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“Júlio de Mesquita Filho”

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SARAH VIANA MATTIOLI

IDENTIFICAÇÃO DE NOVAS VIAS DE DISFUNÇÃO ENDOTELIAL ASSOCIADAS À PRÉ-ECLÂMPSIA

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Identificação de Novas Vias de Disfunção Endotelial Associadas à Pré-eclâmpsia

SARAH VIANA MATTIOLI

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para a obtenção do título de Mestre no Programa de Pós-Graduação em Biotecnologia.

Orientadora: *Profa. Dra. Valéria Cristina Sandrim*
Coorientador: Prof. Dr. José Eduardo Krieger

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Palavras-chave: Disfunção endotelial; Hipertensão gestacional; Pré-eclâmpsia; Shear stress.

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Epígrafe

"*At present I absolutely want to paint a starry sky. It often seems to me that night is still more richly colored than the day; having hues of the most intense violets, blues and greens. If only you pay attention to it, you will see that certain stars are lemon-yellow, others pink or a green, blue and forget-me-not brilliance. And without my expatiating on this theme, it is obvious that putting little white dots on the blue-black is not enough to paint a starry sky."*

-Vincent Van Gogh

Resumo

A pré-eclâmpsia (PE), uma das principais síndromes hipertensivas da gravidez, está associada a elevados níveis de morbidade e mortalidade materna-fetal, e sua causa permanece desconhecida. A disfunção endotelial (DE) materna, está associada a fatores circulantes libertados pela placenta isquêmica na circulação, mas a sua avaliação depende da recapitulação de forças físicas tais como diferentes padrões de stress de cisalhamento (SS, do inglês shear stress) que afetam a função e disfunção do endotélio. Estudos prévios avaliaram a incubação *in vitro* de células endoteliais com plasma de pacientes com PE, mas sem ter em conta os diferentes padrões de SS. Para preencher esta lacuna da literatura, desenvolvemos um estudo para avaliar os efeitos do plasma de pacientes com PE, hipertensão gestacional (GH) e do plasma de gestantes saudáveis (HP) sobre a função das células endoteliais sob shear stress laminar (LSS) e oscilatório (OSS). Nós avaliamos as alterações nas vias celulares enriquecidas na análise global do perfil de expressão genica destas células expostas às amostras de plasma de PE, GH e HP sob as duas condições SS, em comparação com um grupo controle sem tratamento com plasma (CT). Os nossos dados indicaram que existe uma dependência dos padrões de SS nos efeitos causados pelos três tratamentos de plasma na modulação das vias celulares avaliadas pelo transcriptoma global em células endoteliais coronárias humanas (HCAECs). Sob LSS, todos os tratamentos diferem, e observamos a diferença entre os grupos de PE e GH. Houve também diferenças entre os grupos associados à hipertensão e HP. Sob OSS, o transcriptoma da células tratadas com plasma de PE e GH apresentam um perfil muito semelhante com relação ao tratamento com plasma de HP. Sob LSS, a exposição ao PE plasmático resultou principalmente na ativação de genes associados à resposta inflamatória, stress do retículo endoplasmático e transição endotelial para mesenquimal (EndMT). Sob OSS, as HCAEC expostas aos três tratamentos plasmáticos tinham um perfil de expressão de genes muito semelhante com algumas ocorrências raras que poderiam indicar que os plasmas de PE e GH poderiam ter um nível de DE aumentado, como níveis aumentados de expressão de *IL1B.* As vias associadas à DE também apresentaram valores de p ajustados e odds ratio mais elevados do que os outros dois tratamentos de plasma para EndMT e TNF-α sinalização através de NF- κB, indicando que o plasma de PE poderia exacerbar estes processos mais do que os outros dois tratamentos no OSS. Em geral, os nossos dados salientam a importância das vias dependentes de SS na gênese e manutenção da DE, com efeitos particularmente pronunciados nos HCAECs tratados com plasma de PE.

Palavras-chave: disfunção endotelial; pré-eclampsia; hipertensão gestacional; *shear stress*.

Abstract

Preeclampsia (PE) is one of the main pregnancy-specific hypertensive disorders, it is associated with high maternal morbidity and mortality, but whose causes remain unknown. Endothelial dysfunction (ED), associated with circulating factors released by the ischemic placenta, is a potential pathogenic mechanism, but its assessment depends on the recapitulation of physical forces such as different patterns of shear stress (SS) that affect circulation differently. There is evidence from studies that evaluated the *in vitro* incubation of endothelial cells with plasma from patients with PE, but without taking into account the different patterns of SS. Therefore, we developed a study to evaluate the effects of PE, gestational hypertension (GH) and healthy pregnant (HP) patients' plasma on endothelial cell function under laminar (LSS) and oscillatory (OSS) shear stress, respectively. We deduced the changes in cellular pathways enriched in these responses from the global analysis of the gene expression profile of these cells exposed to the PE and GH plasma samples under the two SS conditions compared to a control group without plasma treatment (CT). Our data indicated that there is a dependence of SS patterns on the effects caused by the three plasma treatments on the modulation of cellular pathways evaluated by the global transcriptome in human coronary endothelial cells (HCAECs). Under LSS, all treatments differ, and we observed the greatest difference between the PE and GH groups. There were also differences between the groups associated with hypertension disorders of pregnancy and HP. Under OSS, PE and GH transcriptomes had a much similar profile, but still kept their differences from HP transcriptome. Under LSS, exposure to plasma PE resulted mainly in the activation of genes associated with inflammatory response, endoplasmic reticulum stress and endothelial to mesenchymal transition (EndMT). Under OSS, HCAECs exposed to all three plasma treatments had a very similar gene expression profile with some rare occurrences that might indicate that PE and GH might have an increased ED level, like increased levels of *IL1B* expression. ED associated pathways had higher adjusted p values and odds ratio than the other two plasma treatments for EndMT and TNF-α signaling via NF- κB, indicating that this plasma treatment might exacerbate these processes more than the other two plasma treatments in OSS. Overall, our data highlights the importance of SS-dependent pathways in the genesis and maintenance of ED, with particularly pronounced effects in PE plasma-treated HCAECs.

Keywords: endothelial dysfunction; preeclampsia; gestational hypertension; shear stress.

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Lista de abreviaturas e siglas

μL	.....	Microlitro
g	.....	Gramas
mm Hg	.....	Milímetros de mercúrio
IMC	.....	Índice de Massa Corpórea
RMM	.....	Razão de Mortalidade Materna
ODS	.....	Objetivos de Desenvolvimento Sustentável
ODM	.....	Objetivos de Desenvolvimento do Milênio
SIGs	.....	Síndromes Hipertensivas da Gestação
DC	.....	Débito Cardíaco
VS	.....	Volume Sistólico
FC	.....	Frequência Cardíaca
PA	.....	Pressão Arterial
ACOG	.....	*American College of Obstetricians and Gynecologists*
HP	.....	*Healthy Pregnant*
GH	.....	*Gestational Hypertension*
PE	.....	*Preeclampsia*/Pré-eclâmspsia
HELLP	.....	*Hemolysis, elevated liver enzymes, low platelet count*
MEC	.....	Matriz extracelular
TNF-α	.....	*Tumor Necrosis Factor-α*
ROS	.....	*Reactive Oxygen Species*
AT1-AAs	.....	*Autoanticorpos agonistas contra o receptor-1 de angiotensina*
sENG	.....	*Soluble endoglin*
sFLT1	.....	*Soluble fms-like tyrosine kinase 1*
VEGF	.....	*Vascular endothelial growth factor*
VEGFR-2	.....	*Vascular endothelial growth factor receptor*
PlGF	.....	*Placental growth fator*
TGF-β1	.....	*Transforming growth factor-β*
eNOS/NOS3	.....	*Endothelial nitric oxide synthase*
NO	.....	*Nitric oxide*
oxLDL	.....	*Lipídios oxidados*
HMOX1	.....	*Heme oxygenase*
MCP-1	.....	*Monocyte Chemoattractant Protein-1*
ICAM1	.....	*Intracelular Adhesion molecule*
VCAM1	.....	*Vascular Cell-adhesion Molecule 1*
SELE	.....	*E-selectin*
NF-kB	.....	*Nuclear factor Kappa B*

KLF2/KLF4	.....	*Kruppel Like Factor2/4*
SS	.....	*Shear Stress*
LSS	.....	*Laminar Shear Stress*
OSS	.....	*Oscillatory Shear Stress*
PECAM1	.....	*Platelet endothelial cell adhesion molecule-1*
AT1R	.....	*Angiotensin II type 1 receptor*
HIF-α	.....	*Hypoxia-inducible factor 1-alpha*
ERK1/2/5	.....	*Extracellularly Regulated Kinases 1/2/5*
Akt	.....	*Protein kinase B*
AMPK	.....	*AMP-activated protein kinase*
IRAK2	.....	*Interleukin-1 receptor-associated kinase-like 2*
CHOP	.....	*DNA Damage Inducible Transcript 3*
EndMT	.....	*Endothelial to mesenchymal transition*
TIMP3	.....	*TIMP Metallopeptidase Inhibitor 3*
TGF-β	.....	*Transforming growth factor beta*
$CoCl_2$	.....	Cloreto de cobalto
OxPAPC	.....	*Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine*
RLE	.....	*Relative Log Expression*
NUSE	.....	*Normalized Unscaled Standard Errors*
RMA	.....	*Robust Multi-Array Average*
PCA	.....	*Principal Component Analysis*
DEGs	.....	*Differentially Expressed Genes*
FDR	.....	*False Discovery Rate*
GSEA	.....	*Gene Set Enrichment Analysis*
ORA	.....	*Over Representation Analysis*
PPI	.....	*Protein-Protein Interaction*
CO_2	.....	Dióxido de carbono
HCAECs	.....	*Human Coronary Arteryc Endothelial Cells*
HUVECs	.....	*Human Umbilical Vein Endothelial Cells*
IL-1β	.....	*Interleukin 1 beta*

Introdução e justificativa

Introdução

Índices de Mortalidade Materna no Mundo e no Brasil

A razão de mortalidade materna RMM é determinada pelo número de mortes maternas para cada 100.000 nascidos-vivos durante um determinado período. Sendo consideradas mortes maternas aquelas que têm causa relacionada ou agravada pela gestação ou seu tratamento, salva a exceção de causas acidentais, durante estado gravídico (gravidez, parto ou puerpério). Um nascido-vivo conta como a separação completa do recém-nascido da mãe acompanhado de respiração ou qualquer outra evidência de vida, como batimentos cardíacos ou movimento definitivo dos músculos voluntários. O cálculo de RMM é obtido dividindo o número de mortes maternas registradas (ou estimadas) pelo total de nascidos-vivos registrados (ou estimados) no mesmo período de tempo, multiplicando por 100.000 ("Maternal mortality ratio (per 100 000 live births")

A morte materna pode acontecer por causas diretas, quando as complicações se desenvolvem durante a gravidez, ou indiretas, com o agravamento de condições pré-existentes. Atualmente, as principais causas de mortalidade materna (~75%) no mundo incluem, em ordem de incidência: (1) quadro de hemorragia severa; (2) sindormes hipertensivas da gestação (SIGs); (3) infecção e/ou sepse; (4) complicações durante o parto; (5) aborto ilegal (SAY et al., 2014).

Somente no ano de 2017, mais de 295 000 mulheres perderam suas vidas durante ou após a gestação. A RMM mundial é inadmissivelmente elevada e ocorre, em grande maioria (94%), em áreas de poucos recursos econômicos, sendo que a maioria dessas mortes são evitáveis. Com isso em mente, desde 2015, foi estabelecido, dentro da lista de 17 Objetivos de Desenvolvimento Sustentável (ODS), a meta de redução da RMM global para menos de 70 mortes para cada 100.000 nascidos vivos até 2030. Essa, na verdade, é uma extensão do prazo dos Objetivos de Desenvolvimento do Milênio (ODM), que teve seu início no ano 2000 e tinha prazo final para o ano de 2015 (UNFPA et al., 2019).

No Brasil, segundo o boletim epidemiológico nº 20 do Ministério da Saúde, houve redução da RMM desde 1990 até 2018 (**Figura 1**). No entanto, em 2018 foram registradas 59.1 mortes para cada 100.000 nascidos-vivos. Esse número ainda é considerado acima para meta proposta nos ODM e, para ficar dentro da meta da ODS, o país precisa reduzir a mortalidade em 46.6% até 2030 tomando uma série de medidas (MOTTA; MOREIRA, 2021). Investigando mais a fundo, quando tratamos das causas de mortes obstétricas diretas no Brasil, as SIGs são responsáveis pelo maior número de óbitos no intervalo do estudo, sendo seguida por hemorragia, infecção puerperal e aborto ilegal. Ademais, quando investigadas as causas clínicas indiretas, as doenças do aparelho circulatório lideram, mais uma vez, como causa de morte mais frequente.

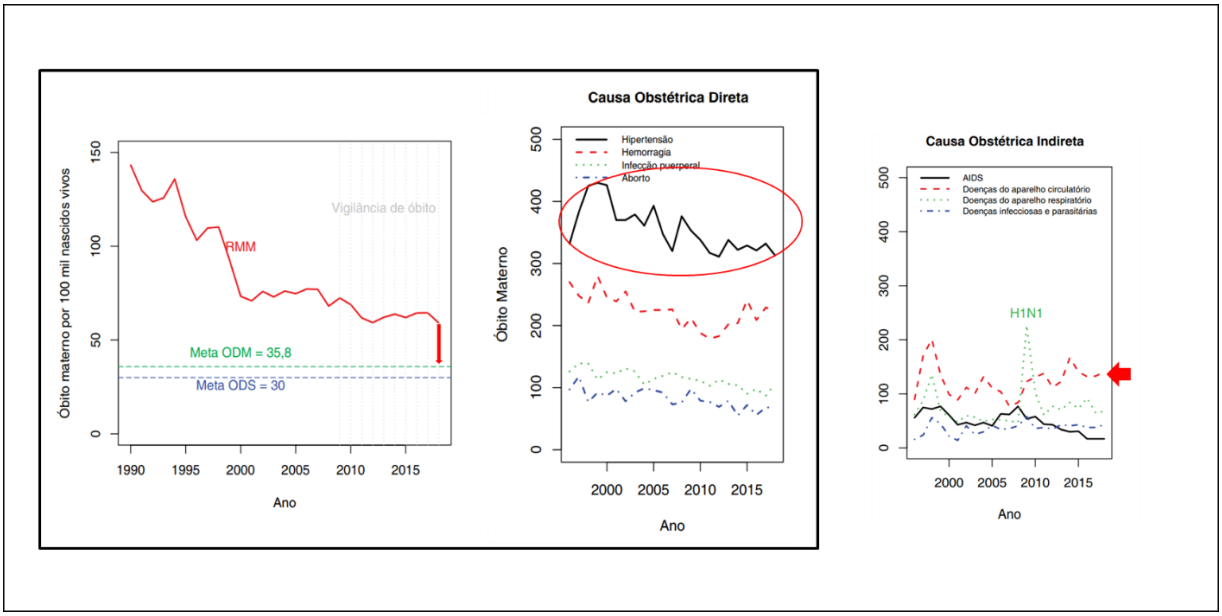

Figura 1 - Fonte: Boletim Epidemiológico N° 20. Volume 51. Ministério da Saúde. Secretaria de Vigilância em Saúde. Boletim Epidemiológico N° 20. Volume 51. maio/2020.

Alterações Fisiológicas do Sistema Cardiovascular Durante a Gestação

Desde o início da gestação até o puerpério, o corpo materno passa por diversas adaptações anatômicas, fisiológicas e metabólicas regidas, em grande parte, por alterações hormonais. Essas adaptações são profundas e contemplam praticamente todos os sistemas do corpo materno de forma a garantir a manutenção da gestação, sendo a maioria delas completamente revertida nas semanas que seguem o fim da gestação. Tendo isso em mente, a compreensão de tais mudanças é fundamental para diferenciar as alterações fisiológicas de processos patológicos, sendo importante para guiar o manejo clínico das mulheres, bem como para direcionar a pesquisa de base nessa área.

O sistema cardiovascular é um dos mais extensamente afetados por essas alterações fisiológicas devido ao aumento da demanda de oxigênio para suprir as necessidades da mãe e do feto. Tais mudanças têm início logo após a implantação da célula-ovo no endométrio, com as adaptações na vasculatura que decorrem do processo de placentação, algumas seguem até mesmo após terminada gestação (HUPPERTZ; PEETERS, 2005; KOLOVETSIOU-KREINER et al., 2018). A incapacidade do organismo materno de se adequar às alterações decorrentes dessas novas demandas pode trazer à tona doenças cardiovasculares, como as SIGs, tendo como consequência o aumento da morbimortalidade materno-fetal.

Dentre essas adaptações podemos citar:

a) O **aumento da volemia**, com aproximadamente ~ 45% a mais do volume sanguíneo pré-gestacional. Neste caso, o volume de plasma é proporcionalmente maior em relação aos eritrócitos, o que resulta na chamada "anemia fisiológica" por hemodiluição (SANGHAVI; RUTHERFORD, 2014; VRICELLA, 2017);
b) O **aumento do débito cardíaco** (DC), o qual é definido como o produto do **volume sistólico** (VS.) e a **frequência cardíaca** (FC) (DC = FC x VS.) e apresenta aumento de 30% a 50% em relação aos níveis pré-gravídicos. No início da gestação o aumento do débito cardíaco é associado ao aumento do volume sistólico, enquanto mais ao final da gestação, o aumento é atribuído ao aumento da frequência cardíaca (ROVINSKY; JAFFIN, 1966; SANGHAVI; RUTHERFORD, 2014);

c) Também ocorre em gestantes a **hipertrofia cardíaca excêntrica**, caracterizada por uma parede cardíaca delgada e aumento do diâmetro do ventrículo esquerdo. Esse tipo de hipertrofia é diferente de uma hipertrofia patológica e, se precisássemos compará-la, podemos dizer que se assemelha à hipertrofia cardíaca adaptativa em resposta ao exercício físico. Ela está associada à necessidade de acomodar o aumento fisiológico do volume sanguíneo e permite que o coração da gestante funcione de forma mais eficiente frente às novas demandas fisiológicas (CHUNG; LEINWAND, 2014);

d) As alterações previamente citadas ocorrem sem que haja aumento da pressão arterial porque, a partir do primeiro trimestre de gestações saudáveis, ocorre a **diminuição substancial da resistência vascular periférica** (com decréscimo de ~35% a 40% com relação a níveis não gravídicos) promovida pelo aumento da vasodilatação e redução da resposta aos vasoconstritores (CHESLEY et al., 1965; SANGHAVI; RUTHERFORD, 2014);

e) Uma parcela da diminuição da resistência vascular periférica está associada ao aumento do **remodelamento da vasculatura uterina e à vasculogênese placentária**. De forma geral, a vasculatura em adultos encontra-se quiescente, as exceções não patológicas são a gestação e o ciclo menstrual (OSOL; MANDALA, 2009; RIZOV; ANDREEVA; DIMOVA, 2017).

Síndromes Hipertensivas da Gestação (SIGs)

Em algumas mulheres, a gravidez não é acompanhada por estes mecanismos adaptativos do sistema cardiovascular, o que pode levar ao surgimento das SIGs (KALAFAT; THILAGANATHAN, 2017). A *American College of Obstetricians and Gynecologists* (ACOG) identifica quatro categorias de SIGs, são elas: a) Hipertensão gestacional (GH, do inglês *Gestational Hypertension*); b) a Pré-eclâmpsia (PE); c) Hipertensão crônica; e d) a PE sobreposta à hipertensão crônica (YING; CATOV; OUYANG, 2018).

Ainda segundo a ACOG, a hipertensão crônica é caracterizada pela elevação da PA (>140/90 mm Hg) antes da 20ª semana de gestação ou persistência do aumento da PA além de 12 semanas pós-parto. Já a GH é definida como elevação da PA (>140/90 mm Hg) a partir da 20ª semana gestacional sem a presença de outras disfunções de outros sistemas do organismo da gestante. Por sua vez, a PE é definida pelo aumento da PA (>140/90 mm Hg), também a partir da 20ª semana, associada à proteinúria e/ou dano de órgãos finais (fígado, rins, cérebro). PE sobreposta à hipertensão crônica é caracterizada pelo aumento da PA (>140/90 mm Hg), previamente observado, que a partir da 20ª semana gestacional passa a ser acompanhado de proteinúria e/ou dano de órgãos vitais. Além dessas, existem duas complicações graves da PE, são elas: a) a síndrome HELLP (do inglês *hemolysis, elevated liver enzymes, low platelet count*) na qual a gestante desenvolve hemólise, níveis elevados de enzimas hepáticas e baixa contagem de plaquetas; e b) a eclâmpsia quando a gestante passa a ter convulsões ou entra em coma (OPICHKA et al., 2021; YING; CATOV; OUYANG, 2018). A **Figura 2**, elaborada por Opichka *et al*., representa um esquema que indica a cronologia de desenvolvimento das SIGs durante a gestação junto ao grau de gravidade da doença e sua incidência

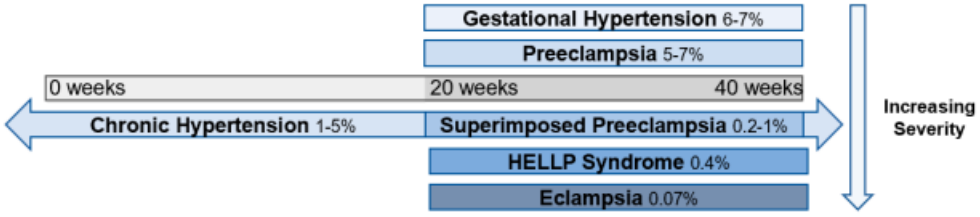

Figura 2 – Esquema de Opichka et al. indicando as SIGs de acordo com sua cronologia, grau de gravidade e incidência. A GH é definida pelo aumento da PA (>140/90 mmHg) após a 20ª semanas de gestação, enquanto que a PE, além do aumento da PA, é acompanhada de proteinúria e disfunção dos órgãos vitais A hipertensão crônica consiste no aumento da PA antes da 20ª semana de gestação ou quando o aumento continua 12 semanas após o parto e pode ocorrer em sobreposição com a PE. A síndrome HELLP, caracterizada pela hemólise, níveis elevados de enzimas hepáticas e baixa contagem de plaquetas, e a eclâmpsia, caracterizada por convulsões e coma, classificadas como complicações da PE.

Pré-Eclâmpsia

O conhecimento da PE data de ~400 a.C quando Hipócrates, apesar do conhecimento e tecnologia limitados, indicou que a dores de cabeça acompanhadas de convulsões durante a gravidez eram consideradas como uma complicação/"algo ruim". No entanto, o termo (pré)-eclâmpsia surgiu apenas em 1619 no Tratado de Ginecologia de Varandaeus, e desde então tem sido associado a diversas etiologias, e, apesar de termos evoluído muito nesse aspecto, atualmente ainda não se sabe a causa específica e nem a "cura" da PE (BELL, 2010). As únicas soluções atuais são o tratamento com fármacos anti-hipertensivos seguros para ao período gestacional, combinados ao acompanhamento médico, ou, em casos graves, a indução do parto (AMARAL et al., 2017).

Fatores de risco e fisiopatologia

Atualmente, há consenso na literatura de que a etiologia da PE seja multifatorial e os principais fatores de risco incluem histórico de PE, hipertensão crônica, diabetes mellitus pré-gestacional, gravidez gemelar, síndrome anti-fosfolípide, e obesidade, além de outros fatores como lúpus eritematoso, idade materna avançada, nuliparidade, histórico de doença renal crônica, e utilização de tecnologias de reprodução assistida. Por fim, alguns outros fatores de riscos mais raros incluem o histórico familiar de PE e fetos com trissomia do cromossomo 13 (RANA et al., 2019). Independentemente de um mecanismo específico, desenvolveu-se o modelo de dois estágios da doença, apontando-se a isquemia placentária como fator incipiente para seu desenvolvimento (**Figura 3**) (BELL, 2010; HLADUNEWICH; KARUMANCHI; LAFAYETTE, 2007; RANA et al., 2019; ROBERTS; HUBEL, 2009).

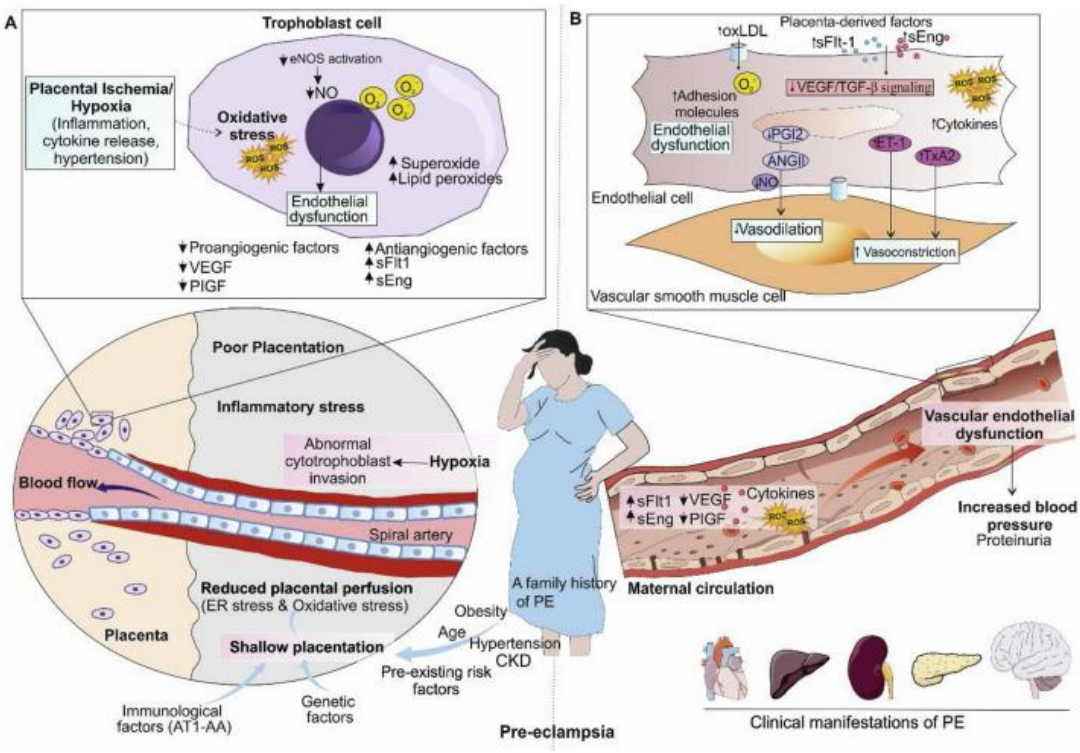

Figura 3 – Fisiopatologia da pré-eclâmpsia, por Ahmadian et al: A) causas multifatoriais, como fatores genéticos, ambientais e imunológicos levam ao desenvolvimento da PE, que tem como primeiro estágio da doença, a remodelação deficiente da arteríolas espiraladas uterinas, resultando em isquemia/hipóxia placentária e inflamação. (B) Com isso, a placenta passa a liberar fatores anti-angiogênicos (sFlt-1, sEng) que reduzem os níveis de fatores pró-angiogênicos circulantes (VEGF, PlGF), alem de fatores pró-inflamatórios e ROS, culminando na disfunção endotelial. Essa disfunção endotelial é caracterizada principalmente pela redução da produção e biodisponibilidade de NO, bem como aumento da expressão de moléculas de adesão de células inflamatórias, o aumento da atividade do tromboxano A2 (TxA2) e da endotelina (ET-1) resultando em maior constrição vascular. As consequências dessa disfunção endotelial sistêmica levam às manifestações clínicas da PE que envolvem orgãos como cérebro, rins, fígado e, a longo prazo, o coração. ET-1: endotelina 1, TxA2: tromboxano A2; oxLDL: lipoproteína de baixa densidade oxidada; ROS: espécies reativas de oxigênio; LDL: lipoproteína de baixa densidade; sFlt-1: tirosina quinase-1 solúvel; sEng: endoglina solúvel; VEGF: fator de crescimento endotelial vascular; PlGF: fator de crescimento placentário; NO: óxido nítrico; TGF-β: fator de transformação do crescimento beta (AHMADIAN et al., 2020).

A. Primeiro estágio isquemia/hipóxia placentária:

No início do primeiro trimestre da gestação, entre a 18ª e a 20ª semanas, instala-se o processo referido como pseudovasculogênese. Esse processo é caracterizado pela migração dos citotrofoblastos do blastocisto para a decídua e miométrio em direção as arteríolas uterinas espiraladas, seguida de diferenciação fenotípica do perfil fetal epitelial para endotelial (JI et al., 2013; MCMASTER; ZHOU; FISHER, 2004; POLLHEIMER et al., 2018). Essa migração e diferenciação dos citotrofoblastos ocorre em resposta a diversos mecanismos como, alterações no perfil de expressão de citocinas, moléculas de adesão, constituintes da matriz extracelular (MEC), metaloproteinases e o antígeno de histocompatibilidade e tem como produto final o remodelamento das arteríolas, resultando no aumento de seu calibre (**Figura 4 A**) (DAMSKY et al., 1994; DAMSKY; FITZGERALD; FISHER, 1992; FISHER; DAMSKY, 1993; WALLACE et al., 2014). Esse processo é essencial para uma gestação saudável e mulheres com PE apresentam alterações, de forma que apenas ~30-50% das arteríolas espiraladas sofrem remodelamento, o que culmina na redução da transferência de oxigênio entre a circulação materna e fetal e isquemia/hipóxia placentária (KNÖFLER; POLLHEIMER, 2013; MCMASTER; ZHOU; FISHER, 2004). Os mecanismos já identificados e propostos para essa placentação inadequada, característica da PE, têm sido aplamente estudados nos ultimos anos e foram revisados rescentemente por Opichka et al. (**Figura 4 B**). No entanto a causa dessa disfunção permanece desconhecida.

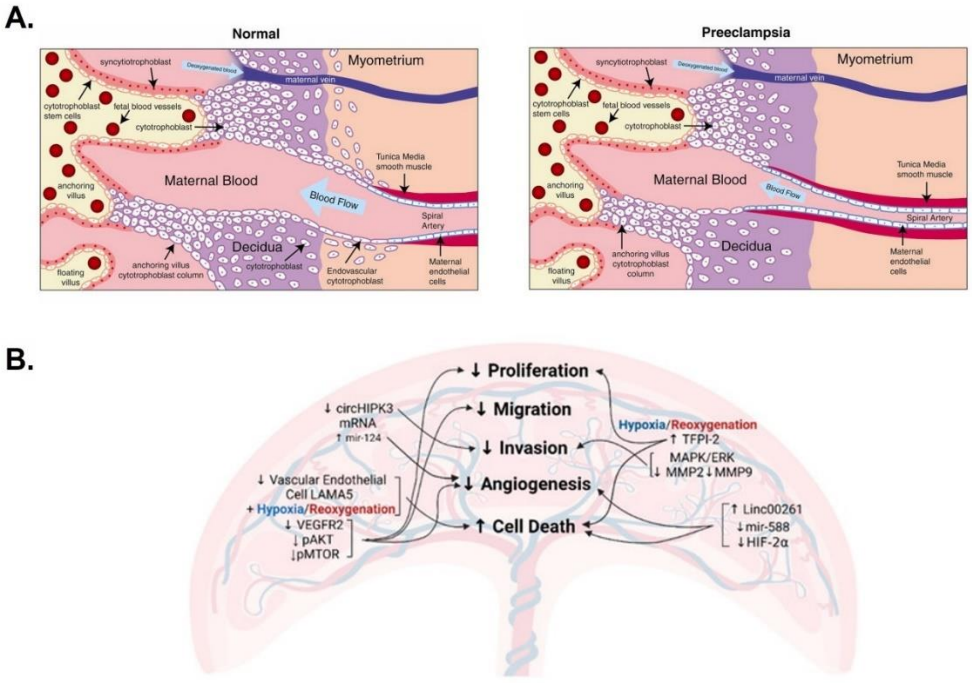

Figura 4 – Esquemas adaptados de Lam et al. e Opichka et al.. A) Processo de placentação normal vs. processo de placentação disfuncional da PE: Durante o processo de invasão vascular da decídua e miométrio, os citotrofoblastos diferenciam-se de um fenótipo epitelial para um fenótipo endotelial, um processo referido como pseudovasculogênese (imagem à esquerda). Na PE, os citotrofoblastos não conseguem diferenciar-se para o fenótipo endotelial invasivo. Com isso, a invasão das arteríolas espiraladas uterinas é superficial, e os vasos mantém calibre reduzido (imagem à direita). B) Mecanismos associados à placentação disfuncional da PE: indica alguns mecanismos moleculares associados à redução de processos como a proliferação, migração, invasão e angiogênese além do auamento da morte celular relacionados à disfunção da placentação em PE. Siglas do inglês HIPK3: circular RNA homeodomain interacting protein kinase 3; LAMA5: laminin subunit alpha-5; VEGFR2: vascular endothelial growth factor receptor 2; pAKT, phosphorilated protein kinase B; pMTOR, phosphorylated mammalian target of rapamycin; TFPI-2: tissue factor pathway inhibitor-2; MAPK: mitogen-activated protein kinase; ERK: extracellular-signal-regulated kinase; MMP: matrix metalloproteinase; HIF-2α: hypoxia-induced factor-2α. ↑ refere-se ao aumento e ↓ à redução.

B. Segundo estágio ou síndrome materna:

Em 1989, Roberts e Taylor et al. propuseram pela primeira vez que a PE é causada pela liberação de fatores desconhecidos pela placenta isquêmica/hipóxica na circulação materna. Esses fatores circulantes somados aos efeitos de fatores de risco constitutivos maternos, como fatores genéticos, comportamentais e ambientais contribuem para o desenvolvimento do segundo estágio da doença, conhecido como síndrome materna, e marcado por disfunção endotelial sistêmica (ROBERTS et al., 1989). Desde então, diversos fatores circulantes já foram identificados e continuam sendo descobertos. Alguns deles são, o fator de necrose tumoral α (TNF-α, do inglês *tumor necrosis factor-α*), as interleucinas IL-6, IL-1α e IL-1β, o ligante Fas, espécies reativas de oxigênio (ROS, do inglês *Reactive Oxygen Species*), lipídios oxidados e autoanticorpos agonistas contra o receptor-1 de angiotensina (AT1-AAs) (CONRAD; MILES; BENYO, 1998; LAM; LIM; KARUMANCHI, 2005; WALLUKAT et al., 1999). No entanto, não existem evidências suficientes de que estas moléculas estejam diretamente associadas à causa da síndrome materna. A descoberta mais recente e robusta foi a dos fatores anti-angiogênicos em sua forma solúvel como a sENG (do inglês s*oluble endoglin*) e o sFLT1 (do inglês *soluble fms-like tyrosine kinase 1*), pelo grupo do Dr. Karumanchi em 2003 (MAYNARD et al., 2003; MUTTER; KARUMANCHI, 2008). O sFLT1 e a sENG são responsáveis pelo sequestro de moléculas pró-angiogênicas como VEGF (do inglês *vascular endothelial growth factor*), PlGF (do inglês *placental growth fator*) e TGF-β1 (do inglês *transforming growth factor-β*) na circulação (**Figura 5**). Além de estimular a angiogênese, o VEGF, quando ligado a seu receptor VEGFR-2 do endotélio, também é capaz de ativar a óxido nítrico sintase endotelial (eNOS, do inglês *endothelial nitric oxide synthase*) pela via de sinalização da fosfatidilinositol-3-quinase (PI3K)/proteína quinase b (AKT) o que resulta na produção de oxido nítrico (NO, do inglês *nitric oxide*), um potente vasodilatador endógeno e marcador de função endotelial (GRUMMER et al., 2009; PANDEY et al., 2018). Por sua vez, o PlGF atua ligando-se aos VEGFRs e é capaz de aumentar sua afinidade pelo VEGF além de potencializar sua cascata de sinalização através da trans fosforilação do receptor (CHAU; HENNESSY; MAKRIS, 2017). O TGF-β liga-se a ENG endotelial regulando processos de diferenciação, migração, e sobrevivência do endotélio (LEBRIN et al., 2005; MA et al., 2020).

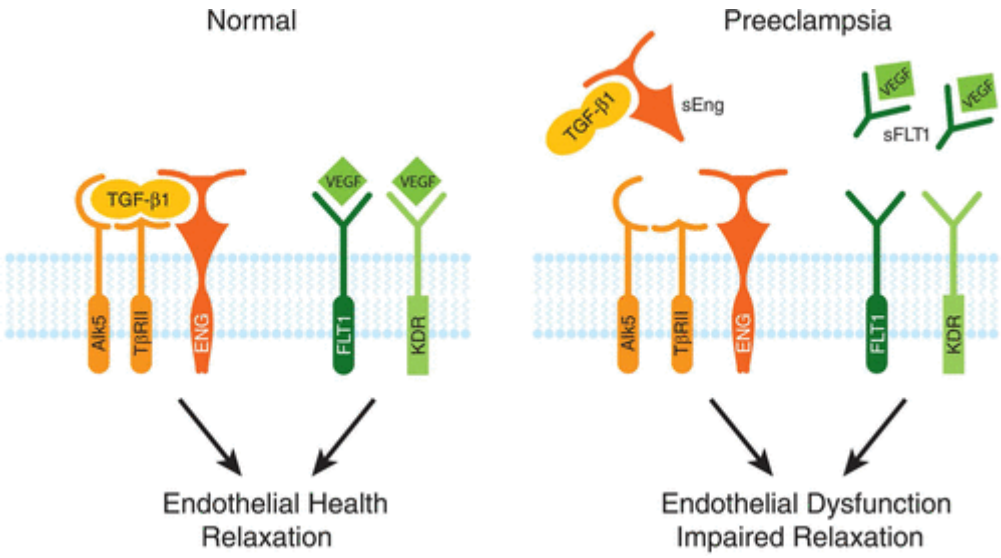

Figura 5 – Esquema de Powe et al., 2011. Disfunção endotelial causada por sFLT1 e sENG: durante a gestação os níveis normais de sinalização VEGF e TGF- β1 são fundamentais para a manutenção da homeostase vascular. A secreção placentária de sFLT1 e sENG (proteínas antiangiogênicas endógenas circulantes) suprime a sinalização de VEGF e TGF- β1 na vasculatura de mulheres com PE. Como resultado, tem-se o desenvolvimento da disfunção endotelial, com redução dos níveis de óxido nítrico.(RANA et al., 2019)

Além da hipertensão, a proteinúria, os problemas hepáticos, e as complicações, tais como edema cerebral também são indicativos do papel central e multi-sistêmico da disfunção do endotélio nas formas graves da doença (LAM; LIM; KARUMANCHI, 2005; LAMARCA, 2012; CIPOLLA, 2007; EUSER; CIPOLLA, 2007). Todos esses efeitos deixam de existir após o parto, no entanto, o histórico de PE tem sido associado ao risco elevado para aterosclerose e outras doenças cardiovasculares ao longo da vida dessas mulheres, sendo referido como um "primeiro evento de doença vascular" e que, portanto, merece ser considerado para acompanhamento e prevenção (DINIZ; PAES; DINIZ, 2020; MUIJSERS et al., 2019).

Similaridades e diferenças com relação à fisiopatologia da hipertensão gestacional

Até hoje não é claro se GH tem uma etiologia distinta da PE ou se ambas as condições são parte de um fenômeno comum (YING; CATOV; OUYANG, 2018). GH e PE apresentam fatores de risco semelhantes e compartilham uma probabilidade próxima (de 39% e 32%, respectivamente) de desenvolvimento de hipertensão após aproximadamente dois anos e meio do fim da gestação (comparado a 1% em HP) (VEERBEEK et al., 2015). Entretanto, o risco de resultados adversos, como parto prematuro, é aproximadamente 3 vezes maior em PE em comparação à GH (SHEN et al., 2017), e pode estar associado a diferenças com relação ao perfil de assinatura inflamatória de cada condição, com GH tendo um perfil compensatório mais efetivo em relação à PE (TANGERÅS et al., 2015). Tendo em vista as similaridades citadas, é possível que, apesar de apresentarem resposta patológica distinta, a origem da hipertensão na gestação seja comum entre PE e GH. Reforçando essa ideia, um estudo indicou que fatores risco de pré-gestacionais são responsáveis por mais da metade dos casos de SIGs, além de estarem associados aos níveis de pressão arterial, Índice de Massa Corpórea (IMC), e lipídios plasmáticos (ROMUNDSTAD et al., 2010).

A (Dis)Função do Endotélio: além do óxido nítrico

O endotélio consiste em uma monocamada de células endoteliais que revestem o lúmen de todos os leitos vasculares desde artérias e veias, até capilares e vasos linfáticos. Por estar localizado entre o sangue e diversos tecidos e órgãos, o endotélio apresenta papel fundamental e clássico, integrando e coordenando a relação entre eles através do controle da permeabilidade, da hemostasia e regulação do tônus vascular (LÜSCHER; BARTON, 1997). Além disso, estudos recentes também têm associado ao endotélio um papel muito mais amplo (**Figura 6**), incluindo a seu *hall* de atividades, processos como a regulação do metabolismo, autorregulação da angiogênese e reparo de lesões e mecanotransdução, associada às forças hemodinâmicas levando à regulação da transcrição gênica (HASAN; FISCHER, 2021c; XU et al., 2021).

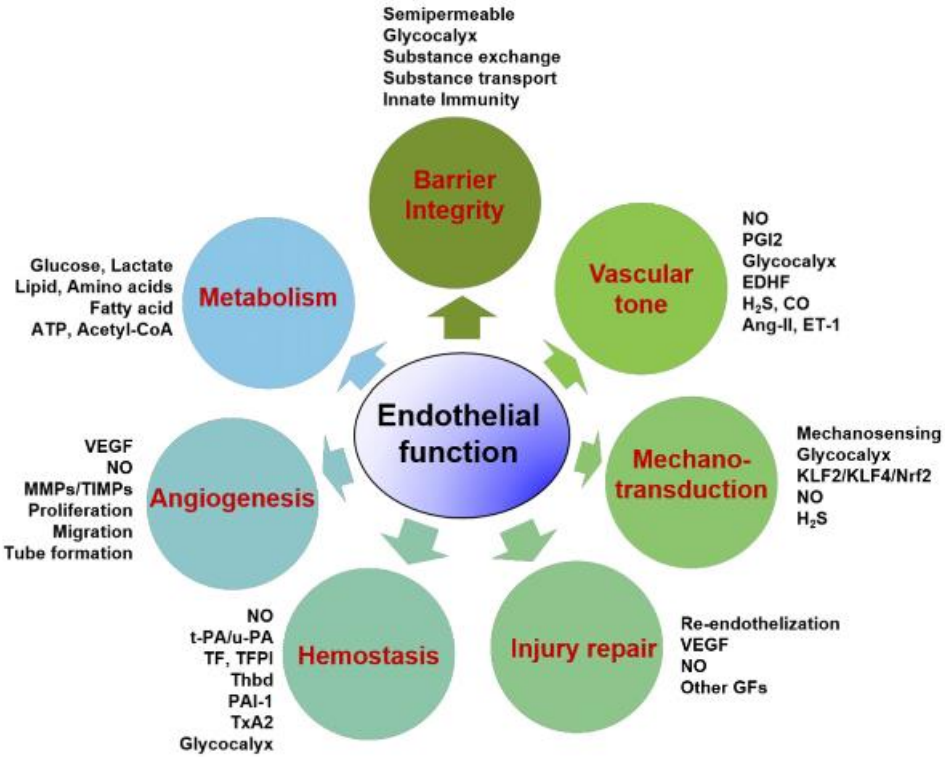

Figura 6 – As funções do endotélio saudável, por Xu et al., 2021: **1) Barreira semipermeável:** papel na regulação da troca e transporte de substâncias e células do sistema imune; **2) regulação do tônus vascular:** através do equilíbrio da produção de vasodilatadores (NO, PGI2, H2S e EDHF) e vasoconstritores (ET-1 e Ang-II); **3) mecanotransdução:** através de mecanosensores em resposta às forças hemodinâmicas levando a alterações do perfíl de expressão gênica pela ação de fatores de transcrição (KLF2/KLF4/Nrf2) que por sua vez, promovem aumento da produção de NO e H2S; **4) reparo de lesão vascular:** através da reendotelização e mediante o auxílio do VEGF, NO, e outros fatores de crescimento; **5) regulação da hemostasia**: através da secreção de moléculas antiplaquetárias e anticoagulantes tais como NO, o ativador do plasminogênio tecidual (t-PA), ativador do plasminogênio do tipo u-PA, o TFPI, e a Thbd e PAI-1; **6) regulação da angiogênese:** através de moléculas como o VEGF, NO, MMP/TIMPs, que desencadeiam a proliferação e a migração endotelial levando à formação de tubos; **7) Metabolismo:** atuação do endotélio no metabolismo de glicose, lípidos, aminoácidos, ácidos graxos, ATP, lactato, e acetil-CoA auxiliando na manutenção do fornecimento de oxigênio e nutrientes para todos os tecidos do organismo. PGI2: prostaciclina; H2S : sulfeto de hidrogênio; EDHF: fator hiperpolarizante derivado do endotélio; ET-1: endotelina-1; Ang-II: angiotensina II; KLF2/4: Kruppel Like Factor 2/4; Nrf2: fator nuclear derivado de eritróide 2; t/u-PA: ativador de plasminogênio tecidual/do tipo uriquinase; TFPI : inibidor da via do factor tecidual; Thbd trombomodulina; PAI-1: inibidor-1 do ativador do plasminogênio; MMP: metaloproteinases da matriz; TIMPs: inibidores da MMPs.

O termo “disfunção endotelial” foi proposto por Gimbrone em meados da década de 80 e definido como a redução da capacidade de vasodilatação observada em diversas patologias que envolviam o sistema cardiovascular (POREDOŠ, 2002). Sua definição pode ser atribuída, de forma reducionista, à redução da vasodilatação, principalmente associada à redução da produção e biodisponibilidade de NO, ou a uma definição mais ampla e atual que engloba

qualquer alteração que comprometa a homeostasia do endotélio (XU et al., 2021). Essa disfunção está associada a uma vasta gama de insultos que podem ter tanto caráter físico quanto bioquímico, além de fenótipos múltiplos (**Figura 7**) Alguns exemplos são a alteração de diferentes mediadores da vasodilatação e vasoconstrição além do NO, a perda do glicocálix, aumento da permeabilidade, rigidez endotelial (vascular), inflamação, resposta endotelial pró-trombótica e comprometimento da fibrinólise (DAIBER; CHLOPICKI, 2020). É importante ressaltar que cada uma dessas alterações não ocorre individualmente e que, pelo contrário, o seu conjunto é o que leva a quadros patológicos e às doenças cardiovasculares.

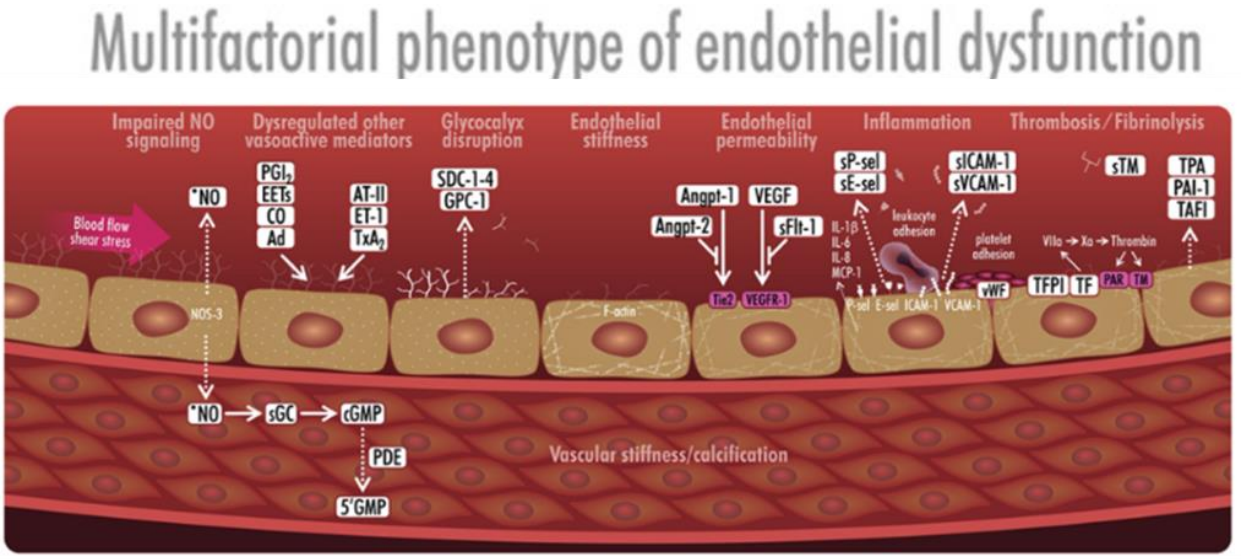

Figura 7 – O fenótipo multifatorial da disfunção endotelial, por Daiber e Chlopicki., 2020: Os processos que contribuem para a disfunção endotelial não se limitam à função do NO e envolvem a desregulação de outros fatores vasoativos, perda do glicocálix, aumento da rigidez endotelial, aumento da permeabilidade, inflamação, além de alterações dos mecanismos trombóticos.

Espécies reativas de oxigênio e macromoléculas oxidadas

ROS (superóxido $O_2^{\bullet-}$, peroxido de hidrogênio H_2O_2, ânions e radicais hidroxil $OH^{-/\bullet}$) são moléculas intermediárias do oxigênio que atuam como segundos mensageiros importantes das células. No entanto, desequilíbrio entre agentes antioxidantes, como a superóxido dismutase, a catalase e a glutationa peroxidase, e ROS, ou radicais livres, pode levar ao dano celular pelo estado de estresse oxidativo (EGEA et al., 2017). Esse dano ocorre devido à oxidação de macromoléculas fundamentais como as proteínas e lipídios, o que pode resultar em dano da membrana celular, além de ácidos nucleicos, causando alterações na estrutura de DNA (XU et al., 2021). A maior parte das doenças cardiovasculares está associada não só ao aumento de ROS, mas também à formação de espécies reativas de nitrogênio (RNS, do inglês *reactive nitrogen species*) que inclui produtos derivados do NO e suas principais fontes são as NADPH oxidases, a mitocôndria e a enzima eNOS desacoplada (GRYGLEWSKI; PALMER; MONCADA, 1986; SCHULZ et al., 2014). O dano causado pelas macromoléculas oxidadas está relacionado ao desenvolvimento de um perfil inflamatório, via expressão de moléculas de adesão da membrana (MCP-1, ICAM1, VCAM, SELE) alterações do citoesqueleto, através da oxidação de proteínas reguladoras da actina ou modificação de redes de sinalização envolvidas com a polimerização do citoesqueleto de actina, e redução da vasodilatação pelo sequestro de NO e consequente formação de peroxinitritro (MITTAL et al., 2014; NAVAB et al., 1991; SPRINGSTEAD et al., 2012). Além disso, o LDL oxidado (oxLDL), acumula-se no espaço subendotelial do endotélio disfuncional, onde é fagocitado por macrófagos que passam à células espumosas, processo fundamental para o desenvolvimento de lesões ateroscleróticas

(JOVINGE et al., 1996). Ademais, indo de acordo com seu papel na aterosclerose, um grupo demonstrou que o oxLDL, junto a mieloperoxidase, é capaz de inibir a angiogênese *in vitro* através do aumento de miR-22 e heme-oxigenase 1 (HMOX1, do inglês *Heme oxygenase*) sugerindo que altos níveis de oxLDL prejudicam a capacidade do endotélio de reparar danos, o que pode contribuir para o desenvolvimento e progressão da formação de placa de ateroma (DAHER et al., 2014).

Inflamação

A inflamação do endotélio tem sido amplamente investigada na literatura devido à sua correlação com o início, a progressão e o prognóstico de doenças cardiovasculares (GONZALEZ; SELWYN, 2003; HAYBAR et al., 2019) . A cascata de efeitos colaterais do estresse oxidativo culmina no início da inflamação crônica e na disfunção do endotélio, como apontado no item anterior. Porém, essa condição ainda pode ser exacerbada, em casos patológicos, pela atuação de mediadores pró-inflamatórios como a IL-1β e o TNF-α que agem no endotélio induzindo a liberação local de IL-6 que induz a produção e secreção de fibrinogênio e proteína c-reativa levando ao aumento do quadro inflamatório (LIBBY, 2017).

Os fatores de transcrição NF-kB (do inglês *nuclear factor kappa B*) e KLF2/KLF4 possuem papel fundamental na inflamação vascular. O NF-kB regula o aumento da expressão de genes pró inflamatórios *IL6*, *IL1β*, *CCL2* (MCP-1), *ICAM1*, *VCAM1* e e-selectina (*SELE*) enquanto KLF2 e KLF4 reduzem a *CCL2* e *VCAM1* (SUN et al., 2019). O aumento dessas citocinas inflamatórias e de moléculas de adesão levam à migração e adesão de leucócitos iniciando o processo de diapedese (para ou trans celular). Esse processo pode ser fisiológico, e simplesmente levar o leucócito ao sítio inflamatório tecidual, ou pode ser o ponto chave para o início da formação da placa de ateroma já que, uma vez na íntima, os leucócitos passam a liberar mais citocinas, recrutando mais células do sistema inflamatório e colaborando para a formação da neointima (BRAUNERSREUTHER; MACH, 2006; MULLER, 2013).

Shear Stress

As células endoteliais encontram-se sobre a ação constante das forças de fricção, provenientes do bombeamento do sangue pelo coração, conhecidas como estresse de cisalhamento (SS, do inglês *Shear Stress*). O SS é afetado pelas características anatômicas dos vasos sanguíneos, sendo laminar (LSS) em regiões lineares e turbilhonado ou oscilatório (OSS) em regiões curvas ou com bifurcações (**Figura 8**). As células endoteliais apresentam mecanosensores em sua superfície que são capazes de detectar e traduzir esses estímulos mecânicos em respostas biológicas e fisiopatológicas (URSCHEL et al., 2021). Alguns dos mecanosensores conhecidos são as proteínas integrinas, NOTCH1, o complexo mecanosensor formado pelas proteínas PECAM1 (do inglês *platelet endothelial cell adhesion molecule-1*), VE-caderina e VEGFR, o receptor de Angiotensina Tipo 1 (AT1R), o canal iônico Piezo 1, bem como componentes estruturais como o glicocálix e o citoesqueleto (BARAUNA et al., 2013; CURRY; ADAMSON, 2012; LI et al., 2014; MACK et al., 2017; TZIMA et al., 2001). A partir desses mecanosensores o SS é capaz de regular a função e disfunção do endotélio, com LSS protegendo o endotélio por sua ação anti-inflamatória e vasodilatadora, além de promover a quiescência, estabilidade da barreira e a homeostasia. O OSS, por sua vez, leva as células endoteliais ao perfil disfuncional, caracterizado pela inflamação, vasoconstrição, aumento na permeabilidade e proliferação, indução de apoptose e aquisição de um fenótipo mesenquimal (EndMT, do inglês *endothelial to mesenchymal transition*) (DEMOS; TAMARGO; JO, 2021).

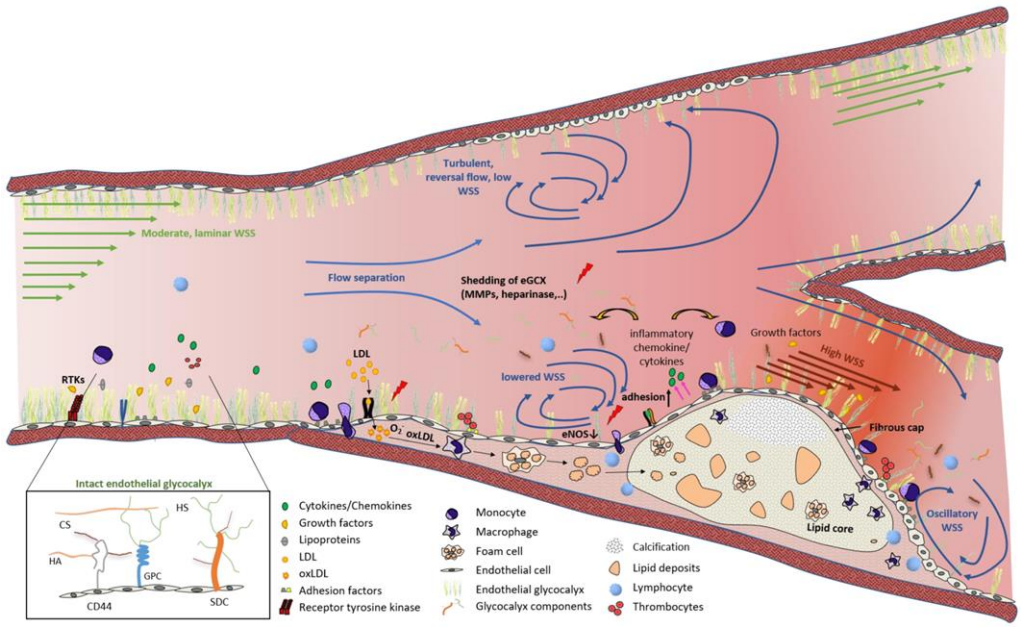

Figura 8 - Padrões de SS ao longo dos vasos, por Urschel et al., 2021: Em segmentos retos dos vasos o LSS leva a um fenótipo endotelial homeostático, quiescente, anti-inflamatório, com capacidade vasodilatadora, integridade da barreira e da permeablidade. Em locais de bifurcação, a separação do fluxo provoca o OSS, um fluxo turbulento e inverso. O OSS resulta em disfunção endotelial, caracterizada pela indução de cascatas de sinalização inflamatória, vasoconstrição, aumento da permeabilidade, proliferação, apoptose e fenótipo mesenquimal, o que culmina na formação da placa de ateroma nessas regiões.

Os principais responsáveis pelo perfil inflamatório nas regiões de OSS são NF-kB, e BMP4 aumentados, e o KLF2/KLF4, reduzidos (JIANG et al., 2014; MOHAN; MOHAN; SPRAGUE, 1997; PINHEIRO-DE-SOUSA et al., 2022; VAN DER HEIDEN et al., 2010). Com relação à vasoconstrição, enquanto o LSS aumenta a expressão da eNOS, o OSS eleva a expressão de NADPH oxidases e de xantinas oxidases e promove o desacoplamento da eNOS, levando ao aumento ROS e reduzindo a disponibilidade do NO (LI et al., 2011). Além disso, estudos demonstraram que o OSS é capaz de reverter o perfil quiescente do endotélio adulto saudável através da ativação de HIF-α e do consequente aumento do VEGF e da angiogênese (FENG et al., 2017), além de ativar a ERK1/2 e Akt enquanto inibe a cip1 e a AMPK (GUO; CHIEN; SHYY, 2007; LI et al., 2011). O aumento da proliferação do endotélio, associado ao OSS, pode estar relacionado à formação de neovascularização em placas de ateroma características dessas regiões (SLUIMER; DAEMEN, 2009). Com relação à apoptose, a literatura indica que o LSS seja capaz de proteger as células endoteliais contra a apoptose pelo aumento da expressão de eNOS e de superóxido dismutase (DIMMELER et al., 1999). Já o OSS apresenta perfil pró-apoptótico por aumentar a expressão de IL-1β nas células endoteliais que, por usa vez, exacerba o estresse do retículo endoplasmático pela via de sinalização IRAK2/CHOP (do inglês *Interleukin-1 receptor-associated kinase-like 2*/ *DNA Damage Inducible Transcript 3*) (PAN et al., 2017).

A permeabilidade da barreira endotelial também é afetada na condição de OSS pela fosforilação das proteínas de junção PECAM1, VE-caderina e VEGFR e remodelamento do citoesqueleto levando à redução da ligação célula-célula e favorecendo a ligação da célula endotelial à membrana basal (KOMAROVA et al., 2017). O aumento da permeabilidade associado ao OSS também pode estar associado ao remodelamento da matriz, o que pode ocasionar seu espessamento e endurecimento. Esse remodelamento deve-se à ativação das catepsinas K, L e S, diminuição da TIMP3 e consequente aumento de MMPs, desintegrinas e metaloproteinases (ADAMs) (PLATT et al., 2007; PLATT; ANKENY; JO, 2006).

O fenômeno de EndMT implica na perda de contato célula-célula e afeta a polaridade fazendo com que as células migrem para a íntima (SOUILHOL et al., 2018). Esse processo se dá a partir de uma série de mecanismos, envolvendo as vias do TGF-β e Smad, que levam à perda de marcadores endoteliais e ao aumento de marcadores de fibroblastos e células musculares lisas (MAHMOUD et al., 2017). O OSS induz esse fenótipo em células endoteliais diretamente pelo aumento da ERK5 e HIF1α (MOONEN et al., 2015).

Além disso, recentemente demonstramos que o perfil biológico derivado da análise do transcriptoma de células endoteliais submetidas a fatores de risco para doenças cardiovasculares [hipóxia química ($CoCl_2$), lipídios oxidados (OxPAPC), inflamação (IL-1β) e OSS] individualmente não recapitula os efeitos da combinação desses fatores *in vitro* (PINHEIRO-DE-SOUSA et al., 2022). Isso ressalta a importância do uso de metodologias *in vitro* que contemplem o uso de SS para a melhor compreensão de doenças cardiovasculares e, principalmente em casos como a PE, em que ainda não há uma completa compreensão da fisiopatologia, o uso do SS nos aproxima, ainda que de forma limitada, dos reais efeitos que os fatores circulantes podem causar *in vivo*.

Modelos experimentais de Pré-eclâmpsia: vantagens e limitações

A carência de modelos animais espontâneos em PE limita o entendimento da doença (BURTON et al., 2019; CHAU et al., 2021). Outro fator limitante é a dificuldade de obtenção de amostras biológicas maternas, o que acaba direcionando a maioria dos estudos na área para o tecido placentário (MARTINEZ-FIERRO et al., 2018). Como alternativa, considerando a característica fisiopatológica de fatores presentes na circulação materna, o uso do modelo *in vitro* de incubação de células endoteliais ou modelo *ex-vivo* com o plasma/soro de pacientes com PE tem se mostrado viável para a compreensão de diversos mecanismos relacionados a disfunção endotelial nessa síndrome. Uma das características mais comumente avaliada nesse modelo é a produção de NO, medida de forma direta ou indireta (dosagem dos metabólitos nitrito e nitrato) e mediante a determinação da atividade da eNOS. Com relação a esse marcador, há discordância nos efeitos do plasma sobre o endotélio *in vitro*, com estudos relatando aumento (DAVIDGE; BAKER; ROBERTS, 1995), ausência de alteração (SILVER et al., 1996) e, em estudos mais recentes do nosso grupo e outros, utilizando técnicas de medidas indiretas tradicionais somadas a técnicas de detecção *real time* diretas, indicando redução (CALDEIRA-DIAS et al., 2019, 2021; MURUGESAN et al., 2022; ROCHA-PENHA et al., 2017; VIANA-MATTIOLI et al., 2020). É importante ressaltar que um estudo que avaliou os efeitos do plasma de PE em quatro tipos celulares [células endoteliais de microvasculatura dérmica, células endoteliais de veia umbilical humana (HUVECs), células endoteliais humanas primárias de microvasculatura da decídua e células endoteliais bovinas de microvasculatura coronária] indicou aumento de nitrito apenas em células endoteliais bovinas, associando o plasma de PE ao aumento de NO *in vitro* (WELLINGS; BROCKELSBY; BAKER, 1998). Além disso, estudos demonstraram a redução do NO na circulação materna de mulheres com PE em comparação com HP, o que reforça os resultados encontrados por nosso grupo (AYDIN et al., 2004; BERNARDI et al., 2015; DIEJOMAOH et al., 2004; SANDRIM et al., 2008; TASHIE et al., 2020).

Diversos estudos utilizando o modelo *in vitro* apontam alterações desde a expressão gênica até a atividade de proteínas, bem como modificações de processos biológicos (CALDEIRA-DIAS et al., 2018; CALICCHIO et al., 2013; ENGLISH et al., 2013; FAAS et al., 2010; LIP, 2020; LUIZON et al., 2016; MURUGESAN et al., 2022; ROCHA-PENHA et al., 2017; SANKARALINGAM; XU; DAVIDGE, 2010). No entanto, ainda são raros os estudos que utilizam

esse modelo combinado à técnicas exploratórias avançadas como as "ômicas". Com relação a técnica de transcriptoma, um estudo relatou não ter encontrado alterações na expressão gênica de HUVECs e de células endoteliais de microvasculatura glomerular incubadas com plasma de PE grave pela técnica de *microarray* (DONKER et al., 2016). Em outro estudo, que avaliou a expressão de HUVECs incubadas com plasma de mulheres com PE obtidos em dois momentos diferentes (16 e 28 semanas), foi demonstrado aumento na expressão do gene c-Fos (FOS) quando em comparação a gestantes saudáveis (HP, do inglês *Healthy Pregnant*) utilizando as técnicas de *microarray* e qRT-PCR (MACKENZIE et al., 2012). Por sua vez, Calicchio et al., que também utilizou a técnica de microarray, porém com técnicas de análise mais avançadas, identificou 116 genes diferencialmente expressos (75 deles aumentados e 41 reduzidos em PE) em HUVECs incubadas com o plasma de gestantes com PE vs. o plasma de GH, sendo que alguns deles estavam associados a vias biológicas importantes como à homeostasia do endotélio, à regulação de processos apoptóticos associados ao estresse do reticulo endoplasmático, e à biossíntese de ácidos graxos (CALICCHIO et al., 2013). Dentre os genes encontrados por esse último grupo podemos citar como principais o *EDN1* e o *JDP2* (com *fold change* de 1.88 e −2.67, respectivamente). O gene *EDN1* codifica a endotelina 1, um potente fator vasoativo secretado por células endoteliais que está associado a diversas doenças cardiovasculares e à PE (SALEH et al., 2016; SCHIFFRIN, 2001). Já o gene *JDP2*, foi indicado como um importante fator de transcrição associado à regulação de um subgrupo de genes que possuem sua expressão regulada pelo plasma de PE nas HUVECs (seu papel foi confirmado por inibição por siRNA).

Vale a pena ressaltar, que, até o presente momento, apenas um estudo avaliou o efeito do SS no modelo de incubação de células endoteliais em cultura com plasma de mulheres com PE, demonstrando reversão induzida pelo fluxo laminar nos efeitos do plasma observados no modelo (BAKER et al., 1996). Porém, esse estudo restringiu-se a avaliação de apenas três parâmetros, sendo eles a produção de NO por nitrito no sobrenadante de cultura, a produção de prostaciclina e de endotelina, limitando a melhor compreensão dos processos subjacentes afetados pela exposição ao plasma de paciente dom PE (BAKER et al., 1996). Apesar de não terem encontrado diferenças nos níveis de NO entre as células endoteliais incubadas com os plasmas de PE e HP a conclusão do estudo foi a de que o SS é capaz de alterar os efeitos do plasma de PE em células endoteliais e que, talvez por esse motivo, muitos dos estudos *in vitro* não retratassem o que é observado *in vivo.*

Justificativa para esta dissertação

Apesar da grande influência da PE na morbidade e mortalidade materno-fetal, até hoje não se sabe a causa específica dos surgimento dos sintomas clínicos dessa doença e poucas frentes de tratamento estão disponíveis. Conhecer os mecanismos pelos quais a disfunção endotelial é promovida na PE é essencial para propor tratamentos mais efetivos e desfechos mais favoráveis às gestantes e aos recém-nascidos acometidos. A ausência de modelos animais espontâneos em PE, a dificuldade de obtenção de amostras biológicas maternas sem o uso de metodologias invasivas e as limitações da comparação da função vascular do modelo *in vitro* com as alterações encontradas *in vivo* são os maiores desafios para melhor compreensão da doença. Nesse contexto, a implementação de novas tecnologias que aproximem o modelo *in vitro* de disfunção endotelial associada à PE com o ambiente hemodinâmico original do sistema cardiovascular humano é de suma importância. Ademais, considerando que o SS ativa diversas vias de mecanotransdução essenciais para a regulação do funcionamento, do fenótipo das células endoteliais e, consequentemente, modula a manutenção do tônus vascular, faz se necessário incluir simulações de fluxo sanguíneo

nos modelos de estudo *in vitro* de avaliação da função endotelial em PE. Assim, com o presente projeto, exploramos os efeitos do plasma de gestantes com PE, GH e HP em células endoteliais submetidas a dois padrões de SS encontrados na circulação, para identificação de vias celulares envolvidas com base na análise de expressão gênica global em células endoteliais primárias humanas e seleção de potenciais alvos terapêuticos para a disfunção endotelial associada às SIGs e, em particular, a PE.

Objetivo

Testar os efeitos dos plasmas de pacientes com PE, hipertensão gestacional (GH) e gestantes saudáveis sob a indução e/ou exacerbação de vias celulares associadas à função e disfunção do endotélio na dependência de SS laminar (LSS) e oscilatório (OSS), respectivamente.

Objetivos específicos

I. Identificar o perfil global de mRNAs alterados em HCAECs pelos plasmas de PE, GH e HP em comparação a um controle sem plasma nos diferentes regimes de SS.

II. Integrar o perfil global e de genes diferencialmente expressos em HCAECs, obtidos no objetivo anterior, à bases de dados de resposta biológica para identificar potenciais novas vias e associadas a disfunção endotelial materna em PE.

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Capitulo 1

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Missing links in preeclampsia cell model systems of endothelial dysfunction

Authors: Sarah Viana-Mattioli [1,2], Miriam Helena Fonseca-Alaniz [2], Iguaracy Pinheiro-de-Sousa [2,3], José Eduardo Krieger [2], Valéria Cristina Sandrim [1].

[1] Department of Biophysics and Pharmacology, Institute of Biosciences, Sao Paulo State University (UNESP), Botucatu, São Paulo, Brazil.

[2] Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), University of Sao Paulo Medical School, São Paulo, Sao Paulo, Brazil.

[3] European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, United Kingdom

Abstract:

Preeclampsia is one of the main hypertensive disorders of pregnancy associated with circulating factors released by the ischemic placenta and systemic endothelial dysfunction. The preeclampsia etiology remains poorly understood and is associated with high maternal and fetal mortality levels and increased cardiovascular disease risk. Most of the cell model systems used to gain additional insights into the endothelial dysfunction have not taken into account hemodynamic physical factors, such as shear-stress forces, which may prevent extrapolating cell data into *in vivo* settings. Here we discuss the role of hemodynamic forces on endothelial cell function and strategies to incorporate this biological characteristic *in vitro* to improve the understanding of endothelial dysfunction associated with preeclampsia.

Keywords: preeclampsia; endothelial dysfunction; shear stress; omics.

Modeling Endothelial Dysfunction in Preeclampsia: Current Understanding and Future Directions

Preeclampsia is one of the main hypertensive disorders of pregnancy and is an independent risk factor for cardiovascular disease even decades later. There are several subtypes of preeclampsia as well as a wide range of risk factors associated with the disease (Figure 1 and Box 1). Although the comprehensive mechanisms of preeclampsia remain unclear, circulating components released by the ischemic placenta and systemic endothelial dysfunction are involved in the preeclampsia pathophysiology (Box 2). Furthermore, several studies have indicated that preeclampsia is an independent risk factor for cardiovascular disease even decades later, suggesting that these women may experience long-term endothelial dysfunction [1–3]. The lack of specific therapeutic targets alongside the increasing association between preeclampsia and future cardiovascular risk highlights the need to increase the understanding of maternal endothelial dysfunction causes, molecular profile, and progression.

In this review, we aim to present a comprehensive discussion on the role of endothelial dysfunction in preeclampsia etiology, highlighting the existing knowledge and limitations. We also focus on the gaps in current study *in vitro* models that need to be addressed, going through the importance of **shear stress (SS) (see Glossary)** in endothelial function and its incorporation in preeclampsia research. Ultimately, our goal is to emphasize the need for applying new biotechnology tools to fill current gaps in knowledge on endothelial dysfunction in etiology of preeclampsia.

Preeclampsia as a hypertensive disorder of pregnancy

Hypertensive disorders of pregnancy stand as one of the three leading causes of maternal and fetal morbidity and mortality worldwide affecting 18.08 million pregnancies globally by 2019, with a total increase in incidence of 10.92 % from 1990 to 2019 [4]. The American College of Obstetricians and Gynecologists classifies hypertensive disorders of pregnancy into four categories: gestational hypertension, preeclampsia, chronic hypertension, and superimposed preeclampsia [5]. To all the aforementioned, hypertension can be defined as a systolic blood pressure of 140 mmHg or higher and a diastolic blood pressure of 90 mmHg or higher. The different diagnosis relies on the time of hypertension onset and of other major symptoms. Gestational hypertension is characterized by the development of high blood pressure after 20 weeks of gestation in women who had previous normal blood pressure readings. In contrast, preeclampsia can be diagnosed when hypertension, diagnosed after the 20th week of gestation or postpartum, is accompanied by proteinuria and/or end-organ damage. Chronic hypertension is diagnosed when women present with hypertension before pregnancy or develop it before 20 weeks of pregnancy. Moreover, women with chronic hypertension have an increased risk of developing preeclampsia, which is known as superimposed preeclampsia. In this case, women develop proteinuria and other symptoms after the 20th week of gestation [5].

Out of all the hypertensive disorders of pregnancy preeclampsia is usually associated with worst outcomes for it can involve renal, hepatic, pulmonary, and neurological complications, as well as hemolysis, **elevated liver enzymes and low platelet count (HELLP)** syndrome, and placental abruption [6,7]. Preeclampsia alone is responsible for about 4.6% of maternal deaths annually, with this percentage varying according to countries' income [4] .

The role of endothelial dysfunction in preeclampsia

The endothelium is composed of a single layer of endothelial cells that lines the lumen of all vascular beds, including arteries, veins, capillaries, and lymphatic vessels. Being placed between both the blood and the tissues, the endothelium plays an essential role in integrating and regulating their connection by balancing permeability, coagulation, and vascular tone [8]. Recently, the endothelium has also been linked to a broader range of functions, including metabolism regulation, autoregulation of angiogenesis, and damage repair [9,10].

Gimbrone coined the term "endothelial dysfunction" in the mid-1980s, characterizing it as the reduction of vasodilation found in numerous cardiovascular diseases [11]. However, endothelial dysfunction can be

attributed to any alteration that compromises endothelial homeostasis, linking it to various physical, molecular, and metabolic stressors and distinct phenotypes [10]. In addition to a reduction in classical **nitric oxide (NO)** production and bioavailability, endothelial dysfunction can manifest as alterations in the levels of other mediators of vasodilation and vasoconstriction, loss of glycocalyx, increased permeability, endothelial (vascular) stiffness, inflammation, oxidative stress, and pro-thrombotic response [12].

Our group has recently demonstrated that, the combination of insults, such as pro-inflammatory signaling molecule, oxidized lipids, chemical hypoxia inducer, and oscillatory blood flow patterns, rather than each one of them alone, is necessary to fully recapitulate the complexity of endothelial dysfunction [13]. Similarly, it is unlikely that the endothelial dysfunction observed in preeclampsia, which is evidenced by impaired **flow-mediated dilation (FMD)**, can be explained by a single circulating bioactive factor targeting endothelial cell function [14]. Some of the circulating factors that were previously reported and associated with the preeclamptic placenta are summarized in **Table 1**.

However, in this table, we did not include some of the factors associated with maternal alterations, such as: circulating lipids, [15,16] inflammatory and autoimmune molecules, like **tumor necrosis factor α (TNF-α)** and **interleukins (IL-1β, IL-2, IL-6, IL-8)** [17–23] and the **angiotensin II type 1 receptor agonistic autoantibody (AT1-AA)** [24,25], which are also altered in preeclampsia circulation and present an important impact on endothelial cell function. These molecules increase endothelial inflammatory phenotype systemically by augmenting the levels of IL-6, **monocyte chemoattractant protein-1 (MCP-1)**, **intercellular adhesion molecule-1 (ICAM-1)**, **vascular cell adhesion molecule-1 (VCAM-1)**, **plasminogen activator inhibitor (PAI)-1**, and **E-selectin (SELE)**, besides increasing vasoconstriction and oxidative stress [26,27]. Particularly, AT1-AA activates the **angiotensin II receptor type 1 (AT1R)**, inducing the expression of endothelial **nuclear factor kappa B (NF-κB)**, a transcription factor considered the main regulator of innate immunity [28].

While we briefly describe some of the altered circulating factors shed by preeclamptic placenta and their association with endothelial dysfunction, more detailed descriptions about mechanisms of endothelial dysfunction in preeclampsia are provided in previous reviews [29–31].

Soluble antiangiogenic factors imbalance is sustained by the overexpression of **hypoxia-inducible factor 1 alpha (HIF-1α)** in the placenta. This imbalance results in a reduction of free circulating **vascular endothelial growth factor (VEGF)** and **placental growth factor (PlGF)**, two important pro-angiogenic factors crucial for normal placental development, and endothelial function. The development of this imbalance is induced by an increase in **soluble fms-like tyrosine kinase-1 (sFlt-1)** levels and a decrease in free **transforming growth factor beta (TGF-β)** levels, promoted by **soluble endoglin (sENG)** [32,33].

The reduction of VEGF and PlGF, in turn, leads to decreased angiogenesis and impaired production of vasodilators such as NO and prostacyclin by the endothelium, which can contribute to the hallmark features of preeclampsia, such as hypertension and proteinuria [34]. Additionally, the decrease in TGF-β1 affects endothelial cell migration, differentiation, and survival, as well as **endothelial nitric oxide synthase (eNOS)**

activation and NO production [35]. Thus, the dysregulation of these angiogenic factors is thought to play a critical role in the pathogenesis of preeclampsia and is a promising noninvasive clinical marker for prediction [36].

Circulating syncytiotrophoblast-derived microparticles (STBMs) are extracellular vesicles released from the syncytiotrophoblasts membrane. In normal pregnancies STBMs are responsible for activating peripheral mononuclear leukocytes, then leading to the shift toward type 2 T cell responses, characterized by reduced production of **interferon gamma (IFNγ)** and **Tumor Necrosis Factor alpha (TNFα)**, characteristic of type 1 immune response and increased levels of IL-4 and IL-6 [37,38].This process is crucial for uncomplicated pregnancies [37,38]. In preeclampsia, STBMs levels are elevated compared to normal pregnancies [39–41]. Besides being associated with systemic inflammatory activation, STBMs were also shown to interact directly with endothelial cells inducing cell death and the expression of endothelial adhesion molecules while reducing angiogenesis and endothelial-dependent vasodilation [42–44].

Cell-free nucleic acids are also altered in maternal circulation during preeclampsia. The literature suggests an increase in **total cell-free DNA (cfDNA)** and **microRNAs (miRNA)**, with varying levels **of fetal cfDNA (cfDNAff)** and miRNA reported in previous studies, and no consensus has been reached yet regarding their role and if they can be used as predictor markers of PE [45–47]. These alterations can have consequences as an increase in cfDNA of placental origin can trigger toll-like receptor 9, which activates endothelial cells to express pro-inflammatory molecules like NF-κB, leading to augmented expression of adhesion molecules and higher vascular permeability [30,48]. Similarly, the total level of miRNAs is elevated in preeclampsia circulation, with some miRNAs such as miRNA-16 and miRNA-155, which regulate angiogenesis and NO levels respectively, being altered in the preeclamptic placenta and maternal circulation [49]. These and other miRNAs altered in preeclampsia are still under investigation and more studies in different populations are necessary to confirm their role in the disease [50–52]. miRNAs have emerged as critical regulators of gene expression and are known to play essential roles in various biological processes, including disease pathogenesis.

The presence of circulating markers of placental origin in the circulation of preeclamptic women has been reported in several studies. However, the precise mechanisms underlying the effects of these markers on the maternal endothelium and their contribution to the pathogenesis of preeclampsia is still lacking. The identification of these markers and their association with maternal symptoms has not yet led to clear diagnostic or therapeutic targets for this complex and multifaceted condition. In the subsequent sections, we will highlight the current challenges and limitations in this field and discuss the potential avenues for future research to address these gaps in knowledge.

The missing links in Preeclampsia study models

Since preeclampsia is a complex multifactorial disease that only occurs spontaneously in humans and some primates, animal models have some disadvantages that must be taken into consideration. Moreover, humans have a unique process of placentation compared to other mammals [53].

Animal models

In general, rodents are commonly used for laboratory experiments due to their low costs for caging and feeding, as well as their high reproductive rates. These animals are particularly useful for studying placentation, as they exhibit interstitial and endovascular trophoblast invasion that results in artery remodeling, similar to humans [54]. Additionally, rodents have hemochorial placentation, which further mirrors the human condition [54]. While rodents are a good choice for investigating isolated molecular pathways, they do not fully replicate the complex pathophysiology of preeclampsia for not presenting all risk factors and the exact pathophysiology as humans. Nonetheless, several animal models are available for studying preeclampsia, including spontaneous genetic models (e.g., BPH/5 mouse), transgenic animals (e.g., those overexpressing sFLT1, human renin/angiotensin, or human STOX1), induced models (e.g., reduced uterine perfusion pressure), pharmacologically induced models (e.g., the NO inhibitor N^{ω}-nitro-L-arginine methyl ester or L-NAME, and lipopolysaccharide), as well as models induced by human preeclamptic serum/extracellular vesicles or administration of sFLT1. Overall, these models provide valuable insights into the mechanisms underlying preeclampsia [55].

In vitro models

Given the characteristic pathophysiology of preeclampsia, the use of *in vitro* models that incubate endothelial cells with preeclamptic women's serum/plasma is a suitable approach for understanding maternal endothelial dysfunction and has been used in many studies, including studies from our group [56–58]. Although these models are suitable for studying preeclampsia, they do have limitations. For instance, they use healthy endothelial cells and vessels, which fail to reproduce genetic and epigenetic variations and they not fully capture the complex interactions between endothelial cells and their surrounding environment like other cell types and hemodynamic forcers. Although it is substantially more difficult to have access to endothelial cells from maternal origin and ensure genetic replication of the disease, we can still control for the other factors.

For example, hemodynamic forces, specifically SS, constantly interacts with endothelial cells and may vary depending on the vascular architecture. SS can take on several forms, including steady **laminar (LSS)**, **oscillatory (OSS)**, and turbulent flow. LSS occurs when blood flow occurs in one direction at a constant magnitude, whereas OSS is characterized by bidirectional flow. Turbulent flow is chaotic and moves in all directions simultaneously. Straight arterial segments are usually exposed to LSS, while branches, curvatures, and bifurcations of the arterial tree experience OSS or turbulent flow [59].

In the following sections we will discuss how SS can regulate endothelial cell function and might add missing information to preeclampsia *in vitro* models.

Endothelial cell function and shear stress

Cell surface mechanosensors can detect and transform mechanical inputs from SS into functional and dysfunctional cell responses (Figure 2) [59,60]. This process is called mechanotransduction and strongly regulates the endothelial cell phenotype [59]. Mechanosensors include integrins, Notch 1, the complex

formed by the platelet endothelial **cell adhesion molecule-1 (PECAM-1)**, VE-cadherin, and **vascular endothelial growth factor receptor (VEGFR)**, besides the AT1R, Piezo 1, other ion channel, and structural components (components of glycocalyx and cytoskeleton) [59].

This process of transforming mechanical inputs from SS into functional and dysfunctional cell responses is called mechanotransduction and it strongly regulates the endothelial cell phenotype [59]. LSS is considered protective of the endothelium for promoting an anti-inflammatory and vasodilatory phenotype, as well as quiescence, barrier stability, and homeostasis. On the other hand, OSS leads to endothelial dysfunction through enhanced inflammation, vasoconstriction, permeability, proliferation, apoptosis, and acquisition of a mesenchymal phenotype (**EndMT**) [61]. These modulations are majorly accomplished by the regulation of key molecules. In endothelial cells exposed to OSS or turbulent SS, for example, there are great increases in the levels of NF-kB and **bone morphogenetic protein 4 (BMP4)**, which are key contributors to the inflammatory profile. On the other hand, in LSS areas, there is an upregulation of the transcription factors **Kruppel Like Factor 2 and 4 (KLF2/KLF4)**, which are greatly responsible for the regulation of the healthy and functional endothelial phenotype [62,63].

Laminar shear stress modulation of endothelial function

The mechanosensitive complex composed of PECAM-1, VE-cadherin, and VEGFR is located at the junctions between endothelial cells and regulates the expression of KLF2, KLF4, and NRF2 in steady laminar flow [64,65]. This complex activates the PI3K/Akt pathway and protein kinase C, which in turn regulate downstream targets. KLF2, in addition to its role in regulating eNOS expression, also promotes NRF2 nuclear translocation and activation [64]. This increases the levels of components of the antioxidant response, such as NAD(P)H quinone oxidoreductase 1, heme oxygenase-1, and superoxide dismutase, ultimately leading to the reduction of oxidative stress and inflammation [66,67]. Additionally, KLF2 regulates the expression of thrombomodulin and tissue plasminogen activator, which promote vasodilation and modulate coagulation, respectively. It also down-regulates plasminogen activator inhibitor-1 and tissue factor, further reducing the risk of coagulation [68,69].

Oscillatory shear stress modulation of endothelial function

OSS promotes an increase of NF-κB, a transcription factor that plays a critical role in the regulation of inflammatory and coagulation responses in the endothelium, [70] through two different pathways: (1) the phosphorylation of VE-cadherin, which then binds to G protein signaling modulator 2 polarity protein activating NF-κB signaling, and (2) matrix-mediated mechanosignaling, where fibronectin binds to integrin 5 and connects with phosphodiesterase-4D5 augmenting this activation [71,72]. In addition to NF-κB, OSS also activates the Yes-associated protein and its cofactor TAZ by suppressing their phosphorylation at Ser127 [73]. This leads to an increase in HIF1α as a consequence of NF-κB regulation and is further sustained by the imbalance in **reactive oxygen species (ROS)** [74,75]. These alterations culminate in a pro-inflammatory profile, marked by an increase in endothelial markers MCP-1, ICAM-1, VCAM-1, SELE, and others. This

profile is further enhanced by the increase of oxidative stress, which promotes eNOS uncoupling and reduces NO bioavailability [63].

Moreover, endothelial cells under OSS present a pro-apoptotic profile that is sustained by the increased expression of IL-1β in endothelial cells [76]. This, in turn, exacerbates endoplasmic reticulum stress through the Interleukin-1 receptor-associated kinase-like 2/ DNA Damage Inducible Transcript 3 signaling pathway. The permeability of the endothelial barrier is also affected by OSS through the phosphorylation of the junction complex PECAM-1, VE-cadherin, and VEGFR, and remodeling of the cytoskeleton. This leads to reduced cell-cell binding and favors endothelial cell binding to the basement membrane [77]. This increased permeability in OSS may also be associated with matrix remodeling, which leads to its thickening and hardening. The remodeling occurs due to the activation of cathepsins K, L, and S, decreased TIMP3 levels, and a consequent increase in MMPs, disintegrins, and metalloproteinases [78,79].

OSS may also directly induce the EndMT phenomenon, a process that promotes the loss of cell-cell contact and affects cell polarity, causing cells to migrate to the intima [80]. EndMT occurs in a series of mechanisms involving the TGF-β and Smad pathways, which lead to the loss of endothelial markers and augments fibroblast and smooth muscle cell markers [81].

Incorporating shear stress in preeclampsia research

According to the aforementioned effects of SS on endothelial function, it is possible to consider that the complete profile of preeclamptic endothelium is not being properly explored since, as demonstrated by our group, the combination of biochemical stimuli to OSS to induce endothelial dysfunction *in vitro*, is not fully recapitulated by each stimulus alone [13]. Besides OSS, studies using LSS applied to cells with preeclamptic plasma certainly have a lot of potential for understanding the endothelial dysfunction profile of this disease. This is further evidenced by the reduced vasodilation in preeclamptic women in comparison to uncomplicated pregnancies in a study that assessed FMD [82]. This reduction in FMD was observed before the development of preeclampsia (20-29 weeks gestation), during preeclampsia onset, and for the following three years postpartum. Furthermore, many of the endothelial alterations associated with SS, specifically OSS, also play a part in endothelial dysfunction associated with preeclampsia, which might exacerbate the endothelial dysfunction in these women and might be associated with later cardiovascular risk and current understanding of preeclampsia pathophysiology, enabling the discovery of new pharmacological targets.

Available models of in vitro shear stress

Fortunately, significant advances in combining *in vitro* modeling with vascular forces have been developed in the past decades. There are two-dimensional models using the cone-plate device and hydrostatic pumps; 3D models using fabricated three-dimensional micro scaffolds that can be entirely made of poly(dimethylsiloxane), poly(dimethylsiloxane), and hydrogel hybrids or only hydrogel combined to hydrostatic pumps; and organ-on-a-chip models [83]. The latter, despite still having some limitations in terms

of vasodilation evaluation, presents significant technological advances that allows the evaluation of processes such as drug transport and endothelial cell migration [83–85].

When these many models are compared, it is clear that each one of them has advantages and disadvantages. Although 2D models are easy and inexpensive, they still lack physiological features. 3D models allow more precise simulation of *in vivo* situations; however, their complexity and expense may be a limiting issue. Organ-on-a-chip models provide the most accurate representation of *in vivo* circumstances, but their complexity makes them more difficult to operate and more expensive to build. The model used to analyze shear stress *in vitro* will be determined by the individual research issue and the available resources. To choose the best model for their study, researchers must assess the strengths and limitations of each model.

Preeclampsia studies incorporating shear stress

Despite the critical role of SS in endothelial cell function, limited research has been conducted on the effects of SS in preeclampsia. In 1996, Baker et al. utilized the cone-plate system to investigate the effect of SS on preeclampsia. The study was limited to the assessment of NO production, prostacyclin, and endothelin secretion in the cell culture supernatant [86]. Although the authors found no differences in nitrite levels in endothelial cells incubated with preeclamptic and uncomplicated pregnancies plasma, they concluded that LSS was able to alter the effects of preeclamptic plasma on endothelial cells, and that this could be a reason why many *in vitro* studies did not reflect *in vivo* findings. Later, Kublickiene et al. found the loss of SS-mediated dilation in *ex vivo* myometrial arteries from gravid women with preeclampsia compared to healthy control [87]. Another study by Rowe et al. measured supernatant nitrate/nitrite levels from decidual endothelial cells and Human Umbilical Vein Endothelial Cells incubated with preeclamptic and normal pregnancy plasma after 30 minutes of SS. The results indicated higher NO production by endothelial cells in the preeclamptic group. However, to our knowledge, no other studies have utilized SS to investigate the effect of preeclampsia plasma on endothelial cell phenotype since then [88]. Further research is required to investigate these aspects not only in protective LSS but also in OSS, and in endothelial cells of other vascular bed besides the ones originating from the placenta. To enhance our knowledge of preeclampsia endothelial dysfunction, comprehensive global analysis such as multi-omics should be integrated with these SS models whenever feasible.

Advanced omics technologies have been used for large-scale analysis of DNA or DNA-associated proteins (epigenomics), genes (genomics), RNA expression (transcriptomics), proteins (proteomics), and metabolites (metabolomics). Recently, Benny et al. reviewed preeclampsia studies associated with omics studies [e.g., genomics, transcriptomics, proteomics, and metabolomics] [89]. In this study, it becomes clear that most researchers are not looking at maternal endothelial dysfunction since no study, in the vast list of articles evaluated in this review paper, evaluated endothelial cells that were not from placental origin. The combination of these complex data might allow us to get insights to identify novel molecular diagnostics and therapeutic targets by evaluating how endothelial cells respond, in a global context, to preeclampsia circulating factors, as well as these factors' identity, origin, and their association with disease progression, even in post pregnancy [90].

Concluding remarks and future perspectives

The limited knowledge of endothelial dysfunction in preeclampsia precludes the advances in effective targeted intervention for prevention and therapeutic treatment and the understanding of preeclampsia-associated future cardiovascular risk. In this review, we addressed the current data on preeclampsia pathogenesis and its study models and highlighted the importance of including hemodynamic physical factors in cell disease models to gain additional insight into the underlying mechanisms that can be extrapolated for *in vivo* complex settings.

Considering preeclampsia pathophysiology and the current limitations on fully recapitulating this human disease in animal models, *in vitro* incubation of endothelial cells with preeclamptic serum/plasma offers a great opportunity to gain additional insights (see clinician's corner). However, conventional static cell culture hardly represents the *in vivo* environment experienced by endothelial cells in different vascular beds. The use of modern biotechnology approaches to simulate different mechanical forces, such as LSS and OSS, aligned to omics approaches, may provide an understanding of some of the remaining questions regarding preeclampsia and its endothelial dysfunction (see Outstanding questions). These novel insights may contribute towards improving understanding of preeclampsia implications *in vivo* and the development of novel diagnostic and therapeutic approaches for this unmet clinical need.

Author contributions

S.V.M, I.P.S, M.H.F.A, J.E.K., and V.C.S contributed to the conception and design of the review and drafted the manuscript. All authors critically revised, read, and approved the final manuscript.

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Declaration of interests

The authors have no interest to declare that might be relevant to the content of this Feature Review.

Box 1

Preeclampsia subtypes and risk factors

The heterogeneous forms of the disease, and the diversity of risk factors, hampers the advances in the discovery of a cause, early diagnosis criteria, and definitive treatment targets for this disease. For example, the **International Society for the Study of Hypertension in Pregnancy (ISSHP)** classifies preeclampsia

subtypes into preterm (delivery prior to 37 weeks of gestation), term (delivery at or after 37 weeks of gestation) and postpartum preeclampsia [91]. Preeclampsia can also be classified as early-onset (defined by premature delivery before or at 34 weeks), and late-onset (delivery after 34 weeks of gestation), but this last classification determined not reflect fetal and maternal clinical outcomes well enough [7,55].

Preeclampsia is also associated with various risk factors, including genetic, maternal, obstetric history, and environmental factors [55,92]. However, the predictive power of these factors is limited when evaluated individually or in combination [93]. Genetic risk factors include family history, maternal and fetal gene alleles and mutations, fetal trisomy 13, and sequence variants in **FLT1 (encoding Fms-related receptor tyrosine kinase 1; FLT1)**, **FTO (encoding α-ketoglutarate-dependent dioxygenase)**, and **ZNF831 (encoding zinc finger protein 831)** [55]. Maternal factors include age (< 20 years or ≥35 years), race, high BMI (>30 kg/m^2), chronic hypertension, diabetes, chronic kidney disease, systemic lupus erythematosus, and antiphospholipid syndrome [55]. Obstetric history of preeclampsia and fetal growth restriction, multiple pregnancy, nulliparity, and *in vitro* fertilization are also risk factors [55]. Environmental factors such as high altitudes and air quality can also contribute to the risk of preeclampsia [55].

Box 2

Preeclampsia pathophysiology

Despite ongoing research efforts, the pathophysiology of preeclampsia remains not completely understood. For example, the main questions remaining regarding the subtypes of the disease, are if preeclampsia causes or exacerbates a maternal predisposition to cardiovascular disease, and what are the most accurate ways to predict preeclampsia [94]. However, the current scientific consensus is that preeclampsia is a two-stage disease involving both placental hypoxia/ischemia, and systemic endothelial dysfunction, which play critical and interdependent roles in the pathophysiology and maternal symptomatology (Figure 1). This two-stage model was first proposed by Redman over three decades ago and has since been supported by a growing body of literature, with some refinements [95–97].

In this model, the first stage of preeclampsia is marked by shallow placentation. This occurs due to insufficient decidual trophoblast invasion and differentiation during pseudovasculogenesis, resulting in reduced spiral artery remodeling and inadequate placental perfusion [96]. As a consequence, chronic hypoxia and ischemia can occur in the placenta [96]. In some cases, these conditions may be exacerbated by alterations in blood flow, which can cause a pulsatile, high-pressure, and turbulent SS profile [98]. In some cases, these flow alterations, combined with placental and maternal inflammation, as well as genetic predisposition, may lead to the development of acute atherosis [99,100]. This condition is characterized by subendothelial lipid-filled foam cells, fibrinoid necrosis of the arterial wall, and perivascular lymphocytic infiltration [100]. Histologically, acute atherosis is similar to early-stage atherosclerosis.

The second stage of preeclampsia is believed to be linked to the first stage, and it involves the development of maternal clinical manifestations, also known as maternal syndrome. This includes hypertension, proteinuria, and end-organ damage, which are known to be associated with endothelial dysfunction [101–

104]. The systemic endothelial dysfunction is believed to be triggered by circulating factors released by the placenta that target endothelial cells [96]. In a study by Mourafarrej et al., differentially expressed genes were identified in cell-free RNA from women with preeclampsia compared to normotensive pregnant women [105]. Approximately 40% of this gene set had less evident changes differ in the postpartum period, possibly reflecting the placental contribution. Analysis of their biological function revealed an association with immune system response [105]. This theory is supported by the fact that delivery of the placenta usually alleviates maternal symptoms.

However, women who have experienced preeclampsia are at increased risk of developing chronic hypertension, even after delivery and separation from the placenta. These women also have a 32% higher incidence of chronic hypertension over the following years [3].

Glossary

Angiotensin II receptor type 1 (AT1R): receptor involved in the regulation of blood pressure and cardiovascular function.

Angiotensin II type 1 receptor agonistic autoantibody (AT1-AA): autoantibody that activates AT1R, mimicking the effects of angiotensin II.

Endothelial nitric oxide synthase (eNOS): enzyme expressed in endothelial cells that produces nitric oxide.

Endothelial to mesenchymal transition (EndMT): process in which endothelial cells lose their typical features and acquire mesenchymal characteristics.

E-selectin (SELE): cell adhesion molecule expressed on endothelial cells in response to pro-inflammatory stimuli.

Fetal cfDNA (cfDNAff): DNA fragments from the fetus circulating in maternal bloodstream.

Flow-mediated dilation (FMD): non-invasive measure of the ability of blood vessels to dilate in response to blood flow.

Hypoxia-inducible factor 1 alpha (HIF-1α): transcription factor that regulates cellular response to hypoxia.

Intercellular adhesion molecule-1 (ICAM-1): cell surface glycoprotein that is involved in leukocyte adhesion to the endothelium.

Interleukin (IL) :group of cytokines produced by cells of the immune system involved in regulating immune responses.

Kruppel Like Factor 2 and 4 (KLF2/KLF4) :transcription factors that are critical regulators of vascular homeostasis.

Laminar shear stress (LSS): mechanical force exerted on endothelial cells generated by blood flow. LSS is unidirectional and constant flow and is considered protective for the endothelium.

MicroRNAs (miRNA): small, non-coding RNA molecules that play important regulatory roles in gene expression.

Monocyte chemoattractant protein-1 (MCP-1): chemokine responsible for the recruitment of monocytes to sites of inflammation.

Nitric oxide (NO): endogenous vasodilator and plays a critical role in regulating vascular tone.

Nuclear factor kappa B (NF-κb): transcription factor that plays a critical role in the regulation of immune and inflammatory responses.

Oscillatory Shear stress (OSS): shear stress that occurs when blood flow is irregular and fluctuating. OSS has harmful effects on endothelial cell function.

Placental growth factor (PlGF): protein that belongs to the VEGF family. It is produced by placental cells and plays an important role in angiogenesis during fetal development.

Platelet endothelial cell adhesion molecule-1 (PECAM-1): adhesive stress-response protein that maintains endothelial cell junctions.

Shear Stress (SS): physical force that is exerted by the flow of fluids, such as blood, over endothelial cells.

Soluble endoglin (sENG): soluble form of endoglin that sequestrates TGF-β in the circulation.

Soluble fms-like tyrosine kinase-1 (sflt-1): is the soluble form of VEGFR that sequestrates circulating the angiogenic factors VEGF and PlGF.

cell-free DNA (cfDNA): DNA that circulates freely in the blood, not contained within cells.

Transforming growth factor beta (TGF-β): cytokine involved in cell growth, differentiation, apoptosis, and immune regulation.

Tumor necrosis factor α (TNF-α): proinflammatory cytokine and potent agonist for endothelial cell activation.

Vascular cell adhesion molecule-1 (VCAM-1): surface protein that interacts with leukocytes allowing them to adhere to the endothelium and migrate.

Vascular endothelial growth factor (VEGF): critical protein for endothelial cells survival and the angiogenesis

Vascular endothelial growth factor receptor (VEGFR): a receptor surface protein plays a key role in VEGF signaling.

Table 1. Maternal circulating factors associated with the PE placenta. Vascular endothelial growth factor (VEGF)

Circulating Biomarker	**Samples**	**Alteration (up or downregulated):** **PE vs Normal Pregnancy**	**Sample Population**	**References**
VEGF	serum/plasma	Up[106,107] Down[24,108–112]	India, Ireland, Turkey, Greece, Korea, Uganda, USA	[24,106–112]
PlGF	serum/plasma	Decreased	India, USA	[24,108,110–113]
sFlt-1	serum/plasma	Increased	India, Greece, Korean, Uganda, USA	[24,108–112,114]
sENG	serum/plasma	Increased	Chile, USA, Brazil	[45,115–118]
STBMs	plasma	Increased	England, China	[40,41]

cfDNA	serum/plasma	Increased	Egypt, Spain, Israel	[45,119,120]
cfDNAff	serum/plasma	Increased	Switzerland, Egypt, China, USA	[45–47,121]
miRNA	serum/plasma	15 increased and 7 decreased[50] 13 increased and 2 decreased[51] 1 increased[52]	China, Brazil	[50–52]

Placental growth factor (PLGF); Soluble fms-like tyrosine kinase 1 (sFlt-1); Soluble endoglin (sENG); circulating syncytiotrophoblast-derived microparticles (STBMs); total cell-free DNA (cfDNA); fetal fraction cfDNA (cfDNAff); and microRNAs (miRNA).

Figure legend

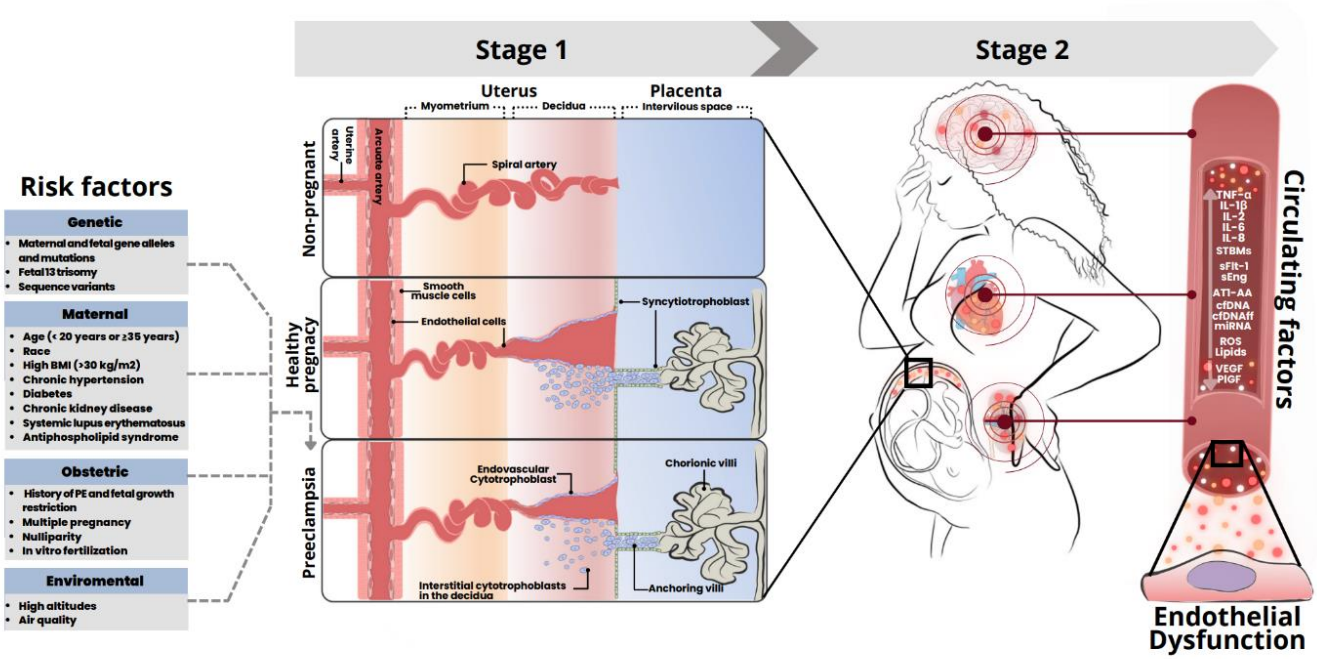

Figure 1. Preeclampsia pathophysiology according to the two-stage model. Multiple risk factors proposed for preeclampsia (genetic, maternal, obstetric history, and environmental factors) may lead to placental ischemia/hypoxia (stage 1), which leads to the release of antiangiogenic factors [E.g., soluble fms-

like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng)] inflammatory mediators [E.g., tumor necrosis factor α (TNF-α), interleukins (IL-1β, IL-2, IL-6, IL-8), and circulating syncytiotrophoblast-derived microparticles (STBMs)], total cell-free DNA (cfDNA), microRNAs (miRNA), fetal cfDNA (cfDNAff) and miRNAs, combined to systemic circulating levels of angiotensin II type 1 receptor agonistic autoantibody (AT1-AA), reactive oxygen species (ROS), and circulating lipids induce maternal syndrome (stage II).

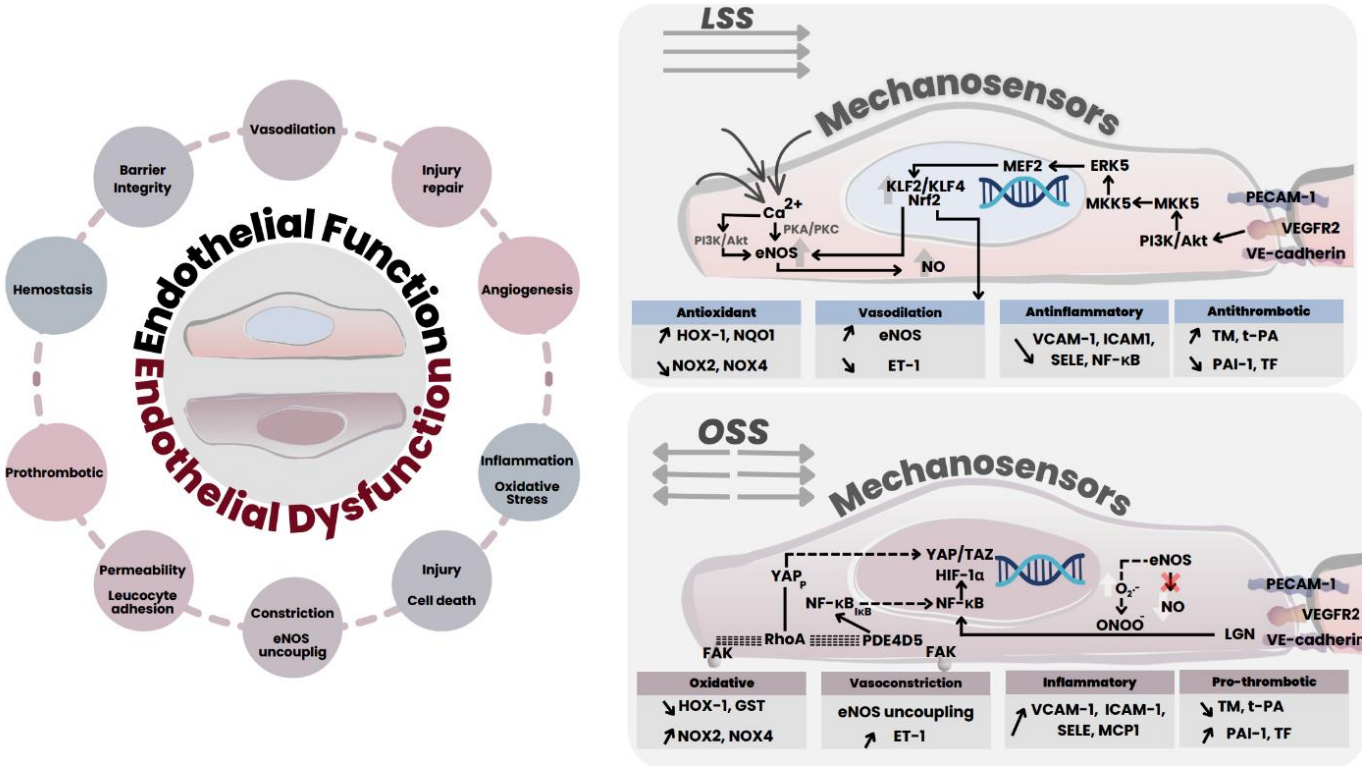

Figure 2. **Endothelial function and dysfunction: the role of shear stress.** Endothelial cell responses to LSS (upper right) and OSS (bottom right). LSS induces endothelial homeostasis through mechanotransduction-mediated calcium influx and upregulation of transcription factors Kruppel Like Factor 2 and 4 (KLF2/KLF4), and nuclear factor erythroid 2-like 2 (NRF2) through the PI3K/Akt signaling pathway. These transcription factors then reduce oxidative stress, inflammatory, and thrombotic processes while increasing vasodilation. OSS promotes an increase in transcription factor NF-κB, which regulates inflammatory and coagulation endothelial responses. Two pathways have been identified: (1) the phosphorylation of VE-cadherin, which then binds to G protein signaling modulator 2 (LGN) polarity protein activating NF-κB signaling, and (2) matrix-mediated mechanosignaling, where fibronectin binds to integrin 5 and connects with phosphodiesterase-4D5 (PDE4D5) augmenting this activation. Additionally, OSS also activates the Yes-associated protein (YAP) and its cofactor TAZ by suppressing their phosphorylation at Ser127, leading to increased HIF1α as a consequence of NF-κB regulation, which is further sustained by the imbalance in reactive oxygen species (ROS). These alterations lead to a pro-inflammatory profile marked by increased endothelial markers MCP-1, ICAM-1, VCAM-1, SELE, and others. This profile is further enhanced by the increase of oxidative stress, which promotes eNOS uncoupling and reduces NO bioavailability. MKK5 (Mitogen-activated protein kinase kinase 5); ERK5 (extracellular signal-regulated kinase 5); myocyte enhancer factor-2 (MEF2); NRF2 (nuclear factor erythroid 2 like 2); PDE4D5 (phosphodiesterase-4D5); YAP (Yes-associated protein); FAK (focal adhesion kinase); LGN (G protein signaling modulator 2).

Clinician's corner

- Although there were many advances in preeclampsia knowledge in the past decades, the specific cause, early diagnosis criteria, and definitive treatment targets for this disease are still not identified.
- Novel circulating biomarkers are continuously being discovered in different pregnancy time windows and populations; however, information on how they can affect endothelial function is still pending.
- The association between preeclampsia and future cardiovascular risk is still under-investigated, and endothelial dysfunction is the common link between them.
- The discovery of therapeutic targets is hampered by ethical and experimental limitations; considering preeclampsia pathophysiology, *in vitro* models may provide more accurate information on the molecular profile of preeclampsia endothelial dysfunction and help in the identification of drug targets.

Capítulo 2

Exploring the Role of Shear Stress in Endothelial Dysfunction Induced by Preeclampsia Plasma: A Human Coronary Endothelial Cell *in vitro* Study.

Authors

Sarah Viana-Mattioli[1,2], Iguaracy Pinheiro-de-Sousa[2,3], Miriam Helena Fonseca-Alaniz[2], Ricardo Rosa Junior[2], Ricardo de Carvalho Cavalli[4], Jose E. Krieger[2], Valéria Cristina Sandrim[1]*.

[1] Department of Biophysics and Pharmacology, Institute of Biosciences of Botucatu, Universidade Estadual Paulista (UNESP), Distrito Rubião Júnior, Botucatu, São Paulo, Brazil

[2] Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), Hospital das Clínicas, Medical School, University of São Paulo, São Paulo, SP, Brazil.

[3] European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, UK.

[4] Department of Gynecology and Obstetrics, Hospital das Clínicas, Medical School of Ribeirão Preto – University of São Paulo, São Paulo, SP, Brazil.

Abstract

Preeclampsia (PE) is a serious pregnancy disorder characterized by hypertension, proteinuria, and organ damage. Despite being a leading cause of maternal morbidity and mortality, the underlying cause of PE remains unknown. Evidence suggests that endothelial dysfunction (ED), caused by circulating factors released by the ischemic placenta, is associated with PE-related symptoms and subsequent cardiovascular disease. Previous *in vitro* studies have not taken into account the important role that *in vivo* factors, such as shear stress (SS), may play in the development of ED. To address this gap in knowledge, the aim of our study was to investigate the effects of plasma from healthy pregnant (HP), gestational hypertension (GH), and PE patients on the regulation, induction, and exacerbation of ED in human coronary endothelial cells (HCAECs) exposed to both laminar (LSS) and oscillatory (OSS) shear stress for 48 hours. Our results showed that under LSS, all plasma treatments produced distinct effects, with the greatest difference observed between PE and GH plasma treated HCAECs. However, this difference was reduced under OSS, where the global transcriptomic profiles of the PE and GH groups shared some similarities. This suggests that SS patterns have a significant impact on the effects of plasma treatments. Additionally, our transcriptomic analysis of differentially expressed genes (DEGs) revealed distinct biological profiles for each treatment when compared to a control group that did not receive plasma. Overall, our data highlights the importance of SS-dependent pathways in the genesis and maintenance of ED, with particularly pronounced effects in PE plasma-treated HCAECs.

Keywords

Endothelial dysfunction; Preeclampsia; Gestational hypertension; Shear stress.

* correspondence should be addressed to Valéria Cristina Sandrim: valeria.sandrim@unesp.br and Jose E. Krieger: j.krieger@hc.fm.usp.br

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Abbreviations

PE – Preeclampsia

ED – Endothelial dysfunction

CVDs – Cardiovascular diseases

SS – Shear stress

LSS – Laminar shear stress

OSS- Oscillatory shear stress

HCAECs - Human coronary artery endothelial cells

HP - Healthy pregnant

GH - Gestational hypertension

CT – Control samples

ACOG - American college of obstetricians and gynecologists

HCFMRP-USP - Hospital das Clínicas de Ribeirão Preto – Universidade de São Paulo

BH - Benjamini-Hochberg

FDR - False discovery ratio

RMA - Robust multichip average

DEGs - Differentially expressed genes

MSigDB - Molecular signatures database

GSVA - Gene set variation analysis

ROS - reactive oxygen species

INF- α - Interferon alpha

LPS – lipopolysaccharides

GO - Gene Ontology

IL-8 - interleukin-8

OSM - oncostatin M

EndMT - Endothelial to mesenchymal transition

Introduction

Preeclampsia (PE) is one of the leading causes of maternal and fetal morbidity and mortality worldwide.[1] This gestational disease is characterized by the onset of de novo hypertension along with proteinuria and organ damage.[2,3] It can cause severe complications for both mothers and their babies, including maternal organ damage, and fetal growth restriction.[4] The available treatments for PE cases are limited to monitoring and controlling the symptoms with antihypertensive drugs.[5] However, sometimes pregnant women will not respond to medical therapy. In these situations, providers will bring forward the delivery date to protect the mother.[6]

PE pathophysiology is believed to involve the placenta hypoxic-ischemic injury, the release of pro-inflammatory cytokines and antiangiogenic factors in the circulation, and systemic endothelial dysfunction (ED).[7,8] History of PE, as well as ED, is a risk factor for developing subsequent cardiovascular diseases (CVDs) later in life.[9–11] The exact mechanisms underlying the association between PE and CVDs are not fully

understood, but there seems to be an association with long-term damage of the maternal vasculature after the end of the gestation period.[12,13] Understanding the underlying mechanisms of maternal ED is, therefore, vital to ensure advances in preventive care and pharmacological therapy. *In vitro* studies are particularly interesting, considering PE's known pathophysiological feature of maternal circulating factors and their association with systemic ED. Indeed, many studies have combined *in vitro* endothelial cell culture with preeclamptic women's plasma/serum.[14–18] However, these models may present several limitations. For instance, conventional one-cell-type static *in vitro* models do not fully replicate the complex interactions and microenvironments these cells are exposed to in the human body. This includes direct and indirect contact with multiple other cell types, and blood mechanical forces.[19] Fortunately, thanks to biotechnological advances, we are currently able to partially replicate the *in vivo* environment of endothelial cells by combining complex fluidic systems, and multicellular structured organ-on-a-chip models. [20,21] However, only few studies have explored these technologies in the PE research field, and most of these studies are focused on endothelial cells of placental origin.[22–24]

Of these aforementioned *in vitro* technologies, fluidic systems are more reasonably accessible in terms of general costs, complexity, and maintenance. Moreover, many studies have already demonstrated how strongly different types of shear stress (SS) can modulate endothelial cell function.[25–29] For example, laminar shear stress (LSS), which occurs in the straight portions of the vascular tree where blood flow is smooth and steady, promotes several beneficial effects on endothelial cells, such as proliferation suppression, increase in the expression of endothelial nitric oxide synthase (eNOS), and suppression of inflammation.[30,31] Furthermore, the type of flow that occurs in bifurcation areas is turbulent and oscillatory and is therefore called oscillatory shear stress (OSS). It is commonly associated with areas of atherosclerotic plaque formation, and it regulates endothelial cells' inflammatory profile, oxidative stress, and overall general damage.[27–29]

To address current gaps regarding PE models of ED, we compared the global transcriptomic profile of human coronary artery endothelial cells (HCAECs) incubated with healthy pregnant women's (HP), gestational hypertension (GH) or PE plasma, while exposing them to LSS and OSS. This allowed us to identify the different patterns of global gene expression levels in PE conditions in both a protective and a harmful SS scenario. Our findings reveal new insights into how endothelial cells may respond in a PE condition and the mechanisms involved in its characteristic vascular damage.

Methods

Plasma samples collection

We collected plasma from 10 HP women, 10 patients with GH, and 8 patients with PE. PE and GH patients' diagnosis were based on the American College of Obstetricians and Gynecologists (ACOG) guidelines. Exclusion criteria consisted in twin pregnancy, chronic hypertension, hemostatic abnormalities, diabetes mellitus, history of cancer, cardiovascular disease, and autoimmune diseases. All samples were collected at the Hospital das Clínicas de Ribeirão Preto (HCFMRP-USP), and all study participants signed an informed consent. The study was approved by the Research Ethics Committee of the Faculdade de Medicina de Ribeirão Preto, São Paulo, Brazil (CAAE-37738620.0.0000.5440, October 19, 2020), following the principles of the Helsinki Declaration.

We collected 15 mL of venous blood from each patient in a standard vacutainer tube containing heparin (Becton Dickinson). We then centrifuged the tubes at room temperature at 3,200 g for 10 minutes. Aliquots of 500 µL were stored together at -80 °C until their use. We assembled plasma pools for each group using equal parts of each individual sample before experiments.

Cell culture

We used HCAECs purchased from LONZA (Lonza, Walkersville, MA, USA) cultured in specific complete medium (Lonza, EGM-2MV) in a humidified incubator (at 37°C and 5% CO_2). All experiments were started with cells at 5th passage and finished at the 7th passage.

Shear stress model

After reaching approximately 90% confluence, we resuspended the cells and seeded them in μ-Slides I Luer 0.4 (IBIDI, Gräfelfing, Germany) coated with a uniform layer of 0.1% gelatin at a density of approximately 1 x 10^5 cells/cm^2. Then the μ-Slides were left in the incubator (37°C with 5% CO_2) for 3 hours in static conditions to reach full confluence. The cells were exposed to LSS of 20 dyn/cm^2, and OSS at 1 Hz ($0 \sim \pm 5$ dyn/cm^2) for 48 hours in replicates of 4 μ-Slides per group (HP, GH, and PE). We have also added a control group (CT) without any plasma.

RNA isolation and Microarray gene expression profiling

At the end of the 48 hours, the cells were washed with 1X PBS and total RNA isolation was performed using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). We followed the manufacturer's protocol, and the steps consisted of: (1) lysis of the samples in a buffer (Buffer RLT) coupled with 1% β-mercaptoethanol; (2) addition of ethanol to the lysate followed by addition of the DNAse enzyme next to the Buffer RDD; (3) the lysate was added to the RNeasy MinElute centrifugation column, where the RNA binds to the membrane but, DNase and any contaminants are washed away; and (4) elution by adding 14 μL of water to the obtained RNA. We verified the total RNA concentration using NanoDrop (Thermo Fisher Scientific, Massachusetts, USA).

Microarray analysis and quality control

We used raw intensity data in CEL file format, obtained from GeneTitan™. After importing the files to the R platform, we used the ArrayQualityMetrics package to identify apparent outliers and compute measures of signal-to-noise ratio. After that, using robust multichip average (RMA) normalization method, the probe sets with no or low expression (log2 signal intensity < 2) were removed from further analysis. We have also corrected the batch effect between samples by running BatchQC SVA. Human Clariom s assays annotation (version 8.7.0) was used for gene annotation and NA and multiple mapping probes were removed.

Differential expression analysis

We obtained differentially expressed genes (DEGs) lists using limma package and significance was defined using the criteria of absolute |log2 fold change| ≥1.25 and false discovery ratio (FDR) <0.05 using Benjamini-Hochberg (BH) correction.

Enrichment analysis

Enrichment of the whole transcriptome was obtained utilizing Gene Set Variation Analysis (GSVA) package and the Molecular Signatures Database (MSigDB) hallmark gene set collection. The enrichment score values of each sample were analyzed using limma package and hallmark gene sets were considered significant when |log2 fold change| ≥ 0.05 and p value was <0.05.

Enrichment analysis of DEGs were carried out using the Enrichr package MSigDB 2020 and Bioplanet 2019 libraries. Pathways were considered significant when adjusted p-value of <0.1.

Results

Clinical profile of plasma samples

Table 1 summarizes the clinical parameters of each group of pregnant women whose plasma were collected for this study. Gestational age at sample collection, gestational age at delivery, newborn and placental weights were reduced in the PE group compared to the other two groups. Both groups associated with hypertension, GH and PE, had increased systolic and diastolic blood pressure compared to HP. Finally, as expected, PE had higher levels of creatinine and proteinuria compared to GH, as well as reduced APGAR 2 score compared to HP.

Parameters	Healthy Pregnant	Gestational Hypertension	Preeclampsia
N	10	10	8
Age (years)	24.0 [17.0-32.0]	25.4 [21.0-34.0]	25.38 [17.0-32.0]
Self-declaration of color (White) (%)	70.0	90.0	87.5
Primiparity (%)	50.0	40.0	**75.0**
Smoking (smoker) (%)	10.0	0	12.5
GA Sampling (weeks)	37.8 [36.0.-39.0]	38.0 [34.0-40.0]	**31.0 [27.0-39.0]** *†
SBP (mmHg) at sampling	111.6±11.7	**120.0 ±14.0***	**138.25±18.25 ***
DBP (mmHg) at sampling	70.6 ± 6.9	**81.0 ±7.1***	**84.13 ±7.86 ***
BMI (Kg/m^2)	28.38 ±3.1	30.03 ±3.8	29.63 ±5.1
Hemoglobin (g/dL)	11.1 ±2.2	11.8 ±1.8	11.6±1.3
Hematocrit (%)	34.47 ±6.2	35.1±5.0	35.0 ±3.85
Platelets (x10^3/mm^3)	252.2±90.4	201.0±56.6	201.1±57.9
Sodium (mmol/L)	N/D	138.1±1.5	138.3±3.3
Potassium (mmol/L)	N/D	3.8±0.4	4.6±0.4
Urea (mg/dL)	N/D	13.7±5.0	28.5±11.7
AST (U/L)	N/D	20.0 [8.0-31.0]	29.25 [15.0-55.0]
ALP (U/L)	N/D	15.3±8.1	32.6±16.7
Creatinine (mg/dL)	N/D	0.6 [0.5-0.9]	**0.81± [0.7-1.0]**†
24 h-Pr	N/D	152.4±3.6	**3681.5± 3075.9**†
Women taking methyldopa (%)	N/A	70	87.5
Women taking nifedipine (%)	N/A	20	25
Nonresponsive to antihypertensive therapy (%)	N/A	0	**75**
GA delivery (weeks)	40.2 ±1.5	39.8 ±1.3	**32.0 ±5.0** *†
Newborn weight (g)	3408 ±379.6	3395.0±360.0	**1541±948.2** *†
Placental Weight (g)	605.5± 146.9	620±74.0	**390±140.2** *†
APGAR Score 1 (1 min) <7	9.0	7.0	7.0
APGAR Score 2 (5 min) <7	10.0	9.0	**6.0 ***

Table 1- Clinical characteristics of pregnant women involved in the study. N/D, not determined; N/A, not applicable; GA, gestational age; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; AST, aspartate aminotransferase; ALP, alanine aminotransferase; 24 hr-Pr, 24-hour proteinuria. Values in mean ± standard deviation

for parametric data and median [minimum and maximum] for nonparametric data; frequency (percentage) for categorical variables; differences were considered significant when $p<0.05$, and * indicates that the value was significant in PE versus HP and † indicates difference versus GH.

Validation of the endothelial cells profile and evaluation of classical SS genes

HCAECs were subjected to LSS or OSS to check the effect of the plasma pools from each study group on global gene expression in HCAECs. In **Supplementary figure 1a**, we evaluated the expression levels of classic SS response markers, such as *HIF1A*, *CCL2*, *ICAM1*, *VCAM1*, *SELE*, *PECAM1*, *CDH5*, *KLF4*, *KLF2*, and *NOS3*, in our CT samples. In accordance with what has been reported in the literature, our LSS samples presented increased levels of *NOS3*, *KLF2*, *KFL4*, *CDH5*, and *PECAM1*, as well as decreased expression of *HIF1A*, *CCL2*, *ICAM1*, *VCAM1*, and *SELE* in comparison to OSS.[32–37]

Moreover, we evaluated the morphology and alignment of the cells right after the end of 48 hours of treatment in the Ibidi system to verify the alignment of the cells when exposed to LSS (**Supplementary figure 1b**). In all treatments (CT, HP, GH, and PE), cells subjected to LSS showed alignment in the direction of flow (indicated by the arrow in the lower right corner of the figure). Cells submitted to OSS showed a "cobblestone-like" morphological appearance. This endothelial cell shape is characteristic of proatherogenic areas of vessels where OSS occurs, such as bifurcation areas, and was observed across all treatments.[38] There were no visible morphological changes between plasma treatments and CT in both SS conditions.

We have also evaluated the difference of the aforementioned classical markers between CT samples and *plasma* treatments for both LSS (**Supplementary figure 1c**) and OSS (**Supplementary figure 1d**) average expression. In LSS, *NOS3* and *ICAM1* were increased in both GH and PE in comparison to CT and HP. PE also had increased expression levels of *KLF4*, *VCAM1*, *ICAM1*, and *CCL2* in comparison to all other groups in LSS. There were no differences in *KLF2* and *SELE* expression and only HP had an increased expression of *HIF1A* and *CDH5* in comparison to CT and GH in LSS.

In OSS samples, PE had a decreased expression of *NOS3* in comparison to all other groups. *KLF2*, *KLF4*, *PECAM1* and *ICAM1* were increased in all the groups treated with plasma in comparison to CT with HP *KLF2* expression also being increased in comparison to GH and PE. *SELE*, *VCAM1* and *CCL2* expression were increased in CT in comparison to the plasma groups with GH presenting also decreased expression of *CCL2* in comparison to HP and PE. *CDH5* was increased in HP and PE in comparison to CT and *HF1A* had no difference in expression between groups.

Briefly, we have found that GH and PE present an increase in *NOS3* expression in LSS, which can be a compensatory mechanism for the increase in *KLF4* expression observed in PE treated cells. However, there is an induction of classical inflammatory markers of PE plasma treated cells, such as *VCAM1*, *ICAM1*, and *CCL2*. This increase, which can be indicative of ED, happens even in a protective environment of LSS and is still consistent in comparison to HP and GH treated cells. In OSS, there is a decrease in *NOS3* expression only for PE plasma treated HCAECs, which can be an indicator of increased ED, since OSS is already associated with ED and *NOS3* reduced expression. The reduction of *SELE*, *VCAM1* and *CCL2* can indicate the existence of compensatory mechanisms associated with plasma incubation, and this response can be associated with the increase in the expression of *KLF2*, KLF4, and PECAM1 observed in all plasma treated groups. However, there is still an increase in *ICAM1* expression in all groups in comparison to CT.

The effect of different plasma treatments on HCAECs global transcriptome

In order to evaluate the global transcriptome alterations in the HCAECs exposed to maternal plasma in both LSS and OSS we performed a principal component analysis (PCA) (**Figure 1a** and **1c**, respectively). In the LSS samples PCA the sum of PC1 and PC2 represent 33.6 % of the expression variance between groups, while in OSS they represent 52.8% of the total expression variance. In LSS PC1 explains the difference between CT and plasma treated groups, consisting of 19.4% of the variance. Equally important, PC2 mainly

explains the difference between GH and the three other groups, as well as the difference between HP and PE. Moreover, in OSS, PC1 represents 42% of the expression variance, which is mainly explained by the difference between the CT group, and the three groups treated with plasma. OSS PC2 mainly explains the difference (10.8%) of variance between HP and the two groups treated with plasma associated to hypertensive disorders. We have used GSVA package and MSigDB hallmark gene set collection for the whole transcriptome enrichment analysis of all samples submitted to both LSS and OSS (**Figure 1b** and **1d**, respectively), and significant pathways and statistics are summarized in **Supplementary tables 1** and **2**. In LSS (**Figure 1b**), endothelial cells treated with HP plasma compared to CT, had decreased scores for genes associated to the hallmark lists of E2F targets and G2M checkpoint and an enhanced score for interferon alpha (INF- α) response and reactive oxygen species (ROS) pathway. When comparing cells treated with PE plasma to CT, we observed a reduction of genes associated to the hallmarks list of MYC targets (v1) and increased expression of genes associated to TNF-α signaling via NF-κb, INF- α response and Hedgehog signaling. The GH plasma treated group had no significant differences in relation to CT samples, but when compared to HP plasma treated samples it presented an increase in the hallmarks lists of genes associated with E2F targets and G2M checkpoint. PE plasma treated endothelial cells had no significant differences to the cells treated with HP plasma regarding hallmarks lists, but had a decreased score of genes associated to E2F and MYC (v1) targets, and an increased score associated to TNF-α signaling via NF-κb in comparison to GH.

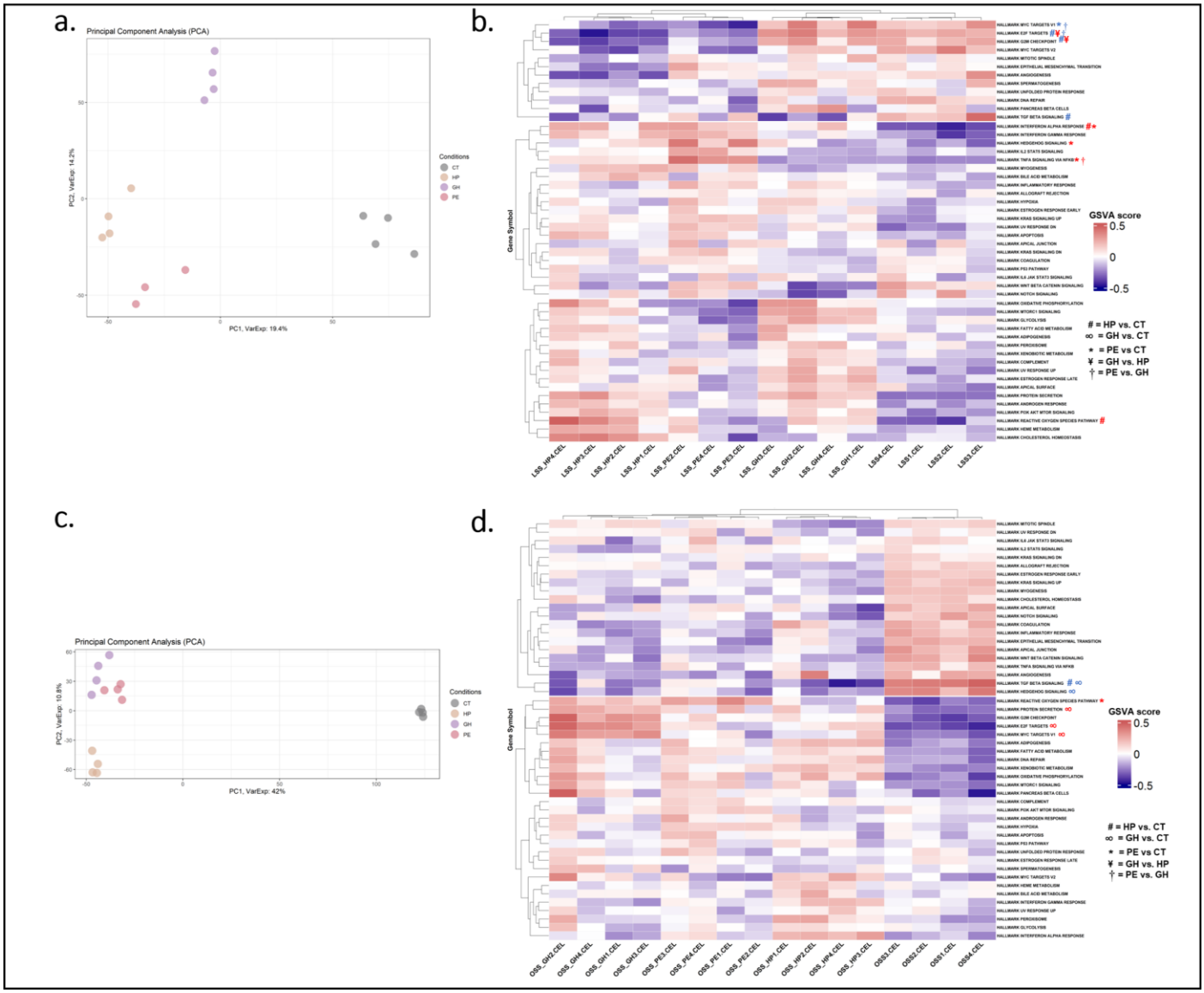

Figure 1- Global gene expression analysis. A) Principal component analysis (PCA) for LSS: presents the PCA of global gene expression profile from each sample of each group. PC1 explained 19.4% of the expression variation between groups while PC2 explained 14.2%. **B) LSS global expression enrichment analysis:** GSVA enrichment of

LSS global expression using MSigDB hallmark gene set collection. Statistically different pathways are represented in the figure for each group according to the legend. **C) Principal component analysis (PCA) for OSS:** presents the PCA of global gene expression profile from each sample of each group. PC1 explained 42% of the expression variation between groups while PC2 explained 10.8%. **D) OSS global expression enrichment analysis:** (**Figure 1d**), endothelial cells treated with HP plasma had reduced GSVA score for the list of genes associated with TGF-β signaling in comparison to CT cells. GH plasma treated HCAECs also had a decreased score for TGF-β signaling, as well as hedgehog signaling, and decreased scores for E2F and MYC (v1) targets and G2M checkpoint. PE treated cells presented an increased score for ROS in comparison to CT cells. There were no significant altered pathways for the comparisons between cells treated with the three different maternal plasma.

In summary, these results indicate that, in LSS condition, PE and HP treated endothelial cells have a very similar global expression, while GH presents a greater difference in comparison to the last two. Our enrichment results indicate that this difference resides mainly in the increased proliferation process in both GH and CT groups, evidenced by the increase in E2F, MYC and G2M pathways. HP treated cells seem to have an increase in inflammation, evidenced by the increased score of INF- α response in comparison to CT. However, PE treated cells present an even more inflammatory/dysfunctional enrichment profile with increased scores for not only INF- α response, but also TNF-α signaling via NF-Kb and Hedgehog signaling, which when over activated can lead to endothelial injury. In OSS, both HP and GH had decreased scores for TGF-β signaling in comparison to CT, which can be mediated by an increase in VEGF.[39] Furthermore, in contrast to LSS results, GH treated cells presented reduced scores for proliferation in comparison to CT cells exposed to OSS, and PE treated cells have increased ROS compared to CT, which can indicate extended damage in an already dysfunctional status.

DEGs and biological pathways associated with each condition.

Using CT samples as reference, we compared the HCAECs exposed to each SS stimulus and the plasma treatment to evaluate differential gene expression between groups. In LSS, we had 55 DEGs for endothelial cells treated with HP plasma, 14 DEGs for cells treated with GH plasma, and 50 DEGs for cells treated with PE plasma (**Figure 2a**). There were 32 genes that were uniquely differentially expressed in HP plasma treated cells, 4 genes uniquely expressed in GH, and 27 genes uniquely expressed by PE plasma treated endothelial cells in comparison to the CT cells.

Of the unique genes with increased expression in HP treated cells we have an overall cytoprotective antioxidant profile mediated by the increased level of *ALDH3A1,* and reduced expression levels of genes associated to endothelial proliferation, such as *DLGAP5*, *RRM2*, *AURKB*, *MYBL2*, and *MKI67*.[40–44] [REF] HP plasma treated cells also presented increased levels of *LYVE-1*, a marker of lymphatic endothelial cells.[45] Although not expected this process of blood vascular cells to lymphatic endothelial cells phenotype transformation have been reported to happen *in vitro*, and is considered reversible. Regarding the most regulated unique genes associated to GH plasma treatment we found increased levels in *ENTPD2*, *AOX1*, and *SCN9A*, which are associated to hypoxia-mediated angiogenesis, aldehyde oxidation regulated by Nrf2, and cell migration in endothelial cells of rat injured aortas, respectively.[46–48] Furthermore, GH plasma treated cells also presented reduced expression of *CADN11*, which is associated with angiogenesis.[49] In PE, top unique upregulated genes include *CHAC1* and *SLC7A5*, which encode proteins associated with endoplasmic reticulum stress and apoptosis, *PTGDS*, whose increased expression is associated with prostaglandin D2 production by the endothelium, a vasodilator associated with inflammatory profile.[50] There were also increased levels of *PSAT1*, a gene that encodes a protein associated serine biosynthesis pathway that protects endothelial cells from ROS, and is also associated with increased nitric oxide levels and is protective against endoplasmic reticulum stress.[51] *INHBB* expression levels were also increased in PE plasma treated HCAECs, although there is little information on the role of this gene in endothelial cells, polymorphisms of this gene are associated with PE and eclampsia development.[52] Moreover, top down-regulated genes included *NOX4*, *FAM11B* and *TENT5A*, whose reduced levels are associated with decreased endothelial cells proliferation and metabolic homeostasis.[53–55]

Next, we have enriched the complete set of DEGs from each group utilizing two enrichment databases, Bioplanet (2019) and MSigDB (2020). Results are represented in **figures 2b** and **2c**, respectively.

HP plasma treatment reduces endothelial cell proliferation in LSS.

Evidenced by multiple pathways in both enrichment databases (e.g., FOXM1 transcription factor network, cyclin A/B1-associated events during G2/M transition, E2F networks, mitotic spindle), genes associated to endothelial proliferation are markedly reduced in HP plasma treated HCAECs when compared to CT. Although LSS is already considered to promote endothelial cell quiescence, this protective feature, which is associated with cellular homeostasis, is further exacerbated by HP treatment.[26,56,57] Quiescence is not quantifiable, however the reduced expression of this set of genes might indicate reduced proliferation, motility, and increased resistance to apoptosis, meaning that endothelial cells are less susceptible to signals associated to endothelial cell injury, which promotes angiogenesis or wound healing.[26] Some of the genes associated with this profile that were most altered are *DLGAP5*, *RRM2*, *AURKB*, *MYBL2*, and *MKI67*, and all of them were previously associated with endothelial proliferation.[42]

Inflammatory response is increased in all plasma treated groups, but the increase is more consistently in PE groups under LSS.

The inflammatory response is also evidenced by multiple pathways. Some of these pathways were significant to the three plasma treatments (e.g., Interleukin-1 processing, and cytokines and inflammatory response), or to only GH and PE plasma treatment (e.g., adhesion and diapedesis of lymphocytes, and cells and molecules involved in local acute inflammatory response). Some pathways were exclusively associated with PE (e.g., cytokine-cytokine receptor interaction, and MSigDB inflammatory response pathway). However, even when inflammatory pathways were shared with the other two plasma treatments, PE-treated HCAECs had a greater score for them, which indicates they have more DEGs associated with this process. The only DEG shared by all conditions was *IL1A*. Moreover, some classic genes associated with inflammatory processes, such as *CCL1*, and *IL1B* were differentially expressed in cells treated with both HP and PE plasma. Interestingly, PE-treated cells also presented increased levels of classic inflammatory genes, namely *VCAM1*, *CXCL8*. The IL-1 system components, *IL1A* and *IL1B*, and their signaling is associated with severe acute or chronic inflammation.[58] Indeed, it was not expected to find this proinflammatory profile in the HCAECs treated with HP plasma. More interestingly, PE plasma induced the expression of *CXCL8* in endothelial cells, corroborating with a previous *in vitro* study with an endothelial cell lineage showing a 3-fold increase in interleukin-8 (IL-8) levels in comparison to HP and non-gravid healthy plasma, and that was associated to increased transendothelial migration of neutrophils.[59]

Besides classical markers of inflammatory response, PE plasma also induced alterations in genes involved in the oncostatin M (OSM) pathway, which is a relatively unknown cytokine that has been associated to endothelial activation.[60,61] This pathway is also marked by increases in *CCL1* and *VCAM1*, besides *ANGPT2*, which can be associated with angiogenesis and the induction of inflammation by increasing endothelial cells sensitivity to TNF- α.[60–62]

Endoplasmic reticulum stress induced apoptosis is increased in PE plasma treated HCAECs under LSS.

This phenotype was exclusively associated with PE plasma treated HCAECs and might be associated with augmented inflammatory response previously described. It is evidenced by the pathways of TGF- β regulation, PERK-regulated gene expression, RAGE, TNF-alpha effects on cytokine activity, cell motility and apoptosis, interleukin 4 and 5 regulated apoptosis and unfolded protein response. These pathways are marked by increased expression of *CHAC1*, *ATF3*, and *SLC7A5* which are genes reportedly associated to inflammatory apoptosis in endothelial cells exposed to oxidized lipids.[63] Moreover, PERK trans-autophosphorylation activates and phosphorylates the eukaryotic translation initiation factor 2 alpha (eIF2) and the transcription

factor NRF2 in response to ER stress, which induces a reduction in global translation, lowering the folding load on the ER.[64] The PERK pathway is also marked by increases in *ASNS* gene expression, whose expression is also increased by PE plasma treatment.[65]

Furthermore, this process is also associated with the RAGE pathway. The genes involved with this pathway (e.g., *FABP4* and *IL1B*) evidence a possible specific profile of metabolic dysfunction, such as insulin resistance, that might be associated to PE.[66,67]

Endothelial to mesenchymal transition is increased in HCAECs treated with PE plasma in LSS.

EndMT is also regulated by inflammatory process, mainly TGF-β induced inflammation, although TGF- β alone might not be sufficient to induce and sustain EndMT.[68] In Bioplanet enrichment it was demonstrated that PE group was associated to TGF-beta regulation of extracellular matrix and EndMT pathway was evidenced in MSigDB enrichment. This process is evidenced in PE plasma treated endothelial cells by the increased expression of *PDGFRB2*, which is a potent inducer of EndMT.[68] However, PE plasma, as well the other two plasma treatments, reduced *VCAN* expression, which is a marker of mesenchymal cells.[69]

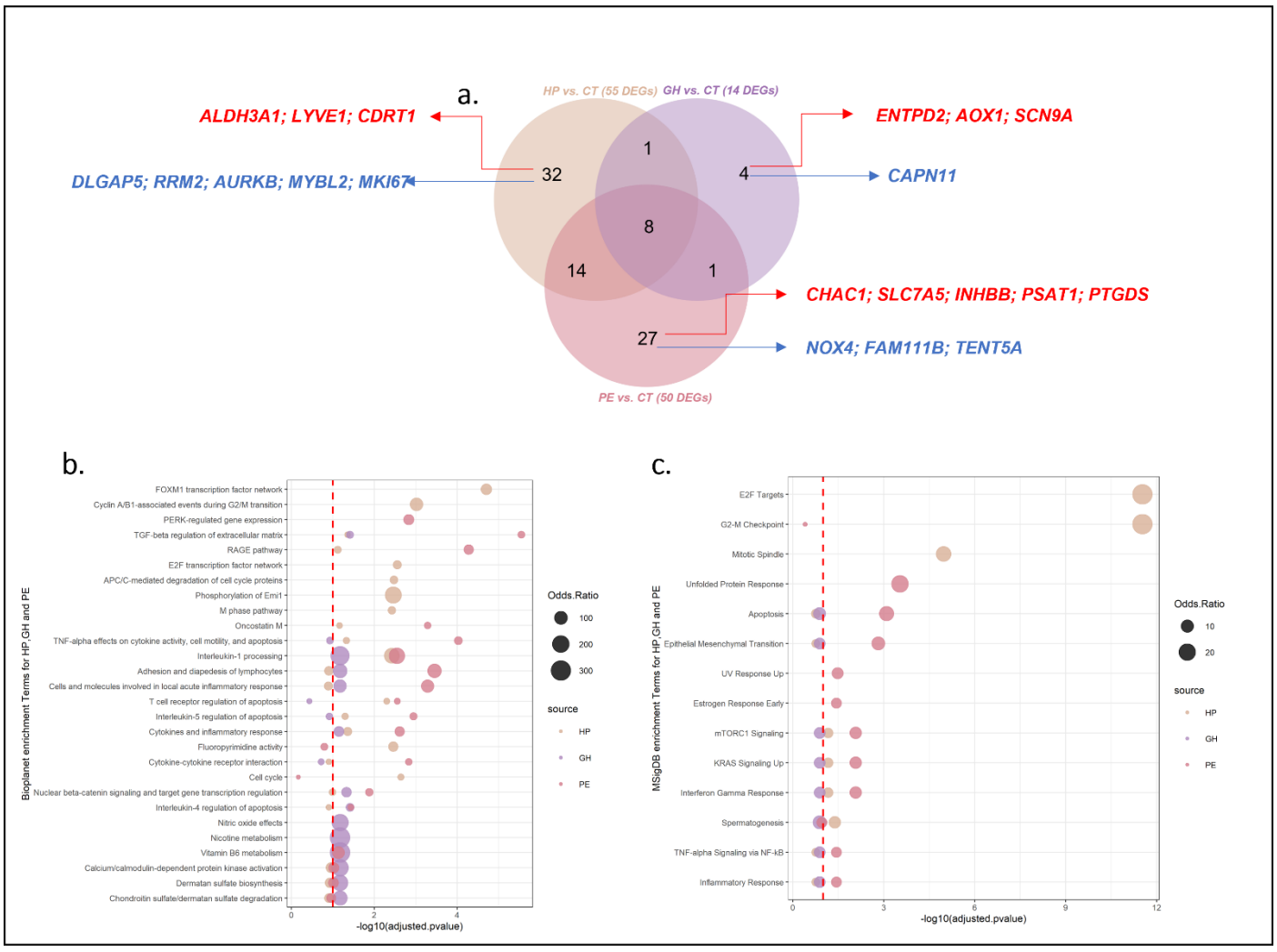

Figure 2 - LSS DEGs analysis. A) Venn diagram of DEGs from each treatment: genes were considered DEGs when adjusted p-value ≤ 0.05 and |Log2foldchange| ≥ 1.25; in total there were 55 DEGs for HP, 14 DEGs for GH, and 50 DEGs for PE, all in comparison to CT. Top up and down regulated genes are represented for each **B) Bioplanet enrichment for HP, GH, and PE plasma treatments *versus* CT in LSS:** depicts the enrichment results for the three plasma treatments in comparison to CT. We used the Bioplanet library to get insights of the overall biological function of the genes associated with each plasma cellular response. **C) MsigDB enrichment for HP, GH, and PE plasma treatments *versus* CT in LSS:** depicts the enrichment results for the three plasma treatments in comparison to CT. Pathways presented were all considered significant (adjusted p-value of <0.1). We used the MsigDB hallmark library

to get insights of the overall biological function of the genes associated with each plasma cellular response. Treatment comparison is indicated by color as indicated in the legend. Pathways presented were all considered significant (adjusted p-value of <0.1). To better visualize both enrichment analysis, pathways are ordered according to $-\log_{10}$ of the adjusted p value and the enrichment score. The dashed red line indicates the established limit of significance.

For OSS, we had 265 DEGs for endothelial cells treated with HP plasma, 251 DEGs for cells treated with GH plasma, and 173 DEGs for cells treated with PE plasma (Figure 3a). 80 genes were unique to the HP plasma treated group, 54 were unique to GH, and 13 were unique to PE plasma treated cells. In total, we had 126 DEGS shared by the three groups.

For the unique genes of each condition, we have selected the top 5 up and down regulated genes for each plasma treatment in comparison to CT in OSS. The genes up regulated in HP plasma treatment includes *P3H2*, *MICAL1*, and *SPARCL1*, which are involved with angiogenesis process, as well as *SEC16A*, that is involved with endoplasmic reticulum cargo traffic, and *FGD1* that is involved with TGF-β- induced matrix degradation.[70–74] Top down-regulated HP plasma associated genes included *AQP1*, which when down-regulated is associated with increased endothelial permeability, *ADAMTS4*, that has its increased levels associated with CVDs and atherosclerosis, and *SLC26A4*, *PIFO* and *SAMD12*, that lack information regarding their activity in endothelial cells.[75,76] GH plasma treatment exclusively augmented the expression of *MET1E*, *GPX3*, *FAM107A*, *ZNF1175* and *H2AC13*, while reducing the expression of *DENNDB*, *FBXO32*, *KIT*, *ADAM19* and *RHOD*. *GPX3* has an antioxidant role in disturbed flow regions of porcine aortas, where it coexists with pro-inflammatory genes.[77] *KIT* encodes a transmembrane receptor with tyrosine-kinase1 activity structurally related to platelet-derived growth factor receptor, and macrophage-stimulating factor receptor. Studies have shown that reduced expression levels of this gene are associated with increased levels of IL-1 α and β.[78,79] *RHOD* is associated with endothelial cell motility.[80] PE plasma top 5 upregulated genes include *RRAGD*, *GGA2*, *FBXO33*, *ZNF420*, *SELENOI*, and down regulated genes include *POSTN*, *ADAMTS10*, *TRPM4*, *LRP5*, and *ZNF687*. There were reports that associated *POSTN* and *LRP5* reduced levels to an angiogenic endothelial phenotype and associated its expression levels reduction to inhibition of HIF-1α.[81,82] TRPM4 is associated to ROS modulated cell death in endothelial cells.[83]

The enrichment analysis using both Bioplanet and MsigDB was also performed for these samples and results are represented in **figures 3b** and **3c**, respectively. Significance was defined when adjusted p value was greater than 0.1 (dashed redline in plots). However, for these enriched pathways, gene expression was very homogenous between groups and few genes had a great difference in fold change values although they were considered significant to only one or two of the groups. Therefore, even though they were considered statistically significant we are going to explore the pathways that were associated with relevant gene alteration.

Endothelial to Mesenchymal transition phenotype is altered in all plasma treated groups when compared to CT in OSS.

This process was considered significant for all plasma treated groups in comparison to control and most gene expression alterations seem to be indicative of a protective phenotype for EndMT. However, *RGS4*, whose augmented expression is considered to reduce EndMT is only significantly increased in HP and GH plasma treatments in comparison to CT and not in PE. Moreover, augmented expression is associated to an antihypertensive effect linked to significantly reduced Angiotensin II and endothelin 1 effect.[84] *POSTN* is also associated to a protective phenotype, where its reduced expression enhanced hemodynamic and cardiac responses, inhibited growth factor and hypoxia-inducible factor (HIF-1) release, and reversed downregulated BMPR (bone morphogenetic protein receptor)-2 expression in pulmonary artery endothelial cells from pulmonary hypertension patients and in mice model.[81] This gene was exclusively downregulated in PE treated HCAECs which might indicate a compensatory mechanism activated in the PE plasma.

GH and PE plasma treated HCAECs present increased inflammatory response associated with IL1B and SERPIN2B expression.

Although a great part of the genes associated with increased inflammatory pathways (e.g., inflammatory response, TNF-α signaling via NF-κB) were down regulated in all plasma treatments, both GH and PE

presented increased levels of *IL1B* and *SERPINB2*. Enhanced endothelial IL-1β expression has already been reported in atherosclerotic coronary arteries and it is believed that several inflammatory cytokines, including IL-1β itself, can stimulate the vascular endothelium to produce IL-1β in an autocrine cycle, leading to functional alterations of the endothelium such as suppression of endothelial cell proliferation, increased expression of adhesion molecules, favoring coagulation and thrombosis.[85–87] Considering that OSS already causes a markedly increased expression of IL-1β in endothelial cells, this augmentation in HCAECs treated with the plasma of the two groups associated to hypertensive phenotype is even more interesting.[88]

Furthermore, *SERPINB2* expression, which is augmented by GH an PE plasma in OSS, has been linked to proteasome interaction in activated endothelial cells, and its endothelial release may be induced in response to a variety of stimuli, including LPS, thrombin, and low-density lipoprotein.[89]

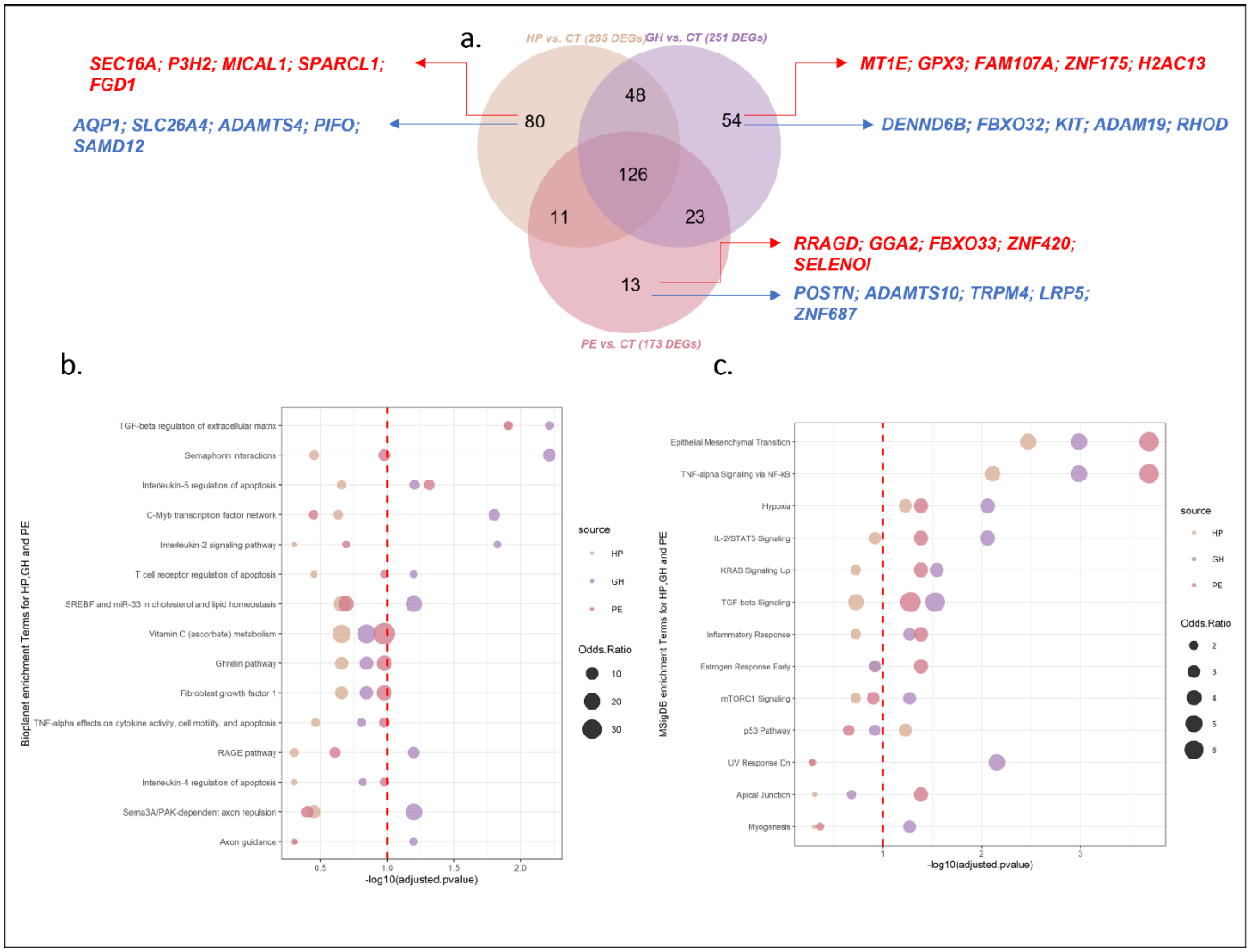

Figure 3 - OSS DEGs analysis. A) Venn diagram of DEGs from each treatment: genes were considered DEGs when adjusted p-value ≤ 0.05 and |Log2foldchange| ≥ 1.25; in total there were 55 DEGs for HP, 14 DEGs for GH, and 50 DEGs for PE, all in comparison to CT**. B) Bioplanet enrichment for HP, GH, and PE plasma treatments versus CT in OSS:** depicts the enrichment results for the three plasma treatments in comparison to CT. We used the Bioplanet library to get insights of the overall biological function of the genes associated with each plasma cellular response**. C) MsigDB enrichment for HP, GH, and PE plasma treatments versus CT in OSS:** depicts the enrichment results for the three plasma treatments in comparison to CT. Pathways presented were all considered significant (adjusted p-value of <0.1). We used the MsigDB hallmark library to get insights of the overall biological function of the genes associated with each plasma cellular response. Treatment comparison is indicated by color as indicated in the legend. Pathways presented were all considered significant (adjusted p-value of <0.1). To better visualize both enrichment analysis, pathways are ordered according to -log10 of the adjusted p value and the enrichment score. The dashed red line indicates the established limit of significance.

Discussion

Our study combined *in vitro* and *in silico* strategies to expand the understanding of ED associated with PE and GH conditions. We have employed plasma samples from HP, GH and PE patients to evaluate the effects of the circulating factors associated with each pathological or physiological state, in HCAECs exposed to two mechanical forces regimes: 1) an endothelium-protective flow pattern, LSS; and 2) a flow pattern that contributes to pathological situations, OSS. With that, we have demonstrated for the first time that the global transcriptome of HCAECs is influenced by plasmas in a blood flow pattern codependent way. For example, in LSS exposure, all treatments differed from each other and from CT, with PE and HP having a more similar global transcriptome profile while also keeping their singularities. However, in OSS exposed HCAECs, PE and GH have a more similar transcriptome profile while HP presents a more singular pattern.

In silico analysis gave us further information on the global expression and DEGs associated biological profile. In both analyses, the enrichment results were consistent, indicating that treatment with HP plasma reduces HCAECs proliferation and increased inflammatory processes despite blood flow regimen in all endothelial cell groups treated with plasma. However, the proinflammatory profile was more distinguished in PE plasma treated cells because it was associated with a larger number of altered inflammatory markers and accompanied by processes associated with inflammatory response, like endoplasmic reticulum stress and EndMT. Although it seems contradictory to find such results in a protective LSS ambient, they are consistent with clinical observations of PE and GH women. For example, until today, it is unclear whether GH has a distinct etiology from PE or whether both conditions are part of a common phenomenon.[90] Also, GH and PE have similar risk factors and share a close probability (of 39% and 32%, respectively) of developing hypertension after approximately two and a half years from the end of gestation (compared to 1% in HP).[11] However, the risk of adverse outcomes, such as preterm birth, is approximately 3 times higher in PE compared to GH, and may be associated with differences with respect to the inflammatory signature profile of each condition, with GH having a more effective compensatory profile compared to PE.[91,92] Moreover, a previous study that evaluated the effect of PE plasma in static Human Umbilical Vein Endothelial Cells (HUVECs) also indicated increased expression of genes associate to apoptosis end endoplasmic reticulum stress.[17] The inflammatory response in PE treated plasmas under LSS was associated with *IL1A*, *IL1B*, *CXCL8*, and OSM, which were all reported to be increased in the plasma/serum of PE patients alongside circulating levels endothelial inflammatory markers, such as *CCL1* and *VCAM1*.[93–95]

Our findings of PE plasma associated endothelial endoplasmic reticulum stress in LSS can be associated to its increased inflammatory profile, as evidenced by augmented expression of *CHAC1*, *ATF3*, and *SLC7A5*, and/or a metabolic inflammation stress response, evidenced by *FABP4* and *IL1B* and RAGE pathway.[64,66] Although the levels of endoplasmic reticulum stress were reported to be increased in early-onset PE placenta, and in a trophoblast cells lineage treated with PE women serum, This effect has not been observed in endothelial cells until now.[96,97]

EndMT, process was indicated as significant for PE group and this group was marked by increased expression of *PDGFRB2* in LSS, which is a potent inducer of mesenchymal phenotype.[69] However, in OSS, although EndMT was activated for all plasma treatments in comparison to CT, RGS4, a protective gene for this process, was increased only in GH and HP but not in PE plasma treated HCAECs. *RGS4* was also associated to an antihypertensive effect, which makes it even more interesting in a PE modulate ED profile, a context where it has not been explored yet.[84]

We have identified an overall anti-inflammatory profile being modulated by all plasma groups under OSS stimulus. However, PE and GH plasma treatments increased *IL1B* endothelial expression. For PE, the higher *IL1B* response profile was consistent in both LSS and OSS conditions, which is surprising considering that they are associated with the reduction and augmentation of this gene expression, respectively.[88] This indicates that PE plasma might not only induce, but also exacerbate *IL1B* associated inflammatory ED. As mentioned

before, augmented *IL1B* was shown to be localized in atherosclerotic lesions, and its endothelial expression can be modulated by multiple inflammatory pathways, but also in response to circulating levels of IL-1β.[85–87]

In summary, our exploratory study results indicated for the first time that different SS regimes, one protective and the other pathologic, can influence HCAECs response to pregnant women plasma. We have also identified how cells' biological processes are modulated by the three plasma treatments, HP, GH, and PE in comparison to CT. Our data indicates increased inflammatory response of HCAECs in response to PE plasma in both LSS and OSS, indicating that these women's plasma can induce and exacerbate ED. The PE-associated ED was also characterized by increased endoplasmic reticulum stress and EndMT process. We also would like to highlight the consistent upregulation of IL1B expression by PE plasma in both SS conditions, which may bring future insights to PE ED understanding.

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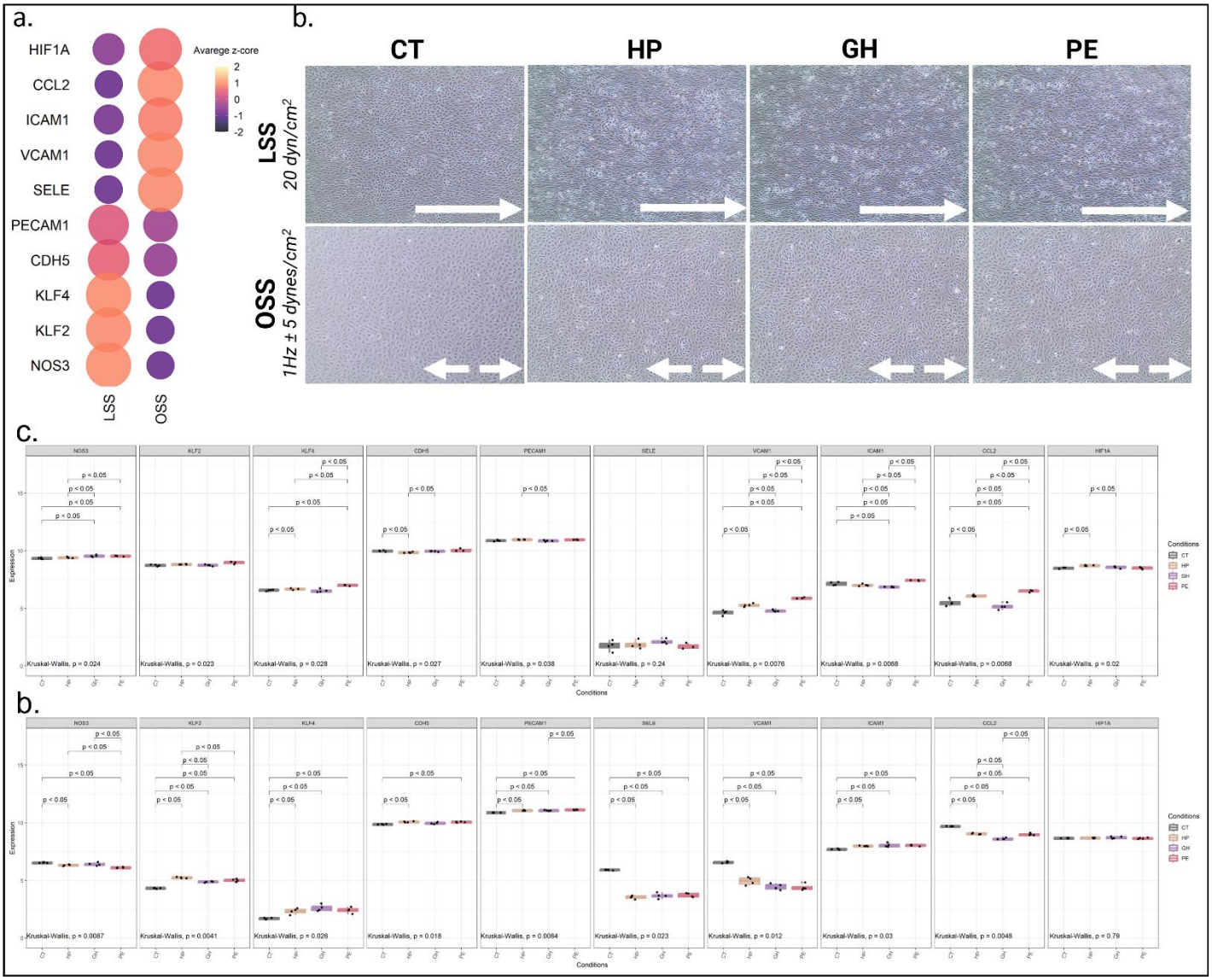
Supplementary file



Supplementary Figure 1- ***Evaluation of the expression of classical shear stress markers and cell morphology. A) Average z-score of SS markers in control samples (cells incubated with complete medium only):*** HIF1A, CCL2, ICAM1, VCAM1 *and* SELE *were increased in OSS stimulus in comparison to LSS while* PECAM1, CDH5, KLF4, KLF2, and NOS3 *were increased in LSS in comparison to OSS.* ***B) Evaluation of endothelial cell morphology according to SS:*** *All endothelial cells with the different treatments submitted to LSS showed alignment in the flow direction, while endothelial cells submitted to OSS showed cobblestone-like appearance morphology.* ***C) Evaluation of the average expression patterns of SS markers across treatments in LSS****:* PE also had increased expression levels of KLF4, VCAM1, ICAM1, and CCL2 in comparison to all other groups in LSS. There were no differences in KLF2 and SELE expression and only HP had an increased expression of HIF1A and CDH5 in comparison to CT and GH in LSS. ***D) Evaluation of the average expression patterns of SS markers across treatments in LSS:*** PE had a decreased expression of NOS3 in comparison to all other groups. KLF2, KLF4, PECAM1 and ICAM1 were increased in all the groups treated with plasma in comparison to CT with HP KLF2 expression also being increased in comparison to GH and PE. SELE, VCAM1 and CCL2 expression were increased in CT in comparison to the plasma groups with GH presenting also decreased expression of CCL2 in comparison to HP and PE. CDH5 was increased in HP and PE in comparison to CT and HF1A had no difference in expression between groups. *CT: control samples; HP: healthy pregnant; GH: gestational hypertension; PE: preeclampsia. * All values were considered significant when $p < 0.05$.*

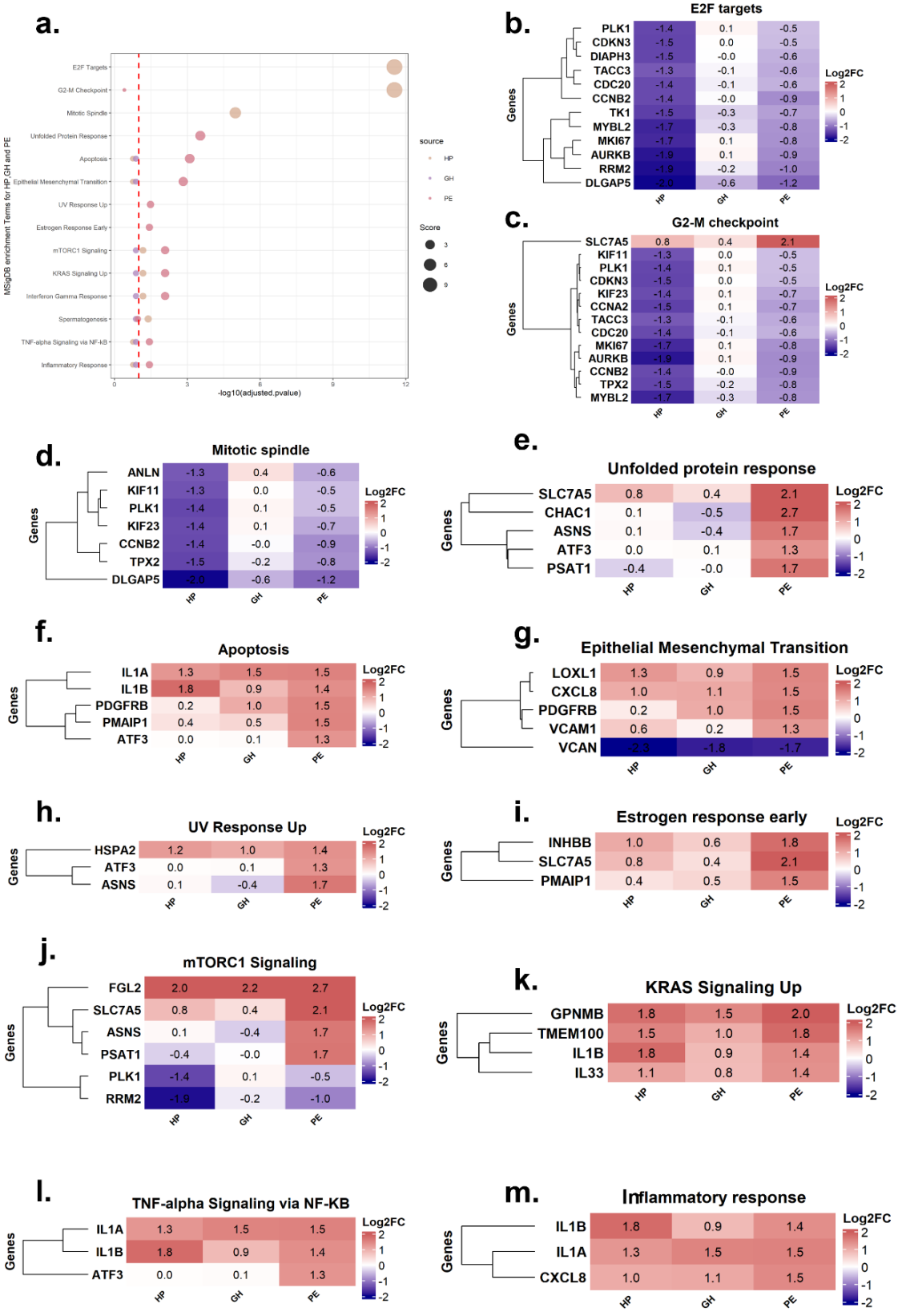
Hallmarks	*LogFC*	*P value*	*Status*
HP vs. CT			
HALLMARK E2F TARGETS	*-0,623922222*	*8,579E-11*	*Down*
HALLMARK G2M CHECKPOINT	*-0,531838101*	*2,05E-08*	*Down*
HALLMARK INTERFERON ALPHA RESPONSE	*0,557389161*	*2,17E-08*	*Up*
HALLMARK REACTIVE OXYGEN SPECIES PATHWAY	*0,565302762*	*2,59E-06*	*Up*
PE vs. CT			
HALLMARK MYC TARGETS V1	*-0,536268522*	*7,51E-07*	*Down*
HALLMARK TNFA SIGNALING VIA NFKB	*0,592201657*	*2,93E-10*	*Up*
HALLMARK INTERFERON ALPHA RESPONSE	*0,564666778*	*6,94E-08*	*Up*
HALLMARK HEDGEHOG SIGNALING	*0,507750047*	*3,34E-06*	*Up*
GH vs HP			
HALLMARK E2F TARGETS	*0,690052181*	*1,00E-11*	*Up*
HALLMARK G2M CHECKPOINT	*0,5531806*	*9,81E-09*	*Up*
PE vs GH			
HALLMARK E2F TARGETS	*-0,532131031*	*9,58E-09*	*Down*
HALLMARK MYC TARGETS V1	*-0,615158234*	*7,26E-08*	*Down*
HALLMARK TNFA SIGNALING VIA NFKB	*0,550813123*	*1,26E-09*	*Up*

Supplementary table 1- Indicates significant GSVA enrichment pathways of LSS global expression using MSigDB hallmark gene set collection. Significance was defined as p-value ≤ 0.05 and $|\text{Log2foldchange}| \geq 0.5$.

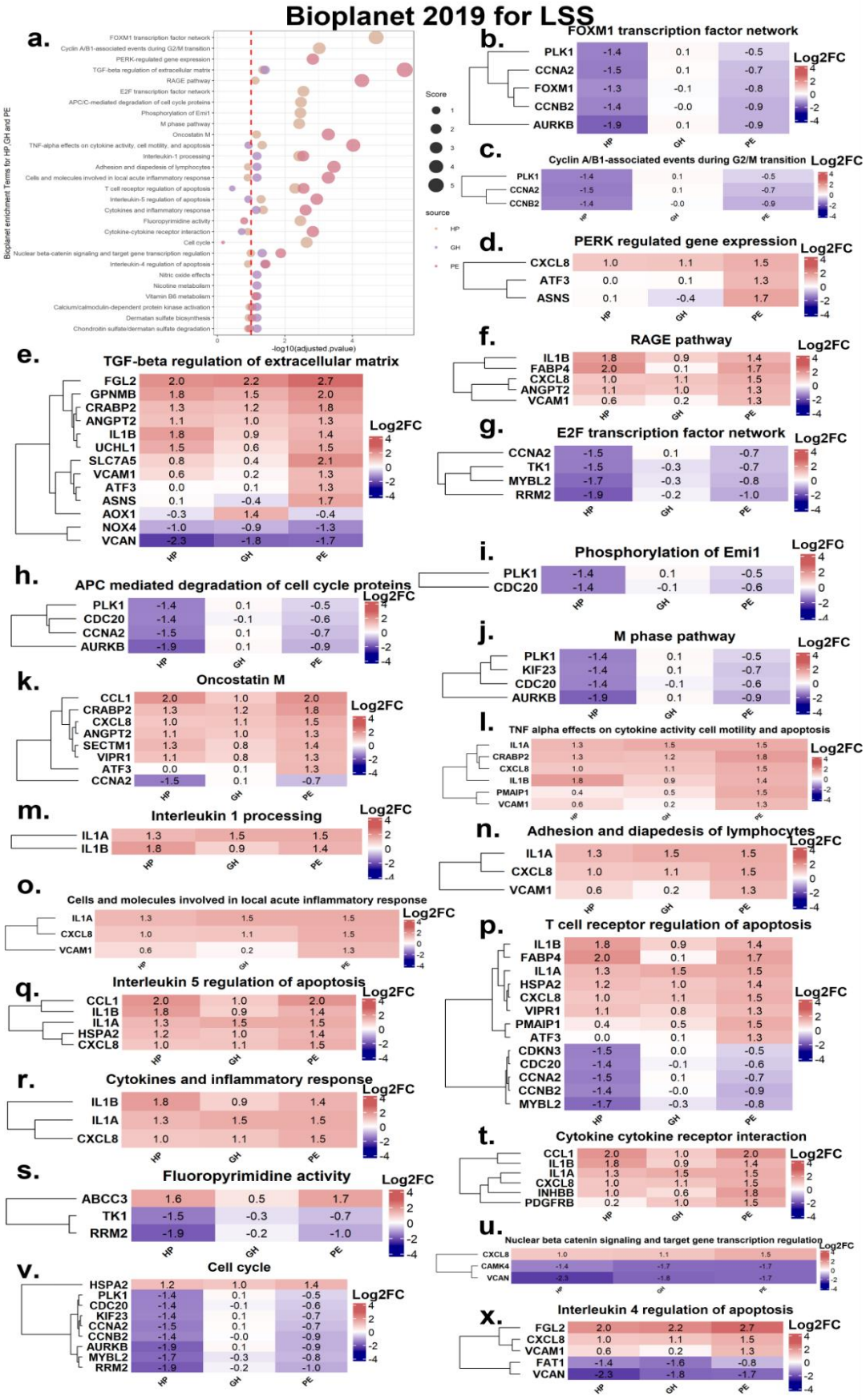
Hallmarks	*LogFC*	*P value*	*Status*
HP vs. CT			
HALLMARK TGF BETA SIGNALING	-0,741117951	5,75E-09	*Down*
GH vs. CT			
HALLMARK TGF BETA SIGNALING	-0,606419207	1,72E-07	*Down*
HALLMARK HEDGEHOG SIGNALING	-0,555589497	2,55E-07	*Down*

HALLMARK E2F TARGETS	0,730408858	4,41E-11	*Up*
HALLMARK G2M CHECKPOINT	0,661518862	8,46E-11	*Up*
HALLMARK MYC TARGETS_V1	0,603995059	6,15E-09	*Up*
PE vs. CT			
HALLMARK REACTIVE OXYGEN SPECIES PATHWAY	0,590832959	4,80E-10	*Up*

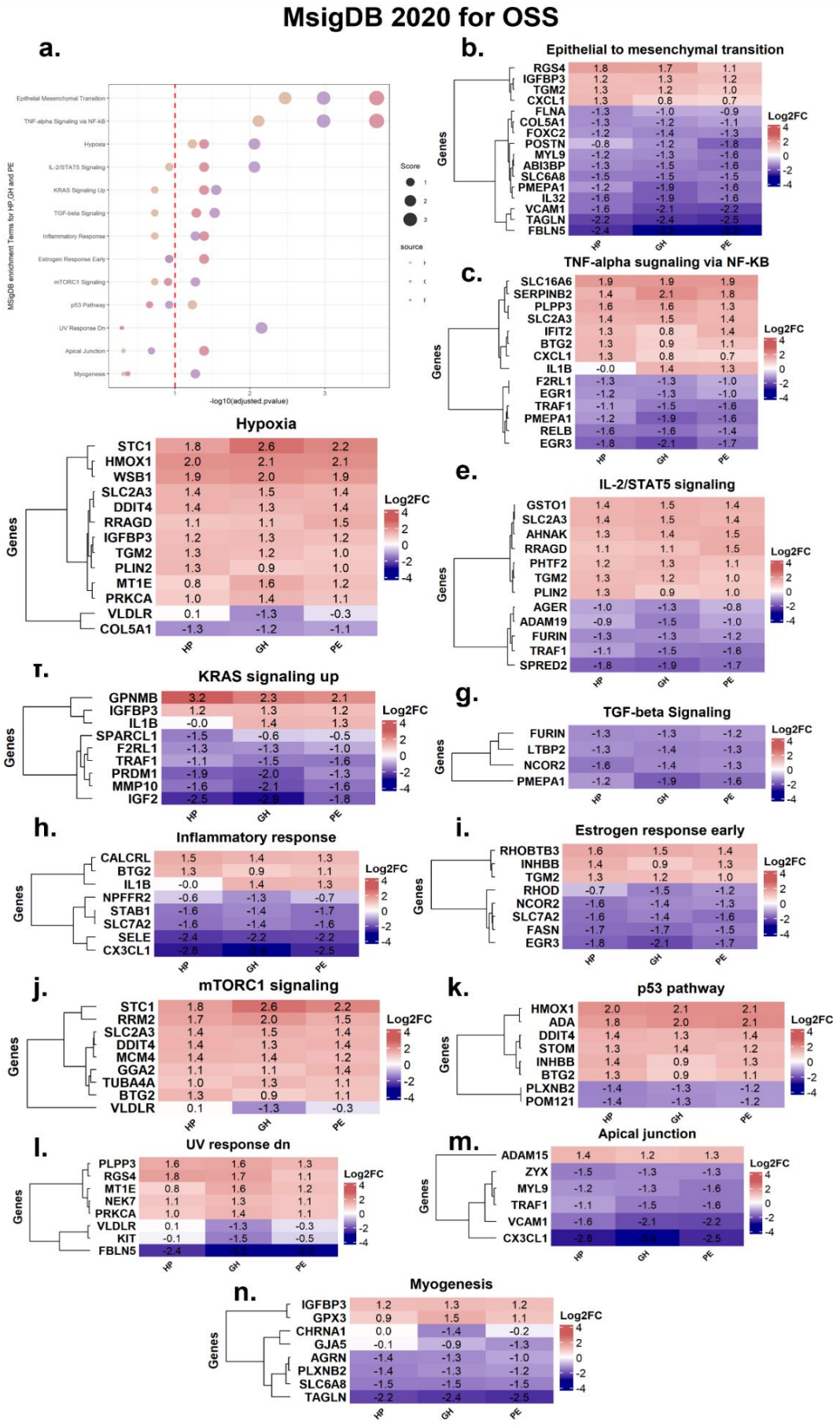
Supplementary table 2- Indicates significant GSVA enrichment pathways of LSS global expression using MSigDB hallmark gene set collection. Significance was defined as p-value ≤ 0.05 and $|Log2foldchange| \geq 0.5$.



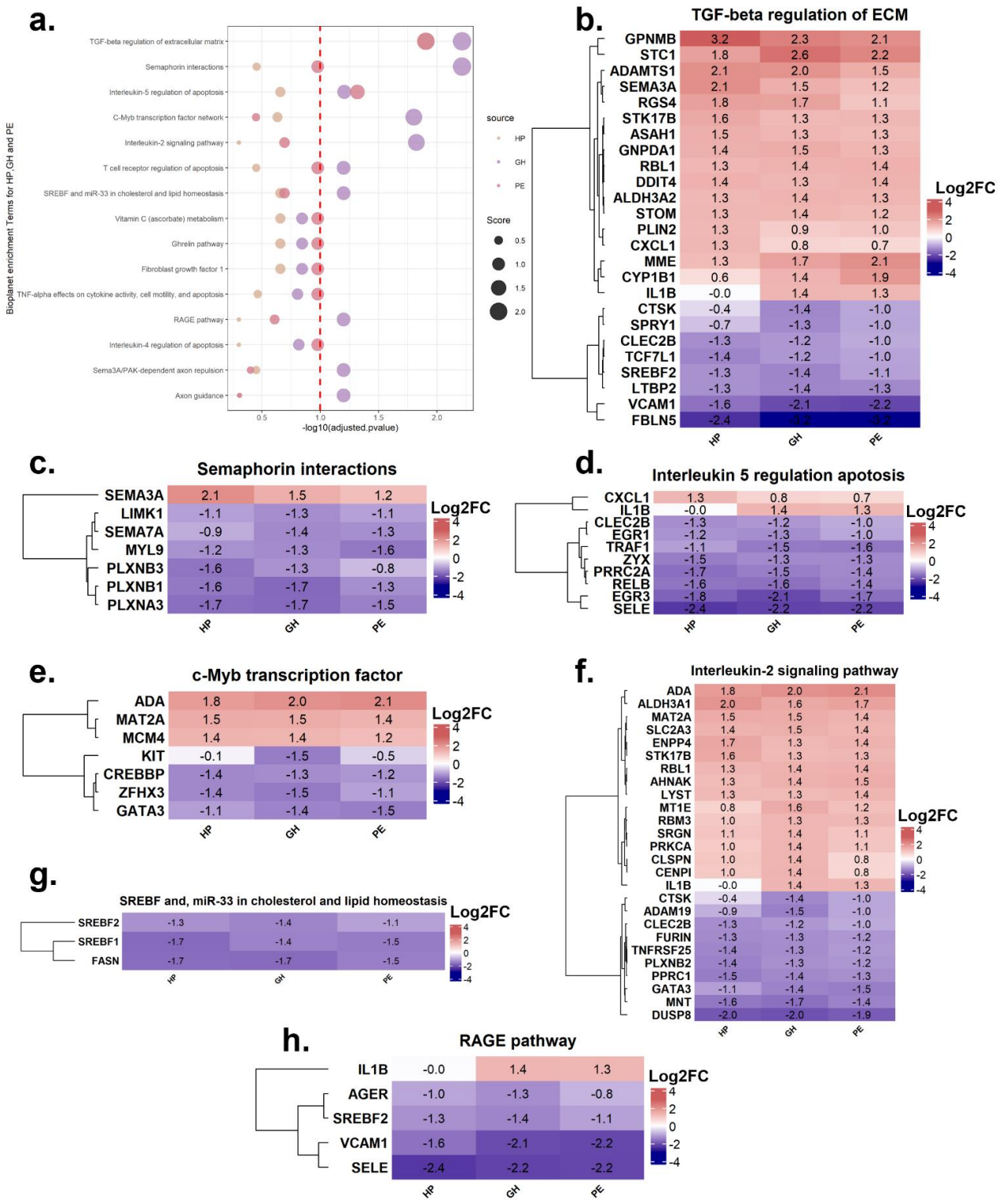
Supplementary Figure 2- MsigDB enrichment pathways for LSS – presents genes involved in each pathway and their respective fold-change.



Supplementary Figure 3- Bioplanet enrichment pathways for LSS – presents genes involved in each pathway and their respective fold-change



Supplementary Figure 4- MsigDB enrichment pathways for OSS – presents genes involved in each pathway and their respective fold-change.



Supplementary Figure 5- Bioplanet enrichment pathways for OSS – presents genes involved in each pathway and their respective fold-change.

Conclusões Finais

Considerando os resultados obtidos em nosso estudo, este trabalho pode elucidar que o efeito de todos os plasmas é influenciado pelo tipo de regime SS. Além disso o plasma de PE induz vias de disfunção endotelial no LSS, e exacerba a disfunção endotelial no OSS em HCAECs. A partir de dados de transcriptoma, identificamos vias moleculares envolvidas com a PE em pelo menos um dos dois regimes de SS, como as vias associadas a respostas inflamatórias, vias de estresse do reticulo endoplasmático e EndMT. Dessa forma, esses resultados possibilitaram a obtenção de novas informações e melhor compreensão dos mecanismos moleculares e celulares que regulam a disfunção endotelial mediada por fatores circulantes no plasma de PE. Além disso, os dados apresentados poderão ser úteis para o desenvolvimento de novas estratégias terapêuticas em pesquisas futuras e nosso estudo poderá orientar pesquisas futuras e melhorar a obtenção de resultados, aproximando modelos *in vitro* com plasma de PE do *in vivo*.

Atividades complementares

Estágio Nacional

60 horas de estágio no laboratório de genética e cardiologia molecular do instituto do coração

Atividade em Docência

Estágio docência de 30 horas na disciplina de farmacodinâmica (1º semestre de 2022) para o curso de Ciências Biomédicas com a Professora Valéria Sandrim.

Artigos publicados em periódicos

NUNES, PRISCILA REZECK ; **MATTIOLI, SARAH VIANA** ; SANDRIM, VALERIA CRISTINA . NLRP3 Activation and Its Relationship to Endothelial Dysfunction and Oxidative Stress: Implications for Preeclampsia and Pharmacological Interventions. Cells, v. 10, p. 2828, 2021.

BERTOZZI-MATHEUS, MARIANA ; BUENO-PEREIRA, THAINA OMIA **; VIANA-MATTIOLI, SARAH** ; CARLSTRÖM, MATTIAS ; CAVALLI, RICARDO DE CARVALHO ; SANDRIM, VALERIA CRISTINA . Different profiles of circulating arginase 2 in subtypes of preeclampsia pregnant women. CLINICAL BIOCHEMISTRY, v. 92, p. 25-33, 2021.

LUIZON, MARCELO R. ; CONCEIÇÃO, IZABELA M. C. A. ; **VIANA-MATTIOLI, SARAH** ; CALDEIRA-DIAS, MAYARA ; CAVALLI, RICARDO C. ; SANDRIM, VALERIA C. . Circulating MicroRNAs in the Second Trimester From Pregnant Women Who Subsequently Developed Preeclampsia: Potential Candidates as Predictive Biomarkers and Pathway Analysis for Target Genes of miR-204-5p. Frontiers in Physiology, v. 12, p. 1, 2021.

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“Missing links in preeclampsia cell model systems of endothelial dysfunction”, **Sarah Viana-Mattioli**, Iguaracy Pinheiro-de-Sousa, Miriam Helena Fonseca-Alaniz, José Eduardo Krieger, Valéria Cristina Sandrim. (Accepted in Cell-Trends in molecular medicine on April 13th 2023)

Artigos submetidos

“In silico network-based screening reveals candidates for endothelial dysfunction therapy”, Iguaracy Pinheiro-de-Sousa, Girolamo Giudice, Miriam Helena FonsecaAlaniz, Silvestre Massimo Modestia, **Sarah Viana Mattioli**, Yun Fang, Evangelia Petsalaki, Jose E. Krieger (Submited to Molecular Systems Biology in November 2022)

“Low lead levels-induced hypertension and endothelial dysfunction in rats are attenuated by sildenafil: evidence of pleiotropic antioxidant effects”, Ediléia Souza Paula Caetano, **Sarah Viana Mattioli**, Maria Luiza Santos da Silva, Laisla Zanetoni Martins, Alaor Aparecido Almeida, Ananda Lini Vieira da Rocha, Gabriela Palma Zochio, and Carlos Alan Dias Junior. (Submited to Basic & Clinical Pharmacology & Toxicology in February 2023)