fins, que estou de acordo com o desenvolvimento do projeto intitulado "PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO" da Faculdade de Farmácia da UFMG, no posto de saúde UBSF Guanabara Betim.

Betim, 31 de agosto de 2010.

Wilson Ribeiro de Meireles
Gerente UBSF Guanabara

ANEXO E - Termo de Consentimento Livre e Esclarecido - grupos I, II e III

UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
DEPTO. ANÁLISES CLÍNICAS E TOXICOLÓGICAS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Para o grupo de mulheres não gestantes)

PROJETO DE PESQUISA: **"PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO"**

Prezada Sra,

Você está sendo convidada para participar de uma pesquisa que tem por objetivo investigar as alterações da coagulação que ocorrem na pré-eclâmpsia e,dessa forma, contribuir para o maior entendimento desta doença. Você será incluída no grupo-controle, ou seja, de mulheres não gestantes.

Para realizar este estudo, gostaríamos de colher 10mL do seu sangue para realização dos exames e armazenamento em um banco de amostras biológicas para estudos genéticos futuros. Esclarecemos que este banco de amostras está aprovado e registrado no Comitê de Ética/UFMG sob o nº ETIC 0216/06.

Na coleta de sangue pode ocorrer uma leve dor localizada e formação de um pequeno hematoma. Para minimizar o risco de formação de hematomas, a coleta de sangue será realizada por um profissional experiente. Serão utilizados agulhas e tubos descartáveis.

Seu nome e os resultados dos exames serão mantidos em segredo.

Esclarecemos que caso não queira participar deste estudo, não haverá nenhum problema.

Para qualquer dúvida sobre esta pesquisa você deverá entrar em contato com as pessoas responsáveis pela mesma, cujos nomes estão abaixo relacionados.

Se você estiver de acordo, por favor, assine esta folha.

Professores responsáveis:
Luci Maria Sant'Ana Dusse – telefone: 3409-6880
Karina Braga Gomes Borges – telefone: 3409-4983
Ana Paula Salles Moura Fernandes – telefone: 3409-6884
Maria das Graças Carvalho – telefone: 3409-6881
Melina de Barros Pinheiro – telefone: 3409-6900
Comitê de Ética em Pesquisa – COEP: Av. Antônio Carlos, nº. 6627 – Pampulha – Campus UFMG, Unidade Administrativa II. CEP: 31270-901. Telefone: 3409-4592.

NOME: _____

Carteira de identidade:_____
Assinatura: _____ DATA: ____/____/____
Agradecemos sua valiosa participação!

UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
DEPTO. ANÁLISES CLÍNICAS E TOXICOLÓGICAS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Para o grupo de gestantes normotensas)

PROJETO DE PESQUISA: "PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO"

Prezada Sra,

Você está sendo convidada para participar de uma pesquisa que tem por objetivo investigar as alterações da coagulação que ocorrem na pré-eclâmpsia e, dessa forma, contribuir para o maior entendimento desta doença. Você será incluída no grupo de gestantes controle, ou seja, que não apresentam a doença.

Para realizar este estudo, gostaríamos de colher 10mL do seu sangue para realização dos exames e armazenamento em um banco de amostras biológicas para estudos genéticos futuros. Esclarecemos que este banco de amostras está aprovado e registrado no Comitê de Ética/UFMG sob o nº ETIC 0216/06.

Na coleta de sangue pode ocorrer uma leve dor localizada e formação de um pequeno hematoma. Para minimizar o risco de formação de hematomas, a coleta de sangue será realizada por um profissional experiente. Serão utilizados agulhas e tubos descartáveis.

Seu nome e os resultados dos exames serão mantidos em segredo.

Esclarecemos que caso não queira participar deste estudo, não haverá nenhum comprometimento ao seu atendimento e tratamento.

Para qualquer dúvida sobre esta pesquisa você deverá entrar em contato com as pessoas responsáveis pela mesma, cujos nomes estão abaixo relacionados.

Se você estiver de acordo, por favor, assine esta folha.

Professores responsáveis:
Luci Maria Sant'Ana Dusse – telefone: 3409-6880
Karina Braga Gomes Borges – telefone: 3409-4983
Ana Paula Salles Moura Fernandes – telefone: 3409-6884
Maria das Graças Carvalho – telefone: 3409-6881
Melina de Barros Pinheiro – telefone: 3409-6900

Comitê de Ética em Pesquisa – COEP: Av. Antônio Carlos, nº. 6627 – Pampulha – Campus UFMG, Unidade Administrativa II. CEP: 31270-901. Telefone: 3409-4592.

NOME:_____

Carteira de identidade:_____

Assinatura: _____ DATA: ____/____/____
Agradecemos sua valiosa participação!

UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
DEPTO. ANÁLISES CLÍNICAS E TOXICOLÓGICAS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(Para o grupo de gestantes com pré-eclâmpsia)

PROJETO DE PESQUISA: "PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO"

Prezada Sra,

Você está sendo convidada para participar de uma pesquisa que tem por objetivo investigar as alterações da coagulação que ocorrem na pré-eclâmpsia e, dessa forma, contribuir para o maior entendimento desta doença.

Para realizar este estudo, gostaríamos de colher 10mL do seu sangue para realização dos exames e armazenamento em um banco de amostras biológicas para estudos genéticos futuros. Esclarecemos que este banco de amostras está aprovado e registrado no Comitê de Ética/UFMG sob o nº ETIC 0216/06.

Na coleta de sangue pode ocorrer uma leve dor localizada e formação de um pequeno hematoma. Para minimizar o risco de formação de hematomas, a coleta de sangue será realizada por um profissional experiente. Serão utilizados agulhas e tubos descartáveis.

Seu nome e os resultados dos exames serão mantidos em segredo.

Esclarecemos que caso não queira participar deste estudo, não haverá nenhum comprometimento ao seu atendimento e tratamento.

Para qualquer dúvida sobre esta pesquisa você deverá entrar em contato com as pessoas responsáveis pela mesma, cujos nomes estão abaixo relacionados.

Se você estiver de acordo, por favor, assine esta folha.

Professores responsáveis:
Luci Maria Sant'Ana Dusse – telefone: 3409-6880
Karina Braga Gomes Borges – telefone: 3409-4983
Ana Paula Salles Moura Fernandes – telefone: 3409-6884
Maria das Graças Carvalho – telefone: 3409-6881
Melina de Barros Pinheiro - telefone: 3409-6900

Comitê de Ética em Pesquisa – COEP: Av. Antônio Carlos, nº. 6627 – Pampulha – Campus UFMG, Unidade Administrativa II. CEP: 31270-901. Telefone: 3409-4592.

NOME: _____

Carteira de identidade:_____

Assinatura: _____ DATA: ____/____/____

Agradecemos sua valiosa participação!

ANEXO F - Fichas clínicas dos grupos I, II e III

FICHA CLÍNICA			
Projeto: **PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO**			
Data:			
Paciente nº:			
Grupo: III - Mulheres não gestantes			
1. Identificação			
Nome:			
Nacionalidade:		Naturalidade:	
Data de nascimento:		Idade:	
Estado civil:			
Endereço:			
Rua/Avenida:			
Número:		Complemento:	
Bairro:			
Cidade:		Estado:	
CEP:			
Telefone: ( )			
Escolaridade:			
2. Anamnese			
Presença de doenças intercorrentes? (distúrbios da coagulação, doenças cardiovasculares, doenças renais, doenças autoimunes, doenças hepáticas, diabetes, câncer, sangramento, história familiar)			
Fumante? ☐ SIM ☐ NÃO			
Consumo de álcool? ☐ SIM ☐ NÃO		Quantidade:	
Pratica exercício físico? ☐ SIM ☐ NÃO			
Freqüência:		Modalidade:	
Uso de medicamentos? ☐ SIM ☐ NÃO			
SE SIM. Quais medicamentos?			
Gestações? ☐ SIM ☐ NÃO			
Se SIM. Quantas?			
Intercorrências durante a gestação? (hipertensão, pré-eclâmpsia, aborto, parto prematuro			
3. Exame físico			
Altura: _____ cm			
Peso: _____ Kg			
IMC:			
Pressão arterial: _____/_____ mmHg			

FICHA CLÍNICA			
Projeto: **PRÉ-ECLÂMPSIA: INTER-RELAÇÃO DOS SISTEMAS HEMOSTÁTICO E INFLAMATÓRIO**			
Data:			
Paciente nº:			
Grupo: ☐ I - Pré-eclâmpsia			
Diagnóstico de pré-eclâmpsia dado em: _____/_____/_____			
Médico responsável:			
☐ II – Normotensas			
1. Identificação			
Nome:			
Prontuário número:			
Nacionalidade:		Naturalidade:	
Data de nascimento:		Idade:	
Estado civil:			
Número de parceiros:			
Endereço:			
Rua/Avenida:			
Número:		Complemento:	
Bairro:			
Cidade:		Estado:	
CEP:			
Telefone: ( )			
Escolaridade:			
2. Anamnese			
Presença de doenças intercorrentes? (distúrbios da coagulação, doenças cardiovasculares, doenças renais, doenças autoimunes, doenças hepáticas, diabetes, câncer, sangramento, pré-eclâmpsia na família, complicações em gravidez anterior)			
Fumante? ☐ SIM ☐ NÃO			
Consumo de álcool? ☐ SIM ☐ NÃO		Quantidade:	
Pratica exercício físico? ☐ SIM ☐ NÃO			
Freqüência:		Modalidade:	
3. Informações sobre a(s) gestação(ões)			
Idade gestacional**: _____** semanas			
Pré-natal? ☐ SIM ☐ NÃO			
Gravidez múltipla? ☐ SIM ☐ NÃO			
GPA (Gravidez Parto Aborto): **_____/_____/_____**			
Partos vaginal (PN) ou cirúrgico (PC)?			
Intervalo interpartal (meses):			
Parto prematuro?			
Filhos vivos:			
Prinicipais queixas:			
☐ Cefaléia ☐ Epigastralgia ☐ Escoltoma ☐ Reflexo patelar			
☐ Outros			

4. Uso de medicamentos		
☐ Nifedipina	☐ Metildopa	☐ Sulfato de magnésio
☐ Outros		
5. Informações clínicas e laboratoriais		
Altura: _____ cm		
Peso: _____ Kg		
Ganho de peso na gravidez:		
Exames laboratoriais: Hm: Hb: Ht: Global: b N E B L M Plaquetas:		TGO: TGP: Bilirrubina total: Bilirrubina direta: Bilirrubina indireta: Ac. Úrico: LDH: Outros:
Acompanhamento:		

Data	Pressão arterial	Proteinúria (24 horas)	Edema