

Syncytiotrophoblast Derived Extracellular Vesicles in Relation to Preeclampsia

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Abstract

The syncytiotrophoblast, a fused single-cell layer between mother and fetus, constitutively releases extracellular vesicles (STBEV) directly into the maternal circulation. STBEV contain a variety of proteins and RNA which can be targeted to specific cells. In preeclampsia, asymptomatic placental oxidative stress is a precursor to later multi-organ dysfunction in the mother. Increased STBEV release in preeclampsia is considered a manifestation of syncytiotrophoblast stress, which may play a key role in signaling between fetus and mother. STBEV release in preeclampsia changes, both in terms of volume and content. In this review, we outline the latest advances in STBEV isolation and detection. We consider evidence for differential STBEV release, protein cargo and RNA content in preeclampsia, highlighting common pitfalls in study design. We summarise studies to date demonstrating STBEV actions on target cells. Ultimately, we consider how STBEV fit into the pathophysiology of the heterogeneous syndrome of preeclampsia. The key unifying concept in early- and late-onset preeclampsia is syncytiotrophoblast stress. We submit that STBEV are the key stress signal in preeclampsia. We believe that further investigation of STBEV release, content, and actions may offer valuable insights into preeclampsia pathophysiology and potential new clinical diagnostics and therapeutic targets.

Keywords: Biomarkers; Diagnostics; Extracellular vesicles; Placenta; Preeclampsia; Signalling; Stress; Syncytiotrophoblast

Understanding preeclampsia (PE)

PE is a syndrome unique to pregnancy, affecting one in 20 women.¹ It is characterized by new onset maternal hypertension after 20 weeks of gestation associated with multi-organ dysfunction. Typically, this can result in renal impairment with proteinuria, hepatic dysfunction with associated clotting abnormalities, hemolysis, reduced platelet counts, cardiovascular remodeling, seizures (eclampsia) and, in extreme cases, maternal death. At the cellular level, endothelial dysfunction is a cardinal feature of PE. The fetus is also affected by the consequences of placental dysfunction, with growth restriction and intrauterine death being correlates. Treatment is supportive; the only cure is delivery of the placenta, making iatrogenic preterm birth a common consequence (though PE can continue or indeed first present in the postpartum period).

The placenta is the cause of PE. This is demonstrated most simply by the observation that women with molar pregnancies (where no fetal tissue exists) can still develop

PE.² Understanding the pathophysiology of PE is a substantial challenge for several reasons. As with all syndromes it is defined by consensus not understanding; individual features of PE are non-specific, but when they occur together (in various combinations), a diagnosis is made and treatment initiated. Moreover, PE occurs as early-onset disease (usually defined as needing delivery <34 weeks of gestation), which is more severe, resulting in substantial preterm neonatal morbidity; late-onset disease (≥34 weeks) is milder and clinical intervention can influence diagnosis. Some researchers now regard the two syndromes to be different diseases.¹ Indeed, other obstetric syndromes may be part of the same spectrum, including intrauterine growth restriction, placental abruption, stillbirth, gestational diabetes, and preterm birth.³

Redman first proposed the “two stage model” of early-onset preeclampsia (EOPE), shown in Figure 1.⁴ During normal placentation (weeks 8–18), the invading extra-villous cytotrophoblast in the placental bed remodels the smooth muscle in uterine spiral arteries, creating high-volume, low-pressure perfusion of the intervillous space where there is interchange between the maternal and fetal circulations. In the first symptomless “preclinical” stage of EOPE, a failure of spiral artery remodeling results in increased resistance in placental and uterine arteries and so creates an oxidatively stressed placenta. In the second “clinical” stage, diffuse maternal endothelial dysfunction affects multiple organs leading to the clinical features of PE. A parallel model of late onset PE (LOPE) suggests that villous overcrowding and placental senescence are the first-stage source of oxidative stress which results in subsequent clinical manifestations of PE.⁵ A blood-borne factor has long been sought as the stress signal linking the two stages of PE.

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Maternal-Fetal Medicine (2021) 3:2

Received: 21 December 2020

<http://dx.doi.org/10.1097/FM9.0000000000000093>

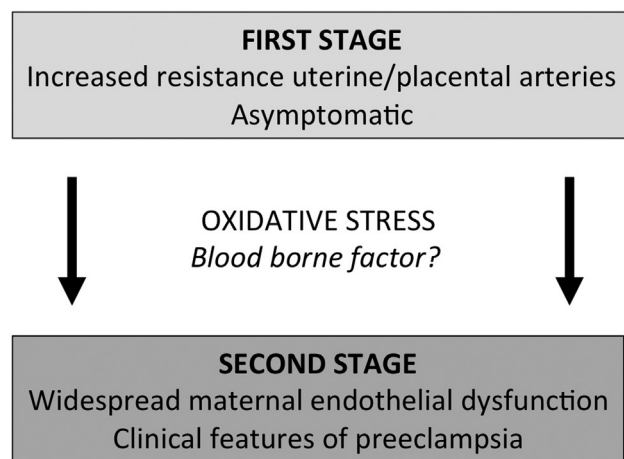


Figure 1. The two stage model of preeclampsia.

Altered levels of placental derived circulating angiogenic factors are an important manifestation of placental stress in PE.⁶ Soluble fms-like tyrosine kinase (sFlt-1) is released by the placenta into the maternal circulation in greater amounts in PE. It acts as a decoy receptor, binding vascular endothelial growth factor and placental growth factor (PlGF), causing inactivation of important factors that maintain endothelial health, hence resulting in endothelial dysfunction. In animal models, administering sFlt-1 induces the PE phenotype, which can subsequently be rescued by PlGF.⁷ sFlt-1:PlGF ratios have transformed clinical diagnosis of EOPE,⁸ however they are not the single causative agent in PE. Dextran-sulfate plasma apheresis to remove sFlt-1 from women with PE has had mixed results.^{9,10} Moreover, sFlt-1:PlGF is less able to differentiate LOPE from normal pregnancy.⁶ This is probably because of the changing background of normality, since advancing pregnancy is associated with increasing development of PE only curtailed by inevitable delivery around term.⁵ Syncytiotrophoblast derived extracellular

vesicles (STBEV) have demonstrated significant potential as a more complex and refined stress signal from placenta to mother in PE.

Understanding extracellular vesicles

Most cells release extracellular vesicles (EVs) of which there are three main types: microvesicles (0.1–1 μ m) that bud directly from the external cell membrane, smaller exosomes (around 100 nm) which are secreted from multivesicular bodies within the cell and the largest, apoptotic bodies (0.5–5 μ m), which are products of cells undergoing programmed cell death. Constitutive release, mainly of exosomes, facilitates physiological intercellular signaling to maintain unstressed cellular and tissue homeostasis. EVs are released as part of cellular stress responses. For example, increases in intracellular cell calcium, a ubiquitous intracellular second messenger is a non-specific stress response, which potently provokes shedding of microvesicles from the external cell membrane. Vesicular cargo is actively enriched in certain types of molecules: signaling proteins, microRNA (miRNA), messenger RNA (mRNA), and lipids (see Fig. 2). To transport this cargo to surrounding cells implies a docking mechanism to facilitate specific interactions with recipient cells and allow internalization of the cargo in a bioactive form. This involves molecules enriched at the surface of EVs engaging with specific receptors at the plasma membrane of recipient cells.^{11,12} Exosomes are too small to study by conventional microscopy or flow cytometry. Microvesicles are easier but may be confused with non-vesicular microparticles such as lipoproteins or chylomicrons, in plasma or serum for example. Within the circulation platelet microvesicles are the most numerous by one order of magnitude.

Syncytiotrophoblast releases all three categories of EV into the maternal circulation (STBEV). Apoptotic STBEV are filtered in the maternal pulmonary microcirculation so can only be detected in uterine vein blood. Under all circumstances STBEV are a small part of the total

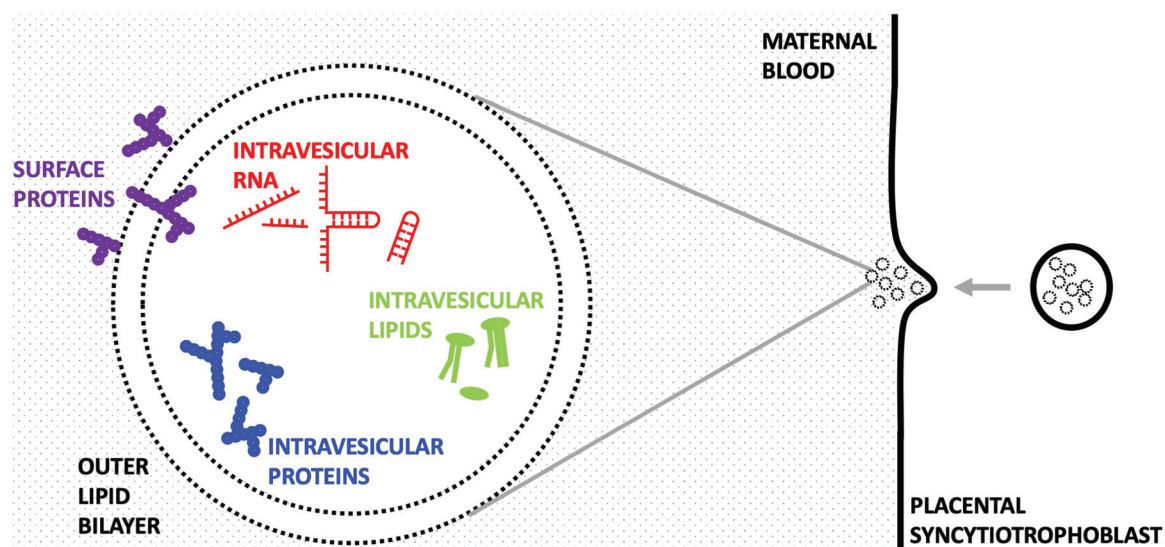


Figure 2. Schematic diagram showing release, structure, and content of syncytiotrophoblast-derived extracellular vesicles.

circulating burden of EVs. Whereas they are potentially living biopsies of the placenta from the tissue that is known to stimulate the maternal syndrome, there are substantial technical challenges before they can be used clinically in that capacity. In the ensuing sections we summarise latest advances that characterize their detection and function.

STBEV in pregnancy and PE

The syncytiotrophoblast is the outermost layer of the placental villi, a fused single-cell barrier between maternal blood and the developing fetus.¹³ At term, the syncytiotrophoblast is estimated to have a surface area of about 10 m².¹⁴ EVs were first isolated from the placenta as an experimental model to understand the immunological properties of the syncytiotrophoblast in 1974.¹⁵ Placentas were agitated in cold water to create microvillous fragments, a technique still currently in practice.¹⁶ These artificial particles were subsequently discovered to be similar to those circulating in vivo in the plasma of pregnant women by Knight *et al.* in 1998 and were termed STBEV.¹⁷

Knight's seminal paper isolated EV from plasma using differential centrifugation, and quantified STBEV using immunoassays and flow cytometry, using a panel of known syncytiotrophoblast surface proteins. STBEV levels were higher in uterine vein plasma compared to peripheral plasma, confirming their placental origin. Comparisons were made between plasma from women with EOPE and normal pregnancy. There were significantly higher levels of STBEV in the EOPE patients; these levels increased between diagnosis and delivery.

STBEV are present in early pregnancy plasma from 6 to 12 weeks; at term the placenta releases an estimated 3 g of STBEV over 24 hours.¹⁸ STBEV offer potential diagnostic insights, being referred to as "circulating biopsies" of the distressed placenta.¹⁹ Surface proteins target STBEV to specific cells, where they dock and can transfer their proteins and RNA to the recipient cells to initiate cascades of functional changes. STBEV represent a complex stress signal in PE, originating directly from the placenta and reaching the full spectrum of dysregulated maternal tissues in PE.

Challenges in studying STBEV

Source material

Samples that best represent in vivo conditions are the most challenging to acquire, and consequently feature in relatively few studies. Ex vivo, the richest source of STBEV is blood from the uterine vein. This can only be taken at the time of cesarean section, which limits experimental design. The most commonly used ex vivo material is peripheral venous blood, usually taken from the antecubital fossa. STBEV in these samples are a different population: they are outnumbered 100:1 by EV from platelets, erythrocytes, leucocytes, and other tissues.^{20,21} Assuming STBEV are destined for specific target cells, it is reasonable to assume that many of the most functionally active STBEV reach their target organ (eg, endothelium, heart, kidney, liver, circulating blood cells) before they reach the venous circulation. The yield of STBEV from

peripheral plasma is poor; other models are needed to obtain STBEV in sufficient quantities to analyze.

We favor dual-lobe placental perfusion as the most physiological ex vivo source of STBEV.²² Fetal vessels from a freshly delivered placenta are cannulated and perfused; the maternal surface is perfused with medium through small tubes placed in the substance of the placenta. STBEV are collected in the maternal perfusate. There are limitations to this model: the placenta is post-partum meaning disease is often end-stage; gestational age-matched samples from healthy controls are impossible to obtain for preterm disease. Nevertheless, these models have been well validated in plasma samples, making them a valuable resource.^{23,24} Cultured placental explants are also extensively used to derive STBEV.²⁵ Villous samples are taken after cesarean section and bathed in culture medium, from which EV are isolated. In addition to the limitations identified for placental perfusion, the contact between culture medium and non-syncytial trophoblast layers in this model must be considered. The more historic mechanical isolation techniques already discussed are less physiological than perfusion or explant models, but can still be useful. The most straightforward (and least valid) source of STBEV are immortalized cell lines, such as BeWo cells. Whilst commonly cited, clearly caution should be used when extrapolating findings from an immortalized cultured cell to human disease.

Isolation and detection of STBEV

STBEV can be isolated and detected by their size, density, or by their characteristic surface proteins using immune-affinity purification. A recent systematic review identified 1038 unique protocols used to retrieve EV from biofluids.²⁶ The earliest studies used differential ultracentrifugation to separate STBEV by size; modifications exploited particle density/buoyancy using density gradients (eg, sucrose). Newer techniques include size-exclusion chromatography, sequential membrane filtration, precipitation using proprietary polymers (eg, Exo-Quick), and proprietary membrane-based affinity binding (eg, ExoRNeasy).²⁷ Immune-affinity techniques involve extracting EV using antibody-coated beads or lateral flow assays.²⁸ These two properties can be combined in technologies such as the ExoCounter, which uses capture antibodies in wells with size limits.²⁹ Downstream detection assays dictate the optimal isolation techniques. For example, larger STBEV can be counted in neat plasma using immune devices such as flow cytometers²⁰; alternatively RNA yield is better when affinity binding is used compared to differential centrifugation.²⁷

Detection techniques include electron microscopy, nanoparticle tracking analysis (NTA) or immune-detection techniques such as enzyme-linked immunosorbent assay (ELISA) or flow cytometry. Optimal detection depends on the source biofluid, the isolation techniques, the size and surface proteins of the EV to be studied, and the background of EV from cells, other than syncytiotrophoblast, which are not the focus of interest. For example, a combination of physical and immune-affinity properties is often used to quantify STBEV in plasma samples, yet no group has been able to isolate STBEV from plasma for use in downstream experiments such as RNA

isolation. Total plasma or serum EV are often misleadingly labeled as STBEV, despite the fact that other blood cell-derived vesicles far outnumber their sparse STBEV content. Placental alkaline phosphatase (PLAP), a unique marker of the syncytiotrophoblast, is often used to quantify STBEV. However, populations of PLAP-negative STBEV have been isolated from placental perfusion models.³⁰ Moreover, PLAP expression on PE STBEV is reported to be lower than on healthy pregnancy STBEV, suggesting that PLAP ELISA alone might underestimate STBEV in PE.³¹ Caution and critical thinking are required when appraising studies of STBEV.

STBEV release in PE

Studies quantifying plasma STBEVs in PE are summarised in Table 1. Their findings can seem contradictory and depend significantly on disease definition (eg, EOPE *vs.* LOPE), sample demographics, isolation methods, and detection techniques: different EV populations are being detected in each paper. Several studies use NTA to quantify total plasma EV. NTA is a technique that determines the size of nanoparticles by their Brownian motion in suspension. It uses ultramicroscopy to detect refracted of light from nanoparticles, under laser illumination. It cannot distinguish membrane bound vesicles from other biological nanoparticles such as liposomes and lipoproteins, which are of a similar size. Hence circulating post-prandial chylomicrons confound the results. Standardized sampling in fasting subjects is to be preferred.^{32–34} Some studies are underpowered; few contain a power calculation.^{21,35} There is consensus that both small and medium-large STBEV release in EOPE is greater in women with PE than gestation-matched controls; most studies report that total plasma EV release is also increased. Pillay *et al.* go further and demonstrate that proportionally small STBEV are increased less in early-onset PE than other plasma EV.³⁶ The significance of this finding is uncertain, but it clearly demonstrates the challenges scientists face in isolating the STBEV signal.

The picture in late-onset PE is much less clear: some studies find total plasma EV and plasma STBEV increase in PE, some find they are reduced. This could represent differential release of smaller and larger STBEV; it could simply be a consequence of differences in experimental design; there are insufficient studies to be certain. A challenge in studying LOPE is that the control “normal” placenta is no longer normal, but senescent.⁵ STBEV release is a manifestation of cellular stress; if control and disease samples are both stressed then inconsistent results between studies are to be anticipated.

If it is a challenge to confidently quantify STBEV release in PE it is even more difficult to elucidate the controlling mechanisms. We previously found that STBEV release is increased in labor, which might explain the postpartum worsening of maternal symptoms seen in some women with PE.³⁷ We postulated that STBEV are released in response to the hypoxia and oxidative stress induced by labor. Other studies consider that actin may play a role in STBEV release. Supplementation with plasma gelsolin, an actin scavenger, limits STBEV release in placental explants.³⁸ Upregulation of RhoB/ROCK, regulators of actin rearrangement, increased STBEV release in vitro;

moreover RhoB/ROCK knockdown reduced STBEV release in a hypoxic model of PE.³⁹ Vitamin D supplementation has been shown to reduce ROCK1 activation in trophoblast cultures, which could contribute to the clinically preventative effect of vitamin D supplementation in PE.⁴⁰ Substantially more work is needed to confirm this theory.

STBEV proteins in PE

Two methods have been employed to determine proteins expressed by STBEVs: measurements of specific proteins (which test hypotheses) or proteomic analyses (which generate hypotheses). The key studies are summarised in Table 2. Some dysregulated proteins in PE STBEV can be linked intuitively to its pathophysiology: for example, downregulated endothelial nitric oxide synthase in relation to endothelial dysfunction or upregulated neprilysin or sFlt-1 in relation to vasoconstriction. In our study of reduced eNOS expression, the findings could be confirmed in STBEV obtained from multiple sources (placental perfusion, peripheral plasma, uterine vein plasma), offering a high degree of confidence in differential expression in vivo.²³ Specific proteins that are increased in PE might be valuable as biomarkers. So far, only sFlt-1 has been shown to have clinical utility⁸; it is reviewed extensively elsewhere.⁶

In three proteomics studies, differentially regulated proteins have been sought in PE. It is important to scrutinize the design of these studies before accepting the findings. If the goal is to understand STBEV actions, then samples from the uterine vein best represent the signal as it leaves the placenta; the best experimental model would be dual-lobe placental perfusion. If the goal is biomarker discovery, then analysis of peripheral blood with minimal processing is the most appropriate: this is most applicable when development of a subsequent clinical assay is anticipated. In two proteomics studies, STBEV from placental explants have been investigated, to compare PE placentas to normal.^{41,42} In the first study 421 proteins were identified in STBEV, of which 25 were differentially expressed in PE⁴¹; none of these results have so far been validated by Western blotting or ELISA. The other identified 3317 proteins, of which 194 were differentially expressed⁴²; just four of these were validated by Western blotting of STBEV derived from explants. Pathways identified were broad, including: cell death, cell organization, immune response, lipid and carbohydrate metabolism, glycolysis, endothelial dysfunction, intercellular junctions, and ubiquitination. These studies demonstrate both the complexity of the STBEV signal in PE, and the need for further work to translate the findings to clinical diagnostics. A third study used cholera toxin B and annexin V antibodies, which bind to components of the EV lipid bilayer, to pull out plasma EV.⁴³ Samples from women with EOPE and healthy controls were compared, with the goal of biomarker discovery. The vesicles separated by the two different methods differed substantially in their cargos. Their sizes were not determined. Moreover, as discussed previously, STBEV would be a small proportion of all the plasma EV in these samples. Nevertheless, for discovery alone, the study identified 104 proteins unique to EOPE vesicles from annexin V and 87

Table 1**Studies quantifying difference in peripheral plasma STBEV load in preeclampsia.**

Mean PE gestation (weeks)	Control gestation (weeks)	EV isolation technique	EV identification technique	Size of EV isolated	All plasma EV detection assay	All plasma EV (PE vs. normal)	STBEV detection assay	STBEV (PE vs. normal)	References
33.3	33.1	Centrifuged at 150,000 × g for 45 minutes	None	Mixed: mostly medium-large	—	—	ELISA using two PLAP antibodies (H317 and NDO62)	6.3 ng protein/mL more in PE ($P=0.01$)	17
30.9	32.7	Centrifuged at 150,000 × g for 45 minutes	None	Mixed: mostly medium-large	—	—	PLAP ELISA using NDO62 antibody	More in PE ($P=0.005$)	35
35.5	32.7	Centrifuged at 150,000 × g for 45 minutes	None	Mixed: mostly medium-large	—	—	PLAP ELISA using NDO62 antibody	More in PE (non-sig)	35
33.5	33.4	Centrifuged at 150,000 × g for 45 minutes	None	Mixed: mostly medium-large	—	—	PLAP ELISA using NDO62 antibody	More in PE ($P<0.01$)	62
28,32,36	Matched	Centrifuged at 18,890 × g for 30 minutes	None	Medium-large	Flow using annexin-V antibody	Reduced in PE ($P=0.04$)	Flow using STB antibody (ED822)	More in PE (non-sig)	21
33.5	33.9	Centrifuged at 14,000 × g for 90 minutes	None	Medium-large	Flow using annexin-V antibody	3.56 μL more in PE ($P=0.004$)	Flow using STB antibody (NDO62)	1.37 μL more in PE (non-sig)	72
30.5	30.8	Centrifugation at 110,000 × g for 2 hours, then using sucrose gradient	NTA, TEM, Western blot for CD63	Small	NTA and CD63 ELISA using ExoELISA™ commercial kit	7.65 × 10 ⁹ /mL more in PE ($P<0.05$)	PLAP ELISA using Elabscience commercial kit	216 pg/mL more in PE ($P<0.05$)	36
36.7	38	Centrifugation at 110,000 × g for 2 hours, then using sucrose gradient	NTA, TEM, Western blot for CD63	Small	NTA and CD63 ELISA using ExoELISA™ commercial kit	2.26 × 10 ⁹ /mL more in PE ($P<0.05$)	PLAP ELISA using Elabscience commercial kit	149 pg/mL less in PE ($P<0.05$)	36
Three groups combined: 11–14, 22–28, 32–38	Matched	Centrifuged at 100,000 × g for 2 hours, then on iodixanol gradient for 20 hours	NTA	Small	NTA	More in PE ($P<0.0005$)	PLAP ELISA using Elabscience commercial kit	More in PE ($P<0.0005$)	33
35.8	36.2	Centrifuged at 10,000 × g for 30 minutes	NTA	Medium-large	NTA	8.74 × 10 ¹⁰ /mL more in PE ($P<0.0001$)	PLAP ELISA using Elabscience commercial kit	355.5 pg/mL more in PE ($P<0.005$)	34
33.1	32.7	None – platelet-free plasma directly analyzed	None	Medium-large	—	—	Flow using PLAP and annexin-V antibodies	7379 μL more in PE ($P<0.001$)	73

EV: Extracellular vesicles; ELISA: Enzyme-linked immunosorbent assay; NTA: Nanoparticle tracking analysis; PE: Preeclampsia; PLAP: Placental alkaline phosphatase; STBEV: Syncytiotrophoblast-derived extracellular vesicles; TEM: Transmission electron microscopy; —: Not applicable.

Table 2**Studies reporting STBEV protein expression in preeclampsia.**

Protein	STBEV source	STBEV isolation technique	EV identification technique	STBEV size	Protein detection assay	Up/down-regulated in PE vs. normal	Putative actions relating to PE	References
Tissue factor	Normal and PE perfused placentas	Centrifuged at $150,000 \times g$	Not done	Mixed: small and medium-large	Thrombin generation by calibrated automated thrombography	Upregulated	Disordered haemostasis	74
Synovitin 1	Term PE placental villous explants	Centrifuged at $70,000 \times g$ for 90 minutes	Not done	Medium-large	Western blot	Not quantified	Peripheral blood mononuclear cell activation	75
sFlt-1	Normal and PE: mechanical isolation; placental villous explants; plasma	Centrifuged at $415,000 \times g$ for 90 minutes	Not done	Small	Western blot; ELISA	Upregulated	Binds VEGF, reducing endothelial cell nitric oxide production and causing vasoconstriction	76
PLAP	Normal and PE: plasma	Centrifuged at $150,000 \times g$ for 60 minutes	NTA	Mixed: small and medium-large	ELISA; Flow cytometry	Upregulated	Unknown	20
Flt-1; Endoglin; LAMP 1; Alix; CD63; CD9	Term normal and PE: mechanical isolation; placental perfusion	Centrifuged at $150,000 \times g$ for 60 minutes	NTA	Mixed: small and medium-large	Flow cytometry; Western blot	CD63/Flt-1 upregulated	Exosome markers (LAMP1, Alix, CD63, CD9); Endoglin/Flt-1 bind VEGF	77
Transferrin	Normal and PE placental explants	Centrifuged at $20,000 \times g$ for micro-vesicles and $100,000 \times g$ for nano-vesicles	Not done	Small and medium-large	Western blot; Immunofluorescence; ELISA	Upregulated	Induces ER stress in recipient cells	78
eNOS	Normal and PE perfused placentas and plasma	Centrifuged at $10,000 \times g$ for micro-vesicles and $150,000 \times g$ for exosomes	NTA; Western blot	Small and medium-large	Western blot; Flow cytometry; ELISA	Downregulated	Reduced endothelial nitric oxide synthesis causing vasoconstriction	23
HLA-DR	Normal and PE perfused placentas	Centrifuged at $10,000 \times g$ for 30 minutes	Not done	Medium-large	Flow cytometry	Upregulated (absent in normal)	Uncertain	79
PP13	Normal and PE perfused placentas	Centrifuged at $10,000 \times g$ for micro-vesicles and $150,000 \times g$ for exosomes	NTA	Small and medium-large	Western blot	Downregulated	Multiple signaling pathways: inflammation, arterial stiffness, immune tolerance	80
TPGB	Normal and PE perfused placentas	Centrifuged at $10,000 \times g$ for micro-vesicles and $150,000 \times g$ for exosomes	NTA	Small and medium-large	Western blot; qPCR	Upregulated	Modulation of maternal immune system	81
NEP	Normal and PE perfused placentas and plasma	Centrifuged at $10,000 \times g$ for micro-vesicles and $150,000 \times g$ for exosomes	NTA	Small and medium-large	Western blot; Immunoprecipitation; NEP activity assay; Flow cytometry	Upregulated	Degrades vasoactive peptides which usually maintain vasodilatation and diuresis	24
Siglec-6	Normal and PE perfused placentas	Centrifuged at $10,000 \times g$ for micro-vesicles and $150,000 \times g$ for exosomes	Not done	Small and medium-large	Western blot; Mass spectrometry	Upregulated	Transmembrane receptor which binds sialylated glycans – significance uncertain	82
Synovitin-1	Normal and PE plasma	Centrifuged at $120,000 \times g$ for exosomes	Electron microscopy; Western blot; NTA	Small	Western blot; qPCR	Downregulated	Peripheral blood mononuclear cell activation	83

ELISA: Enzyme-linked immunosorbent assay; eNOS: Endothelial nitric oxide synthase; ER: Endoplasmic reticulum; EV: Extracellular vesicles; Flt-1: Fms-like tyrosine kinase; HLA-DR: Human leucocyte antigen-DR; LAMP 1: Lysosomal associated membrane protein 1; NEP: Neprilysin; NTA: Nanoparticle tracking analysis; PE: Preeclampsia; PLAP: Placental alkaline phosphatase; PP13: Placental protein 13; qPCR: Quantitative polymerase chain reaction; Siglec-6: Sialic acid-binding immunoglobulin-like lectin-6; sFlt-1: Soluble fms-like tyrosine kinase; STBEV: Syncytiotrophoblast-derived extracellular vesicles; TPGB: Trophoblast glycoprotein; VEGF: Vascular endothelial growth factor.

unique to cholera toxin B vesicles. The putative biomarkers were validated by referring to the published literature, not using other techniques within the study.

In summary, STBEV-related proteins can offer important insights into STBEV signalling, as well as be potential diagnostic and therapeutic targets. It is clear that much more work is needed to elucidate the complexity of STBEV-protein signaling pathways in PE. Published studies are limited by experimental design: specifically, STBEV source materials, isolation and detection techniques, and validation with different techniques in different sample types.

STBEV RNA in PE

EVs carry complex cargos, which are not simply subsets of the content of the parent cell, but enriched in particular factors that are delivered to remote cellular targets. As already mentioned, the cargo includes RNA of which non-coding RNA (ncRNA), that is, RNA that is not translated into proteins, is important. ncRNAs are heterogeneous and have wide-ranging signaling and regulatory functions. The best known is miRNA, of about 22 nucleotides, which post-transcriptionally regulates gene expression and can silence RNA by specific complementary binding. Hence EVs are pervasive intercellular signals with roles in normal physiology and disease of which their RNA content is a major component.

RNA was first identified within STBEV in 2004. Gupta and colleagues used villous explant culture, mechanical dissection, and placental perfusion to harvest STBEV via ultracentrifugation and found corticotropin-releasing hormone mRNA in STBEV from all three preparations using reverse-transcription polymerase chain reaction.⁴⁴ Interest in the functionality of STBEV RNA cargo was sparked by the discovery of plasma miRNAs unique to pregnancy.⁴⁵ Co-staining of miR-517B (in-situ hybridization) and exosome markers (CD63-immunostain) in placental sections, suggested that placenta-derived miRNAs might be released in exosomes. This was confirmed by transfecting BeWo trophoblast cells with miR-517A, demonstrating a higher level of miR-517A in the exosomal fraction of supernatant compared with controls. In addition to miRNA, tRNA fragments have recently been described as the most abundant short RNA within STBEV from placental perfusion.⁴⁶

miRNA production is dysregulated in PE STBEV derived from experimental models and plasmas. Placentas perfused with medium containing cell-free fetal hemoglobin (to mimic PE) released less miR-517a, miR141 and miR-517b in STBEV than controls.⁴⁷ STBEV from placental explants of preeclamptic and normotensive women revealed 16 differentially expressed miRNAs.⁴⁸ It is possible that plasma EV miRNA profiles could be used as a clinical biomarker for PE. In one study, miR-486-1-5p and miR-486-2-5p were most able to differentiate PE from normotensive pregnancies.⁴⁹ Differential RNA expression in PE STBEV has since been reported by others, looking at both miRNA and other non-coding RNA, in plasma, serum and EV fractions at both early and late gestations.^{50–53} An important criticism of these studies is that they do not distinguish STBEV from other plasma EV. So, whilst they may identify clinically useful biomarkers for

PE, their contributions to our understanding of placental signaling via STBEV-RNA are limited. Differential expression of tRNA fragments in PE samples suggests they too may prove a clinically useful biomarker,⁵⁴ though there are fewer studies in this field.

Several miRNAs delivered to target cells by STBEV in PE may have functional roles. An elegant study by Cronqvist and colleagues used fluorescent-protein labeled EV and gold-labeled primers to visualize PE STBEV uptake in human coronary artery endothelial cells and miR-517a deposition in the endoplasmic reticulum.⁵⁵ This correlated with increased miR-517a expression in the endothelial cells following application of STBEV. Endothelial cells treated with miR-517a had altered expression of several genes including upregulation of FLT1. The authors also noted endothelial damage with the STBEV and suggested STBEV miRNAs might be one mechanism for underlying endothelial dysfunction in women with PE. Another group postulate that miR-155 signaling in serum EV may underlie reduced NO production in PE.⁵⁶ Serum exosomes from women with PE inhibited NO production and eNOS expression in endothelial cells. PE exosomes contained over 50% more miR-155 than controls. Overexpression of miR-155 in BeWo cells produced exosomes which also suppressed eNOS expression.

tRNA halves, a small ncRNA influence protein synthesis in cultured fibroblasts⁴⁶; they are generated by angiogenin in response to oxidative stress and regulate both transcription and translation in other organisms.⁵⁷ They are consequently a component of significant interest in PE STBEV, where the syncytiotrophoblast is very much affected by oxidative stress.

STBEV actions in PE

Several studies of proteins and RNA from PE STBEV have demonstrated putative target signaling actions for these molecules in PE. Other groups have taken a less reductionist approach and looked at the actions of PE STBEV as a complete entity. These experiments are also difficult to interpret, because the actions of cells in vitro do not necessarily correlate with disease pathophysiology. Nevertheless, they are a useful tool for understanding the molecular pathways underlying PE.

Endothelial dysfunction is pathognomonic of PE. Several groups have treated endothelial cells in culture with PE STBEV demonstrating: suppressed endothelial cell proliferation,^{17,58} disruption to the cell monolayer,⁵⁸ decreased nitric oxide production and eNOS expression,⁵⁶ and activation including ICAM-1 expression.⁵⁹ Neutrophils have been proposed as another mediator of endothelial dysfunction. Mechanically isolated STBEV from PE placentas induced more superoxide production in neutrophils than STBEV from normal placentas.⁶⁰ It was therefore surprising that gene expression in endothelial cells after treatment with placental perfusion PE STBEV showed no significant differences in genes related to blood pressure regulation from treatment with normal STBEV.⁶¹ Significant differences were seen in the PCR array for genes relating to inflammation, though these were not validated.

STBEV have been shown to stimulate production of inflammatory cytokines in peripheral blood mononuclear cells by several investigators.^{62–64} Indeed, PE STBEV were

found to exacerbate the cytokine response to lipopolysaccharide in peripheral blood mononuclear cells. PE STBEV have been shown to increase platelet activation over normal STBEV.⁶⁵ In parallel, STBEV triggered thrombin generation in plasma in a dose dependent manner; this was more pronounced in PE STBEV than normal.

Syncytiotrophoblast stress: a key differentiator between health and disease

The unifying theme in studies investigating the pathophysiology of PE is syncytiotrophoblast stress, which includes oxidative stress.⁵ Placental oxidative stress as a consequence of abnormal spiral artery remodeling is a key step in EOPE. Syncytiotrophoblast stress as a result of villous overcrowding in the growing but aging placenta is thought to underlie LOPE. This stress manifests in the maternal circulation as release of syncytiotrophoblast-derived angiogenic factors and increased release of STBEV. We believe that STBEV are the key stress signal in PE, which could be exploited in clinical differentiation between PE and normal, as well as offering additional targets for therapeutic intervention.

Dysregulated proteins in PE STBEV identified from proteomics analyses influence cellular death, immune cell activation, and endoplasmic reticulum stress. miRNA signaling by stressed STBEV results in dysfunctional endothelial cells. PE STBEV stimulate inflammation, thrombin generation and platelet activation *in vitro*. Indeed, some of the clinical benefit seen in the earlier plasma apheresis studies aiming to remove sFlt-1 from maternal plasma has been attributed to removal of STBEV.⁶⁶

In addition to established markers of syncytiotrophoblast stress, we have noted the presence of previously unreported stress molecules in STBEV, such as tRNA halves.⁴⁶ If differential release of STBEV tRNA halves were confirmed in PE, this would add to the existing multifaceted stress signal released in STBEV from the preeclamptic placenta. As our understanding of this signal grows, we might be better placed to diagnose, predict, and treat the many shades within the PE spectrum using STBEV as liquid biopsies.

Syncytiotrophoblast stress is not unique to PE. Differential STBEV release and contents have been reported in other placental diseases including gestational diabetes, preterm birth, and fetal growth restriction.^{67–70} Angiogenic factors are being investigated as a predictor of stillbirth in a clinical trial of women with reduced fetal movements.⁷¹ We believe that interrogating STBEV as a more complex and precise manifestation of placental stress may offer new diagnostic and therapeutic insights across the field of obstetrics, as well as in PE.

Funding

William R. Cooke receives a doctoral training fellowship from the Wellcome Trust.

Author Contributions

William R. Cooke, Christopher W.G. Redman, and Manu Vatish conceived of the manuscript title and concept. All

authors contributed to drafting of the manuscript and approved the final version.

Conflicts of Interest

None.

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Edited By Dandan Shi

How to cite this article: Cooke WR, Jones GD, Redman CWG, Vatsish M. Syncytiotrophoblast Derived Extracellular Vesicles in Relation to Preeclampsia. *Maternal Fetal Med* 2021;3(2):151–160. doi: 10.1097/FM9.0000000000000093.