

# Identification of Novel Syncytiotrophoblast Membrane Extracellular Vesicles Derived Protein Biomarkers in Early-onset Preeclampsia: A Cross-Sectional Study

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Toluwalase Awoyemi, Shuhan Jiang, Bríet Bjarkadóttir, Maryam Rahbar, Prasanna Logenthiran, Gavin Collett, Wei Zhang, Adam Cribbs, Ana Sofia Cerdeira, Manu Vatish 

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom • Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom

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## Abstract

## Background

Preeclampsia (PE), a multi-systemic hypertensive pregnancy disease that affects 2-8% of pregnancies worldwide, is a leading cause of adverse maternal and fetal outcomes. Current clinical PE tests have a low positive predictive value for PE prediction and diagnosis. The placenta notably releases extracellular vesicles from the syncytiotrophoblast (STB-EV) into the maternal circulation.

## Objective

To identify a difference in placenta and STB-EV proteome between PE and normal pregnancy (NP), which could lead to identifying potential biomarkers and mechanistic insights.

## Methods

Using ex-vivo dual lobe perfusion, we performed mass spectrometry on placental tissue, medium/large and small STB-EVs isolated from PE (n = 6) and NP (n = 6) placentae. Bioinformatically, mass spectrometry was used to identify differentially carried proteins. Western blot was used to validate the identified biomarkers. We finished our investigation with an in-silico prediction of STB-EV mechanistic pathways.

## Results

We identified a difference in the STB-EVs proteome between PE and NP. Filamin B, collagen 17A1, pappalysin-A2, and scavenger Receptor Class B Type 1) were discovered and verified to have different abundances in PE compared to NP. In silico mechanistic prediction revealed novel mechanistic processes (such as abnormal protein metabolism) that may contribute to the clinical and pathological manifestations of PE.

## Conclusions

We identified potentially mechanistic pathways and identified differentially carried proteins that may be important in the pathophysiology of PE and are worth investigating because they could be used in future studies of disease mechanisms and as biomarkers.

## Funding

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### eLife assessment

This study presents **valuable** findings that could be utilized for identifying women at risk for preeclampsia before the onset of the disease. The novel aspect of this study lies in the utilization of exosomes of two different sizes. The data are **solid**: the methods, data, and analysis broadly support the claims. This work will be of interest to medical researchers and clinicians who work on preeclampsia and women's health.

## Introduction

Preeclampsia (PE) is a significant cause of maternal and neonatal morbidity and mortality, affecting 2-8% of all pregnancies (Lisonkova & Joseph, 2013 [↗](#)). It is characterized by hypertension (systolic blood pressure  $\geq 140$ mmHg / diastolic pressure  $\geq 90$ mmHg), and either proteinuria (protein/creatinine ratio of  $\geq 30$  mg/mmol or more), or evidence of maternal acute kidney injury, liver dysfunction, neurological abnormalities, hemolysis, or thrombocytopenia, and/or fetal growth restriction. (“ACOG Practice Bulletin No. 202: Gestational Hypertension and Preeclampsia,” 2019; Brown et al., 2018 [↗](#)) Predicting or early detection of PE is thus of extreme importance to reduce the chance of long term complications but this has been challenging due to the limitations of current predictive models and biochemical tests (which lack in positive predictive value) (Zeisler et al., 2016 [↗](#)). The existing tests perform far better in ruling out rather than ruling in PE. They are also most effective shortly before the onset of the disease and within a specific time frame (1 or 2 weeks) rather than earlier in pregnancy (Thadhani et al., 2022 [↗](#)).

The pathophysiology of PE implicates the placenta. It is known that PE can occur in trophoblastic tumors (without the presence of a fetus); that PE is more common in multiple pregnancy (with greater placental mass) and that it has occurred in ectopic pregnancies (excluding the involvement of the uterus)(Billieux et al., 2004 [\[1\]](#); Hailu et al., 2017 [\[2\]](#); C. W. G. Redman et al., 2022 [\[3\]](#); Soto-Wright et al., 1995 [\[4\]](#)). Finally, delivery of the placenta (irrespective of gestational age) is currently the only cure for the condition(C. Redman, 2014 [\[5\]](#)).

The placental syncytiotrophoblast (STB) layer, the interface between the fetus and the mother which lies in direct contact with the maternal circulation. EVs, including STB-EVs, are membrane-bound and cell-derived particles that carry different cargos, including proteins, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and lipids(Raposo & Stoorvogel, 2013 [\[6\]](#)). EVs are based on size into medium/large STB-EVs (MVs, 201–1000nm) or small STB-EVs ( $\leq 200$ nm)(Dragovic et al., 2015a [\[7\]](#)). The release of syncytiotrophoblast extracellular vesicles (STB-EVs), including both size ranges, into the maternal circulation increases with gestation and is further elevated in PE (Germain et al., 2007 [\[8\]](#); Goswamia et al., 2006 [\[9\]](#)). Variations in cargo content are evident among different subsets of syncytiotrophoblast-derived extracellular vesicles (STB-EVs), with medium/large STB-EVs (m/ISTB-EVs) showing higher levels of total RNA and total protein in comparison to small STB-EVs (sSTB-EVs)(Zabel et al., 2021 [\[10\]](#)). Additionally, distinct miRNA profiles have been noted to differ in abundance between these subsets. While limited studies have investigated disparities in EV subtypes, Keerthikumar et al reported that exosomes and other small extracellular vesicles (EVs) have a more pronounced impact on cell migration and proliferation than the larger EVs known as ectosomes (Keerthikumar et al., 2015 [\[11\]](#)). Furthermore, Minciacchi et al demonstrated that large EVs exhibit greater efficiency in reprogramming fibroblasts and promoting the formation of endothelial cell tubes when compared to small EVs(Minciacchi et al., 2017 [\[12\]](#)).

In addition, when it comes to immune cell-derived EVs, large EVs were found to induce the secretion of Th2-associated cytokines, while both medium and small EVs (pelleted at 10,000g and 100,000g) triggered the release of Th1 cytokines(Tkach et al., 2017 [\[13\]](#)). These functional distinctions were also observed in STB-EVs. Specifically, our investigation unveiled that normal pregnancy (NP) medium/large-sized STB-EVs significantly enhanced the transcriptional expression of pro-inflammatory cytokines in contrast to pathological (PE) medium/large-sized STB-EVs(Awoyemi et al., 2021 [\[14\]](#)). However, there was no significant difference in the small STB-EV population between the two groups, although, in general, NP small STB-EVs exhibited a slight upregulation of the same cytokines(Awoyemi et al., 2021 [\[14\]](#)). It is reasonable to conduct further in-depth exploration into the qualitative functional properties of distinct STB-EVs, with the initial step being a comprehensive characterization of these diverse subtypes. The presence of proteins, RNA species and DNA in EVs combined with their constitutional release as inter-cellular signaling moieties means they also have potential in disease prediction and diagnosis. Notably, these distinct STB-EV subtypes have also been identified in the bloodstream. Nakahara et al extensive review compiles a collection of studies that have specifically isolated various subtypes of EVs from maternal plasma(Nakahara et al., 2020 [\[15\]](#)). In this study, we performed proteomic analysis of placenta and STB-EVs in PE and NP to identify potential placenta-derived biomarkers. We also conducted in silico analysis on the proteomes to detect potential molecular targets, mechanisms, and processes of PE.

## Methods

### Ethics approval and patient information

Oxfordshire Research Ethics Committee C (07/H0606/148) approved this study. Normal pregnancy was a healthy singleton pregnancy with normal maternal blood pressure. PE was defined as new (after 20 weeks) systolic blood pressure 140 mmHg or diastolic pressure 90 mmHg, proteinuria (protein/creatinine ratio of 30 mg/mmol or more). None of our patients had maternal acute kidney

injury, liver dysfunction, neurological features, haemolysis, thrombocytopenia, and/or foetal growth restriction. This study included only early-onset PE patients (diagnosed before 34 weeks gestation). After informed consent, placenta was obtained from women undergoing elective caesarian section without labor were collected.

## Placenta sample preparation and syncytiotrophoblast membrane extracellular vesicles (STB-EVs) enrichment

Placenta biopsies were obtained adjacent to the perfused lobe and immediately frozen at -80°C pending transfer to the Target Discovery Institute (Oxford) for proteomic analysis. We obtained STB-EVs via placental perfusion as previously described (Dragovic et al., 2015b [\[link\]](#)). Our STB-EV enrichment and categorization process has been deposited on EV Track (<http://www.EVTRACK.org> [\[link\]](#)], EV-TRACK ID: EV 220157) with a score of 78% (the average score on EV track for 2021 is 52 %). Full details can be found in the supplemental data.

## Characterization of syncytiotrophoblast membrane extracellular vesicles (STB-EVs)

Enriched STB-EVs were resuspended in filtered phosphate buffered saline (fPBS) and characterized with bicinchoninic acid (BCA) assay (for protein concentration) and nanoparticle tracking analysis (NTA) (for particle number and size profile). We also phenotyped the STB-EVs with transmission electron microscopy (for morphology), flow cytometry (BD Biosciences, LSRII), and western blot (for immunophenotyping). Flow cytometric analysis was performed using antibodies to placental alkaline phosphatase – PLAP-(to confirm syncytiotrophoblast origin), CD 41 (to identify co-isolated platelet EVs, CD235 a/b (to identify co-isolated red blood cell EVs), HLA class I and II (to identify co-isolated white blood cell EVs). Western blot was probed for placental alkaline phosphatase (PLAP [1.667 mg/ml] at 1:1000 dilution in house antibody), the known EV markers CD 63 ([200ug/ml] at 1:1000 dilution, Sc-59286, Santa Cruz Biotechnology), ALIX ([200ug/ml] at 1:1000 dilution, Sc-53538, Santa Cruz Biotechnology) and the known negative EV marker Cytochrome C ([200ug/ml] at 1:1000 dilution, Sc-13156, Santa Cruz Biotechnology) as recommended by the international society for extracellular vesicles (ISEV) and subsequently incubated with the corresponding secondary antibody anti-mouse, or anti-rabbit polyclonal goat immunoglobulins/HRP (at 1: 2000 dilution, Dako UK Ltd, Cambridgeshire UK). Details of nanoparticle tracking analysis and transmission electron microscopy can be found in the supplemental data.

## Sample preparation for Mass Spectrometric analysis and bioinformatic analysis of proteomic data from placenta tissue, medium/large, and small STB-EVs

STB-EVs and placental tissue samples were processed for liquid chromatography mass spectrometry (LC-MS) (Target Discovery Institute, Oxford). Briefly, STB-EVs and placental tissue samples (10 µg total protein) were reduced with dithiothreitol (DTT) (final concentration 5 mM) for 60 min at room temperature, then alkylated with iodoacetamide (final concentration 20 mM) for 60 min at room temperature. After precipitation with methanol/chloroform, the protein pellet was resuspended in 6 M urea, and then the urea concentration was reduced to < 1 M with milliQ H<sub>2</sub>O. Trypsin was added to achieve the final trypsin: protein ratio of 1:50, and the samples were digested overnight at 37°C. According to the manufacturer's instructions, peptides were purified on Waters C18 Sep-Pak cartridges. The purified peptides were dried down in a speed vac, resuspended in 2% acetonitrile/0.1% trifluoroacetic acid, and diluted 1:20 before injection. Peptides were injected into an LC-MS system comprised of a Dionex ultimate 3000 Nano LC (Liquid Chromatography) and a Thermo Q-Exactive mass spectrometer. Peptides were separated on a 50-cm-long EasySpray column (ES803; Thermo Fisher) with a 75 µm inner diameter and a 60-minute gradient of 2% to 35% acetonitrile in 0.1% formic acid and 5% dimethyl sulfoxide (DMSO) at a flow

rate of 250 nl/min. The Top 15 most abundant peaks were fragmented after isolation with a mass window of 1.6 and a resolution of 17,500. The normalized collision energy was 28% (higher collisional dissociation). Raw data was imported and analyzed with Progenesis QI (Waters) using standard settings and manually refined retention time alignment. MS/MS data was searched in Mascot (Matrix Science) against a human database (fused Uniprot/Trembl, 03/2018), with oxidation (Met), deamidation (Gln/Asn) and carbamidomethylation (Cys) fixed as variable modifications. Precursor mass tolerance was set to 10 parts-per-million (ppm) and fragment tolerance to 0.04 Da. Peptide identifications were FDR (False Discovery Rate) adjusted at 1%, and identifications with Mascot score < 20 were discarded.

Raw label-free quantitation (LFQ) data was imported and analyzed in Perseus (Max Planck Institute of Biochemistry). Differential expression was performed by conducting a two-sample independent Student t-test. Multiple testing was corrected via permutation-based FDR (False Discovery Rate) with the default software settings. Proteins were considered differentially expressed if their false discovery rate (FDR) was less than 0.05 and their fold change was greater than or equal to 1 or less than or equal to -1. G: Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) was used for functional enrichment analysis of the differentially expressed proteins (DEPs). The enriched pathways/terms from the KEGG database and Gene ontology database, gene ontology biological process (GO: BP), gene ontology molecular function (GO: MF), and gene ontology clinical component (GO: CC) were ascertained for each set of DEPs by applying hypergeometric testing. Multiple testing was corrected by Benjamin Hochberg correction, and the significance level was set to < 0.05. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031953.132

## Bioinformatic analysis of proteomic data from placenta tissue, medium/large and small STB-EVs

Persus (Max Planck Institute of Biochemistry) was used for the analysis, along with the accompanying documentation and tutorials. To remove invalid data, we pre-processed the raw data by log transforming and filtering. Missing values were imputed at random from a normal distribution using the following parameters: width = 0.3 and downshift = 1.8. To ensure conformity to the normal distribution, the underlying distribution was visually inspected with a histogram before and after missing data imputation. The data was then transformed further by deducting each (transformed) value from the highest occurring protein expression value.

Principal component analysis (PCA), heatmaps, and Pearson correlation matrices were used to further investigate the data. The data was analyzed using the correlation index and hierarchical clustering. A two-sample independent student t-test was used to assess differential expression. Multiple testing was corrected using permutation-based false discovery rate (FDR) with the following parameters, with significance set at less than 0.05.

## Western Blotting

To further characterize and immune-phenotype, we performed western blots on placental homogenates and STB-EV pellets. The relevant primary antibodies were used to probe all STB-EVs (as discussed in the main article). An equal amount of protein (20 micrograms) was mixed with 4 X Laemmli buffer ((180 mM Tris-Cl (pH 6.8), 6% SDS, 30% glycerol, 0.3% 2-mercaptoethanol, and 0.0015% bromophenol blue, BioRad)). After heating the sample mix for ten minutes at 70°C, an equal volume of the sample mix was loaded. Electrophoresis was performed at 150 V for 1.5 hours in a Novex minicell tank (Invitrogen, UK) filled with NuPAGE™ MOPS SDS running buffer under reducing (for PLAP, Cytochrome C, ALIX, SR-BI, Filamin B, PAPP-A2, Collagen 17 A1) and non-reducing (for CD 63) conditions on NuPAGE™ 4-12% Bis-Tris Gel 1.0 mm x 10 well gels (Invitrogen by Thermo Fisher Scientific) (Novex by Life Technologies). As a protein size marker,

Precision plus protein™ dual color standards (Bio-Rad Laboratories Ltd, Hertfordshire, UK) were used. Following protein separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad).

In a Novex semi-dry transfer apparatus, the PVDF membrane and gel were sandwiched between four filter paper sheets pre-soaked in anode one buffer solution (300 mM Tris, 10% methanol pH 10.4) and two filter paper sheets pre-saturated with anode two buffer solution (25 mM Tris, 20% methanol pH 10.4) at the bottom and three filter paper sheets pre-soaked in cathode buffer solution (25 mM Tris, 40 mM (Life Technologies, UK). For 45 minutes, the transfer was run at 25 V. The membranes were blocked for one hour with 5% Blotto (2BScientific) in 0.1% TBST (Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) and 1% Tween-20, Sigma), then incubated overnight with the appropriate antibody.

Antibodies used were SR-BI (ab52629 [0.35 µg/µl] 1:1000 dilution, monoclonal Rabbit, Abcam), Filamin B (GTX387 [1.64 µg/µl] 1: 1000 dilution, monoclonal mouse, Insight Biotechnology), PAPP-A2 (ab59100 [1 µg/µl] 1: 2000 dilution polyclonal Rabbit, Abcam), Collagen 17 A1(ab28440 [1 µg/µl] 1: 2000 dilution polyclonal Rabbit, Abcam), and for characterization, placental alkaline phosphatase (PLAP [1.667 µg/µl] at 1:1000 dilution in house antibody), CD 63 ([200 µg/µl] at 1:1000 dilution, Sc-59286, Santa Cruz Biotechnology), Alix ([200µg/µl] at 1:1000 dilution, Sc-53538, Santa Cruz Biotechnology), and Cytochrome C ([200 µg/µl] at 1:1000 dilution, Sc-13156, Santa Cruz Biotechnology) as recommended by the international society for extracellular Vesicles (ISEV).

After an overnight incubation and three five-minute TBST washes, the membranes were incubated for an hour at room temperature with the corresponding secondary antibody, anti-mouse (P044701-2) or anti-rabbit (P044801-2) polyclonal goat immunoglobulins/ horseradish peroxidase (HRP) (1: 2000 dilution, Dako UK Ltd, Cambridgeshire UK) and rinsed three times in TBST. The membranes were developed using a gel documentation system (G-Box, Syngene, Cambridge UK) running GeneSys (version 1.5.0.0, Syngene) and a chemiluminescence film (Amersham Hyperfilm™ ECL (enhanced chemiluminescence; GE Healthcare Limited, Buckinghamshire UK) to obtain the band intensity for each lane. The fold change (FC) between normal and preeclampsia samples was calculated by normalizing the comparative expression analysis to the total protein loaded (using Amido Black stain) 17,18. The normalized densitometric values were statistically tested using a one-tailed Student t-test, with significance set at less than 0.05. **Tables 1** and **2** show the reagents and antibodies used in western blot analysis, respectively. Details of western blot for STB-EV characterization and proteomic validation experiments can be seen in the supplemental material.

This study utilized 12 samples for initial discovery and 12 for targeted western blot validation

## Results

### Patient demographics and clinical characteristics

PE mothers (**Table 1**), as expected, had a significantly higher average systolic (178.83 mmHg,  $P < 0.001$ ) and diastolic (109.17 mmHg,  $P < 0.001$ ) blood pressure compared to normal pregnant mothers average systolic (129.50 mmHg) and diastolic (67.00 mmHg). PE women are also significantly more likely to deliver prematurely (PE = 32.00 weeks gestation NP = 39.17 weeks gestation,  $P < 0.001$ ) and have proteinuria (PE = 2.58 pluses on urine dipstick NP = 0 pluses on urine dipstick,  $P < 0.001$ ) compared to normal pregnancy. Finally, PE babies significantly weighed less than normal babies (PE = 1515.83g NP = 3912.50 g,  $P < 0.001$ ). Surprisingly, we found no significant difference in body mass index, the gender of the child, and maternal age.



Characteristics	Sub-Classification	Normal Pregnancy	Preeclampsia	P Value
Sample size		6	6	
Maternal age years (mean (SD))		34.5 ± 5.39	36.33 ± 4.13	0.524
Systolic blood pressure mmHg (mean (SD))		129.5 ± 4.93	178.83 ± 12.56	<0.001
Diastolic blood pressure mmHg (mean (SD))		67 ± 6.2	109.17 ± 9.85	<0.001
Body mass index kg/m-2 (mean (SD))		29.92 ± 9.1	31.25 ± 11.12	0.825
Proteinuria plus(es) (mean (SD))		0	2.58 ± 1.2	<0.001
Gestational age at diagnosis in weeks (mean (SD))		NA	30.5 ± 3.50	0.001
Gestational age at delivery in weeks (mean (SD))		39.17 ± 0.98	32 ± 3.52	0.001
Birth weight grams (range)		3912.5 ± 730.4	1515.83 ± 600.57	<0.001
Intrauterine growth restriction (IUGR) = Yes (%)		0(0)	6(100)	0.004
Male new-born sex (%)		2(33.3)	2(33.3)	1.000

**Table 1.**

**General descriptive statistics of sample population**

## Characterization of syncytiotrophoblast membrane extracellular vesicles (STB-EVs)

Flow cytometry (**Figure 1**) was showed that many detected events ( $83-85 \pm 8.0-8.3\%$ ) were negative for CD 235a (Red blood cells), CD41 (platelets) and HLA-I and II (white blood cells) (**Figure 1B**) while  $92 \pm 0.9\%$  (**Figure 1D** and **1G**) of detected events were PLAP<sup>+</sup> extracellular vesicles (BODIPY FL N-(2-aminoethyl)-maleimide (bioM) and placental alkaline phosphatase (PLAP) double-positive). Detergent treatment, which could break down EVs, with NP-40 confirmed that majority (99%) of our samples were largely vesicular since only  $0.1 \pm 0.12\%$  of BODIPY FL N-(2-aminoethyl)-maleimide and PLAP double-positive events were detected (a reduction of 99%) (**Figure 1E** and **1H**).

Transmission electron microscopy on 10K STB-EV pellets (**Figure 2A** and **2B**) and 150K STB-EVs (**Figure 2C** and **2D**) in our sample preparation showed the typical cup-shaped morphology of extracellular vesicles on transmission electron microscopy (TEM) within the appropriate size range. Western blot confirmed they express the classic syncytiotrophoblast membrane marker, placenta alkaline phosphatase (PLAP), the extracellular vesicle markers ALIX and CD 63. In addition, they lacked the negative EV marker cytochrome C (**Figure 3A**). Nanoparticle tracking analysis (NTA) confirmed the homogeneity of the 150K STB-EV pellets (small STB-EVs) (**Figure 3B**) with a modal size of  $(205.8 \pm 67.7)$  nm and the heterogeneity of the 10K STB-EV pellets (medium/large STB-EVs) (**Figure 3C**) with a size range of  $(479.4 \pm 145.6)$  nm.

## Differentially carried proteins (DCPs) in Placenta homogenate, Medium/Large STB-EVs and Small STB-EVs in Preeclampsia versus normal pregnancies

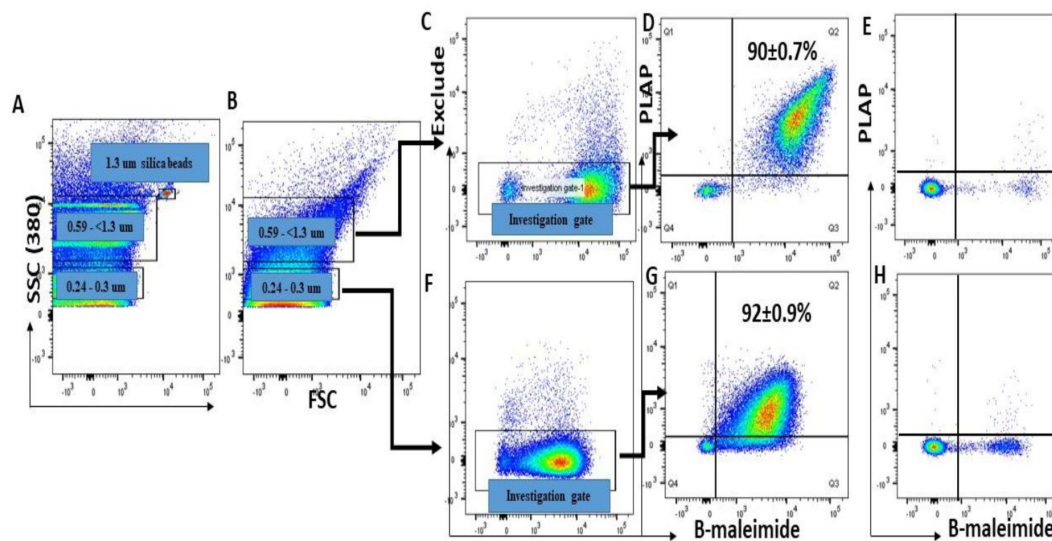
In total, using mass spectrometry, there were fifteen (15) proteins in the placenta, three hundred and four (304) in m/STB-EVs, and seventy-three (73) in sSTB-EVs were differentially expressed between preeclampsia (PE) and normal pregnancy (NP).

In the placenta (**Table 2**), isoform HMG-R of High mobility group protein (HMGA1), Fibrinogen-like protein 1 (FGL1), isoform 1 of Kinesin-like protein (KIF2A), Ig kappa chain C region (IGKC) were the most abundant proteins based on fold change. Concomitantly, serum paraoxonase/arylesterase 1 (PON1) and alpha-1B-glycoprotein (A1BG) were the least abundant proteins.

For m/STB-EVs (**Table 2**), the most differentially abundant proteins were the collagen alpha-1(XVII) chain (COL17A1), isoform 2 of Filamin-B (FLNB), tumor necrosis factor-alpha-induced protein 2 (Fragment) (TNFAIP2) based on fold change. In contrast, the least differentially abundant proteins were sodium-dependent phosphate transporter 1 (SLC20A1), methylthioribose-1-phosphate isomerase (Fragment) (MRI1), prostatic acid phosphatase (Fragment) (ACPP) based on fold change.

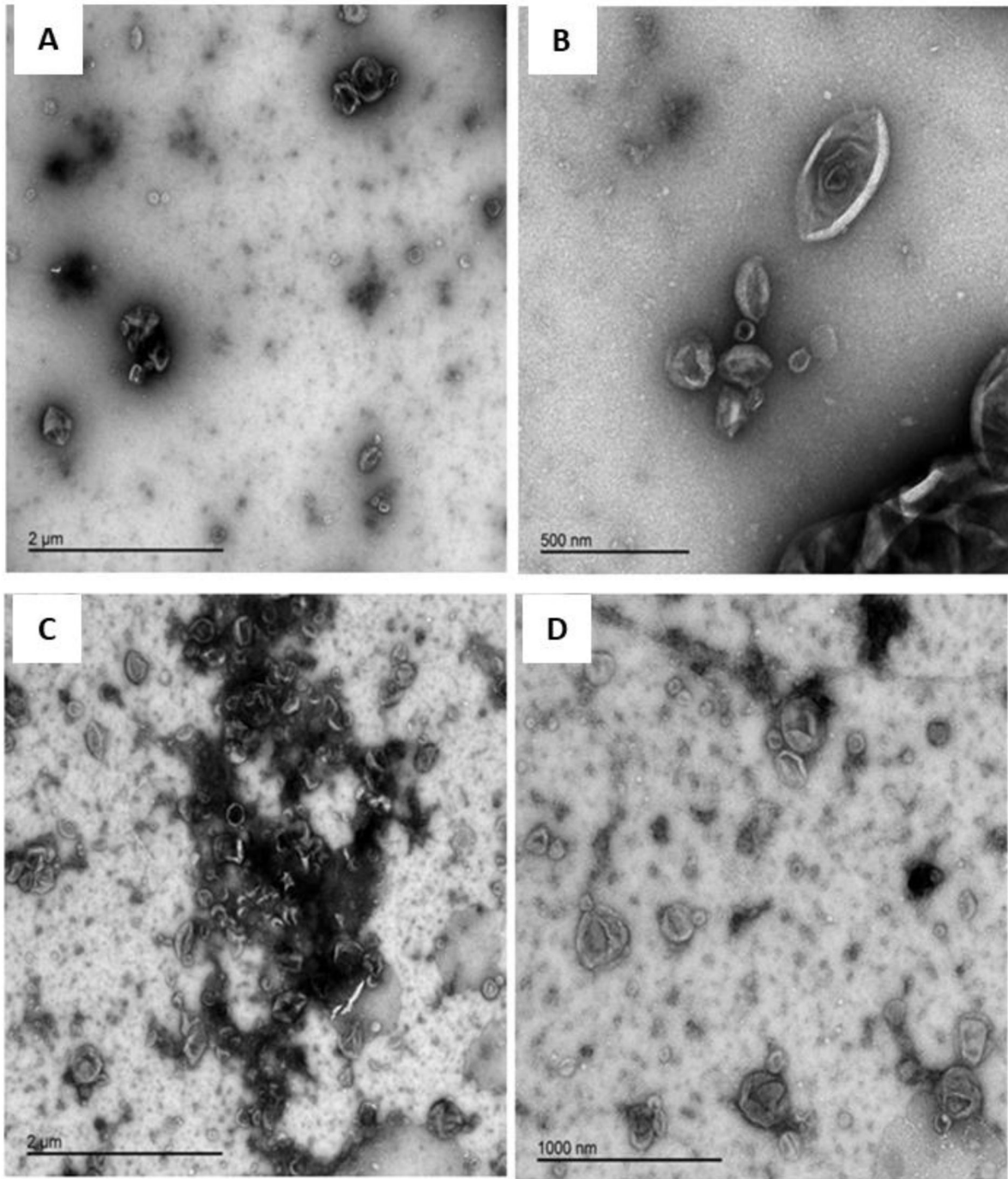
Finally, the sSTB-EVs (**Table 2**) had solute carrier family 2, facilitated glucose transporter member (SLC2A11), v-type proton ATPase 16 kDa proteolipid subunit (ATP6V0C), pappalysin 2 (PAPP-A2) as the most differentially abundant by fold change while sodium-dependent phosphate transporter 1 (SLC20A1) and isoform 2 of ADP-ribosylation factor GTPase-activating protein (ARFGAP3) were the least differentially abundant. There were 25 differentially carried proteins (DCPs) including filamin B (FLNB), ERO1-like protein alpha (ERO1A), endoglin (EGLN), pappalysin-2 (PAPP-A2), siglec6 (SIGLEC6) shared between m/STB-EVs and sSTB-EVs and only one protein, isoform 1 of kinesin-like protein (KIF2A) shared between the placenta and the m/l STB-EVs. Only one protein chloride intracellular channel protein 3 (CLIC3), was found in all three sample sub-types (**Table 3**)





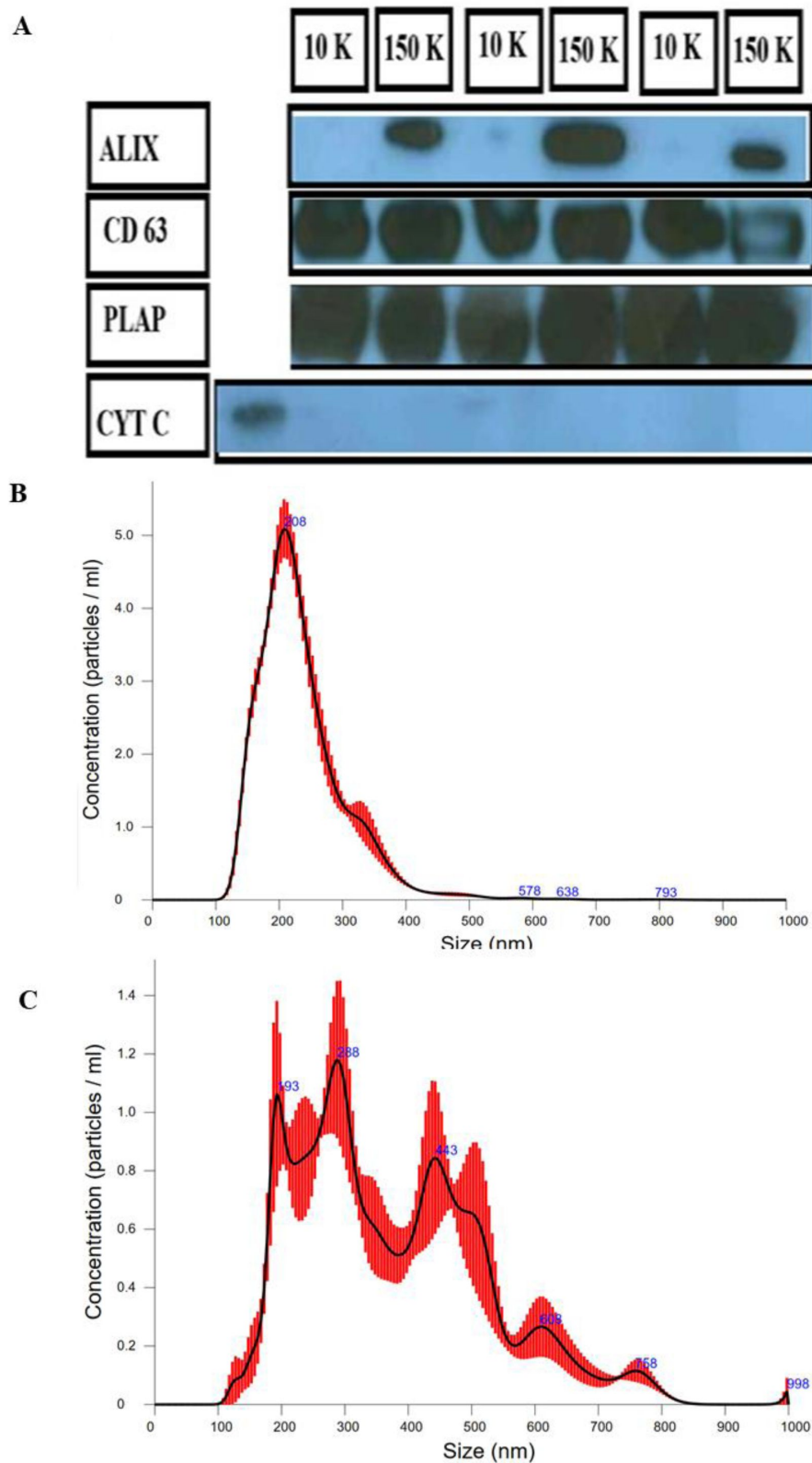
**Figure 1.**

Flow analysis of medium/large STB-EVs in the 10K STB-EV pellet. Apogee beads mix were used to set the flow machine's light scatter resolution to 0.59-1.3  $\mu\text{m}$  and 0.24-1.3  $\mu\text{m}$  silica beads(A). Figure B shows the application of SSC and FSC PMTVs as determined by apogee beads mix for the analysis of m/ISTB-EVs in the 10K pellet. An investigation gate was created to include only medium/large EVs negative for non-placental markers (C & F). Extracellular vesicles from the investigation gate were further analyzed for staining by bioM and expression of PLAP (D & G). Figure E and H shows that the bioM<sup>+</sup> PLAP<sup>+</sup> EVs were sensitive to detergent treatment. The percent of B-Maleimide<sup>+</sup> PLAP<sup>+</sup> from the 0.59-1.3  $\mu\text{m}$  gate is consistent under both SSC conditions.



**Figure 2.**

Results of STB-EV characterization. Figure A,B,C and D displays representative transmission electron microscopy (TEM) images with wide view (A and C), medium/large STB-EVs (B), and Small STB-EVs (D).



**Figure 3.**

Results of STB-EV characterization. The western blot characterization of S STB-EVs and mISTB-EVs (A). 10K refers to mISTB-EVs and 150K refers to s STB-EVs. CYT C refers to cytochrome c. [Figure 2A](#) and [2B](#) show the NTA results of m/ISTB-EVs (B) and sSTB-EVs (C).

Protein	Symbol	LFC	Adjusted P. Value
Placenta			
<b>Isoform HMG-R of High mobility group protein HMG-I/HMG-Y</b>	<b>HMGA1</b>	<b>3.14</b>	<b>0.03</b>
<b>Fibrinogen-like protein 1</b>	<b>FGL1</b>	<b>2.49</b>	<b>0.04</b>
<b>Isoform 1 of Kinesin-like protein KIF2A</b>	<b>KIF2A</b>	<b>1.47</b>	<b>0.02</b>
<b>Chloride intracellular channel protein 3</b>	<b>CLIC3</b>	<b>1.09</b>	<b>0.05</b>
<b>Mesencephalic astrocyte-derived neurotrophic factor</b>	<b>MANF</b>	<b>1.02</b>	<b>0.03</b>
<b>Ig kappa chain V-I region AG</b>	<b>KV101</b>	<b>-1.02</b>	<b>0.05</b>
<b>Haloacid dehalogenase-like hydrolase domain-containing protein 2 (Fragment)</b>	<b>K7EJQ8</b>	<b>-1.23</b>	<b>0.05</b>
<b>Ig kappa chain C region</b>	<b>IGKC</b>	<b>-1.27</b>	<b>0.05</b>
<b>Alpha-1B-glycoprotein</b>	<b>A1BG</b>	<b>-1.34</b>	<b>0.05</b>
<b>Serum paraoxonase/arylesterase 1</b>	<b>PON1</b>	<b>-2.18</b>	<b>&lt;0.01</b>
Medium/Large STB-EVs			

**Table 2**

**Top ten differentially expressed Proteins between normal and preeclampsia placentas (bold font), medium/large STB-EVs (normal font), small STB-EVs(italics)**

Collagen alpha-1(XVII) chain	COL17A1	4.79	<0.01
Isoform 2 of Filamin B	FLNB	3.09	<0.01
Tumor necrosis factor alpha-inducible protein	TNFAIP2	3.03	<0.01
Minor histocompatibility antigen	HMHA1	1.59	0.03
Bridging integrator 2	BIN2	2.33	0.02
Sodium/potassium-transporting ATPase subunit beta-1	ATP1B	1.99	0.02
ERO-1 like protein alpha	ERO1L	1.97	<0.01
Endoglin	ENG	1.82	<0.01
Methylthioribose-1-phosphate isomerase (Fragment)	MRI1	-1.96	0.04
Sodium-dependent phosphate transporter 1	S20A1/SLC17A1	-5.24	0.04
Small STB-EVs			
<i>Solute carrier family 2, facilitated glucose transporter member 11</i>	<i>SLC2A11</i>	<i>8.91</i>	<i>0.01</i>
<i>V-type proton ATPase 16 kDa proteolipid subunit</i>	<i>VATL</i>	<i>7.25</i>	<i>0.02</i>
<i>Pappalysin-2</i>	<i>PAPP2</i>	<i>3.76</i>	<i>0.02</i>
<i>ERO1-like protein alpha</i>	<i>ERO1A</i>	<i>3.29</i>	<i>0.02</i>
<i>Isoform 2 of Creatine kinase U-type, mitochondrial</i>	<i>KCRU</i>	<i>3.20</i>	<i>0.02</i>
<i>SCARB1 protein</i>	<i>B7ZKQ9</i>	<i>2.95</i>	<i>0.05</i>
<i>Isoform 2 of Filamin-B</i>	<i>FLNB</i>	<i>2.92</i>	<i>0.02</i>
<i>Solute carrier organic anion transporter family member 2A1</i>	<i>SO2A1</i>	<i>2.86</i>	<i>0.02</i>
<i>Isoform 2 of Oligaccharyltransferase complex subunit TC</i>	<i>OSTC</i>	<i>2.82</i>	<i>0.02</i>
<i>SCY1-like protein 2</i>	<i>SCYL2</i>	<i>2.71</i>	<i>0.02</i>
<i>Sodium-dependent phosphate transporter 1</i>	<i>S20A1</i>	<i>-2.93</i>	<i>0.01</i>

**Table 2** (continued)

Sample types	Differentially expressed proteins (DEPs)
m/ISTB-EVs and Placenta	Kinesin-like protein
m/ISTB-EVs, sSTB-EVs and Placenta	Chloride intracellular channel protein 3
m/ISTB-EVs and sSTB-EVs	Filamin B
	Endoplasmic Reticulum Oxidoreductase Alpha
	X-linked retinitis pigmentosa GTPase regulator interacting protein 1
	Protein NDRG1
	Collagen alpha 1(XVII) chain
	Monocyte differentiation antigen CD14
	Trophoblast glycoprotein
	Heat shock-related 70 kDa protein 2
	Endoglin
	Hippocalcin-like protein 1
	SCARB1 protein
	Importin subunit alpha 7
	Protein BCAP
	Endothelial protein C receptor
	Caveolin
	Pappalysin-2
	Phosphatidylinositol 3-kinase regulatory subunit alpha
	Keratin, type I cytoskeletal 19
	Siglec6
	Protein disulfide-isomerase

**Table 3.**

**List of overlapping differentially carried proteins in the placenta, medium/large STB-EVs and small STB-EVs**



	Amine oxidase [flavin-containing] A
	Copine-3
	Dolichyl-diphosphooligosaccharide
	Sodium-dependent phosphate transporter 1
	Annexin A4

**Table 3.** (continued)

## Validation of select proteins in the placenta homogenate, medium/Large STB-EVs, and small STB-EVs

We combined all the DCPs identified from the placenta, m/lSTB-EVs, and sSTB-EVs and selected proteins to validate based on fold change and our placenta specificity or enrichment as previously described above in the methods section. These were pappalysin A2 (*PAPP-A2*), *collagen 17A1* (*COL17A1*), *filamin B*, and *scavenger receptor class B type I* (*SR-BI/ SCARB1*). Validation was performed by western blot on all sample sub-types.

In the placenta (**Figure 4A** [↗](#) and **4D** [↗](#)), *PAPP-A2* (FC = 6.39, P Value = 0.0001) and *SR-BI* (FC = 1.72, P Value = 0.04) were all differentially abundant in PE. *Filamin B* (FC = 2.59, P Value = 0.12) was differentially abundant in PE but not significant while *COL17A1* was undetectable. In medium/large STB-EVs (**Figure 4B** [↗](#) and **4E** [↗](#)), pappalysin A2 (FC = 5.09, P Value = 0.004), *collagen 17A1* (FC = 71.21, P Value = 0.002), *filamin B* (FC = 7.60, P Value = 0.014) and *scavenger receptor class B type I* (FC = 2.28, P Value = 0.018) were all significantly differentially abundant in PE compared to normal. In sSTB-EVs (**Figure 4C** [↗](#) and **4F** [↗](#)), only *filamin B* (FC = 7.73, P Value = 0.003) and *SR-BI/SCARB1* (FC = 1.60, P Value = 0.002) were significantly differentially abundant while *COL17A1* (FC = 12.79, P Value = 0.07) and *PAPP-A2* (FC = 1.48, P Value = 0.11) were differentially abundant but not significantly so.

## Functional enrichment of differentially carried proteins (DCPs) in preeclampsia (PE)

We performed a functional enrichment analysis on the list of differentially carried proteins in placenta tissue, medium/large STB-EVs, and small STB-EVs to help better understand their role in preeclampsia (PE). The three sample sub-types did not have overlapping gene ontology terms or KEGG pathways. The top biological processes overrepresented in the placenta (**Table 3A** [↗](#)) were processes that involve *neurotransmitter secretion and transport*. In comparison, the enriched biological processes in the m/lSTB-EVs (**supplemental table 3** [↗](#)) involved *responses to hypoxia*. Finally, *posttranslational protein modification processes* were enriched among small STB-EVs (**supplemental table 3** [↗](#)). *Neurotrophin signaling pathway*, *spinocerebellar ataxia*, and *protein processing pathway* were among the over enriched KEGG pathways in the placenta, m/lSTB-EVs and sSTB-EVs respectively (**Supplemental table 4** [↗](#)).

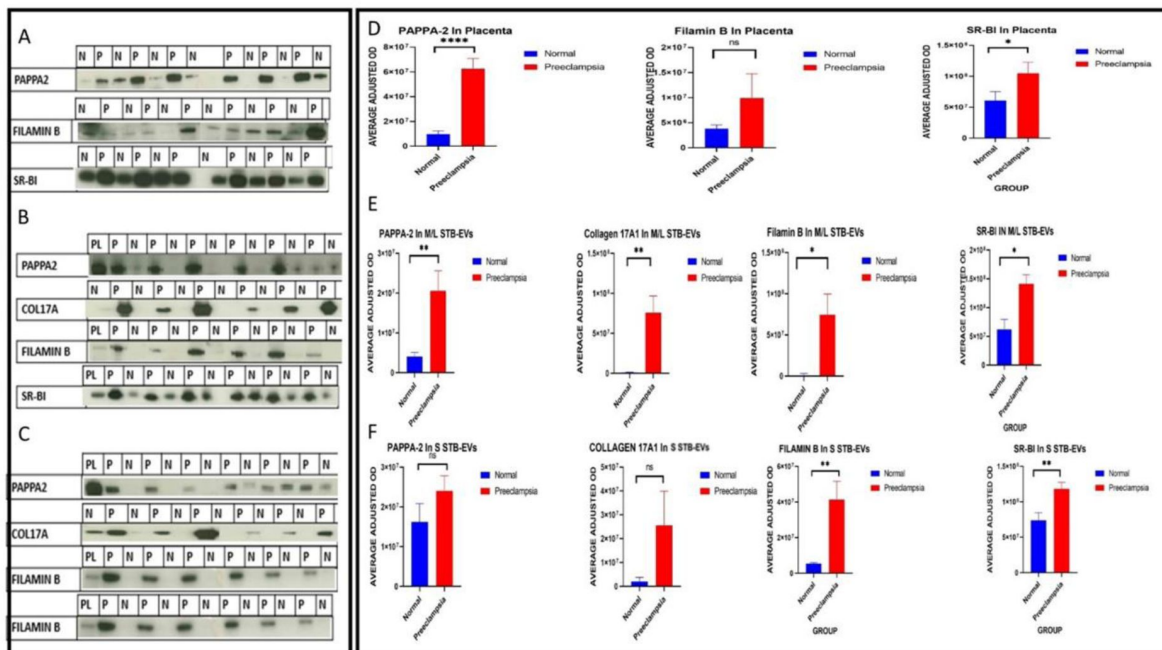
## Discussion

### Principal findings

In our analysis, PE and NP have different placenta and STB-EV proteomes. Four STB-EV biomarkers—filamin B, collagen 17A1, pappalysin-A2, and scavenger Receptor Class B Type 1—were verified for differential abundance. In silico investigation revealed molecular pathways such as abnormal protein metabolism that may contribute to PE's clinical and pathological symptoms and inform future research.

### Results in the context of what is known

Our analysis found chloride intracellular channel 3 (CLIC3) to be the only protein differentially abundant among all sample types. CLIC3 is expressed in the placenta throughout pregnancy (Money et al., 2007 [↗](#)) and is abundant in PE placentas compared to NP, a finding corroborated by our results. However, it has not previously been described in terms of STB-EVs.



**Figure 4.**

Figure showing the results of western blot and densitometric verification of PAPP-A2, COL17A1, FLNB and SR-BI in the placenta (A and D) m/ISTB-EVs (B and E) and sSTB-EVs (C and F). Collagen 17A1 did not show up in the placenta homogenate. \* means < 0.05, \*\* means < 0.01, \*\*\* means < 0.001, \*\*\*\* means less than 0.001 and ns means non-significant.

CLIC3 also recycles activated integrins back to the plasma membrane and facilitates cell migration and invasion(Dozynkiewicz et al., 2012 [DOI](#)) a process that has been identified as abnormal in PE(KHONG et al., 1986 [DOI](#)).

We found *COL17A1* to be more abundant in PE m/ISTB-EVs but not detectable in the placenta in both normal and PE. Although collagen 17 has not been described in preeclampsia, a protein of the same family, collagen 1 is deposited in higher amounts in the PE placenta and can induce preeclampsia-like symptoms by suppressing the proliferation and invasion of trophoblasts. This suppression was reversible by treating with ERK and B-catenin agonists(Feng et al., 2021 [DOI](#)). Likewise, *PAPP-A2* was significantly more abundant in the PE placenta and m/ISTB-EVs. *PAPP-A2* cleaves insulin-like growth factor binding protein (IGFBP-5 and, to a lesser extent, IGFBP-3). *PAPP-A2*'s mRNA and protein are differentially expressed in the placenta and maternal serum in PE patients(Whitehead et al., 2013 [DOI](#)).

Filamin B was significantly more abundant in PE m/ISTB-EVs and sSTB-EVs compared to NP. Filamin B participates in cellular structural mechanics and signal transduction by interacting with ion channels, signaling molecules, transmembrane proteins, and transcription factors(Zhou et al., 2010 [DOI](#)). It also suppresses tumor growth and metastasis(Iguchi et al., 2015 [DOI](#)). Interestingly, in contrast to our study wei et al described Filamin B as being up regulated in the placenta (Wei et al., 2019 [DOI](#)) . Wei et al used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference while we used total protein normalization to Amido black to quantify and compare protein expression. GAPDH is an unreliable internal reference protein, particularly in preeclampsia(Lanoix et al., 2012 [DOI](#)) and this may explain these discordant findings. In addition, in our proteomics data, we found GAPDH to be among the most differentially expressed (upregulated) proteins.

Likewise, we identified scavenger receptor class B, type 1 (SCARB1/SR-BI) to be significantly increased in PE placenta, m/l and sSTB-EVs. SCARB1/SR-BI, is most abundant in the adrenal glands, placenta, liver, and brain(Ganesan et al., 2016 [DOI](#); Shen et al., 2016 [DOI](#)). SR-BI facilitates the uptake of cholesteryl esters from high-density lipoproteins and lipid-soluble vitamin and transthyretin-bound thyroid hormone by placental trophoblast cells(Landers et al., 2018 [DOI](#)).

In terms of potential mechanisms of preeclampsia, we found no overlap in the biological processes and KEGG pathways among the three sample sub-type. In the placenta, gene ontology biological processes (GO: BP) involved in *neurotransmitter secretion and transport* were overrepresented while *platelet activation*, *MAPK* and *Rap 1 signaling* pathways were among the detected KEGG pathways. In m/ISTB-EVs, *protein modification process*, and *decreased oxygen level responses* were among the perturbed GO: BP terms. *Alzheimer's* and *prion disease* were among the identified KEGG pathways. In sSTB-EVs, *post-translational modification processes* and *endoplasmic reticulum protein processing* were the principal GO: BP terms and KEGG pathways.

Recent research has shown that ischemic hypoxia and the release of proinflammatory cytokines in PE can cause protein misfolding and initiate endoplasmic reticulum (ER) stress due to hypoxia-reoxygenation damage to the endoplasmic reticulum(Gathiram & Moodley, 2016 [DOI](#)). PE can also cause posttranslational modifications to proteins, such as changing the isoelectric point, which results in different S-nitrosylation outcomes in placental proteins(Zhang et al., 2011 [DOI](#)). It is thought that the accumulation of these aggregates of unfolded protein response (UPR) or misfolded proteins contributes to the pathophysiology of PE(Gathiram & Moodley, 2016 [DOI](#)). Other processes and KEGG pathways have been previously described in preeclampsia, while others found in our study are new and may warrant further research(Lee et al., 2020 [DOI](#); Wan Shumei, Peng Ping, Qiao Lin, 2019).

## Ideas and Speculation

STB-EVs are liquid biomarkers with real-time information from the damaged placenta in PE due to their placenta-specificity. It would be interesting to test maternal plasma or serum samples for the four STB-EV indicators reported in our study.

It is unclear which comes first: misfolded proteins depositing in trophoblasts and preventing normal invasion, causing ischemia and endoplasmic reticulum stress, which leads to defects in trophoblast invasion, oxidative stress, and endothelial cell dysfunction, or the trophoblast invasion defects, oxidative stress, and endothelial cell dysfunction, or the misfolded proteins because of faulty invasion and oxidative stress. Further studies exploring these PE pathogenic processes would be intriguing.


## Strengths and limitations

Our study explored the difference in the proteome between PE and NP by analyzing the placenta and its extracellular vesicles. This study is one of the few to do so using extracellular vesicles obtained by a physiologic technique, the *ex-vivo* dual lobe placenta perfusion. However, the control population used for this study was not gestationally age-matched because it is impossible to obtain the ideal control for early onset PE patients. Also, our sample size was small (n of 12).

## Conclusion

Our study may have found novel STB-EV-bound protein indicators that are significantly more abundant in PE than normal. Since STB-EVs are present in the circulation from early pregnancy to term and are released more in PE, these STB-EV carried proteins may help with earlier diagnosis and mechanistic insights.

## Data Availability

All data produced are available online at <https://data.mendeley.com/datasets/j2x4h9ddcj/draft?a=8ccd383c-c6e5-495d-b66b-29c691608995> .

## Acknowledgements

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## Competing interests

The authors declare no competing interests.

## Supplemental Material

### Supplemental methods

#### Transmission electron microscopy

The Sir William Dunn School of Pathology was contracted to perform transmission electron microscopy. STB-EV pellets were diluted with fPBS to produce STB-EV solutions with concentrations ranging from 0.1 to 0.3 g/l. For 2 minutes, ten microliter of the STB-EV pellet solution was applied to freshly glowing discharged carbon formvar 300 mesh copper grids, blotted with filter paper, stained with 2% uranyl acetate for 10 seconds, blotted, and air-dried. The grid's STB-EV pellets are negatively stained to increase the contrast between the STB-EV pellets and the background. The grids were imaged with a Gatan OneView CMOS camera on an FEI Tecnai 12 TEM at 120 kV.

#### Nanoparticle tracking analysis

The Nanosight NS500 (instrument equipped with a 405 nm laser [Malvern UK]), sCMOS camera, and nanoparticle tracking analysis (NTA) software version 2.3, Build 0033 (Malvern UK) system was used for the analysis. Instrument performance was tested with silica 100 nm microspheres prior to sample analysis (Polysciences, Inc.). The samples were diluted in fPBS to a concentration to 1/100,000 based on the starting concentration. The samples were automatically injected into the sample chamber with a 1 ml syringe using the EV measurement script: prime, delay of 5, capture of 60, and repeat of 4. Camera images of the analyzed samples were captured at a level of 12. (Camera shutter speed; 15 ms and Camera gain; 350). NTA post-acquisition settings were optimized and maintained constant across samples. Each video recording was analyzed to determine the size and concentration profile of STB-EV.

#### Western blotting

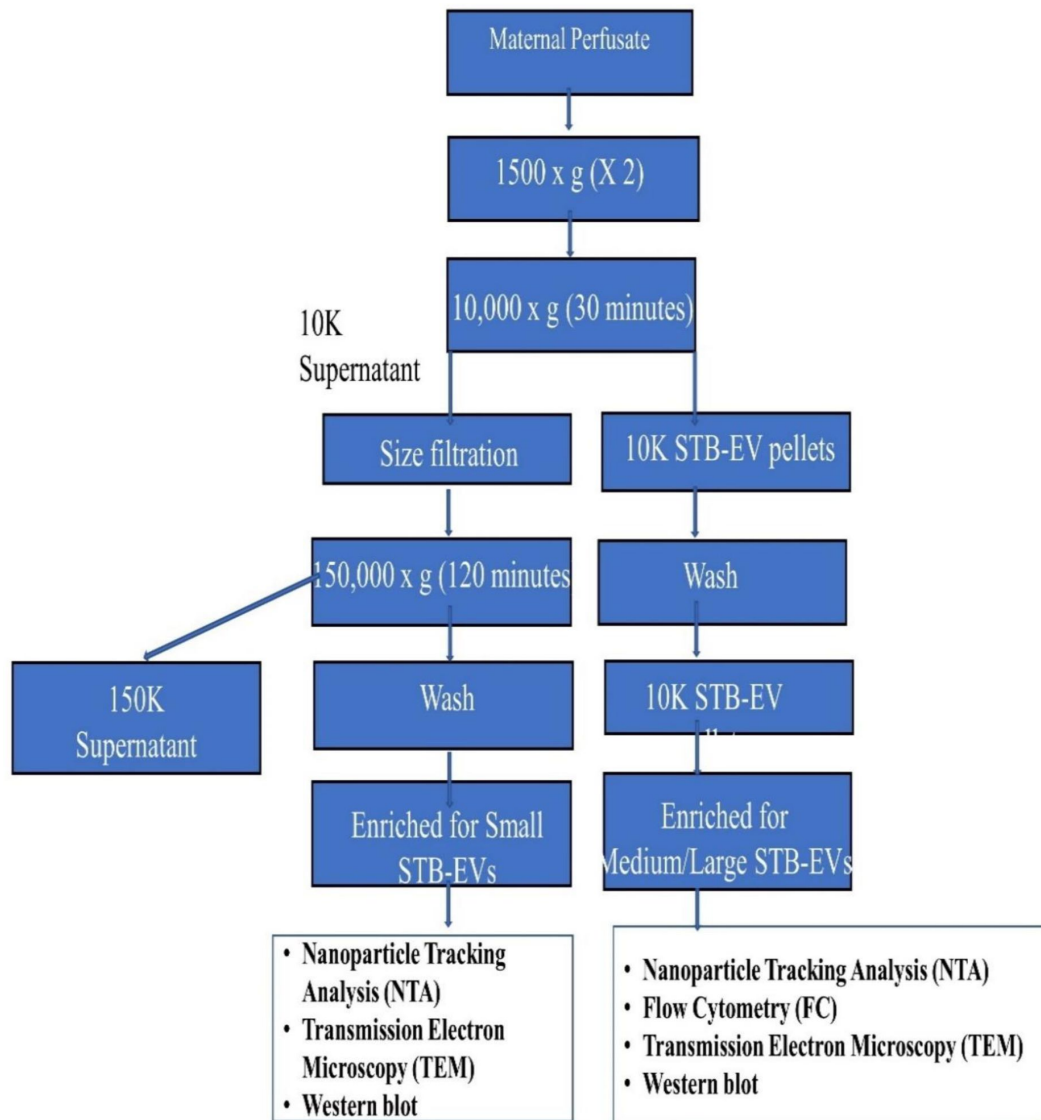
#### Bioinformatic analysis of proteomic data from placenta tissue, medium/large and small STB-EVs

Persus (Max Planck Institute of Biochemistry) was used for the analysis, along with the accompanying documentation and tutorials. To remove invalid data, we pre-processed the raw data by log transforming and filtering. Missing values were imputed at random from a normal distribution using the following parameters: width = 0.3 and downshift = 1.8. To ensure conformity to the normal distribution, the underlying distribution was visually inspected with a histogram before and after missing data imputation. The data was then transformed further by deducting each (transformed) value from the highest occurring protein expression value.

Principal component analysis (PCA), heatmaps, and Pearson correlation matrices were used to further investigate the data. The data was analyzed using the correlation index and hierarchical clustering. A two-sample independent student t-test was used to assess differential expression. Multiple testing was corrected using permutation-based false discovery rate (FDR) with the following parameters, with significance set at less than 0.05.

#### Functional enrichment of differentially expressed proteins (DEPs) in preeclampsia (PE)





### Supplemental figure 1.

Flow chart illustrating the steps involved in characterizing m/ISTB-EVs and sSTB-EVs obtained via differential ultracentrifugation (10,000 and 150,000 g) of maternal perfusate. Nanoparticle trafficking analysis, transmission electron microscopy, flow cytometry, and western blot were used to characterize the pellets from both spins.

Buffers	Power of hydrogen (pH)	Constituents
4X Laemmli reducing buffer	6.8	9 parts 4 X Laemmli buffer
		1 part 2-beta-mercaptoethanol
4X Laemmli non-reducing buffer		4 X Laemmli buffer
Anode buffer 1	10.4	300 mM Tris base (36.34 g/L)
		100 % methanol (20 mL/L)
		980 ml of ddH <sub>2</sub> O
Anode buffer 2	10.4	25 mM Tris base (3.0 g/L)
		100 % methanol (20 mL/L)
		980 ml of ddH <sub>2</sub> O
Cathode buffer	9.4	25 mM Tris base (3.0 g/L)
		40 nM 6-aminocaproic acid (5.2 g/L)
		100 % methanol (20 mL/L)
		980 mL of ddH <sub>2</sub> O
10 X TBS	8	NaCl (87.6g)
		Tris base (12.1g)
		700 ml of ddH <sub>2</sub> O
TBST	8	10 X TBS (100 mL)
		0.1 % Tween-20 (1 mL)
		900 mL of ddH <sub>2</sub> O
5% Milk TBST		5g of Blotto in 100 mL of TBST

**Supplemental table 1.**

**Reagents used for western blot.**

Antibodies	Concentration	Dilution	Antigen	Specificity	Manufacturer
Anti-PLAP (NDOG 2)	1.6 µg/µl	1/1000	PLAP	STB-EV	In house antibody
Anti-CD63	200 µg/µl	1/1000	CD63	STB-EV	Santa Cruz Biotechnology
Anti-ALIX	200 µg/µl	1/1000	ALIX	S STB-EV	Cell Signalling
Anti-Cytochrome C	200 µg/µl	1/500	Cytochrome C	Placenta homogenate	Santa Cruz Biotechnology
Anti-SR-BI	0.35 µg/µl	1/1000	SR-BI	N/A	Abcam
Anti-Filamin B	1.64 µg/µl	1/1000	Filamin B	N/A	Insight Biotechnology
Anti-PAPP-A2	1 µg/µl	1/2000	PAPP-A2	N/A	Abcam
Anti-Collagen 17 A1	1 µg/µl	1/1000	Collagen 17 A1	N/A	Abcam
Polyclonal goat-anti-mouse/rabbit immunoglobulin HRP		1/2000	Mouse and Rabbit Immunoglobulins	N/A	Dako UK Ltd

**Supplemental table 2.**

**Antibodies used for western blot.**

Biological Process (BP)	Adjusted P Values	No of Proteins in BP terms	No of DEP queried	No of DEP found in BP terms
<b>Negative regulation of neurotransmitter secretion</b>	<b>0.02</b>	<b>2</b>	<b>14</b>	<b>2</b>
<b>Negative regulation of synaptic vesicle exocytosis</b>	<b>0.02</b>	<b>2</b>	<b>14</b>	<b>2</b>
<b>Negative regulation of neurotransmitter transport</b>	<b>0.05</b>	<b>3</b>	<b>14</b>	<b>2</b>
Response to decreased oxygen levels	0.00	84	293	30
Cellular response to decreased oxygen levels	0.00	59	293	24
Cellular ketone metabolic process	0.00	65	293	26
<i>Peptidyl-asparagine modification</i>	<i>0.02</i>	<i>10</i>	<i>73</i>	<i>5</i>
<i>Protein N-linked glycosylation via asparagine</i>	<i>0.02</i>	<i>10</i>	<i>73</i>	<i>5</i>
<i>Protein N-linked glycosylation</i>	<i>0.02</i>	<i>16</i>	<i>73</i>	<i>6</i>

### Supplemental table 3

The top three functionally enriched gene ontologies are: biological process (GO: BP) Placenta (bold font), medium/large STB-EVs (normal font), and small STB-EVs (italics).

KEGG Pathways (KP)	Adjusted P Values	No of Proteins in KP terms	No of DEP queried	No of DEP found in KP terms
<b>Neurotrophin signalling pathway</b>	<b>0.00</b>	<b>13</b>	<b>14</b>	<b>3</b>
<b>Long-term potentiation</b>	<b>0.01</b>	<b>6</b>	<b>14</b>	<b>2</b>
<b>Renal cell carcinoma</b>	<b>0.02</b>	<b>11</b>	<b>14</b>	<b>2</b>
Proteasome	0.00	35	293	17
Spinocerebellar ataxia	0.00	48	293	21
Alzheimer disease	0.01	94	293	29
<i>Protein processing in endoplasmic reticulum</i>	<i>0.02</i>	<i>60</i>	<i>73</i>	<i>10</i>

### Supplemental table 4

The top three functionally enriched KEGG Pathways in the placenta, medium/large STB-EVs, and small STB-EVs (only 1 KEGG). Placenta (bold font), medium/large STB-EVs (normal font), and small STB-EVs (italics)

## Major Resources Table

### Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Dilution
PLAP	In house antibody		1.6 µg/µl	1/1000
CD63	Santa Cruz Biotechnology	sc-365604	200 µg/µl	1/1000
ALIX	Cell Signalling	#2171	200 µg/µl	1/1000
Cytochrome C	Santa Cruz Biotechnology	sc-13560	200 µg/µl	1/500
SR-BI	Santa Cruz Biotechnology	ab52629	0.35 µg/µl	1/1000
Filamin B	Insight Biotechnology	GTX387	1.64 µg/µl	1/1000
PAPP-A2	Abcam	ab59100	1 µg/µl	1/1000
Collagen 17A1	Abcam	Ab28440	1 µg/µl	1/2000
Mouse Immunoglobulins	Dako UK Ltd	P044701	1 µg/µl	1/2000
Rabbit Immunoglobulins	Dako UK Ltd	P044801	1 µg/µl	1/2000

## Flow cytometry resources

Markers	Fluorochromes	Clone	CAT	Isotype	Dilution / Concentration
CD41	PE Vio770	REA386	130-105-562	REA	1 in 50 for dump channel analysis.
CD235	PE Vio770	RAE-175	130-100-258	REA	
HLA Class-I (ABC)	PE Vio770	REA230	130-101-460	REA	
HLA Class-II (DRDPDQ)	PE Vio770	RAE-332	130-104-828	REA	
REA control				REA	
CD41	Pacific blue	HIP8	303713	Mouse IgG1	1 in 100
CD235	Pacific blue	HI264	349107	Mouse IgG2a	1 in 100
HLA Class-I (ABC)	Pacific blue	W6/32	311417	Mouse IgG2a	1 in 100
PLAP	PE	Mouse mAb	N/A	IgG1	0.2mg/ml
IgG2a isotype control	Pacific blue	MOPC-173	981904	IgG2a	1 in 500
IgG1 isotype control	Pacific blue	MOPC-21	400131	IgG1	1 in 200
bioM	FITC	NA	NA	NA	0.5-1nM

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## Article and author information

### Toluwalase Awoyemi

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

ORCID iD: [0000-0003-1542-9435](https://orcid.org/0000-0003-1542-9435)

### Shuhan Jiang

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

### Bríet Bjarkadóttir

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Maryam Rahbar**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Prasanna Logenthiran**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Gavin Collett**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Wei Zhang**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Adam Cribbs**

Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom

ORCID iD: [0000-0001-5288-3077](https://orcid.org/0000-0001-5288-3077)

**Ana Sofia Cerdeira**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**Manu Vatish**

Nuffield Department of Women's & Reproductive Health, University of Oxford, Oxford, United Kingdom

**For correspondence:** [Manu.vatish@wrh.ox.ac.uk](mailto:Manu.vatish@wrh.ox.ac.uk)

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**Editors**

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**Reviewer #1 (Public Review):**

The authors primary objective in this study was to identify differences between patients with preeclampsia and normal patients with respect to the placental syncytiotrophoblast extracellular vesicle proteome.

A strength of this study is that the authors identified novel STB-EV protein markers that are more abundant in the placenta of patients with preeclampsia compared with normal controls. This contributes a little more to what is already known about STB-EV markers and preeclampsia. If these markers can be shown to be more abundant in maternal plasma of preeclampsia patients, it would be very useful for identifying patients who are at high risk for developing early-onset preeclampsia.

Weaknesses include:

- (1) The small sample size. There were only 6 patients in the study group and 6 normal controls. However, this can be considered as a pilot study.
- (2) The normal controls were not matched with the study patients and the authors did not state how the controls were selected.
- (3) The authors state that the placenta samples were obtained at the time of elective cesarean section. However, it is likely that all the preeclampsia patients were delivered for clinical indications rather than electively. This should be clarified.

<https://doi.org/10.7554/eLife.88841.2.sa1>

**Reviewer #2 (Public Review):**

Summary:

Preeclampsia is a disorder of pregnancy that affects 4-5% of pregnancies worldwide. Identifying this condition early is clinically relevant as it will help clinicians to make management decisions to prevent adverse outcomes. The placenta holds a key to many pregnancy-related pathologies including preeclampsia and studies have shown many differences in the placenta of women with preeclampsia as compared to controls. However as the placenta cannot be collected directly during pregnancy, the exosomes secreted by it are considered a good alternative to tissue biopsy. In this study, the authors have compared the proteins in different sizes of exosomes from the placenta of women with and without preeclampsia. The idea is to eventually use these as biomarkers for early detection of preeclampsia.

Strengths:

The novelty factor of this study is the use of two different-sized exosomes which has not been achieved earlier.

Weaknesses:

The study measured the proteins at only a single time point after the disease has already occurred. However, the placenta is an ever-changing tissue throughout pregnancy and different proteins can come up at different times in pregnancy. Thus serial measurements are necessary and a single time point measurement. The has not validated the identified biomarkers in plasma or circulating placental exosomes from women with and without preeclampsia. Thus the utility of these findings in real-life situations can not be judged from this work.

<https://doi.org/10.7554/eLife.88841.2.sa0>

## Author Response

The following is the authors' response to the original reviews.

### Reviewer #1

*(1) Since you only included patients with early-onset preeclampsia in the study, I suggest revising the title to "Identification of novel syncytiotrophoblast membrane extracellular vesicle derived protein biomarkers in early-onset preeclampsia...."*

We have changed our title to early-onset preeclampsia.

*(2) Under methods, you state that placenta was obtained from women undergoing elective cesarean section. Was this because all the study patients were delivered before the onset of labor? Or were laboring patients specifically excluded from the study?*

Indeed, labor influences the extracellular vesicles (EVs) generated. To ensure consistency in our samples and avoid this variable, we chose placentas obtained from elective cesarean sections (CS) for our study.

*(3) In Table 1 on page 10, the 8th row (Birth weight grams) needs to be reformatted. The mean birthweights for normal pregnancy and preeclampsia should be the same.*

We have reformatted the table and using ranges instead of brackets.

*(4) In the legend for Table 1, the sentence beginning on page 10, line 227, and continuing onto page 11, line 228, does not make sense. Part of the sentence was omitted inadvertently.*

We have modified this sentence to :

Detergent treatment, which could break down EVs, with NP-40 confirmed that the majority (99%) of our samples were largely vesicular since only  $0.1 \pm 0.12\%$  of BODIPY FL N-(2-aminoethyl)-maleimide and PLAP double-positive events were detected (a reduction of 99%) (Figure 1E and 1H).'

*(5) As you acknowledge, the sample size (12 patients) was small. This is understandable because early-onset preeclampsia occurs in <1% of parturients. You could collaborate with other centers in future studies to increase the sample size.*

Thank you very much for your comment. We are willing to cooperate on future research and will try to expand our sample size in subsequent studies.

### Reviewer #2 (Recommendations For The Authors):

*(1) This is one of the many "catalogue" papers where placental exosome proteins in preeclampsia are profiled. Thus, the manuscript lacks novelty. The only novelty factor is the authors have isolated exosomes by a different method and even separated the small and large exosomes. However, there is no mention of how these exosomes differ from each other in terms of their functionality. Thus it is hard to judge the biological significance of this work.*

We appreciate your insights regarding the novelty of our study. While numerous papers have profiled placental exosome proteins in preeclampsia, our methodology for enriching sSTB-EVs (exosomes) offers a distinct perspective. We believe that the separation of sSTB-EVs

(exosomes) and medium/large STB-EVs (microvesicles) introduces a differentiation that extends beyond mere profiling, with implications for their functionality. There are previous studies showed that the different sizes of placenta EVs have distinct characteristics (Zabel RR, et al. Enrichment and characterization of extracellular vesicles from ex vivo one-sided human placenta perfusion. *Am J Reprod Immunol*. 2021 Aug;86(2)). Furthermore, the way cells internalize and respond to EVs may depend on the size of the EV (Zhuang X et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther*. 2011 Oct;19(10).) Therefore, it would be important for future studies to distinguish different sizes of EVs for the research.

*(2) The authors must demonstrate that these two types of EVs are also produced in vivo by detecting them in the serum of women.*

Thank you for the comment. Many previous studies have shown the two types of placental EVs in women's blood. Nakahara et al.'s (PMCID: PMC7755551) extensive review compiles studies that have specifically isolated various subtypes of placenta-derived EVs from maternal circulation. We have also readdressed it in the introduction.

*(3) The authors must compare the proteomes of serum-derived placental exosomes and the proteome of the STBs isolated from the perfusion experiments to judge how overlapping the outcomes are from those produced naturally and those produced under ex vivo conditions.*

We appreciate the reviewer's suggestion to compare the proteomes of serum-derived placental sSTB-EVs (exosomes) with those from STBs isolated through perfusion experiments. Indeed, such a comparison would provide valuable insights into the similarities and differences between naturally produced and ex vivo-generated sSTB-EVS (exosomes). However, isolating placental EVs from maternal circulation for comprehensive proteomic profiling presents challenges. It requires a significant amount of serum or plasma sample that will be sufficient to enable the isolation of placenta-specific EVs amongst numerous EVs in the circulation. In addition, it will require multiple intricate steps such as ultracentrifugation followed by immunoprecipitation. Each of these steps can potentially lead to the loss of EVs. Additionally, given the high concentration of lipoproteins in plasma relative to EVs, there's a significant risk of obtaining low-purity isolates from the outset. These challenges might compromise the comparability of results between placenta-specific EVs from maternal circulation and those from ex vivo perfusion. Nevertheless, we acknowledge the value of such an endeavor and will consider incorporating this aspect in future studies as the EV and proteomic methodology and technology improve and become more sensitive.

*(4) I have a major issue with the chosen study subjects. While the study title and the manuscript mention preeclampsia, as per the inclusion criteria mentioned in lines 88-90, the patients will be HELLP syndrome. Please clarify what was used and modify the manuscript accordingly.*

Thank you very much for finding this error. Our patients had none of the features that would qualify them for HELLP syndrome. We have edited to:

PE was defined as new (after 20 weeks) systolic blood pressure of 140 mmHg or diastolic pressure of 90 mmHg, proteinuria (protein/creatinine ratio of 30 mg/mmol or more). None of our patients had maternal acute kidney injury, liver dysfunction, neurological features, hemolysis, or thrombocytopenia.

*(5) It is hard to reconcile how only 15 proteins were identified in the placental extract while 300+ in EVs. There is a methodological issue in the mass spec or extraction. With*



*such widely different denominators in the total proteins identified, it is hard to compare the outcomes in terms of the three sample types.*

We acknowledge the reviewer's concerns regarding the disparity in protein counts between the placental extract and the EVs. Ultimately, more is not necessarily better. Several factors might contribute to this discrepancy. Firstly, it is plausible that certain proteins exhibit selective affinity to varying sizes of EVs, leading to a more diverse range of proteins than the placental extract. We were also stringent in our analysis to enable us to select proteins whose biological differences are more likely to be reproducible with a different validity method like a western blot. Additionally, although the placental extract might contain a higher total protein concentration, it doesn't necessarily translate to a richer diversity of disease-specific proteins. Considering these nuances when comparing protein outcomes across sample types is helpful.

*(6) I am unable to understand the terms least differentially expressed and most differentially expressed. Do the authors mean upregulated and downregulated? Please clarify and use the terms appropriately by providing fold change values.*

We appreciate the reviewer's request for clarification. We intended to provide a relative measure of expression for the terms 'least differentially expressed' and 'most differentially expressed'. The terms are roughly equitable to down- and upregulated. Regarding EVs, we avoid using the terms 'upregulated' and 'downregulated' as EVs act as transporters and do not possess regulatory functions per se. However, for the placenta, we recognize the relevance of these terms.

*(7) The data presented is very superficial and lacks methodological details. The authors should provide the total number of targets achieved after mass spec. The cutoffs used the FDRs and other details.*

We apologize for the omission. We have added these details to the method section.

*(8) It is not clear how were these differentially abundant proteins identified. What was the cutoff used? Was it identified in all the replicates?*

We apologize for the omission. We have added these details to the method section.

*(9) How many samples were subjected to the discovery cohort, and how many were in the validation cohort? Were they the same or different? If the samples were different, how many PE samples had differentially abundant proteins by both methods?*

The study utilized 12 samples for initial discovery and another 12 for western blot validation. The validation samples specifically targeted proteins of interest, rather than undergoing another comprehensive mass spectrometry analysis.

*(10) It is striking that the authors report the expression of prostatic acid phosphatase in the placenta. In my understanding of placental biology, this gene or protein is not known to be expressed by the placenta. Please perform immunofluorescence to demonstrate that this protein is indeed produced in the STBs*

Research has revealed that even though it's called prostate-specific antigen, it's created in tissues other than the prostate, such as the placenta. Here are a couple of references to support this claim: PMID: 10634405, PMID: 7533063, PMID: 8939403, and PMID: 8945610. Hence it is likely not beneficial to demonstrate what many researchers have already demonstrated.

*(11) Please validate the differential abundance of these proteins in the exosomes isolated from the plasma of women with and without preeclampsia. A serial measurement will be of high value to determine how early as compared to hypertension, these biomarkers can predict preeclampsia.*

We are validating each EV-carried marker individually in the circulation (plasma or serum), localizing them in the placenta, and performing downstream functional analysis. This article is already lengthy and would likely be too cumbersome to include the details of all individual proteins in this manuscript. However, we have already published papers on Siglec 6 (PMID: 32998819) and Neprilysin (PMID: 30929513), and others will be published soon. We agree that there will be a lot of value to serial measurement, not just in terms of how early as compared to hypertension, these biomarkers can predict preeclampsia but also as potentially a more sensitive or specific test. This would be the subject of subsequent papers.

*(12) The authors are recommended to carry out immunofluorescence to localize the differentially abundant proteins in the placental sections and show that they are specific to STBs.*

We have already provided a similar response earlier (see response to point 11). In addition, while it is preferable, the biomarkers don't necessarily need to be specific to STB. Not all biomarkers are mechanistic agents/targets, and not all mechanistic agents are biomarkers. However, mechanistic agents should preferably be placental-specific. For example, the total sFLT1, the most studied biomarker, is not exclusively synthesized in the placenta, even though the placental-specific isoform represents a small fraction of the total sFLT-1. For example, in the non-placental world, alkaline phosphatase (ALP) is not exclusively produced by the liver but is a 'biomarker' of cholestatic disease.

*(13) Table 1 should give the range and SD could be given as + instead of the bracket.*

Thank you for your suggestion. We have edited it accordingly.

*(14) It is necessary to provide the gestational age of the onset of hypertension to get a judgment of how long these women were preeclamptic, culminating in HELLP.*

We want to emphasize that none of our patients experienced HELLP syndrome. In the results section, we have included the gestational age at the time of diagnosis in the table for preeclampsia. It's crucial to understand that the gestational age at diagnosis is distinct from the gestational age when hypertension initially appeared. Detecting the exact gestational age of hypertension onset would be challenging, and it would likely require a prospective or randomized clinical trial with continuous monitoring, possibly on a daily basis. However, our study is retrospective. Thus we can only comment on the gestational age at diagnosis

*(15) For newborns the term Sex is used and not gender*

Thank you for your suggestion. We have edited it accordingly.

*(16) Figure 2 is stretched and hard to read*

Thank you for your suggestion. We have edited it accordingly by creating two separate images to promote readability.

*(17) Line 278 change the sentence "there fifteen (15) proteins in the placenta" to "there were fifteen (15) proteins in the placenta"*

Thank you for your suggestion. We have edited it accordingly.

| (18) Line 288 you mean *least* and not *lease*

Thank you for your suggestion. We have edited it accordingly.