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Thrombospondin-1 (THBS1) is dysregulated in preeclampsia

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ABSTRACT

Introduction: Preeclampsia involves endothelial dysfunction and impaired angiogenesis. Thrombospondin-1 (THBS1), a pro-thrombotic and anti-angiogenic glycoprotein, may contribute to preeclampsia pathogenesis. This study investigated the role of THBS1 in preeclampsia.

Methods: THBS1 mRNA expression and protein levels were measured in placentas from early-onset preeclampsia (<34 weeks) and gestation-matched controls. Circulating THBS1 was assessed in early-onset preeclampsia and at 36 weeks' gestation preceding diagnosis of preeclampsia at term. *THBS1* was examined during human trophoblast stem cell (hTSC) differentiation into syncytiotrophoblast and extravillous trophoblast (EVT) and compared to a public hTSC organoid dataset. THBS1 regulation was assessed in hTSCs exposed to hypoxia and inflammatory cytokines. Investigating the source of circulating THBS1, we induced endothelial dysfunction in human umbilical vein endothelial cells (HUVECs) with TNFα and treated them with recombinant THBS1.

Results: THBS1 mRNA ($P < 0.0001$, n = 78 vs n = 30 controls) and protein ($P = 0.0039$, n = 43 and n = 21 controls) levels were significantly reduced in early-onset preeclamptic placentas. Contrastingly, circulating THBS1 was elevated in early-onset preeclampsia ($P = 0.011$, n = 35 vs n = 27 controls) and preceding term preeclampsia diagnosis ($P = 0.0025$, n = 21 vs n = 184 controls). *THBS1* decreased during syncytiotrophoblast ($P = 0.0028$) and EVT differentiation ($P = 0.0008$), indicating mainly cytotrophoblast expression. Analysis of a public hTSC organoid dataset confirmed this. Hypoxic (1 % O_2 vs 8 % O_2) and TNFα or IL-6 exposure led to differential expression and secretion of THBS1. We observed no changes in *THBS1* with induced endothelial dysfunction. Recombinant THBS1 had no effect on endothelial dysfunction.

Discussion: THBS1 is dysregulated in preeclampsia and may be regulated by hypoxic stimuli. These findings support THBS1 as a potential mediator in preeclampsia pathogenesis.

1. Introduction

Preeclampsia is a serious complication of pregnancy. The disease is characterised by sudden onset of hypertension, proteinuria, and endothelial dysfunction which can lead to complex multiple end-organ damage [1,2]. Preeclampsia, which affects 3–5 % of pregnancies, is a significant contributor to global maternal and perinatal morbidity and mortality [1,3]. Currently, there is no robust predictive test or effective treatment for preeclampsia with the only cure being delivery of the fetus [2]. This may result in negative consequences for both mother and child.

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In the two-stage theory of preeclampsia, placental insufficiency is thought to be key to the underlying pathophysiology of preeclampsia [4–6]. Placental insufficiency results from poor vascular remodelling, which leads to intermittent placental hypoxia and inflammation [7]. The dysfunctional placenta releases a variety of factors into the maternal circulation, particular anti-angiogenic factors, disrupting angiogenic equilibrium [8–10]. This contributes to the clinical signs and symptoms of preeclampsia through significant maternal endothelial dysfunction [1,11].

Current biomarker strategies for preeclampsia primarily focus on angiogenic imbalance, with the soluble fms-like tyrosine kinase-1 (sFlt-1)/placental growth factor (PlGF) ratio [12] now being widely used in clinical practice as a successful tool to rule out disease. Additional candidates have emerged, including soluble endoglin (sEng) [13], Activin A [14], SIGLEC6 [15], as well as the ratio of pregnancy-associated plasma protein A (PAPPA) and alpha fetoprotein (AFP) [16]. This highlights the expanding pool of potential biomarkers implicated in placental dysfunction. However, most biomarkers for preeclampsia remain at the discovery stage and require validation in large, diverse cohorts. Unless they outperform or add value beyond the sFlt-1/PlGF ratio, they are unlikely to be translated into clinical use.

While current approaches lack the ability to definitively predict preeclampsia, discovering novel biomarkers may hold promise for earlier detection, risk stratification and improved clinical management strategies. They may also contribute to bettering our understanding of the underlying pathophysiology of the disease.

Thrombospondin-1 (THBS1), an adhesive 450 kDa trimeric glycoprotein, is well characterised in the cardiovascular [17–19], metabolic [20,21] and cancer [22–24] fields. THBS1 interacts with extracellular matrix proteins and has been shown to be involved in angiogenesis, wound healing, tissue remodelling, and inflammation [25–30]. Additionally, THBS1 has been shown to inhibit nitric oxide (NO) [31]. Impaired NO signalling has been associated with hypertension observed in preeclampsia [32].

The role of THBS1 in pregnancy and preeclampsia is not well established. Duan. et al., demonstrated that THBS1 signals through the CD36-mediated cAMP pathway to regulate trophoblast differentiation [33]. They suggested that increased THBS1 in preeclamptic placenta may impair placental formation [33]. Most recently, THBS1 has been shown to contribute to the pathogenesis of preeclampsia through the promotion of damage-associated molecular patterns (DAMPs) released from trophoblast cells which contribute to cellular necroptosis [34]. One study has suggested that serum THBS1 levels are reduced in women with severe preeclampsia compared to controls [35].

In this work, we sought to explore the expression of THBS1 in the placenta and levels in maternal circulation from preeclamptic pregnancies, compared to gestation matched controls. We aimed to study THBS1 regulation in the placenta, examine placental cell type expression and compare our results with a publicly available single cell RNA sequencing (scRNA-seq) dataset (GEO accession number GSE174481: GSM5315576 – GSM5315581). We sought to identify the source of circulating THBS1 by investigating *THBS1* mRNA expression in a model of endothelial dysfunction. Finally, we aim to assess the potential role of THBS1 in the pathogenesis of preeclampsia.

2. Methods

2.1. Pregnancies complicated by early-onset preeclampsia (birthing <34 weeks' gestation) cohort

Placental and blood samples were collected for this study following ethics approval by Mercy Health Human Research Ethics Committee (R11/34). Participants presenting to the Mercy Hospital for Women (Melbourne, Australia) gave informed, written consent for sample collection. Diagnosis of early-onset preeclampsia was conducted in accordance with the American College of Obstetricians and Gynaecologists (ACOG) guidelines [36]. To note, healthy term placentas are not gestationally matched and given that preeclampsia typically occurs before term, we selected gestational age matched preterm controls.

For mRNA analysis, placental tissue samples were collected from 78 participants with early-onset preeclampsia (<34 weeks' gestation) and 30 gestation-matched controls delivered via caesarean section. Patient characteristics are shown in Table S1. For protein analysis, placental tissue samples were collected from 43 participants with early-onset preeclampsia (<34 weeks' gestation) and 21 gestation-matched controls who delivered via caesarean section. Patient characteristics are shown in Table S2. Control samples were obtained from women who were delivered preterm without hypertension due to other complications such as placenta praevia or spontaneous preterm rupture of membranes. Samples were excluded from both case and control groups if they exhibited congenital abnormalities or signs of congenital infection confirmed through histopathological examination. Placental tissue samples were collected and processed as previously described [37]. Samples were stored at −80 °C for subsequent RNA or protein extraction.

Plasma was collected from 35 participants with early-onset preeclampsia (<34 weeks' gestation) and 27 normotensive gestation-matched controls who delivered at term without preeclampsia. Patient characteristics are shown in Table S3. Whole blood was collected in a 9 mL ethylenediaminetetraacetic acid (EDTA) BD Vacutainer® K2E tube. Samples were centrifuged at 1000×*g* for 10 min and plasma was aliquoted and stored at −80 °C until analysis.

2.2. Predictive cohort - biomarker and ultrasound measures for preventable stillbirth (BUMPS)

This study used the BUMPS pregnancy cohort [38]. This large-scale prospective study, conducted at Mercy Hospital for Women, involved collecting plasma samples from a general, unselected pregnant population around 36 weeks of gestation. Ethical approval was obtained from the Mercy Health and Human Research Ethics Committee (approval number: 2019-012). Following written informed consent, women aged over 18 years, with a singleton pregnancy and normal mid-trimester fetal morphology examination were eligible to participate. Whole blood was collected in 9 mL EDTA tubes. Plasma was obtained and stored at −80 °C until sample analysis.

The case-cohort of 205 samples was obtained from the first 1000 participants enrolled in the BUMPS study. In this study, 21 women who delivered with preeclampsia at term. Preeclampsia was defined according to established ACOG guidelines [36]. The cohort included 184 participants who delivered without preeclampsia randomly selected from the whole cohort. Detailed characteristics of the study participants are presented in Table S4.

2.3. Culture of human trophoblast stem cells (hTSCs)

First-trimester hTSCs (CT30, female) were obtained from the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan (RCB Cat#RCB4938, RRID: CVCL_A7BB) [39], described in detail by Okae et al., 2018 [39]. The cells were cultured according to the optimised conditions and protocol outlined in the manuscript by Okae et al., 2018 [39].

2.4. Differentiation of hTSCs to extravillous trophoblast cells (EVTs) or syncytiotrophoblast cells [ST(2D)]

48-well or 24-well plates were pre-coated in iMatrix-511 (NovaChem Pty Ltd), incubated and washed as part of the hTSC protocol [39]. For EVT and syncytiotrophoblast differentiation, cells were seeded at 30,000 cells/well in a 48-well plate and 60,000 cells/well in a 24-well plate.

Following seeding, hTSCs were cultured in either EVT media to

induce EVT differentiation or syncytial [ST(2D)] media to induce differentiation across 96 h [39]. 48- and 72-h post-differentiation, cell lysates were collected for RNA extraction. Final lysates were collected at 96 h post-differentiation. Over the course of 96 h, morphological changes and established differentiation markers were assessed to confirm successful differentiation.

2.5. Isolation of primary human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from umbilical cord samples obtained after placenta delivery in select cases, as previously described [40]. Importantly, only HUVECs between passages 2 and 4 were used in experiments. Cells were cultured in HUVEC medium (M199 medium [Life Technologies] supplemented with 20 % fetal calf serum, 1 % antibiotic-antimycotic solution [Life Technologies], 1 % endothelial cell growth factor [Sigma], and 1 % heparin). The treatment solutions were prepared in 10 % fetal calf serum (Sigma) and administered to the cells for a 24-h period.

2.6. Isolation of term primary cytotrophoblast cells

Primary cytotrophoblast cells were isolated from fresh placental tissue collected from consenting participants at Mercy Hospital for Women who underwent uncomplicated term elective caesarean sections [41]. The cells were isolated as previously described [40].

Primary trophoblasts were cultured in DMEM high Glutamax (Life Technologies) containing 10 % Fetal Calf Serum (Sigma, St Louis, United States) and 1 % antibiotic-antimicotic (Life Technologies) on fibronectin coated plates. Treatments were administered at 90 % confluence for 24 h at 8 % (normoxia) or 1 % (hypoxia) O_2 and 5 % CO_2, depending on the experiment. RNA was isolated for subsequent analysis.

2.7. Isolation of placental explants

Placental explants were sampled and cultured according to an established procedure [40]. Small pieces of villous tissue were cut from the mid-portion of the placenta to avoid the maternal and fetal surfaces. Tissues were dissected into small fragments of 1–2 mm size and four pieces put into each well of a 24 well plate.

2.8. Hypoxic stimulation

First trimester hTSCs, placental explants or term primary trophoblast cells were cultured in a hypoxic environment. After 24 h in 8 % oxygen at 37 °C, cells or explants were incubated at either 8 % oxygen for normoxia or 1 % oxygen for hypoxia, for 48 h. Hypoxic and normoxic conditions were established based on previously described protocols [37,42,43]. The media was collected, and cells lysed for RNA extraction.

2.9. Treatment of first trimester hTSCs, primary trophoblast cells and placental explants with IL-6 and TNFα

hTSCs or primary trophoblast cells were seeded at 60,000 cells/well in a 24-well plate or 30,000 cells/well in a 48-well plate in trophoblast medium [39]. Cells were incubated at 37 °C, 8 % O_2, and 5 % CO_2 for 24 h to allow for adherence. Additionally, in the case where placental explants were used, explants were seeded into a 24-well plate for 24 h. Cells were then treated with increasing doses of TNFα (Life Technologies, Carlsbad, CA, USA) or IL- 6 (In Vitro Technologies, Noble Park, VIC, Australia) at 0, 0.1, and 1 ng/mL for 24 h. Conditioned media and cell lysates were collected for subsequent analysis.

2.10. Treatment of primary HUVECs with TNFα to induce endothelial dysfunction

HUVECs were plated at 22,500 cells/well in a 48-well cell culture plate in HUVEC media (M199 medium [Life Technologies] supplemented with 20 % fetal calf serum, 1 % antibiotic-antimycotic solution [Life Technologies], 1 % endothelial cell growth factor [Sigma], and 1 % heparin). Cells were treated with increasing doses of TNFα (Life Technologies, Carlsbad, CA, USA) at 0, 1 ng/mL and 10 ng/mL for 24 h to induce endothelial dysfunction. Treatments were made and cells were incubated in HUVEC media with 10 % fetal calf serum (M199 medium [Life Technologies] supplemented with 10 % fetal calf serum, 1 % antibiotic-antimycotic solution [Life Technologies], 1 % endothelial cell growth factor [Sigma], and 1 % heparin). Conditioned media and cell lysates were collected for subsequent analysis.

2.11. Treatment of primary HUVECs with recombinant THBS1

Cells were treated with vehicle control, TNFα (Life Technologies, Carlsbad, CA, USA) at 1 ng/mL, recombinant THBS1 (R and D Systems, catalog no. 3074-TH) at 8000 ng/mL and TNFα (1 ng/mL) + recombinant THBS1 (8000 ng/mL) for 24 h. Conditioned media and cell lysates were collected for subsequent analysis.

2.12. Ribonucleic acid (RNA) extraction

RNA was isolated from placental samples and cell culture lines with the RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol. Once extracted, RNA was quantified with a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA).

2.13. Reverse transcription to generate complementary deoxyribonucleic acid (cDNA)

mRNA extracted from cells and tissues was converted to complementary deoxyribonucleic acid (cDNA) using the Applied Biosystems high-capacity cDNA reverse transcriptase kit (Life Technologies, Carlsbad, USA) according to manufacturer's instructions. Equal amounts of RNA were reverse transcribed into cDNA on the Applied Biosystems MiniAmp Thermal Cycler (ThermoFisher Scientific) following the manufacturer's instructions. The conversion was performed under the following conditions: 25 °C for 10 min, 37 °C for 60 min and 85 °C for 5 min cDNA was stored at −20 °C prior to processing for quantitative reverse transcriptase polymerase chain reaction (RT-qPCR).

2.14. RT-qPCR analysis

RT-qPCR was conducted to quantify mRNA expression levels of the following genes: *e-cadherin 1* (*CDH1,* Hs01023895_m1), *GATA binding protein 3* (*GATA3,* Hs00231122_m1), *human leukocyte antigen G* (*HLA-G,* Hs03045108_m1), *syndecan-1* (*SDC1,* Hs00896423_m1), *TEA domain transcription factor 4* (*TEAD4,* Hs01125032_m1), *vascular cell adhesion protein 1* (*VCAM-1, Hs01003372_m1*), *Endothelin 1* (*ET-1, Hs00174961_m1*), *intercellular Adhesion Molecule 1* (*ICAM-1, Hs00164932_m1*), and *thrombospondin-1* (*THBS1, Hs00962908_m1*) (all Life Technologies), on a CFX384 (BioRad) machine or Applied Biosystem QuantStudio 5 (ThermoFisher Scientific). The master mix included FAM-labelled Taqman™ Fast Advanced Master Mix (ThermoFisher Scientific) and their respective primer (Life Technologies). The run conditions used were as follows: 95 °C for 20s, (95 °C for 3s and 60 °C for 30s) x 40 cycles. No gene products were detected in non-template controls.

All data were normalised to their respective housekeeper genes, depending on the conditions and cell/tissue type: *Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta* (*YWHAZ,* Hs01122454_m1) was used for HUVEC or *in vitro* experiments,

glyceraldehyde 3-phosphate dehydrogenase (*GAPDH, Hs99999905_m1*) was used for hTSC differentiation to syncytiotrophoblast cells or the geometric mean of *Topoisomerase-1* (*TOP1,* Hs00243257_m1) and *Cyclin-1* (*CYC1,* Hs00357717_m1) which was used for placental samples or for *in vitro* experiments. *CYC1* was also used for hTSC differentiation to EVTs. Each sample was run in duplicate for RT-qPCR analysis, and the average cycle threshold (Ct) value was used for further calculations. To account for variations, gene expression was normalised to the mean Ct value obtained from the control group for each experiment. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative fold change in gene expression relative to controls. This sets the control group as the reference (100 %), resulting in no plotted variability for the control group.

2.15. Protein extraction

To isolate protein from placental tissue, a RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO, USA) and Halt™ phosphatase inhibitor cocktail (Thermo Fisher Scientific; Waltham, MA, USA) was used to lyse cells. The Pierce™ BCA assay (Thermo Fisher Scientific) was performed according to the manufacturer's protocol to quantify protein in each sample. Equal protein amounts were loaded for THBS1 ELISA (see below).

2.16. Enzyme linked immunosorbent assays (ELISAs)

Maternal plasma samples, placental lysates and conditioned media from primary trophoblast, hTSC and explant experiments were measured via ELISA. The large cohort analyses were analysed using an R&D Systems Human Thrombospondin-1 DuoSet ELISA kit (R and D Systems, catalog no. DY3074), following manufacturer's specifications. The early onset preeclampsia and BUMPS plasma samples were diluted 1:300 while explant conditioned media samples were diluted 1:5 for THBS1 measurement following optimisation. Placental lysates, primary trophoblast and hTSC conditioned media samples were run neat.

2.17. scRNA-seq analysis

For our scRNA-seq analysis, the code made available by Shannon et al. (https://github.com/MatthewJShannon), was applied to a publicly available dataset (GEO accession number GSE174481; GSM5315576 – GSM5315581) [44]. For this analysis, the detailed methodology has been previously described using the same pipeline and dataset [43,45].

Characteristic marker genes for each cell type were used to annotate clusters. Each cluster was annotated and resulted in 6 distinct transcriptomic cell identities tailored for this study: CTB, CTBprol, CTBpf, pEVT, iEVT and STB [46–51]. A dot plot including marker genes and general trophoblast markers, confirming cell population identities, is shown in the Supplementary Fig. 1 [52,53]. After cluster annotation, a data subset was created based on EVT-differentiated and undifferentiated samples. Dot plot and feature plots were generated for the gene, *THBS1*, to visualize their respective expression. The full code is available upon request.

2.18. Statistical analysis

Maternal characteristics compared women diagnosed with early-onset preeclampsia, compared to normotensive, gestation-matched controls using a Mann–Whitney *U* test for continuous data and Chi-square test for categorical data. The data obtained was tested for normality using the Anderson–Darling test, D'Agostino & Pearson test, Shapiro–Wilk test, and Kolmogrov-Smirnov test. For two unpaired groups, an unpaired *t*-test (parametric) or Mann–Whitney test (non-parametric) test was used. For analysis of two unpaired groups, a *t*-test was used. For ≥3 groups, either one-way ANOVA (parametric) or a Kruskal Wallis test (non-parametric) was used. For fetal sex correlation, Ordinary two-way ANOVA was performed. Parametric data was represented as mean ± standard error of the mean (SEM) while non-parametric data was represented as a median with interquartile range (IQR). $P < 0.05$ were considered statistically significant. All *in vitro* experiments were performed in technical duplicates or triplicates and repeated at least five times unless stated. All analyses were performed using GraphPad Prism 10.2.3 (GraphPad Software, LLC).

3. Results

3.1. THBS1 gene expression and protein concentration is reduced in placenta from pregnancies complicated by early-onset preeclampsia (< 34 weeks' gestation)

To assess whether *THBS1* mRNA expression is dysregulated in placentas, *THBS1* expression was measured in placental lysates from 78 women with early-onset preeclampsia, compared to 30 gestation-matched controls. *THBS1* mRNA expression was significantly reduced ~2-fold in placenta from patients with early-onset preeclampsia, relative to controls (Fig. 1A, $P = < 0.0001$). Subsequent analysis of *THBS1* expression showed no significant association of the control group with gestational age at delivery (Fig. 1B, green line, $r^2 = 0.1132$, $P = 0.0691$) and fetal birth weight (Fig. 1C, green line, $r^2 = 0.1192$, $P = 0.0617$). The same was observed in the preeclampsia group for gestational age at delivery (Fig. 1B, pink line, $r^2 = 0.0011$, $P = 0.7717$) and fetal birth weight (Fig. 1C, pink line, $r^2 = 0.0002$, $P = 0.9151$). THBS1 mRNA expression was significantly decreased in preeclampsia compared with controls, for those delivering with female ($P < 0.001$) or male ($P < 0.01$) babies (Supplementary Fig. 1A). However, there was no significant association between those delivering with male or female babies and disease status.

We next measured THBS1 protein concentration in placental lysates obtained from 43 women with early-onset preeclampsia and 21 gestation-matched controls. THBS1 protein concentrations were significantly decreased at a mean concentration of 1367 pg/mL [interquartile range (IQR), $1.08x10^4$ pg/mL – 271.6 pg/mL] compared to a concentration of 1864 pg/mL [IQR, 3615 pg/mL – 874.3 pg/mL] (Fig. 1D, $P = 0.0039$) in the control group. Further analysis of placental THBS1 protein concentration showed no significant association of the control group with gestational age at delivery (Fig. 1E, green line, $r^2 = 0.0168$, $P = 0.5756$) and fetal birth weight (Fig. 1F, green line, $r^2 = 0.0144$, $P = 0.6043$). The same was observed in the preeclampsia group for gestational age at delivery (Fig. 1E, pink line, $r^2 = 0.0000$, $P = 0.9768$) and fetal birth weight (Fig. 1F, pink line, $r^2 = 0.0297$, $P = 0.2813$). THBS1 protein levels in placenta showed no significant association between those delivering with male or female babies and disease status (Supplementary Fig. 1B).

3.2. Circulating THBS1 is increased in plasma from pregnancies complicated by early-onset preeclampsia

We next sought to investigate circulating levels of THBS1 in those diagnosed with early-onset preeclampsia. Circulating THBS1 was significantly increased 1.96-fold in plasma obtained from 35 women with early-onset preeclampsia at $1.3x10^7$ pg/mL [IQR, $4.0x10^7$ pg/mL – $6.4x10^5$], compared to 27 gestation-matched controls at $6.9x10^6$ pg/mL [IQR, $4.1x10^7$ pg/mL – $9.5x10^5$ pg/mL] (Fig. 2A, $P = 0.01$). Further analysis of circulating THBS1 showed no significant changes in circulating levels across different gestational ages for the control group (Fig. 2B, green line, $r^2 = 0.2152$, $P = 0.0148$) and the preeclampsia group (pink line, $r^2 = 0.0065$, $P = 0.6456$). There were no significant differences in plasma THBS1 when comparing fetuses that were designated male or female at birth in the control or preeclamptic group (Supplementary Fig. 1C).

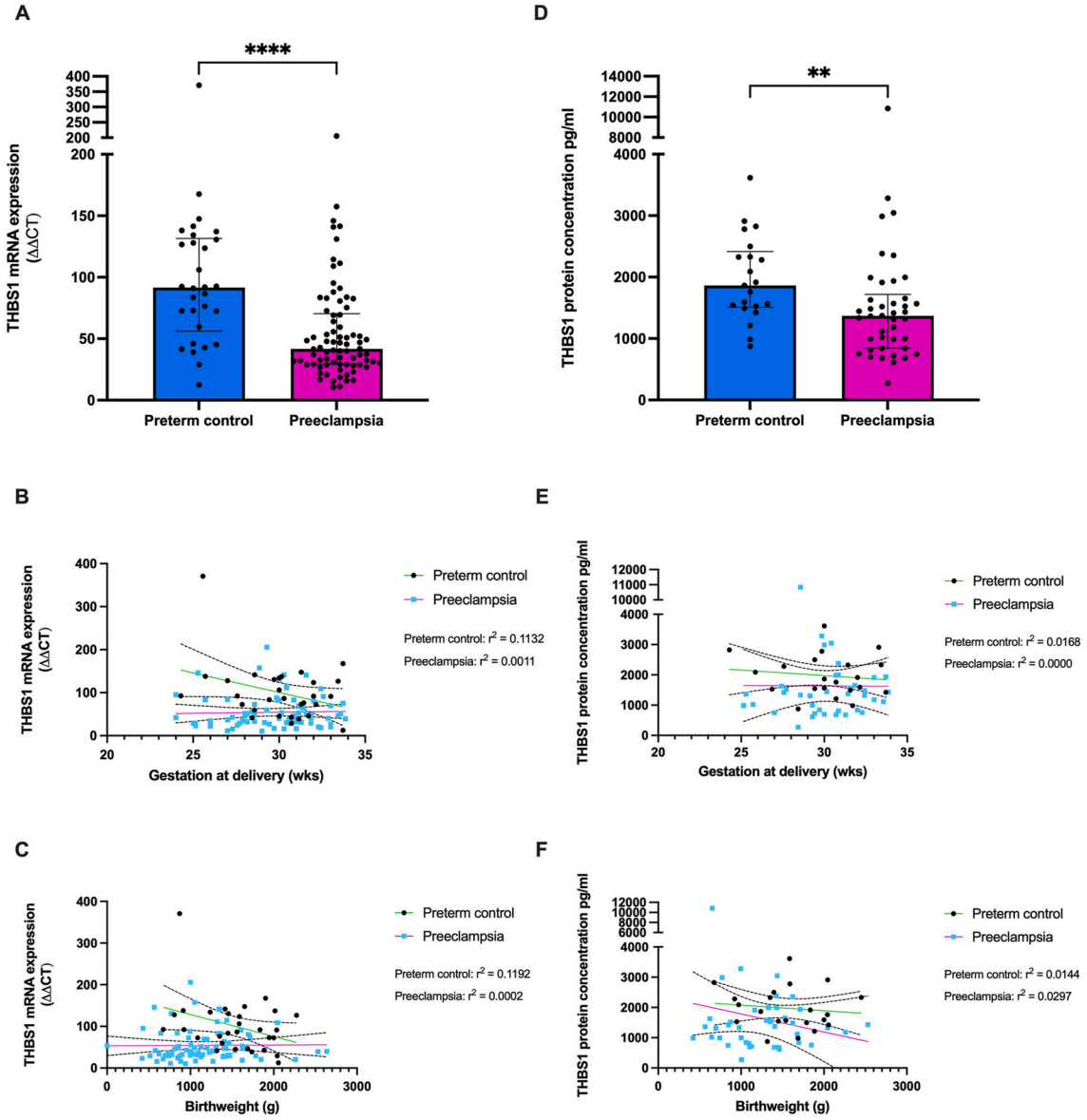

Fig. 1. THBS1 mRNA expression and protein concentration is reduced in placental lysates from pregnancies complicated by early-onset preeclampsia. (A) THBS1 mRNA expression was examined via RT-qPCR in placentas obtained from patients with early-onset preeclampsia (n=78, blue bar), relative to gestation-matched controls (n=30, orange bar). THBS1 expression was significantly reduced, with no significant association in the control group with gestational age at delivery (green line, $r^2 = 0.1132$, P = 0.0691) **(B)** or fetal birthweight **(C)** (green line, $r^2 = 0.1192$, P = 0.0617). The same was observed for the preeclampsia group for gestational age at delivery (B, pink line, $r^2 = 0.0011$, P = 0.7717) or fetal birthweight (C, pink line, $r^2 = 0.0002$, P = 0.9151). **(D)** THBS1 protein concentration was measured via ELISA in placentas obtained from patients with early-onset preeclampsia (n=43, blue bar), relative to gestation-matched controls (n=21, orange bar). THBS1 expression was significantly reduced and THBS1 protein concentration showed no significant association of the control group with gestational age at delivery **(E)** (green line, $r^2 = 0.0168$, P = 0.5756) and fetal birth weight **(F)** (green line, $r^2 = 0.0144$, P = 0.6043). The same was observed in the preeclampsia group for gestational age at delivery (E, pink line, $r^2 = 0.0000$, P = 0.9768) and fetal birth weight (F, pink line, $r^2 = 0.0297$, P = 0.2813). Each dot represents individual participants. The significance of the data was determined using a Mann–Whitney U test and a simple linear regression was used to determine the association of THBS1 expression/concentration with fetal birthweight and gestation age at delivery. Data is expressed as median ± interquartile range. ****p<0.0001, **p<0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Circulating THBS1 is elevated at 36 Weeks in patients preceding diagnosis of term preeclampsia

To evaluate the predictive potential of THBS1, we compared circulating THBS1 in plasma from 21 women at 36 weeks' gestation, who were later diagnosed with preeclampsia, to 184 gestation-matched controls who did not (Fig. 3A). THBS1 was significantly increased in patients preceding diagnosis of term preeclampsia at $4.37x10^6$ pg/mL [IQR, $1.46x10^7$ pg/mL – $1.01x10^6$ pg/mL] ($P = 0.0025$), compared to gestation-matched controls at $2.40x10^6$ pg/mL [IQR, $2.33x10^7$ pg/mL – $8.93x10^5$ pg/mL].

The area under the receiver operating characteristic curve (AUC) showed modest discriminatory performance for THBS1 (Fig. 3B, AUC = 0.70, [95 % Confidence Interval (CI), 58.32 to 81.33]). The optimal cutoff, determined by Youden's index, corresponded to a sensitivity of 71.4 % and a specificity of 71.6 %. There was no significant association between those delivering with male or female babies and disease status (Supplementary Fig. 1D).

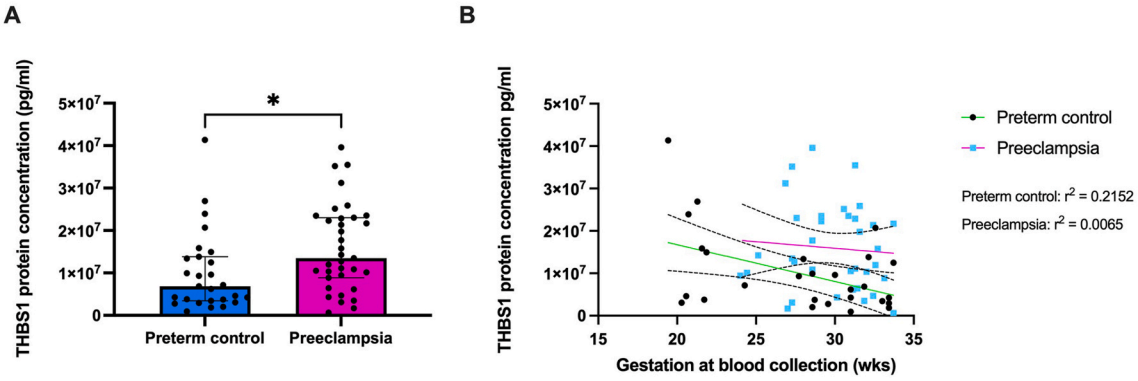

Fig. 2. THBS1 protein concentration is increased in plasma from pregnancies complicated by early-onset preeclampsia. (A) Circulating THBS1 protein concentration was measured via ELISA in blood obtained from patients with early-onset preeclampsia (n = 35, blue bar), relative to gestation-matched controls (n = 27, orange bar). THBS1 levels were significantly increased with no significant changes in circulating levels across different gestational ages for the control group **(B)** (green line, $r^2 = 0.2152$, P = 0.0148) and the preeclampsia group (pink line, $r^2 = 0.0065$, P = 0.6456). The same was observed for fetal sex (Supplementary Fig. 1C). Each dot represents individual participants. The significance of the data was determined using a Mann–Whitney *U* test and a simple linear regression was used to determine the association of THBS1 expression/concentration with gestation at blood collection. Data is expressed as median ± interquartile range. *p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

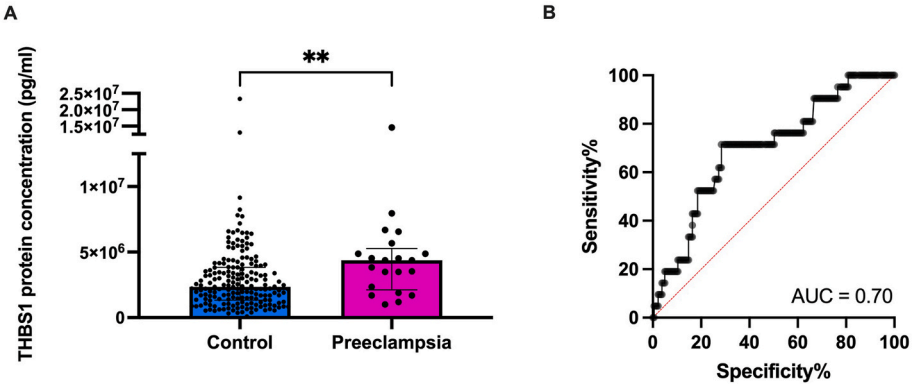

Fig. 3. THBS1 protein concentration in plasma was increased in patients preceding diagnosis of preeclampsia at term (36 weeks' gestation). (A) In the BUMPS cohort, circulating THBS1 was increased in women who developed preeclampsia at term (36 weeks' gestation) (n = 21) compared to gestation-matched controls (n = 184). **(B)** Area under the receiver operator curve (AUC) of 0.70 (95 % CI, 58.32–81.33). Each dot represents individual participants. The significance of the data was determined using a Mann–Whitney *U* test. Data is presented as median ± interquartile range. **P < 0.01.

3.4. *THBS1 is highly expressed in hTSCs*

Given that THBS1 is differentially expressed in placenta from patient with preeclampsia, a hTSC differentiation model was employed [39] to assess THBS1 expression in various trophoblast cell lineages, including hTSCs, syncytiotrophoblast cells, and EVT cells.

Successful differentiation and fusion of hTSCs into syncytiotrophoblast cells was confirmed by a significant loss of cell surface marker, *CDH1* (Fig. 4A, $P < 0.0001$) and cytotrophoblast progenitor marker, *TEAD4* (Fig. 4B, $P < 0.0001$) and a significant increase in syncytiotrophoblast marker, *SDC1* (Fig. 4C, $P < 0.0001$). *THBS1* mRNA expression was significantly reduced as hTSCs differentiated into syncytialised cells (Fig. 4D, P < 0.0001).

Successful differentiation of hTSCs into EVTs was confirmed by a significant increase in established EVT marker, *HLA-G* (Fig. 4E, $P = 0.0264$ at 72 h and $P = 0.0002$ at 96 h) and significant loss of cytotrophoblast progenitor marker, *TEAD4* (Fig. 4F, $P = < 0.0001$ across 96 h*)*. *THBS1* mRNA expression was significantly reduced with EVT differentiation (Fig. 4G, $P = 0.0001$ across 96 h during EVT differentiation).

3.5. *scRNA-seq analysis identifies THBS1 expression in an organoid model of trophoblast differentiation*

To further evaluate THBS1 gene expression in trophoblast differentiation and to validate our *in vitro* and *in vivo* observations, *THBS1* expression was assessed in a publicly available scRNA-seq dataset.

Following relevant quality control procedures, 6354 high-quality cells were obtained from the scRNA-seq dataset. Uniform Manifold Approximation and Projection (UMAP) was used to visualize the relationships between these cells based on their gene expression profiles as previously described (Fig. 5A and B) [45,54]. Cells with similar gene expression patterns were clustered together, with closer physical proximity on the plot signifying greater transcriptional similarity. Six distinct cell subtypes were identified within these clusters and assigned labels (Fig. 5A and C)**:** cytotrophoblast (CTB), proliferative cytotrophoblast (CTBprol), pre-fusion cytotrophoblast (CTBpf), progenitor EVT (pEVT), invasive EVT (iEVT) and syncytiotrophoblast (STB). A population of cells within both undifferentiated and differentiated organoid cultures exhibited transcriptional characteristics resembling STB. Supplementary Fig. 2 depicts a heatmap of marker of known trophoblast genes identifying each cluster [44].

Transcriptomic analysis showed moderate expression of *THBS1* within identified cytotrophoblast cell identities (Fig. 5C). *THBS1*

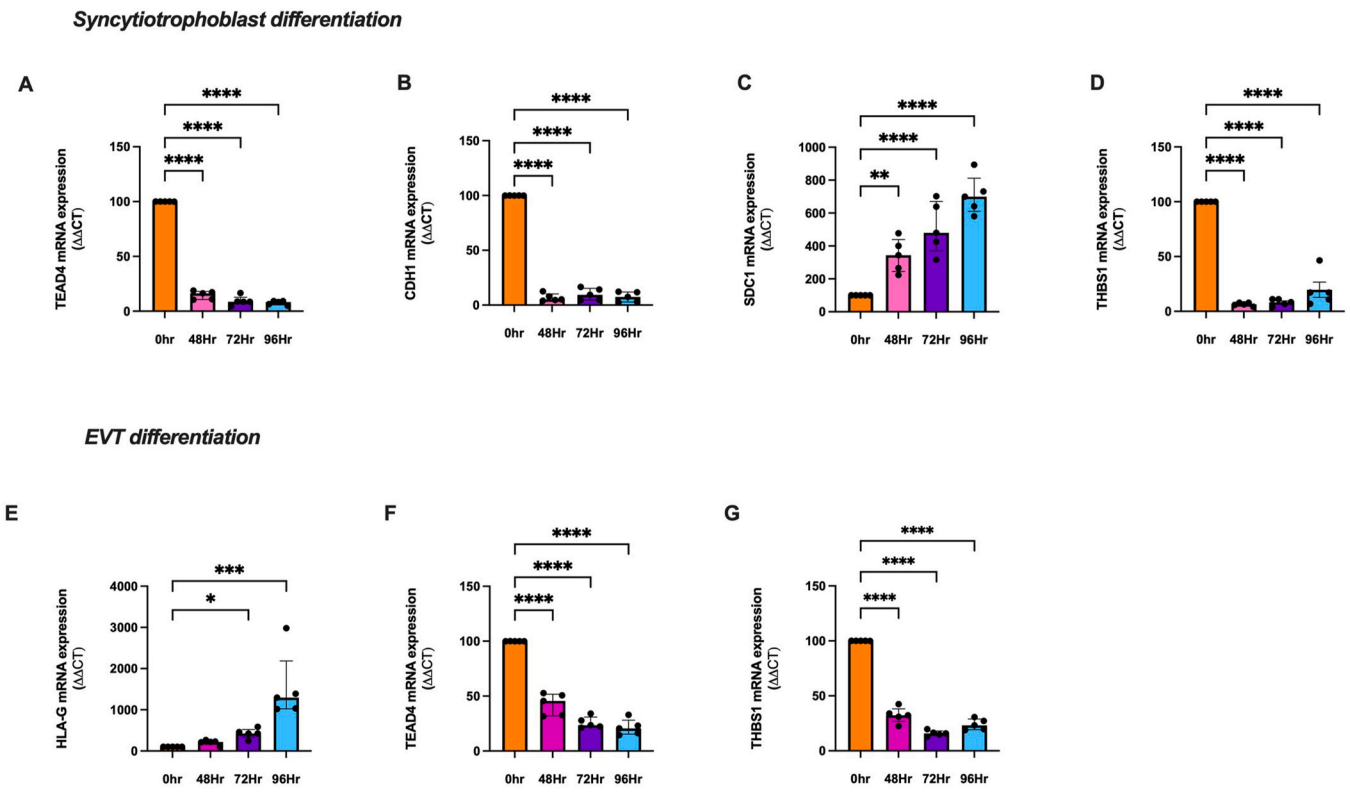

Fig. 4. THBS1 expression with differentiation of human trophoblast stem cells (hTSCs) to extravillous trophoblasts and syncytiotrophoblasts. hTSCs were differentiated to extravillous trophoblasts (EVT) or syncytiotrophoblasts and measured across 0, 48, 72 and 96 h mRNA expression was assessed via qRT-PCR. Syncytiotrophoblast differentiation was confirmed by a significant loss in CDH1 **(A),** significant reduction in TEAD4 **(B)** and significant increase in SDC1 **(C).** Following differentiation of hTSCs into syncytiotrophoblasts, there was a significant loss of THBS1 expression across 96 h **(D)**. EVT differentiation was confirmed by a significant increase of HLA-G **(E)** and significant loss of TEAD4 **(F)**. **(G)** Following hTSC differentiation into EVT, THBS1 expression was reduced across 96 h mRNA expression was normalised to the geometric mean of housekeeper gene(s). Statistical significance was assessed using either one-way ANOVA (parametric) or Kruskal–Wallis test (non-parametric), as appropriate. Data are presented as mean ± SEM for experiments (A, B, C, D, F, and G), or as median ± IQR for experiment (E). Experiments were repeated with n = 5 biological replicates, each performed in technical triplicate. *p < 0.05, ***p < 0.001, ****p < 0.0001.

expression was identified in the CTB identity (~6 % of total population within both undifferentiated and EVT differentiated organoid cultures) and CTBprol cell identity (~6 % of total population within undifferentiated and ~4 % in EVT differentiated organoid cultures). This expression was found in both undifferentiated and differentiated populations and correlates with our *in vitro* observations. *THBS1* was found to be lowly expressed in pEVT (~2 % of total population) and iEVT (~4 % of total population) in undifferentiated organoid culture.

3.6. *THBS1 expression is upregulated in hTSCs and primary cytotrophoblasts but unchanged in placental explants following inflammatory exposure*

Preeclampsia is associated with placental inflammation. Using the main placental cell type expressing THBS1, we next assessed the regulation of THBS1 in hTSCs exposed to increasing doses of inflammatory cytokines, tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6). THBS1 mRNA expression was significantly increased following treatment with 10 ng/mL of IL-6 (Supplementary Fig. 3A, $P = 0.0267$) relative to vehicle control. Protein secretion in conditioned media was not affected by IL-6 treatment at $1.26x10^3$ pg/mL [IQR, $1.15x10^5$ pg/mL – $4.83x10^2$ pg/mL] (Supplementary Fig. 3B) relative to vehicle control at $8.89x10^2$ pg/mL [IQR, $1.13x10^3$ pg/mL – $5.60x10^2$ pg/mL]. In hTSCs treated with TNFα, THBS1 expression was significantly increased (Supplementary Fig. 3C, $P = 0.0253$) relative to vehicle control. THBS1 protein levels were not affected by TNFα treatment at $1.56x10^3$ pg/mL [IQR, $4.39x10^3$ pg/mL – $1.08x10^3$ pg/mL] (Supplementary Fig. 3D) relative to vehicle control at $7.72x10^2$ pg/mL [IQR, $3.33x10^3$ pg/mL – $7.14x10^2$ pg/mL].

To compare the findings from *in vitro* placental cell line exposure to inflammation, we assessed the regulation of THBS1 in primary cytotrophoblast cells, isolated from term placenta, exposed to the same conditions. THBS1 mRNA expression was unchanged when treated with increasing doses of IL-6 (Supplementary Fig. 3E). The same was observed for THBS1 protein secretion with IL-6 treatment at $1.89x10^3$ pg/mL [IQR, $4.59x10^3$ pg/mL – $1.72x10^3$ pg/mL] (Supplementary Fig. 3F) relative to vehicle control at $2.63x10^3$ pg/mL [IQR, $3.80x10^3$ pg/mL – $1.98x10^3$ pg/mL]. THBS1 mRNA expression was significantly increased following treatment with 10 ng/mL of TNFα (Supplementary Fig. 3G, $P = 0.003$) relative to vehicle control. THBS1 protein levels were unchanged following TNFα treatment at $3.29x10^3$ pg/mL [IQR, $6.35x10^3$ pg/mL – $2.28x10^3$ pg/mL] (Supplementary Fig. 3H) relative to vehicle control at $2.27x10^3$ pg/mL [IQR, $3.39x10^3$ pg/mL – $2.22x10^3$ pg/mL].

Finally, we investigated the regulation of THBS1 in placental explants, isolated from term placenta. THBS1 mRNA expression was unchanged when treated with IL-6 (Supplementary Fig. 3I) at 10 ng/ml relative to control. THBS1 protein levels were also unchanged following treatment with IL-6 at $8.67x10^4$ pg/mL [IQR, $1.25x10^5$ pg/mL – $7.49x10^4$ pg/mL] (Supplementary Fig. 3J) relative to vehicle control at $9.60x10^4$ pg/mL [IQR, $1.09x10^5$ pg/mL – $7.46x10^4$ pg/mL]. THBS1 expression was unchanged when treated with TNFα (Supplementary Fig. 3K) at 10 ng/ml relative to vehicle control while THBS1 protein secretion remained unchanged between TNFα treated explants at $7.87x10^4$ pg/mL [IQR, $1.74x10^5$ pg/mL – $6.93x10^4$ pg/mL] (Supplementary Fig. 3L) relative to vehicle control at $8.77x10^4$ pg/mL

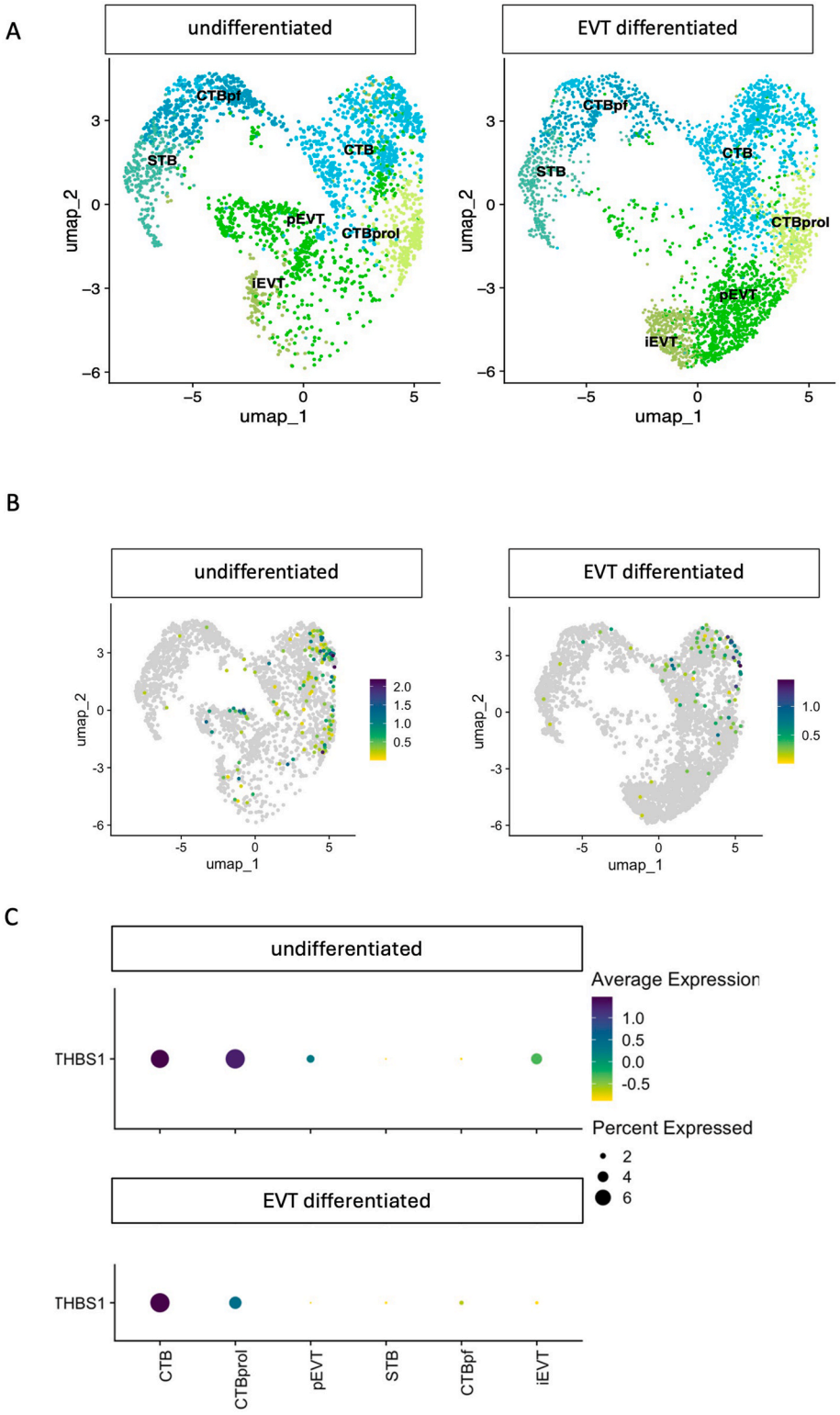

Fig. 5. Single cell RNA sequencing analysis of THBS1 in hTSC organoids and differentiated EVT. Transcriptomic analysis of a publicly available scRNA-seq dataset of three-dimensional hTSC-derived organoids (n = 3) treated under hTSC conditions or induced to differentiate to EVTs across 21 days [43]. Analysis resulted in 6 distinct transcriptomic cell identities: cytotrophoblast (CTB), proliferative cytotrophoblast (CTBprol), pre-fusion cytotrophoblast (CTBpf), progenitor EVT (pEVT), invasive EVT (iEVT) and syncytiotrophoblast (STB). **(A)** UMAP plot of cell identities in undifferentiated and EVT differentiated hTSC organoids. **(B)** UMAP plot of THBS1 expression relative to cell identities. **(C)** Dot plot of THBS1 to indicate levels of gene expression in cell proportions of specific cell identities for each condition.

[IQR, 1.19x10^5 pg/mL – 7.27x10^4 pg/mL]

3.7. THBS1 expression and secretion is differentially regulated in hTSCs, placental explants and primary cytotrophoblast cells exposed to a hypoxic environment

Preeclampsia is associated with placental hypoxia. Therefore, we assessed the regulation of THBS1 in hTSCs, primary trophoblast cells and placental explants when exposed to a hypoxic environment (1 % O_2). In hTSCs, THBS1 mRNA expression was significantly increased following exposure to hypoxia (Fig. 6A, $P = 0.0004$) relative to normoxic control (8 % O_2). THBS1 protein levels in conditioned media was significantly increased following hypoxic exposure at 2.56x10^4 pg/mL [IQR, 3.28x10^4 pg/mL – 1.71x10^4 pg/mL] (Fig. 6B, $P = 0.0079$) relative to vehicle control at 2.16x10^3 pg/mL [IQR, 5.79x10^3 pg/mL – 8.94x10^2 pg/mL].

In primary trophoblast cells, THBS1 mRNA expression was significantly increased when exposed to hypoxia (Fig. 6C, $P = 0.028$) relative to control. THBS1 protein levels were unchanged following hypoxic exposure at 3.72x10^3 pg/mL [IQR, 4.04x10^3 pg/mL – 2.77x10^3 pg/mL] (Fig. 6D) relative to vehicle control at 3.90x10^3 pg/mL [IQR, 6.81x10^3 pg/mL – 2.90x10^3 pg/mL].

Finally, in placental explants, THBS1 mRNA expression was significantly decreased when exposed to hypoxia (Fig. 6E, $P = 0.0001$) relative to control. Contrastingly, THBS1 protein secretion was significantly increased following hypoxic exposure at 1.71x10^5 pg/mL [IQR, 2.34x10^5 pg/mL – 1.37x10^5 pg/mL] (Fig. 6F, $P = 0.0079$) relative to vehicle control at 1.18x10^5 pg/mL [IQR, 1.32x10^5 pg/mL – 7.45x10^4 pg/mL].

3.8. THBS1 expression is not affected by endothelial dysfunction and recombinant THBS1, alone, has no effect on endothelial dysfunction

As preeclampsia is associated with endothelial dysfunction, we measured *THBS1* expression in HUVECs exposed to increasing doses of inflammatory cytokine TNFα, to induce endothelial dysfunction. We confirmed successful endothelial dysfunction through significant increase of markers *ICAM1* (Supplementary Fig. 4A, $P = 0.0014$ at 10 ng/mL TNFα), *VCAM1* (Supplementary Fig. 4B, $P = 0.0031$ at 10 ng/mL TNFα) and *ET-1* (Supplementary Fig. 4C, $P = 0.0034$ at 10 ng/mL TNFα). There was no change in *THBS1* across all concentrations of TNFα (Supplementary Fig. 4D), highlighting that endothelial dysfunction has no effect on THBS1 expression.

Given the elevated levels of THBS1 in maternal circulation both preceding term preeclampsia and in established early onset disease, we exposed HUVEC cells to recombinant THBS1 to observe the effects on endothelial cells. While TNFα alone (1 ng/ml) did not significantly enhance ICAM1 expression in HUVECs, we observed significant induction when both TNFα and THBS1 were added together (Supplementary Fig. 4E, $P = 0.019$), with no effect of recombinant THBS1 alone (Supplementary Fig. 4E). We observed significant induction of markers *VCAM1* (Supplementary Fig. 4F, $P = 0.007$ at 1 ng/mL TNFα alone) while treatment with recombinant THBS1 and TNFα together led to a reduced but still significant induction of *VCAM1* ($P = 0.024$).

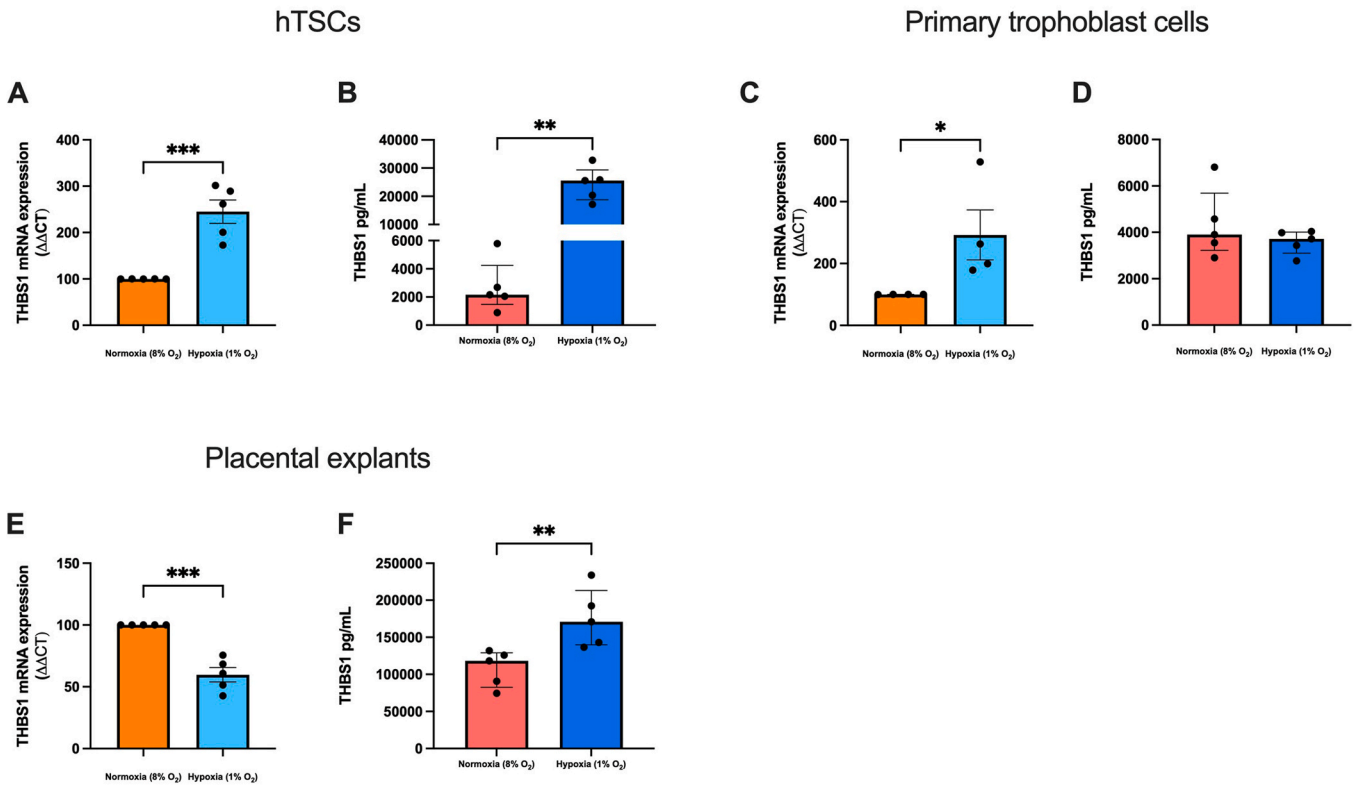

Fig. 6. Hypoxic regulation of THBS1 expression and secretion in trophoblast cells and placental explants. In hTSCs: THBS1 mRNA expression **(A)** and protein levels **(B)** were significantly increased when exposed to a hypoxic environment (1 % O_2) compared to control normoxic environment (8 % O_2). **In primary trophoblast cells: (C)** THBS1 mRNA expression was significantly increased when incubated in a hypoxic environment (1 % O_2) compared to control normoxic environment (8 % O_2). **(D)** THBS1 protein levels were unchanged in primary trophoblast cells exposed to hypoxia. **In placental explants:** THBS1 mRNA expression **(E)** and protein levels **(F)** were significantly increased when exposed to a hypoxic environment (1 % O_2) compared to control normoxic environment (8 % O_2). mRNA expression was normalised to the geometric mean of housekeeper gene(s) depending on the treatment. To assess statistical significance, either an unpaired *t*-test (parametric) or Mann-Whitney *U* test (non-parametric) was used, as appropriate. Data are presented as mean ± SEM for experiments (A, C, and E), or median ± IQR for experiments (B, D, and F). Experiments were performed with $n = 4$–5 biological replicates, as indicated by the data points, and each was conducted in technical triplicates. *p < 0.05, **p < 0.01, ***p < 0.001.

Recombinant THBS1 alone had no effect on *VCAM1*. TNFα treatment led to a significant induction of *ET-1* (Supplementary Fig. 4G, $P = 0.01$) while recombinant THBS1 had no effect on *ET-1*. Treatment with recombinant THBS1 and TNFα led to a significant induction of *ET-1* ($P = 0.0009$). Taken together, recombinant THBS1 in combination with TNFα, leads to increased *ICAM1*, *VCAM1* and reduced *ET-1* in HUVECs.

4. Discussion

The aim of this study was to investigate the potential role of THBS1 in preeclampsia. We showed significant dysregulation of THBS1 in both placenta and circulation in patients with preeclampsia compared to gestation matched controls.

THBS1 protein and mRNA expression levels were significantly reduced in the placenta of women with early-onset preeclampsia (<34 weeks' gestation). This correlates with recent findings where Hu et al., observed reduced THBS1 levels in the placenta of 20 women with severe late-onset preeclampsia when compared to 30 normal pregnancies [34]. However, these results contrast with observations by Duan et al. and Li et al., who found that THBS1 is increased in preeclamptic placenta [33,55]. Notably, the study included a smaller cohort of placentae from normal pregnancies (n = 5 and n = 6) and from pregnancies with late-onset preeclampsia (n = 3 and n = 6). Together, we provide insight into the expression of THBS1 in the placenta in early-onset preeclampsia.

It is important to consider how THBS1 may be regulated in the placenta and its role in the pathophysiology of preeclampsia. Conflicting reports exist regarding placental THBS1 regulation in preeclampsia, with some studies describing upregulation linked to impaired syncytialisation via CD36/cAMP inhibition [33]. Hu et al., demonstrated in HTR8/SVneo cells, an *in vitro* model for EVT cells, that THBS1 knockdown impaired cell viability and proliferation [34]. THBS1 inhibition triggered necroptosis, a programmed cell death pathway, but not caspase-dependent apoptosis in these cells [34]. These divergent findings may reflect differences in disease severity or experimental design. This highlights the context-dependent nature of THBS1 regulation in and heterogeneity of preeclampsia. These findings suggest that dysregulated placental THBS1 expression in preeclampsia might contribute to the exacerbated placental ischemia and systemic inflammation identified with the disease.

In this study, THBS1 levels were significantly elevated in maternal circulation of women both preceding diagnosis of preeclampsia (36 weeks' gestation) and those with established disease (<34 weeks' gestation). This contrasts with our findings of decreased expression within the placenta. Circulating immune cells can secrete THBS1 during systemic inflammation [56] which is a hallmark of preeclampsia [57]. This may contribute to increased THBS1 accumulation in maternal circulation in preeclampsia. Additionally, there may be impaired clearance of circulating THBS1 resulting from renal impairment [58]. Preeclampsia often involves renal impairment and, in severe cases, hepatic dysfunction [58,59]. Notably, increased THBS1 has been linked to kidney and liver disease [60–62]. Therefore, even moderate renal/hepatic impairment could reduce THBS1 clearance and exacerbate organ dysfunction observed in preeclampsia.

THBS1 is involved in a variety of processes including haemostasis [63], platelet activation [63–65], vascular function [66] and inflammation [27,67]. Upregulation of THBS1 has been shown to contribute to endothelial and vascular dysfunction [66–69] as well as pulmonary hypertension [70]. Additionally, THBS1's ability to antagonise NO signalling has been shown to influence vascular function [65] and angiogenesis [71]. Elevated THBS1 levels in maternal circulation may exacerbate endothelial dysfunction and vascular dysfunction.

In contrast to our findings in maternal circulation, a study by Ulu et al., showed that lower maternal serum THBS1 levels might be a promising indicator for preeclampsia [35]. In this case control study they assessed 84 pregnant women in the third trimester of which 41 were healthy and 43 had a diagnosis of preeclampsia split into two groups - mild and severe [35]. They suggest that THBS1 levels are increased in early-onset preeclampsia and decreased in late-onset preeclampsia. This is interesting given our observations that THBS1 remains increased in maternal circulation at 36 weeks' gestation preceding the diagnosis of preeclampsia at term. Stenczer et al., had a case control cohort of 45 pregnant women with early-onset and 43 with late-onset preeclampsia, 21 patients with HELLP-syndrome and 45 women with uncomplicated pregnancies with an additional 20 non-pregnant controls [72]. Stenczer et al., focused on THBS1 in HELLP syndrome and observed only a trending increase in THBS1 levels between all patients with preeclampsia and the control group [72]. Taken together, we highlight the dynamic regulation of THBS1 throughout gestation in both early-onset and late-onset preeclampsia. Therefore, THBS1 secretion may precede clinical onset of preeclampsia, however this requires validation in an appropriate longitudinal and validation cohort.

Further research is necessary to explore the impact of blood sampling (serum vs plasma) on THBS1 measurement. Serum, obtained after clotting, removes platelets and fibrin [73], which express and interact with THBS1 [65,74]. Therefore serum-based assessment of THBS1 might underestimate actual concentrations. As such, this current study investigates THBS1 in plasma, containing clotting factors, which might provide a more accurate quantification of THBS1 levels.

We observed downregulation of THBS1 as cells differentiated into syncytiotrophoblast cells or EVT cells. Concordantly, Duan et al., reported high levels of THBS1 in the CK7-positive cytotrophoblast cells from five healthy term placenta, showing low THBS1 expression in syncytial knots [33]. Additionally, it was shown in primary cytotrophoblasts from healthy placentae that were differentiated into syncytiotrophoblast cells, that THBS1 expression in the cytotrophoblast cells was higher than in the syncytiotrophoblast [33]. Hu. et al., also reported that THBS1 was mainly located in placental extravillous trophoblasts [34]. These findings also show some concordance with our transcriptomic analysis where low THBS1 expression was observed in iEVT and pEVT cell populations in undifferentiated hTSC organoid culture. Notably, this expression was lost in the EVT differentiated organoid culture. It would be important to assess THBS1 in specific placental cell types across gestation to characterise *THBS1* expression and further establish THBS1's role in trophoblast differentiation and function.

It is known that preeclampsia is characterised by a significant shift in angiogenic factors to favour anti-angiogenesis [10]. THBS1 exhibits potent anti-angiogenic and pro-thrombotic activities and may therefore contribute to this angiogenic shift [75,76]. Additionally, THBS1 is known to regulate matrix metallopeptidase and metalloproteinase activity, thereby regulating extracellular matrix remodelling [74,77,78]. A recent review has highlighted that aberrant expression of secreted and matricellular proteins is a consistent feature of the disease [79]. Therefore, we may suggest that the loss of THBS1 in preeclamptic placenta may result in impaired cytotrophoblast fusion and differentiation due to defective extracellular matrix remodelling contributing to disease pathology.

Placental hypoxia is a well-established feature of preeclampsia, particularly in early-onset cases, and hypoxia has been shown to regulate the expression of THBS1 [80–83]. Additionally, THBS1 activates TGFβ and is associated with pulmonary arterial hypertension due to chronic hypoxia [84,85]. Hypoxia appears to play a key regulatory role in modulating THBS1 expression and secretion across our *in vitro* models. In hTSCs, hypoxic exposure significantly upregulated THBS1 at both the transcript and protein levels, whereas in primary trophoblast cells, only mRNA expression was elevated. Interestingly, in placental explants, hypoxia reduced THBS1 mRNA expression but increased its secretion into conditioned media. This pattern aligns with our *in vivo* findings, where <34 weeks' placental tissue exhibited reduced THBS1 mRNA and protein levels, yet maternal plasma levels were elevated. The

concordance between hypoxia-exposed term explants and early gestation tissue suggests a gestational age-independent mechanism of hypoxic regulation. These findings highlight potential post-transcriptional regulation of THBS1 secretion under low-oxygen conditions. These processes may be crucial for both physiological adaptation and the pathogenesis of preeclampsia. In contrast, exposure to inflammatory cytokines led to only a modest increase in THBS1 mRNA in hTSCs and primary trophoblasts, without affecting protein secretion, and had no effect on either expression or secretion in placental explants. A recent study by Tosetta et al. revealed that SPARC, a functionally analogous matricellular regulator, is significantly reduced in placentas from preeclamptic pregnancies - with or without fetal growth restriction [86]. The reduction was shown to be associated with hypoxic stress in trophoblasts, suggesting a failure of normal extracellular matrix remodelling and invasion. Collectively, this suggests that THBS1 is differentially regulated by hypoxia and inflammation, with hypoxia emerging as a more robust and consistent modulator of both THBS1 transcription and secretion. Ultimately, this may contribute to and further emphasises THBS1's potential role in defective extracellular matrix remodelling which is characteristic of preeclampsia.

THBS1 exhibits widespread expression across diverse cell types including fibroblasts, leukocytes, activated platelets, endothelial and vascular smooth muscle cells [87]. The influence of other modulators on THBS1 function in preeclampsia requires further exploration and may explain why we have reduced levels of THBS1 in preeclamptic placenta and placental explants despite increased expression in perturbed trophoblast cell monoculture. Therefore, the main source of increased circulating THBS1 in preeclampsia may not stem from the placenta. We suggest that the increased circulatory THBS1 in preeclampsia may stem from activated platelets [65,88] and immune cells [89] which have been shown to drive THBS1 production in other diseases.

Our *in vitro* model of endothelial dysfunction in preeclampsia did not change THBS1 expression in HUVECs. Treatment with recombinant THBS1 led to modest enhancement of endothelial dysfunction when treated in combination with TNFα. These contrasting findings further highlight the complexity of THBS1's role in preeclampsia. Michaeli et al., showed that HUVEC and human umbilical arterial cells (HUAC) differ in terms of how they respond to varying perturbations [90]. This indicates a possible different regulation of THBS1 in endothelial cells derived from arteries, which would need to be investigated. Therefore, While THBS1 may contribute to endothelial dysfunction through its known inhibitory effects on vasodilation and platelet activity, its cellular localisation and potential context-dependent effects within the entire physiological system warrant further investigation.

This study offers further insight into THBS1's role in preeclampsia and benefitted significantly from the inclusion of two independent, well-characterised cohorts. However, limitations inherent to our *in vitro* models necessitate further investigation. An *in vitro* and *in vivo* THBS1 knockout model could provide a more relevant approach to assess THBS1 function in placental cells, given the reduced expression of THBS1 in the placenta. An additional limitation of this study is that preterm controls, may not fully represent physiological placental states. Although samples were carefully selected, preterm delivery and associated stressors could influence trophoblast biology. This should be considered when interpreting these findings. It is important to note that reduced THBS1 expression in preeclampsia may reflect not only a relative decrease in cytotrophoblast abundance, but also altered trophoblast function and microenvironmental influences from immune and stromal cells. Our *in vitro* models cannot fully recapitulate the heterogeneity and functional complexity of placental tissue *in vivo*. Although longitudinal data on THBS1 expression across gestation are limited, our study design matched cases and controls for gestational age, making it unlikely that the observed differences simply reflect normal gestational dynamics. Future studies assessing cell-type-specific THBS1 expression in term placental tissue, particularly in the context of preeclampsia, are warranted.

Further exploring THBS1's interaction with its receptors CD36, CD47, and integrins, and its impact on angiogenesis within the placental microenvironment could yield crucial insights into its role in preeclampsia. Additional immunofluorescence investigation of placental tissues across gestation would allow for more accurate identification of THBS1 localisation and quantification in the placenta. This analysis would complement our *in vitro* findings in conjunction with our transcriptomic data.

There are currently no biomarkers that effectively rule in which women are going to develop preeclampsia. A ratio of sFlt-1/PlGF is used clinically to rule out preeclampsia, reflecting the anti-angiogenic nature of this complication [12]. While many emerging candidates have been identified, most have not been validated in large cohorts none have been successfully translated into clinical use. Emerging candidates such as Activin A [14], SIGLEC6 [15], sEng [13], PAPPA [16], Pregnancy-Specific β-1 Glycoproteins 7 and 9 (PSG7/9) [91], broaden this landscape but require further validation. Thus, there is a significant dearth in candidates that meet the clinical bar needed for successful translation. Our study demonstrates consistent dysregulation of THBS1 at both tissue and circulating levels in preeclampsia, reflecting its possible involvement in the pathology of preeclampsia. However, THBS1 showed only a moderate predictive capacity for disease classification, suggesting it is not a robust standalone biomarker for preeclampsia. Whether it might add to a multi-marker test for this disease requries further investigation.

In conclusion, our findings show that THBS1 is dysregulated in preeclampsia. The findings of this study extend our current understanding of placental dysfunction in preeclampsia by linking dysregulated THBS1 expression across tissue and circulating compartments in different cohorts. We suggest that placental hypoxia may modulate THBS1 levels, as it is observed in preeclampsia. Further studies are required to clarify the mechanisms and role of THBS1 in placental and endothelial dysfunction. This work provides an additional basis for further functional investigation of THBS1 in preeclampsia.

CRediT authorship contribution statement

Stefan M. Botha: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lucy A. Bartho:** Writing – review & editing, Methodology, Formal analysis. **Sunhild Hartmann:** Writing – review & editing, Methodology, Formal analysis. **Ping Cannon:** Writing – review & editing, Methodology. **Anna Nguyen:** Writing – review & editing, Methodology. **Tuong-Vi Nguyen:** Writing – review & editing, Methodology. **Natasha Pritchard:** Writing – review & editing, Methodology. **Ralf Dechend:** Writing – review & editing, Funding acquisition. **Olivia Nonn:** Writing – review & editing, Funding acquisition. **Stephen Tong:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Tu'uhevaha J. Kaitu'u-Lino:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval

Ethical approval was obtained from the Mercy Health Human Research Ethics Committee (R11/34 and 2019–012) before we commenced the study. Participants provided written informed consent to participate in this study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2025.11.013.

Data availability

All data generated or analysed during this study are included in this publication and supplementary file. Other resources used or highlighted in this study are available from the corresponding author upon reasonable request. The full code used for scRNA-seq analysis is available upon request.

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