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Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia

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ABSTRACT

The release of extracellular vesicles (EV) by the syncytiotrophoblast (STB) may be an important mechanism by which the placenta signals to the mother. STB derived EV (STBEV) are comprised predominantly of exosomes (50–150 nm) and microvesicles (100–1000 nm) that contain bioactive mediators such as proteins, nucleic acids and lipids. They, along with larger syncytial nuclear aggregates are released by the STB into the maternal circulation throughout gestation in normal pregnancy where they appear to have an immunoregulatory role, inhibiting T cell and NK cell responses. In pre-eclampsia (PE) STBEV are released in significantly increased numbers and have pro-inflammatory, anti-angiogenic and procoagulant activity, implicating them in the maternal systemic inflammation, endothelial dysfunction and activation of the clotting system which typifies the disorder. Research has focused on understanding the biological significance of STBEV by measuring their size and repertoire of molecules carried and how they differ in normal pregnancy and PE, using techniques such as Nanoparticle Tracking Analysis, flow cytometry and mass spectrometry. We have also found alterations in STBEV surface glycans associated with PE. The goal is to better understand the role STBEV play in normal pregnancy and PE and whether they are potential biomarkers of placental pathology and therapeutic targets in PE.

Contents

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1. Introduction

The syncytiotrophoblast (STB); a multinucleated, terminally differentiated, polarised epithelium that covers the entire surface of human placental villi, is one of the largest cell types in human biology (Burton and Fowden, 2015). It forms during the early stages of embryo development by the initial fusion of mononuclear cytotrophoblasts and then is maintained throughout gestation by a process of turnover, with spent material shed from the apical surface being replenished by incorporation of underlying cytotrophoblast cells (Huppertz et al., 2006). It is the largest and most critical fetal-maternal interface, responsible for nutrient uptake, gas exchange, waste removal, protein and steroid hormone production and modulation of maternal physiology. The STB is also specially adapted to shield the fetus from the maternal immune system. The semi-allogeneic STB is unique in being human leukocyte antigen (HLA) null and therefore immunologically inert to prevent allorecognition and rejection by maternal T cells (Nancy and Erlebacher, 2014). While this lack of HLA expression might be expected to render the STB open to attack by maternal natural killer cells (NK cells; which are programmed to destroy HLA negative tumour cells), there is no evidence that this occurs. This may be due to the presence of a glycocalyx on the STB membrane which could prevent interactions with NK cells (Arkwright et al., 1994). The STB communicates with the maternal immune system using both soluble factors, such as chemokines, cytokines and steroid and protein hormones, and factors carried by extracellular vesicles (EV).

EV are cell-derived membranous vesicles and potent mediators of both physiological and pathological processes (Colombo et al., 2014). The term EV encompasses three main vesicle types: exosomes, microvesicles (also known as ectosomes and microparticles) and apoptotic bodies. EV biology has been extensively reviewed for comprehensive reviews see (Colombo et al., 2014; Kalra et al., 2016), so briefly, exosomes are the smallest vesicle type (~50–150 nm) and are produced in a constitutive manner, using machinery of the endocytic pathway, in structures called multivesicular bodies (MVB), which enable loading with a targeted cargo followed by release of exosomes into the extracellular environment by fusion with the plasma membrane and exocytosis. Microvesicles (~100 nm–1 μm) are released directly from the plasma membrane in response to stimuli that cause a rise in intracellular calcium levels and cytoskeletal remodeling such as cellular activation or stress. Also included under the umbrella of EV are apoptotic bodies which overlap in size with microvesicles (~200 nm–5 μm) and are released from apoptosing cells, but only once in the life of a cell as it is a terminal event in the apoptotic pathway. As such, research into the role of EV in cell–cell communication has tended to focus on microvesicles and exosomes. EV carry proteins, lipids and RNAs (such as mRNA, miRNA, vaultRNA and tRNAs) and are thought to signal to their target cells via surface interactions including protein or lipid ligand-receptor binding, by fusing and releasing their contents into the cytosol of the target cell and finally via endocytosis and subsequent fusion with endosomes (Raposo and Stoorvogel, 2013).

EV release appears to be evolutionarily conserved, involving the coordinated activity of numerous proteins (comprehensively reviewed by (Colombo et al., 2014)). The transmembrane proteins tetraspanins, including CD9, CD81, CD82 and CD63, clustered in tetraspanin enriched domains (TEMs) in the plasma membrane and the endosomal sorting complex required for transport (ESCRT) complex, made up of around 30 different proteins including ALIX, TSG101, syntenin and multiple RAB proteins, are major components of EV biogenesis, involved at multiple stages in a cell specific manner (Henne et al., 2011; Friand et al., 2015; Stuffers et al., 2009). TEMs act as specialized scaffolds, enabling the compartmentalization of proteins from the plasma membrane into EVs and the downstream sorting of proteins and possibly RNA and lipids into EV cargo (Villarroya-Beltri et al., 2014; Mazurov et al., 2013). Components of the ESCRT complex are required for endocytosis of the endosomal membrane wall to form exosomes, targeting of MVB for fusion with the plasma membrane for exosome exocytosis and EV release (Hanson and Cashikar, 2012). The selective recruitment of proteins, such as adhesion molecules, glycoproteins and externalization of phosphatidylserine (PS) also enables the targeting of EV to particular recipient cells following their release, while pathological cellular changes lead to characteristic alterations in EV cargo (Colombo et al., 2014; Andreu and Yanez-Mo, 2014).

The STB is the primary source of placenta derived EV, that may constitute a major signaling mechanism between fetus and mother, augmenting maternal physiology to allow the presence and meet the demands of the developing fetus. Pregnancy is an ideal system to study EV as the entire process has a definite start and end point, with specific STB markers, principally placental alkaline phosphatase (PLAP), distinguishing STB derived EV (STBEV) from those produced by other cell types, and availability at the end of pregnancy of the STBEV source, the placenta. This is particularly relevant to the investigation of pregnancy disorders driven by placental dysfunction, such as preeclampsia (PE); a problem of human pregnancy and leading cause of maternal mortality (Tannetta and Sargent, 2013; Redman et al., 2012). PE affects 2–5% of women worldwide and carries a substantial risk of long-term cardiovascular health for both the mother and baby. It is characterized by the maternal signs of hypertension, proteinuria and hypercoagulation, triggered by the release of placental proinflammatory, antiangiogenic and procoagulant factors in response to ischemia reperfusion and downstream inflammatory and endoplasmic reticulum (ER) stresses (Redman and Sargent, 2005; Burton and Yung, 2011).

This review will outline our current understanding of STBEV subtypes, interactions of STBEV with maternal cells, potential novel mediators of these interactions including altered STBEV surface glycan groups and their possible role in pregnancy and PE.

2. STBEV release and composition in normal pregnancy and PE

It has been known for many years that release of membranous material into the maternal circulation by the STB is a feature of normal pregnancy (Burton and Jones, 2009). This material, ranging from multinucleated syncytial sprouts and knots (known as syncytial nuclear aggregates (SNA)) and viable trophoblast cells to STBEV has, until relatively recently, been regarded as inert STB debris of little consequence. However, the demonstration of their immunomodulatory activities has increased interest in their role in both normal and pathological pregnancies, particularly PE.

Given their size, SNA can easily be isolated using low speed centrifugation (Abumaree et al., 2006b). However, the subcellular nature of STBEV means that specialised isolation protocols are required. There is no consensus in the field of EV research on protocols for the isolation of specific EV subtypes. Efforts to standardise isolation procedures may also not be practical given the range of biological fluids that contain EV (e.g. plasma, urine, saliva, cerebrospinal fluid, breast milk) and *in vitro* culture systems used in EV research (Witwer et al., 2013). Methods routinely used to isolate EV include precipitation, differential centrifugation, density gradient ultracentrifugation, filtration, size exclusion chromatography and immunocapture on beads or chips. Sample type (ranging from complex biological fluids such as plasma to *in vitro* derived samples such as conditioned media) and downstream analyses (e.g. cargo determination using transcriptomic and proteomic approaches or EV characterization techniques such as electron microscopy, flow cytometry and nanoparticle tracking analysis (NTA)) will

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determine the choice of isolation technique due to effects of sample complexity, sample volume, EV yield, enrichment of certain EV subtypes and purity of the resultant preparation (Xu et al., 2016). Each isolation method has its advantages and disadvantages that have been comprehensively discussed in recent reviews (Momen-Heravi et al., 2013; Xu et al., 2016; Gudbergsson et al., 2016; Van Deun et al., 2014). Briefly, centrifugation techniques are most commonly used to isolate EV and have proved useful to fractionate large and small STBEV from placental perfusate (Dragovic et al., 2015). However, as with precipitation techniques, ultracentrifugation can lead to contamination of pelleted EV with protein aggregates and, in the case of blood derived samples such as plasma, also high density lipoproteins, that can interfere in downstream analyses (Yuana et al., 2014; Momen-Heravi et al., 2013). Size exclusion chromatography (SEC) is quick and gives high EV yields with minimal contaminating soluble and aggregated proteins (Boing et al., 2014). For even greater purity and enrichment of particular EV phenotypes, immunocapture using antibodies to specific surface antigens has proved effective in the field of cancer biomarker research (An et al., 2015). Similar strategies may enable the routine isolation of STBEV from plasma and use as circulating biomarkers of placental status.

Efforts to characterize EV are also hampered by the lack of specific features unique to each EV subtype, therefore measurement of multiple parameters is required to allow a general assessment of EV cellular and subcellular origin. EV size can be measured routinely using NTA, to determine whether EV are in the expected size range of exosomes (~100 nm) or microvesicles (~100 nm–1 μm) (Dragovic et al., 2011). Given their role in exosome biogenesis, tetraspanins, particularly CD63 and ESCRT components such as ALIX and TSG101 are routinely employed as exosome markers, in techniques such as Western blotting and immunocapture assays. However, doubts have been raised regarding CD63 exosome specificity, which is not unexpected as tetraspanin enriched microdomains and all EV both originate from the plasma membrane (Colombo et al., 2014). A buoyant density of 1.13–1.19 g/mL is also reported to be indicative of exosomes, but again this is not definitive (Colombo et al., 2014). Much of the characterization of EV has been carried out on EV prepared from cancer derived cell lines and the general findings applied to STBEV. No in-depth studies of expression of the known TEMs and ESCRT complex components by STB have been reported. This is critical considering the unique nature of STB biology and the heterogeneity already reported amongst different cell types.

Given the caveats outlined above, the general consensus is that STB release the full range of EV; exosomes, microvesicles and apoptotic bodies (Dragovic et al., 2015) (Fig. 1). Unique to STB, multinucleated material, in the form of SNA, are also released (Fig. 1). The biological significance of each remains to be determined. Examination of *in vitro* placenta explant culture supernatant, *ex vivo* placental lobe maternal side perfusate and uterine vein plasma gives an insight into the full repertoire of EV and SNA released by the STB (Chamley et al., 2011; Dragovic et al., 2015; Johansen et al., 1999). Larger STB material, such as SNA can easily be isolated from placenta explant culture supernatants and maternal side placenta perfusates by low speed centrifugation (~400×*g*) (Fig. 1). Meanwhile, differential centrifugation has predominantly been used to isolate both total STBEV preparations and fractions enriched in microvesicles or nanovesicles (Dragovic et al., 2015) (Fig. 1). Larger STB derived vesicles and fragments have been found in uterine vein blood but not in peripheral blood, reflecting their sequestration in the pulmonary capillary bed (Johansen et al., 1999). However, smaller STBEV pass unimpeded into the peripheral circulation (Knight et al., 1998). Using a PLAP based ELISA to distinguish STBEV from other EV in the circulation, STBEV have been found circulating at progressively higher levels with advancing pregnancy (Germain et al., 2007) and labour (Reddy et al., 2008), returning to non-pregnant levels around 48 h post-delivery. PLAP and CD63 double positive EV were detected from as early as 6 weeks gestation and as with total PLAP positive EV, levels increased over the course of pregnancy (Salomon et al., 2014). Flow cytometry, using STB specific monoclonal antibodies NDOG2 (recognizes PLAP) and ED822 (antigen unknown), has also been used to analyse STBEV pelleted from maternal plasma but, with a detection threshold of ~300 nm, discriminates in favour of EV in the size range of large MV and apoptotic bodies, unlike immunoassay based methods that measure all STB marker positive EV irrespective of size (Dragovic et al., 2013).

Maternal plasma STBEV, measured by ELISA, are increased in PE. Increased levels tend to reflect disease severity. Increased levels were not detected in normotensive fetal growth restriction, another pregnancy complication commonly associated with placental dysfunction (Knight et al., 1998; Goswami et al., 2006; Chen et al., 2012a,b). A significant increase in circulating STBEV in PE women during labour, may also play a part in worsening maternal symptoms in the postpartum period (Reddy et al., 2008). Results of flow cytometry studies have been less consistent with both increased plasma levels of STBEV in PE and no difference between PE and normal pregnancy being found, perhaps reflecting the need to differentiate severe from mild forms of the disease (Lok et al., 2008; Orozco et al., 2009; Marques et al., 2012; Dragovic et al., 2013; Germain et al., 2007). Given the bias of flow cytometric analysis towards larger EV, poor consistency also suggests increases found with immunoassay based measurements are due to changes in STBEV subtypes below the detection threshold of flow cytometry. Indeed, NTA analysis showed a significant increase in placental perfusate derived total STBEV median size in PE, compared to normal pregnancy (201 nm vs 166 nm respectively) suggesting a shift towards release of more microvesicles and a potential impact on overall functional effects (Tannetta et al., 2013).

3. Cellular interactions of STBEV in normal and PE pregnancies

Adaptation of maternal immune responses to accommodate and support the development of the semiallogeneic fetus are critical for a successful pregnancy. Failure of the maternal immune system to respond appropriately to pregnancy may contribute to serious obstetric complications such as miscarriage, preterm labour and PE. Evidence supporting a role for STB derived EV and larger material in the complex modulation of maternal immune cell function continues to grow. Results derived from *in vitro* studies suggest a tolerising effect of STB derived exosomes and SNA on the maternal adaptive immune response from the first trimester onwards. Published data also support a role for STBEV in activation of maternal innate immunity seen during normal pregnancy and the exaggerated systemic inflammatory response, vascular dysfunction and hypercoagulation associated with PE. However, confirmation using *in vivo* models have not been performed. Results of published studies investigating the biological effects of STBEV on maternal cells in normal pregnancy and PE are summarized below.

3.1. Neutrophils

Neutrophilia is one of the first clinical changes of early pregnancy. In the second trimester it is associated with infiltration of the decidua by a unique subset of neutrophils (N2) that endow immunosuppressive and angiogenic functions (Amsalem et al., 2014). Their importance is underlined by pregnancy pathology, in mildly hypertensive BPH/5 mice, which have maternal decidual arteriopathy, abnormal placentation and fetal growth restriction. These problems are mediated by classical neutrophils (N1) which are recruited by complement activation to areas of aberrant

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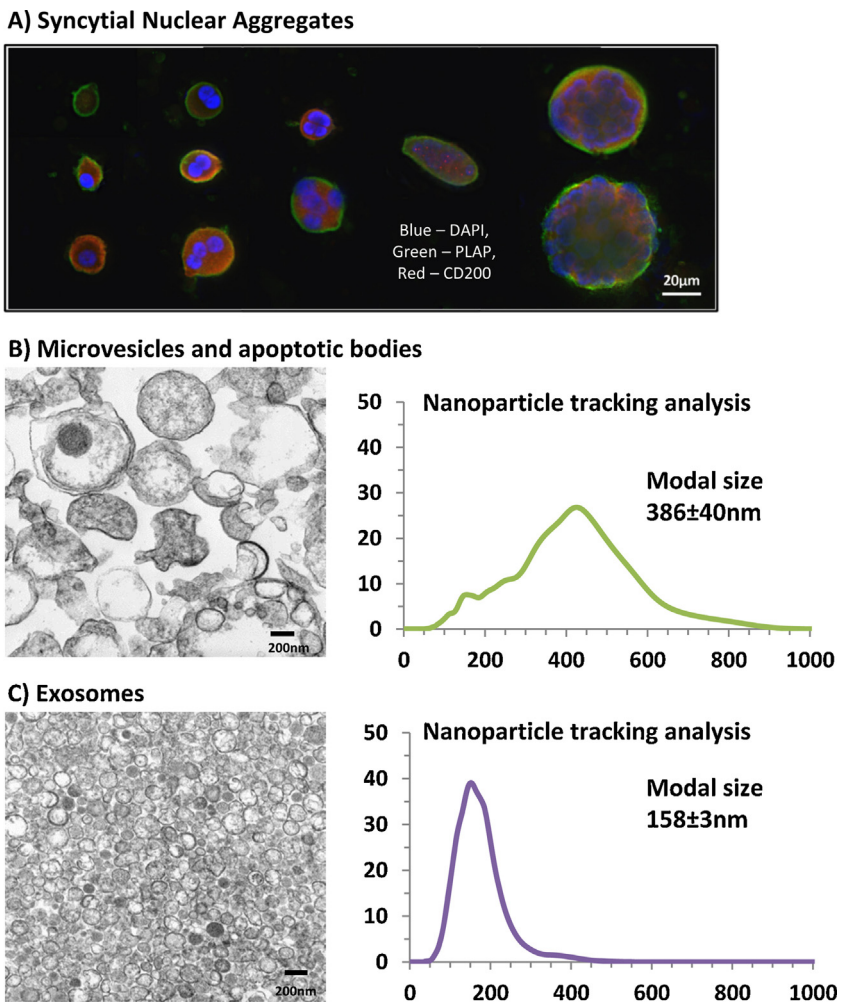

Fig. 1. Syncytial nuclear aggregates (SNA) and syncytiotrophoblast (STB) derived extracellular vesicles (STBEV) isolated by *ex vivo* placental perfusion method. A) SNA (20–500 μm) are released from the STB on the placental surface. Here SNA have been fluorescently labelled for STB marker placental alkaline phosphatase (PLAP; green), anti-inflammatory molecule CD200 (red) and nuclei (blue). B) Microvesicles (100 nm–1 μm) are produced by direct budding of the plasma membrane in response to stimuli that trigger an increase in intracellular calcium levels. Apoptotic bodies (1–5 μm) are released from cells undergoing apoptosis. C) Exosomes (50–150 nm) are generated from reverse budding of the endosome membrane, resulting in a multivesicular body which fuses with the plasma membrane of the cell and releases exosomes by exocytosis. Adapted from Dragovic et al., 2015.

decidual inflammation (Gelber et al., 2015). In the third trimester of normal human pregnancy circulating neutrophils are activated but more so in pre-eclampsia (Sacks et al., 1998). STBEV prepared from pre-eclampsia placentas induce increased superoxide production by neutrophils (Aly et al., 2004) and may contribute to the increased NETs in the intervillous space of PE placentae (Gupta et al., 2005a). These studies, implicate STBEV activated neutrophils in the etiology of placental pathology and the exaggerated systemic inflammation of PE. Interactions of STBEV with other granulocytes have not been investigated.

3.2. Monocytes and macrophages

There is strong evidence of interactions between STBEV and SNA and monocytes/macrophages in normal pregnancy and PE. Primary monocytes rapidly bind and internalise STBEV both *in vitro* and *in vivo* (Germain et al., 2007; Southcombe et al., 2011). When monocytic U937 cells or primary macrophages are incubated with SNA anti-inflammatory changes are induced. These include increased anti-inflammatory cytokine release such as IL-10 and indoleamine 2,3-dioxygenase (IDO) expression and decreased pro-inflammatory cytokine secretion and MHC class II molecule surface expression (Abumaree et al., 2006a; Abumaree et al., 2012), ascribed to the apoptotic nature of the SNA. The authors suggest that the capture and clearance of larger apoptotic STB debris by alveolar macrophages and circulating monocytes may tolerise maternal immune cells to fetal alloantigens (Chamley et al., 2011). Down regulation of innate immune response may also occur through the actions of STBEV and syncytial nuclear aggregate associated CD200, an anti-inflammatory molecule. We have found high expression of CD200 associated with SNA and STBEV (Fig. 1) (Sargent, 2013).

Suppression of maternal adaptive immune cell activity might contribute to a mechanism by which the placenta avoids immune rejection. However, a systemic inflammatory state due to controlled activation of maternal innate immune responses is characteristic of normal pregnancy, perhaps a physiological compensation for suppressed T and NK cell mediated immune responses (Sacks et al., 1998). That STBEV could stimulate this maternal systemic inflammatory response is suggested by studies showing that *ex vivo* preparations of STBEV stimulate peripheral blood mononuclear cells (PBMCs) and monocytes to release proinflammatory cytokines (Messerli et al., 2010; Southcombe et al., 2011; Atay et al., 2011; Germain et al., 2007). Moreover, treatment of PBMC with STBEV from PE placenta explants stimulate significantly increased release of pro-inflammatory cytokines and

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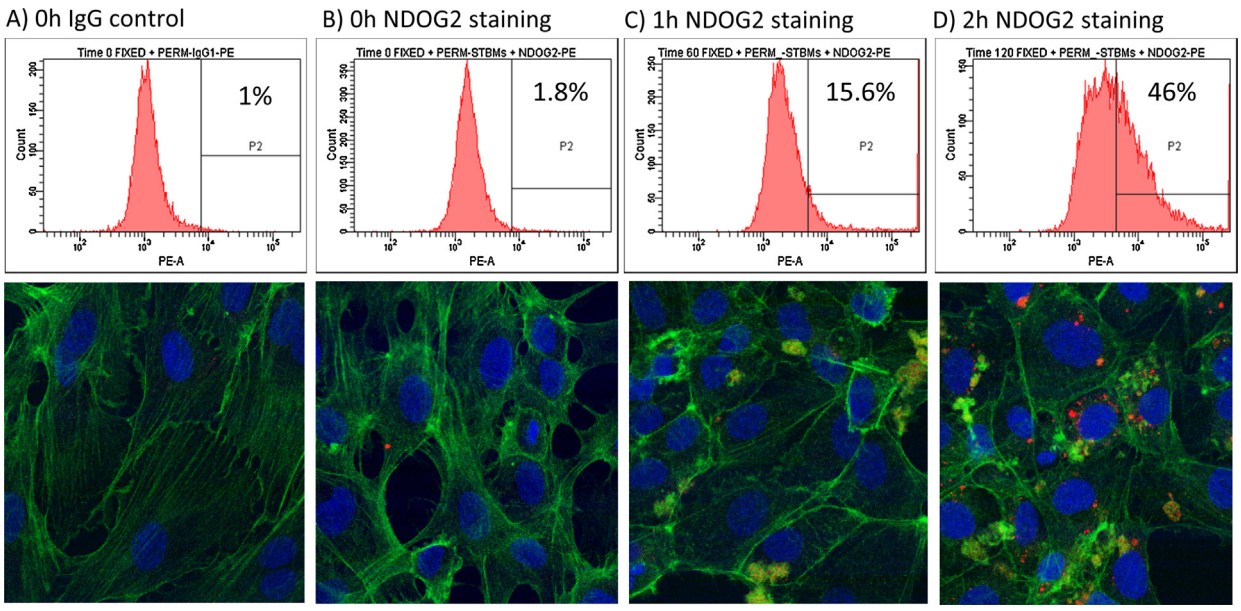

Fig. 2. Internalisation of syncytiotrophoblast extracellular vesicles (STBEV) over time by human umbilical vein endothelial cells (HUVEC). Flow cytometric and immunofluorescence confocal microscopy assessment of STBEV internalisation over time, using the monoclonal antibody NDOG2 which binds to the STB marker placental alkaline phosphatase and an appropriate IgG negative control. For immunofluorescence microscopy PLAP was labelled with NDOG2-phycoerythrin (red), actin with phalloidin-FITC (green) and nuclei with DRAQ-5 (blue).

chemokines, including IL-1β, when compared to normal placenta STBEV (Holder et al., 2012). This suggests qualitative as well as quantitative differences in STBEV shed in PE.

3.3. Dendritic cells

Although dendritic cells (DC) are major phagocytosing and antigen presenting cells, no studies have been published to date investigating interactions of DC with STBEV or SNA. When undertaking these types of studies, maturation of DC due to placenta derived soluble cytokines should be avoided by careful isolation and washing of STBEV.

3.4. B cells

Largely ignored in reproductive immunology, B cells are capable of more than just mediating humoral immunity through the production of antibodies (Arck et al., 2015). Although vital for passive immunization of the neonate for early life protection against pathogens, recent work has also identified antibody independent effects mediated by B cell subsets that secrete distinct cytokine profiles and modulate DC, natural killer cell (NK) and T cell immune responses (Bao and Cao, 2014; Moulin et al., 2000). B cells rapidly bind and internalise STBEV, suggesting possible *in vivo* interactions, although the functional consequences of such interactions are not known (Southcombe et al., 2011).

3.5. NK cells and T cells

STBEV have broad down-regulatory effects on T cell responses such as inhibition of proliferation induced by PHA, the mixed lymphocyte response (Gupta et al., 2005b; Thibault et al., 1991) or by CD3-zeta loss (Sabapatha et al., 2006; Taylor et al., 2006) although the effect depends on how the STBEV are prepared (Gupta et al., 2005b). STBEV express MHC class I chain-related (MIC A/B) and UL-16 binding proteins (ULBPs) that are ligands and inhibitors of the NKG2D receptor of NK cells, cytotoxic T lymphocytes and γd T cells (Hedlund et al., 2009; Mincheva-Nilsson et al., 2006). STBEV associated Fas ligand and TRAIL also promote lymphocyte and PBMC apoptosis (Stenqvist et al., 2013), and allogeneic reactivity is suppressed by N-linked oligosaccharides of STB membrane glycoproteins (Arkwright et al., 1994).

As well as general inhibition of T and NK cell cytotoxicity, factors expressed by STBEV may also tolerise maternal adaptive immune responses. Although HLA null, the STB does express paternal derived minor histocompatibility antigens (mHAgs), to which reactive maternal T-cells can be produced and whose numbers expand over the course of normal pregnancy, to persist for many years afterwards. The mHAgs DDX3Y and HA-1 are released by placental explants bound to SNA and may constitute a mechanism by which the maternal immune system is exposed to fetal alloantigens and induces antigen specific regulatory cytotoxic T cells, conferring maternal immunological tolerance (Holland et al., 2012; Linscheid et al., 2015). Interestingly, in a transgenic mouse model expressing ovalbumin as a surrogate fetal alloantigen, although pregnancy alone suppressed the induction of ovalbumin reactive CD8+ T cells when primed with soluble ovalbumin, presentation of ovalbumin incorporated into shed placental membranous material blocked its immunogenicity completely (Tay et al., 2013). Therefore, alloantigen association with STB derived membrane may also block immunogenicity in contrast to the effects of the soluble antigens.

These studies suggest involvement of STBEV in the mechanisms that regulate maternal cell mediated immunity in normal pregnancy. The possible involvement of these STBEV driven mechanisms in the pathophysiology of PE has not been investigated.

3.6. Platelets

Excessive activation of the coagulation system and increased platelet activation are features of PE (Macey et al., 2010). PE placental perfusion derived STBEV have higher tissue factor activity and stimulate higher levels of platelet activation than those isolated from normal placentas, (Gardiner et al., 2011; Reverdiau et al., 1995; Tannetta et al., 2015). Furthermore, aspirin treatment, which is recommended for women at high risk of developing PE to prevent platelet aggregation, blocked PE derived STBEV induced platelet

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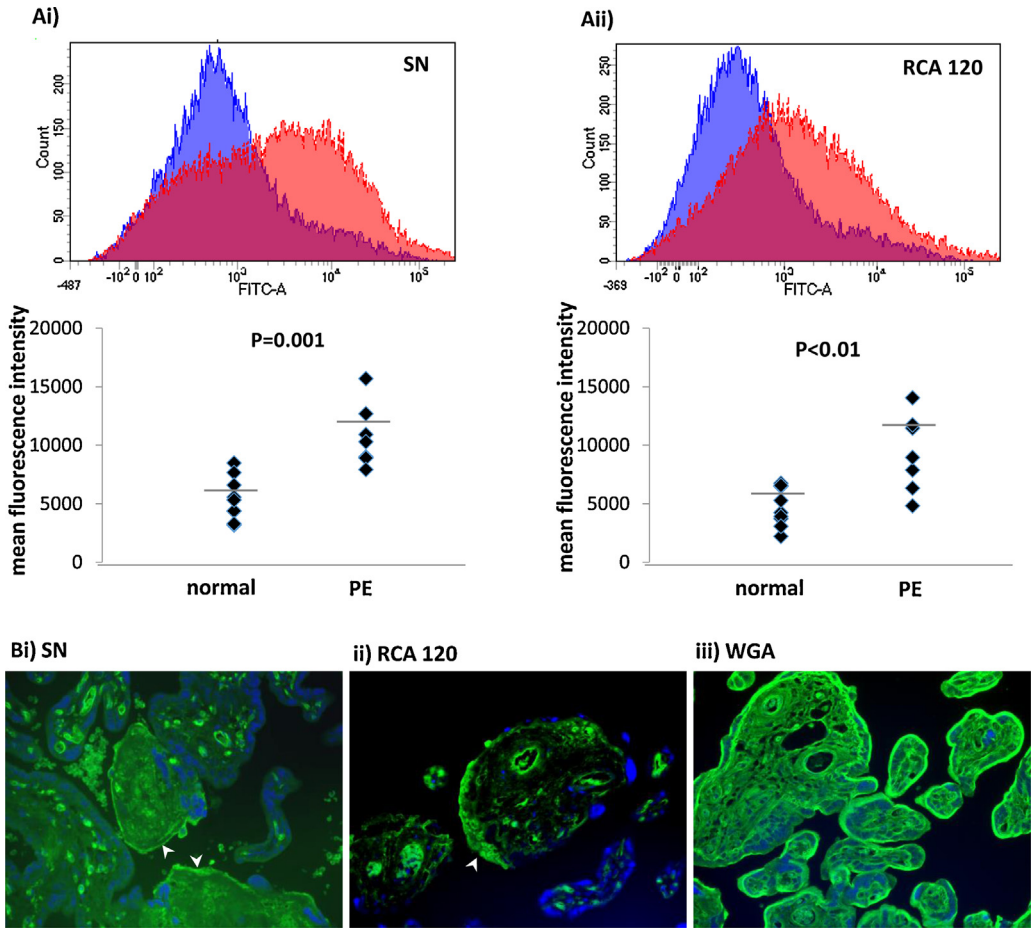

Fig. 3. Analysis of syncytiotrophoblast extracellular vesicle (STBEV) and placental section glycans. A) Flow cytometric analysis of STBEV glycan expression using plant lectin binding. STBEV preparations from normal (n = 10) and preeclampsia derived placentas (PE; n = 10) were labelled with FITC conjugated *Sambucus nigra* (SN; binds sialic acid) and *Ricinus communis* (RCA 120; binds galactose) and mean fluorescence intensity measured. Representative histograms for normal (blue) and PE (red) STBEV and the corresponding mean fluorescence data for all the samples analysed are shown. B) Fluorescence microscopy images of PE placenta sections labelled with FITC conjugated plant lectins Bi) SN, Bii) RCA 120 and Biii) wheat germ agglutinin (WGA; binds *N*-acetylglucosamine). Arrow heads indicate discrete areas of lectin staining.

aggregation, providing a potential explanation for the beneficial clinical effect of aspirin (Tannetta et al., 2015).

3.7. Endothelial cells

Given the endothelial dysfunction that is a central feature of PE, several studies have investigated the effects of SNA and STBEV on endothelial cells. In normal pregnancy, trophoblastic material, such as SNA, released by apoptotic like processes during the normal turnover of the STB, may have a suppressive effect on endothelial immune responses. Phagocytosis of apoptotic trophoblast debris by microvascular and pulmonary artery derived endothelial cells *in vitro* reduced their subsequent response to pro-inflammatory stimuli, such as necrotic trophoblast debris, previously shown to induce endothelial cell activation (Chen et al., 2006, 2010, 2012a). This may be important in regulating maternal endothelial responses to low levels of STB derived necrotic material released during normal pregnancy, especially in the pulmonary capillary bed which traps a significant amount of the large STB derived material. Furthermore, the transcriptome and proteome of endothelial cells exposed to normal placentae derived SNA showed changes that may also contribute to maternal cardiovascular adaptations to pregnancy (Wei et al., 2016).

We have found that human umbilical vein endothelial cells (HUVECs) readily bind and internalise STBEV (Fig. 2). STBEV have also been shown to have antiangiogenic and hypertensive effects, inhibiting endothelial cell monolayer growth *in vitro* and inhibiting the relaxation of pre-constricted blood vessels *ex vivo* (Smarason et al., 1993; Gupta et al., 2005c; Hoegh et al., 2006; Cockell et al., 1997). Furthermore, culture supernatants from HUVECs treated with STBEV can activate neutrophils, suggestive of a role for STBEV in both the endothelial cell dysfunction and excessive inflammatory effects in PE (Von Dadelszen et al., 1999). Unlike PE placentae derived SNA, thought to be more necrotic in nature and which strongly activated microvascular endothelial cells *in vitro* (Shen et al., 2014), no differential effects of PE derived STBEV on endothelial cells have been shown, however the disruptive effects of STBEV on endothelial cell function demonstrated *in vitro* and *ex vivo* suggest a direct contribution of increased circulating levels of STBEV to the endothelial dysfunction characteristic to the maternal syndrome of PE. Indirect actions of PE derived STBEV activating immune cells and platelets, such as those highlighted above, may also cause divergent reactions in maternal endothelial cells.

4. STBEV functional moieties and novel PE STBEV biomarkers

STBEV, like many EV studied, carry a complex cargo of proteins, lipids and miRNAs (recently reviewed by (Tannetta and Sargent, 2013; Tannetta et al., 2014; Mouillet et al., 2015), whilst in PE both qualitative and quantitative differences in shed STBEV have been found, such as changes in the size of EV released and the molecular

cargo they carry, that could alter their biological function (Tannetta and Sargent, 2013; Tannetta et al., 2013). As with apoptotic cells and apoptotic bodies, 'eat me' signals that are recognized by phagocytic cells may also play a role in EV-cell interactions and be altered in PE (Biermann et al., 2013). 'Eat me' signals include clustered externalized PS, PS binding molecules such as annexin A1 and MFG-E8 and modified glycosylation, such as reduced sialylation of extracellular glycans (Biermann et al., 2013; Franz et al., 2007; Meesmann et al., 2010).

Patterns of protein glycosylations affect numerous biological processes including protein folding, cell adhesion and clearance, molecular trafficking and immune cell responses, to name a few (Lyons et al., 2015; Alavi and Axford, 2006). Membrane proteins are richly glycosylated. This is also the case for EV membranes and is used as a mechanism for sorting certain proteins into EV (Batista et al., 2011; Liang et al., 2014). Disease states can lead to characteristic alterations in cellular glycosylation profiles, including, diabetes, atherosclerosis, neurodegenerative and immune disorders and cancer, where tumour associated glycans have led to the development of numerous diagnostic and prognostic markers (Almeida and Kolarich, 2016). Again, this has also been found for EV isolated from diseased patients (Gerlach et al., 2013; Staubach et al., 2012). Using flow cytometry, we investigated sugar moieties found on the surface of normal pregnancy and PE placental perfusion derived STBEV, by staining with a panel of FITC conjugated plant lectins (Fig. 3). Results showed increased staining of PE derived STBEV with lectins *Sambucus nigra,* which binds preferentially to sialic acid attached to terminal galactose by α-2,6 linkage (SN; $p = 0.001$) and *Ricinus communis agglutinin I,* which binds to galactose or *N*-acetylgalactosamine residues (RCA 120; $p = 0.001$) (Fig. 3A). Fluorescence microscopy analysis of PE placenta sections with SN and RCA 120 showed discrete areas of STB staining (Fig. 3Bi and Bii), very different to the universal staining obtained with wheat germ agglutinin (Fig. 3Biii). As with cell–cell interactions, surface glycans may also play a significant role in EV-cell interactions. In PE, altered surface glycans may affect functions such as cell targeting, clearance and immune activity and may also have potential use as markers of placental stress.

5. Conclusions

Our understanding of the importance of STBEV in the establishment and maintenance of a healthy pregnancy continues to grow. Whether *in vitro* studies reveal the biological effects of STBEV *in vivo* is not known. Recreating the cytokine, chemokine and hormonal milieu of pregnancy for *in vitro* studies is technically very challenging. Also, where primary immune cells have been used, these have sometimes been isolated from non-pregnant individuals. Better understanding of the biological activity of STBEV in normal pregnancy and PE may uncover new therapeutic targets such as neutralizing or removing specific pathogenic STBEV, using immunotherapy or apheresis, or increasing the levels of advantageous STBEV, by either increasing their release or administering the beneficial bioactive factor. The ultimate goal would be to reduce the underlying causes of the maternal syndrome and enable the pregnancy to continue for longer, avoiding early pre-term delivery, thus lessening the associated risks to both the baby and mother.

It is clear that much more work is needed to understand the intricacies of STBEV subtype biogenesis, mechanisms of action and their role over the course of gestation. There is clearer evidence that STBEV contain thousands of proteins, many of which are altered in PE. The examination of STBEV cargo in the maternal circulation could assist with the detection and management of PE by giving a real time read out of placental health. Enrichment of placental stress indicators, such as altered glycosylation, affecting multiple molecules and their enrichment in STBEV, could also amplify biomarker signals giving improved sensitivity. However, clinical use of STBEV requires better isolation methods, precise markers of EV subtypes and the development of more sensitive techniques to aid their detection and characterisation.

Ethics statement

Collection of placentas from normal and preeclamptic pregnancies and the isolation of STBEV were approved by the Oxfordshire Research Ethics Committee C and informed written consent was obtained from all participants.

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