

Reduced placental protein 13 (PP13) in placental derived syncytiotrophoblast extracellular vesicles in preeclampsia – a novel tool to study the impaired cargo transmission of the placenta to the maternal organs

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Author contribution: The study was conceived, designed, conducted and led by SM. DT and RD isolated, purified and characterized the STB-EVs. DT, IS and MV supervised all the experimental work. Patients were enrolled at John Radcliffe hospital, Oxford by CR. HM provided essential reagents including PP13 specific antibodies and PP13 ELISA kits and was involved in the ELISA testing, data analysis and statistical modeling. ASN performed the statistical analysis and mathematical modeling. All authors were involved in writing the manuscript, data analysis and discussions.

Declaration of interest: - HM holds a patent for the potential therapeutic use of PP13 in pregnancy complications. All other authors declare no conflicts of interest.

Abstract (250 words)

Introduction: Placental syncytiotrophoblast (STB) release extracellular vesicles (STB-EVs) that communicate physiological and pathological placental signals to the maternal organs. STB-EV release also increases in preeclampsia (PE). Here we explored the cargo of PP13 in STB-EVs from PE versus control placentas.

Methods: Placentae were harvested following caesarean section deliveries, and dual placental lobe perfusion was used to harvest STB-EV. Maternal side perfusate was centrifuged at 10,000xg to yield the STB microvesicles, and then at 150,000xg to yield STB exosomes. Total STB-EVs (tSTB-EVs) were collected using a one step 150,000xg centrifugation. Placental origin and size distribution were assessed by Western blotting and Nanoparticle Tracking Analysis, respectively. PP13 expression was determined by Western blot and ELISA.

Results: Placental alkaline phosphatase (PLAP; a STB specific marker) was present in all preparations. Total tSTB-EVs and STB-EXs also expressed the exosome markers such as the Apoptosis-Linked Gene 2-Interacting Protein X (Alix) and the cluster differentiation protein 9 (CD9). PP13 was localized to the outer surface and intra-vesicular compartments of all fractions. Surface to total PP13 ratios were ~1:1 for all STB-EV preparations.

In contrast to the previously reported higher circulating concentrations of soluble PP13 in PE, significantly lower levels of PP13, normalised to total vesicular protein, were observed in PE samples. PP13 reduction in all STB-EVs' sub-populations may be attributed to differences in gestational age (GA). A simple correction for GA suggested that PE may be an important influence.

Conclusions: PP13 is located in and on all types of STB-EVs. Circulating PP13 may therefore be either soluble or associated with extracellular vesicles with different pathophysiological effects in the maternal circulation.

Introduction (3308 words)

The release of extracellular vesicles (EVs) by viable cells is a feature of many activated cell types, including nerve cells, hormonal glands, tumours, immune cells and fetal and placental cells [1-4]. The placental syncytiotrophoblast (STB) releases EVs (STB-EVs) within the placenta or routed to the uterine veins and released into the maternal circulation. Increasing evidence suggests they play a role in placental communication with the maternal organs in normal and pathological pregnancies [3-7].

As with EVs, the STB-EVs can be sub-divided into exosomes (STB-EXs; ~30-200nm), microvesicles (STB-MVs; ~100-1000nm), and apoptotic vesicles (~1-5 μ m). The STB also releases syncytial nuclear aggregates (SNA; ~20-500 μ m) [6-10], and in pathological situations, necrotic debris. EVs can only be roughly classified on the basis of their size due to overlap between sub-types. STB-EV sub-types are formed by specific mechanisms, and as a result may carry a different protein cargo. STB-MVs are formed by direct budding from the plasma membrane in response to cellular activation or stress. STB-EXs are formed from internalized endocytic vesicles, and are constitutively released. Apoptotic vesicles are released as a terminal event of the apoptosis process. [4-12]. Once released, SNAs get lodged in the pulmonary capillaries and are cleared by the alveolar macrophages. They are transcriptionally active and can secrete proteins into the maternal circulation [13]. The smaller apoptotic bodies, STB-MVs and STB-EXs can pass through the pulmonary bed and into the systemic circulation. Animal models suggest that EV clearance occurs quickly (within 5 min), mostly in the liver and spleen, but this process is not yet fully understood [15].

Circulating STB-EVs are detectable from week 6 of gestation [3] and their release is increased by stress factors such as hypoxia and ischemia. The liberation of STB-EVs is increased from the first to the third trimester in proportion to the placental mass. Several studies have found some correlation between the level of their release

and impaired placentation [7-16]. While the complete functional significance of placental STB-EVs remains to be elucidated, available data supports a role in placentation, fetal-maternal interactions, angiogenesis, proliferation, maternal immune-tolerance to the pregnancy, and in communication between the placenta and the maternal organs [3, 17-19].

Preeclampsia (PE) is a disorder associated with impaired placental function. There are approximately 2.5%–7.0% of pregnant women who are affected by PE of which 0.5-1.8% develop severe PE associated with reduced placental perfusion. PE is a major cause of morbidity and mortality of mothers and babies [20-22]. It was found to be accompanied by long term cardiovascular diseases, diabetes and obesity for both the mother and baby [23]. While the underlying etiology is unclear, STB-EVs have been implicated in the pathophysiology of PE [6, 9, 12-14]. Housekeeping proteins of STB-EVs (such as actin) can be used to standardize levels of other STB-EVs' components according to gestational age (GA) or other attributes [11]. In addition, the cargo of STB-EVs subpopulations can be used to track changes in PE and to investigate how STB-EVs communicate with the maternal organs [11, 13-14].

PP13 is a galectin that is specifically expressed in the placenta [24] and its role in pregnancy has not been fully elucidated. Than et al [24] have shown that its specific placental expression in primate is associated with unique insertion of DNA sequence in the promoter region of the placental cells that occurs in the placenta of primates in relation to the deep placentation in these species. It is released into the maternal circulation and can be detected from week 5 of gestation [25]. Two meta-analyses have shown that reduced PP13 levels in the 1st trimester can predict the risk of later PE development [26-27]. PP13 was found to be involved in the mother's immune tolerance to pregnancy [28-29]. In early pregnancy, lower PP13 levels expose trophoblasts to immune rejection [27-28]. Reduced PP13 mRNA in PE was found in the placenta [29-30], in cultured placental cells, and in circulating maternal free mRNA throughout pregnancy [30-32]. During established PE, PP13 is elevated as revealed by

immunohistochemistry [25, 27]. STB derived apoptotic vesicles of the large diameters (~1-5µm) were already shown to have increased shading from the placenta at PE, but due to their size, they release their contents into the blood, leading to elevated circulating free PP13 [25, 27, 34]. However, to date there is no report of PP13 presence in the smaller STB-EVs (STB-MVs and STB-EXs).

Here we used the placental lobe dual perfusion model and sequential centrifugation, of the maternal side perfusate, to collect STB-EVs from control and PE pregnancies and characterized their subpopulations by nanoparticle tracking analysis (NTA) and examination of their molecular cargo [6]. The verified subpopulations (tSTB-EVs, STB-MVs and STB-EXs) were analyzed for the presence and distribution of PP13.

Materials and methods

Placentas

Women were enrolled at the John Radcliffe Hospital, Oxford. The Central Oxford Research Ethics Committee C approved this study (07/H0607/74), and written informed consent was obtained from each patient prior to placental collection.

Placentae were harvested from 18 control women with uncomplicated pregnancies that were scheduled to undergo delivery by elective cesarean (C) - section, and 20 women who developed PE and delivered by emergency C-sections. All women were non-labouring at enrolment and time of delivery. Placentae were only used if collected within 10min of delivery. The enrolment criteria for control pregnancy were singleton pregnancies without known fetal abnormalities, no maternal chronic diseases such as chronic hypertension, diabetes, kidney or cardiovascular illness, coagulation disorder or phospholipid syndrome. All women conceived spontaneously and had no history of previous PE.

PE was defined as new hypertension (blood pressure $\geq 140/90$ mm Hg on two consecutive occasions) and new proteinuria (24hr secretion of ≥ 300 mg), in the absence of urinary tract infection, developed after 20 weeks gestation in women with no prior hypertension or proteinuria before pregnancy [35-36].

Placental lobe dual perfusion methodology

STB-EVs were prepared using the placental lobe dual perfusion model, modified as previously described [37-38]. Placentas were processed immediately and, following an equilibration period, they were perfused for 3hr. All perfusion media were passed through $0.1\mu\text{m}$ filters to minimize background particles that would interfere in subsequent STB-EV analysis. At the end of the 3hr perfusion period, the maternal-side perfusate was centrifuged first at $1,500\times g$ for 10min at 4°C (Beckman Coulter Avanti J-20XP centrifuge and Beckman Coulter JS-5.3 swingout rotor) to remove contaminating red blood cells, other cells and large cellular debris. This procedure was repeated and the maternal side perfusate, cleared of all cells and cellular debris, was used to isolate total STB-EVs (tSTB-EVs) or was subjected to sub-fractionation (figure 1) to obtain STB-MVs and STB-EXs.

Accordingly, the following STB-EV preparations were obtained:

Study group 1 - tSTB-EV of ten control and ten PE placentae

Study group 2 - processed for STB-MV using nine control and ten PE cases.

Study group 3 - processed for STB-EX using five control and seven PE cases.

Purification of the tSTB-EVs

Maternal side perfusate, prepared from placentae of study group 1, was centrifuged at $150,000\times g$ for 1hr at 4°C (Beckman L80 ultracentrifuge and Sorvall TST28.39 swingout rotor) to isolate a pellet enriched for tSTB-EVs. The resultant pellet was washed in sterile PBS (Sigma, Poole, UK) and then re-suspended in sterile PBS.

Preparation of STB-MV and STB-EX

Fresh cleared maternal side perfusate was centrifuged at 10,000xg for 30min at 4°C (same centrifuge as above)) to pellet a fraction enriched for STB-MVs [6, 39] . The supernatant was removed and retained for STB-EX isolation, and the pellet (STB-MVs) was then washed, and re-suspended in sterile PBS (Fig. 1).

The supernatant retained following STB-MV removal was passed through a 0.22µm stericup filtration device (Millipore, Watford, UK), and then centrifuged at 150,000xg for 2hr at 4°C. The resultant STB-EX pellet was washed and resuspended in sterile PBS (Fig 1).

Protein Concentration and sample storage:

The total protein content of each STB-EV preparation was determined by BCA protein assay kit (Pierce, Paisley, UK), and aliquots then stored at -80°C until subsequent use (39).

Molecular Cargo identification by Western blotting

We used Western blots to confirm STB origin of the isolated STB-EVs, enrichment of STB-MV and STB-EX, and the presence of PP13, using modifications of previously described methods [24,39]. Briefly, tSTB-EVs, STB-MVs and STB-EXs (25µg protein) were diluted in Laemmli buffer and loaded onto 4-12% bis-Tris gradient gels (Life-Technologies, San Diego, CA, USA). Separated proteins were then electro-transferred onto PVDF membrane (Bio Rad, Hercules, CA, USA). Nonspecific binding sites were blocked with 5% blotto (Santa Cruz, San Diego, CA, USA). The blocked membranes were then incubated overnight at 4°C to identify the following antigen with their respective antibodies: 1) PP13 was detected by monoclonal antibody clone 534 [28], 2) Placental alkaline phosphatase (syncytiotrophoblast marker; PLAP) PLAF was detected by NDOG2 mAb (Oxford group in house mAb) [37], 3) Apoptosis-Linked Gene 2-Interacting Protein X (Alix) was detected by mAb clone 3A9 as used as exosomes specific marker (Cell Signaling Technology) [6, 16], 4) the cluster

differentiation protein 9 (CD9) was detected by its anti mAb to confirm exosome enrichment, and 5) Actin was detected by anti-actin mAb as a loading control [6]. The membranes were washed thrice and then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP, Dako, Losning, Denmark). The immune-complexes were washed again and incubated with an enhanced chemiluminescence substrate (Pierce). Signals were detected by membrane exposure to Hyper-film ECL (GE Health Care, Minnesota, USA).

Nanoparticle Tracking Analysis

Size distribution profiles of STB-MVs and STB-EXs from control and PE placentas were obtained using NTA as described previously [6,38-39], with minor modifications. All samples were analysed using an NS500 instrument equipped with 405nm laser and software version 2.3 (Malvern Instruments, UK). Each sample was diluted in sterile PBS to generate approximately 5×10^8 vesicles/mL. The samples were automatically introduced into the sample chamber and five video recordings of 1min in duration were captured for each sample as previously described [11]. NTA post acquisition settings were also optimised and kept constant between samples. Each video was processed to obtain the mean, and modal EV size [37-39].

Enzyme-linked immune sorbent assay (ELISA)

Surface PP13 levels were determined using Hylabs ELISA kits (EL96W003) with minor modifications [25]. Standards and 50ng total protein of individual tSTB-EVs, STB-MVs and STB-EXs samples were loaded in duplicate onto the ELISA micro-titer plate coated with PP13 specific 1st mAb. The complex was completed with biotin conjugated PP13-specific mAb 215-28-3 and further reacted with Streptavidin-HRP. The reaction was developed with TMB substrate and stopped with 2M HCl, and the optical density was determined at 450 versus 650 nm using Flouorostar (OPTIMA, Perkin Elmer Turku, Finland). Values were converted to PP13 levels using the standard curve.

To obtain the total PP13 concentration (inside and outside), each sample was solubilized with 0.1% Triton-X₁₀₀ for 30 min on ice [39]. PP13 was then determined by ELISA as described above.

PP13 adjustment to gestational age

Median PP13 for each gestational age (and the number of cases used to calculate it) were depicted against the gestational weeks for Total PP13 for PE and control. The weighted regression line was calculated considering the number of cases used to calculate each median. The regression line equation was defined as

- 1) $Y_{PP13} = aX + b$, where a is the slope of the curve, X the gestational week, and b the value of Y_0 .

For adjustment we used a Ratio (R) that was obtained as

- 2) $R = PP13_{\text{measured}} / PP13_{\text{calculated}}$

The measured value of an individual sample was divided by the calculated value derived of the regression curve of equation 1 above.

All values were then set to gestational week 40 (full term) according to:

- 3) $Y_{\text{adj}} = R \times aX + b$

In equation 3 the value of X was set to 40 weeks (term).

Statistical Methods

Comparisons of categorical variables between PE and control groups were made using Fisher's exact test. Kruskal-Wallis or Mann Whitney non-parametric tests were used for continuous variables. P-values ≤ 0.05 were considered significant. The data were analyzed using SPSS/V-24 (SPSS Inc., Chicago, IL, USA).

Results

Participant characteristics

Control and PE groups showed no difference in maternal age, BMI and gravidity (Table 1). At booking the PE group had significantly higher mean arterial blood pressure (MAP) compared to controls. Maximum MAP at delivery was even higher for PE patients with more severe proteinuria (data not shown). Gestational age at delivery was significantly earlier for PE cases compared to control, with corresponding significantly smaller birthweight and placental size. PE baby weights were in the lowest 10-centile for gestational age (Table 1).

Nanoparticle Tracking Analysis

The size distribution of STB-MVs and STB-EXs isolated from control and PE placentas was determined using NTA (Fig. 2). The profiles clearly confirmed that the isolation protocol produced enriched preparations of EVs in the size range of MVs and EXs, with no significant difference in size between control and PE preparations (Fig. 2).

Analysis of the protein cargo of STB-EVs and their sub-populations

Initial Western Blot analysis of pooled samples confirmed the presence of PLAP in the tSTB-EVs, STB-MVs and STB-EXs, verifying their placental origin [15] (Fig 3A). The house keeping protein actin was also detected in all three pools (Fig. 3A).

Exosome enrichment in STB-EX was demonstrated by the presence of Alix and CD9, which were absent from STB-MVs and only weakly detected in tSTB-EVs (Fig 3A).

Finally, PP13 was detected in all study groups by Western blotting, supporting previous observations of a placental origin of PP13 (Fig 3A).

Comparison of marker distribution between PE and Control

Expression of PLAP, PP13, and Alix was then investigated in individual samples from tSTB-EVs (upper panel), STB-MVs (middle panel) and STB-EXs (lower panel) study groups (Fig 3B). High PLAP expression was found in all samples of both PE and control groups (Fig.3B). Alix was expressed in all STB-EXs but not in STB-MVs and tSTB-EVs (Fig 3B). Finally, PP13 was highly expressed in the tSTB-EVs, STB-MVs and STB-EXs (Fig. 3B). A semi-quantitative densitometry analysis of PP13 normalized to either actin or PLAP did not show significant differences between control and PE samples (data not shown), presumably due to low sensitivity of this method.

PP13 level in the STB extracellular vesicles sub-fractions

The total PP13 (surface and intravesicular), measured by ELISA, was significantly reduced in PE tSTB-EVs ($p=0.001$), and in STB-MVs ($p=0.003$), compared to controls (Table 2). For STB-EX, the trend was towards lower PP13 in PE, although the differences did not reach significance, most likely due to the small sample size ($p=0.109$) (Table 2).

Surface level of PP13 was significantly reduced in PE STB-MVs compared to controls ($p=0.001$) (Table 2). The difference did not reach significance for STB-MVs ($p=0.076$) and STB-EXs ($p=0.286$), (Table 2).

In all three preparations, the ratio of surface PP13 versus total PP13 for PE compared to controls was not significantly different (Table 2). Interestingly, the ratio was approximately half of the total PP13, indicating a 1:1 ratio of surface to intravesicular PP13 except for control STB-MVs where the ratio was 0.8 (Table 2).

PP13 level of Total PP13 in the tSTB-EVs was had a correlation coefficient $r=0.3162$ and for PE it $r=0.696$ (Figure 4). Adjusting PP13 to GA have shown that the level of total PP13 was lower in PE compared to control after adjustment to GA (Table 3) and that for tSTB-EVs and the STB-MVs, that could maximize the numbers of PE cases, the differences was also found before and after adjustment to GA (Figure 5,

Table 2 &3).

Discussion

PP13 has been previously identified in maternal serum and plasma, and in the placental tissue [24, 26]. In this study, we have demonstrated, for the first time that PP13 is also a part of the protein cargo of STB-EVs and more specifically STB-MVs and STB-EXs, verified by size and molecular cargo. The study showed the presence of PP13 on the surface and inside the exosomes and microvesicles. Also, in PE, PP13 expression is reduced in both the STB-MV and STB-EX, and the reduction is compartment proportional (e.g. – the surface and intravesicular levels are reduced in a similar manner).

Figure 6 shows a schematic drawing of STB-MVs and STB-EXs, with the novel positioning of PP13 added. Once released into the intravillous space, the STB-EVs travel along the uterine vein, and into the maternal circulation via the lungs, where, if they behave as other tested EVs do, they are rapidly cleared predominantly by the liver and spleen [1, 13]. Thus, there is potentially only a short window of opportunity for the surface PP13 to interact with maternal vasculature and organs.

PE is a heterogeneous syndrome that is often sub-divided into term and pre-term cases. The latter are accompanied by decreased blood perfusion to the pregnancy and inadequate uterine spiral artery remodeling [19, 20], and is often associated with fetal growth restriction [19], while term cases are mainly associated with maternal obesity, cardiac dysfunction, and other maternal disorders [20]. Significantly reduced PP13 expression in the tSTB-EVs was found for all PE cases. The adjustment to GA in our data suggests that reduced PP13 in tSTB-EVs and STB-MVs in PE cases may represent changes associated with PE rather than with earlier GA at delivery. In this study the number of cases was relatively small and sub-division to early (<34 weeks),

preterm (<37 weeks) and term (>37 weeks) was not meaningful. A larger study cohort is required to confirm the current findings and further investigate the significance of soluble and STB-EV PP13 in term and preterm PE. Also, severity of PE tends to be greater the earlier the disorder develops, and therefore other confounders may contribute to the changes in PP13 earlier in gestation.

We have conducted testing and analysis of *ex vivo* perfusion derived samples from the maternal side. Work performed in Oxford by part of the co-authors (Vatish et al. unpublished observation) suggests that PLAP positive STB-EVs that are derived from the apical surface of the syncytiotrophoblast are not transported into the fetal circulation. However transportation of STB-EV released from the syncytiotrophoblast basal membrane into the fetal circulation, has not been studied and can therefore not be ruled out. Thus, we couldn't rule out release of EVs from the syncytiotrophoblast basal membrane.

The reduced level of PP13 following *ex-vivo* perfusion may not reflect *the situation in the in vivo* pathophysiology [4]. Circulating levels of STB-EVs are a reflection of the rate of clearance versus the rate of production. In the study we determined a decreased level of PP13 in the cargo of the STBs in the PE cases compared to Control. Accordingly, it is difficult to say what will be the overall amount of PP13 delivered to the circulation, and whether there is an overall decrease or increase in the amount of PP13 linked to STBs that reached the maternal circulation through this pathway, even though the placentas are smaller. Qualitative changes in the cargo of STB-EV cargo, eg. 'eat me' surface markers such as desialylated proteins are decreased on PE derived STB-EV [40], which could be a mechanism leading to decreased clearance. Further studies are thus required to evaluate the *in-vivo* situation and to determine STB-EVs-linked PP13 in the circulation

In the past we have determined the level of free circulating PP13 in pregnant

women comparing control to PE cases [25-27]. In this study we have found that there is STB-EVs-PP13 that may also reach the maternal circulation. Thus - PP13 may be either soluble or associated with STB-EVs, with different pathophysiological effects in the maternal circulation. These dual PP13 compartments raise the question of their potential differential role in communicating the PE pathology to maternal cells and organs [46].

Given that PP13 is associated with multiple signaling pathways [43-44], reduced level of PP13 in STB-EVs could contribute to the exacerbation of the PE state through impaired immune tolerance [23,26,43-45], increased inflammation [27], susceptibility to PE among people with blood groups B and AB [46-47], fetal growth restriction [49], and arterial stiffness [50]. If this is the case, then PP13 administration as a targeted therapy for PE could warrant further investigation [49]. Our main future undertaking is to evaluate how PP13 on the surface of STB-MVs and STB-EXs and in their intra-vesicular compartment influences the maternal organs in PE [51].

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Tables

Table 1: Patients' characteristics

<u>Parameter</u>	<u>Control</u> <u>(n=18)</u>	<u>PE</u> <u>(n=20)</u>	<u>P*</u>
<u>Enrolment</u>			
Maternal age (Yrs, Mean, [95%CI])	33.4[30.4-36.5]	33.7[30.8-36.6]	0.963
BMI (kg/h², (Mean [95%CI])	24.4[20.8-28.0]	28.7[25.2-32.3]	0.102
Gravidity (Mean, [95%CI])	1.9[1.3-2.7]	1.5[1.0-1.9]	0.373
Previous PE (%)	0	50.0	0.002
MAP (mm Hg, Mean [95%CI])	77.6[73.9-81.3]	91.6[84.2-99.0]	0.002
<u>At Delivery</u>			
<u>Max MAP</u> (mm Hg, Mean [95%CI])	90.9[86.5-95.2]	130.8[124.4-137.2]	<0.001
<u>Max Urine protein (g/dL (Mean [95%CI])</u>	0.17[0.00-0.36]	2.70[2.39-3.01]	<0.001
<u>Gestational Age (wks, Mean [95%CI])</u>	39.9[39.4-40.5]	34.0[32.4-35.6]	<0.001
<u>Baby birthweight (g, Mean [95%CI])</u>	3759[3519-3998]	1978[1602-2354]	<0.001
<u>Placental Weight (g, Mean [95%CI])</u>	733[680-786]	408[331-485]	<0.001

MAP – Mean Arterial blood pressure, g – grams, wks- weeks, yrs-years, mm HG- millimeter mercury, CI- confidence interval, dL - deciliter

* Mean Values, 95% CI and P values were calculated according to Mann-Whitney non-parametric test

Table 2 – PP13 in the different STB-EV study groups

<u>Parameter</u>	<u>Sub-</u>	<u>n</u>	<u>Controls</u>	<u>PE</u>	<u>P*</u>
<u>population</u>					
<u>Total PP13</u>	tSTB-EVs	9,10	638[514-761]	301[181-920]	0.001
	STB-MVs	7,10	402[218-465]	216[140-292]	0.003
	STB-EXs	4,6	284[43-525]	176[111-241]	0.109
<u>Outside PP13</u>	tSTB-EVs	8,10	308[199-418]	189[92-287]	0.076
	STB-MVs	7,10	342[218-465]	125[77-173]	0.001
	STB-EXs	4,6	106[28-185]	78[22-135]	0.286
<u>Ratio **</u>	tSTB-EVs	9,10	0.49[0.34-0.63]	0.58[0.45-0.72]	0.286
	STB-MVs	7,10	0.80[0.65-0.95]	0.62[0.43-0.63]	0.079
	STB-EXs	4,6	0.39[0.22-0.63]	0.42[0.21-0.63]	0.831

tSTB-EVs- total syncytiotrophoblast extracellular vesicles

STB-MVs – syncytiotrophoblast micro vesicles

STB-EXs- syncytiotrophoblast exosomes

Values are pg PP13/μg Protein, Means [95%CI]

*- Mann Whitney Non-parametric test

** -Outside PP13/Total PP13

Table 3 – Total PP13 in the STB-EV study groups after adjustment to GA

<u>Parameter</u>	<u>STB</u>	<u>n</u>	<u>Controls</u>	<u>PE</u>	<u>P*</u>
<u>Total PP13</u>	tSTB-EVs	9,10	624[510-737]	391[264-518]	0.001
	STB-MVs	7,10	399[277-522]	223[151-296]	0.004
	STB-EXs	4,6	348[34-62]	161[100-222]	0.039

tSTB-EVs- total syncytiotrophoblast extracellular vesicles

STB-MVs – syncytiotrophoblast micro vesicles

STB-EXs- syncytiotrophoblast exosomes

Values are pg PP13/μg Protein, Means [95%CI]

Mann Whitney Non-parametric test

Figure Legends

Figure 1: Fractionation steps of placental perfusate to obtain the different sub-populations of STB-EVs

Schematic diagram of the sequential centrifugation and filtration protocol for the isolation and fractionation of placental STB- EV from maternal perfusate collected using the *ex vivo* dual placental lobe perfusion method. SN=supernatant. More details are provided in the materials and methods.

Figure 2: Nanoparticle tracking analysis of size distribution profile for controls and PE of STB-MVs and STB-EXs

Mean Nanoparticle Tracking Analysis size distribution profile for STB-MVs and STB-EXs from A) Control and B) PE affected pregnancies. Values in the tables show mean \pm SE for the mean and modal size (nm).

Figure 3: Biochemical characterization of STB-EVs, STB-MVs and STB-EXs

A) Representative immunoblots for Alix, CD9, placental alkaline phosphatase (PLAP), PP13 and actin in pooled samples of STB-MVs, STB-EXs and tSTB-EVs and placental lysate as a positive control.

B) Representative immunoblots of PP13, PLAP and Alix in individual control and PE tSTB-EVs (upper panel), STB-MVs (middle panel) and STB-EX (lower panel) samples. Densitometry analyses showed no statistically significant differences.

Figure 4: Median total PP13 for STB-EVs according to gestational age

Median PP13 is depicted against the gestational weeks for Total PP13. Blue- Control, (n=9), Red- PE (n=10). The weighted regression line was taking into consideration the number of cases used to calculate each median.

The regression lines equations for control and PE were:

1) $Y_{\text{control}} = 36.19X - 800$, $r^2=0.1$, $p=0.686$.

2) $Y_{\text{PE}} = 25.45X - 597.08$, $r^2=0.422$, $p=0.041$.

Where is is the gestational week.

3) $R = \text{PP13}_{\text{measured}} / \text{PP13}_{\text{calculated}}$ according to equations 1 and 2 above.

The adjusted value was then made for each measured value according to the following way

4) $Y_{\text{adjusted}} = R * Y_{\text{control}}$, or

5) $Y_{\text{adjusted}} = R * Y_{\text{PE}}$

In equations 4 and 5, the value of X was set to 40 weeks (terms) .

Figure 5. Box Plot of PP13 in PE and Control before and after adjustment to gestational age

Total PP13 of the different study groups of STB-EV are depicted for PE cases (green) and Controls (blue). The left pairs are raw PP13 value (pgPP13/ μ g protein). The right pair PP13 with the PP13 adjusted to protein before (left pair) or after adjustment to gestational age (right pair). Horizontal line – medians, boxes – the values between 25-75%, lines represent the 5-95%.

The significant differences are detailed in Table 2 top part for the unadjusted values and in Table 3 for the adjusted values

Figure 6: Distribution of PP13 on STB-MVs and STB-EXs.

STB-EXs formed as the end-product of the endocytic recycling pathway. PP13 position

on the surface and the intravesicular space is added to the known localization of exosome markers including tetraspanins family (CD9 and CD63), Alix, Tsg101, PLAP, heat shock proteins (HSPs), and galectin-1.

STB-MVs bud off the plasma membrane of the syncytiotrophoblast. The known localization of actin, PLAP, and other bioactive molecules is supplemented by the presence of PP13 in the intravesicular and on the surface of the STB-MVs.

Figure 1:

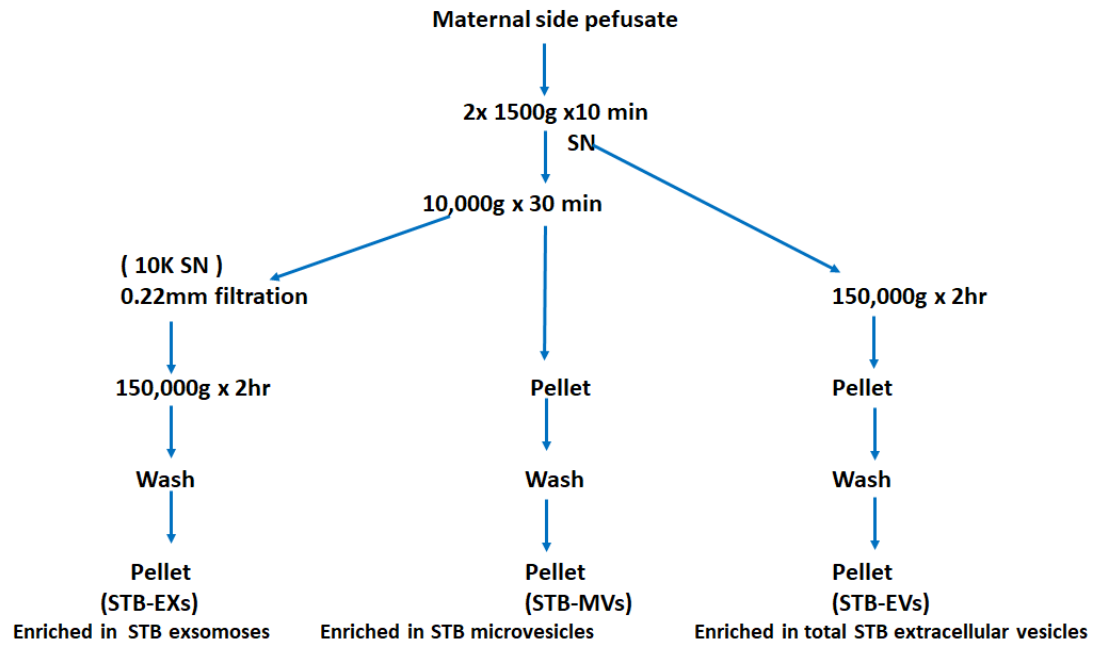
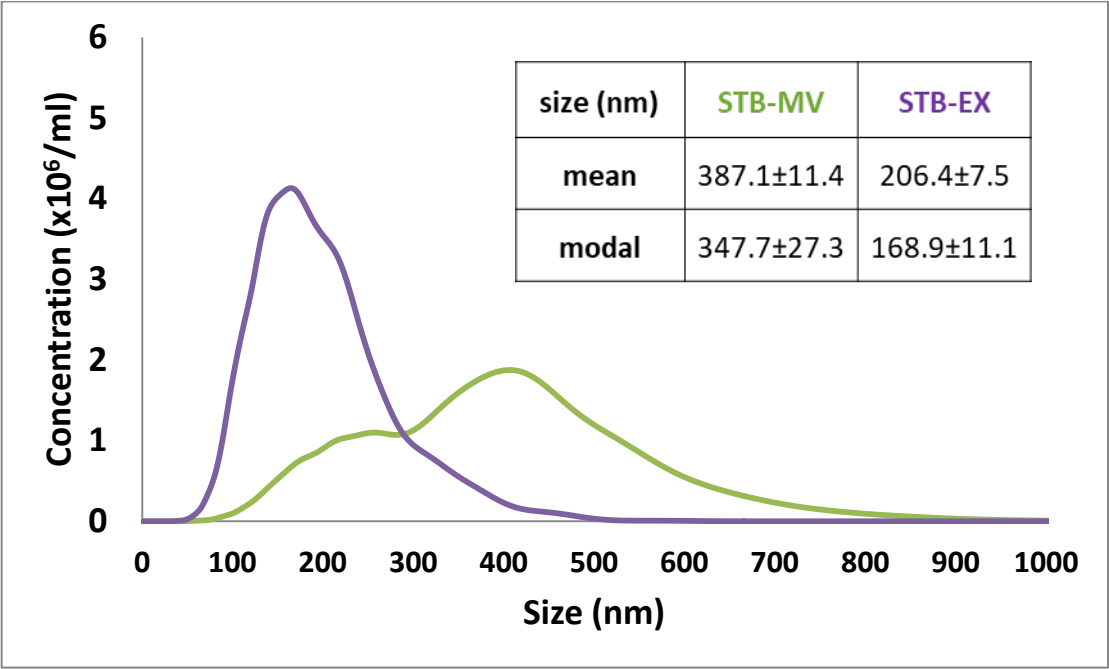


Figure 2:

A)



B)

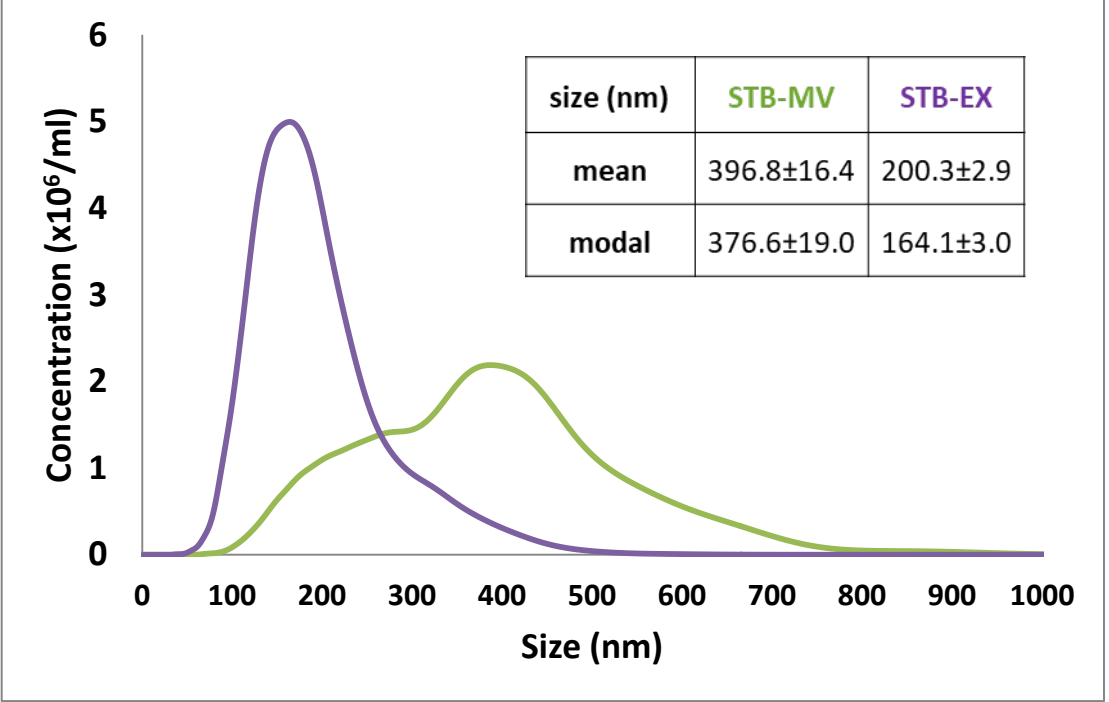


Fig.3A:

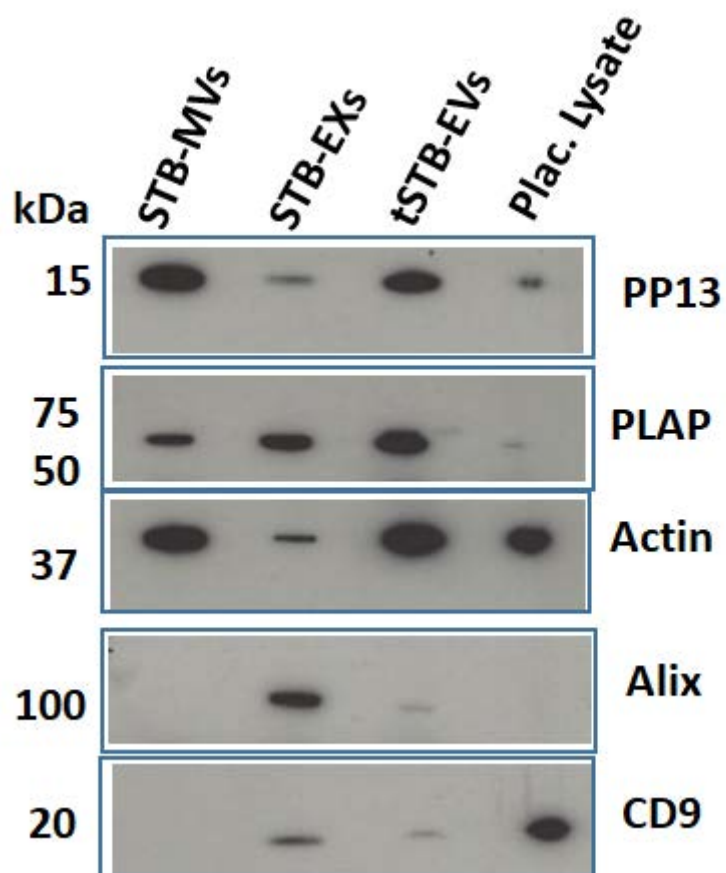


Figure 3B

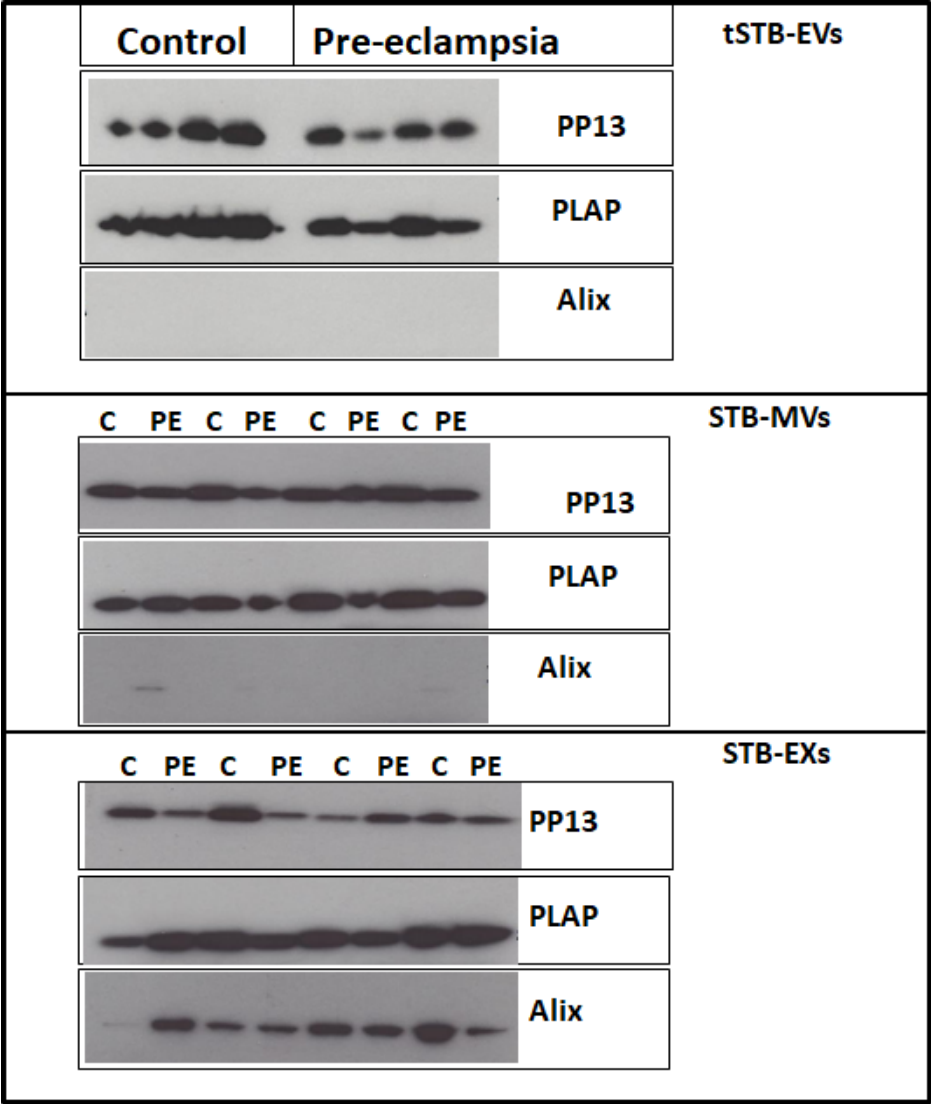


Figure 4: Median total PP13 for STB-EVs according to gestational age

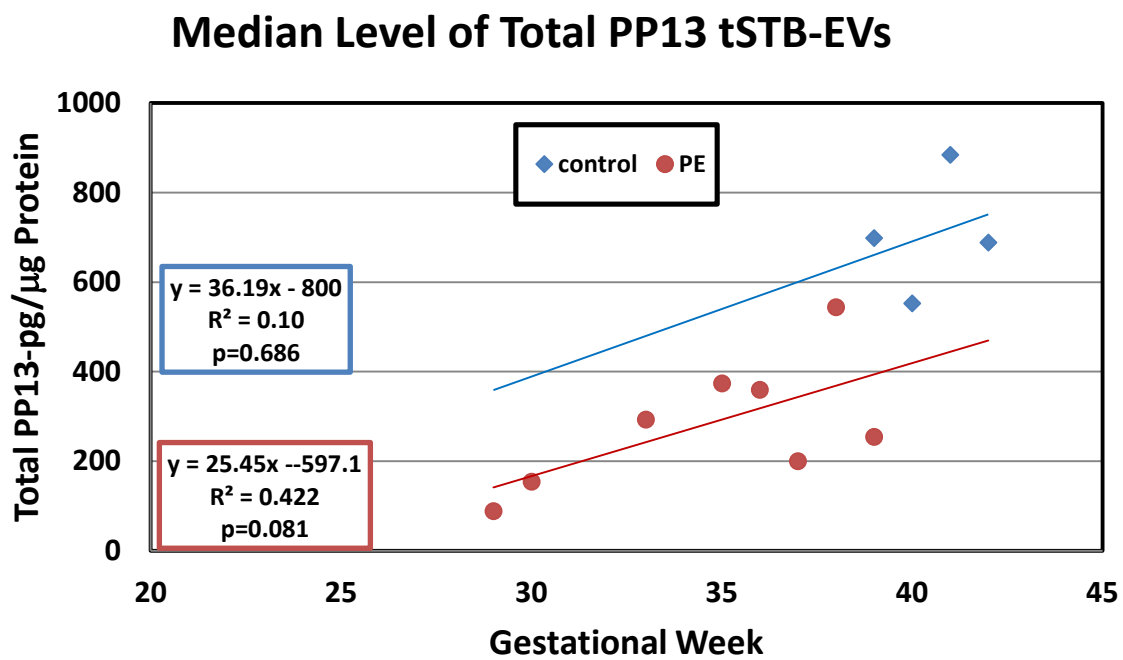


Figure 5: Box Plot of measured Total PP13 in PE and Control before and after PP13 adjustment to gestational age.

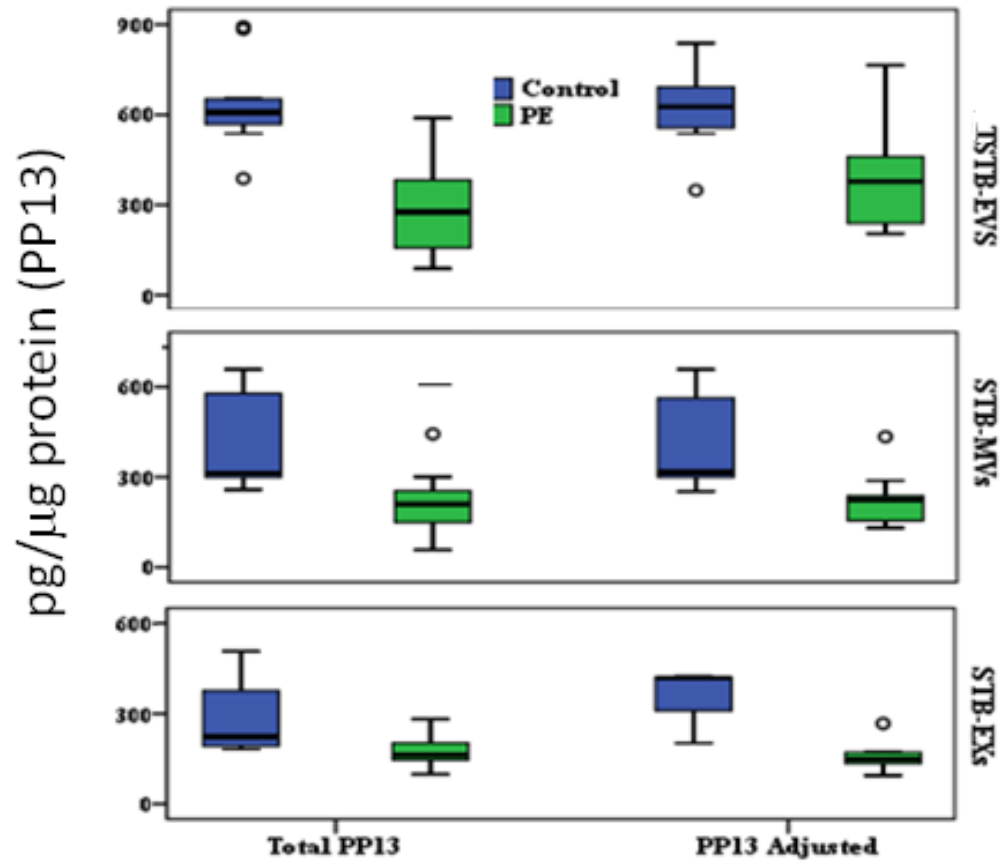


Figure 6: Distribution of PP13 on STB-MVs and of STB-EXs

