VNIVERSITAT ID VALÈNCIA

Programa de Doctorado en Biomedicina y Biotecnología

Departamento de Bioquímica y Biología Molecular. Facultad de Ciencias Biológicas

TESIS DOCTORAL

Molecular investigation on the etiology of preeclampsia and new strategies for its early diagnosis

Doctoranda:

Nerea Castillo Marco

Directores:

Dra. Tamara Garrido Gómez

Dr. Carlos Simón Vallés

Tutor académico:

Dr. Luis Francisco Pascual Calaforra

Valencia, Enero 2023

El Dr. Carlos Simón Vallés, catedrático de Pediatría, Obstetricia y Ginecología de la Universidad de Valencia y presidente de la Fundación Carlos Simón para la Investigación de la Salud de la Mujer; la Dra. Tamara Garrido Gómez, investigadora principal en el departamento de investigación de la Fundación Carlos Simón y directora científica de iPremom; y el Dr. Luis Francisco Pascual Calaforra, docente e investigador titular de la Universidad de Valencia

CERTIFICAN:

Que el trabajo de investigación titulado: "Molecular investigation on the etiology of preeclampsia and new strategies for its early diagnosis" ha sido realizado íntegramente por Dña. Nerea Castillo Marco bajo su dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

Y para que así conste a los efectos oportunos, firman la presente certificación en:

Valencia, a 11 de Enero de 2023

Fdo: Carlos Simón
Vallés (Director)

Fdo: Tamara Garrido
Gómez (Directora)

Fdo: Luis Francisco
Pascual Calaforra (Tutor)

Publications

Garrido-Gómez, T*., **Castillo-Marco, N***., Muñoz-Blat, I., Cordero, T., Clemente-Ciscar, M., Monfort-Ortiz, R., Perales-Marin, A., Simon, C. (2021). Defective decidualization after severe preeclampsia is connected to dysregulation of progesterone receptor B and estrogen receptor 1 [Abstract]. *Fertility and Sterility*. 116(3):E39-E40.

Garrido-Gómez, T*., **Castillo-Marco, N*.,** Clemente-Ciscar, M*., Cordero, T., Muñoz-Blat, I., Amadoz A., Jimenez-Almazan, J., Monfort-Ortiz, R., Climent R., Perales-Marin, A., Simon, C. (2021). Disrupted PGR-B and ESR1 signaling underlies defective decidualization linked to severe preeclampsia *eLife,* 10:e70753

Review and Book chapter

Garrido-Gómez, T*., **Castillo-Marco, N***., Cordero, T., Simón, C. (2022). Decidualization resistance in the origin of preeclampsia. *Am J Obstet Gynecol.* 226(2S):S886-S894.

Muñoz-Blat, I., Castillo-Marco, N., Cordero, T., Simón, C., Garrido-Gómez, T., (2021). Decidualization resistance: A new identified condition in severe preeclampsia. In

Simón & Rubio (Eds.) *New Genetic Diagnostic Technologies in Reproductive Medicine* (pp. 256-265) Florida: CRC Press.

Congresses

Congress 1

Title of the work*:* Defective decidualization after severe preeclampsia is connected to dysregulation of progesterone receptor b and estrogen receptor 1.

Name of the conference: ASRM 2021 American Society of Reproduction Medicine.

Session Date*:* October 18, 2021.

Type of presentation: Oral (On-Demand Prize Paper Session)

Authors: Garrido-Gómez, T*., **Castillo-Marco, N*.,** Muñoz-Blat, I., Cordero, T., Clemente-Ciscar, M., Monfort-Ortiz, R., Perales-Marin, A., Simon, C.

Award: In-Training Award

Congress 2

Title of the work*:* Single-cell atlas of defective decidualization in preeclampsia.

Name of the conference: SRI 2022 Society for Reproductive Investigation 69th Annual Scientific Meeting.

Session Date*:* March 18, 2022.

Type of presentation: Oral

Authors: Garrido-Gómez, T*., Muñoz-Blat, I*., **Castillo-Marco, N**., Roson, B., Perez, R., Cordero, T., Lozano, C., Ochando A., Monfort-Ortiz, R., Climent, R., Simon, C.

AGRADECIMIENTOS

ABSTRACT

Endometrial decidualization is critical for pregnancy establishment and maintenance. This process promotes endometrial remodeling allowing placental cytotrophoblasts (CTBs) invasion up to an appropriate depth to form the maternal-fetal interface. A shallow CTBs invasion compromises healthy pregnancy and it is associated with the development of severe preeclampsia (sPE). Our working hypothesis is that defective decidualization impairs the adaptation of the maternal "soil" —the decidua— to be invaded by CTBs and plays a pivotal role in the etiology of sPE. Previously, we have demonstrated the existence of an *in vitro* defective decidualization of endometrial stromal cells isolated from women who suffered sPE. The present thesis doctoral aims to dilucidated the existence of a *in vivo* footprint and the molecular mechanisms encoding endometrial defective decidualization that might identify the maternal contribution to sPE.

To that purpose global RNA sequencing was applied to obtain the transcriptomic profile of endometrium collected from non-pregnant women who suffered sPE (n=24) in a previous pregnancy versus women who did not develop this condition (n=16). Samples were randomized in two cohorts, the training set to identify the fingerprint encoding defective decidualization in sPE and the test set for its subsequent validation. Transcriptional analysis revealed 593 differentially expressed genes in sPE compared to controls. From those, 120 (≥1.4-fold; FDR<0.05) genes were selected to formulate the footprint encoding defective decidualization. This signature allowed us to effectively segregate samples into sPE and control groups in both cohorts. Gene Ontology enrichment and an interaction network were performed to deeper in pathways impaired by genetic dysregulation in sPE. Major biological processes affected were associated with decidualization and sPE pathogenesis such as extracellular matrix and immune response. Since decidualization is tightly regulated by progesterone and estrogen, we assessed the link between this hormonal signaling and the gene expression imbalance encoding defective decidualization. We identified the footprint was composed by 94 genes highly expressed in the endometrium of which 47.9% are modulated by estrogen receptor 1 (ER1) and 45.7% by progesterone receptor (PR). An interactome network confirmed these receptors were strongly interconnected with the proteins codified by the genes encoding defective

decidualization. Moreover, gene expression and protein abundance of ER1 and PR was significantly downregulated in sPE. Specifically, the PR isoform B was impaired in sPE, while the isoform A was not affected.

Our data support the concept that *in vivo* defective decidualization is responsible, at least in part, for the maternal contribution to sPE. Further, we postulate that impaired ER1 and PR-B are potential drivers of compromised decidualization, including dysregulation of endothelial and immune response observed in sPE. These findings open new horizons in the search for early detection, prevention, and therapy strategies since the origin of sPE could lie in maternal endometrial health.

RESUMEN

Introducción

La preeclampsia (PE) es una complicación del embarazo propia de humanos que se caracteriza por la aparición de hipertensión, proteinuria y signos de daño en diferentes órganos que se manifiestan después de las 20 semanas de gestación ("Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy," 2000). Afecta a 8 millones de mujeres embarazadas en todo el mundo cada año, y se considera una de las principales causas de mortalidad y morbilidad tanto para la madre como para el feto (Winn et al., 2011). De acuerdo con la guía ACOG, la preeclampsia grave (sPE) se diagnostica cuando la presión arterial supera un umbral preestablecido (sistólica ≥160 o diastólica ≥100 mm Hg) y cursa con cualquiera de los siguientes: trombocitopenia, deterioro de la función hepática, insuficiencia renal progresiva, edema pulmonar o aparición de trastornos cerebrales o visuales ("Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy," 2013).

Hay dos grandes desafíos que dificultan el diagnóstico y tratamiento de la PE, la heterogeneidad de su presentación clínica y la manifestación tardía de síntomas durante el embarazo (Burton et al., 2019). Identificar a las pacientes con riesgo de desarrollar PE durante las primeras semanas del embarazo es crítico para aplicar estrategias de prevención efectivas (Rana et al., 2019). No obstante, para desarrollar herramientas de diagnóstico temprano es necesario entender el origen de la esta complicación obstétrica. Descubrir los mecanismos moleculares que acontecen en estadios iniciales no solo permitiría a un diagnóstico meses antes de la aparición de los síntomas, sino que también permitiría desarrollar terapias efectivas para tratar la enfermedad.

Aunque los agentes desencadenantes de la PE siguen siendo desconocidos, se han producido grandes avances en los mecanismos que subyacen a su fisiopatología (Ives et al., 2020). La placentación anormal ha sido el centro de la mayoría de los estudios sobre la patogénesis de la PE, dando lugar a un modelo en dos etapas en el que una placentación defectuosa —etapa 1, preclínica— conduce a una disfunción del endotelio vascular que provoca la aparición de síntomas —etapa 2, clínica— (Redman & Sargent, 2005). Según este modelo, las células de trofoblasto invaden la decidua sin alcanzar la profundidad suficiente y, como consecuencia, un gran número de arterias maternas no se remodelan adecuadamente. Este fallo en la remodelación provoca una situación de hipoxia y daño en el tejido de origen

fetal. Como resultado, se liberan al torrente circulatorio materno factores que provocan una disfunción del endotelio vascular y, en última instancia, aparecen los síntomas clínicos asociados a la PE. Por ende, el inicio de la enfermedad y su manifestación clínica están muy separados en el tiempo, dificultando el actual diagnóstico, pero abriendo la posibilidad de diseñar estrategias de detección precoz.

En este contexto, se han llevado a cabo investigaciones sobre la contribución del útero en la salud del embarazo, puesto que la decidua es el tejido especializado en el que tiene lugar la invasión de las células del trofoblasto y donde se remodelan las arterias para establecer una óptima comunicación materno-fetal (Garrido-Gómez et al., 2022; Ng et al., 2020).

La formación de la decidua empieza antes del embarazo, durante la fase secretora tardía del ciclo menstrual, el cual se divide en dos fases dominantes: (i) la fase proliferativa, sigue a la menstruación y se produce en respuesta al aumento de los niveles de estrógeno que precede a la ovulación; (ii) la fase secretora, que se inicia después de la ovulación debido al incremento de los niveles progesterona (Vollman, 1977). Durante la fase secretora tardía, el endometrio sufre una serie de transformaciones que permiten la implantación e invasión embrionaria. Esta transformación del tejido se denomina decidualización y es fundamental para el establecimiento del embarazo y la posterior formación de la decidua (Gellersen et al., 2007; Okada et al., 2018).

La decidualización, es un proceso de diferenciación que sufre el tejido endometrial caracterizado por un proceso clave que es la reprogramación transcripcional, morfológica y bioquímica de las células estromales, las cuales pasan de un fenotipo fibroblástico transformándose en células deciduales secretoras (Garrido-Gomez et al., 2011; Wang et al., 2020). Mediante el secretoma, las células deciduales dirigen y regulan el remodelado de resto de células endometriales incluyendo el reclutamiento y activación de las células inmunitarias, y a la vez, promueven la invasión del trofoblasto (Santos et al., 2021). La decidualización es un proceso altamente coordinado gobernado cíclicamente por factores hormonales, bioquímicos, inmunológicos y locales en cada ciclo menstrual (Ng et al., 2020). La progesterona (P4) es el principal impulsor de la decidualización. Las vías de transducción de señales inducidas por P4 están mediadas principalmente por el receptor de progesterona (PR), que también responde a la señalización de cAMP/PKA (Wu et al., 2018). El PR activa una red de señalización compleja compuesta por una gran cantidad de reguladores como IHH, FOXO1, transductores de señal y activadores de transcripción (STAT1, STAT3, STAT5), BMP2, y WNT, entre otros (Mazur et al., 2015). Además, el PR consta de dos isoformas PR-

A y PR-B y se considera que los efectos de P4 representan actividades combinadas de las dos isoformas. Sin embargo, PR-B juega un papel predominante durante la decidualización (Kaya et al., 2015).

El estradiol (E2), es fundamental en la preparación del útero para el embarazo puesto que induce la expresión de LIF a través de la activación del receptor de estrógeno 1 (ER1) (Wang et al., 2017). En las células del epitelio, LIF promueve la activación de los genes que participan en la implantación del embrión. Además, el ER1 también regula la proliferación del epitelio en respuesta a los niveles de E2. Esta regulación es paracrina, puesto que es el ER1 de las células estromales el que controla los cambios en las células epiteliales (Cooke et al., 1997; Winuthayanon et al., 2017). Esta comunicación epitelio-estroma juega un papel clave en la preparación del útero para el embarazo a través de regulación de la expresión de los receptores ER1 y PR en ambos compartimentos. La activación de PR en el epitelio promueve la expresión de mediadores de la decidualización en el estroma; mientras que, como se ha comentado, el ER1 en el estroma regula proliferación del epitelio (Wang et al., 2017). Esta comunicación entre compartimentos del mismo tejido, requiere una señalización hormonal equilibrada para la óptima preparación del endometrio al embarazo. Puesto que, con la decidualización el endometrio se transforma en la decidua, alteraciones en este proceso a cualquier nivel pueden conllevar a una formación aberrante de la decidua, lo cual condicionaría la posterior placentación y, por ende, la salud del embarazo.

Diferentes problemas de infertilidad y complicaciones obstétricas se han vinculado a una decidualización defectuosa como la infertilidad asociada con la endometriosis (Marquardt et al., 2019), el aborto recurrente (Lucas et al., 2020), el deterioro reproductivo asociado al envejecimiento (Woods et al., 2017), la restricción del crecimiento intrauterino (Dunk et al., 2019), la placenta accreta (McNally et al., 2020) y la sPE (Garrido-Gomez et al., 2017).

Estudios recientes han asociado la decidualización defectuosa con la etiología de la PE aportando evidencias de este defecto en estadios tempranos del embarazo, en el momento del parto y años después del embarazo afectado. También, se ha demostrado que hasta seis meses antes de la aparición de los síntomas de la PE, las vellosidades coriónicas ya muestran un perfil transcriptómico aberrante, el cual incluye genes que participan en la decidualización (Rabaglino et al., 2015). Además, en el momento del parto, las células de la decidua basalis y parietales muestran una decidualización defectuosa comprobada mediante marcadores morfológicos, transcriptómicos y proteómicos. Además, la decidualización *in vitro* de estas células mediante estímulos hormonales, reveló que no respondían a dichos estímulos. Por

último, la decidualización *in vitro* de células del estroma endometrial aisladas de biopsias procedentes de mujeres que sufrieron sPE en un embarazo previo permitió demostrar que el defecto en decidualización persiste años después del embarazo afectado (Garrido-Gomez et al., 2017).

La implicación de la decidualización en el éxito reproductivo es ampliamente reconocida. Es por ello, que muchas investigaciones han estado dirigidas a descifrar los mecanismos moleculares que subyacen a la decidualización, revelando reguladores y mediadores críticos de la misma (Gellersen & Brosens, 2014; Okada et al., 2018)). Este conocimiento ha sido un recurso valioso para comprender tanto la biología del endometrio como su impacto en la salud del embarazo, lo que ha contribuido al progreso significativo en los campos de la fertilidad y la medicina reproductiva (Deryabin et al., 2020; Haller et al., 2019). Sin embargo, muchos enfoques experimentales se han basado en modelos *in vitro* de células del estroma endometrial y tecnologías dirigidas al estudio de marcadores concretos. Las principales limitaciones del tipo de diseños experimentales son la caracterización de un tipo de célula aislada de su nicho biológico, y la evaluación de transcritos y proteínas específicas.

Durante la última década, los avances tecnológicos han proporcionado herramientas de adquisición de datos de alto rendimiento y, en paralelo, la bioinformática ha evolucionado para permitir el análisis de los resultados ómicos obtenidos. Este avance ha sido más efectivo en el campo de la transcriptómica, destacando la tecnología de secuenciación masiva del ARN (RNA-seq) (Wang et al., 2009). El RNA-seq permite la cuantificación de todos los transcritos presentes en una muestra de forma relativamente sencilla y con un coste reducido a partir de pequeñas cantidades de material genético. En consecuencia, ahora es posible caracterizar el transcriptoma global de los sistemas biológicos. Anteriormente, este abordaje se llevaba cabo con *arrays* de ARN, sin embargo, está técnica solamente permite identificar transcritos prestablecidos que hibridarán con los oligonucleótidos presentes en la matriz.

En este escenario, el análisis del transcriptoma global e *in vivo* durante la decidualización revelaría un amplio abanico de nuevos hallazgos en este campo; puesto que, superaría las limitaciones asociadas con los modelos *in vitro* y las estrategias dirigidas. Por lo tanto, el presente estudio se basa en el análisis de biopsias endometriales de pacientes con sPE y controles mediante la aplicación de RNA-seq, con el objetivo de identificar el perfil transcriptómico que codifica la decidualización defectuosa de sPE *in vivo* y dilucidar el mecanismo molecular que subyace a este fenotipo. Este descubrimiento abriría la puerta a futuros estudios sobre métodos de detección de la sPE enfocados en la contribución de la

decidua permitiendo un diagnóstico más temprano que el que ofrecen las estrategias basadas en la placentación aberrante. Por otro lado, la identificación de biomarcadores de decidualización aberrante es un gran avance hacia el diseño de terapias que reviertan el fenotipo condicionante antes de desarrollar la patología.

Hipótesis

La hipótesis del presente trabajo es que una decidualización defectuosa que se encuentra en el origen de la sPE se mantiene tras el embarazo y tiene un perfil transcriptómico único que puede ser detectado *in vivo* durante la fase secretora del ciclo menstrual. La caracterización de dicho perfil puede ser un avance significativo hacia el desarrollo de nuevas estrategias que permitan una evaluación temprana del riesgo de sPE y terapias efectivas para reducir la morbilidad y la mortalidad asociadas.

Objetivos

El objetivo general de esta tesis doctoral es:

Identificar la decidualización defectuosa presente mujeres que sufrieron sPE y determinar posibles mecanismos moleculares implicados en dicha patología endometrial, creando bases sólidas para desarrollar una herramienta dirigida a la evaluación del riesgo y la detección temprana, así como terapias dirigidas a biomarcadores específicos.

Los objetivos específicos son:

- Identificar las alteraciones del transcriptoma endometrial durante la fase secretora tardía de pacientes que han sufrido sPE mediante el enfoque global de RNA-seq en endometrio.

- Formular y validar la firma transcriptómica que codifica la decidualización defectuosa en sPE *in vivo* que persiste años después del embarazo afectado.

- Describir las rutas moleculares asociadas a la decidualización defectuosa.

- Explorar las interacciones de las proteínas codificadas por la firma de decidualización defectuosa con aquellas proteínas reportadas como afectadas en la interfase materno-fetal de pacientes con sPE.

- Modelar la red que conecta la decidualización defectuosa con la señalización hormonal en pacientes que han sufrido sPE.

Metodología

Para llevar a cabo el presente estudio se obtuvieron biopsias endometriales de 40 mujeres no embarazadas que habían tenido un embarazo previo entre 1 y 8 años antes. Esta cohorte incluyó 24 mujeres que sufrieron sPE y 16 mujeres sin antecedentes de complicaciones obstétricas como control. La cohorte se dividió en dos conjuntos de muestras de forma aleatoria y siguiendo una proporción 70:30: un conjunto de muestras se utilizó para identificar la firma transcriptómica vinculada a la decidualización defectuosa a partir del transcriptoma global (n=29) y el otro conjunto para su validación (n=11). Todas las biopsias fueron procesadas para la extracción del ARN y su posterior secuenciación.

En primer lugar, se caracterizó el transcriptoma global del endometrio durante la fase secretora tardía. Las lecturas se asignaron al transcriptoma del genoma humano hg19 utilizando el alineador de lectura STAR (versión 2.4.2a) (Dobin et al., 2013). El paquete de R FastQC (versión 0.11.2) se utilizó para determinar la calidad de los archivos FASTQ. La manipulación de los archivos SAM y BAM se realizó con el software SAMtools (versión 1.1) (Li et al., 2009). Para contar la cantidad de lecturas que podrían asignarse a cada gen, usamos HTSeq (versión 0.6.1p1) (Anders et al., 2015) y el software BEDtools (versión 2.17.0) (Quinlan & Hall, 2010) para obtener genes cobertura y trabajo con bedFiles. Se utilizaron filtros de control de calidad en cada programa siguiendo las recomendaciones del paquete de software, y las lecturas se filtraron por calidad de mapeo superior al 90%. Los datos transcriptómicos se depositaron en la base de datos Gene Expression Omnibus (número de acceso GSE172381). Se utilizó el paquete Bioconductor edgeR (versión 3.24.3) (Robinson et al., 2010) para analizar genes expresados diferencialmente.

Una vez identificadas todas las alteraciones genéticas en el grupo de casos en comparación con el grupo control, se llevó a cabo un estudio de ontología genética para identificar los procesos biológicos afectados en base a la información transcriptómica disponible utilizando la función goana del paquete de R EdgeR. Seguidamente, se comparó este conjunto de datos del transcriptoma *in vivo* con el publicado previamente por nuestro grupo del transcriptoma *in vitro* de células estromales aisladas de biopsias endometriales pacientes que sufrieron una sPE previa (Garrido-Gomez et al., 2017). Para corroborar la expresión genética *in vivo* de los genes determinantes de la decidualización defectuosa *in vitro* (Garrido-Gomez et al., 2017), se construyó un panel customizado para la secuenciación del ARN de los 129 previamente

descritos. El procesamiento de las lecturas de secuenciación se llevó a cabo como se ha detallado para el transcriptoma global. Gracias a la reciente publicación del atlas transcriptómico del endometrio a nivel de célula única (Wang et al., 2020), se llevó a cabo una deconvolución del tejido utilizando el transcriptoma descrito para los distintos tipos celulares que componen el tejido. Con ello, fue posible inferir las poblaciones celulares más afectadas en las biopsias endometriales de las pacientes. El siguiente paso fue formular la firma representativa codificante de la decidualización defectuosa seleccionando genes en base a su significancia estadística y biológica. La utilidad de esta firma en un grupo diferente de muestras se confirmó en el grupo de validación mediante la aplicación de análisis de componentes principales y de agrupamiento jerárquico. Se llevó a cabo un enriquecimiento funcional de estos genes para su caracterización biológica y se evaluó su interacción con los receptores hormonales ER1 y PR por su importancia en la regulación de la decidualización. Las interacciones se obtuvieron con String (Jensen et al., 2009) y se visualizaron con Cytoscape (Shannon et al., 2003). El nivel de expresión y la abundancia de estos receptores se cuantificó mediante RT-qPCR e inmunofluorescencia respectivamente. Además, se cuantificó la expresión de *STAT3, PIAS3* y *PLZF*, por ser mediadores de la decidualización fundamentales que participan en la ruta de señalización de PR.

Finalmente, se profundizó en el posible impacto de esta decidualización defectuosa durante el embarazo. Para ello se identificaron las interacciones entre los genes de la firma y las alteraciones transcriptómicas descritas en la interfase materno-fetal de pacientes con PE en el primer trimestre de la gestación. Para ello, se utilizó el transcriptoma de vellosidades coriales obtenidas de pacientes que desarrollaron preeclampsia meses más tarde y en las cuales se corroboró la presencia de tejido materno (Rabaglino et al., 2015). De nuevo, las interacciones se obtuvieron con la herramienta String y se visualizaron con Cytoscape.

Resultados y discusión

La secuenciación global de ARN de biopsias endometriales extraídas en el momento de la fase secretora tardía nos permitió identificar 593 genes expresados diferencialmente (DEGs) en sPE en comparación con el grupo control. Se identificaron un total de 155 DEGs aumentados y 438 DEGs reducidos en el transcriptoma endometrial de las pacientes con sPE en el momento de la decidualización. Entre los transcritos con expresión disminuida encontramos mediadores de decidualización, como *PRL, IL-6 e IHH*; y otros genes asociados con la biología que explica este proceso de diferenciación como señalización (p. ej*., NR4A3 e IL8*), proliferación (p. ej., *FGF1* y *FGF7*), angiogénesis (p. ej., *EDN2* y *TMEM215*) y

respuesta inmunitaria (*CCL20* y *CXCL3*). Los genes regulados al alza están involucrados en los procesos metabólicos/catabólicos de aminoácidos (*IDO2* y *CAPN3*), el transporte y la actividad de la oxidorreductasa. Este resultado refleja la diversidad de alteraciones detectadas en nuestro enfoque basado en el análisis de tejido endometrial mediante tecnología RNA-seq.

El resultado de la superposición de este conjunto de datos con los resultados de nuestro estudio *in vitro* anterior (Garrido-Gomez et al., 2017) fue muy modesta, habiendo solo nueve coincidencias. Para investigar la desregulación de los genes previamente reportados *in vitro* en las biopsias procedentes de la cohorte del presente estudio. Para ello, se realizó una estrategia de RNA-seq dirigida a la amplificación de los 129 DEGs detectados durante la decidualización defectuosa *in vitro*, la cual reveló la misma tendencia de expresión para esos genes en comparación con el enfoque *in vivo*. Además, se obtuvieron 21 DEGs en el grupo sPE en comparación con controles. La expresión genética de estos genes permitió el agrupamiento de las muestras del estudio en casos y controles, tanto en un análisis de componentes principales como aplicando un análisis de agrupamiento jerárquico. Por lo tanto, en este estudio corroboramos nuestros hallazgos anteriores y describimos por primera vez un conjunto novedoso de alteraciones transcriptómicas en sPE *in vivo*, las cuales han podido ser identificadas por la alta complejidad celular en el tejido endometrial (excluida en los estudios *in vitro*).

El RNA-seq de un tejido proporciona la expresión promedio de cada gen activo en una muestra. Este valor es un resultado valioso para identificar las características distintivas de los tejidos asociadas con condiciones específicas. Sin embargo, no permite detectar anomalías a nivel de tipos celulares, lo que resulta interesante para descubrir los mecanismos moleculares que rigen los eventos biológicos en estudio. En este contexto, se ha desarrollado una estrategia de deconvolución combinando datos de RNA-seq y scRNA-seq con el objetivo de inferir las proporciones celulares que componen un tejido (Wang et al., 2019). Debido a que scRNA-seq requiere la disgregación de tejidos y el aislamiento de diferentes tipos de células, la proporcionalidad celular que compone la muestra está sesgada. Por lo tanto, la combinación de datos de ambas estrategias puede superar las limitaciones intrínsecas de cada enfoque. Este enfoque proporcionó resultados que sugieren que un gran número de los genes identificados mediante RNAseq son expresados por fibroblastos y linfocitos, y que, además, la proporción de estos últimos está incrementada en las pacientes del grupo sPE.

Respecto a la determinación de genes clave en la firma transcriptómica codificante del defecto en decidualización *in vivo* en sPE, se seleccionaron 120 genes. Éstos permitieron distinguir con alta eficacia las muestras controles de las procedentes de casos de sPE perteneciente al conjunto de muestras del set de entrenamiento. Su validación en el segundo conjunto de datos permitió separar las muestras en los grupos sPE y control, agrupando correctamente el 90,9% de las muestras. Seguidamente, obtuvimos los procesos y rutas biológicas enriquecidas en estos genes. Este análisis reveló que la firma se asocia con la biología de la decidualización y la patogénesis de la sPE, incluyendo procesos como la organización de la matriz extracelular, la regulación de la señalización a través de receptores, el metabolismo de los lípidos y la aterosclerosis. Sorprendentemente, el análisis de enriquecimiento reveló que los genes que codifican la decidualización defectuosa estaban involucrados en la respuesta inmune a patógenos, incluidas bacterias y virus. Hecho que es consistentes con teorías que vinculan la PE e infecciones patógenas (Ishimwe, 2021; Papageorghiou et al., 2021).

Con el objetivo de entender el origen de la desregulación genética observada, evaluamos la relación de los genes de la firma con los receptores hormonales ER1 y PR. Identificamos los genes enriquecidos de endometrio, hallando un conjunto de 94 genes, de los cuales el 47,9% se incluyeron en el transcriptoma modulado por ER1 (Hewitt et al., 2010), y el 45,7% se superpusieron con el transcriptoma y cistroma asociado con PR (Mazur et al., 2015). Tras dichos análisis, se realizó la construcción de una red incluyendo las interacciones de las proteínas codificadas por los genes de la firma junto con ambos receptores. Dicho interactoma reveló que ER1 y PR estaban integrados en la red y altamente conectados con las proteínas codificadas por los genes de la firma, destacando su interacción con mediadores de decidualización claves como IHH y MSX2 validados por RT-qPCR. Además, el interactoma demostró una interacción directa entre ambos receptores, ER1 y PR. Estos resultados sustentan el vínculo entre los genes presentes en la firma codificante de la decidualización defectuosa y la señalización anómala de receptores hormonales en sPE. Posteriormente, el análisis por RT-qPCR e inmununofluorescencia de los receptores ER1 y PR-B mostró que el grupo sPE presentaba niveles más bajos de expresión génica junto con una menor abundancia de proteínas de ambos receptores, especialmente en las glándulas secretoras.

Además, se comprobó que los genes incluidos en la firma interaccionan directamente con genes alterados en la interfase materno-fetal en semana 11.5 de embarazo de mujeres que desarrollaron sPE y el número de interacciones detectadas fue estadísticamente significativo. Este resultado refuerza la contribución materna a la sPE a través de la decidualización

defectuosa; puesto que, proteínas codificadas por genes desregulados durante la fase secretora interaccionan con biomarcadores de sPE determinados en el primer trimestre de la gestación.

En base a estos resultados, postulamos que el desequilibrio de la señalización hormonal conduce a la resistencia a la decidualización en mujeres que sufrieron sPE. La baja expresión de *PGR* y *ESR1* ─los genes que codifican PR y ER1, respectivamente─ desregula la acción de P4 y E2 activando el programa de decidualización en el estroma de forma anómala. En consecuencia, la variedad de procesos involucrados en la decidualización endometrial podría verse comprometida. Esto incluye no solo la diferenciación de células estromales, sino también la comunicación estroma-epitelio (Wang et al., 2017), la degradación de la matriz extracelular (Itoh et al., 2012), la respuesta del sistema inmunitario y la función endotelial (Okada et al., 2018). El sistema inmunitario parece estar notablemente desregulado debido a la expresión alterada de interleucinas, citoquinas, quimioquinas e inmunoglobulinas que podrían alterar el microambiente tolerante en la interfaz materno-fetal durante el embarazo (Erlebacher, 2013; Harris et al., 2019). Para compensar este defecto en la señalización hormonal, reguladores de *PGR* como *STAT3* aumentan su expresión, lo que lleva a la activación de efectores posteriores como *PLZF* derivando en una decidualización aberrante.

Puesto que, en humanos, la decidualización ocurre mensualmente en cada ciclo menstrual, la decidualización defectuosa podría identificarse incluso antes del embarazo. Además, defectos en este proceso da lugar a alteraciones que se manifiestan antes de la placentación aberrante y sus señales como el desequilibrio angiogénico. Por ello, comprender la decidualización defectuosa en la sPE brinda la oportunidad de desarrollar nuevos métodos para un diagnóstico más temprano que podrían aplicarse incluso antes de la concepción.

Si bien es cierto que el presente estudio tiene la limitación de estar basado en un diseño retrospectivo al estudiar el endometrio años después de un embarazo (normal o con sPE). Esta limitación surge de la imposibilidad de recolectar muestras endometriales una vez establecido el embarazo. Además, una investigación prospectiva requeriría la recolección de miles de biopsias endometriales de mujeres nulíparas antes del embarazo y el seguimiento de todas las participantes hasta que den a luz para registrar al menos 30 casos de sPE, lo cual supondría un esfuerzo titánico en términos de recursos humanos y económicos. Sin embargo, existen evidencias que apoyan que este fenotipo puede estar presente antes de la concepción y ser una causa más que una consecuencia de la sPE. Se ha sugerido la contribución materna a la sPE a través de una inmadurez endometrial antes y después de la

implantación (Rabaglino et al., 2015), y se ha demostrado sólidamente en el momento del parto y años después (Garrido-Gomez et al., 2017). Las células estromales del endometrio aisladas de pacientes con sPE mostraron una decidualización defectuosa *in vitro* y, además, los factores secretados por dichas células se asociaron a una invasión del trofoblasto más superficial (Garrido-Gomez et al., 2017). Los resultados de estas investigaciones junto con el papel de la decidualización para transformar el endometrio en decidua, respaldan la hipótesis de que la maduración endometrial alterada es anterior a la sPE (Garrido-Gómez et al., 2022). En esta tesis doctoral proporcionamos nuevas evidencias para reforzar la contribución materna a la sPE a través de la decidualización defectuosa y presentamos una firma transcriptómica que puede identificar esta condición. Además, a partir de estos hallazgos, profundizamos en los mecanismos moleculares que subyacen al fallo en decidualización con el fin de entender la biología e identificar factores desencadenantes de esta alteración. El descubrimiento de la relación entre la decidualización defectuosa y   la alteración de la ruta de señalización de ER1 y PR-B abre un campo para desarrollar estrategias para la terapia de sPE basadas en moléculas capaces de restaurar estas señales.

Recientemente, los perfiles de ARN circulante (C-ARN) se han propuesto como una herramienta prometedora para monitorizar la salud del embarazo y detectar pacientes con alto riesgo de sufrir PE (Munchel et al., 2020; Rasmussen et al., 2022). Sin embargo, se requieren más estudios para evaluar la viabilidad de la firma C-RNA como una herramienta de diagnóstico, especialmente en relación con cuántas semanas se puede predecir la preeclampsia con dicho método. En este sentido, el estudio llevado a cabo en 2020 (Munchel et al., 2020) utilizó muestras obtenidas en el momento del diagnóstico; mientras que, el de 2022 (Rasmussen et al., 2022) utilizó muestras anteriores a la aparición de síntomas pero sin especificar cuántas semanas antes fueron obtenidas. Por ello, actualmente faltan por definir parámetros relevantes para la traslación clínica como cuántas semanas antes de la aparición de síntomas es posible predecir el riesgo y si la estratificación de PE subtipos es posible en base al perfil de C-ARN.

Nuestra hipótesis sobre que la decidualización defectuosa, durante la fase secretora tardía, conduce a la sPE, brinda la posibilidad de identificar un perfil de C-RNA asociado con esta condición a principios del primer trimestre del embarazo. La decidualización defectuosa implica una decidua aberrante y, por ende, una comunicación materno-fetal alterada. En consecuencia, moléculas de ARN pueden liberarse al torrente sanguíneo materno que reflejen los eventos afectados y los daños asociados. El presente estudio sienta las bases sólidas para alentar un proyecto dirigido a identificar el perfil de C-RNA asociado con la

decidualización defectuosa para estratificar el riesgo de desarrollar sPE. Además, fallos en la decidualización también se asocian con otros trastornos reproductivos y de la fertilidad, como endometriosis, fallos de implantación y aborto espontáneo. Por lo tanto, evaluar la decidualización a través de una prueba no invasiva basada en el C-RNA en sangre podría proporcionar datos valiosos para monitorizar la salud obstétrica en mujeres embarazadas con riesgo de sufrir complicaciones.

Conclusiones

1. El tejido endometrial de mujeres que sufrieron sPE presenta 593 genes expresados diferencialmente en la fase secretora tardía en comparación con los controles años después del embarazo afectado. El perfil transcriptómico identificado muestra un amplio número de genes que participan en la decidualización defectuosa como *PRL, CCL20, IGHG1*, *FGF7, EDN2,* y *IDO2.*

2. El transcriptoma global combinado con el transcriptoma de célula única reveló que el ~45% de genes desregulados en sPE se expresan en fibroblastos endometriales y la proporción de linfocitos durante la decidualización es significativamente mayor en el grupo de sPE en comparación con los controles.

3. La firma que codifica la decidualización defectuosa está compuesta por 120 genes incluyendo *IHH, MSX2, FGF1, EL6,* y *TNF.* El análisis de ontología genética de estos genes reveló 151 procesos biológicos enriquecidos, incluyendo rutas relacionadas con la biología de la decidualización y la patogénesis de la sPE como organización de la matriz extracelular, regulación de la señalización mediada por receptores y migración celular.

4. Los 120 genes de la firma permiten separar las muestras en dos grupos, sPE y control de manera efectiva en la cohorte de entrenamiento y en la de validación, evidenciando que estrategias dirigidas a evaluar la decidualización defectuosa mediante estos marcadores pueden tener un gran potencial en la predicción del riesgo a desarrollar sPE.

5. Las proteínas codificadas por los genes incluidos en la firma están interconectados con biomarcadores que se expresan en la interfaz materno-fetal durante el primer trimestre de embarazos que desarrollaron sPE.

6. La firma que codifica la decidualización defectuosa está compuesta por genes enriquecidos en el endometrio y modulados por ER1 y PR. Además, estos receptores hormonales están

embebidos y situados en una posición central en el interactoma resultante de las proteínas codificadas por los genes incluidos en la firma. Ello sugiere que, una desregulación en ER1 y PR podría tener un gran impacto en el interactoma.

7. La expresión génica de *ESR1* y *PGR-B* está reducida en el grupo de casos, así como su abundancia a nivel de proteína. En cambio, *STAT3* y *PLZF*, mediadores fundamentales de la decidualización, muestran niveles más elevados de expresión genética en sPE.

8. El perfil del transcriptoma endometrial nos permite postular un modelo que vincula la decidualización defectuosa inducida por señalización hormonal alterada con sPE, lo que abre un campo para desarrollar nuevas estrategias para la terapia de sPE.

INDEX

LIST OF FIGURES

LIST OF TABLES

ABREVIATIONS

ACOG: American College of Obstetricians and Gynecologists

ANXA2: Annexin A2

BMI: Body mass index

BMP2: Bone Morphogenesis Protein 2

cAMP: Cyclic Adenosine Monophosphate

cDNA: complementary Deoxyribonucleic Acid

CNR1: Cannabinoid Receptor-1

COX2: Cyclooxygenase-2

CPM: Counts Per Million

C-RNA: Circulating Ribonucleic Acid

CTBs: Cytotrophoblasts

CVS: Chorionic villous samples

DAVID: Database for Annotation, Visualization and Integrated Discovery

DB: Decidua basalis

DD: Defective Decidualization

DEGs: Differentially Expressed Genes

DOCK4: Dedicator Of Cytokinesis 4

DP: Decidua Parietalis

E2: Estradiol

EOPE: Early-Onset Preeclampsia

ER1: Estrogen Receptor 1 (protein)

ESCs: Endometrial Stromal Cells

ESR1: Estrogen Receptor 1 (gene)

EVT: Extravillous Trophoblast

FC: Fold Change

FDR: False Discovery Rate

FOSL1: FOS Like 1

FOXO1: Transcription Factor Forkhead Box 1

GWAS: Genome-Wide Association Studies

IGFBP1: Insulin-Like Growth Factor-Binding Protein 1

IHH: Indian Hedgehog

IL1β, IL-6, IL-8, IL-11, IL-15: Interleukin-1β, -6, -8, -11, -15

IUGR: Intrauterine Growth Restriction

KEGG: Kyoto Encyclopedia of Genes and Genomes

LIF: Leukemia Inhibitory Factor

LOPE: Late-Onset Preeclampsia

MCC: Maximal Clique Centrality

MMP1, MMP3, MMP9: Matrix Metallopeptidase-1, -3, -9.

MNC: Maximum Neighborhood Component

NK: Natural Killer

P4: Progesterone

PCA: Principal Component Analysis

PE: Preeclampsia

PGE2: Prostaglandin E2

PGR: Progesterone Receptor (gene)

PGRMC1, PGRMC2: Progesterone Receptor Membrane Component-1, -2

PIGF: Placental Growth Factor

PPI: Protein–Protein Interaction

PR: Progesterone Receptor (protein)

PRL: Prolactin

PVCA: Principal Variance Component Analysis

RNA: Ribonucleic Acid

RNA-seq: Ribonucleic Acid sequencing

ROS: Reactive Oxygen Species

scRNA-seq: Single-Cell Ribonucleic Acid Sequencing

sFLT1: soluble fms-like tyrosine kinase-1

sPE: Severe Preeclampsia

STAT1, STAT3, STAT5: Signal Transducer and Activator of Transcription-1, -2, -3.

STB: Syncytiotrophoblast

TGF-β: Transforming Growth Factor-beta

1. INTRODUCTION

1. INTRODUCTION

1.1. Preeclampsia and severe preeclampsia

1.1.1. Definition

Preeclampsia (PE) is a multisystemic disorder specific to human pregnancy characterized by the new onset of hypertension, proteinuria, and signs of organ damage developing after 20 weeks of gestation ("Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy," 2000). It impacts 8 million mother–infant pairs worldwide each year, being considered as a major cause of mortality and morbidity for both mother and infant (Winn et al., 2011). Severe preeclampsia (sPE) is diagnosed based on further increased of blood pressure (systolic ≥160 or diastolic of ≥100 mm Hg) or any of the following: thrombocytopenia, impaired liver function, progressive renal insufficiency, pulmonary edema, or the onset of cerebral or visual disturbances according ACOG guidelines ("Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy," 2013).

PE has been documented for approximately 200 years. However, since the end of the 20th century, many researchers have attempted to identify the origin of this condition. Although, the triggering agents that turn on the molecular mechanisms underlaying PE remain elusive, much progress has been made in its pathophysiology (Bell, 2010; Ives et al., 2020).

1.1.2. Pathophysiology

The central role of abnormal placentation in its pathophysiology has been widely studied and described (Fisher, 2015). The placenta is composed by projections termed chorionic villi that mediates the transport of nutrients, gases and wastes between maternal and fetal blood. This exchange occurs in the placental bed where the uteroplacental circulation is completely adapted to establish and maintain the

maternal blood supply. Defective vascularization of placental bed results in great obstetrical syndromes including PE (Brosens et al., 2019).

- **Physiology of placentation**

Placentation begins when implantation initiates and the first trophoblast lineages emerge (Ruane et al., 2017). However, until the 11 week's gestation, the blastocyst is under hypoxic conditions and nourished via histotrophic support from the endometrial gland's secretions. Then, the uteroplacental blood flow is stablished and supports the conceptus development (Burton et al., 2002; Jones et al., 2015).

During the first week after conception, the blastocyst interacts with the uterus. Consequently, the trophectoderm proliferates and differentiates, developing invasive syncytial masses and the mononuclear cytotrophoblasts (CTBs) (Aplin & Ruane, 2017). Later, CTBs form multinucleated syncytiotrophoblast (STB) or become extravillous trophoblast (EVT) in the trophoblastic plate (Kliman et al., 1986; Tarrade et al., 2001). The EVT transform into invasive cells through a partial epithelial mesenchymal transition, involving changes in expression of genes associated with cytoskeletal organization, junctional interactions between epithelial cells, migration, and invasion (DaSilva-Arnold et al., 2015). Then, EVT deeply migrate into the maternal spiral arteries and the myometrium through the decidua, the specialized endometrial tissue that controls trophoblast invasion during pregnancy (Pijnenborg et al., 1983).

The EVT invade the maternal spiral arteries walls by break down extracellular matrix and replacing the musculoelastic structure with amorphous fibrinoid material (Brosens et al., 1967; Pijnenborg et al., 1983) (Figure 1). Endothelial cells release cytokines such as IL-6 and IL-8 in response to EVT diffusive signals, thereby uterine natural killer (NK) cells and macrophages are recruited into vascular smooth muscle in the decidua basalis to disrupt vessels media (Choudhury et al., 2017). Subsequently, the EVT embedded in the fibrinoid aggregates and plug the lumen of the spiral arteries. This vessel wall remodeling allows blood supply into the intervillous space at high volume, low velocity and pulsatility (Burton et al., 2009; Dickey & Hower, 1995). The invasion progresses deeply, until the myometrium reaching the inner third, where anchor the

placenta to the uterus and form multinucleated giant cells which likely act as endocrine cells (Velicky et al., 2016).
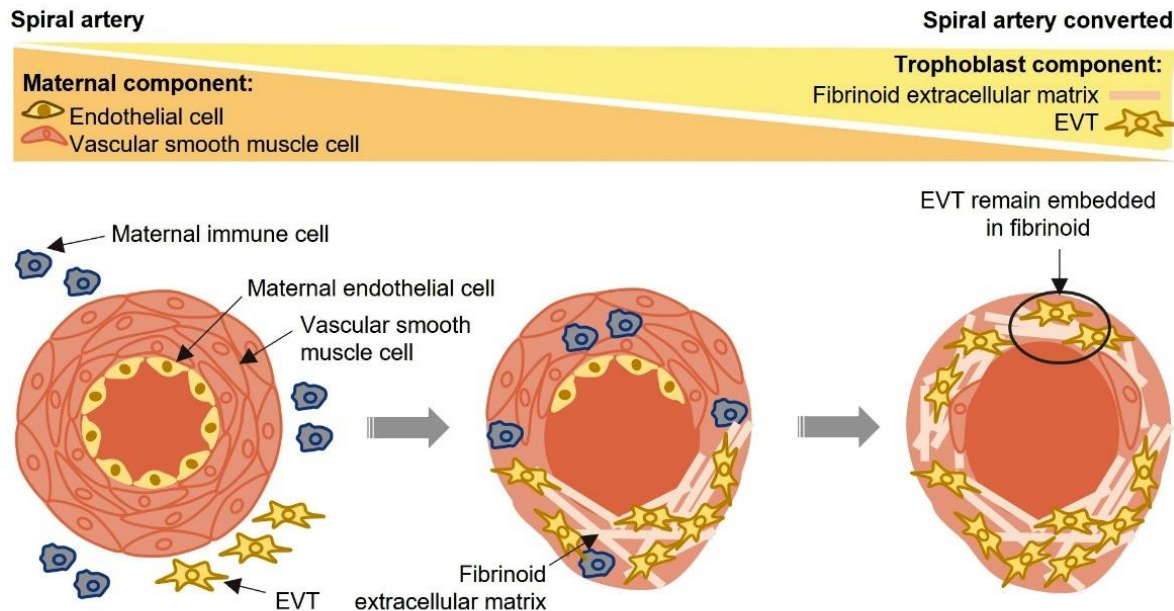
Figure 1. Maternal spiral artery remodelling by EVT. The maternal components of the vascular structure, maternal endothelial and smooth muscle cells, is replaced by trophoblast components: fibrinoid extracellular matrix within which residual EVT remains embedded. Consequently, spiral arteries become expanded vessels with decreased resistance index and increased blood flow.

▪ **Placentation in preeclampsia**

The connection between placentation and PE syndrome is explained by a two-stage model in which poor placentation −stage 1, preclinical− leads to vascular endothelium dysfunction, resulting in maternal signs −stage 2, clinical− (Redman & Sargent, 2005).

Histologic studies of placental bed biopsies demonstrate a shallow trophoblast invasion in PE patients. The invasion depth into the decidual stromal varies, while the endovascular invasion is restricted to superficial portions (Redman & Sargent, 2005). In addition, the percentage of decidual and myometrial segments with trophoblast invasion was lower in placental bed biopsies from sPE compared with normal

pregnancies (Meekins et al., 1994). In sPE, the lack of spiral arteries conversion is sharper and only a few spiral arteries in the centre of the placental bed are converted (Brosens et al., 2011). Consequently, spiral arteries remodelling is defective and high-resistance vessels remain. Moreover, the unconverted arteries are prone to suffer pathological inflammation resulting in acute atherosis, accelerated atherosclerosis and chronic processes probably due to defective haemodynamics (Burton et al., 2009; Harris et al., 2019).

The interaction between CTBs and spiral arteries have been studied in PE in detail (Zhou et al., 1997). It revealed that CTBs have a reduce invasive capability and even and altered morphology if they gain access to the lumen of vessels. This abnormal CTBs fail to express properly integrin, cadherin, and Ig superfamily members, all of them hallmarks of vascular adhesion phenotype; instead, CTBs retain the expression of epithelial-like molecules. Vascular mimicry is fundamental to replace the maternal endothelial lining of uterine vessel during placentation. Thus, this could be at least one cause of defective vascular invasion. Strikingly, CTBs isolated from preeclamptic placentas and cultured *in vitro* express vascular-like molecules (Zhou et al., 2013). Thus, deregulated genes in CTBs return to control values, suggesting the decidua environment *in vivo* impacts in the defective invasion observed in PE.

These failures results in a defective spiral arterial remodelling leading to reduced placental perfusion and turbulent blood flow that could damage the villous STB through hypoxia-reoxygenation processes and oxidative stress (Figure 2). Maternal malperfusion is associated with focal necrosis of the STB, along to swelling of the mitochondria and dilation of the endoplasmic reticulum cisternae (Holland et al., 2017; Jones & Fox, 1980). These disruptions cause the unfolded protein response and reactive oxygen species (ROS) production, leading to the attenuation of non-essential protein synthesis, the increase of phospholipid biosynthesis and the activation of inflammatory responses (Schröder & Kaufman, 2005). The damaged STB releases multiple factors into maternal circulation including extracellular vesicles, which contain proinflammatory, proangiogenic and antiangiogenic molecules (Redman & Staff, 2015).
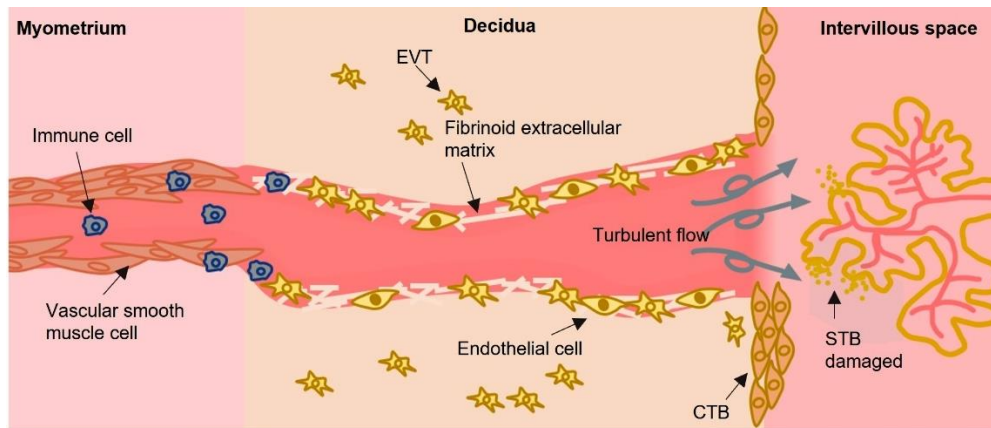
Figure 2. Defective maternal spiral remodelling in PE. Shallow EVT invasion results in unconverted maternal spiral arteries. Thus, arteries supply blood with high velocity and low volume creating a turbulent flow that damage the STB allocated in the villous placenta. Immune system cells are recruited in response to endothelium activation increasing inflammatory signals. CTB: cytotrophoblast; EVT: extravillous trophoblast; STB: syncytiotrophoblast.

It has been described an imbalance in ROS and antioxidants in placentas from PE patients. The increased ROS inhibits the β-catenin signaling pathway that promotes CTBs invasion (Zhuang et al., 2015). Even more, ROS induce the transcription of soluble fms-like tyrosine kinase-1 (sFLT1) (Huang et al., 2013), the angiogenic factor which is a classic biomarker of PE with potential to predict adverse maternal and perinatal outcomes (Lim et al., 2021).

1.1.3. Clinical challenges and perspectives

PE is associated with life-threatening consequences for both mother and infant (Table 1). It has been reported a 2-fold increased risk of suffering cardiovascular diseases and death in women who suffered PE (Bellamy et al., 2007). This risk increases from 6- to 9-fold ifpon PE develops in more than one pregnancy (Mongraw-Chaffin et al., 2010). Infants show a higher risk for hypertension, cardiovascular disease (Davis et al., 2012), stroke (Kajantie et al., 2009), and other related with fetal growth restriction such as diabetes or obesity (Burton et al., 2016). The Healthcare Cost and Utilization Project revealed the number of PE patients raised up to 21% from 2005 to 2014. It is noteworthy that 37% of cases diagnosed in 2014 were sPE (Fingar et al., 2017). Thus,

the impact of PE and specially sPE in public health is an issue that need to be address urgently.

Table 1. Highlights of clinical features and consequences of PE (Adapted from Ives et al. 2020).

Clinical feature	Clinical consequence
Hypertension	Heart failure; pulmonary edema; renal dysfunction; neurological injury.
Proteinuria	Hypertension; ischemic heart disease; stroke; chronic kidney disease; end-stage renal disease.
Renal dysfunction	Hypertension; chronic kidney disease; end-stage renal disease.
Neurological abnormalities	Seizures; posterior reversible encephalopathy syndrome; permanent blindness.
Eclampsia	Permanent neurological dysfunction.
Cardiac dysfunction	Heart failure; peripartum cardiomyopathy.
Pulmonary edema	Acute hypoxemic respiratory failure.
Hapatic dysfunction	Liver failure; hepatic rupture.
Hematologic dysfunction	Thromocytopenia; disseminated intravascular coagulation.
Fetal growth restriction	Fetal growth <10th percentile.

There are two major challenges that hamper PE diagnosis and treatment, the heterogeneity of its clinical presentation since it is now considered a syndrome and the manifestation of symptoms later in pregnancy. The stressed STB release soluble factors such as sFLT1 and placental growth factor (PlGF) that damage the endothelium resulting in a systemic syndrome and affecting multiple organs including liver, kidney, and central nervous system (Burton et al., 2019). Hence, the PE pathogenesis begins during the first trimester of pregnancy although symptoms appear in an advance stage. Additionally, there are maternal factors that predispose to an exacerbated endothelia activation or susceptibility to vascular damage in response to placental factors (Myatt & Roberts, 2015). In this context, well-stablished risk factors are high body mass index (BMI), chronic renal disease, pre-gestational diabetes, and high blood pressure (Rana et al., 2019).

According to timing of the symptom's onset considering 34 weeks of gestation as cut-off, two subtypes of PE are considered: early and late onset. Diagnostic criteria applied are the same for both subtypes although they are thought to be different entities based on pathophysiology. Evidence suggest early-onset PE (EOPE) is due to defective placentation and late-onset PE (LOPE) arise owing to maternal constitutional factors such as diabetes mellitus or obesity (Lisonkova & Joseph, 2013; McLaughlin et al., 2018; Valensise et al., 2008). However, vascular malperfusion and STB stress that lead to excessive systemic vascular inflammation is a hallmark shared by the two subtypes (Aplin et al., 2020). This complexity hinders the discovery and assessment of effective biomarkers for an early diagnose during the first trimester, being variability the major concern. In fact, a prospective study using maternal risk factors proposed by the National Institute for Health and Clinical Excellence as test predictors, revealed a detection rate of 37% and 28.9% cases in early and late onset preeclampsia respectively (Poon et al., 2010).

Researchers attempt to develop a predictive method of PE and its outcomes suitable for first trimester pregnancies whose performance include cost effective, high sensitivity and specificity. Evidence suggests that currently the most promising method for PE screening is the sFLT1 to PlGF ratio (Rana et al., 2019). A meta-analysis of 15 studies (N= 534 PE patients; N= 587 controls) obtained the ratio has a sensitivity of 80% and a specificity of 92% in both high- and low-risk patients (Agrawal et al., 2018). The performance of these biomarkers for EOPE and LOPE could not be calculated since many studies make no distinction between subtypes. Additionally, the cut-off values to rule out or rule in the disease are not clear. Unfortunately, these results were obtained analysing blood samples collected after 19 gestational weeks. The reason was that previous studies concluded that sFLT1 and PlGF levels change from the second half of pregnancy on (Chaiworapongsa et al., 2004; Levine et al., 2004). Recently, a meta-analysis of 33 studies (N=9,426 patients) assessed the sFlt-1/PlGF ratio as a prognostic method showing a promising potential for predicting adverse outcomes such as preterm birth and fetal growth restriction (Lim et al., 2021). However, heterogeneity among studies was high, limiting their clinical translation.

Identify women at high risk of developing PE and specifically sPE would allow clinicians to apply a closer monitoring and/or prophylactic treatment to improve patient

outcomes. However, predictive methods based on placental dysfunction biomarkers could offer delayed results, during the second trimester of pregnancy when applying preventive strategies is not effective. Thus, other strategies are being explored to assess the risk in an early stage when prevention improve the outcome. The ongoing research is focus on combine cardiovascular, immunologic, or inflammatory-related biomarkers trying to catch the complex pathophysiology of PE (Chaemsaithong et al., 2022). Thereby, current strategies are focus on assessing the consequences rather than the origin, which can provide results close to the symptoms appearance.

1.2. Etiology and maternal contribution

It is well known that placenta plays a central role in the pathogenesis of PE and its delivery is the unique existing cure. This fact encouraged researchers to investigate placentation and embryo invasion, both considered as the main drivers of pregnancy health. In contrast, the maternal contribution to PE remains poor explored and understood, although it is supported by epidemiology. The unsolved question is: what is the primary driver of the placental bed dysfunction observed in PE? To answer this question and to unveil the molecular pathways underlying PE heterogeneity and complexity, some hypothesis has been proposed whose focus shift to the maternal contribution.

1.2.1. Epidemiology

Epidemiological studies of PE revealed risk factors that support the maternal contribution to this condition (Table 2).

Table 2. Risk factors for PE according to the ACOG.

Risk factors for women at HIGH risk	PE in a past pregnancy.
	Carrying more than one fetus.
	Chronic high blood pressure.
	Kidney disease.
	Diabetes mellitus.
	Autoimmune conditions.

Risk factors for women at MODERATE risk	First pregnancy.
	Being pregnant more than 10 years after your previous pregnancy.
	BMI > 30.
	Family history of preeclampsia (mother or sister).
	Age ≥ 35.
	Previous pregnancy complications.
	In vitro fertilization (IVF).
	Black ancestry (because of racism and inequities that increase risk of illness).
	Lower income (because of inequities that increase risk of illness).

Familial aggregation of preeclampsia and eclampsia cases was described for first time in the 1960s (Chesley et al., 1968). From then on, genetic predisposition to PE has been widely studied (Cnattingius et al., 2004; Esplin et al., 2001; Lie et al., 1998; Mogren et al., 1999; Nilsson et al., 2004). Based on these studies, heritability of PE is estimated at 55% approximately, being attributable the 30-35% to maternal contribution.

However, the identification of genetic variants that increased the risk of PE remains elusive. Maternal and fetal genes interact during pregnancy, hampering the detection of risk variants. Moreover, currently it is known that thousands of samples need to be included in genome-wide association studies (GWAS) to find significant and accurate variant-phenotype associations. Recently, it was published a meta-analysis of eight GWAS including European and Central Asian mothers (N=9515 preeclamptic women; N=157,719 controls) (Steinthorsdottir et al., 2020). This study identified sequence variants associated with PE in the maternal genome. Interestingly, these variants were allocated in regions associated with blood pressure and BMI ─ZNF831/20q13, FTO/16q12, MECOM/3q26, FGF5/4q21 and SH2B3/12q24─.

Beyond identifying specific genetic variants that explain the phenotype observed in PE, there are researchers investigating if defective functionality of complex systems ─such as the immune and cardiovascular system─ or the decidua is underlying this condition. Healthy pregnancy requires profound adaptation of the maternal body to

allow embryo implantation, invasion, and development. In addition, the complex array of adaptations must evolve in concert with the fetus and placental growth through a crosstalk strongly coordinated.

1.2.2. Cardiovascular risk predisposition

The pathophysiological model based on the triad of defective placentation, malperfusion and vascular reactivity is the most acknowledged model to explain PE development. However, other great obstetrical syndromes such as intrauterine growth restriction (Brosens et al., 1970), preterm premature rupture of membranes (Kim et al., 2002), and preterm labor (Kim et al., 2003) are associated with defective placentation. Different degrees of abnormal remodelling and obstructive lesions have been suggested to explain the range of pregnancy complications which share this pathophysiology (Brosens et al., 2011). Likewise, it has been proposed this defect is not sufficient to cause PE. According to this, placental villous and vascular histopathological lesions were not identified in some PE patients, although they were 4- to 7-fold higher in preeclamptic than in normal pregnancies (Falco et al., 2017).

Epidemiological studies report that PE and cardiovascular diseases shared risk factors, including chronic hypertension, metabolic syndrome, dyslipidemia, a family history of myocardial infection before 60 years old, obesity, diabetes, chronic kidney disease or psychological stress, among others (Egeland et al., 2016; Giannakou et al., 2018; Tangren et al., 2017). Furthermore, it is well known that the impact of PE in the cardiovascular system prints a hallmark that persists years after the affected pregnancy and increases the risk of developing cardiovascular diseases in those affected women later in life (Kaaja & Greer, 2005). Consequently, cardiovascular system has been considered as a victim disrupted by the poor placentation that try to maintain pregnancy in a hostile environment, being more damaged in its effort.

However, vascular function in preeclamptic women has been monitored before and after the onset of the condition, revealing an altered prepregnancy hemodynamic phenotype could precedes PE (Foo et al., 2018; Weissgerber et al., 2016). In the preclinical phase of preeclampsia, vascular reactivity, hemodynamic indices, and left ventricular properties are subtly impaired (Melchiorre et al., 2014). Impaired function

of vascular physiology has been detected in not only in uteroplacental circulation but also in maternal peripheral arteries, suggesting a generalized vascular maladaptation to pregnancy (Foo et al., 2018; Kalafat et al., 2018; Lopes van Balen et al., 2017; Weissgerber et al., 2016). Vascular dysfunction early in pregnancy could lead placental malperfusion and activate systemic inflammation in response to STB stress (Figure 3).
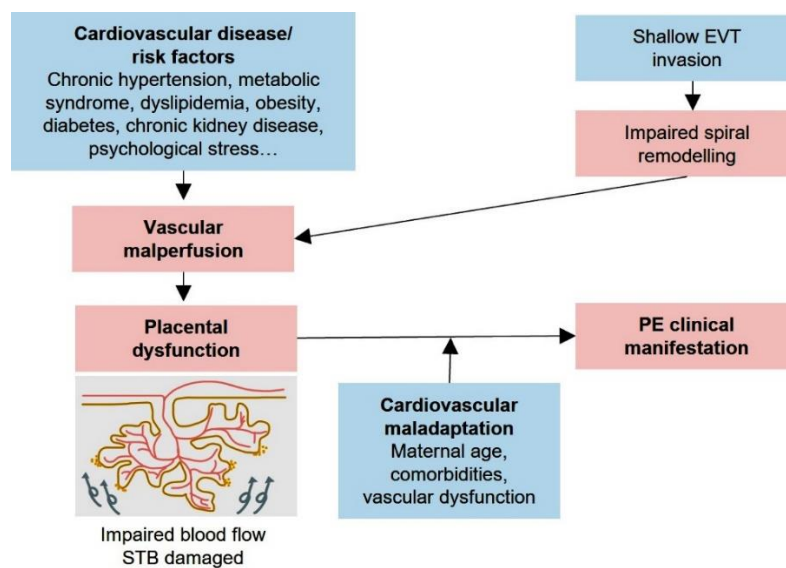
Figure 3. Cardiovascular system in the pathogenesis of PE. Cardiovascular dysfunction could be unmasked in response to the high demand of the growing fetus leading to vascular malperfusion and placental dysfunction. But also, placental malperfusion and dysfunction could be originated by impaired spiral remodelling. In these cases, cardiovascular maladaptation could be manifested in response to the factors released by the STB damaged.

1.2.3. Abnormal maternal immunological adaptation

Epidemiologic observations regarding nulliparity (Hernández-Díaz et al., 2009), autoimmune conditions (Schreiber et al., 2018), and assisted reproduction (Sites et al., 2017) as risk factors of PE suggest an abnormal maternal immunological adaptation in response to pregnancy.

During pregnancy, the immune system must ensure protection from pathogens and induce tolerance to the semi-allogeneic fetus, simultaneously. Furthermore, immune

cells are actively involved in spiral arteries remodelling, promote the trophoblast proliferation as well as supress apoptosis, and secrete natural IgM antibodies to clearance apoptotic tissue (Bonney, 2016; Mor & Cardenas, 2010). Thus, a tight balance of cell interactions, signalling, and functions is required, whose vary in a timely manner through pregnancy stages (Yang et al., 2019).

An imbalance in this system could result in defective placentation or malperfusion leading to PE. Disturbances in innate immune system activation that promote inflammation have been extensively described in PE (Aneman et al., 2020), including higher number of M1 macrophages and neutrophils, releasing of histamines by intensive degranulation of mast cells, activation of natural cytotoxicity receptors in NK cells, higher induction of T cell proliferation induced by dendritic cells, overactivation of innate B-1a cells and the complement system (Figure 4).
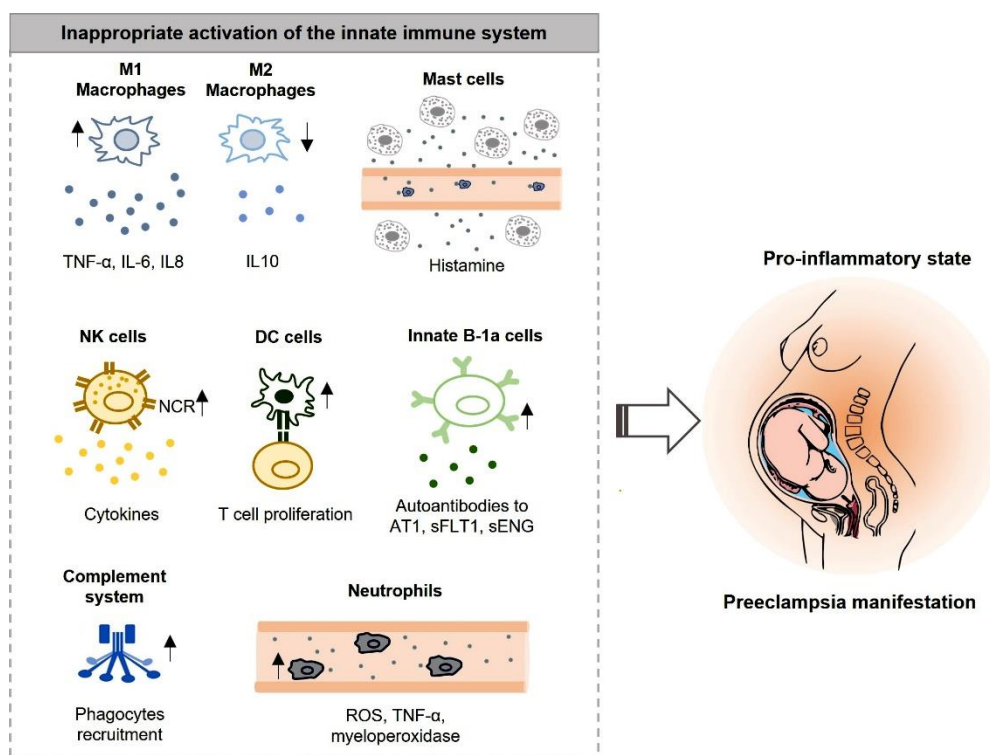
Figure 4. Summary of the abnormal maternal immunological activation. In PE pregnancies, mechanisms of key innate immune cells have been found inappropriately activated. The disrupted imbalance in the immune system response during pregnancy lead to a pro-inflammatory state and finally to PE symptoms such as hypertension and multisystemic damage.

1.2.4. The role of the uterus: decidua perspective

Pregnancy health is determined not only by the embryo and the placenta but also by the quality of the maternal soil —the decidua—, where the EVT invasion and maternal spiral arteries remodelling occur (Cha et al., 2012; Norwitz et al., 2001; Sharma et al., 2016; Su & Fazleabas, 2015). To successfully form the decidua, the endometrium transforms into an adapted tissue for pregnancy establishing and maintenance through the process known as decidualization. In humans, decidualization starts during the late-secretory phase of the menstrual cycle independent of the presence or absence of the conceptus —explained in detail in the following section—.

Although decidua formation starts before pregnancy, it completely develops   at the time of the blastocyst is attached to the uterine wall, when physical and humoral changes orchestrate a drastic tissue remodeling. The decidua embraces the embryo during pregnancy allowing its vital support through essential roles: protecting the embryo from maternal immune system and infections, nourishing the embryo via histotrophic support under hypoxic conditions, and controlling EVT invasion until the inner third of the myometrium (Mori et al., 2016). Recently, the single-cell atlas of the early- maternal interface has been published, revealing decidua-trophoblast interactions that are critical for placentation and for reproductive outcomes. EVT first invade the decidua compacta, where stromal cells secrete molecules that interact with inhibitory receptors of decidual NK cells and suppressing inflammatory reactions. Also, macrophages express immunomodulatory molecules that could interact with receptors in the EVT and decidual cells. Decidualized fibroblasts secrete ligands that promote EVT invasion, such as prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1) (Vento-Tormo et al., 2018). Thus, early alterations in decidua differentiation and function could compromise placenta formation and perfusion, what can manifest as pregnancy complications in later stages.

Transcriptomic studies revealed striking deregulation in decidua transcriptome of PE patients six months before symptoms appeared and at the time of delivery (Garrido-Gomez et al., 2017; Løset et al., 2011; Rabaglino et al., 2015). Chorionic villous samples (CVS) with decidual portion obtained at ~11.5 gestational weeks collected from women who develop PE later (N=4) and matched controls (N=8) exhibited 396

differentially expressed genes (DEGs). Thus, defective decidual gene expression preceded the development of PE clinical manifestation up to six months (Rabaglino et al., 2015). It is noteworthy that 40% of deregulated genes were involved in endometrial maturation before blastocyst implantation. Deregulated transcriptome found included genes associated with NK cells (Rabaglino et al., 2015), the most abundant leucocyte type in early maternal-fetal interface involved in spiral artery remodelling and EVT invasion (Vacca et al., 2011). Moreover, decidua at the time of delivery in PE and sPE patients show hallmarks of oxidative stress, metabolism impairment, defective cell communication and immune response, all of them consistent with PE biology (Garrido-Gomez et al., 2017; Løset et al., 2011).

In a nutshell, the decidua is the maternal tissue where the immune and vascular systems activation converge to prepare the uterus for successful implantation and placentation. To orchestrate all adaptations in a timely manner, endometrial decidualization is a critical step.

1.3. Decidualization and pregnancy health

The endometrial cycle is divided in two dominant phases: the proliferative phase, which follows menstruation in response to increased levels of estrogens preceding ovulation; and the secretory phase, which occurs after ovulation due to progesterone rise (Vollman, 1977).

1.3.1. Decidualization biology

Decidualization is the remodeling of the maternal endometrium driven by the progesterone secreted after ovulation and local cyclic Adenosine Monophosphate (cAMP) (Brar et al., 1997; Gellersen & Brosens, 2014), which stimulate the synthesis of a complex network of intracellular and secreted proteins via progesterone receptor (PR) activation (Garrido-Gomez et al., 2011; Wu et al., 2018). Endometrial decidualization involves: (i) morphological (Dunn et al., 2003), biochemical (Giudice et al., 1998; Jabbour & Critchley, 2001) and transcriptional (Wang et al., 2020) reprogramming of the endometrial stromal cells; (ii) secretory transformation of the

uterine glands (Filant & Spencer, 2014; Kelleher et al., 2019); (iii) influx of specialized immune cells (Gellersen et al., 2007); (iv) vascular remodeling and angiogenesis (Blois et al., 2011).

Decidualization starts in the mid secretory menstrual phase, geographically surrounding the terminal portion of the spiral arteries and expand through the entire tissue in the late secretory phase (Gellersen & Brosens, 2014). Morphologically, is characterized by the transformation of endometrial stromal cells (ESCs) from elongated fibroblast-like to enlarged polygonal/round cells shaped by a complex intracellular cytoskeleton rearrangement (Dunn et al., 2003). Decidualized ESCs secrete biomarkers such as PRL and IGFBP1 (Giudice et al., 1998; Jabbour & Critchley, 2001), involved in promoting EVT invasion and the classical markers of decidualization (Ramathal et al., 2010) (Figure 5).
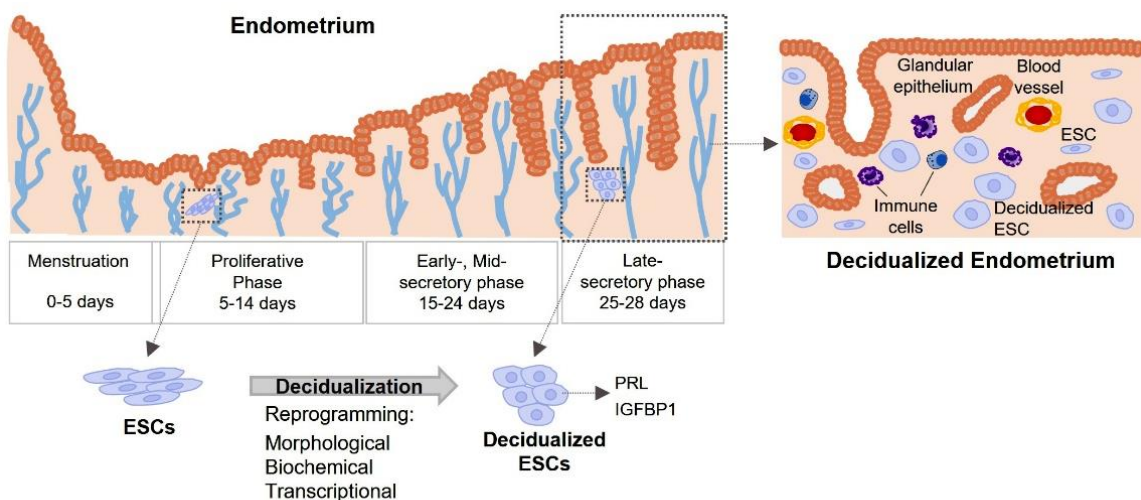
Figure 5. Menstrual cycle and decidualization. During the secretory phase, the ESCs undergo a morphological, biochemical, and transcriptional reprogramming termed decidualization. As a result, they transform into decidual cells specialized in secrete factors, such as PRL and IGFBP1. In the late-secretory phase, the endometrium is completely remodeled including transformation of blood vessels, immune and decidual cell populations and secretory glands. ESC: Endometrial Stromal Cells.

Recently, transcriptomics of the decidualization process has been characterized at single cell resolution, discovering that is initiated gradually after ovulation with a direct interplay between stromal fibroblasts and lymphocytes collaborating in the widespread decidualized features by the end of the menstrual cycle (Wang et al., 2020). This data revealed that the decidualized ESCs are less heterogeneous compared to those in early pregnancy, supporting that fully decidualization is completed in response to embryo implantation.

1.3.2. Master regulators

Decidualization is a highly coordinated process governed periodically by hormonal, biochemical, immunological, and local factors in each menstrual cycle (Ng et al., 2020).

- **Progesterone and estradiol**

Progesterone (P4) is the major driver of decidualization. After ovulation, P4 is produced by the corpus luteum, which regresses in 14 days in the absence of a conceptus. However, in case of pregnancy, the STB produces human chorionic gonadotropin to support pregnancy preventing menstruation. From 6-8 weeks of gestation, the placenta become the main source of P4 production (Carp, 2020; Tuckey, 2005).

The signal transduction pathways induced by P4 are mainly mediated by PR activation. PR consists of two isoforms PR-A and PR-B, effects from P4 are considered to represent combined activities of the two isoforms, that responds also to cAMP/PKA signaling (Wu et al., 2018). PR activates a complex signalling network comprised by high number of downstream regulators such as Indian Hedgehog (IHH), transcription factor Forkhead Box 1 (FOXO1), Signal Transducer and Activator of Transcription (STAT1, STAT3, STAT5), Bone Morphogenesis Protein 2 (BMP2), and WNT signaling, among others (Mazur et al., 2015; Ng et al., 2020). PR-B plays a predominant role during decidualization (Kaya et al., 2015). The proper temporal and cell-specific expression of *PGR*—the gene encoding PR—, is critical for uterine function

and especially for decidualization (Patel et al., 2017). In contrast, P4 can mediate non-genomic functions via progesterone receptor membrane component 1 and 2 (PGRMC1 and PGRMC2) to activate downstream signal pathways smartly (Pru & Clark, 2013).

Estradiol (E2) acting through their cognate receptor estrogen receptor 1 (ER1) is critical to prepare the uterus to support pregnancy. ER1 has been widely studied in the regulation of embryo attachment via expression of leukemia inhibitory factor (LIF) (Chen et al., 2000). Moreover, ER1 in stromal cells induce the proliferation of the epithelium in response to E2 in a paracrine manner (Cooke et al., 1997; Winuthayanon et al., 2010). This epithelial-stromal crosstalk plays an important role in the regulation of PR and ER1. *PGR* expression is enhanced by ER1, which gene expression is inhibited by PR, feedback that ensure the balanced hormonal signalling for optimal uterine function (Haluska et al., 1990; Tsai & O'Malley, 1994) (Figure 6).

Protein abundance of ER1 and PR is high in the endometrial epithelium upon proliferative phase and disappear in the secretory phase. In contrast, the expression of both genes encoding the receptors, *ESR1* and *PGR,* is retained in the stromal compartment during the mid- and late-secretory phase (Classen-Linke et al., 1998). Regarding decidualization, recently, it has been described more in depth the role of ER1 during decidualization in humans. *In vitro* decidualization of human primary ESCs revealed that E2 activates *ESR1*, which induces the expression of FOS-like 1 (*FOSL1*), matrix metallopeptidase 1 and 9 (*MMP1* and *MMP9*) (Chen et al., 2020). MMPs and cell motility is a feature acquired by decidualized cells required for embryo implantation and encapsulation (Grewal et al., 2010; Grewal et al., 2008).
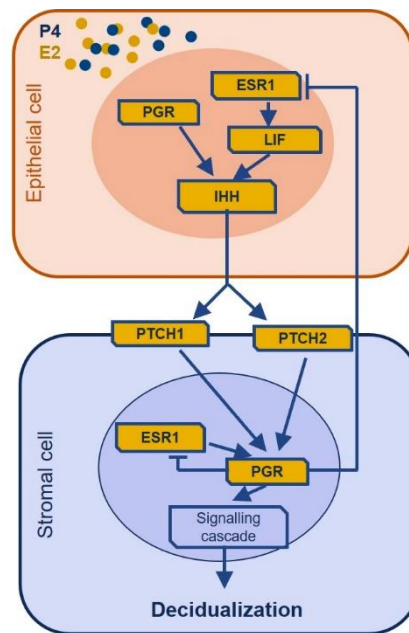
Figure 6. Epithelial-stromal crosstalk in response to progesterone and estradiol in decidualization. P4 and E2 are the major ovarian steroids governing fertility, including decidualization at the time of late-secretory phase. IHH is activated by PR and ER1 to transduce the differentiation stimulus to the stromal compartment, where receptors PTCH1 and PTCH2, and ER1 activate PR. Then, the signaling cascade to promote decidualization and to stop epithelial proliferation start simultaneously.

▪ **Biochemical factors**

Biochemical factors have been described as main modulators of decidualization. Among these, lipid mediators, interleukins and transforming growth factor-beta (TGF-β) superfamily members are highlighted (Ng et al., 2020).

Lipid signaling includes the cyclooxygenase-2 (COX2), prostaglandin E2 (PGE2) and prostacyclin released from endothelial cells in response to hemodynamic forces, emphasising the vascular endothelium role (Gnecco et al., 2019). In addition, lipid substances like endocannabinoids or phosphatidic acid are involved (Gellersen & Brosens, 2014). The expression of cannabinoid receptor-1 (*CNR1*) enhances upon decidualization and exerts a spatiotemporal control (Moghadam et al., 2005). Regarding interleukins, *IL-11* expression is highest during decidualization and could be detected in decidualized stromal cells, glandular epithelial cells, endothelial and

vascular smooth muscle cells (Dimitriadis et al., 2000). IL-6 is expressed by ESCs and plays a role in control EVT invasion and placental morphogenesis. Reduced levels in response to E2 and P4 are associated with impaired decidualization in patients with polycystic ovary syndrome, along with *IL-8* and *IL-11* downregulation (Piltonen et al., 2015).

TGF-β superfamily members include TGF-β1, activin, and BMP2 that are involved in regulating EVT invasion (Li et al., 2021). Regarding decidualization, evidence suggest these growth factors play a significant role in promoting decidualization, cAMP signaling, extracellular matrix reorganization and angiogenesis regulation (Jones, Findlay, et al., 2006; Jones, Stoikos, et al., 2006; Kim et al., 2005; Li et al., 2007).

▪ **Immunological factors**

Immunological priming of the endometrium is driven by intrinsic and extrinsic factors through, endocrine and autocrine/paracrine signals (Evans et al., 2016; Harris et al., 2019; Lane et al., 2014).

During the secretory phase the most abundant leucocytes in the uterus are NK cells that do not express *PGR* (Henderson et al., 2003). Thus, P4 condition the local leucocyte profile indirectly via ESCs secreted factors including cytokines such as IL-15 (Kitaya et al., 2005). Moreover, decidual leucocytes show specific phenotypes unlike those peripheral due to the influence of ESCs (Nancy et al., 2012). The single cell atlas of the human endometrial cycle revealed that decidualization is characterized by direct interplay between lymphocytes and stromal fibroblasts (Wang et al., 2020).

Regarding extrinsic factors, the seminal fluid contains signalling agents that promote the production of cytokines and growth factors by the female reproductive tract to induce leukocytes recruitment and regulatory T cells production (Guerin et al., 2011; Robertson, 2005). Consequently, seminal fluid induces vascular adaptation, suppression of inflammation, and tolerance to fetal antigens. This mechanism could offer a plausible explanation to some risk factors for PE, such as nulliparity and IVF conception (Robertson et al., 2003). Thus, the exposition to seminal fluid prior and

approximately at the time of implantation is involved in endometrial priming (Saftlas et al., 2014).

1.4.   Decidualization and pregnancy complications

Due to the prime role of the decidualization in pregnancy health, defects in this process can lead to obstetric complications in late stages, although the failure occur before implantation (Cha et al., 2012). Some pregnancy complications in which it has been described decidualization disruptions are intrauterine growth restriction (IUGR) (Dunk et al., 2019), infertility associated with endometriosis (Marquardt et al., 2019), age–reproductive decline (Woods et al., 2017) recurrent pregnancy loss (Lucas et al., 2020), placenta accreta (McNally et al., 2020), and sPE (Garrido-Gomez et al., 2017) (Figure 7).
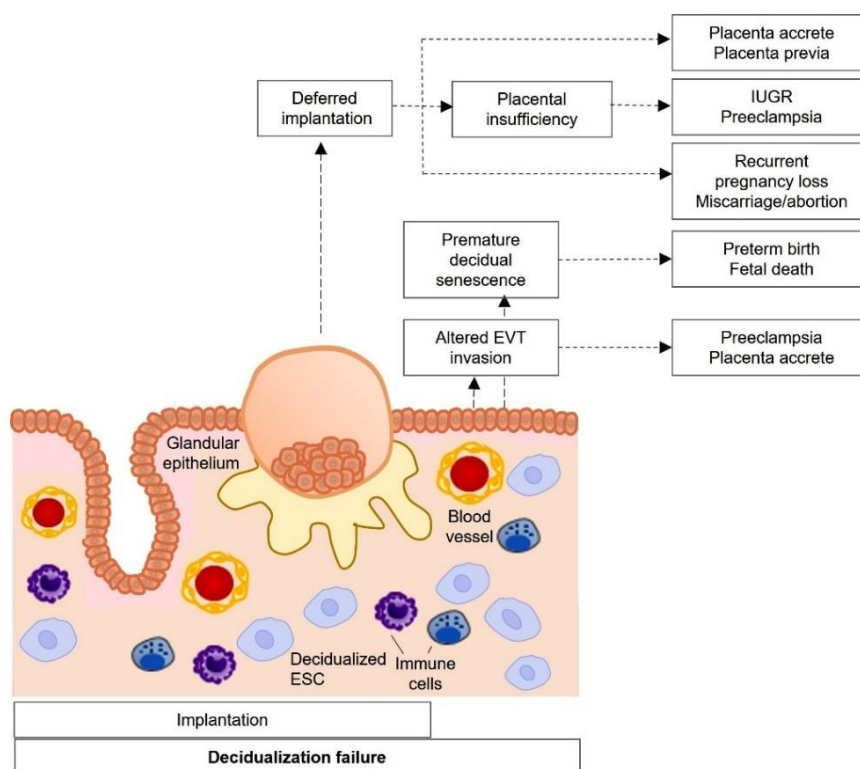
Figure 7. Decidualization failure and pregnancy complications. Decidualization starts gradually after ovulation. Stromal and epithelial compartment establish a crosstalk to prepare the uterus for embryo implantation and invasion. Failures in decidualization could impact pregnancy establishment, leading to obstetric complications in later stages. Adapted from Cha et al. 2012.

- Women affected by **endometriosis** have a reduced fertility (Macer & Taylor, 2012). Continuous inflammation due to endometriosis impairs signaling from E2 and P4 affecting decidualization of ESCs through overexpression of sex steroid hormone receptors (Wu et al., 2017). Progesterone resistance leads to deregulation stopping proliferation of the epithelium and impairing stromal decidualization. Consequently, these patients have an increased risk of infertility and miscarriage (Gellersen & Brosens, 2014; Marquardt et al., 2019).

- **Age-related reproductive decline** has been associated with decidualization and placentation defects as a major cause in those cases independent to karyotypic abnormalities (Woods et al., 2017). Maternal age is a major risk factor for pregnancy complications such as miscarriage, preeclampsia, stillbirth, and extremely preterm birth (Jacobsson et al., 2004; Jolly et al., 2000). Embryo chromosomal abnormalities is widely recognized as the underlying cause of negative reproductive outcome which increase with age. However, many complications occur in the absence of karyotype abnormalities. In these cases, the evidence from murine models suggests that the cause is a failure in decidualization leading to aberrant placentation, which in turn affect the normal development of the embryo (Woods et al., 2017). Leucocytes cell composition in the maternal-fetal interface is affected by maternal age since macrophages and dendritic cells are reduced. This alteration in immune populations was related to ESC instead of uterine NK cells based on differentially expressed genes in the decidua from young and old pregnant mice. Up to the 50% of the transcriptional changes observed were associated with an Er1 and/or Pr binding site. The expression of *Esr1* and *Pgr-A* isoform show differences in uteri distribution in aged compared young females, being the highest levels expressed in the primary decidual zone which suggests a slower decidualization (Woods et al., 2017).

- In **recurrent pregnancy loss** decidualized ESCs isolated from patients show lower expression of decidualization markers, higher vulnerability to apoptosis by oxidative stress, disrupted responses to chorionic gonadotropin and defective discrimination between high- and low-quality human embryos (Gellersen & Brosens, 2014). The aberrant decidua is not able to support the dramatic changes of oxygen tension produced in response to active maternal perfusion of the

placenta, and finally the miscarriage occurs (Salker et al., 2010; Salker et al., 2011; Salker et al., 2012; Weimar et al., 2012). Recently, aberrant decidualization and limited cell-cell communication between ESC and other decidual cell populations —such as NK and macrophages— has been described at single-cell resolution (Du et al., 2021).

- **IUGR** has been linked to failure of decidualization and maternal immune tolerance by uterovascular resistance (Dunk et al., 2019). This study evidence a defective P4 response early in pregnancy impairs ESCs decidualization leading to alterations in vascular transformation (even affecting myometrial segments), EVT invasion and maternal leukocyte recruitment. Altogether lead to the uteroplacental pathology observed in IUGR pregnancies. Consistent with this, decidua of IUGR pregnancies show a wide variety of alterations in immune cell populations during the second trimester of gestation such as increase CD4 T helper cells and T-reg, immature phenotype of dendritic cells and atypical phenotype of macrophages (Kwan et al., 2014). Alterations that can be a consequence of defective decidualization since decidualized cells modulate the immune response since the endometrial cycle and the first trimester of pregnancy (Vento-Tormo et al., 2018; Wang et al., 2020).

- **Placenta accreta spectrum** is characterized by the overly deep EVT invasion into the myometrium and beyond due to the failure of restriction mechanisms (Goh & Zalud, 2016). The maternal-fetal interface in pregnancies affected by this condition shows a thin decidua characterized by impaired decidualization (McNally et al., 2020). Additionally, the transcriptomic study of isolated CTBs from placenta accreta spectrum patients evidenced the uncontrolled invasiveness results from aberrant gene expression in EVT, affecting a total of 118 DEGs. Among these DEGs there were genes encoding molecules associated with cancer (McNally et al., 2020). The most up-regulated gene was dedicator of cytokinesis 4 (*DOCK4),* which is involved in cell invasion and angiogenesis, its overexpression in primary CTBs increased the invasion by ~3.7-fold. Thus, the primary cause of the genetic pattern described in CTBs from patients is the lack of decidualization. Consistent with it, cesarean section is a major risk factor of this condition, a surgery that impacts directly in the endometrial stroma.

- **sPE** has been associated with failure in decidualization of ESCs isolated from women who suffered the condition, suggesting that a deficient decidua is an important contributor of the pathophysiology observed in the disease (Garrido-Gomez et al., 2017). Evidence to support sPE-decidualization connection is detailed in the next section.

1.5. Defective decidualization in the origin of severe preeclampsia

The hypothesis that a decidualization failure is underlying the etiology of preeclampsia began as a logic deduction due to the intimate crosstalk between the EVT and the decidua early in pregnancy (Wallace et al., 2012). The maternal contribution of this condition is consistent with risk factors reported in epidemiological studies, being a major risk factors nulliparity, suffering PE in a previous pregnancy, and a family history of PE (Boghossian et al., 2014; Boyd et al., 2013; Cho et al., 2015; Hernández-Díaz et al., 2009).

Investigations aimed to understand decidualization and its role in PE and sPE development later in pregnancy have revealed evidence to support maternal contribution to these conditions through defective decidualization (Garrido-Gómez et al., 2022). Our hypothesis is based on defective decidualization occurs before pregnancy, being the cause of sPE rather than a consequence, and persisting years afterwards (Figure 8).
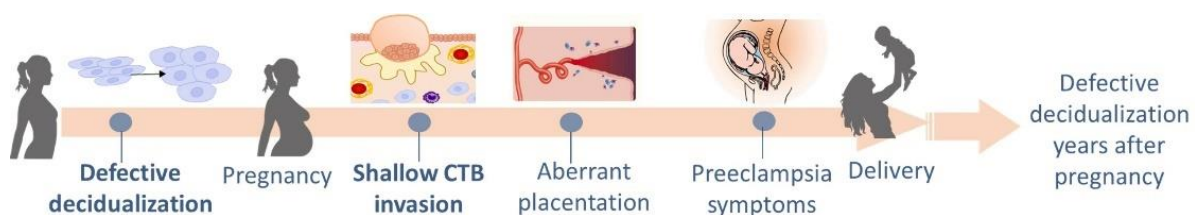
Figure 8. Model of defective decidualization in the origin of sPE. The pathogenesis of sPE is conceptualize in the next model: defective decidualization, which begins before implantation, leads to shallow CTB invasion and subsequent aberrant placentation. This would cause the release of inflammatory factors resulting in systemic endothelial dysfunction and clinical manifestation of PE. The phenotype of defective decidualization persist years after the affected pregnancy.

1.5.1. Evidence in early pregnancy

The first research to explore the link between impaired decidualization and shallow EVT invasion in PE was based on a bioinformatic approach, and it included CVS collected in the first trimester of pregnancy six months before preeclampsia was develop (Rabaglino et al., 2015). Although the sample size of this study was limited (sPE, n=4; controls, n=8), it provides signs of defective endometrial maturation precedes sPE. This study revealed 396 DEGs in the decidua from women who suffer sPE later compared with women with normal pregnancies at ~11.5 gestational weeks. This large number of deregulated genes were overlapped with gene expression data from datasets of endometrial decidualization before and after implantation −GSE4888 (Talbi et al., 2006) and GSE6364 (Burney et al., 2007)−. Strikingly, 116 of those 396 DEGs were identified as decidualization mediators in the absence of conceptus and 112 change in the opposite direction expected. Among the altered transcriptome associated with sPE, there were 16 downregulated genes associated with NK cells. These findings point towards deregulated expression of genes involved in endometrial maturity before embryo implantation are contributors of sPE development in early pregnancy and likely even before pregnancy.

Prospective research about defective decidualization during the first trimester of pregnancy implies taking a biopsy of CVS. This is a major limitation since these biopsies can be collected in pregnancies with high risk of abnormal genetic composition of the embryo because of the risk of miscarriage. Moreover, the experimental design requires the investment of largely resources to follow up thousands of patients for recruiting dozens of pregnant women who develop sPE months later.

1.5.2. Evidence at the time of delivery

Decidual samples collected at delivery from women who had sPE and spontaneous preterm birth with no signs of infection have been investigated at the level of transcriptional profiling to provide insight into decidualization deficiency when disease is present (Garrido-Gomez et al., 2017).

To that purpose, laser microdissection to isolate portions of decidua −basalis (DB) and parietalis (DP)− from tissue section of maternal–fetal interface was applied (Garrido-Gomez et al., 2017). The transcriptional profile revealed 79 DEGs in the decidua basalis DP associated with immune systems response and metabolism. In contrast, decidua basalis DB of sPE cases showed 227 DEGs dysregulated in sPE cases that were associated with affected immune system functions, oxidative stress, metabolisms, signaling and intercellular communication. Interestingly, the vast majority of those deregulated genes were decidual instead of trophoblastic. Decidualization analysis assessed in situ using PRL and IGFBP-1 immunostaining revealed the expression of these classical markers were reduced or even absent in DB and DP from sPE samples.

Furthermore, freshly isolated decidual cells from DB and DP in both cases and controls were cultured to be specifically characterized by immunostaining (Garrido-Gomez et al., 2017). Rhodamine-phalloidin immunostaining showed isolated cells had an elongated morphology with a fibroblast-like F-actin organization instead of the expected distribution in polygonal/round cells. Anti-PRL and anti-IGFBP1 immunostaining revealed much lower antibody reactivity in decidual cells isolated from sPE patients. Additionally, secreted PRL and IGFBP1 was highly reduced in cells isolated from sPE in the two compartments DB and DP. In addition, stromal cells isolated from cases failed to decidualize *in vitro*, whereas those from controls were able to undergo decidualization in response to hormonal stimulus in culture. Furthermore, the impact of decidualization failure in CTBs invasion was assessed using conditioned media from decidual cells of cases and controls cultured *in vitro*, showing cells from sPE did not support CTBs invasion.

These results, provide evidence that impaired decidualization was present at the time of delivery in women who suffer sPE and impact negatively in CTBs invasion. Thereby, this is a proof-of-concept for defective decidualization coexists with sPE symptoms and it is a phenomenon mechanistically related to reduced CTBs invasion.

1.5.3. Evidence years after the affected pregnancy

Prospective exploration of defective decidualization before pregnancy has limitations due to the nature of the sample type needed. This approach would require collecting a large number of endometrial biopsies in the order of thousands from nulliparous non-pregnant women to include a few dozen of sPE cases —taking into account the incidence of severe cases and the experimental drop out. Furthermore, endometrial biopsies are collected by invasive methods which hamper the volunteer donation. In this context, the assessment of defective decidualization years after the affected pregnancy has been key to link this failure with the pathogenesis of sPE.

Defective decidualization has been associated with sPE in early pregnancy and at the time of delivery. Strikingly, this failure persists years after the affected pregnancy (Garrido-Gomez et al., 2017) (Figure 9A). ESCs isolated from biopsies samples of women who suffered sPE and women who had normal obstetric outcomes were decidualize *in vitro* and assessed by stage-specific antigens, morphological criteria and transcriptionally profiling (Garrido-Gomez et al., 2017). Surprisingly, ESCs from cases failed to undergo decidualization showing an elongated morphology with a fibroblast-like F-actin organization, low secretion of PRL and IGFBP1, and 129 DEGs at least twofold compared to ESCs of the control group. Upregulated genes in sPE were involved in hormone conversion, extracellular structure organization, vascular development, and response to peptides. In contrast, downregulated genes in sPE included decidualization mediators —such as *IGFBP1*, *CNR1*, and *IL-1B—,* and genes involved in cytokine–receptor interactions, inflammation, TFG-β signaling, and decidualization.

Interestingly *annexin A2* (*ANXA2*) was among those 129 deregulated genes identified in ESCs from women who had sPE, showing lower expression in cases compared to controls (Garrido-Gomez et al., 2017). This gene is highly expressed in placenta and its impaired activity could cause fibrinolytic deficiency associated with increased thrombosis, which predispose to PE (Xin et al., 2012). *In vitro* and *in vivo* models of *ANXA2* deficiency were developed to investigate its relevance in decidualization and embryo invasion reveling striking findings (Garrido-Gomez et al., 2020) (Figure 9B).

ESCs isolated from women with previous sPE showed reduced gene expression and protein abundance of ANXA2 in response to decidualization stimulus *in vitro*. This finding was corroborated inducing *ANXA2* inhibition by *siRNA* in ESCs collected from fertile donors. Furthermore, ESCs with failed decidualization were not able to support embryo invasion. *In vivo*, *Anxa2*-null mice display a phenotype characterized by impaired decidualization, abnormal placentation and fetal growth restriction. These findings highlight a maternal contribution to sPE, suggesting defective decidualization as a first event that hampers placentation and embryo invasion.

Defective decidualization during and after sPE

A
Placenta
Decidua basalis
Decidua parietalis
Laser capture and global transcriptional profiling

B
Endometrial stromal cells
In vitro decidualization
Non-Decidualized Decidualized
Global transcriptome profiling

Annexin A2 role in defective decidualization and pregnancy outcome

C
Endometrial stromal cells
ANXA2
siRNA
+
In vitro decidualization
Control siRNA
ANXA2 siRNA
Decidualization assessment

D
In vivo model
Anxa2$^{+/+}$ Anxa2$^{-/-}$
vs
Endometrial tissue
Placental weight
Fetal weight
Decidualization, placentation and embryo development assessment

Figure 9. Experimental approaches to investigate maternal contribution to the etiology of sPE through defective decidualization. (A). Defective decidualization during and after sPE was demonstrated. Laser capture and global transcriptome profiling was applied to decidua basalis and parietalis of patients and controls at the time of delivery. **(B)**. Then, endometrial stromal cells from non-pregnant women were isolated and decidualized *in vitro*. Global transcriptome profiling allowed the description of defective decidualization *in vitro* in sPE patients vs controls (Garrido-Gomez et al., 2017). **(C)**. The functional role of *ANXA2* in decidualization was investigated *in vitro* and *in vivo*, using endometrial stromal cells and a murine model respectively (Garrido-Gomez et al., 2020). *ANXA2* deficiency was associated with defective decidualization in human primary cells and murine models. **(D)**. Additionally, pregnant mice with *Anxa2* deficiency showed impaired placentation and reduced fetal weight compared with the wild type.

1.6. Transcriptomics of decidualization

Many investigations have attempted to unveil the underlying pathway of decidualization in humans, revealing critical regulators and mediators of decidualization (Gellersen & Brosens, 2014). This knowledge has been an important resource to understand the endometrial biology and its impact in pregnancy health and disease, contributing to the significant progress in the fields of fertility and reproductive medicine (Ng et al., 2020). However, many approaches were based on *in vitro* models of endometrial stromal cells and targeted technologies. The main limitations of this type of experimental designs emerge from the characterization of one cell type isolated from its biological niche and the assessment of specific transcripts and proteins.

During the last decade, technological advances have provided high-throughput data acquisition tools and, in parallel, bioinformatics has evolved to allow the analysis of the omics results obtained. Consequently, global approaches are now available to characterise the global transcriptome and the proteome profile of biological systems. This progress has been more effective in the field of transcriptomic, since lower amounts of biological material is required, and gene expression assessment is less complex than proteomic mapping and quantification due to the nature of this biomolecules.

Gene expression arrays have been applied to describe the transcriptome profile of ESCs before and after decidualization in culture, characterising the genetic reprogramming of fibroblasts in response to hormonal stimuli (Cloke et al., 2008; Lu et al., 2008). Even more, this approach was applied for profiling the transcriptome of ESCs during *in vitro* decidualization from women who suffered sPE as it was detailed in a previous section (Garrido-Gomez et al., 2017). These studies were an important step forward in the global characterisation of endometrial differentiation in health and disease. However, microarray results are limited to detect transcripts that hybridize in pre-designed probes. This limitation was solved by RNA sequencing (RNA-seq) technology based on next generation sequencing (Figure 10).
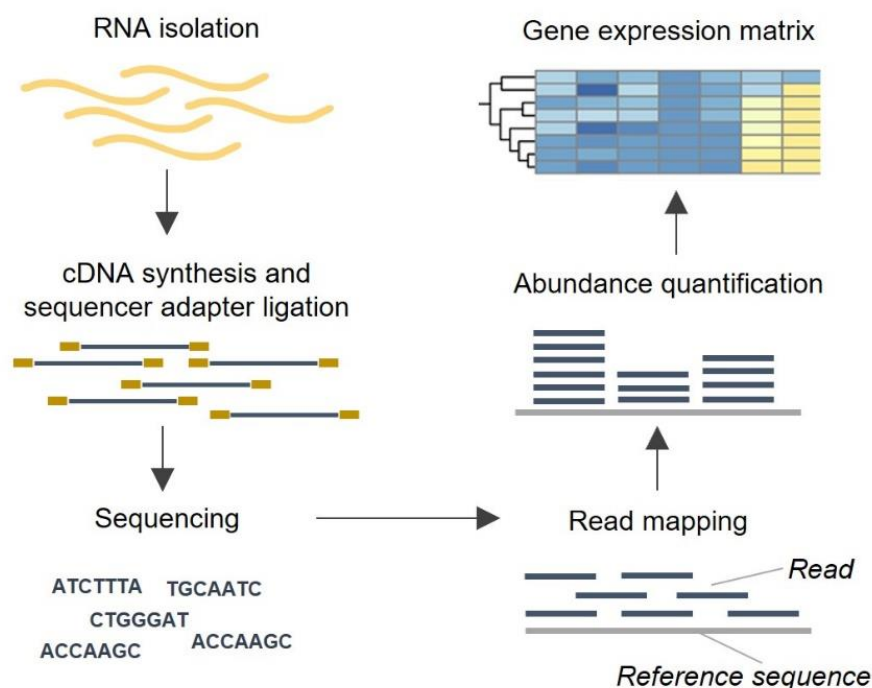
Figure 10. RNA-seq workflow. RNA-seq is designed to capture any transcript present in the sample. RNA is isolated and used as a template for cDNA synthesis. Then sequencer adapters are ligated to each cDNA fragment depending on the sequencing platform used. All sequences obtained are mapped against a reference genome using bioinformatic tools. Thus, RNA-seq provides absolute quantification rather than relative expression values of every transcript present in the sample and it allows hypothesis-free experimental design.

RNA-seq is a comprehensive tool that allow to describe the transcriptome with robustness and high resolution (Wang et al., 2009). Briefly, this technique consists in RNA isolation and purification, conversion of RNA molecules into complementary DNA (cDNA) and ligation of sequencer adapters. The set of cDNA fragments attached to specific adapters is called library. Then, this library should be quantified and denaturalized before sequencing. These general steps have specifications depending on the manufacturer, the RNA types of interest and the platform chose for sequencing, but generally all strategies need a low amount of RNA input, require a short time of handling, and offer a good performance. The last advances allow to apply the RNA-seq to single cells (scRNA-seq). It is an emerging and promising tool whose application is being extended. However, currently it requires expensive resources in terms of reagents, devices, and data analysis, consequently, is not widely used yet. Although scRNA-seq has been applied to decipher the transcriptomic atlas of the endometrium along the menstrual cycle, the sample size of women included was reduced due to the technical challenge and affordability (Wang et al., 2020).

In this context, the analysis of the global and *in vivo* transcriptome during decidualization would reveal a broad of new findings in this field. Since, it would overcome the limitations associated with *in vitro* models and targeted strategies. Thus, this study applies RNA-seq to endometrial biopsies from sPE patients and controls, with the aim of identifying the *in vivo* transcriptome profile encoding defective decidualization in sPE.

2. HYPOTHESIS

2. HYPOTHESIS

Endometrial decidualization precedes embryo implantation to prepare the uterus for pregnancy establishment and maintenance. Hence, failures in this tissue remodelling could lead to obstetric complications that manifest in later stages of gestation. Defective decidualization has been associated with sPE at the time of delivery and *in vitro* models demonstrate this defect lingers years after the affected pregnancy.

Here, we hypothesize that an *in vivo* defective decidualization encoded by an aberrant transcriptome profile during the secretory phase of the endometrial cycle in non-pregnant women who suffered sPE should exist. The characterization of the molecular defect/s orchestrating defective decidualization is a significant progress toward the development of new strategies that enable early assessment of risk for sPE and therapies to reduce its morbidity and mortality associated.

3. OBJECTIVES

3. OBJECTIVES

3.1. General objective

- To determine *in vivo* the fingerprint and molecular mechanisms underlying defective decidualization in the endometrium of women who suffered sPE, creating strong foundations for developing a screening tool targeting to its risk assessment and early detection.

3.2. Specific objectives

- To identify the endometrial transcriptome alterations during the late-secretory phase in sPE using global RNA-seq approach.

- To formulate and validate the transcriptomic fingerprint that encodes defective decidualization (DD fingerprint) in sPE *in vivo* which persist years after the affected pregnancy.

- To investigate the molecular pathways associated with the defective decidualization footprint.

- To explore the protein interactions encoded by the DD fingerprint within the maternal-fetal interface of sPE patients.

- To model the network that connect DD with hormonal signaling in sPE patients that persist over time.

4. MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Study design

A total of 40 non-pregnant women who experienced a previous pregnancy were enrolled in this study for endometrial RNA-sequencing analysis. Endometrial samples were obtained for research purposes during late secretory phase in 24 women who had developed sPE in a previous pregnancy and in 16 women with no history of sPE with full term (n=8) and preterm pregnancies (n=8) as controls. sPE was clinically defined based on elevated blood pressure (systolic ≥160 or diastolic of ≥100 mm Hg) or thrombocytopenia, impaired liver function, progressive renal insufficiency, pulmonary edema, or the onset of cerebral or visual disturbances. Endometrial biopsies were processed to obtain RNA and then converted to cDNA for library generation to perform next generation sequencing. The experimental design was based on a stratified random sampling with a 70:30 proportion in two cohorts: a training (n=29) and validation (n=11) set of samples. The training set of samples was analyzed by RNA-seq to identify the global transcriptomic profiling changes between control (n=12) and sPE (n=17) samples. Selection criteria were applied to define the DD fingerprint in sPE. These criteria were based on statistical and biological significance: false discovery rate (FDR) < 0.05, fold change (FC) > 1.4. This strategy allows to obtain a filtered list of genes that suffer the major changes in gene expression. Additionally, we just selected those genes with an Entrez ID assigned to ensure the selection of genes with annotated features for further analysis. Finally, targeted analysis of the defective decidualization (DD) fingerprint was validated in the test set composed of controls (n=4) and sPE (n=7) (Figure 11).
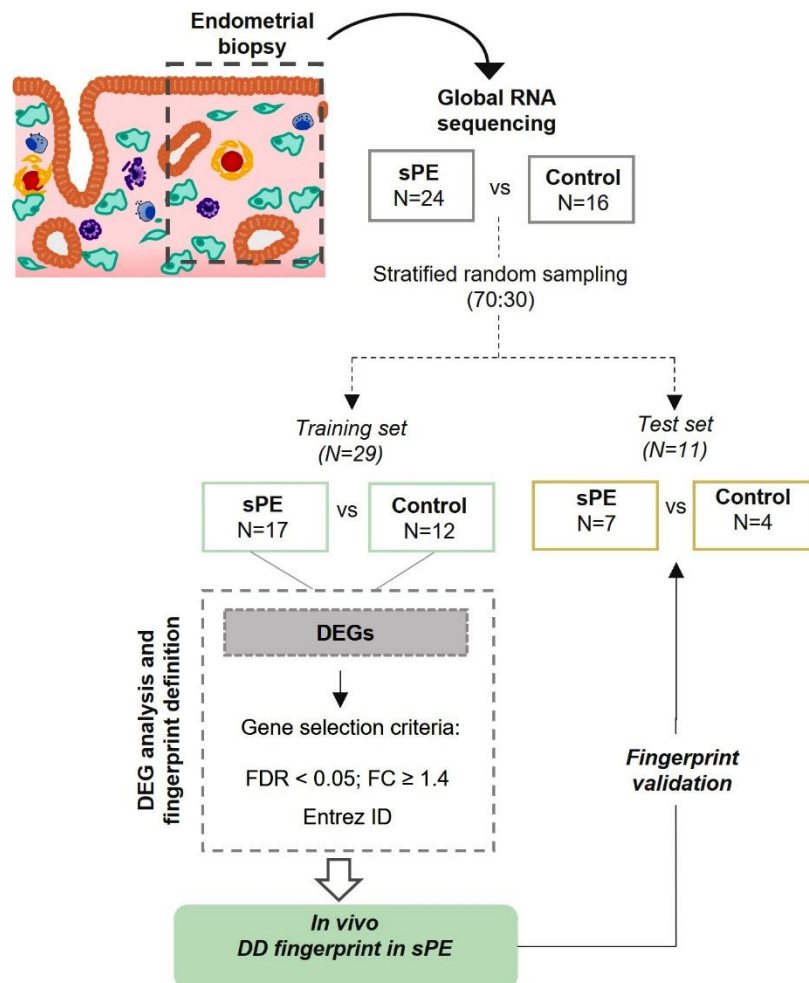
Figure 11. Schematic drawing of the study design used to identify and validate DD fingerprint in sPE. Endometrial samples were collected at the time of late secretory phase from 24 sPE cases and 16 controls. Random sampling was performed to stratify the cohort in the training and the test set, following a proportion of 70% and 30% from the total of patients, respectively. As a result, 29 patients were included in the training set to obtain the fingerprint and 11 patients in the test set for validation.

4.2. Human donors

Endometrial samples were collected from women aged 18–42 without any medical condition who had been pregnant 1–8 years earlier. All participants had regular menstrual cycles (26-32 days) with no underlying gynecological pathologic conditions and had not received hormonal therapy in the 3 months preceding sample collection. After the inclusion criteria were applied, endometrial biopsies were obtained by pipelle catheter (Genetics Hamont-Achel,

Belgium) under sterile conditions in the late secretory phase (cycle days 22-32). Specimens were kept in preservation solution until processing. Maternal and neonatal characteristics of women with sPE and controls are summarized in Table 3. Biological and technical variables for each donor were considered to discard confounding effects on the transcriptomic profile (Table 4). This study was approved by the Clinical Research Ethics Committee of University and Polytechnic La Fe Hospital (Valencia, Spain) (2011/0383), and written informed consent was obtained from all participants before tissue collection and all samples were anonymized.

Table 3. Maternal and neonatal characteristics for endometrial donors.

Biological variables	sPE (n=24)	Term pregnancy (n=8)	Preterm pregnancy (n=8)	P* (sPE vs term)	P* (sPE vs preterm)
Maternal age (years)	37.3 (0.8)	37.6 (1.1)	34.9 (2.4)	n.s.	n.s.
Systolic blood pressure (mm Hg)	164.8 (3.5)	124.0 (6.6)	113.1 (7.7)	< 0.001	< 0.001
Diastolic blood pressure (mm Hg)	99.0 (2.1)	69.4 (2.4)	62.8 (3.0)	< 0.001	< 0.001
Proteinuria (mg/dL)	280.2 (42.5)	0 or NA	0 or NA	NA	NA
Gestational age at delivery (weeks)	31.9 (0.6)	39.5 (0.4)	33.9 (1.2)	< 0.001	n.s.
Birth weight (g)	1651.6 (153.3)	3200.1 (160.3)	2403.8 (218.3)	< 0.001	< 0.05
Parity (n)	1.7 (0.2)	3.4 (0.7)	2.8 (0.7)	< 0.01	< 0.05
Interval from last pregnancy to endometrial biopsy (years)	3.4 (0.3)	4.7 (1.0)	3.3 (1.0)	n.s.	n.s.

Mean ± SEM* Wilcoxon test.
NA: Not available
n.s.: Not significant

Table 4. Biological and technical variables of interest for controlling confounding effects in the RNA-seq analysis.

#Donor	Group	(i) Age	(ii) Time since last pregnancy	(iii) Day of cycle	(iv) Processing batch	(v) RIN of RNA	(vi) rRaw reads per library	(vii) Percent mapped reads	(viii) Ethnicity
C1	Control	39	1.5	4	1	8.4	16025682	94.89	Caucasian
C2	Control	37	4.4	1	2	6.3	11096416	94.36	Caucasian
C3	Control	40	4.8	4	2	7	8164469	92.22	Caucasian
C4	Control	34	8.9	2	2	8.4	11031261	93.79	Caucasian
C5	Control	41	1.5	4	1	5.2	8312541	92.61	Caucasian
C6	Control	38	8.7	6	1	5.3	14394669	93.67	Latin
C7	Control	28	1.4	3	2	7.9	25243090	97.21	African
C8	Control	43	2.9	4	2	7.4	12967178	94.52	Caucasian
C9	Control	36	1.8	5	2	8.6	21756039	96.26	Latin
C10	Control	30	4	2	2	8.7	15334997	96.95	Caucasian
C11	Control	41	2.3	2	1	8.9	20486156	94.51	Caucasian
C18	Control	38	1.4	1	3	8	8397993	97.08	Caucasian
C20	Control	41	8.8	5	3	9.3	5122956	96.18	Caucasian
C21	Control	36	5.3	6	3	9.1	8748652	97.61	Caucasian
C22	Control	33	4.2	4	3	9.4	8415149	96.36	Caucasian
C25	Control	25	1.5	2	3	8.1	6989298	95.83	Latin

#Donor	Group	(i) Age	(ii) Time since last pregnancy	(iii) Day of cycle	(iv) Processing batch	(v) RIN of RNA	(vi) rRaw reads per library	(vii) Percent mapped reads	(viii) Ethnicity
sPE1	sPE	37	2.6	5	3	8.4	10612006	97.22	Caucasian
sPE2	sPE	30	2.7	1	3	9.1	16534887	94.04	Caucasian
sPE3	sPE	36	4	3	3	9.3	11852535	96.30	Caucasian
sPE4	sPE	38	4.5	4	3	9.2	9192303	92.70	Caucasian
sPE5	sPE	41	2.6	6	3	8.7	7652629	95.22	Caucasian
sPE6	sPE	40	7.5	1	3	8.3	6719499	96.67	Caucasian
sPE7	sPE	40	3.8	1	3	9.2	14618494	97.37	Latin
sPE8	sPE	39	2.9	1	3	9.6	11476573	96.59	Caucasian
sPE9	sPE	40	4	2	3	9.4	17113411	97.52	Latin
sPE10	sPE	30	3.2	5	2	8.4	27246424	95.16	Caucasian
sPE11	sPE	40	5.8	3	1	7.6	16305782	95.66	Caucasian
sPE12	sPE	42	1.6	1	2	8.5	11722265	97.06	Caucasian
sPE13	sPE	41	5.1	4	1	4.9	20164331	96.01	Caucasian
sPE14	sPE	29	3.3	4	2	7.4	22636735	97.00	Caucasian
sPE15	sPE	39	2.9	3	1	8	31426841	93.87	Latin
sPE16	sPE	34	3.5	5	1	7	12101006	93.21	Latin
sPE17	sPE	30	3	4	1	8	21158390	93.78	Caucasian
sPE18	sPE	37	3.25	6	1	8.7	27905439	96.26	Caucasian
sPE19	sPE	36	1.92	1	2	6.9	10813702	94.98	Caucasian
sPE20	sPE	34	1.88	3	2	8.1	22361473	93.86	Caucasian
sPE21	sPE	40	2	3	2	8.4	21147093	96.31	Caucasian
sPE22	sPE	43	1.29	1	1	8.7	30957526	95.72	Caucasian
sPE23	sPE	37	4.42	2	1	7.5	23875845	94.79	Caucasian
sPE24	sPE	41	2.8	2	2	7.5	22885895	96.86	Caucasian

4.3. RNA extraction

Total RNA from endometrial biopsies was isolated using QIAsymphony RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentrations were quantified using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Waltham, US) at a wavelength of 260 nm. The integrity of the total RNA samples was evaluated by the RNA integrity number (RIN) and DV200 metrics using an Agilent high-sensitivity RNA ScreenTape in a 4200 TapeStation system (Agilent Technologies Inc., Santa Clara, CA). Samples used for the global RNA-seq showed RIN values ranging from 4.9 to 9.2.

4.4. Targeted RNA sequencing (custom panel)

RNA customized panel was built to sequence those 129 genes previously detected as *in vitro* sPE signature (Garrido-Gomez et al., 2017) (Table 5), using the Ion Ampliseq Designer (www.ampliseq.com) (Thermo Fisher Scientific, MA, USA). Fourteen nanograms of the RNA were taken for reverse transcription reaction and the resulting amplicons were used to generate the libraries with Ion Ampliseq Library kit 2.0 (Thermo Fisher Scientific, MA, USA). The average size of the cDNA libraries was approximately 120 bp (including the adapters). The pooled libraries were introduced in the Ion Chef System (Life Tech, USA) for clonal amplification and enrichment, the resulting pool was loaded in an Ion 540 chip kit (Thermo Fisher Scientific) and next generation sequencing run was performed in an Ion S5 system (Life Tech, USA).

Table 5. *In vitro* sPE signature. (Garrido-Gomez et al., 2017).

Gene symbol			
AADAC	COL8A1	IGFBP1	PITX1
ABLIM2	CPE	IGFBP5	PPAP2B
ADAMTS19	CRLF1	IL15	PRUNE2
ADAMTS8	DBC1	IL1B	RASGRP2
ADRA2A	DCN	IRS2	RASL11B
ALDH1A1	DDIT4	ISM1	REEP2
ANGPT2	DENND2A	ITGA11	RGS16
ANXA2	DES	KCNJ8	RGS20
ARHGDIB	DMKN	KLF2	RHOU
ATCAY	DUSP6	KRTAP17-1	RLN2
BAIAP2L2	EDNRA	LAMA5	RSPO3
BDNF	EDNRB	LOC728392	SBSN
C10orf10	EFEMP1	LOXL4	SCARA5
C14orf37	EGR1	LPAR1	SCG5
C17orf107	EHD3	LPL	SERPINA3
C1orf133	ENST00000313664	LRRC15	SERTAD4
C1QTNF7	ENST00000380464	LSAMP	SIPA1L2
C4orf49	ERAP2	LTBP1	SLC35F3
C6orf176	ERP27	LYPD1	SLC7A2
CA12	F2RL2	MEST	SLITRK6
CCDC81	FAM19A2	MFAP2	SPARCL1
CCL8	FAM38B	MRVI1	SSTR1
CFD	FAT1	MYCN	SULF1
CHI3L2	FBXO2	MYLK	TMEM132C
CHODL	FST	NANOS3	TMEM25
CHST7	GAL	NCKAP5	TNFAIP6
CLEC3B	GALNT14	NKAIN1	TNFRSF10C
CLIC3	GALNTL2	NPR1	TNFRSF8
CNIH3	GBP2	NPTX1	TTR
CNR1	GGT5	OLFML1	WNT6
COCH	GRP	OXTR	
COL14A1	HMCN1	P2RY14	
COL15A1	HSD17B2	PDGFD	

4.5. Global RNA-seq library preparation and transcriptome sequencing

cDNA libraries from total RNA samples (n=40) were prepared using an Illumina TruSeq Stranded mRNA sample prep kit (Illumina, San Diego, CA) following a balanced batch-group design. Three micrograms of total RNA were used as the RNA input according to the manufacturer's protocol. mRNAs were isolated from the total

RNAs by purifying the poly-A containing molecules using poly-T oligo attached to magnetic beads. The RNA fragmentation, first and second strand cDNA syntheses, end repair, single 'A' base addition, adaptor ligation, and PCR amplification were performed according to the manufacturer's protocol. The average size of the cDNA libraries was approximately 350 bp (including the adapters). cDNA libraries were quantified using an Agilent D1000 ScreenTape in a 4200 TapeStation system (Agilent Technologies Inc., Santa Clara, CA). Libraries were normalized to 10 nM and pooled in equal volumes. The pool concentration was quantified by qPCR using the KAPA Library Quantification Kit (Kapa Biosystems Inc.) before sequencing in a NextSeq 500/550 cartridge of 150 cycles (Illumina, San Diego, CA). Indexed and pooled samples were sequenced 150-bp paired-end reads on the Illumina NextSeq 500/550 platform according to the Illumina protocol.

4.6. RNA-seq analysis

Reads were mapped to the hg19 human genome transcriptome using the STAR (version 2.4.2a) read aligner (Dobin et al., 2013). FastQC (version 0.11.2) was used to determine the quality of FASTQ files. The manipulation of SAM and BAM files was done with the software SAMtools (version 1.1) (Li et al., 2009). To count the number of reads that could be assigned to each gene, we used HTSeq (version 0.6.1p1) (Anders et al., 2015) and BEDtools software (version 2.17.0) (Quinlan & Hall, 2010) to obtain gene coverage and work with bedFiles. Quality control filters in each program were used following the software package recommendations, and reads were filtered by mapping quality greater than 90%. Transcriptomic data were deposited in the Gene Expression Omnibus database (accession number GSE172381). The Bioconductor package edgeR (version 3.24.3) (Robinson et al., 2010) was used to analyze differentially expressed genes. The trimmed mean of M-values normalization method was applied to our gene expression values. The glmTreat function was used to find differentially expressed genes between groups. The p-value adjustment method was FDR with a cut-off of 0.05 (FDR<0.05) and the fold-change threshold was 1.2. edgeR analysis was carried out in R version 3.5.1. A volcano plot was created to visualize differentially expressed genes (DEGs). Custom scripts are available on GitHub at link https://github.com/mclemente-igenomix/garrido_et_al_2021.

4.7. Deconvolution analysis

The endometrial transcriptome obtained by RNAseq was deconvoluted using the single-cell atlas of the endometrium at the time of late-secretory phase (Wang et al., 2020). Therewith, deregulated genes in sPE expressed by fibroblast and lymphocytes were obtained overlapping our bulk tissue dataset with the transcriptome atlas of these cell types. Moreover, the proportion of each major cell type that composes the endometrium (ciliated and unciliated epithelium, stromal fibroblasts, macrophages, endothelial cells and lymphocytes) was estimated. This estimation was calculated using MuSiC R package (version 0.2.0) (Wang et al., 2019). MuSiC enables the transfer of cell type-specific gene expression information from one dataset to another, weighting genes based on their variance among samples. Those genes that show high consistency between subjects are considered informative to deconvolute the transcriptomics of bulk tissue estimating cell proportions.

4.8. Transcriptomic fingerprinting definition and validation

Genes with assigned EntrezID with an FDR cut-off of 0.05 and an expression ≥1.4-fold higher in the sPE vs. control training set samples were selected to define a fingerprint associated with DD in sPE. Targeted analysis of the fingerprint genes was performed using the validation set of samples. PCA and unsupervised hierarchical clustering with a Canberra distance based on gene signature were performed comparing sPE to control specimens. Custom scripts are available on GitHub at https://github.com/mclemente-igenomix/garrido_et_al_2021.

4.9. Enrichment analysis

GO analyses were conducted to obtain biological processes using the *goana* function in edgeR (Robinson et al., 2010). The input genes were those 120 included in the fingerprinting. The p-value adjustment method was FDR with a cut-off of 0.05 (FDR<0.05).

Kyoto Encyclopedia of Genes and Genomes annotation was obtained using The Database for Annotation, Visualization and Integrated Discovery (DAVID). The input genes were those 120 included in the fingerprint. Clustering is based on Kappa statistic score to measure relationships among the annotation terms based on the degrees of their co-association genes and a novel fuzzy clustering algorithm to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups. Statistical significance is calculated using the EASE Score and the p-value adjustment method was FDR with a cut-off of 0.05 (FDR<0.05). To establish the gene list is enriched in a specific pathway the EASE score considers the proportion of genes in the list involved compared with the proportion of genes in the human genome that participate. The Hypothetical example provided by DAVID: "In the human genome background (30,000 genes total; Population Total (PT)), 40 genes are involved in the p53 signaling pathway (Population Hits (PH)). A given gene list has found that three genes (List Hits (LH)) out of 300 total genes in the list (List Total (LT)) belong to the p53 signaling pathway. Then we ask the question if 3/300 is more than random chance compared to the human background of 40/30000. Exact p-value = 0.007. Since p-value < 0.05, this user's gene list is specifically associated (enriched) in the p53 signaling pathway by more than random chance."

4.10. Interaction network

An interaction network between proteins encoded by DD fingerprint genes was created using the functional analysis suite String (Jensen et al., 2009). To construct the network, the interactions included were from curated databases and included experimentally determined and predicted interactions, textmining, co-expression information. The clustering algorithm k-means was applied based on the distance matrix obtained from the String global scores. The network was visualized using Cytoscape software (Shannon et al., 2003). Hub genes were extracted using the maximal clique centrality (MCC) and maximum neighborhood component (MNC) from the cytoHubba plugin (Chin et al., 2014). The overlapping genes identified by the two topological analysis methods were selected as the hub genes.

4.11. qRT-PCR

To validate our transcriptomic results, a selection of differentially expressed genes was validated by qRT-PCR in a subgroup of samples from the experimental cohort [controls (n=9) and sPE (n=14)]. *IHH, MSX2, ESR1*, and *PGR* isoforms were assessed to obtain their gene expression values in the endometrial tissue. Specific primers for each gene are described in Table 6. cDNA was generated from 400 ng of RNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, US). Template cDNA was diluted 5 in 20 and 1 µL was used in each PCR. Real-time PCR was performed in duplicate in 10 µL using commercially validated Kapa SYBR fast qPCR kit (Kapa biosystems Inc, Basilea, Switzerland) and the Lightcycler 480 (Roche Molecular Systems, Inc, Pleasanton, CA) detection system. Samples were run in duplicate along with appropriate controls (i.e., no template, no RT). Cycling conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 1 s. A melting curve was done following the product specifications. Data were analyzed using the comparative Ct method (2−ΔΔCT). Data were normalized to the housekeeping gene β-actin, changes in gene expression were calculated using the ΔΔCT method with the control group used as the calibrator; values are illustrated relative to median in the control group. The relative expression of *PGR-A* mRNA was calculated by subtracting the relative expression of *PGR-B* mRNA from that of *PGR* total.

Table 6. RT-qPCR primer list.

Sequence name	Sequence
ERP27_FW	ACAAGGCCTCCCCAGAGTAT
ERP27_RV	CTTCTGCTGTGGGCAGTGTA
ESR1_FW	ATGTGCCTGGCTAGAGATCC
ESR1_RV	CAAACTCCTCTCCCTGCAGA
IHH_FW	CTCGCCTACAAGCAGTTCAG
IHH_RV	CCTGTGTTCTCCTCGTCCTT
ISM1_FW	GACCTGTGACCGTCCAAACT
ISM1_RV	AGAACTCGCTTTTGCAGCTC
MEST_FW	CGCAGGATCAACCTTCTTTC
MEST_RV	CATCAGTCGTGTGAGGATGG
MFAP2_FW	CCAGATCGACAACCCAGACT
MFAP2_RV	GCAAGGCCTGTGTATGGAGT
MSX2_FW	ATATGAGCCCTACCACCTGC
MSX2_RV	GCTTTTCCAGTTCTGCCTCC
PGR_FW	GTGGGAGCTGTAAGGTCTTCTTTAA
PGR_RV	AACGATGCAGTCATTTCTTCCA
PGRB_FW	TCGGACACCTTGCCTGAAGT
PGRB_RV	CAGGGCCGAGGGAAGAGTAG
PIAS3_FW	GAAGCGCACTTTACCTTTGC
PIAS3_RV	GCACAGTTTCCCATTGACCT
PLZF_FW	CATGATCCAGCTGCAGAACC
PLZF_RV	AAGGTCTTTGGCGAGAGGAA
REEP2_FW	GGGTGCTGTCAGAGAAGCTC
REEP2_RV	TGTCTCCCATGTCATCCTCA
STAT3_FW	TTTCACTTGGGTGGAGAAGG
STAT3_RV	GCTACCTGGGTCAGCTTCAG

4.12. Immunofluorescence of tissue sections

Endometrial tissue samples were fixed in 4% paraformaldehyde and preserved in paraffin-embedded blocks. For immunostaining, tissue sections were deparaffinated and rehydrated. Antigen retrieval was performed with buffer citrate 1x at 100°C for 10 min. Then, non-specific reactivity was blocked by incubation in 5% BSA/0.1% PBS-Tween 20 at room temperature for 30 min. Sections were incubated at room temperature for 1.5 h with primary antibodies (1:50 rabbit monoclonal anti-human progesterone receptor, Abcam, Cambridge, UK) and 1:50 mouse monoclonal anti-human estrogen receptor 1 (Santa Cruz Biotechnology, CA, USA) diluted in 3%

BSA/0.1% PBS-Tween 20. Then, slides were washed two times for 10 min with 0.1% PBS-Tween 20 before they were incubated for 1 h at room temperature with AlexaFluor-conjugated secondary antibodies diluted in 3% BSA/0.1% PBS-Tween 20. Finally, slides were washed two times in 0.1%PBS-Tween 20. To visualize nuclei, 4′,6-diamidino-2-phenylindole at 400 ng/uL was used. Tissue sections were examined using an EVOS M5000 microscope.

4.13. Statistical analysis

Clinical data are expressed as mean ± standard error mean (SEM). Clinical data were evaluated by Wilcoxon test for comparisons between sPE and control samples. Statistical significance was set at $p < 0.05$. Differential expression analysis was performed using the R package edgeR, the p-value adjustment method was FDR with a cut-off of 0.05 (FDR<0.05).

4.14. Data sets previously published

Different data sets previously published have been used to complete analyses along this doctoral thesis, the detailed information is presented in Table 7.

Table 7. Data set information and results section in which analysis is explained.

Data set description	Reference	Results section
Transcriptome-wide analyses of hESC decidualized *in vitro*.	(Garrido-Gomez et al., 2017)	5.2
Single-cell transcriptomic atlas of the human endometrium during the menstrual cycle.	(Wang et al., 2020)	5.3
The human protein atlas.	(Uhlén et al., 2015)	5.5
Progesterone receptor transcriptome and cistrome in decidualized human endometrial stromal cells.	(Mazur et al., 2015)	5.5
Biological and biochemical consequences of global deletion of exon 3 from the ER alpha gene.	(Hewitt et al., 2010)	5.5
Database for Regulations of Human Transcription Factors and Their Targets.	(Zhang et al., 2020)	5.5
Differentially expressed genes between chorionic villous samples obtained from preeclamptic and normal pregnant women.	(Rabaglino et al., 2015)	5.6

5. RESULTS

5. RESULTS

5.1. *In vivo* DD transcriptome associated to sPE

Having reported the ESCs *in vitro* DD associated with sPE in our previous work, we then wanted to investigate this phenotype *in vivo*. We applied global RNA-seq to endometrial samples obtained during the late secretory phase from women who developed sPE in a previous pregnancy (n=24) versus controls who never had sPE (n=16) (GSE172381). Clinical maternal and neonatal characteristics of the participants are summarized in Table 3.

After quality trimming and filtering, the DNA sequences from each fragment (known as reads) were aligned to the reference genome hg19. The forty samples produced 56,638 raw sequencing reads; After quality filtering and normalization, 18,301 genes were kept in the analysis. Confounding effects on the transcriptomic profile were discarded considering biological and technical variables for each donor (Table 4). A principal variance component analysis (PVCA) was applied to fit a mixed linear model using biological and technical variables as random effects to estimate and partition the total variability (Figure 12). The results demonstrated that the effect of biological and technical variables evaluated individually is lower than 0.05, and their confounding effects are negligible. By contrast, the variable group (control and sPE) is the most plausible variable compared with the rest of the individual or double-interaction between variables (lower than 0.1)
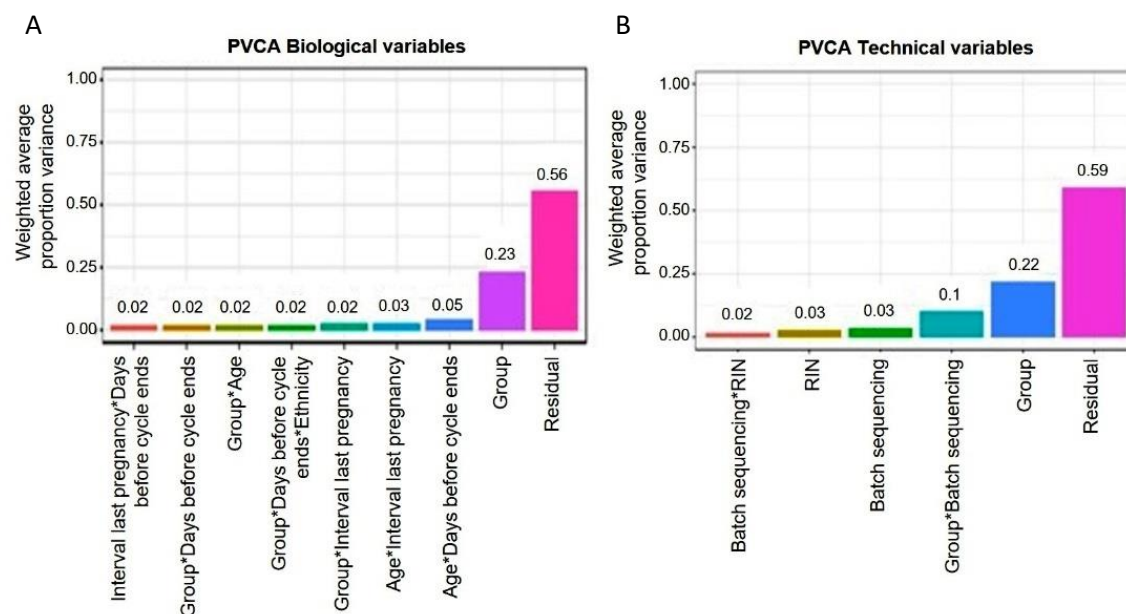
Figure 12. Principal variance component analysis (PVCA). (A). Biological variables. **(B)**. Technical variables. Axis y: proportion of the variance attributed to each variable. Axis x: variables.

The control group included women who had a preterm birth with no signs of infection (n=8), and women who gave birth at full term with normal obstetric outcome (n=8). First, we tested that these patients did not present transcriptional differences to ensure they can be considered as a unique group. Transcriptomic profiles were compared by differential expression analysis, revealing no significant changes in the endometrial transcriptome between preterm and term controls (FDR ≥0.05) (Figure 13A). After reducing the dimensionality of our dataset by principal component analysis (PCA), it was revealed the variance among samples is weakly explained by the transcriptomic data (Figure 13B) supporting no underlying pattern of distribution depending on gestational age at delivery.
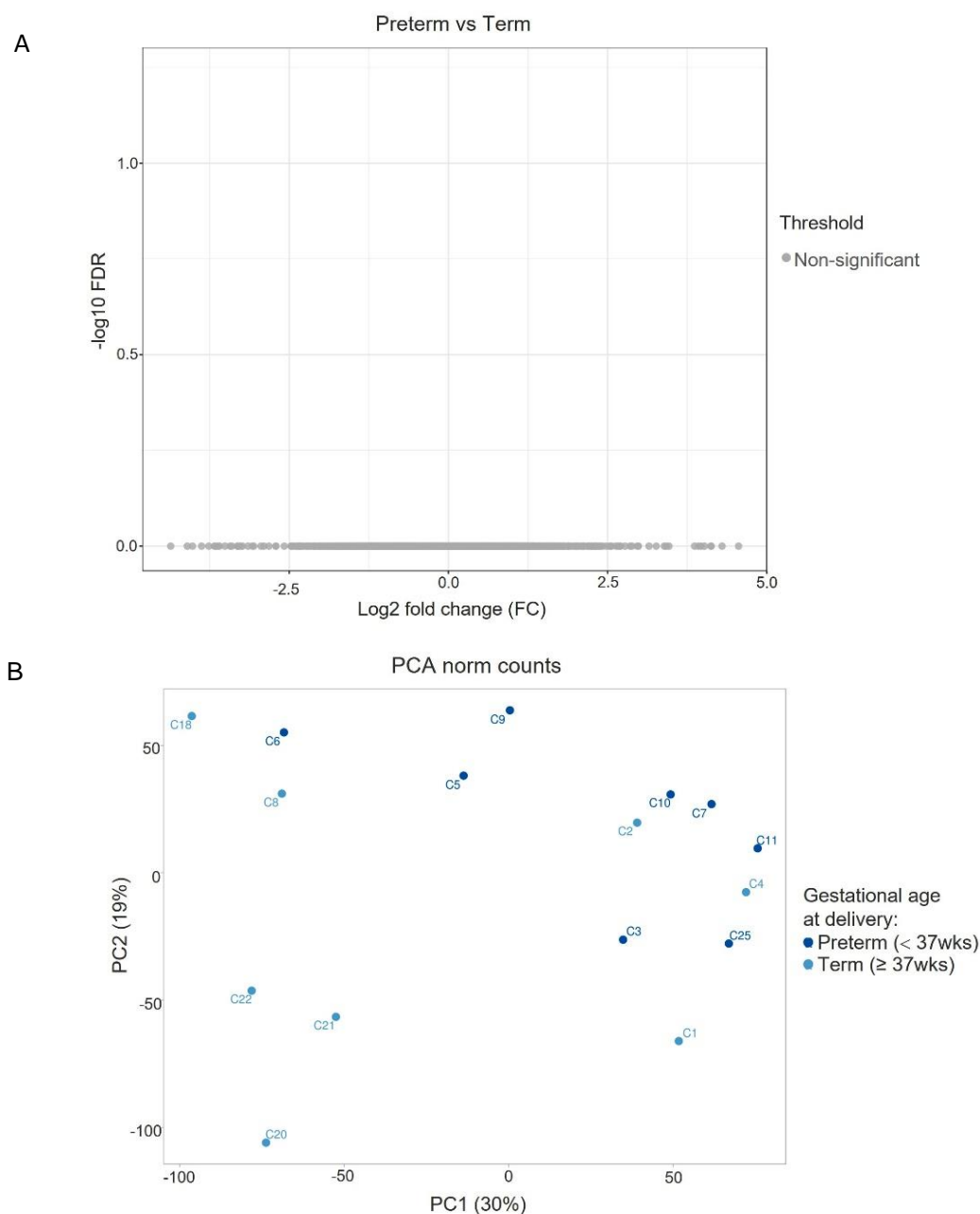
Figure 13. Transcriptomic analysis based on gestational age at delivery of control samples. **(A).** Volcano plot showing there were not significant DEGs between controls according to gestational age at delivery. Labels show the two criteria that we used to define the differentially expressed genes: p-value adjusted (FDR < 0.05) and fold-change (FC ≥ 1.2). Legend: Not significant (FDR ≥ 0.05). **(B).** PCA based on 18,476 genes after filtering out lowly expressed genes do not demonstrate clustering based on gestational age.

Once we ruled out bias on controls due to gestational age at delivery, we randomly split samples into two cohorts, a training set (70%) and a test set (30%). Random

sampling occurred within each class (sPE and controls), so overall class distribution of the data was preserved. The training set (n=29) was used for the identification of the molecular fingerprint encoding defective decidualization in sPE, while the test set (n=11) was used to confirm our findings. All samples in both cohorts were processed and sequenced equally.

Transcriptional analysis in the training set was performed by comparing gene expression patterns in sPE (n=17) and controls (n=12). This comparison revealed 593 DEGs based on FDR<0.05 and with at least 1.2 fold-change between groups (FC≥1.2) (Figure 14).
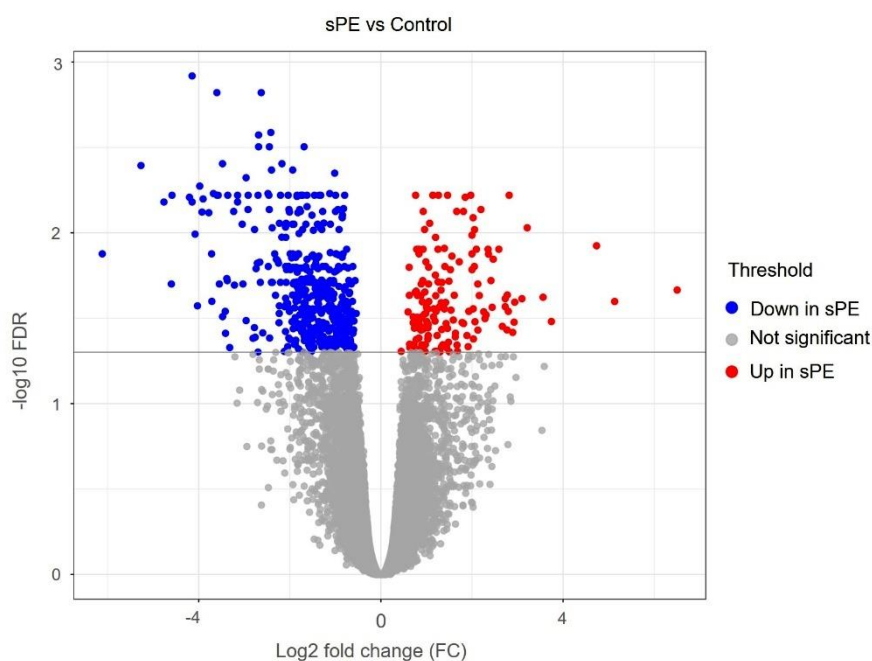
Figure 14. Statistical significance (-log10 FDR) vs. gene expression log2 fold change (FC) is displayed as a volcano plot of global RNA-seq results. Label indicates downregulated in sPE (blue dots); upregulated in sPE (red dots); not significant genes (grey dots).

A total of 155 upregulated and 438 downregulated DEGs were identified in the endometrial transcriptome of the sPE patients at the time of decidualization (Figure 15; Table 8*).* Among the downregulated transcripts we found mediators of decidualization, such as *PRL*, *IL-6*, and *IHH*; and other genes associated with the biology that accounts in this differentiation process like signaling (e.g., *NR4A3* and

IL8), growth (e.g., *FGF1* and *FGF7*), angiogenesis (e.g., *EDN2* and *TMEM215*), and immune response (*CCL20*, *CXCL3*, and *IGHG1*). Upregulated genes are involved in amino acid metabolic/catabolic processes (*IDO2* and *CAPN3*), transport, and oxidoreductase activity.
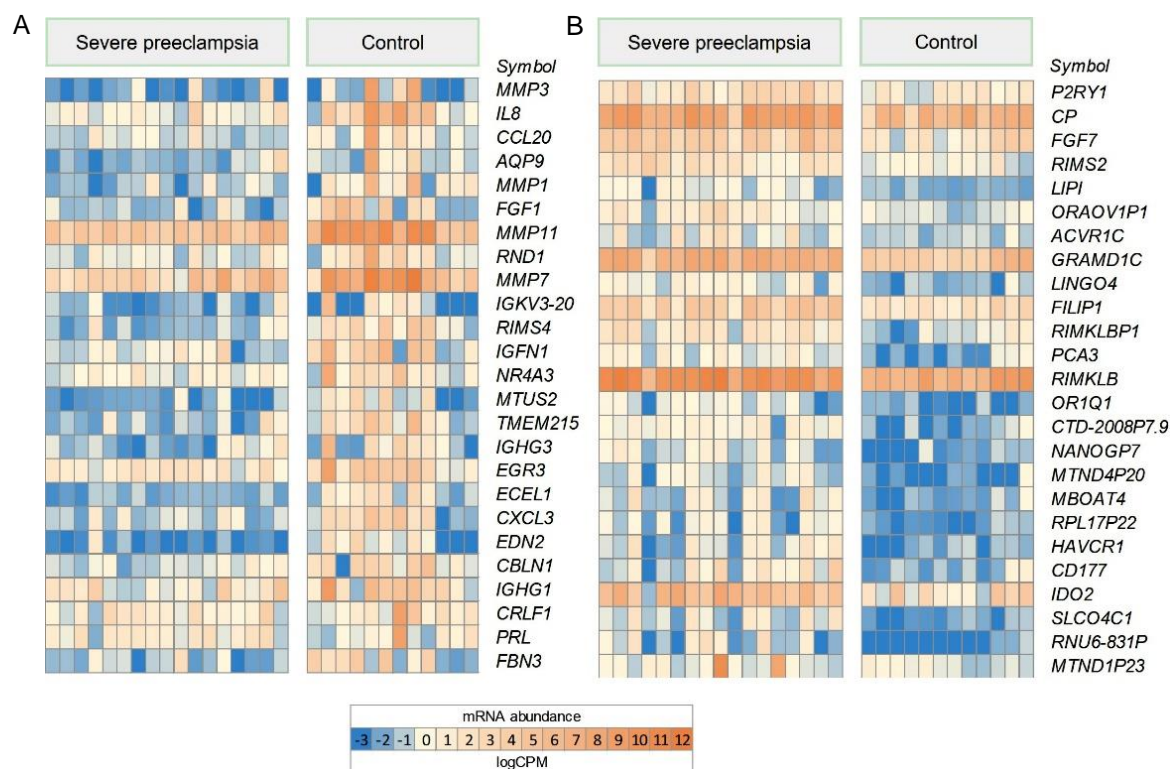
Figure 15. Heatmap showing the 50 most deregulated genes in sPE compared to controls. (A). The 25 most upregulated genes. **(B).** The 25 most downregulated genes. Gradient color shows mRNA abundance as the log counts per million, which represents the expression level.

Table 8. Top 50 differential expressed genes (FDR < 0.05) with at least 1.2-fold change (FC ≥ 1.2) in sPE vs control cases obtained from RNA-seq analysis.

Gene	FDR	logFC	FC*	Gene	FDR	logFC	FC*
MMP3	0.013	-6.12	-69	*P2RY1*	0.034	1.82	4
IL8	0.004	-5.27	-39	*CP*	0.040	1.89	4
CCL20	0.007	-4.76	-27	*FGF7*	0.046	1.91	4
AQP9	0.020	-4.60	-24	*RIMS2*	0.013	2.00	4
MMP1	0.006	-4.59	-24	*LIPI*	0.010	2.00	4
FGF1	0.006	-4.20	-18	*ORAOV1P1*	0.016	2.00	4
MMP11	0.001	-4.14	-18	*ACVR1C*	0.042	2.03	4
RND1	0.007	-4.14	-18	*GRAMD1C*	0.016	2.05	4
MMP7	0.010	-4.08	-17	*LINGO4*	0.010	2.05	4

IGKV3-20	0.027	-4.03	-16	*FILIP1*	0.012	2.10	4
RIMS4	0.005	-3.98	-16	*RIMKLBP1*	0.020	2.13	4
IGFN1	0.008	-3.93	-15	*PCA3*	0.037	2.13	4
NR4A3	0.006	-3.90	-15	*RIMKLB*	0.023	2.16	4
MTUS2	0.008	-3.78	-14	*OR1Q1*	0.029	2.26	5
TMEM215	0.013	-3.72	-13	*CTD-2008P7.9*	0.013	2.36	5
IGHG3	0.025	-3.71	-13	*NANOGP7*	0.027	2.44	5
EGR3	0.006	-3.68	-13	*MTND4P20*	0.027	2.73	7
ECEL1	0.002	-3.60	-12	*MBOAT4*	0.037	2.77	7
CXCL3	0.006	-3.57	-12	*RPL17P22*	0.023	2.78	7
EDN2	0.020	-3.55	-12	*HAVCR1*	0.029	2.80	7
CBLN1	0.004	-3.48	-11	*CD177*	0.038	2.89	7
IGHG1	0.031	-3.48	-11	*IDO2*	0.025	2.94	8
CRLF1	0.029	-3.42	-11	*SLCO4C1*	0.009	3.21	9
PRL	0.039	-3.41	-11	*RNU6-831P*	0.012	4.73	27
FBN3	0.019	-3.38	-10	*MTND1P23*	0.022	6.50	91

*FC: indicates downregulated genes calculated as -POWER(2,-logFC)

5.2. *In vivo* transcriptomics corroborates previous *in vitro* findings and reveals novel genes involved in DD in sPE

Our previous study about defective decidualization was restricted to the endometrial stromal cell population based on a culture model (Garrido-Gomez, Dominguez, Quinonero, et al. 2017). Here, we obtained the transcriptomic hallmark of this phenotype in endometrial biopsies collected at the time of late secretory phase. Comparing these two datasets we confirm the identification of new players in this phenotypic alteration and corroborate our previous findings.

First, we compared the differentially expressed genes in sPE and controls reported by the two datasets *in vitro* (n=129 DEGs) versus *in vivo* (n=593 DEGs). Nine genes were overlapped between the two datasets (Figure 16A); *ERP27* was the only upregulated gen, and the rest eight genes were downregulated. The expression pattern of common genes is presented as a box plot using counts per million, corroborating significant differential expression between sPE and control (Figure 16B). Expression was validated by RT-qPCR for *ERP27, ISM1, MEST, MFAP2*, and *REEP2*, and a high correlation (R=0.99) was observed with the global RNA-seq results (Figure 16C and 16D). Thus, *in vivo* assessment reveals a broad spectrum of dysregulated transcripts

compared with previous *in vitro* findings providing new mediators of decidualization biology.
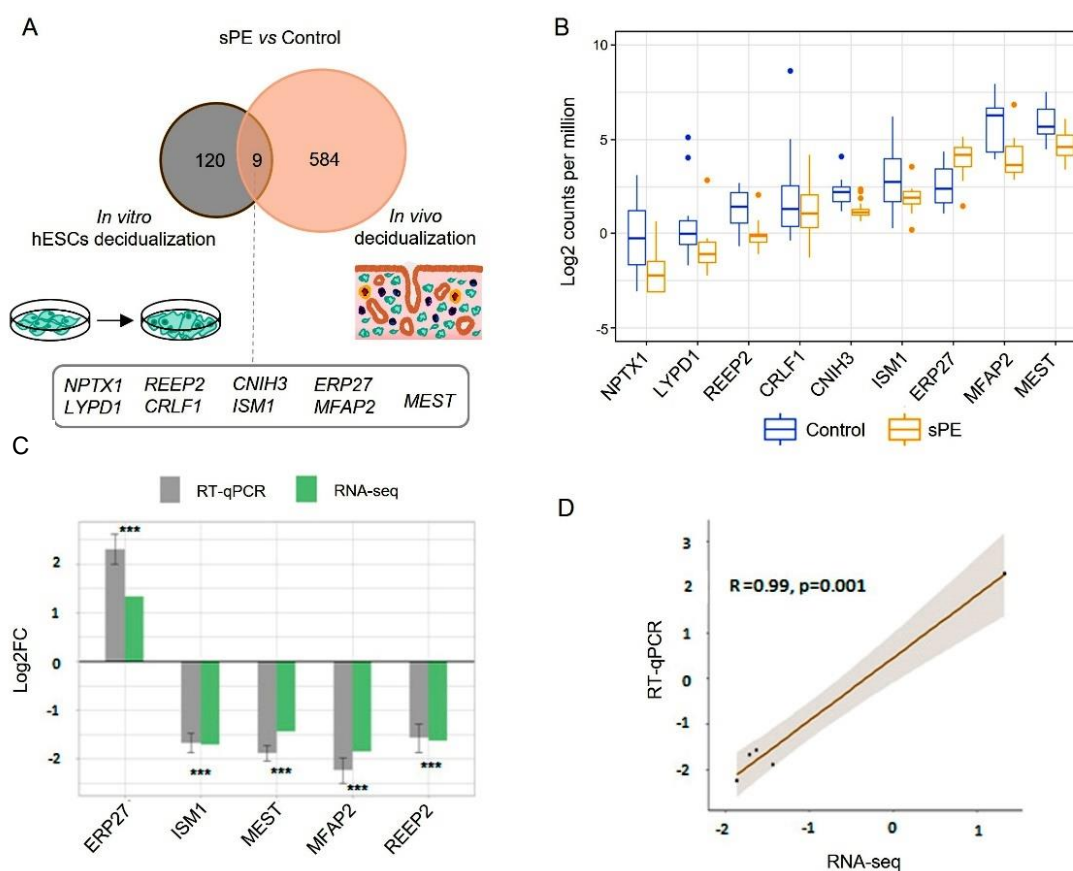
Figure 16. DD transcriptomics *in vitro* vs *in vivo*. (A). Common genes between previous *in vitro* (left) and current *in vivo* approach analyzing decidualization (right). Nine genes overlap in both approaches. **(B).** Box plot showing the average expression of the nine common genes between control (blue boxes) and severe preeclampsia (sPE) (orange boxes) samples**. (C).** Fold change between control and sPE was validated by RT-qPCR (gray bars) and sequencing (green bars) for five transcripts from the 18 common transcripts identified in both approaches. **(D)**. Correlation plot for RT-qPCR and RNA-seq (Pearson R = 0.99, p = 0.001). RT-qPCR values are expressed Mean± SE. *** p $\leq$ 0.001.

Once we characterized the global *in vivo* transcriptome of DD linked to sPE, we assessed those 129 genes previously reported in our *in vitro* study with a targeted RNA-seq strategy (Garrido-Gomez et al., 2017). The objective was to corroborate the deregulated genes during *in vitro* ESCs decidualization are also impaired in the endometrial environment *in vivo*. For this purpose, a mRNA customized panel was build including the 129 genes previously reported by our group (Garrido-Gomez et al.,

2017) using the Ion Ampliseq Designer (Table 5, Material and methods section). This panel was applied to sequence a subset of samples composed by sPE (n=15) and controls (n=11). Amplicons for 127 out of 129 genes were successfully sequenced since the 94% of the samples had coverage for these genes. The amplicons not successfully sequenced corresponded to *BDNF* and *LRRC15* and, consequently, they were not considered for the analysis.

Then, a differential gene expression analysis was performed comparing sPE vs. controls. This analysis revealed 21 DEGs between groups based on a p-value ≤0.05 and at least two-fold differentially expressed (Table 9). They include genes known to be involved during *in vitro* decidualization, some of them were up-regulated [*CFD, DMKN, ERP27* and *ANXA*)], and others were down-regulated [e.g. *COL8A1, MFAP, CNIH3, ISM1, REEP2,* and *MEST*]. A PCA was performed to reduce the dimensionality of the dataset including the information of the 21 DEGs. It shows a clustering between sPE and control samples, being a 75% of the variance explained by the transcriptome (Fig. 17A). Further, unsupervised hierarchical clustering was performed, and the dendogram reflects two main clusters composed by most of the sPE and control samples, except in two cases (Fig. 17B).

Using a bioinformatic approach, the expression pattern of the 127 genes were assessed by targeted and global RNA-seq combined to evaluate if the expression changes follow the same trend in the two datasets. Box plot showed the counts per million (CPM) in total for each gene in global and targeted strategies demonstrated the same trend of gene expression in both approaches (Fig. 18). Also, correlation analysis revealed a strong gene expression association between both strategies (Pearson´s value= 0.89).

Targeted RNA-seq is a simple strategy based on the gene amplification of a closed number of genes that were defective during decidualization restricted to an *in vitro* model applied on endometrial stromal cells, while global RNA-seq analysis gather more depth analysis of all genes that are expressed in the whole endometrium composed by different cell types. Nevertheless, PCA demonstrated a clear clustering of controls and sPE samples based on the DEGs identified in both approaches. Although the number of overlapped genes was nine, we corroborated that the rest of

genes targeted with the customized panel showed a same trend in the global RNA-seq results. This finding can explain the strong correlation between both approaches.

In conclusion, targeted and global RNA sequencing revealed a DD transcriptome *in vivo* associated with sPE that could be used to define a specific fingerprint that encodes this alteration.

Table 9. Targeted RNA-seq results (21 DEGs).

| Gene name	Gene description	LogFC	PValue
CFD	Complement factor D (adipsin)	3,08	3,9279E-09
DMKN	Dermokine	3,00	4,2881E-05
CHODL	Chondrolectin	2,93	0,00035327
C10orf10	Chromosome 10 open reading frame 10	2,30	1,0609E-05
PRUNE2	Prune homolog 2 (Drosophila)	2,20	8,1517E-06
ERP27	Endoplasmic reticulum protein 27	2,04	3,5078E-06
RGS20	Regulator of G-protein signaling 20	1,96	0,0001299
ANXA2	Annexin A2	1,24	6,2187E-05
C14orf37	Chromosome 14 open reading frame 37	-1,16	9,3736E-05
LTBP1	Latent transforming growth factor beta binding protein 1	-1,34	4,3015E-05
MEST	Mesoderm specific transcript	-1,43	7,9664E-06
CPE	Carboxypeptidase E	-1,75	0,00021461
REEP2	Receptor accessory protein 2	-1,83	8,2257E-06
COCH	Cochlin	-2,00	1,1927E-05
F2RL2	Coagulation factor II (thrombin) receptor-like 2	-2,10	3,0733E-05
ISM1	Isthmin 1, angiogenesis inhibitor	-2,34	1,8411E-07
CNIH3	Cornichon family AMPA receptor auxiliary protein 3	-2,35	7,4491E-06
MFAP2	Microfibrillar-associated protein 2	-2,47	1,1545E-09
C4orf49	Chromosome 4 open reading frame 49	-2,56	2,9421E-06
COL8A1	Collagen, type VIII, alpha 1	-2,66	3,4056E-05
MYCN	V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog	-2,82	2,376E-07
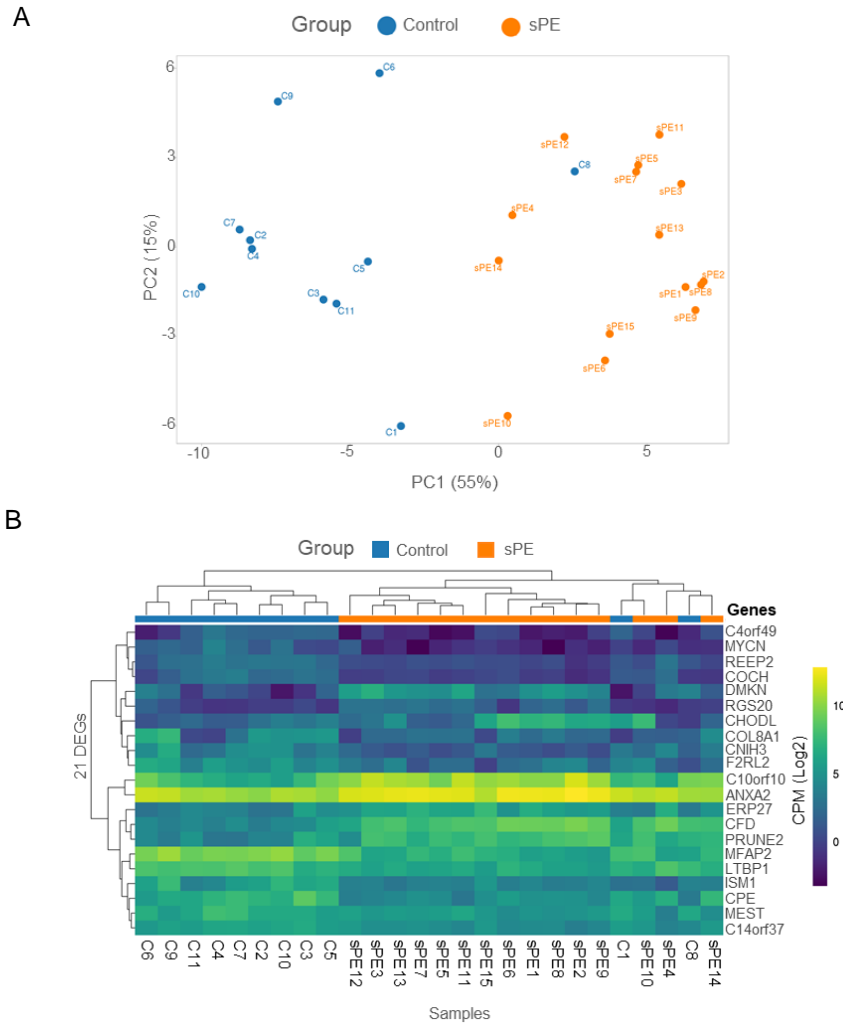
Figure 17. Targeted transcriptomics RNA-seq results. **(A)**. PCA based on the 21 differential expressed genes (DEGs). **(B)**. Hierarchical cluster analysis of 26 samples (columns) using a set of 21 differentially expressed genes (rows). The normalized expression value for each gene is coded by color: yellow indicates high expression and dark blue indicates low expression.
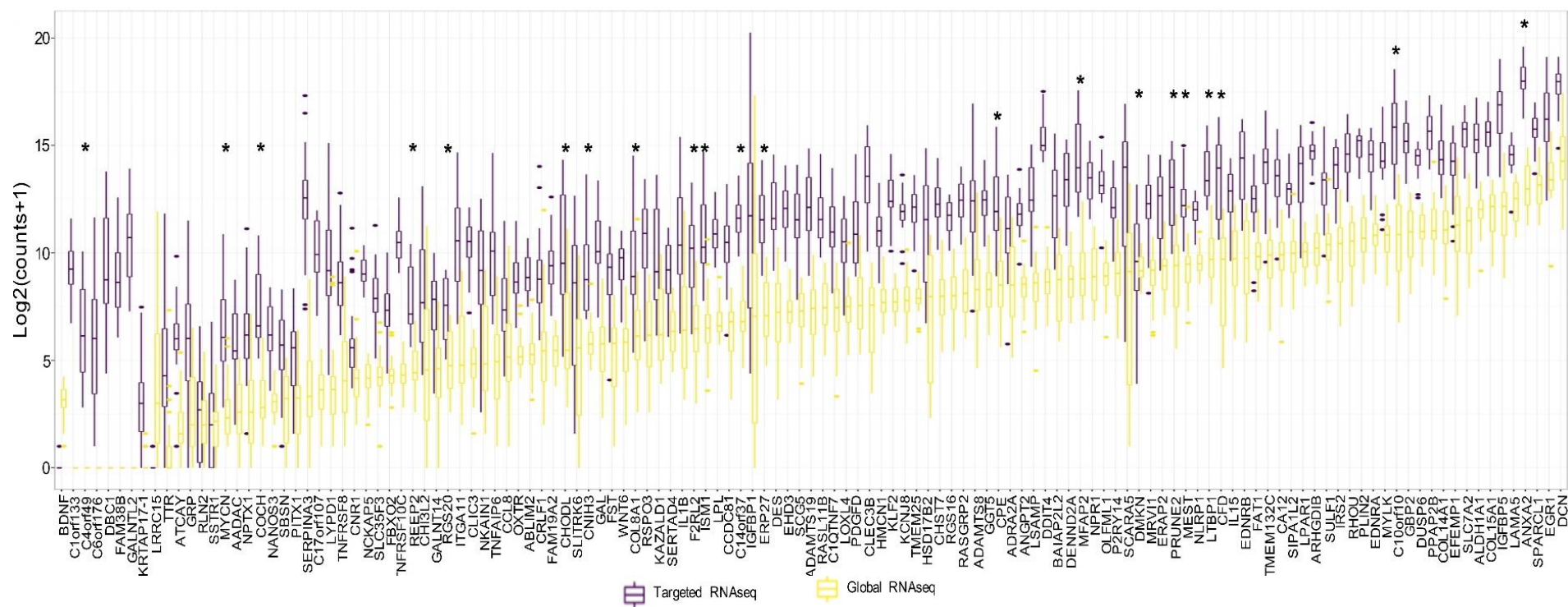
Figure 18. Comparison between targeted and global RNA sequencing data. Box plot showing average expression genes of both approximations after normalization: Targeted RNA-seq (purple boxes) and Global RNA-seq (yellow boxes). Asterisks show the 21 DEGs identified by Targeted RNA-seq.

5.3. Stromal cells and lymphocytes are affected in sPE

In vivo transcriptomics of the human endometrium at single-cell resolution across the menstrual cycle has been characterized (Wang et al., 2020). Overlapping the transcriptome profile of stromal fibroblasts in the secretory phase with the global endometrial transcriptome presented here, allowed the identification of deregulated genes in sPE as associated to ESC. This match revealed that 263 DEGs in our dataset are expressed by ESC (Figure 19). Thus, although the bulk tissue assessment provides a broad spectrum of dysregulated transcripts due to the cell heterogeneity in the sample, our dataset includes a high concordance with *in vivo* ESC genes.
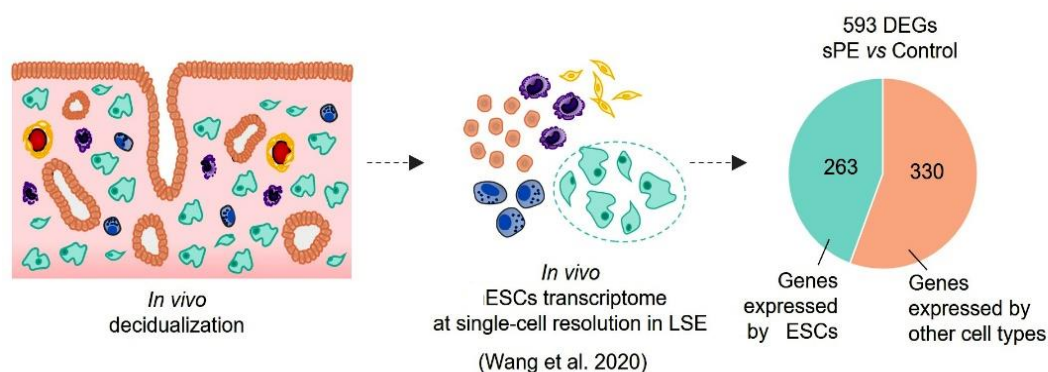
Figure 19. Genes affected in sPE expressed by ESCs. From the 593 differentially expressed genes (DEGs) obtained by global RNA-seq, a subset of 263 DEGs were identified as genes with a human endometrial stromal cell (ESC) origin using the scRNA-seq data published by Wang et al., 2020.

Then, we wonder if cell type ratios were affected in sPE patients compared controls. To answer this question we performed a bulk tissue deconvolution with endometrial single-cell expression reference (Wang et al., 2020) using the MuSiC R package (Wang et al., 2019) to characterise the cellular heterogeneity in our cohort. MuSiC enables the transfer of cell type-specific gene expression information from a single-cell dataset to global transcriptome data for estimating cell type proportions in bulk tissue. This R package weight genes based on the expression variance among samples: up-weighing genes with low variance (informative genes) and down-weighing genes with high variance (non-informative genes). This principle is critical to have enough consistency transferring data from different datasets.

The deconvolution analysis revealed that cases and controls did not show significant differences in terms of cell proportions for ciliated and unciliated epithelium, stromal fibroblasts, macrophages, and endometrial cells. However, lymphocytes were increased in sPE patients (p-value <0.05) (Figure 20).

Then, we explored how many deregulated genes in our dataset were expressed by this cell population. For this purpose, we overlapped the global transcriptome dataset with the genetic profile of lymphocytes reported by the single-cell dataset (Wang et al., 2020), revealing 162 DEGs in the global transcriptome of the decidualized endometrium expressed by lymphocytes.

Altogether, these results suggest that stromal cells and lymphocytes are the major cell types involved in DD disrupted in sPE. Both cell types showed an aberrant gene expression and even lymphocytes were present in a higher proportion in these patients, corroborating an aberrant signaling and intercellular communication during decidualization in sPE.
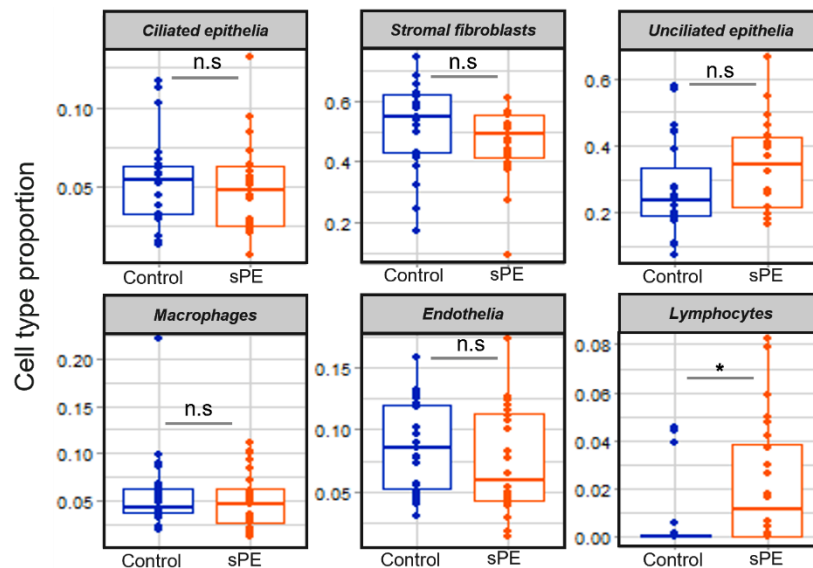
Figure 20. Bulk tissue cell type deconvolution with endometrial single-cell expression reference. Boxplot shows cell proportions for each cell type included in the dataset (ciliated and unciliated epithelium, stromal fibroblasts, macrophages, endothelial cells, and lymphocytes). Cell proportions are represented in y-axis and x-axis shows the group (control, blue boxes; sPE, orange boxes). scRNA-seq data used was published by Wang et al., 2020. *p<0.05.

5.4. Identification of the fingerprint encoding endometrial DD

5.4.1. Genes encoding DD fingerprint

We aim to identify the highlighted genes among the 593 DEGs to formulate the transcriptomic signature that encodes DD detected in sPE *in vivo*. For this purpose, we decided to select genes based on its statistical (FDR < 0.05) and biological (FC > 1.4) significance, since higher changes in expression can have higher impact in decidualization. Additionally, we selected those genes with an Entrez ID assigned to ensure the selection of genes with annotated features for further analysis. After applying these criteria, we obtained the DD fingerprint composed by 120 DEGs, 106 downregulated and 14 upregulated (Figure 21; Table 10). Interestingly, the number of downregulated genes was higher than the number of upregulated genes in sPE compared to controls, suggesting that, *in vivo*, DD may be induced by the lack of expression of a subset of genes.
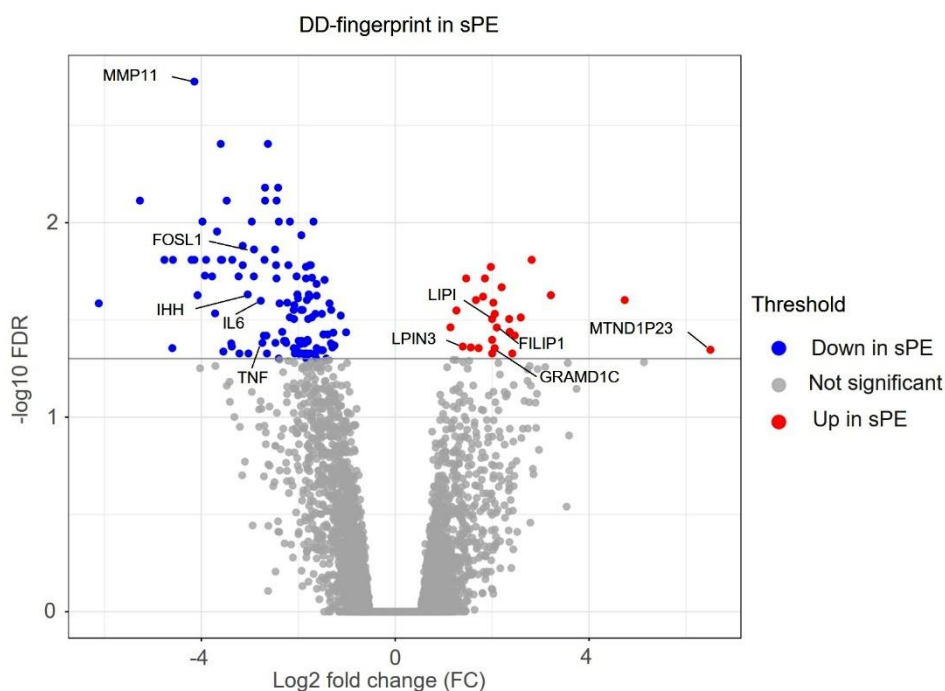
Figure 21. DD fingerprint in sPE. Volcano plot showing downregulated (blue) and upregulated (red) genes in sPE from the DD fingerprint. Each point represents one gene; gray points are the rest of the genes obtained in the global RNA-seq analysis.

Table 10. List of genes selected as the DD signature in sPE (120 DEGs with FDR < 0.05 and FC ≥ 1.4).

| Gene	FDR	logFC	Gene	FDR	logFC	Gene	FDR	logFC
MMP3	0.026	-6.1	CNTN1	0.041	-2.5	ENC1	0.023	-1.8
IL8	0.008	-5.3	KCNK15	0.017	-2.5	GINS2	0.024	-1.8
CCL20	0.016	-4.8	GJB3	0.019	-2.5	TK1	0.017	-1.8
AQP9	0.044	-4.6	FJX1	0.007	-2.4	CCNB2	0.047	-1.8
MMP1	0.016	-4.6	EDAR	0.050	-2.4	PTTG1	0.031	-1.7
FGF1	0.016	-4.2	TMSB15A	0.010	-2.4	DERL3	0.019	-1.7
MMP11	0.002	-4.1	LAMP5	0.026	-2.4	XRCC2	0.047	-1.6
RND1	0.016	-4.1	NTM	0.036	-2.3	PAG1	0.029	-1.6
MMP7	0.024	-4.1	KLK2	0.040	-2.3	REEP2	0.050	-1.6
RIMS4	0.010	-4.0	TNFAIP3	0.041	-2.3	PODXL2	0.021	-1.6
IGFN1	0.019	-3.9	CPM	0.042	-2.2	E2F1	0.044	-1.6
NR4A3	0.016	-3.9	PBK	0.026	-2.2	CDH2	0.024	-1.6
MTUS2	0.019	-3.8	COL22A1	0.017	-2.2	CCNB1	0.045	-1.5
TMEM215	0.029	-3.7	PFKFB4	0.031	-2.2	UBE2T	0.029	-1.5
EGR3	0.011	-3.7	MSX2	0.010	-2.2	MAD2L1	0.045	-1.5
ECEL1	0.004	-3.6	MYBL2	0.028	-2.1	ITIH5	0.038	-1.5
CXCL3	0.016	-3.6	PRRG3	0.044	-2.1	CDH24	0.020	-1.5
EDN2	0.046	-3.5	EXO1	0.031	-2.1	NAT8L	0.050	-1.4
CBLN1	0.008	-3.5	C2CD4C	0.027	-2.1	AUNIP	0.038	-1.4
FBN3	0.042	-3.4	TUBB2B	0.047	-2.1	CENPH	0.026	-1.4
CXCL2	0.043	-3.4	E2F7	0.045	-2.0	GGH	0.042	-1.3
COL9A1	0.016	-3.4	FAM222A	0.019	-2.0	MDK	0.028	-1.3
POSTN	0.019	-3.2	CDC25C	0.046	-2.0	POC1A	0.037	-1.3
SFRP4	0.047	-3.2	VASH2	0.023	-2.0	FOLH1	0.043	-1.3
EGR2	0.013	-3.1	RTKN2	0.024	-2.0	RNASEH2A	0.030	-1.1
ARSI	0.017	-3.1	KCNN1	0.040	-2.0	TIPIN	0.037	-1.0
IHH	0.023	-3.0	DEPDC1	0.047	-2.0	AASS	0.035	1.1
NPTX1	0.047	-3.0	LAMA1	0.028	-1.9	LPIN3	0.043	1.4
UCN2	0.010	-3.0	MND1	0.012	-1.9	RIMBP2	0.044	1.7
OVGP1	0.019	-2.9	SKA3	0.047	-1.9	PRKXP1	0.024	1.8
FOSL1	0.014	-2.9	ADAMTS14	0.028	-1.9	RIMS2	0.040	2.0
IL6	0.025	-2.8	CDC45	0.042	-1.9	LIPI	0.031	2.0
TNF	0.042	-2.7	FBN2	0.041	-1.9	ORAOV1P1	0.047	2.0
WISP1	0.038	-2.7	CDC20	0.047	-1.9	GRAMD1C	0.044	2.1
DPP10	0.016	-2.7	CDT1	0.017	-1.8	LINGO4	0.029	2.1
PMAIP1	0.007	-2.7	UHRF1	0.019	-1.8	FILIP1	0.035	2.1
RAB3B	0.008	-2.7	BIRC5	0.047	-1.8	CTD-2008P7.9	0.036	2.4
ADRA2B	0.038	-2.7	ANO7	0.040	-1.8	SLCO4C1	0.024	3.2
KMO	0.047	-2.6	NREP	0.041	-1.8	RNU6-831P	0.025	4.7
NKD1	0.004	-2.6	ZNF367	0.031	-1.8	MTND1P23	0.045	6.5

5.4.2. Biological process and KEGGs pathways enriched in the DD footprint

To understand the major biological impacts of the DD footprint and describe the main events impaired during the late-secretory phase of the menstrual cycle in sPE cases, we performed a gene ontology analysis revealing 151 downregulated biological processes (FDR < 0.05). These biological processes were associated with cell cycle, DNA damage response, cell signaling, cellular response, cell motility, extracellular matrix, immune response, and reproductive process (Figure 22). All correspond to hallmarks of impaired decidualization and sPE pathogenesis.
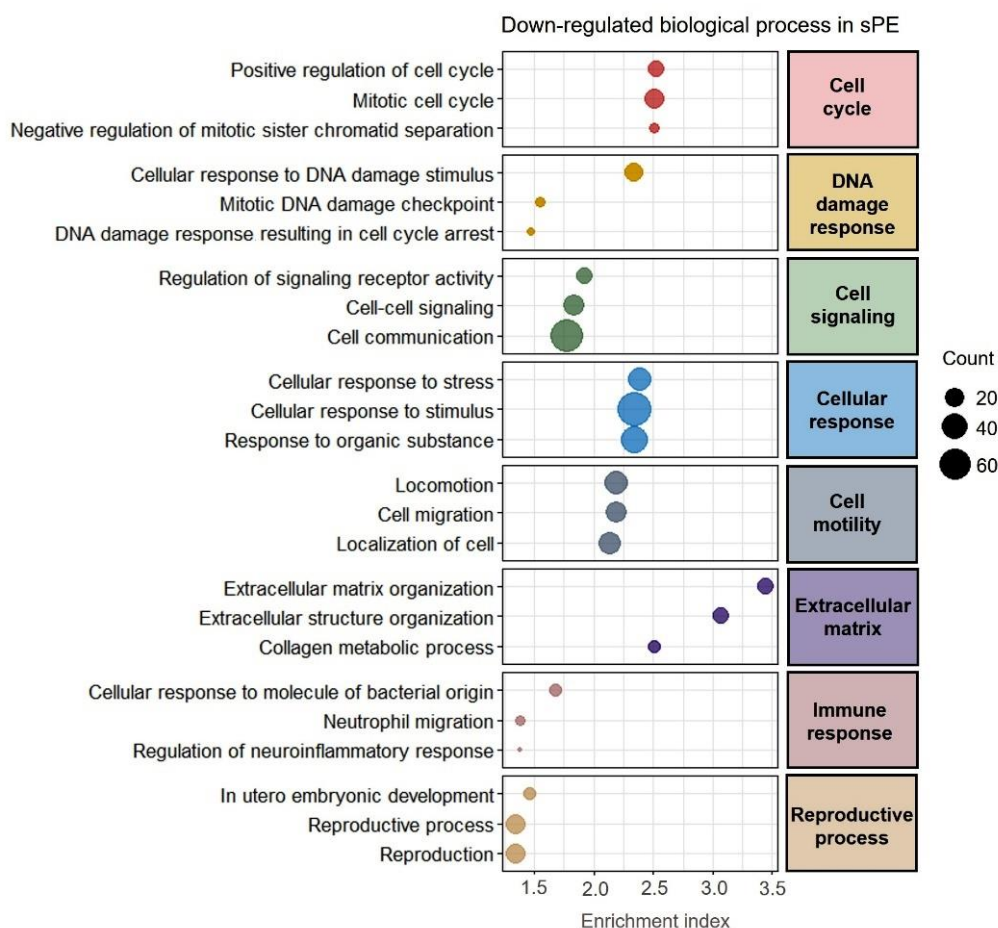
Figure 22. Downregulated biological processes involved in sPE. The three most highly downregulated biological process for each major category (*red*, cell cycle; *yellow*, DNA damage response; *green*, cell signaling; *blue*, cellular response; *gray*, cell motility; *purple*, extracellular matrix; *pink*, immune response; *brown*, reproductive process). Enrichment index was calculated by -log(p-value).

We identified that fingerprint genes are representative of the altered processes described in sPE, such as *MMP3* and *MMP1* participating in the extracellular matrix organization, and *TNF*, *IL8,* and *FGF1* implicated in the downregulated receptor signaling. Interestingly, the biological process of response to bacterial molecules was enriched in sPE including *IL6* and *TNF* (Figure 23). Functional analysis revealed that the 120 DEGs included in DD fingerprint are implicated in pathways related to decidualization, corroborating the maternal contribution to sPE.
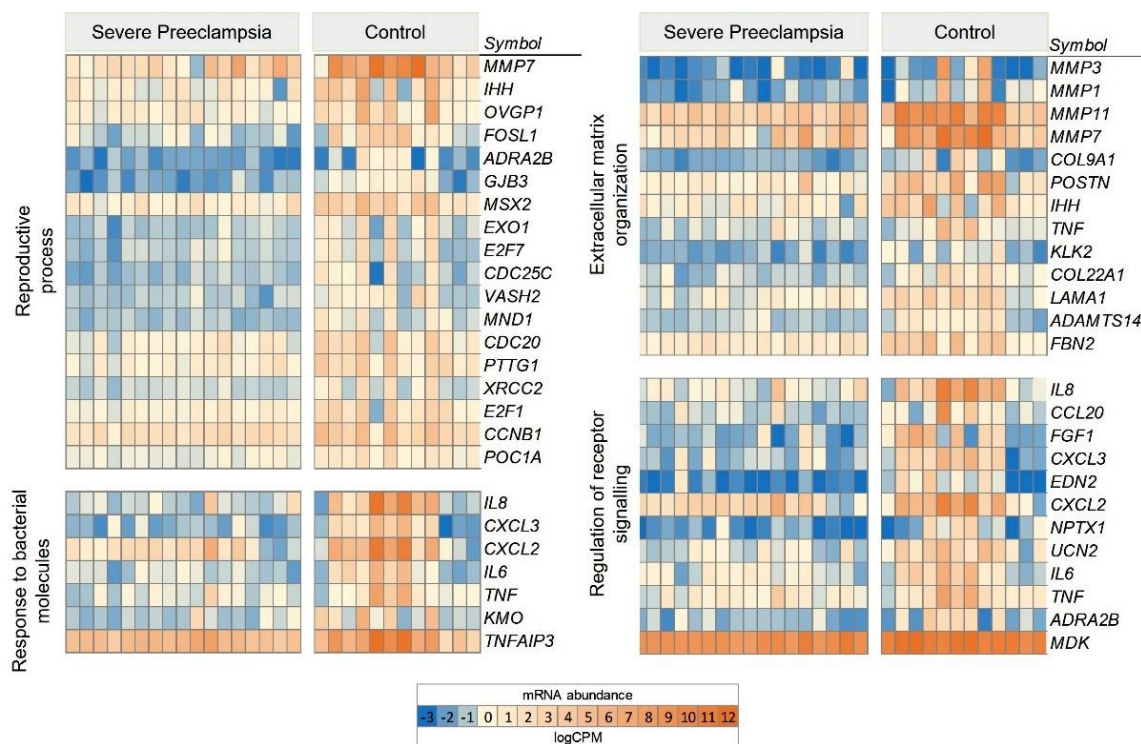
Figure 23. Highlighted biological processes downregulated in sPE associated with sPE and decidualization biology. Clustering of DD fingerprint genes shown for reproductive process, response to bacterial molecules, extracellular matrix organization, regulation of receptor signaling, and response to hormones.

To identify high-level functions of the 120 genes encoding the DD footprint, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conduct using the Database for Annotation, Visualization and Integrated Discovery (DAVID) with medium stringency classification (Figure 24). This tool automatically generated three clusters of pathways to reduce the burden of associating similar redundant terms.

Cluster 1 was mainly associated with cell cycle, whereas clusters 2 and 3 with immune system. Interestingly, cluster 2 was highly related with immune system and microbial infections such as IL17 and TNF signaling. Specially, *IL6, TNF* and *CXCL8* participate in most pathways reported that act in pathogen response. However, some of these pathways were not significantly enriched due to the high total number of genes participating in them compared to the number of genes in the fingerprint (see point 4.9 from material and methods section for the statistical algorithm). Consequently, 5 genes of the DD fingerprint are involved in the pathway described for coronavirus disease, although this KEGG is no significantly enriched due to it is composed by 232 genes. The same is observed for other pathways associated with infections such as Influenza A, Epstein-Barr and Yersina, among others. This result reinforces immune system is deregulated in patients with sPE, highlighting those genes that are involved in response to infections.
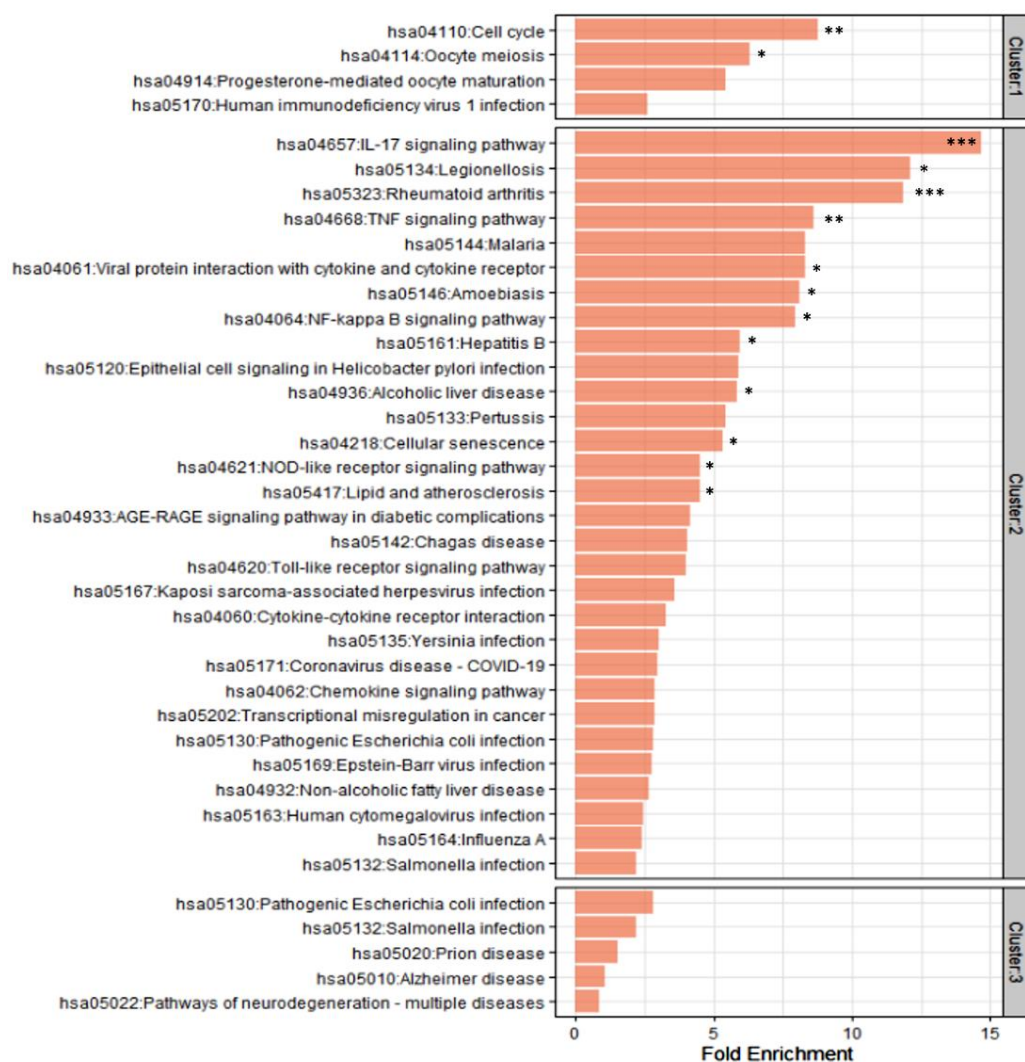
Figure 24. Functional annotation clustering based on KEGG pathways from DAVID database. Pathways are represented on the y-axis and the fold enrichment to each is detailed on the x-axis. Clusters 1 to 3 represent modules of related genes and terms to reduce redundant results. Significant enriched pathways are shown with an asterisk, *** $p<0.001$, ** $p<0.01$, *$p<0.05$ (FDR).

5.4.3. Functional validation of the DD fingerprint

We applied the DD fingerprint to segregate samples from the training set. The samples distribution obtained shows a subset of 120 genes highly representative of the biological variance associated with DD in sPE. PCA showed that sPE and control clustered separately in two groups, except for three control samples (C20, C21, and C22) and a high variance between groups was effectively captured in the first two principal components, 68% specifically (Figure 25A). Unsupervised hierarchical clustering analysis confirmed the DD fingerprint effectively segregates the two groups: one clustering mainly controls and the other mainly sPE samples (Figure 25B).

A
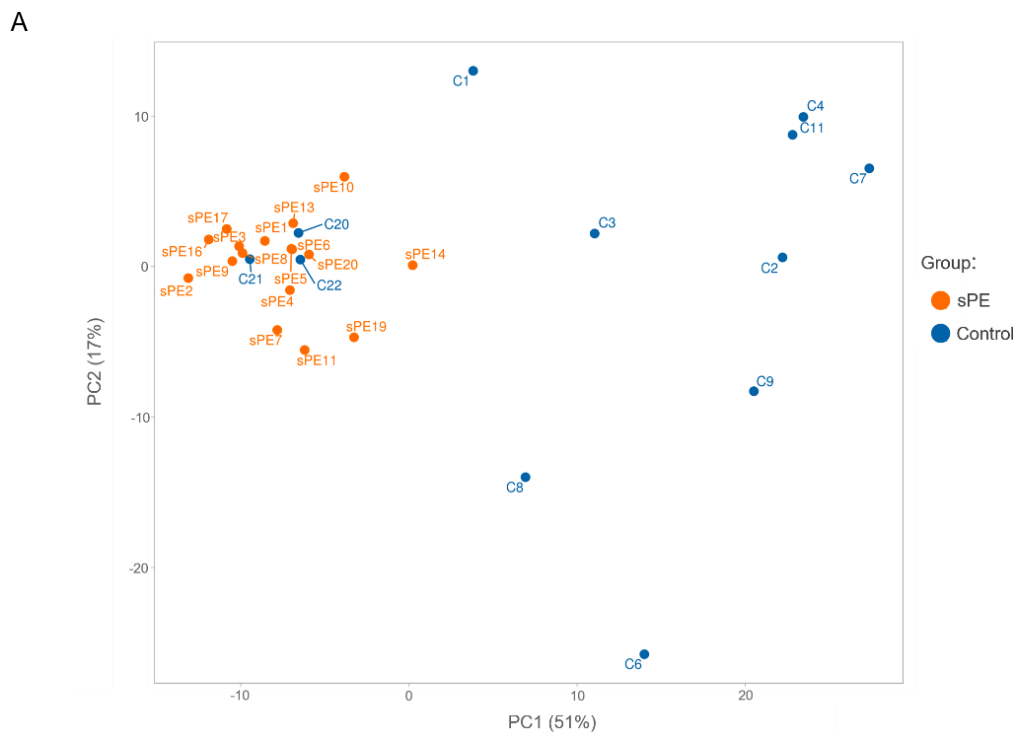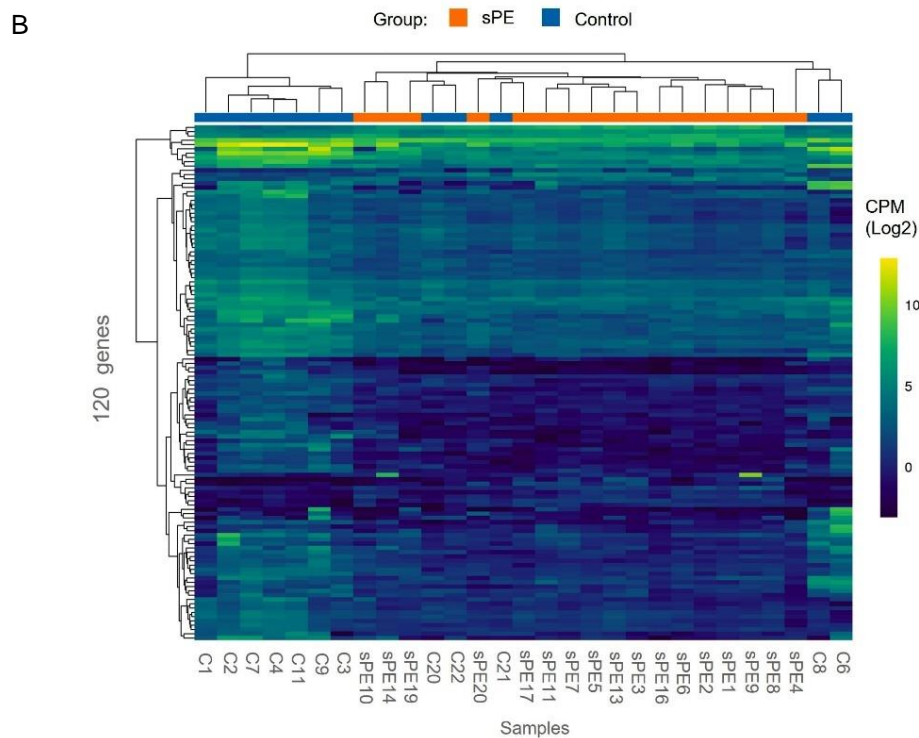
Figure 25. Validation of DD fingerprint in sPE from the training set. (A). PCA based on 120 genes included in the fingerprinting in the training set. Each sample is represented as a colored point (*blue*, control; *orange*, sPE). **(B)**. Heatmap dendrogram of expression of the 120 genes included in the final fingerprint for each sample of the training set (control, n = 12; sPE, n = 17).

To validate the DD fingerprint in an independent cohort of samples [sPE (n=7), control (n=4)] we also performed a PCA and a hierarchical clustering. The PCA based on these transcripts effectively segregated samples in two homogeneous groups (Figure 26A), corroborated by hierarchical clustering (Figure 26B). These genes successfully grouped 100% of controls and 85.7% of sPE cases supporting DD in sPE, reinforcing the fingerprint presented here is representative of the defective decidualization observed in these patients.
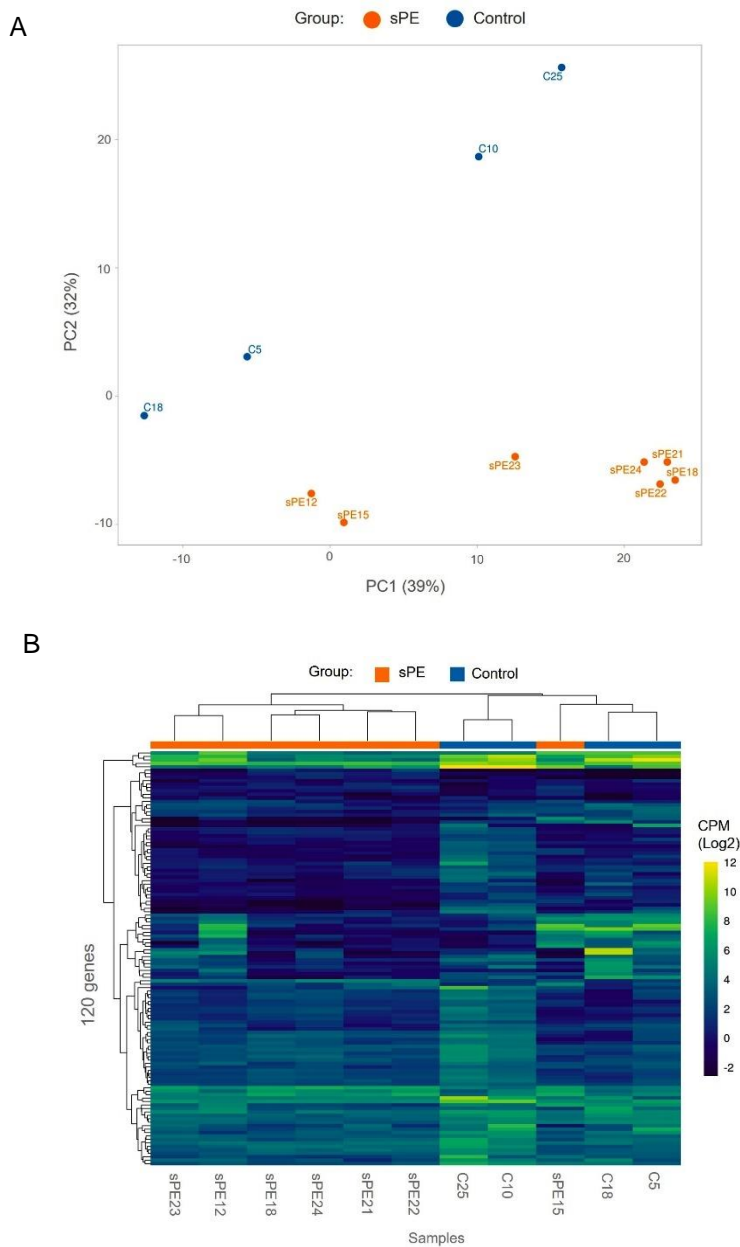
Figure 26. Validation of DD fingerprint in sPE from the test set. (A). PCA based on the fingerprinting in the test set. Each sample is represented as a colored point (*blue*, control; *orange*, sPE). **(B)**. Heatmap dendrogram of expression of the 120 genes included in the final fingerprint for each sample of the test set (control, n = 4; sPE, n = 7).

5.5. DD fingerprint in sPE is connected to ER1 and PR-B

We investigated the molecular mechanisms underlying this hallmark in sPE. Since decidualization is tightly regulated by progesterone and estrogen, we focus on assessing the link between this hormonal signaling and the gene expression imbalance encoding DD.

5.5.1. Genes regulated by progesterone and estradiol

First, we identified those genes that are enriched in the endometrium compared to other tissues based on the Human Protein Atlas database (Uhlén et al., 2015). Of the 120 genes in the DD fingerprint, 94 were endometrial enriched genes. Then, due to the key role of progesterone and estradiol in controlling the endometrial cycle progression, we investigated how much genes in the DD fingerprint are modulated by the cognate receptors, PR and ER1. Interestingly, 45 of those genes (47.9%) were included in the transcriptome modulated by ER1 (Hewitt et al., 2010), and 43 genes (45.7%) overlapped with the transcriptome and cistrome associated with PR (Mazur et al., 2015) (Figure 27). Regarding target genes of ER1 and PR, the database of Human Transcription Factor Targets (hTFtarget) reported 17 genes responsive to ER1 and 50 responsive to PR, based on epigenomic, CHIP-seq, or motif evidence (Zhang et al., 2020).
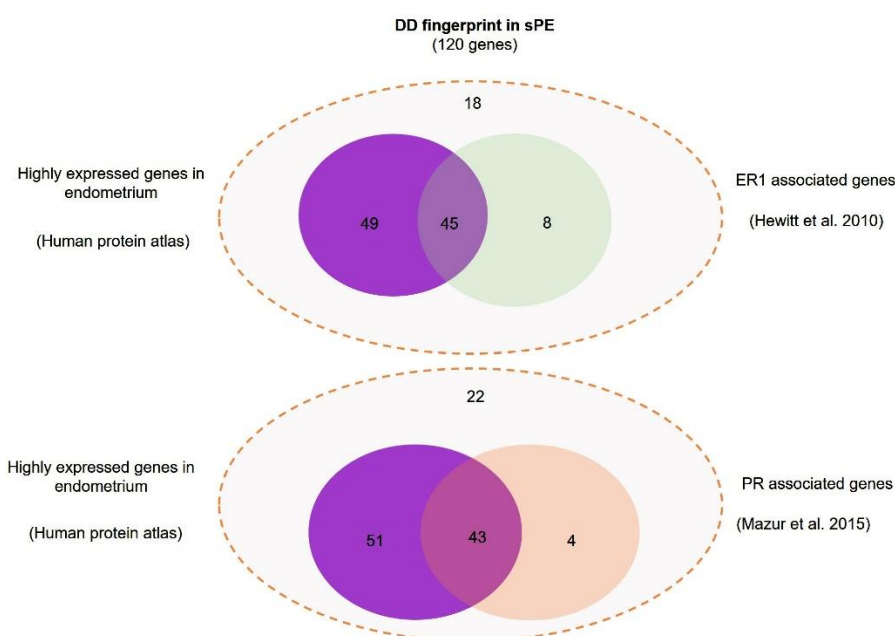
Figure 27. Number of genes in the DD fingerprint highly expressed in the endometrium associated with ER1 or PR. Venn diagram displaying genes included in the fingerprint (120) predominantly expressed in the endometrium based on Human Protein Atlas data that overlap with genes modulated by ER1 described by Hewitt et al., 2010 and genes associated with PR silencing described by Mazur et al., 2015.

Then, we evaluated the interaction between steroid receptor signaling and the proteins encoded by DD fingerprint by building a dynamic network including ER1 and PR. String software (Jensen et al., 2009) was used to construct network connections that was visualized with Cytoscape software (Shannon et al., 2003). The interactome contained 117 nodes directly interconnected by 361 edges (Figure 28).
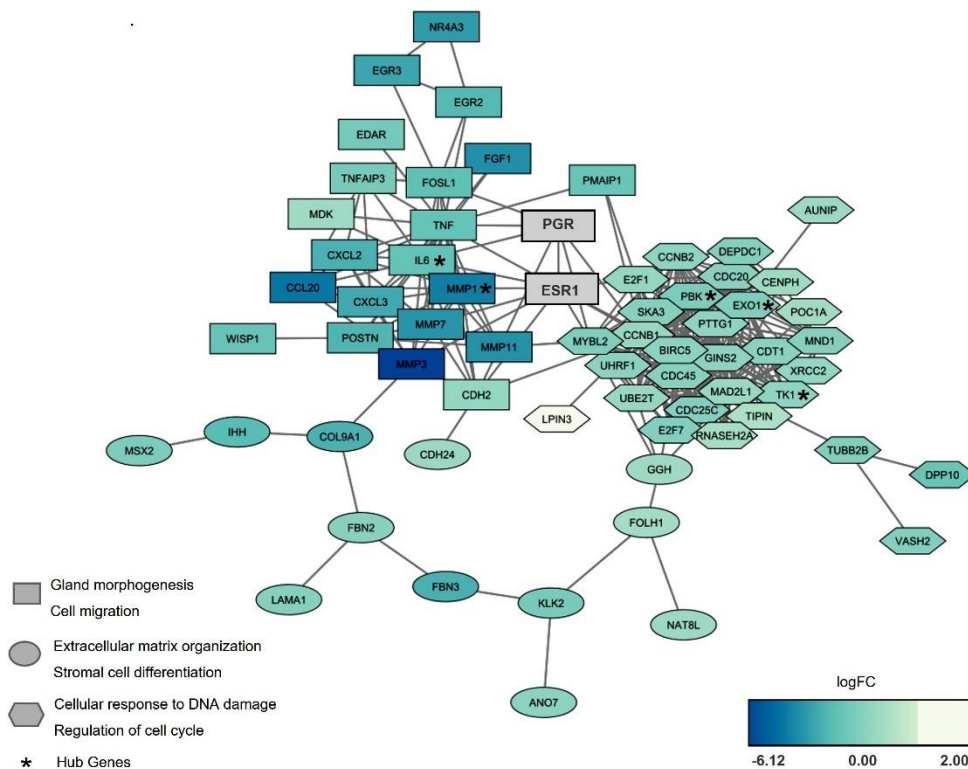
Figure 28. Network of the connections between proteins codified by DD fingerprint and the hormonal receptors, ER1 and PR. Shapes indicate different clusters established by String k-means method. *Squares*, cluster involved in gland morphogenesis and cell migration; *circles*, cluster involved in extracellular matrix organization and stromal cell differentiation; *hexagons*; cluster involved in cellular response to DNA damage and regulation of cell cycle. Color gradients indicate gene expression in terms of log2FC. Hub genes are shown with an asterisk.

This DD fingerprint network showed a highly enriched protein–protein interaction (PPI) in sPE. Indeed, the interconnection between nodes was significantly higher than the 93 edges expected (PPI enrichment p<1.0e-16). Clustering revealed three main modules based on their connectivity degree, with functionally relevant genes involved in gland morphogenesis, cell migration, extracellular matrix organization, stromal cell differentiation, cellular response to DNA damage stimulus, and regulation of cell cycle. The hub genes were determined by overlapping the top 10 genes obtained using two topological analysis methods in the cytoHubba plugin (Chin et al., 2014), MCC and MNC. Five genes were selected, all of which were downregulated.

As expected, both ER1 and PR were strongly embedded in the network and highly connected with DD fingerprint highlighting the interaction of hormonal receptors with notable decidualization mediators such as IHH and MSX2 validated by RT-qPCR (Figure 29A and 29B). Furthermore, the interactome demonstrated a direct interaction between ER1 and PR. These results support the transcriptomic dysfunction of the genes present in the DD signature through imbalanced hormonal receptor signaling in sPE.

5.5.2. Gene expression and protein abundance

To confirm our hypothesis, we analyzed the expression of *ESR1* and *PGR* in the endometrial tissue. We investigate the gene expression of these two receptors in a subset of endometrial biopsies collected from women who suffered sPE (N=13) versus controls (N=9) by RT-qPCR. We found reduced expression of *ESR1* (p<0.05) and *PGR* (p<0.001) in sPE patients (Figure 29C and 29D). In-depth expression analyses revealed that the isoform *PGR-B* was significantly downregulated in sPE vs controls (p<0.01), while the isoform *PGR-A* was unaffected (p>0.05) (Figure 29E and 29F). These results were confirmed at the protein level by immunohistochemical analysis of ER1 and PR-B in endometrial biopsies collected in the late secretory phase from women with previous sPE (n=4) and controls (n=4) (Figure 29I and 29J).
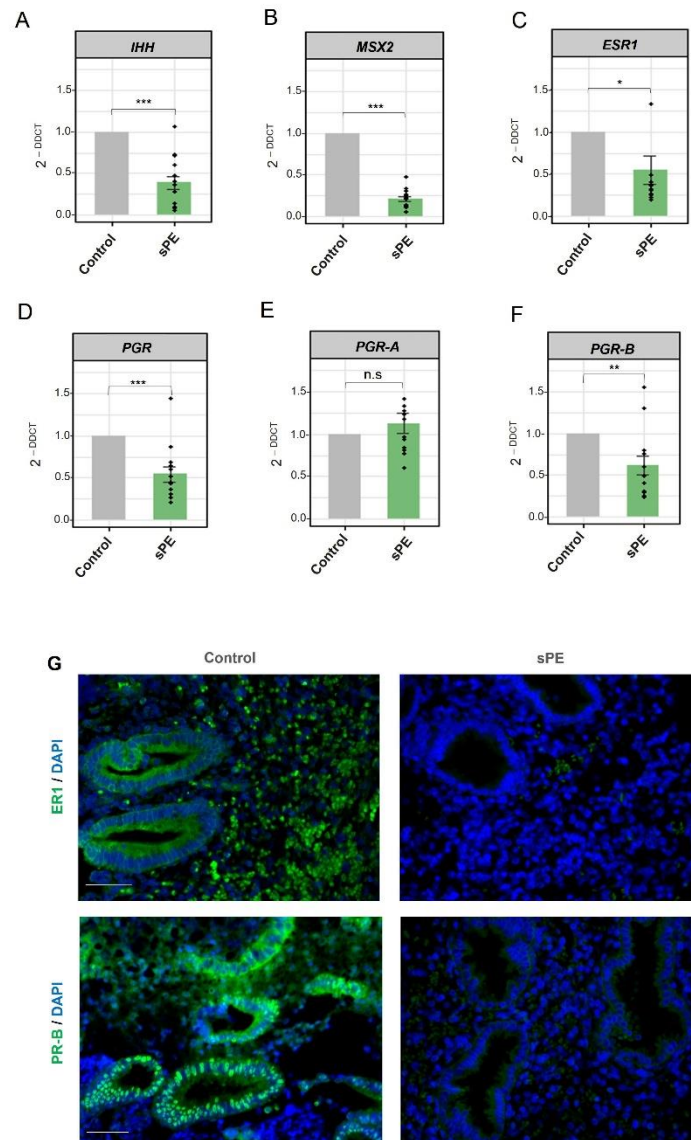
Figure 29. ER1 and PR-B are linked to DD fingerprint in sPE. (A-F). Gene expression levels of *IHH, MSX2, ESR1, PGR, PGR-A,* and *PGR-B* assessed for sPE (n=13) vs. controls (n=9) by RT-qPCR (gray bars, control; green bars, sPE). RT-qPCR values are expressed as mean± SE. *** $p<0.001$, ** $p<0.01$, *$p<0.05$. **(G)**. Tissue sections of control (n=4) and sPE (n=4) endometrium during late secretory phase were immunostained with antibody against ER1 or PR. Nuclei were visualized with DAPI. Scale bar: 50 μM.

ER1 and PR-B were highly expressed through the normal decidualized endometrium, especially in the secretory glands. In contrast, their expression was greatly reduced or absent in sPE samples. These results suggest that the DD transcriptomic signature implicates dysregulated ER1 and PR-B signaling in the late secretory phase.

5.6. Unveiling DD biology

5.6.1. DD and the maternal-fetal interface

Our experimental design allowed as to test rigorously DD in non-pregnant women who suffered sPE. However, we wonder whether the footprint formulated here could impact prospectively in the maternal-fetal interface establishment in early pregnancy. Impaired endometrial maturation and deficient decidual NK was previously reported in the decidual fraction of CVS collected at ~11.5 gestational weeks from pregnant women who developed sPE 6-months later (Rabaglino et al., 2015). Specifically, these alterations were encoded by 396 DEGs in cases compared to controls. Thus, we built a protein interaction network including those proteins encoded by the DD fingerprint and those encoded by the 396 DEGs in CVS using String software (Jensen et al., 2009). Also, ER1 and PR were included in the network due to its relevance in the processes assessed.

The network was composed of 496 nodes and 1531 edges, showing a higher interconnection than the 998 expected (PPI enrichment $p < 1.0e-16$). Then, we selected just those direct connections between fingerprint-CVS proteins and visualize the resulting filtered network in Cytoscape (Shannon et al., 2003) (Figure 30). Proteins codified by DD fingertprint interact with those disrupted in CVS collected prospectively from preeclampsia patients. We found proteins associated with immune system and inflammation (IL6, TNF, PMAIP1, CCL20 and CXCL2) highly embedded in CVS nodes. This result shows that genes deregulated in late-secretory phase, and before embryo invasion, could impair the maternal-fetal formation and suggests a remarkable role of immune system mediators.
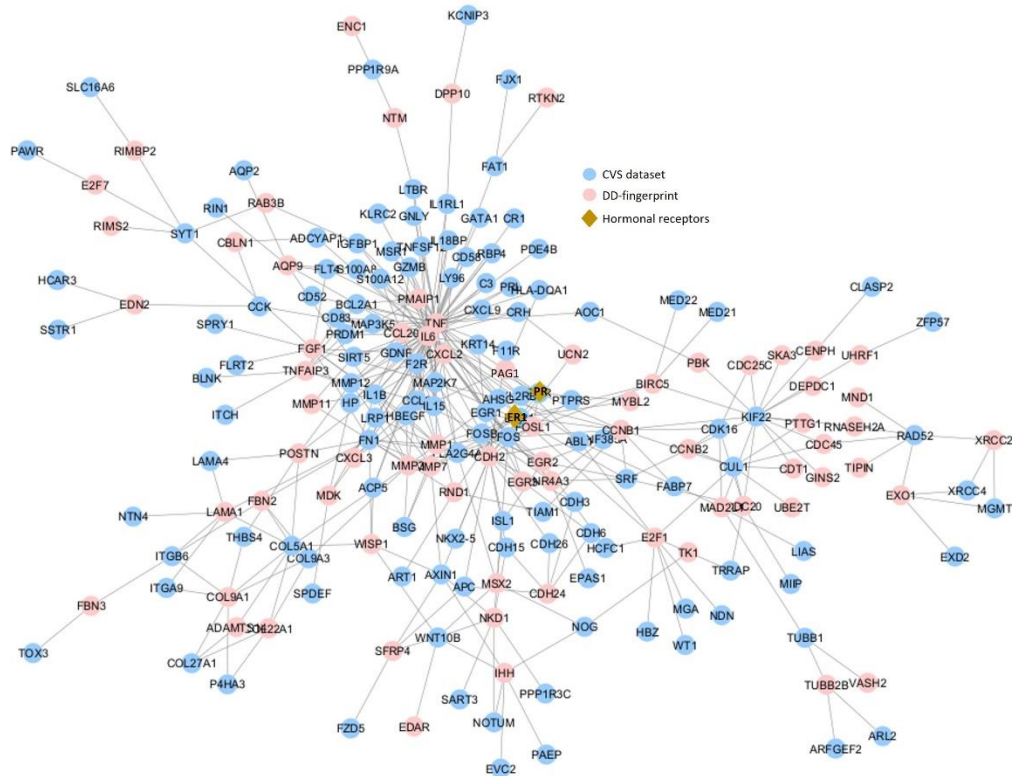
Figure 30. Deregulated genes encoding DD could impact biomarkers of impaired maternal-fetal interface in sPE patients prior to symptoms. Network showing direct the connections between proteins codified by DD fingerprinting and by the 396 deregulated in CVS form preeclamptic patients, and the hormonal receptors, ER1 and PR. *Blue*, nodes from CVS dataset; *pink*, nodes from DD fingerprint; *yellow*, hormonal receptors ER1 and PR.

5.6.2. Molecular mechanisms might be activated to revert the DD phenotype

STAT3 is activated by a variety of cytokines, including IL6 (Zhong et al., 1994), and interact with PR to promote decidualization (Kaya et al., 2015; Wang et al., 2012). Furthermore, mice uteri with ablated *Stat3* showed decreased levels of stromal PR and lower expression of PR target genes (Lee et al., 2013). In primary ESC, analysis of motifs within a ±100-bp window from the summits of target sequences of *PGR-A* and *PGR-B* revealed that STATs motifs were unique to *PGR-B* (Kaya et al., 2015).

Thus, we sought to assess *STAT3* gene expression by qRT-PCR to understand mechanisms that could be operating in *PGR* downregulation. Additionally, we test gene expression of PIAS3 which blocks the DNA-binding activity of STAT3 (Chung et

al., 1997). We found *STAT3* was significantly upregulated in sPE compared to controls, whereas *PIAS3* was not affected (Figure 31A). This result suggests some molecular mechanisms are activated to increase *PGR-B* expression and promote decidualization. Next, we tested the gene expression of PLZF, a direct target of PR that is an essential effector in decidualization (Szwarc et al., 2018). We found *PLZF* was significantly upregulated in sPE (Figure 31B), demonstrating a major downstream effector of PR is aberrantly activated.

These results suggest that in response to reduced levels of PR, decidualization mediators such as STAT3 are upregulated to revert the failure. Consequently, downstream effectors of PR like *PLZF* are increased, leading to DD in sPE patients.
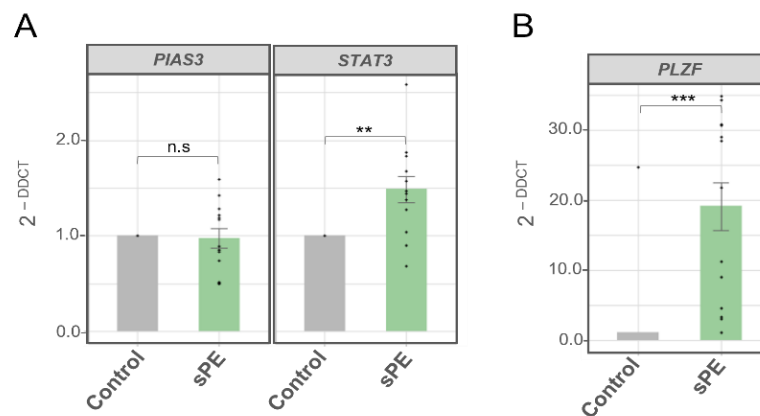
Figure 31. Upstream decidualization mediators are upregulated in sPE. (A). Gene expression levels of *PIAS3* and *STAT3* assessed for sPE (n=13) vs. controls (n=9) by RT-qPCR (*gray bars*, control; *green bars*, sPE). **(B)**. Gene expression level of *PLZF* assessed for sPE (n=13) vs. controls (n=9) by RT-qPCR (*gray bars*, control; *green bars*, sPE). RT-qPFCR values are expressed as mean± SE. *** p<0.001, ** p<0.01.

5.6.3. Modeling DD in sPE

Based on our findings, we postulate that in non-sPE pregnancies balanced hormonal signaling leads to proper decidualization, which in turn interacts with immune and endothelial cells to control CTBs invasion (Figure 32A). P4 and E2 activate their receptors in the epithelium and signal to the stromal compartment. Likewise, stromal PR is activated by ER1, which induces target genes involved in decidualization such

as *MSX2*, *CCNB1*, and *IL6*. In contrast, in sPE, endometrial *ESR1* and *IHH* are downregulated, decreasing *PGR* expression (Figure 32B) and leading to compromised decidualization, endothelial dysfunction, and local immune dysregulation.

Our findings reveal significant gene expression dysregulation underlying DD in the late secretory phase in women who have had sPE. The potential origin of sPE may lie in the downregulation of *ESR1* and *PGR-B*. Both receptors are strongly coordinated to regulate decidualization and *PGR* expression is induced by ER1, which is inhibited by PR (Patel et al., 2015). E2 and P4 act through ER1 and PR in the epithelium and stroma, modulating the transcriptome and promoting the crosstalk between both compartments (Winuthayanon et al., 2010). Epithelial ER1 regulates stromal decidualization via paracrine mechanisms mediated by leukemia inhibitory factor (LIF), which controls *IHH* expression that transduce the signal activation of PR in the stroma (Pawar et al., 2015). Also, stromal ER1 activates PR in the same compartment (Kaya Okur et al., 2016). These findings could inform about the development of therapeutic targets to restore optimal decidualization in sPE.

A
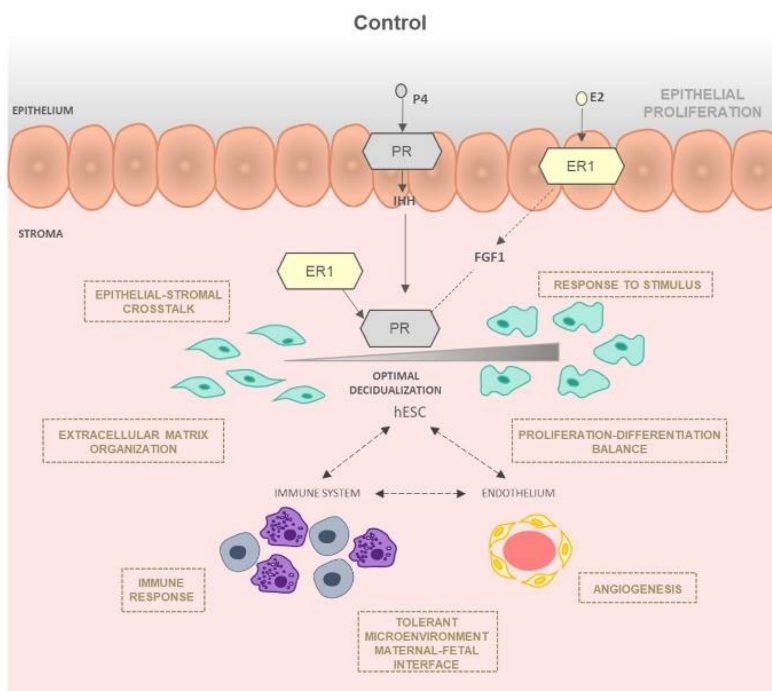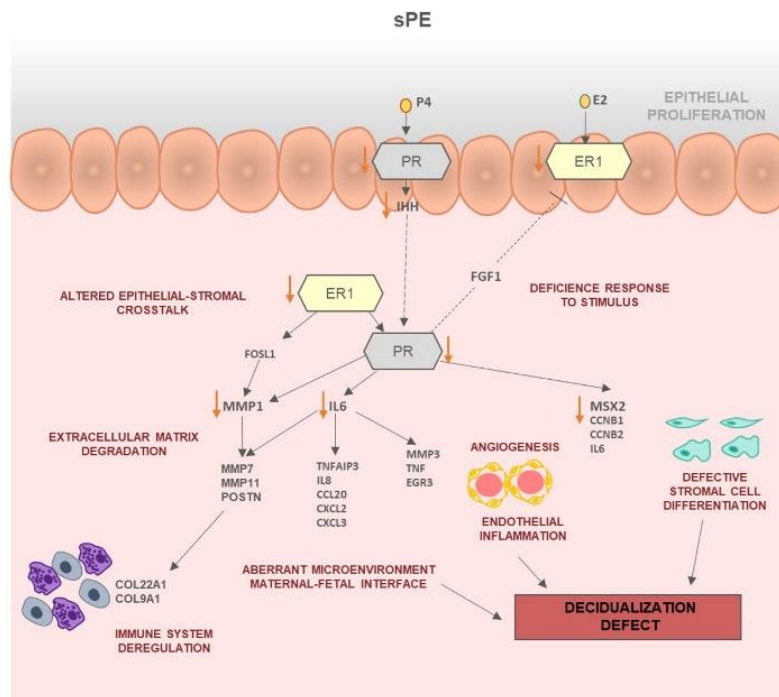
Figure 32. Modeling of the molecular mechanism for DD in sPE. (A). Decidualization induced by P4 and E2 in control pregnancy including the interaction of immune response and endothelium. **(B)**. Hypothetical network that could link DD and dysregulated hormone signaling in sPE. All genes were downregulated. Biological processes specified are candidates to be impaired based on functions associated with the observed dysregulation. *Red arrows* show the downregulation of decidualization modulators.

6. DISCUSSION

6. DISCUSSION

Decidualization of the endometrium during the late-secretory phase of the menstrual cycle is a primary driver for pregnancy health. Thus, failures in this differentiation program that transform the endometrium into decidua are associated with pregnancy complications. DD during and years after sPE has been previously demonstrated using a cell culture model of ECSs decidualized *in vitro* profiling 129 deregulated genes. Although this was a highlighted step forward in the understanding of the uterine contribution to sPE, the experimental approach had limitations. This investigation was focus in one cell type isolated from its *in vivo* environment, subjected to decidualization using controlled time-dose of inductors and the transcriptome profile was obtained with microarray technology, detecting just those genes that can hybridize with predefined proofs. In the present thesis, we move to the next step overcoming these main limitations by applying RNA-seq technology to endometrial biopsies collected at the time of decidualization in women who suffered sPE and women who had a healthy pregnancy. This approach allows to sequence any RNA in the tissue and characterise the abnormalities in the transcriptome *in vivo*, reporting a broad range of novel alterations in gene expression involved in defective decidualization in sPE patients.

Here, the global RNA-seq of endometrial biopsies collected at the time of late-secretory phase detected 593 genes differentially expressed in sPE compared to controls. This dataset includes remarkably decidualization mediators such as *PRL*, *IL-6*, and *IHH*, and other genes involved in biological process that account for tissue remodeling like angiogenesis and immune response. These results reflect the diversity of alterations detected in our approach based on endometrial tissues and RNA-seq technology. The overlapping of this dataset with our previous *in vitro* study was modest, reporting just nine coincidences. However, we investigated deeper the gene expression of the *in vitro* biomarkers in the current cohort of donors. A targeted RNA-seq including the 129 DEGs of *in vitro* DD fingerprint was perform, revealing the same expression trend for those genes compared with the *in vivo* approach. Thus, in this study we corroborate our previous findings and provide a new broad range of transcriptomic disruptions in sPE *in vivo*, that arise from the high cellular complexity in the endometrial tissue and lingers for years afterward.

Bulk RNA-seq provides the average expression of each gene active in a tissue sample. This value is a powerful result to identify tissue hallmarks associated with specific conditions. However, it does not allow to detect abnormalities at the cell type level, which is key to unveil the molecular mechanisms of biological events. In this context, it has been developed a deconvolution strategy combining data from bulk RNA-seq and scRNA-seq with the aim of inferring cell proportions that compose a tissue. Due to scRNA-seq requires tissue disaggregation and isolation of different cell types, the cell proportions are bias. In contrast, bulk RNA-seq include handling steps that can alter this feature in the sample, since the RNA is extracted from the whole tissue. Thus, combining data from both strategies can overcome the intrinsic limitations of each approach.

Deconvolution analysis was performed using the single-cell atlas of the endometrium published by our group (Wang et al., 2020) to unveil major cell types affected in the bulk RNA-seq dataset. This revealed a subset of 263 DEGs that were expressed by stromal fibroblasts during decidualization, and the proportion of lymphocytes was significantly higher in sPE specimens. Highlighting the main role of these cell types in the pathological phenotype. These results are consistent with the decidualization biology and sPE pathogenesis. Fibroblasts are the major cell type in the stromal compartment that decidualize in response to hormone stimuli during decidualization. Then, the secretome released by decidual cells execute immunoregulatory functions, orchestrating immune cell recruitment and activity. Thus, it is expected that both the proportion and gene expression of lymphocytes are impaired in sPE biopsies. During decidualization stromal fibroblasts and lymphocytes interact, and if one partner is disrupted the other most probably will be affected. Although, this result should be considered with caution since the sample size of the single-cell atlas of the endometrium during menstrual cycle is reduced (N=20).

Then, we identified the major gene expression changes associated with defective decidualization in sPE among the hundreds of affected genes reported. The aim was to select a subset composed by those dysregulated transcripts with higher biological impact based on the higher FC. A reduced list of the main genes would allow the development of a customized RNAseq panel for detecting this phenotype. Targeted assessments provide more comprehensive results as well as require a lower

investment in terms of time and resources, allowing a widespread use. Hence, this tool could be interesting for future investigations related with defective decidualization and pregnancy complications. Thus, we formulate the DD fingerprint associated with sPE comprising 120 genes.

The signature presented here allows effectively to segregate samples from the training and test set between sPE and controls. One sPE was misclustered in the test set, such that 90.9% of samples from an independent cohort were properly clustered in the dendrogram. Having controlled for confounding effects of biological and technical variables, we consider that this misclustering is consistent with the nature of decidualization and its inherent variability. Decidualization is a highly dynamic process governed by: (i) the inter-individual variability inherent to the endometrial menstrual cycle, which was supported with the displacement of the window of implantation (Díaz-Gimeno et al., 2011; Wang et al., 2018), (ii) the physiology of the spatial expansion of this process, starting in some areas around spiral arteries and extending to the entire endometrium during the last days of the menstrual cycle (Gellersen & Brosens, 2014), (iii) and the random sampling taking the endometrial biopsy, that is inherent to the experimental strategy used in our investigation. Taken together, we detected that the fingerprinting associated to defective decidualization showed its highest potential to segregate the samples in two groups during the last three days of menstrual cycle.

Once the global transcriptome of the endometrium was characterised and the DD fingerprint formulated, we wanted to go deep in the understanding of the molecular mechanisms that are driving defective decidualization. Enriched biological processes and pathways were associated with decidualization biology and sPE pathogenesis such as extracellular matrix organization, regulation of receptor signaling, lipids and atherosclerosis. Strikingly, enrichment analysis revealed that genes encoding defective decidualization were involved in immune response to pathogens, including bacteria and virus. Consistent with this, an increase in PE-like obstetric pathology has recently been observed in pregnant COVID-19 patients (Mendoza et al., 2020). The INTERCOVID prospective longitudinal study enrolled 2184 pregnant women —including 123 PE patients of which 59 were in the COVID-19 diagnosed group—, shed light in the association among both pathological conditions (Papageorghiou et al., 2021). The study demonstrates that COVID-19 is strongly associated with PE and

this association is independent of risk factors and pre-existing conditions. Authors suggest PE precedes SARS-CoV-2 and increased the risk of COVID-19, since this relationship has been seen especially nulliparous women and the severity of COVID-19 symptoms not increase the association. In this line, the deregulation genes involved in immune response could be a link between the two syndromes. Additionally, evidence supporting associations between microbiome and PE are emerging and suggest potential roles of oral, gut, placental, and vaginal microbiome (Ishimwe, 2021). Since the imbalance of immune response genes can impair decidualization and lead to PE, in case of infections immune imbalance can be intensified, increasing the risk of both PE and pathogenic diseases, giving rise these associations.

Regarding the potential mediators of the broad dysregulations observed at the level of gene expression, we explored the function of ER1 and PR based on its role in regulate the phases along menstrual cycle. We found genes of the DD fingerprint were interconnected and modulated by ER1 and PGR. Moreover, sPE specimens showed downregulation of *ESR1* and *PGR-B* and lower protein abundance of both receptors, especially in secretory glands. Furthermore, proteins codified by the DD fingerprint interact directly with those deregulated in CVS collected from women who develop sPE 6-months later. This result reinforces maternal contribution to sPE through DD during menstrual cycle.

Based on these results, we postulate that imbalanced hormonal signaling drives to decidualization resistance in women who suffered sPE. Low expression of *PGR* and *ESR1* dysregulate the action of P4 and E2 activating the decidualization program into the stroma. Consequently, the variety of process involved in endometrial decidualization could be compromised. This includes not only stromal cell differentiation but also stromal–epithelial crosstalk (Wang et al., 2017), extracellular matrix degradation (Itoh et al., 2012), immune system response and endothelial function (Okada et al., 2018) might be affected. Remarkably, immune system appeared notable deregulated due to the altered expression of interleukins, cytokines, chemokines and immunoglobulin that could disrupt the tolerant microenvironment at the maternal-fetal interface in pregnancy (Erlebacher, 2013; Harris et al., 2019). To compensate the failure in hormonal signaling and support pregnancy, regulators of PR such as *STATS3* are upregulated leading to the activation of downstream effectors

like *PLZF*. Consequently, ESC suffers an aberrant transcriptional reprograming that ends in defective decidualization and sPE.

In a nutshell, we have characterized the transcriptomics of the late-secretory endometrium from women who suffered sPE compared to women with normal obstetric outcomes. As a result, we demonstrated *in vivo* defective decidualization and provided a signature encoding it that allowed segregate samples in sPE and controls effectively. Further, we postulate impaired ER1 and PR-B as potential driver of compromised decidualization and suggest upregulation of decidualization mediators as a mechanism that try to revert the defective phenotype leading to an aberrant transcriptome reprogramming.

The challenge in the development of prevention and diagnostic strategies in PE is that its etiology remains poorly understood and it is widely separated in time from the onset of disease symptoms. To date, most diagnostic efforts based on molecular test for early detection of high-risk patients have been focused on biomarkers of angiogenic imbalance derived from placental malperfusion (Burton et al., 2019; Rana et al., 2019).

The most promising screening methods with a high sensitivity and specificity are with combined biomarkers sFLT1 and PlGF (Rana et al., 2019). The sFLT1/PlGF ratio has been proposed as a biomarker showing a positive predictive value of 36.7%, with 66.2% sensitivity and 83.1% specificity but its application is effective only 4 weeks before PE symptoms manifest (Zeisler et al., 2016). Multiparametric models were developed to detect pregnancies at risk of PE by screening at 11 to 13 weeks' gestation. These models combine data from medical history with biophysical and biochemical markers. However, when applied to different populations, they showed poor performance. A multivariate Gaussian distribution model was recently proposed including maternal factors, early PlGF determination, and biophysical variables, but this model is not validated in different populations (Serra et al., 2020). Thus, predictive strategies based on placental dysfunction provide delayed results to significantly reduce morbidity and mortality associated with PE.

In this scenario, the goal is obtaining an effective early screening based on molecular events that precedes placental damage. Recent advances in decidualization as a primary driver of pregnancy health and disease open new avenues to develop predictive tools for pregnancy complications including sPE (Garrido-Gómez et al., 2022; Ng et al., 2020). In humans, decidualization occurs monthly in each menstrual cycle regardless the presence of a conceptus. Thus, defective decidualization could be identified even before pregnancy. Moreover, decidualization precedes placentation leading to alterations that manifest before placental damage and its signals such as angiogenic imbalance. Altogether, understanding defective decidualization in sPE brings the opportunity of developing new methods for earlier diagnosis which might be applied even preconceptionally.

To analyse samples retrospectively, years after the event (normal or pathological pregnancy), is a limitation that arises from the inability of collect endometrial samples once pregnancy is stablished. Additionally, a prospective investigation would require the collection of thousands of endometrial biopsies from nulliparous women before pregnancy and follow up all participants until they give birth to enroll at least 30 sPE cases. This would mean a huge effort in terms of human and financial resources. Previously, the maternal contribution to sPE through defective decidualization has been suggested before (Garrido-Gomez et al., 2017) and after implantation (Rabaglino et al., 2015), and robustly demonstrated at the time of delivery and years after (Garrido-Gomez et al., 2017). Furthermore, ESCs isolated from sPE patients were not able to decidualize *in vitro* and to support CTB invasion (Garrido-Gomez et al., 2017). Results from these experimental investigations along with the role of decidualization to prepare the "maternal soil" for pregnancy support the hypothesis that impaired endometrial maturation predates sPE (Garrido-Gómez et al., 2022). Here, we provide new evidence to reinforce this maternal contribution to sPE and a footprint that might identify this condition. DD is a potential footprint that may be leverage for screening of sPE risk. Further, the connection with ESR1 and PGR-B signaling impairment opens a field to develop strategies for sPE therapy based on molecules capable of restoring these signaling.

Recently, analysis of circulating RNA (C-RNA) has been proposed as a promising tool to monitor pregnancy health and to detect patients at high risk of suffering PE (Munchel

et al., 2020; Rasmussen et al., 2022). At the time of severe early-onset diagnosis PE patients show a hallmark of 49 C-RNA transcripts that allow its identification with 85-89% of accuracy. In contrast, late-onset PE had a potentially weaker or a different C-RNA signature (Munchel et al., 2020). The last work on this field conducted by Rassmusen and colleagues (Rasmussen et al., 2022) demonstrated C-RNA profile can monitor pregnancy progression at the placental, maternal, and fetal levels and identify women at risk of PE with a sensitivity of 75% and a positive predictive value of 32.3% (s.d., 3%). This study includes eight independent cohort which is an advantage for investigate the effect of multiple ethnicities and to have a large sample size (N=1840 pregnancies; N=72 PE), but also it a disadvantage to have detailed clinical information about the cohort. Consequently, there is a lack of important data to assess the feasibility of the C-RNA signature as a diagnostic tool such as how long the blood was drawn before clinical symptoms manifested and PE stratification in clinical phenotypes. PE is a complex and heterogenous obstetric complication stratified into the commonly used clinical phenotypes related with severity and with timing of symptoms manifestation being early- or late-onset. The potential of C-RNA transcripts to diagnose different types of PE remains unclear, since the signature reported by Munchel et al., 2020 showed a good performance for severe early-onset PE but no for late-onset, whereas Rasmussen et al., 2022 did not stratify samples.

Our hypothesis about DD during the late secretory phase leading to sPE, brings the possibility to identify a C-RNA profile associated with this condition early in the first trimester of pregnancy. DD implies a disrupted maternal-fetal crosstalk during EVT invasion, consequently RNA transcripts might be release to maternal bloodstream reflecting the impaired events and associated damages. The present study set strong foundations to encourage a research focus on identify the C-RNA profile associated with defective decidualization to stratify the risk of developing sPE. Furthermore, decidualization failure is also associated with infertility and reproductive disorders including endometriosis, failed implantation, miscarriage, IUGR, among others. Hence, evaluating decidualization through a non-invasive test based on C-RNA in blood might provide valuable data to assess fertility —avoiding endometrial biopsies— and to monitor obstetric health in pregnant women with risk of suffering complications.

Thus, future studies will be focus on two main aims: (i) to demonstrate translational potential of the DD fingerprint to develop new noninvasive strategies based on C-RNA to improve diagnosis and prognostication for women with sPE early enough to support clinical value; (ii) to investigate the reversion of the defective decidualization targeting the DD fingerprint genes and the downregulation of both *ESR1* and *PGR-B*.

7. CONCLUSIONS

7. CONCLUSIONS

1. Endometrial tissue from women who suffered sPE years after the affected pregnancy in the late-secretory phase have a differential transcriptomic signature composed by 593 genes. This transcriptome profile identified a broad number of genes involved in defective decidualization in sPE such as *PRL, CCL20, IGHG1, FGF7, EDN2,* and *IDO2.*

2. The endometrial transcriptome combined with single-cell data revealed that ~45% genes deregulated in sPE are expressed in endometrial fibroblast and the lymphocytes ratio during decidualization is significantly increased in cases compared controls.

3. DD fingerprint is composed by 120 genes such as *IHH, MSX2, FGF1, IL6,* and *TNF*. Gene ontology analysis revealed 151 biological processes enriched, including pathways related with decidualization biology and sPE pathogenesis such as extracellular matrix organization, regulation of receptor signaling and regulation of cell migration.

4. DD fingerprint allows to segregate samples in sPE and control group effectively in both the training and test sets, showing the potential to be leverage for developing early screaming tools based on defective decidualization.

5. DD fingerprint is highly interconnected with biomarkers reported of impaired maternal-fetal interface in sPE patients prior to symptoms manifestation, reinforcing the maternal contribution to this condition through defective decidualization.

6. DD fingerprint is composed by genes enriched in the endometrium and modulated by ER1 and PR. Furthermore, these hormonal receptors are embedded and central in the interactome network resulting from the proteins encoded by the DD fingerprint genes. This suggests that disrupted signaling of ER1 and PR might impact deeply the network.

7. *ESR1* and *PGR-B* gene expression and protein abundance are downregulated in sPE. In contrast, *STAT3* and *PLZF*, essential decidualization mediators, are upregulated in sPE.

8. The transcriptome profiling of the endometrium allows us to postulate a model that links DD induced by impaired hormonal signaling with sPE, opening the field to develop new strategies for sPE therapy.

8. REFERENCES

8. REFERENCES

Agrawal, S., Cerdeira, A. S., Redman, C., & Vatish, M. (2018). Meta-Analysis and Systematic Review to Assess the Role of Soluble FMS-Like Tyrosine Kinase-1 and Placenta Growth Factor Ratio in Prediction of Preeclampsia: The SaPPPhirE Study. *Hypertension*, *71*(2), 306-316. https://doi.org/10.1161/HYPERTENSIONAHA.117.10182

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166-169. https://doi.org/10.1093/bioinformatics/btu638

Aneman, I., Pienaar, D., Suvakov, S., Simic, T. P., Garovic, V. D., & McClements, L. (2020). Mechanisms of Key Innate Immune Cells in Early- and Late-Onset Preeclampsia. *Front Immunol*, *11*, 1864. https://doi.org/10.3389/fimmu.2020.01864

Aplin, J. D., Myers, J. E., Timms, K., & Westwood, M. (2020). Tracking placental development in health and disease. *Nat Rev Endocrinol*, *16*(9), 479-494. https://doi.org/10.1038/s41574-020-0372-6

Aplin, J. D., & Ruane, P. T. (2017). Embryo-epithelium interactions during implantation at a glance. *J Cell Sci*, *130*(1), 15-22. https://doi.org/10.1242/jcs.175943

Bell, M. J. (2010). A historical overview of preeclampsia-eclampsia. *J Obstet Gynecol Neonatal Nurs*, *39*(5), 510-518. https://doi.org/10.1111/j.1552-6909.2010.01172.x

Bellamy, L., Casas, J. P., Hingorani, A. D., & Williams, D. J. (2007). Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ*, *335*(7627), 974. https://doi.org/10.1136/bmj.39335.385301.BE

Blois, S. M., Klapp, B. F., & Barrientos, G. (2011). Decidualization and angiogenesis in early pregnancy: unravelling the functions of DC and NK cells. *J Reprod Immunol*, *88*(2), 86-92. https://doi.org/10.1016/j.jri.2010.11.002

Boghossian, N. S., Yeung, E., Mendola, P., Hinkle, S. N., Laughon, S. K., Zhang, C., & Albert, P. S. (2014). Risk factors differ between recurrent and incident preeclampsia: a hospital-based cohort study. *Ann Epidemiol*, *24*(12), 871-877e873. https://doi.org/10.1016/j.annepidem.2014.10.003

Bonney, E. A. (2016). Immune Regulation in Pregnancy: A Matter of Perspective? *Obstet Gynecol Clin North Am*, *43*(4), 679-698. https://doi.org/10.1016/j.ogc.2016.07.004

Boyd, H. A., Tahir, H., Wohlfahrt, J., & Melbye, M. (2013). Associations of personal and family preeclampsia history with the risk of early-, intermediate- and late-onset preeclampsia. *Am J Epidemiol*, *178*(11), 1611-1619. https://doi.org/10.1093/aje/kwt189

Brar, A. K., Frank, G. R., Kessler, C. A., Cedars, M. I., & Handwerger, S. (1997). Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. *Endocrine*, *6*(3), 301-307. https://doi.org/10.1007/BF02820507

Brosens, I., Pijnenborg, R., Vercruysse, L., & Romero, R. (2011). The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. *Am J Obstet Gynecol*, *204*(3), 193-201. https://doi.org/10.1016/j.ajog.2010.08.009

Brosens, I., Puttemans, P., & Benagiano, G. (2019). Placental bed research: I. The placental bed: from spiral arteries remodeling to the great obstetrical syndromes. *Am J Obstet Gynecol*, *221*(5), 437-456. https://doi.org/10.1016/j.ajog.2019.05.044

Brosens, I., Robertson, W. B., & Dixon, H. G. (1967). The physiological response of the vessels of the placental bed to normal pregnancy. *J Pathol Bacteriol*, *93*(2), 569-579. https://doi.org/10.1002/path.1700930218

Brosens, I. A., Robertson, W. B., & Dixon, H. G. (1970). The role of the spiral arteries in the pathogenesis of pre-eclampsia. *J Pathol*, *101*(4), Pvi.

Burney, R. O., Talbi, S., Hamilton, A. E., Vo, K. C., Nyegaard, M., Nezhat, C. R., . . . Giudice, L. C. (2007). Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology*, *148*(8), 3814-3826. https://doi.org/10.1210/en.2006-1692

Burton, G. J., Fowden, A. L., & Thornburg, K. L. (2016). Placental Origins of Chronic Disease. *Physiol Rev*, *96*(4), 1509-1565. https://doi.org/10.1152/physrev.00029.2015

Burton, G. J., Redman, C. W., Roberts, J. M., & Moffett, A. (2019). Pre-eclampsia: pathophysiology and clinical implications. *BMJ*, *366*, l2381. https://doi.org/10.1136/bmj.l2381

Burton, G. J., Watson, A. L., Hempstock, J., Skepper, J. N., & Jauniaux, E. (2002). Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab*, *87*(6), 2954-2959. https://doi.org/10.1210/jcem.87.6.8563

Burton, G. J., Woods, A. W., Jauniaux, E., & Kingdom, J. C. (2009). Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta*, *30*(6), 473-482. https://doi.org/10.1016/j.placenta.2009.02.009

Carp, H. J. A. (2020). Progestogens in luteal support. *Horm Mol Biol Clin Investig*, *42*(2), 143-148. https://doi.org/10.1515/hmbci-2019-0067

Cha, J., Sun, X., & Dey, S. K. (2012). Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*, *18*(12), 1754-1767. https://doi.org/10.1038/nm.3012

Chaemsaithong, P., Sahota, D. S., & Poon, L. C. (2022). First trimester preeclampsia screening and prediction. *Am J Obstet Gynecol*, *226*(2S), S1071-S1097.e1072. https://doi.org/10.1016/j.ajog.2020.07.020

Chaiworapongsa, T., Romero, R., Espinoza, J., Bujold, E., Mee Kim, Y., Gonçalves, L. F., . . . Edwin, S. (2004). Evidence supporting a role for blockade of the vascular endothelial growth factor system in the pathophysiology of preeclampsia. Young Investigator Award. *Am J Obstet Gynecol*, *190*(6), 1541-1547; discussion 1547-1550. https://doi.org/10.1016/j.ajog.2004.03.043

Chen, C., Li, C., Liu, W., Guo, F., Kou, X., Sun, S., . . . Zhao, A. (2020). Estrogen-induced FOS-like 1 regulates matrix metalloproteinase expression and the motility of human endometrial and decidual stromal cells. *J Biol Chem*, *295*(8), 2248-2258. https://doi.org/10.1074/jbc.RA119.010701

Chen, J. R., Cheng, J. G., Shatzer, T., Sewell, L., Hernandez, L., & Stewart, C. L. (2000). Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive

uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology*, *141*(12), 4365-4372. https://doi.org/10.1210/endo.141.12.7855

Chesley, L. C., Annitto, J. E., & Cosgrove, R. A. (1968). The familial factor in toxemia of pregnancy. *Obstet Gynecol*, *32*(3), 303-311.

Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. W., Ko, M. T., & Lin, C. Y. (2014). cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst Biol*, *8 Suppl 4*, S11. https://doi.org/10.1186/1752-0509-8-S4-S11

Cho, G. J., Kim, L. Y., Min, K. J., Sung, Y. N., Hong, S. C., Oh, M. J., . . . Kim, H. J. (2015). Prior cesarean section is associated with increased preeclampsia risk in a subsequent pregnancy. *BMC Pregnancy Childbirth*, *15*, 24. https://doi.org/10.1186/s12884-015-0447-x

Choudhury, R. H., Dunk, C. E., Lye, S. J., Aplin, J. D., Harris, L. K., & Jones, R. L. (2017). Extravillous Trophoblast and Endothelial Cell Crosstalk Mediates Leukocyte Infiltration to the Early Remodeling Decidual Spiral Arteriole Wall. *J Immunol*, *198*(10), 4115-4128. https://doi.org/10.4049/jimmunol.1601175

Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., & Shuai, K. (1997). Specific inhibition of Stat3 signal transduction by PIAS3. *Science*, *278*(5344), 1803-1805. https://doi.org/10.1126/science.278.5344.1803

Classen-Linke, I., Alfer, J., Hey, S., Krusche, C. A., Kusche, M., & Beier, H. M. (1998). Marker molecules of human endometrial differentiation can be hormonally regulated under in-vitro conditions as in-vivo. *Hum Reprod Update*, *4*(5), 539-549. https://doi.org/10.1093/humupd/4.5.539

Cloke, B., Huhtinen, K., Fusi, L., Kajihara, T., Yliheikkilä, M., Ho, K. K., . . . Brosens, J. J. (2008). The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. *Endocrinology*, *149*(9), 4462-4474. https://doi.org/10.1210/en.2008-0356

Cnattingius, S., Reilly, M., Pawitan, Y., & Lichtenstein, P. (2004). Maternal and fetal genetic factors account for most of familial aggregation of preeclampsia: a population-based Swedish cohort study. *Am J Med Genet A*, *130A*(4), 365-371. https://doi.org/10.1002/ajmg.a.30257

Cooke, P. S., Buchanan, D. L., Young, P., Setiawan, T., Brody, J., Korach, K. S., . . . Cunha, G. R. (1997). Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proc Natl Acad Sci U S A*, *94*(12), 6535-6540. https://doi.org/10.1073/pnas.94.12.6535

DaSilva-Arnold, S., James, J. L., Al-Khan, A., Zamudio, S., & Illsley, N. P. (2015). Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial-mesenchymal transition. *Placenta*, *36*(12), 1412-1418. https://doi.org/10.1016/j.placenta.2015.10.013

Davis, E. F., Lazdam, M., Lewandowski, A. J., Worton, S. A., Kelly, B., Kenworthy, Y., . . . Leeson, P. (2012). Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. *Pediatrics*, *129*(6), e1552-1561. https://doi.org/10.1542/peds.2011-3093

Deryabin, P., Griukova, A., Nikolsky, N., & Borodkina, A. (2020). The link between endometrial stromal cell senescence and decidualization in female fertility: the art of balance. *Cell Mol Life Sci*, *77*(7), 1357-1370. https://doi.org/10.1007/s00018-019-03374-0

Dickey, R. P., & Hower, J. F. (1995). Ultrasonographic features of uterine blood flow during the first 16 weeks of pregnancy. *Hum Reprod*, *10*(9), 2448-2452. https://doi.org/10.1093/oxfordjournals.humrep.a136317

Dimitriadis, E., Salamonsen, L. A., & Robb, L. (2000). Expression of interleukin-11 during the human menstrual cycle: coincidence with stromal cell decidualization and relationship to leukaemia inhibitory factor and prolactin. *Mol Hum Reprod*, *6*(10), 907-914. https://doi.org/10.1093/molehr/6.10.907

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., . . . Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15-21. https://doi.org/10.1093/bioinformatics/bts635

Du, L., Deng, W., Zeng, S., Xu, P., Huang, L., Liang, Y., . . . Chen, D. (2021). Single-cell transcriptome analysis reveals defective decidua stromal niche attributes to recurrent spontaneous abortion. *Cell Prolif*, *54*(11), e13125. https://doi.org/10.1111/cpr.13125

Dunk, C., Kwan, M., Hazan, A., Walker, S., Wright, J. K., Harris, L. K., . . . Lye, S. J. (2019). Failure of Decidualization and Maternal Immune Tolerance Underlies Uterovascular Resistance in Intra Uterine Growth Restriction. *Front Endocrinol (Lausanne)*, *10*, 160. https://doi.org/10.3389/fendo.2019.00160

Dunn, C. L., Kelly, R. W., & Critchley, H. O. (2003). Decidualization of the human endometrial stromal cell: an enigmatic transformation. *Reprod Biomed Online*, *7*(2), 151-161.

Díaz-Gimeno, P., Horcajadas, J. A., Martínez-Conejero, J. A., Esteban, F. J., Alamá, P., Pellicer, A., & Simón, C. (2011). A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril*, *95*(1), 50-60, 60.e51-15. https://doi.org/10.1016/j.fertnstert.2010.04.063

Egeland, G. M., Klungsøyr, K., Øyen, N., Tell, G. S., Næss, Ø., & Skjærven, R. (2016). Preconception Cardiovascular Risk Factor Differences Between Gestational Hypertension and Preeclampsia: Cohort Norway Study. *Hypertension*, *67*(6), 1173-1180. https://doi.org/10.1161/HYPERTENSIONAHA.116.07099

Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annu Rev Immunol*, *31*, 387-411. https://doi.org/10.1146/annurev-immunol-032712-100003

Esplin, M. S., Fausett, M. B., Fraser, A., Kerber, R., Mineau, G., Carrillo, J., & Varner, M. W. (2001). Paternal and maternal components of the predisposition to preeclampsia. *N Engl J Med*, *344*(12), 867-872. https://doi.org/10.1056/NEJM200103223441201

Evans, J., Salamonsen, L. A., Winship, A., Menkhorst, E., Nie, G., Gargett, C. E., & Dimitriadis, E. (2016). Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol*, *12*(11), 654-667. https://doi.org/10.1038/nrendo.2016.116

Falco, M. L., Sivanathan, J., Laoreti, A., Thilaganathan, B., & Khalil, A. (2017). Placental histopathology associated with pre-eclampsia: systematic review and meta-analysis. *Ultrasound Obstet Gynecol*, *50*(3), 295-301. https://doi.org/10.1002/uog.17494

Filant, J., & Spencer, T. E. (2014). Uterine glands: biological roles in conceptus implantation, uterine receptivity and decidualization. *Int J Dev Biol*, *58*(2-4), 107-116. https://doi.org/10.1387/ijdb.130344ts

Fingar, K. R., Mabry-Hernandez, I., Ngo-Metzger, Q., Wolff, T., Steiner, C. A., & Elixhauser, A. (2017). Healthcare Cost and Utilization Project (HCUP). Delivery hospitalizations involving preeclampsia and eclampsia, 2005-2014. . In *Statisttical brief #222*. https://hcup-us.ahrq.gov/reports/statbriefs/sb222-Preeclampsia-Eclampsia-Delivery-Trends.jsp: Agency for Healthcare Research and Quality.

Fisher, S. J. (2015). Why is placentation abnormal in preeclampsia? *Am J Obstet Gynecol*, *213*(4 Suppl), S115-122. https://doi.org/10.1016/j.ajog.2015.08.042

Foo, F. L., Mahendru, A. A., Masini, G., Fraser, A., Cacciatore, S., MacIntyre, D. A., . . . Lees, C. C. (2018). Association Between Prepregnancy Cardiovascular Function and Subsequent Preeclampsia or Fetal Growth Restriction. *Hypertension*, *72*(2), 442-450. https://doi.org/10.1161/HYPERTENSIONAHA.118.11092

Garrido-Gomez, T., Dominguez, F., Antonio Lopez, J., Camafeita, E., Quinonero, A., Antonio Martinez-Conejero, J., . . . Simon, C. (2011). Modeling Human Endometrial Decidualization from the Interaction between Proteome and Secretome. *Journal of Clinical Endocrinology & Metabolism*, *96*(3), 706-716. https://doi.org/10.1210/jc.2010-1825

Garrido-Gomez, T., Dominguez, F., Quiñonero, A., Diaz-Gimeno, P., Kapidzic, M., Gormley, M., . . . Simón, C. (2017). Defective decidualization during and after severe preeclampsia reveals a possible maternal contribution to the etiology. *Proc Natl Acad Sci U S A*, *114*(40), E8468-E8477. https://doi.org/10.1073/pnas.1706546114

Garrido-Gomez, T., Quiñonero, A., Dominguez, F., Rubert, L., Perales, A., Hajjar, K. A., & Simon, C. (2020). Preeclampsia: a defect in decidualization is associated with deficiency of Annexin A2. *Am J Obstet Gynecol*, *222*(4), 376.e371-376.e317. https://doi.org/10.1016/j.ajog.2019.11.1250

Garrido-Gómez, T., Castillo-Marco, N., Cordero, T., & Simón, C. (2022). Decidualization resistance in the origin of preeclampsia. *Am J Obstet Gynecol*, *226*(2S), S886-S894. https://doi.org/10.1016/j.ajog.2020.09.039

Gellersen, B., Brosens, I. A., & Brosens, J. J. (2007). Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med*, *25*(6), 445-453. https://doi.org/10.1055/s-2007-991042

Gellersen, B., & Brosens, J. J. (2014). Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocr Rev*, *35*(6), 851-905. https://doi.org/10.1210/er.2014-1045

Giannakou, K., Evangelou, E., & Papatheodorou, S. I. (2018). Genetic and non-genetic risk factors for pre-eclampsia: umbrella review of systematic reviews and meta-analyses of observational studies. *Ultrasound Obstet Gynecol*, *51*(6), 720-730. https://doi.org/10.1002/uog.18959

Giudice, L. C., Mark, S. P., & Irwin, J. C. (1998). Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant human endometrium and at the decidual-trophoblast interface. *J Reprod Immunol*, *39*(1-2), 133-148.

Gnecco, J. S., Ding, T., Smith, C., Lu, J., Bruner-Tran, K. L., & Osteen, K. G. (2019). Hemodynamic forces enhance decidualization via endothelial-derived prostaglandin E2 and prostacyclin in a microfluidic model of the human endometrium. *Hum Reprod*, *34*(4), 702-714. https://doi.org/10.1093/humrep/dez003

Goh, W. A., & Zalud, I. (2016). Placenta accreta: diagnosis, management and the molecular biology of the morbidly adherent placenta. *J Matern Fetal Neonatal Med*, *29*(11), 1795-1800. https://doi.org/10.3109/14767058.2015.1064103

Grewal, S., Carver, J., Ridley, A. J., & Mardon, H. J. (2010). Human endometrial stromal cell rho GTPases have opposing roles in regulating focal adhesion turnover and embryo invasion in vitro. *Biol Reprod*, *83*(1), 75-82. https://doi.org/10.1095/biolreprod.109.080630

Grewal, S., Carver, J. G., Ridley, A. J., & Mardon, H. J. (2008). Implantation of the human embryo requires Rac1-dependent endometrial stromal cell migration. *Proc Natl Acad Sci U S A*, *105*(42), 16189-16194. https://doi.org/10.1073/pnas.0806219105

Guerin, L. R., Moldenhauer, L. M., Prins, J. R., Bromfield, J. J., Hayball, J. D., & Robertson, S. A. (2011). Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. *Biol Reprod*, *85*(2), 397-408. https://doi.org/10.1095/biolreprod.110.088591

Haller, M., Yin, Y., & Ma, L. (2019). Development and utilization of human decidualization reporter cell line uncovers new modulators of female fertility. *Proc Natl Acad Sci U S A*, *116*(39), 19541-19551. https://doi.org/10.1073/pnas.1907652116

Haluska, G. J., West, N. B., Novy, M. J., & Brenner, R. M. (1990). Uterine estrogen receptors are increased by RU486 in late pregnant rhesus macaques but not after spontaneous labor. *J Clin Endocrinol Metab*, *70*(1), 181-186. https://doi.org/10.1210/jcem-70-1-181

Harris, L. K., Benagiano, M., D'Elios, M. M., Brosens, I., & Benagiano, G. (2019). Placental bed research: II. Functional and immunological investigations of the placental bed. *Am J Obstet Gynecol*, *221*(5), 457-469. https://doi.org/10.1016/j.ajog.2019.07.010

Henderson, T. A., Saunders, P. T., Moffett-King, A., Groome, N. P., & Critchley, H. O. (2003). Steroid receptor expression in uterine natural killer cells. *J Clin Endocrinol Metab*, *88*(1), 440-449. https://doi.org/10.1210/jc.2002-021174

Hernández-Díaz, S., Toh, S., & Cnattingius, S. (2009). Risk of pre-eclampsia in first and subsequent pregnancies: prospective cohort study. *BMJ*, *338*, b2255. https://doi.org/10.1136/bmj.b2255

Hewitt, S. C., Kissling, G. E., Fieselman, K. E., Jayes, F. L., Gerrish, K. E., & Korach, K. S. (2010). Biological and biochemical consequences of global deletion of exon 3 from the ER alpha gene. *FASEB J*, *24*(12), 4660-4667. https://doi.org/10.1096/fj.10-163428

Holland, O., Dekker Nitert, M., Gallo, L. A., Vejzovic, M., Fisher, J. J., & Perkins, A. V. (2017). Review: Placental mitochondrial function and structure in gestational disorders. *Placenta*, *54*, 2-9. https://doi.org/10.1016/j.placenta.2016.12.012

Huang, Q. T., Wang, S. S., Zhang, M., Huang, L. P., Tian, J. W., Yu, Y. H., . . . Zhong, M. (2013). Advanced oxidation protein products enhances soluble Fms-like tyrosine kinase 1 expression

in trophoblasts: a possible link between oxidative stress and preeclampsia. *Placenta*, *34*(10), 949-952. https://doi.org/10.1016/j.placenta.2013.06.308

Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. (2013). *Obstet Gynecol*, *122*(5), 1122-1131. https://doi.org/10.1097/01.AOG.0000437382.03963.88

Ishimwe, J. A. (2021). Maternal microbiome in preeclampsia pathophysiology and implications on offspring health. *Physiol Rep*, *9*(10), e14875. https://doi.org/10.14814/phy2.14875

Itoh, H., Kishore, A. H., Lindqvist, A., Rogers, D. E., & Word, R. A. (2012). Transforming growth factor β1 (TGFβ1) and progesterone regulate matrix metalloproteinases (MMP) in human endometrial stromal cells. *J Clin Endocrinol Metab*, *97*(6), E888-897. https://doi.org/10.1210/jc.2011-3073

Ives, C. W., Sinkey, R., Rajapreyar, I., Tita, A. T. N., & Oparil, S. (2020). Preeclampsia-Pathophysiology and Clinical Presentations: JACC State-of-the-Art Review. *J Am Coll Cardiol*, *76*(14), 1690-1702. https://doi.org/10.1016/j.jacc.2020.08.014

Jabbour, H. N., & Critchley, H. O. (2001). Potential roles of decidual prolactin in early pregnancy. *Reproduction*, *121*(2), 197-205.

Jacobsson, B., Ladfors, L., & Milsom, I. (2004). Advanced maternal age and adverse perinatal outcome. *Obstet Gynecol*, *104*(4), 727-733. https://doi.org/10.1097/01.AOG.0000140682.63746.be

Jensen, L. J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., . . . von Mering, C. (2009). STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res*, *37*(Database issue), D412-416. https://doi.org/10.1093/nar/gkn760

Jolly, M., Sebire, N., Harris, J., Robinson, S., & Regan, L. (2000). The risks associated with pregnancy in women aged 35 years or older. *Hum Reprod*, *15*(11), 2433-2437. https://doi.org/10.1093/humrep/15.11.2433

Jones, C. J., Choudhury, R. H., & Aplin, J. D. (2015). Tracking nutrient transfer at the human maternofetal interface from 4 weeks to term. *Placenta*, *36*(4), 372-380. https://doi.org/10.1016/j.placenta.2015.01.002

Jones, C. J., & Fox, H. (1980). An ultrastructural and ultrahistochemical study of the human placenta in maternal pre-eclampsia. *Placenta*, *1*(1), 61-76. https://doi.org/10.1016/s0143-4004(80)80016-6

Jones, R. L., Findlay, J. K., Farnworth, P. G., Robertson, D. M., Wallace, E., & Salamonsen, L. A. (2006). Activin A and inhibin A differentially regulate human uterine matrix metalloproteinases: potential interactions during decidualization and trophoblast invasion. *Endocrinology*, *147*(2), 724-732. https://doi.org/10.1210/en.2005-1183

Jones, R. L., Stoikos, C., Findlay, J. K., & Salamonsen, L. A. (2006). TGF-beta superfamily expression and actions in the endometrium and placenta. *Reproduction*, *132*(2), 217-232. https://doi.org/10.1530/rep.1.01076

Kaaja, R. J., & Greer, I. A. (2005). Manifestations of chronic disease during pregnancy. *JAMA*, *294*(21), 2751-2757. https://doi.org/10.1001/jama.294.21.2751

Kajantie, E., Eriksson, J. G., Osmond, C., Thornburg, K., & Barker, D. J. (2009). Pre-eclampsia is associated with increased risk of stroke in the adult offspring: the Helsinki birth cohort study. *Stroke*, *40*(4), 1176-1180. https://doi.org/10.1161/STROKEAHA.108.538025

Kalafat, E., Laoreti, A., Khalil, A., Da Silva Costa, F., & Thilaganathan, B. (2018). Ophthalmic artery Doppler for prediction of pre-eclampsia: systematic review and meta-analysis. *Ultrasound Obstet Gynecol*, *51*(6), 731-737. https://doi.org/10.1002/uog.19002

Kaya, H. S., Hantak, A. M., Stubbs, L. J., Taylor, R. N., Bagchi, I. C., & Bagchi, M. K. (2015). Roles of progesterone receptor A and B isoforms during human endometrial decidualization. *Mol Endocrinol*, *29*(6), 882-895. https://doi.org/10.1210/me.2014-1363

Kelleher, A. M., DeMayo, F. J., & Spencer, T. E. (2019). Uterine Glands: Developmental Biology and Functional Roles in Pregnancy. *Endocr Rev*, *40*(5), 1424-1445. https://doi.org/10.1210/er.2018-00281

Kim, M. R., Park, D. W., Lee, J. H., Choi, D. S., Hwang, K. J., Ryu, H. S., & Min, C. K. (2005). Progesterone-dependent release of transforming growth factor-beta1 from epithelial cells enhances the endometrial decidualization by turning on the Smad signalling in stromal cells. *Mol Hum Reprod*, *11*(11), 801-808. https://doi.org/10.1093/molehr/gah240

Kim, Y. M., Bujold, E., Chaiworapongsa, T., Gomez, R., Yoon, B. H., Thaler, H. T., . . . Romero, R. (2003). Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. *Am J Obstet Gynecol*, *189*(4), 1063-1069. https://doi.org/10.1067/s0002-9378(03)00838-x

Kim, Y. M., Chaiworapongsa, T., Gomez, R., Bujold, E., Yoon, B. H., Rotmensch, S., . . . Romero, R. (2002). Failure of physiologic transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes. *Am J Obstet Gynecol*, *187*(5), 1137-1142. https://doi.org/10.1067/mob.2002.127720

Kitaya, K., Yamaguchi, T., & Honjo, H. (2005). Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16(-) natural killer cells into human endometrium. *J Clin Endocrinol Metab*, *90*(5), 2932-2940. https://doi.org/10.1210/jc.2004-2447

Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M., & Strauss, J. F. (1986). Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology*, *118*(4), 1567-1582. https://doi.org/10.1210/endo-118-4-1567

Kwan, M., Hazan, A., Zhang, J., Jones, R. L., Harris, L. K., Whittle, W., . . . Lye, S. J. (2014). Dynamic changes in maternal decidual leukocyte populations from first to second trimester gestation. *Placenta*, *35*(12), 1027-1034. https://doi.org/10.1016/j.placenta.2014.09.018

Lane, M., Robker, R. L., & Robertson, S. A. (2014). Parenting from before conception. *Science*, *345*(6198), 756-760. https://doi.org/10.1126/science.1254400

Lee, J. H., Kim, T. H., Oh, S. J., Yoo, J. Y., Akira, S., Ku, B. J., . . . Jeong, J. W. (2013). Signal transducer and activator of transcription-3 (Stat3) plays a critical role in implantation via progesterone receptor in uterus. *FASEB J*, *27*(7), 2553-2563. https://doi.org/10.1096/fj.12-225664

Levine, R. J., Maynard, S. E., Qian, C., Lim, K. H., England, L. J., Yu, K. F., . . . Karumanchi, S. A. (2004). Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*, *350*(7), 672-683. https://doi.org/10.1056/NEJMoa031884

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Subgroup, G. P. D. P. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352

Li, Q., Kannan, A., Wang, W., Demayo, F. J., Taylor, R. N., Bagchi, M. K., & Bagchi, I. C. (2007). Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human. *J Biol Chem*, *282*(43), 31725-31732. https://doi.org/10.1074/jbc.M704723200

Li, Y., Yan, J., Chang, H. M., Chen, Z. J., & Leung, P. C. K. (2021). Roles of TGF-β Superfamily Proteins in Extravillous Trophoblast Invasion. *Trends Endocrinol Metab*, *32*(3), 170-189. https://doi.org/10.1016/j.tem.2020.12.005

Lie, R. T., Rasmussen, S., Brunborg, H., Gjessing, H. K., Lie-Nielsen, E., & Irgens, L. M. (1998). Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ*, *316*(7141), 1343-1347. https://doi.org/10.1136/bmj.316.7141.1343

Lim, S., Li, W., Kemper, J., Nguyen, A., Mol, B. W., & Reddy, M. (2021). Biomarkers and the Prediction of Adverse Outcomes in Preeclampsia: A Systematic Review and Meta-analysis. *Obstet Gynecol*, *137*(1), 72-81. https://doi.org/10.1097/AOG.0000000000004149

Lisonkova, S., & Joseph, K. S. (2013). Incidence of preeclampsia: risk factors and outcomes associated with early- versus late-onset disease. *Am J Obstet Gynecol*, *209*(6), 544.e541-544.e512. https://doi.org/10.1016/j.ajog.2013.08.019

Lopes van Balen, V. A., van Gansewinkel, T. A. G., de Haas, S., van Kuijk, S. M. J., van Drongelen, J., Ghossein-Doha, C., & Spaanderman, M. E. A. (2017). Physiological adaptation of endothelial function to pregnancy: systematic review and meta-analysis. *Ultrasound Obstet Gynecol*, *50*(6), 697-708. https://doi.org/10.1002/uog.17431

Lu, Z., Hardt, J., & Kim, J. J. (2008). Global analysis of genes regulated by HOXA10 in decidualization reveals a role in cell proliferation. *Mol Hum Reprod*, *14*(6), 357-366. https://doi.org/10.1093/molehr/gan023

Lucas, E. S., Vrljicak, P., Muter, J., Diniz-da-Costa, M. M., Brighton, P. J., Kong, C. S., . . . Brosens, J. J. (2020). Recurrent pregnancy loss is associated with a pro-senescent decidual response during the peri-implantation window. *Commun Biol*, *3*(1), 37. https://doi.org/10.1038/s42003-020-0763-1

Løset, M., Mundal, S. B., Johnson, M. P., Fenstad, M. H., Freed, K. A., Lian, I. A., . . . Austgulen, R. (2011). A transcriptional profile of the decidua in preeclampsia. *Am J Obstet Gynecol*, *204*(1), 84.e81-27. https://doi.org/10.1016/j.ajog.2010.08.043

Macer, M. L., & Taylor, H. S. (2012). Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstet Gynecol Clin North Am*, *39*(4), 535-549. https://doi.org/10.1016/j.ogc.2012.10.002

Marquardt, R. M., Kim, T. H., Shin, J. H., & Jeong, J. W. (2019). Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? *Int J Mol Sci*, *20*(15). https://doi.org/10.3390/ijms20153822

Mazur, E. C., Vasquez, Y. M., Li, X., Kommagani, R., Jiang, L., Chen, R., . . . DeMayo, F. J. (2015). Progesterone receptor transcriptome and cistrome in decidualized human endometrial stromal cells. *Endocrinology*, *156*(6), 2239-2253. https://doi.org/10.1210/en.2014-1566

McLaughlin, K., Zhang, J., Lye, S. J., Parker, J. D., & Kingdom, J. C. (2018). Phenotypes of Pregnant Women Who Subsequently Develop Hypertension in Pregnancy. *J Am Heart Assoc*, *7*(14). https://doi.org/10.1161/JAHA.118.009595

McNally, L., Zhou, Y., Robinson, J. F., Zhao, G., Chen, L. M., Chen, H., . . . Fisher, S. J. (2020). Up-regulated cytotrophoblast DOCK4 contributes to over-invasion in placenta accreta spectrum. *Proc Natl Acad Sci U S A*, *117*(27), 15852-15861. https://doi.org/10.1073/pnas.1920776117

Meekins, J. W., Pijnenborg, R., Hanssens, M., McFadyen, I. R., & van Asshe, A. (1994). A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol*, *101*(8), 669-674. https://doi.org/10.1111/j.1471-0528.1994.tb13182.x

Melchiorre, K., Sharma, R., & Thilaganathan, B. (2014). Cardiovascular implications in preeclampsia: an overview. *Circulation*, *130*(8), 703-714. https://doi.org/10.1161/CIRCULATIONAHA.113.003664

Mendoza, M., Garcia-Ruiz, I., Maiz, N., Rodo, C., Garcia-Manau, P., Serrano, B., . . . Suy, A. (2020). Pre-eclampsia-like syndrome induced by severe COVID-19: a prospective observational study. *BJOG*, *127*(11), 1374-1380. https://doi.org/10.1111/1471-0528.16339

Moghadam, K. K., Kessler, C. A., Schroeder, J. K., Buckley, A. R., Brar, A. K., & Handwerger, S. (2005). Cannabinoid receptor I activation markedly inhibits human decidualization. *Mol Cell Endocrinol*, *229*(1-2), 65-74. https://doi.org/10.1016/j.mce.2004.09.007

Mogren, I., Högberg, U., Winkvist, A., & Stenlund, H. (1999). Familial occurrence of preeclampsia. *Epidemiology*, *10*(5), 518-522.

Mongraw-Chaffin, M. L., Cirillo, P. M., & Cohn, B. A. (2010). Preeclampsia and cardiovascular disease death: prospective evidence from the child health and development studies cohort. *Hypertension*, *56*(1), 166-171. https://doi.org/10.1161/HYPERTENSIONAHA.110.150078

Mor, G., & Cardenas, I. (2010). The immune system in pregnancy: a unique complexity. *Am J Reprod Immunol*, *63*(6), 425-433. https://doi.org/10.1111/j.1600-0897.2010.00836.x

Mori, M., Bogdan, A., Balassa, T., Csabai, T., & Szekeres-Bartho, J. (2016). The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Semin Immunopathol*, *38*(6), 635-649. https://doi.org/10.1007/s00281-016-0574-0

Munchel, S., Rohrback, S., Randise-Hinchliff, C., Kinnings, S., Deshmukh, S., Alla, N., . . . Kaper, F. (2020). Circulating transcripts in maternal blood reflect a molecular signature of early-onset preeclampsia. *Sci Transl Med*, *12*(550). https://doi.org/10.1126/scitranslmed.aaz0131

Myatt, L., & Roberts, J. M. (2015). Preeclampsia: Syndrome or Disease? *Curr Hypertens Rep*, *17*(11), 83. https://doi.org/10.1007/s11906-015-0595-4

Nancy, P., Tagliani, E., Tay, C. S., Asp, P., Levy, D. E., & Erlebacher, A. (2012). Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science*, *336*(6086), 1317-1321. https://doi.org/10.1126/science.1220030

Ng, S. W., Norwitz, G. A., Pavlicev, M., Tilburgs, T., Simón, C., & Norwitz, E. R. (2020). Endometrial Decidualization: The Primary Driver of Pregnancy Health. *Int J Mol Sci*, *21*(11). https://doi.org/10.3390/ijms21114092

Nilsson, E., Salonen Ros, H., Cnattingius, S., & Lichtenstein, P. (2004). The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG*, *111*(3), 200-206. https://doi.org/10.1111/j.1471-0528.2004.00042x.x

Norwitz, E. R., Schust, D. J., & Fisher, S. J. (2001). Implantation and the survival of early pregnancy. *N Engl J Med*, *345*(19), 1400-1408. https://doi.org/10.1056/NEJMra000763

Okada, H., Tsuzuki, T., & Murata, H. (2018). Decidualization of the human endometrium. *Reprod Med Biol*, *17*(3), 220-227. https://doi.org/10.1002/rmb2.12088

Papageorghiou, A. T., Deruelle, P., Gunier, R. B., Rauch, S., García-May, P. K., Mhatre, M., . . . Villar, J. (2021). Preeclampsia and COVID-19: results from the INTERCOVID prospective longitudinal study. *Am J Obstet Gynecol*, *225*(3), 289.e281-289.e217. https://doi.org/10.1016/j.ajog.2021.05.014

Patel, B. G., Rudnicki, M., Yu, J., Shu, Y., & Taylor, R. N. (2017). Progesterone resistance in endometriosis: origins, consequences and interventions. *Acta Obstet Gynecol Scand*, *96*(6), 623-632. https://doi.org/10.1111/aogs.13156

Pijnenborg, R., Bland, J. M., Robertson, W. B., & Brosens, I. (1983). Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta*, *4*(4), 397-413. https://doi.org/10.1016/s0143-4004(83)80043-5

Piltonen, T. T., Chen, J. C., Khatun, M., Kangasniemi, M., Liakka, A., Spitzer, T., . . . Giudice, L. C. (2015). Endometrial stromal fibroblasts from women with polycystic ovary syndrome have impaired progesterone-mediated decidualization, aberrant cytokine profiles and promote enhanced immune cell migration in vitro. *Hum Reprod*, *30*(5), 1203-1215. https://doi.org/10.1093/humrep/dev055

Poon, L. C., Kametas, N. A., Chelemen, T., Leal, A., & Nicolaides, K. H. (2010). Maternal risk factors for hypertensive disorders in pregnancy: a multivariate approach. *J Hum Hypertens*, *24*(2), 104-110. https://doi.org/10.1038/jhh.2009.45

Pru, J. K., & Clark, N. C. (2013). PGRMC1 and PGRMC2 in uterine physiology and disease. *Front Neurosci*, *7*, 168. https://doi.org/10.3389/fnins.2013.00168

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841-842. https://doi.org/10.1093/bioinformatics/btq033

Rabaglino, M. B., Post Uiterweer, E. D., Jeyabalan, A., Hogge, W. A., & Conrad, K. P. (2015). Bioinformatics approach reveals evidence for impaired endometrial maturation before and during early pregnancy in women who developed preeclampsia. *Hypertension*, *65*(2), 421-429. https://doi.org/10.1161/HYPERTENSIONAHA.114.04481

Ramathal, C. Y., Bagchi, I. C., Taylor, R. N., & Bagchi, M. K. (2010). Endometrial decidualization: of mice and men. *Semin Reprod Med*, *28*(1), 17-26. https://doi.org/10.1055/s-0029-1242989

Rana, S., Lemoine, E., Granger, J. P., & Karumanchi, S. A. (2019). Preeclampsia: Pathophysiology, Challenges, and Perspectives. *Circ Res*, *124*(7), 1094-1112. https://doi.org/10.1161/CIRCRESAHA.118.313276

Rasmussen, M., Reddy, M., Nolan, R., Camunas-Soler, J., Khodursky, A., Scheller, N. M., . . . McElrath, T. F. (2022). RNA profiles reveal signatures of future health and disease in pregnancy. *Nature*, *601*(7893), 422-427. https://doi.org/10.1038/s41586-021-04249-w

Redman, C. W., & Sargent, I. L. (2005). Latest advances in understanding preeclampsia. *Science*, *308*(5728), 1592-1594. https://doi.org/10.1126/science.1111726

Redman, C. W., & Staff, A. C. (2015). Preeclampsia, biomarkers, syncytiotrophoblast stress, and placental capacity. *Am J Obstet Gynecol*, *213*(4 Suppl), S9.e1, S9-11. https://doi.org/10.1016/j.ajog.2015.08.003

Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. (2000). *Am J Obstet Gynecol*, *183*(1), S1-S22.

Robertson, S. A. (2005). Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res*, *322*(1), 43-52. https://doi.org/10.1007/s00441-005-1127-3

Robertson, S. A., Bromfield, J. J., & Tremellen, K. P. (2003). Seminal 'priming' for protection from pre-eclampsia-a unifying hypothesis. *J Reprod Immunol*, *59*(2), 253-265. https://doi.org/10.1016/s0165-0378(03)00052-4

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139-140. https://doi.org/10.1093/bioinformatics/btp616

Ruane, P. T., Berneau, S. C., Koeck, R., Watts, J., Kimber, S. J., Brison, D. R., . . . Aplin, J. D. (2017). Apposition to endometrial epithelial cells activates mouse blastocysts for implantation. *Mol Hum Reprod*, *23*(9), 617-627. https://doi.org/10.1093/molehr/gax043

Saftlas, A. F., Rubenstein, L., Prater, K., Harland, K. K., Field, E., & Triche, E. W. (2014). Cumulative exposure to paternal seminal fluid prior to conception and subsequent risk of preeclampsia. *J Reprod Immunol*, *101-102*, 104-110. https://doi.org/10.1016/j.jri.2013.07.006

Salker, M., Teklenburg, G., Molokhia, M., Lavery, S., Trew, G., Aojanepong, T., . . . Brosens, J. J. (2010). Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. *PLoS One*, *5*(4), e10287. https://doi.org/10.1371/journal.pone.0010287

Salker, M. S., Christian, M., Steel, J. H., Nautiyal, J., Lavery, S., Trew, G., . . . Brosens, J. J. (2011). Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure. *Nat Med*, *17*(11), 1509-1513. https://doi.org/10.1038/nm.2498

Salker, M. S., Nautiyal, J., Steel, J. H., Webster, Z., Sućurović, S., Nicou, M., . . . Brosens, J. J. (2012). Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PLoS One*, *7*(12), e52252. https://doi.org/10.1371/journal.pone.0052252

Santos, E. D., Moindjie, H., Sérazin, V., Arnould, L., Rodriguez, Y., Fathallah, K., . . . Dieudonné, M. N. (2021). Preimplantation factor modulates trophoblastic invasion throughout the decidualization of human endometrial stromal cells. *Reprod Biol Endocrinol*, *19*(1), 96. https://doi.org/10.1186/s12958-021-00774-5

Schreiber, K., Sciascia, S., de Groot, P. G., Devreese, K., Jacobsen, S., Ruiz-Irastorza, G., . . . Hunt, B. J. (2018). Antiphospholipid syndrome. *Nat Rev Dis Primers*, *4*, 17103. https://doi.org/10.1038/nrdp.2017.103

Schröder, M., & Kaufman, R. J. (2005). ER stress and the unfolded protein response. *Mutat Res*, *569*(1-2), 29-63. https://doi.org/10.1016/j.mrfmmm.2004.06.056

Serra, B., Mendoza, M., Scazzocchio, E., Meler, E., Nolla, M., Sabrià, E., . . . Carreras, E. (2020). A new model for screening for early-onset preeclampsia. *Am J Obstet Gynecol*, *222*(6), 608.e601-608.e618. https://doi.org/10.1016/j.ajog.2020.01.020

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., . . . Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*, *13*(11), 2498-2504. https://doi.org/10.1101/gr.1239303

Sharma, S., Godbole, G., & Modi, D. (2016). Decidual Control of Trophoblast Invasion. *Am J Reprod Immunol*, *75*(3), 341-350. https://doi.org/10.1111/aji.12466

Sites, C. K., Wilson, D., Barsky, M., Bernson, D., Bernstein, I. M., Boulet, S., & Zhang, Y. (2017). Embryo cryopreservation and preeclampsia risk. *Fertil Steril*, *108*(5), 784-790. https://doi.org/10.1016/j.fertnstert.2017.08.035

Steinthorsdottir, V., McGinnis, R., Williams, N. O., Stefansdottir, L., Thorleifsson, G., Shooter, S., . . . Consortium, G. (2020). Genetic predisposition to hypertension is associated with preeclampsia in European and Central Asian women. *Nat Commun*, *11*(1), 5976. https://doi.org/10.1038/s41467-020-19733-6

Su, R. W., & Fazleabas, A. T. (2015). Implantation and Establishment of Pregnancy in Human and Nonhuman Primates. *Adv Anat Embryol Cell Biol*, *216*, 189-213. https://doi.org/10.1007/978-3-319-15856-3_10

Szwarc, M. M., Hai, L., Gibbons, W. E., Peavey, M. C., White, L. D., Mo, Q., . . . Lydon, J. P. (2018). Human endometrial stromal cell decidualization requires transcriptional reprogramming by PLZF. *Biol Reprod*, *98*(1), 15-27. https://doi.org/10.1093/biolre/iox161

Talbi, S., Hamilton, A. E., Vo, K. C., Tulac, S., Overgaard, M. T., Dosiou, C., . . . Giudice, L. C. (2006). Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology*, *147*(3), 1097-1121. https://doi.org/10.1210/en.2005-1076

Tangren, J. S., Powe, C. E., Ankers, E., Ecker, J., Bramham, K., Hladunewich, M. A., . . . Thadhani, R. (2017). Pregnancy Outcomes after Clinical Recovery from AKI. *J Am Soc Nephrol*, *28*(5), 1566-1574. https://doi.org/10.1681/ASN.2016070806

Tarrade, A., Lai Kuen, R., Malassiné, A., Tricottet, V., Blain, P., Vidaud, M., & Evain-Brion, D. (2001). Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta. *Lab Invest*, *81*(9), 1199-1211. https://doi.org/10.1038/labinvest.3780334

Tsai, M. J., & O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem*, *63*, 451-486. https://doi.org/10.1146/annurev.bi.63.070194.002315

Tuckey, R. C. (2005). Progesterone synthesis by the human placenta. *Placenta*, *26*(4), 273-281. https://doi.org/10.1016/j.placenta.2004.06.012

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., . . . Pontén, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science*, *347*(6220), 1260419. https://doi.org/10.1126/science.1260419

Vacca, P., Moretta, L., Moretta, A., & Mingari, M. C. (2011). Origin, phenotype and function of human natural killer cells in pregnancy. *Trends Immunol*, *32*(11), 517-523. https://doi.org/10.1016/j.it.2011.06.013

Valensise, H., Vasapollo, B., Gagliardi, G., & Novelli, G. P. (2008). Early and late preeclampsia: two different maternal hemodynamic states in the latent phase of the disease. *Hypertension*, *52*(5), 873-880. https://doi.org/10.1161/HYPERTENSIONAHA.108.117358

Velicky, P., Knöfler, M., & Pollheimer, J. (2016). Function and control of human invasive trophoblast subtypes: Intrinsic vs. maternal control. *Cell Adh Migr*, *10*(1-2), 154-162. https://doi.org/10.1080/19336918.2015.1089376

Vento-Tormo, R., Efremova, M., Botting, R. A., Turco, M. Y., Vento-Tormo, M., Meyer, K. B., . . . Teichmann, S. A. (2018). Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature*, *563*(7731), 347-353. https://doi.org/10.1038/s41586-018-0698-6

Vollman, R. F. (1977). The menstrual cycle. *Major Probl Obstet Gynecol*, *7*, 1-193.

Wallace, A. E., Fraser, R., & Cartwright, J. E. (2012). Extravillous trophoblast and decidual natural killer cells: a remodelling partnership. *Hum Reprod Update*, *18*(4), 458-471. https://doi.org/10.1093/humupd/dms015

Wang, W., Taylor, R. N., Bagchi, I. C., & Bagchi, M. K. (2012). Regulation of human endometrial stromal proliferation and differentiation by C/EBPβ involves cyclin E-cdk2 and STAT3. *Mol Endocrinol*, *26*(12), 2016-2030. https://doi.org/10.1210/me.2012-1169

Wang, W., Vilella, F., Alama, P., Moreno, I., Mignardi, M., Isakova, A., . . . Quake, S. R. (2020). Single-cell transcriptomic atlas of the human endometrium during the menstrual cycle. *Nat Med*, *26*(10), 1644-1653. https://doi.org/10.1038/s41591-020-1040-z

Wang, W., Vilella, F., Llama, P., Moreno, I., Mignardi, M., Pan, W., . . . Quake, S. R. (2018). Single cell RNAseq provides a molecular and cellular cartography of changes to the human endometrium through the menstrual cycle. In (Vol. http://dx.doi.org/10.1101/350538). bioRxiv.

Wang, X., Park, J., Susztak, K., Zhang, N. R., & Li, M. (2019). Bulk tissue cell type deconvolution with multi-subject single-cell expression reference. *Nat Commun*, *10*(1), 380. https://doi.org/10.1038/s41467-018-08023-x

Wang, X., Wu, S. P., & DeMayo, F. J. (2017). Hormone dependent uterine epithelial-stromal communication for pregnancy support. *Placenta*, *60 Suppl 1*, S20-S26. https://doi.org/10.1016/j.placenta.2017.07.003

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet*, *10*(1), 57-63. https://doi.org/10.1038/nrg2484

Weimar, C. H., Kavelaars, A., Brosens, J. J., Gellersen, B., de Vreeden-Elbertse, J. M., Heijnen, C. J., & Macklon, N. S. (2012). Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. *PLoS One*, *7*(7), e41424. https://doi.org/10.1371/journal.pone.0041424

Weissgerber, T. L., Milic, N. M., Milin-Lazovic, J. S., & Garovic, V. D. (2016). Impaired Flow-Mediated Dilation Before, During, and After Preeclampsia: A Systematic Review and Meta-Analysis. *Hypertension*, *67*(2), 415-423. https://doi.org/10.1161/HYPERTENSIONAHA.115.06554

Winn, V. D., Gormley, M., & Fisher, S. J. (2011). The Impact of Preeclampsia on Gene Expression at the Maternal-Fetal Interface. *Pregnancy Hypertens*, *1*(1), 100-108. https://doi.org/10.1016/j.preghy.2010.12.001

Winuthayanon, W., Hewitt, S. C., Orvis, G. D., Behringer, R. R., & Korach, K. S. (2010). Uterine epithelial estrogen receptor α is dispensable for proliferation but essential for complete biological and biochemical responses. *Proc Natl Acad Sci U S A*, *107*(45), 19272-19277. https://doi.org/10.1073/pnas.1013226107

Winuthayanon, W., Lierz, S. L., Delarosa, K. C., Sampels, S. R., Donoghue, L. J., Hewitt, S. C., & Korach, K. S. (2017). Juxtacrine Activity of Estrogen Receptor α in Uterine Stromal Cells is Necessary for Estrogen-Induced Epithelial Cell Proliferation. *Sci Rep*, *7*(1), 8377. https://doi.org/10.1038/s41598-017-07728-1

Woods, L., Perez-Garcia, V., Kieckbusch, J., Wang, X., DeMayo, F., Colucci, F., & Hemberger, M. (2017). Decidualisation and placentation defects are a major cause of age-related reproductive decline. *Nat Commun*, *8*(1), 352. https://doi.org/10.1038/s41467-017-00308-x

Wu, D., Kimura, F., Zheng, L., Ishida, M., Niwa, Y., Hirata, K., . . . Murakami, T. (2017). Chronic endometritis modifies decidualization in human endometrial stromal cells. *Reprod Biol Endocrinol*, *15*(1), 16. https://doi.org/10.1186/s12958-017-0233-x

Wu, S. P., Li, R., & DeMayo, F. J. (2018). Progesterone Receptor Regulation of Uterine Adaptation for Pregnancy. *Trends Endocrinol Metab*, *29*(7), 481-491. https://doi.org/10.1016/j.tem.2018.04.001

Xin, H., Zhang, Y., Wang, H., & Sun, S. (2012). Alterations of profibrinolytic receptor annexin A2 in pre-eclampsia: a possible role in placental thrombin formation. *Thromb Res*, *129*(5), 563-567. https://doi.org/10.1016/j.thromres.2011.07.039

Yang, F., Zheng, Q., & Jin, L. (2019). Dynamic Function and Composition Changes of Immune Cells During Normal and Pathological Pregnancy at the Maternal-Fetal Interface. *Front Immunol*, *10*, 2317. https://doi.org/10.3389/fimmu.2019.02317

Zhang, Q., Liu, W., Zhang, H. M., Xie, G. Y., Miao, Y. R., Xia, M., & Guo, A. Y. (2020). hTFtarget: A Comprehensive Database for Regulations of Human Transcription Factors and Their Targets. *Genomics Proteomics Bioinformatics*, *18*(2), 120-128. https://doi.org/10.1016/j.gpb.2019.09.006

Zhong, Z., Wen, Z., & Darnell, J. E. (1994). Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, *264*(5155), 95-98. https://doi.org/10.1126/science.8140422

Zhou, Y., Damsky, C. H., & Fisher, S. J. (1997). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest*, *99*(9), 2152-2164. https://doi.org/10.1172/JCI119388

Zhou, Y., Gormley, M. J., Hunkapiller, N. M., Kapidzic, M., Stolyarov, Y., Feng, V., . . . Fisher, S. J. (2013). Reversal of gene dysregulation in cultured cytotrophoblasts reveals possible causes of preeclampsia. *J Clin Invest*, *123*(7), 2862-2872. https://doi.org/10.1172/JCI66966

Zhuang, B., Luo, X., Rao, H., Li, Q., Shan, N., Liu, X., & Qi, H. (2015). Oxidative stress-induced C/EBPβ inhibits β-catenin signaling molecule involving in the pathology of preeclampsia. *Placenta*, *36*(8), 839-846. https://doi.org/10.1016/j.placenta.2015.06.016