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Multicolor Flow Cytometry and Nanoparticle Tracking Analysis of Extracellular Vesicles in the Plasma of Normal Pregnant and Pre-eclamptic Women¹

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ABSTRACT

Excessive release of syncytiotrophoblast extracellular vesicles (STBMs) from the placenta into the maternal circulation may contribute to the systemic inflammation that is characteristic of pre-eclampsia (PE). Other intravascular cells types (platelets, leukocytes, red blood cells [RBCs], and endothelium) may also be activated and release extracellular vesicles (EVs). We developed a multicolor flow cytometry antibody panel to enumerate and phenotype STBMs in relation to other EVs in plasma from nonpregnant (NonP) and normal pregnant (NormP) women, and women with late-onset PE. Nanoparticle tracking analysis (NTA) was used to determine EV size and concentration. In vitro-derived STBMs and EVs from platelets, leukocytes, RBCs, and endothelial cells were examined to select suitable antibodies to analyze the corresponding plasma EVs. Flow cytometry analysis of plasma from NonP, NormP, and PE showed that STBMs comprised the smallest group of circulating EVs, whereas most were derived from platelets. The next most abundant group comprised unidentified orphan EVs (which did not label with any of the antibodies in the panel), followed by EVs from RBCs and leukocytes. NTA showed that the total number of EVs in plasma was significantly elevated in NormP and late-onset PE women compared to NonP controls, and that EVs were smaller in size. In general, EVs were elevated in pregnancy plasma apart from platelet EVs, which were reduced. These studies did not show any differences in EVs between NormP and PE, probably because late-onset PE was studied.

exosomes, extracellular vesicles, flow cytometry, microvesicles, nanoparticle tracking analysis, placenta, pre-eclampsia, pregnancy

INTRODUCTION

Pre-eclampsia (PE) is a multisystem disorder of pregnancy that can be life threatening to both mother and fetus. Its pathogenesis is unknown, but the presence of trophoblast and placental tissue is mandatory. The maternal syndrome is characterized by new onset of hypertension and proteinuria in the second half of pregnancy. PE is a “two-stage” disease [1]. Stage 1 occurs in the first half of pregnancy, is asymptomatic, and is associated with the development of an inadequate uteroplacental circulation (poor placentation), which leads to ischemia/reperfusion injury and placental oxidative stress [1–3]. In stage 2, the mother's circulation is affected by proinflammatory and antiangiogenic factors released from the dysfunctional placenta (reviewed in [1]).

Many different trophoblast-derived proteins are increased in PE, and may play a role in the pathogenesis of the disorder. These include the antiangiogenic factors soluble fms-like tyrosine kinase-1 and soluble endoglin [4–7]. However, these factors alone cannot account for the diversity of the maternal features. We have proposed that extracellular vesicles (EVs), derived from the syncytiotrophoblast, may play a role [8, 9]. These vesicles (known as syncytiotrophoblast microparticles [STBMs]) are present in the plasma of normal pregnant (NormP) women, with significantly increased levels in PE [9, 10]. There is strong evidence that they play a role in the maternal syndrome of PE. STBMs prepared from placentas ex vivo cause endothelial dysfunction [11–14], activate neutrophils [15, 16], stimulate monocyte proinflammatory cytokine production [10, 17–21], and trigger thrombin generation in vitro [22].

Plasma STBMs were first measured with an ELISA using the syncytiotrophoblast-specific anti-placental alkaline phosphatase (PLAP) monoclonal antibody (NDOG2) [9]. STBMs were detected as early as the late first trimester of normal pregnancy, increased throughout normal pregnancy [10], and were present in significantly higher concentrations in PE [9, 10], more so in early-onset PE (≤ 34 -wk gestation) than in late-onset PE (≥ 34 -wk gestation) [8]. However, although this STBM ELISA is quantitative, it gives no information about the nature of the EVs.

Currently, flow cytometry is the most widely used method to investigate EVs. It is quantitative and allows simultaneous measurement of multiple antigens of the EVs [23–26]. It has been used to measure STBMs in maternal plasma in normal pregnancy and PE with variable results, depending on the anti-syncytiotrophoblast antibody used. Antibodies to various antigens expressed on the syncytiotrophoblast have been used, including: ED822, an antibody to an unknown syncytiotrophoblast epitope [27, 28]; NDOG1, an IgM antibody to an epitope of hyaluronic acid [29–31]; NDOG2, an antibody to PLAP [32]; and AbD Serotec clone H17E2, also an antibody to PLAP [33]. Using NDOG1, circulating STBMs were detected

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in the plasma of nonpregnant (NonP) women, which raises doubts concerning the trophoblast specificity of the antibody [30, 31]. Using ED822 or anti-PLAP, STBMs comprised a small population of the total EVs in NormP and PE women [27, 28, 32, 33], but, in one study, surprisingly more circulating STBMs were detected in NonP controls compared to NormP and PE women [32]. However, all but one of these studies [30] used indirect antibody labeling, which is well known to be more susceptible to nonspecific binding than direct labeling, necessitating more thorough washing, which is more difficult to achieve with suspensions of EVs compared with cells.

To better define STBM populations, we have therefore developed a multicolor flow cytometry assay using directly labelled antibodies, which reduces the problem of nonspecific binding and allows more precise identification of STBMs using multiple markers [14]. We used STBMs prepared from the maternal-side eluate of dual perfused placenta lobes (placental STBMs [pSTBMs]) for assay development [14, 21, 22, 34].

A downstream consequence of the intensified systemic inflammation of PE, to which circulating STBMs may contribute, would be activation and release of EVs from maternal cells, including leukocytes, endothelium, platelets, and red blood cells (RBCs). We speculate that these might further exacerbate the disease. As with the studies of STBMs, there are major inconsistencies in the numbers of circulating EVs reported in previous studies (reviewed in [35]), and many variables that may affect quantification of EVs [36]. One is the specificity of the markers for each EV subpopulation. Some of the previous markers chosen are not absolutely specific and may identify more than one EV type, leading to some EVs being incorrectly identified, particularly if single-color labeling is used [28, 31]. Furthermore, many researchers assume that the antigens expressed on the surface of a particular cell will always be present on EVs derived from that cell, whereas this may not be the case [37, 38]. We therefore prepared EVs from pure populations of the different vascular cell types *in vitro* and examined their angiogenic labeling in relation to the parent cells. We tested each marker for cross-reactivity with pSTBMs and, on this basis, we identified a panel of suitable markers for use in flow cytometry to measure STBMs in relation to EVs from other cell types in maternal plasma in normal pregnancy and PE.

Although conventional flow cytometry is a powerful tool to identify and phenotype EVs, it is limited by its lower limit of detection of the size of single EVs [39, 40]. EVs are comprised of microvesicles and exosomes, which differ with respect to size, protein content, mode of production, and probably function. Microvesicles are larger (100 nm to 1 μ m), and are generated by direct budding or shedding of the plasma membrane. Exosomes are smaller (30–100 nm). They are generated by reverse budding of the endosome membrane into multivesicular bodies, which fuse with the plasma membrane of the cell and are released by exocytosis. Most studies of EVs have used analog flow cytometers, which can only detect EVs \geq 500 nm [36]. In this study, we used a digital flow cytometer, which we have shown can measure EVs \geq 300 nm (sizing based on polystyrene beads) [14, 34], but cannot detect the smaller microvesicles and exosomes (<300 nm) present. We have therefore used nanoparticle tracking analysis (NTA) to size and count EVs. This methodology, which determines a vesicle's size by the degree of its Brownian motion, can visualize and quantify nanoparticles down to 40 nm in size [14, 34]. We have used it here for the first time to size and count EVs in pregnancy plasma.

MATERIALS AND METHODS

Preparation of In Vitro-Derived EVs

Peripheral blood ($n=3$) and normal placentas from Cesarean section ($n=8$) were donated by volunteers after obtaining informed consent. This study was approved by the Oxfordshire Research Ethics Committee.

Placental STBMs were prepared using a dual placental perfusion system as previously described [14, 21, 22, 34]. Platelet EVs were derived by calcium ionophore (A-23187) stimulation as previously described [37]. RBC EVs were derived, with slight modification, as previously described [41]. Aliquots of RBCs ($\sim 1 \times 10^7$ /ml) were stimulated with 5 μ mol/L A-23187 for 30 min at 37°C, then 5 mmol/L EDTA was added to each aliquot to stop the activation process, and samples were centrifuged for 30 sec at $12000 \times g$. The EV-containing supernatant was collected and analyzed by flow cytometry.

Human umbilical vein endothelial cells (HUVECs) were prepared from three human umbilical cords using a previously described method [42]. For EV generation, confluent HUVECs were seeded into six-well plates coated with 1% gelatin at a density of 1×10^5 per well. HUVECs were washed in PBS and cultured in 0.1- μ m filtered medium with 5 μ mol/L Camptothecin (Sigma-Aldrich) for 24 h, culture supernatants were collected, and cells were removed by centrifugation at $1000 \times g$ for 3 min. The supernatant containing the HUVEC EVs was centrifuged ($150000 \times g$ [max], 1 h, 4°C) in a Beckman L8-80M ultracentrifuge. As a control, 0.1- μ m filtered medium was also centrifuged under the same conditions. The pellets were resuspended in PBS and analyzed by flow cytometry. Any background events/contaminating particles found in the control 0.1- μ m filtered medium pellet were subtracted from the final flow cytometry analysis.

Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation over lymphoprep (Axis Shield Diagnostics, Cambridge, UK). PBMCs were counted and separated into monocytes and lymphocytes using immunomagnetic anti-CD14 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Granulocytes were prepared by step density gradient centrifugation over Percoll (GE Healthcare Life Sciences, Little Chalfont, UK) and further enriched using immunomagnetic anti-CD15 MicroBeads (Miltenyi Biotec). Lymphocyte, monocyte, and granulocyte purity was determined using flow cytometry.

Monocytes, lymphocytes, and granulocytes were cultured in 12-well plates in 0.1- μ m filtered RPMI medium (Sigma-Aldrich), supplemented with 10% FBS, 50 IU/ml penicillin-streptomycin, and 2 mmol/L L-glutamine. Monocytes (5×10^5) were treated with 1 μ g/ml lipopolysaccharide (Sigma-Aldrich) for 5 h, and 10 μ mol/L A-23187 was added at the end of the culture for 15 min. Lymphocytes (1×10^6) were treated with 10 ng/ml phorbol myristate acetate (Sigma-Aldrich) plus 2 μ mol/L ionomycin (Sigma-Aldrich) for 4 h. Granulocytes (1×10^6) were cultured for 18 h [43]. After incubation, culture supernatants were removed and cells pelleted by centrifugation at $400 \times g$ for 5 min. The remaining supernatants were centrifuged ($150000 \times g$ [max], 1 h, 4°C) in a Beckman L8-80M ultracentrifuge to pellet the EVs.

Antibody Labeling and Flow Cytometric Analysis of Cells

Cells were resuspended in 1% (v/v) FBS/PBS and incubated for 15 min with the appropriate antibody and its matched isotype control antibody (Table 1) in a total volume of 100 μ l. Following incubation, cells were washed, resuspended in 300 μ l of 1% (v/v) FBS/PBS, and analyzed using a BD LSRII flow cytometer (BD Biosciences). A total of 10000 events were collected for each cell type.

Antibody Labeling and Flow Cytometry of In Vitro-Derived EVs

Prior to use, all antibodies, FcR blocking reagent (Miltenyi Biotec GmbH), and Bio-Maleimide were filtered through Nanosep 0.2- μ m centrifugal filters (Pall Life Sciences, Portsmouth, UK). PBS was filtered through 0.1- μ m filters (Anotop 25; Whatman GmbH, Dassel, Germany). EVs were analyzed using a BD LSRII flow cytometer as previously described [14, 34], using a side-scatter (SSC) threshold of 200 arbitrary units. Fluorescent calibration microspheres of 290 nm (Duke Scientific Corporation, Palo Alto, CA) and 1 μ m (Polysciences Inc., Warrington, PA) were used to establish a vesicle gate (300 nm to 1 μ m), and the SSC and forward scatter (FSC) voltages were set. EV counts were calculated based on the instrument flow rate, measured using standard beads (BD Trucount Tubes; BD Biosciences). Two tubes were analyzed at the beginning of each experiment and one at the end. Samples were analyzed for 2 min using the LO flow rate setting (10 μ l/min). EV suspension (50 μ l) was incubated with 10 μ l FcR blocking reagent for 10 min at 4°C and then labeled with its selected antibody and isotype-matched control antibody for 15 min at

TABLE 1. Fluorescent label, antibodies, and isotype controls used in experiments.

Parameter	Clone	Final concentration	Specificity
Fluorescent label			
Bio-Maleimide (BODIPY FL N-(2-aminoethyl) maleimide) ^a	Not applicable	0.25 µM	Thiol reactive dye. General cell membrane marker
Negative controls			
IgG1-PECy7 ^b	MOPC-21	0.25 µg/ml	
IgG2a-Alexa647 ^c	MRC OX-34	2.5 µg/ml	
IgG2a-APCCy7 ^d	MOPC-173	2.5 µg/ml	
IgG2b-PECy5 ^d	MPC-11	0.05 µg/ml	
IgG1-Alexa647 ^d	MOPC-21	0.125 µg/ml	
IgG1-APC ^e	P3	2.5 µg/ml	
IgG1-PE ^f	11711	0.5 µg/ml	
IgG1-APCCy7 ^g	MOPC-21	5 µg/ml	
IgG1-PE ^d	MOPC-21	0.5 µg/ml	
IgG1-FITC ^c	Not specified	2.5 µg/ml	
IgG1-FITC ^f (conjugated using FITC Lightning Link kit) ^h	11711	3 µg/ml	
Antibodies			
CD61(gpIIb)-PECy7 ^b	SZ21	0.25 µg/ml	Platelets, megakaryocytes, osteoclasts, endothelial cells
CD41(gpIIb)-PECy7 ^b	P2	0.25 µg/ml	Platelets, megakaryocytes, early embryonic hematopoietic stem cells
HLA Class I-Alexa647 ^c	W6/32	2.5 µg/ml	All Hematopoietic cells, except red blood cells. Platelets, endothelial cells
HLA Class I-APCCy7 ^d	W6/32	2.5 µg/ml	All hematopoietic cells, except red blood cells. Platelets, endothelial cells
CD235a/b-PECy5 ^d	HIR2	0.05 µg/ml	Red blood cells, erythroid precursors
CD146-Alexa647 ^d	OJ79c	0.125 µg/ml	Endothelial cells, melanoma cells, Epithelial cells, fibroblasts, activated T cells, mesenchymal stromal cells, activated keratinocytes
CD144-APC ^e	16B1	2.5 µg/ml	Endothelial cells, hematopoietic stem cells, perineurial cells
CD105-PE ^f	166707	0.5 µg/ml	Vascular endothelial cells, activated monocytes, tissue macrophages, stromal fibroblasts (low levels), syncytiotrophoblast
CD45-APCCy7 ^g	2D1	5 µg/ml	Hematopoietic cells except platelets and mature erythrocytes
CD14-Alexa647 ^d	HCD14	2.5 µg/ml	Monocytes, macrophages, granulocytes (low levels)
CD66b-FITC ^c	80H3	2.5 µg/ml	Mature granulocytes, meta-myelocytes
NDOG2-PE ^d	Not applicable	0.5 µg/ml	Syncytiotrophoblast
NDOG2-FITC (conjugated using FITC Lightning Link kit) ^h	Not applicable	3 µg/ml	Syncytiotrophoblast

^a Molecular Probes/Invitrogen, Paisley, UK.
^b Beckman Coulter, High Wycombe, UK.
^c AbD Serotec, Kidlington, UK.
^d Biologend UK Ltd., Cambridge, UK.
^e eBioscience, Ltd., Hatfield, UK.
^f R&D Systems Europe Ltd., Abingdon, UK.
^g BD Biosciences, Oxford, UK.
^h Innova Biosciences Ltd., Cambridge, UK.

room temperature in the dark. EVs derived from platelets, RBCs, HUVECs, lymphocytes, monocytes, and granulocytes were labeled with the same antibody panels as their parent cells, as described above. Five-color flow cytometry was carried out on the pSTBMs, labeled with: Bio-Maleimide; NDOG2-PE; W6/32-Alexa-647; CD41-PECy7; and CD235a/b-PECy5. Fluorochrome compensation was set using BD CompBeads (BD Biosciences) and a single stain using Bio-Maleimide-labeled pSTBMs. Fluorescence minus one (FMO) controls substituting with the respective isotype control antibody were used in order to set gates. Placental STBMs were also double labeled with NDOG2-PE and CD146-Alexa-647, NDOG2-FITC and CD105-PE, NDOG2-FITC and CD144-Alexa-647, or the isotype control antibodies. Following incubation, samples were diluted with PBS and analyzed by flow cytometry.

Subjects

Blood samples were taken from 10 NonP, 10 NormP, and 10 PE women, all recruited to the Oxford Pregnancy Biobank. PE was diagnosed as ≥ 90 mm Hg diastolic blood pressure on at least two occasions within 24 h and proteinuria ≥ 500 mg in a 24-h protein urine collection, 50 mg/mmol protein:creatinine ratio, or at least 2+ on dipstick testing on two consecutive measurements. PE

women were matched to NonP women for age (± 4 yr) and parity (0, 1–3), and to NormP women for age, parity, and gestational age (± 13 days) (Table 2).

Preparation of Platelet Free Plasma EVs

Blood was drawn through a 20-gauge needle into a 4.5-ml 0.105 M buffered sodium citrate vacutainer (BD Biosciences). The first 2 ml of blood was discarded and all samples were processed within 2 h. Platelet-free plasma (PFP) was generated by double centrifugation; blood was first centrifuged at $1500 \times g$ for 15 min and then at $13000 \times g$ for 2 min, and then the PFP (supernatant) was frozen in aliquots at -80°C until use. For analysis, 3 ml of frozen PFP was thawed at 37°C , made up to 11 ml with PBS, and centrifuged ($150000 \times g$ [max], 1 h, 4°C) in a Beckman L8-80M ultracentrifuge. The supernatant was removed and the EV pellet was resuspended in 750 µl of PBS and analyzed by flow cytometry and NTA.

NTA of PFP EVs in NonP, NormP, and PE Samples

PFP EVs in ultracentrifuge pellets from NonP, NormP, and PE samples were analyzed using the NanoSight LM10 instrument (NanoSight Ltd., Amesbury, UK) and NTA software version 2.0, Build 130 (NanoSight Ltd.).

TABLE 2. Patient details for NonP, NormP, and PE women (n = 10).*

Parameter	Nonpregnant	Normal pregnant	Pre-eclampsia	P value [†]
Age (yr)	33.6 (3.63)	33.5 (3.24)	33.6 (3.58)	n.s.
Nulliparity	5/10 (50.0%)	5/10 (50.0%)	5/10 (50.0%)	n.s.
Gestation (days)		255.5 (14.3)	260.2 (17.7)	n.s.
Booking bp (mm Hg)		108/64 (6.88/6.74)	109/69 (10.0/10.39)	n.s.
Max bp (mm Hg)		120/74 (9.97/6.12)	160/106 (14.38/11.39)	<0.001
Max proteinuria (mg/24 h)			3077.41 (4669.49); range, 585–15 340	n.s.
Birth weight (g)		3514.1 (459.96)	2719.6 (705.24)	<0.01

* Data show mean \pm SD.

[†] n.s., not significant.

EVs were diluted in PBS and analyzed as previously described [44]. Videos were captured using a shutter speed of 20 msec and a camera gain setting of 500. Postacquisition settings were based on the manufacturer's recommendations and were kept constant between samples. Each video was analyzed to give the mode and mean EV size together with an estimate of EV concentration.

Flow Cytometry of PFP EVs in NonP, NormP, and PE Samples

PFP EVs in ultracentrifuge pellets from NonP, NormP, and PE samples were analyzed by flow cytometry as described above. PFP EVs were labeled with two separate antibody panels using FMO controls. The first panel aimed to identify the placenta-derived EVs. EVs were triple labeled with Bio-Maleimide, NDOG2-PE, and W6/32-APCCy7. The second panel used five-color labeling to identify EVs derived from platelets, RBCs, leukocytes, and endothelial cells. This panel consisted of Bio-Maleimide, CD41-PECy7, CD235a/b-PECy5, W6/32-APCCy7, and CD146-Alexa-647. Data were analyzed using FACSDiva software (BD Biosciences) and figures were generated using FlowJo version 7.6.5 (Tree Star Inc, Ashland, OR).

Measurement of STBMs in PFP by ELISA

STBMs in NonP, NormP, and PE PFP samples (n = 10) were also measured for comparison with flow cytometry using an in-house ELISA as previously described [8], with some modification. The anti-placental alkaline phosphatase antibody NDOG2 (10 μ g/ml) was used as the capture antibody; 4-methylumbelliferyl phosphate (0.2 mg/ml; Sigma-Aldrich) was used as a fluorescent substrate for the detection of endogenous alkaline phosphatase activity on the surface of the EVs. A pooled preparation of pSTBMs (n = 8) was used to prepare the standards for the ELISA. The plate was read using a FLUOstar Optima (BMG Labtech) plate reader at 1 h. The pSTBM standard curve was used to determine the STBM concentration in each PFP sample (ng/ml).

Statistics

Each data set was first tested for a Gaussian distribution by using a Shapiro-Wilk normality test and for equal variance using a Levene median test. Data sets comparing antigen expression on cells versus in vitro-derived EVs were compared using a Student *t*-test. Total PFP EV counts (flow cytometry and NTA) and NTA sizing data were measured using a one-way ANOVA, and pairwise multiple comparisons were made using a Holm Sidak test. Flow cytometry phenotypic analysis of all PFP EVs and ELISA data were compared using a nonparametric Kruskal-Wallis one-way ANOVA on ranks, and multiple comparisons were made using a Tukey test. Values of *P* < 0.05 were considered to be significant. SigmaPlot 12.0 (Systat Software Inc.) was used for data analysis.

RESULTS

Designing a Panel of Markers to Examine pSTBMs in Plasma

Placental STBMs from eight normal placentas prepared using a dual placental perfusion system were pooled and analyzed by multicolor flow cytometry. Multicolor analysis was necessary to establish: 1) the purity of the pSTBM population, as, in the dual placental perfusion system, pSTBMs may be contaminated with EVs, especially those derived from maternal blood cells and 2) cross-reactivity between markers of

other cell types and pSTBMs, which has not been considered in previous studies [27, 28]. Many such studies use single-color labeling to discriminate different EV populations, assuming that each marker will be specific for each EV type. To address this issue, two-parameter histograms were used for the analysis. Placental STBMs (pool of eight preparations) were labeled with a five-color panel consisting of Bio-Maleimide, NDOG2, CD41 or CD61 (platelets), CD235a/b (RBCs), and W6/32 (leukocytes/platelets/endothelial cells). Over 90% of the pSTBMs were <1 μ m in size (Fig. 1A), and most (>94%) labeled positively with Bio-Maleimide (which labels all EVs) and the trophoblast marker PLAP (Fig. 1B). Placental STBMs also labeled positively for CD61 (>85%; Fig. 1C) and CD41 (27.4%; Fig. 1D). Only 1% of the pSTBMs labeled with CD235a/b (Fig. 1E) and 3.1% with the HLA class I marker W6/32 (Fig. 1F), demonstrating their purity. The endothelial cell-like characteristics of the syncytiotrophoblast have made it difficult to find an endothelial cell-specific marker that does not cross-react with it. Placental STBMs were double labeled with NDOG2 and CD146 (melanoma cell adhesion molecule), CD144 (VE-cadherin), or CD105 (endoglin). CD146 was not expressed on pSTBMs (Fig. 1G), and only a very small number of pSTBMs expressed CD144 (Fig. 1H). In contrast, the vast majority expressed CD105 (Fig. 1I). Together, these data show that pSTBMs prepared using the dual placental perfusion system are useful for defining STBM-specific markers and, in particular, the utility of the NDOG2 antibody for this purpose.

Designing a Panel of Markers to Examine Other Ex Vivo EVs in Plasma

The purpose of this part of the investigation was to find suitable markers to identify plasma EVs from different cell types in the maternal circulation, and to examine antibody specificities. EVs were generated in vitro from a variety of cell types that are found in the circulation, including; platelets, RBCs, endothelium, and leukocytes. Table 3 shows the percentage of positive labeling for each surface antigen examined on the parent cell and the EVs derived from them. All parent cells and EVs labeled positive with Bio-Maleimide (>95%; data not shown).

As expected, platelets, RBCs, HUVECs, monocytes, lymphocytes, and granulocytes all stained positively with antibodies toward the lineage markers used (Table 3). EVs derived from each parent cell also expressed these antigens; however, expression (% positive) was significantly lower in all cases, except for HLA class I, which was variable between lymphocyte EV preparations and did not reach significance (Table 3).

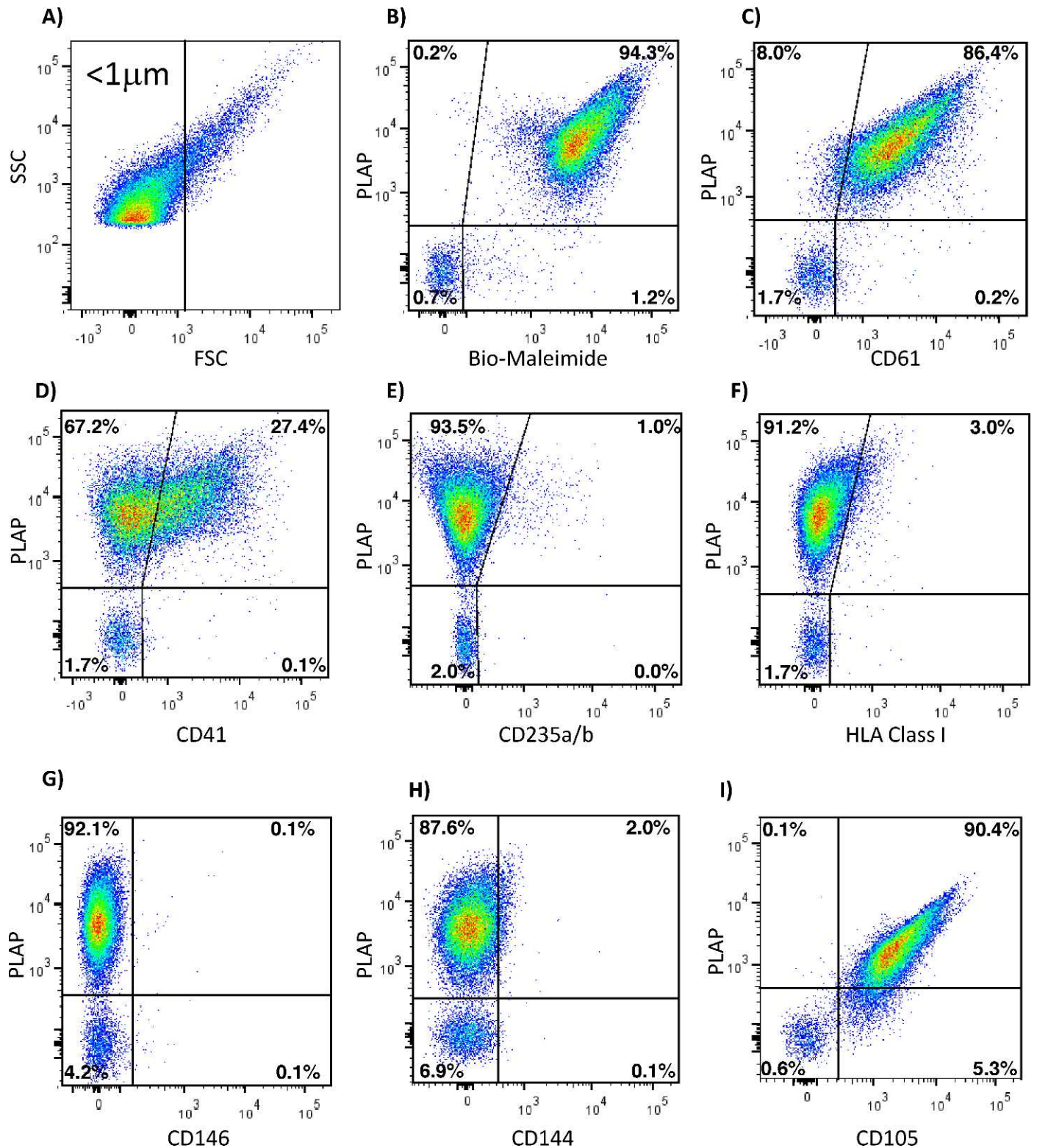


FIG. 1. Flow cytometry analysis of pSTBMs. A) FSC versus SSC dot plot of pSTBMs (>90% are <math>< 1 \mu\text{m}</math>). Double labeling of pSTBMs: Bio-Maleimide versus PLAP (B), CD61 versus PLAP (C), CD41 versus PLAP (D), CD235a/b versus PLAP (E), HLA class I versus PLAP (F), CD146 versus PLAP (G), CD144 versus PLAP (H), and CD105 versus PLAP (I). Data show percentage positive pSTBMs. Percentages have been adjusted to account for background contaminating particulates.

TABLE 3. Flow cytometry analysis of antigen expression on parent cells and the EVs derived from them.*

Surface antigen	Parental cell % positive	EVs % positive	P value [†]
Platelets			
CD41	99.9 ± 0.1	90.6 ± 3.9	P < 0.05
CD61	99.9 ± 0.1	91.2 ± 3.8	P < 0.05
HLA class I	99.9 ± 0.7	82.4 ± 4.7	P < 0.05
Red blood cells			
CD235a/b	99.4 ± 0.8	91.9 ± 3.1	P < 0.05
HUVECs			
CD146	100	30.5 ± 7.3	P < 0.001
CD105	100	70.5 ± 11.6	P < 0.002
CD144	99.8	5.9 ± 4.1	P < 0.001
HLA class I	99.1 ± 0.1	3.7 ± 1.8	P < 0.001
CD61	99.6 ± 0.3	18.5 ± 8.8	P < 0.001
Monocytes			
HLA class I	100	59.6 ± 5.4	P < 0.002
CD45	99.8 ± 0.1	12.3 ± 7.8	P < 0.001
CD14	99.9 ± 0.1	8.5 ± 5.5	P < 0.001
Granulocytes			
HLA class I	100	1.5 ± 0.4	P < 0.001
CD45	100	3.5 ± 1.7	P < 0.001
CD66b	100	9.9 ± 1.2	P < 0.001
Lymphocytes			
HLA class I	100	61.6 ± 39.2	P = 0.06
CD45	99.8 ± 0.2	10.7 ± 14.7	P < 0.001
CD3	87.8 ± 4.5	5.7 ± 5.6	P < 0.001

* Each data set is from three individual experiments showing the percentage of positive staining for each given antigen.

[†] P values compare parent cells and EVs.

Establishing the Final Antibody Panel to Examine In Vivo-Derived STBMs and Other EVs in Plasma

Based on these results, selected markers were chosen to examine STBMs in plasma in relation to EVs from all other cell types. Markers showing a high degree of specificity and positive staining for each EV population were chosen: pSTBMs; NDOG2, platelets; CD41, RBCs; CD235a/b, leukocytes; HLA class I and endothelial cells; CD146. These markers were then tested for cross-reactivity; each marker was first tested against the parent cell and, if positive staining was detected, then this marker was tested on the EV population. The RBC marker CD235a/b, endothelial cell marker CD146, and trophoblast marker NDOG2 showed no cross-reactivity with any other cell type (data not shown). W6/32 was chosen as a general marker of all HLA class I and showed no cross-reactivity with RBCs (data not shown). The platelet marker CD41 showed no cross-reactivity with RBCs or HUVECs, but positive expression was shown on monocytes (>90%), lymphocytes (27%), and granulocytes (10%), probably due to platelet-leukocyte aggregates (data not shown).

Taken together, these results were used to select two separate antibody panels to analyze STBMs and other EVs in vivo in plasma samples from NonP, NormP, and PE women. To identify the trophoblast-derived EVs that are PLAP positive and HLA class I negative, the first panel consisted of Bio-Maleimide, NDOG2, and W6/32. The second panel used five-color flow cytometry to identify EVs derived from platelets, RBCs, leukocytes, and endothelial cells, and consisted of Bio-Maleimide, CD41, CD235a/b, W6/32, and CD146.

A representative gating strategy used to characterize PFP-derived EVs from a NormP woman is shown in Figure 2. A 1- μ m gate on an FSC versus SSC plot defines the EV population

and excludes large debris and any residual platelets (Fig. 2A). Bio-Maleimide staining determines the percentage of cellular-derived EVs (Fig. 2B). Roughly 90% of events from the PFP samples labeled positive with Bio-Maleimide. Bio-Maleimide-positive EVs were then displayed on two-parameter dot plots to determine the phenotype of the EVs. It is important to note that Bio-Maleimide-negative EVs did not label with any of the antibodies examined in this study (data not shown). PFP-derived EVs were identified as follows: STBMs (NDOG2⁺/W6/32⁻; Fig. 2C); RBC EVs (CD235a/b⁺/W6/32⁻; Fig. 2D); platelet EVs (CD41⁺/W6/32⁻ and CD41⁺/W6/32⁺; Fig. 2E); leukocyte EVs (CD41⁻/W6/32⁺; Fig. 2E); and endothelial EVs (W6/32⁺/CD146⁺ or W6/32⁻/CD146⁺), although none were found (Fig. 2F).

STBMs in PFP from NonP, NormP, and PE Women

STBMs per milliliter of PFP and the percentage of STBMs were significantly raised in NormP and PE women compared to background levels observed in NonP women (Fig. 3, A and B, respectively). No difference in total counts or the percentage of positive EVs was observed in PFP from NormP women versus PE women (Fig. 3, A and B, respectively).

The levels of STBMs in the same PFP samples from NonP, NormP, and PE women were also measured using an in-house ELISA, which has been used in our previous studies, for comparison [8–10]. Unlike flow cytometry, which only measures STBMs >290 nm [14], the ELISA measures the entire population of EVs, including those <1 μ m and those >1 μ m. Consistent with the flow cytometry results, STBM levels were significantly increased in PFP in the samples from NormP and PE women compared to the NonP controls (Fig. 3C). No difference in STBM levels in PFP between NormP and PE women was found (Fig. 3C).

Measurement of Total, Platelet, RBC, Leukocyte, and Endothelial EVs in PFP from NonP, NormP, and PE Women

Using flow cytometry, total EV counts (Bio-Maleimide positive) were determined by analyzing all EVs <1 μ m. EV counts (mean \pm SE) were comparable in NonP (1.19×10^6 /ml $\pm 0.18 \times 10^6$ /ml), NormP (1.20×10^6 /ml $\pm 0.21 \times 10^6$ /ml), and PE women (0.84×10^6 /ml $\pm 0.11 \times 10^6$ /ml). The percentage of PFP EVs labeling with Bio-Maleimide was the same across all three groups (~90%), thus indicating that the pellet was enriched with cellular EVs. Most PFP EVs from NonP, NormP, and PE women were derived from platelets (Fig. 4, A and B). Total platelet EV counts and the percentage of positively labeled platelet EVs were significantly reduced in PE women versus NonP women (Fig. 4, A and B, respectively).

The total count of RBC EVs was elevated (although not significantly) in NormP and PE women compared to NonP women (Fig. 4A). The percentage of RBC EVs was significantly increased in NormP and PE women compared to NonP women (Fig. 4B). Total RBC EVs and the percentage labeled positive were similar in NormP and PE women (Fig. 4, A and B, respectively).

Total count and percentage of leukocyte EVs were significantly elevated in PE women compared to NonP women (Fig. 4, A and B, respectively). Leukocyte-derived EVs accounted for <0.1% of the total EV population in NonP women, <0.5% in NormP women, and 2.3% in PE women (Fig. 4B).

Endothelial EVs were not detected in any of the PFP samples from NonP, NormP, or PE women. However, we also

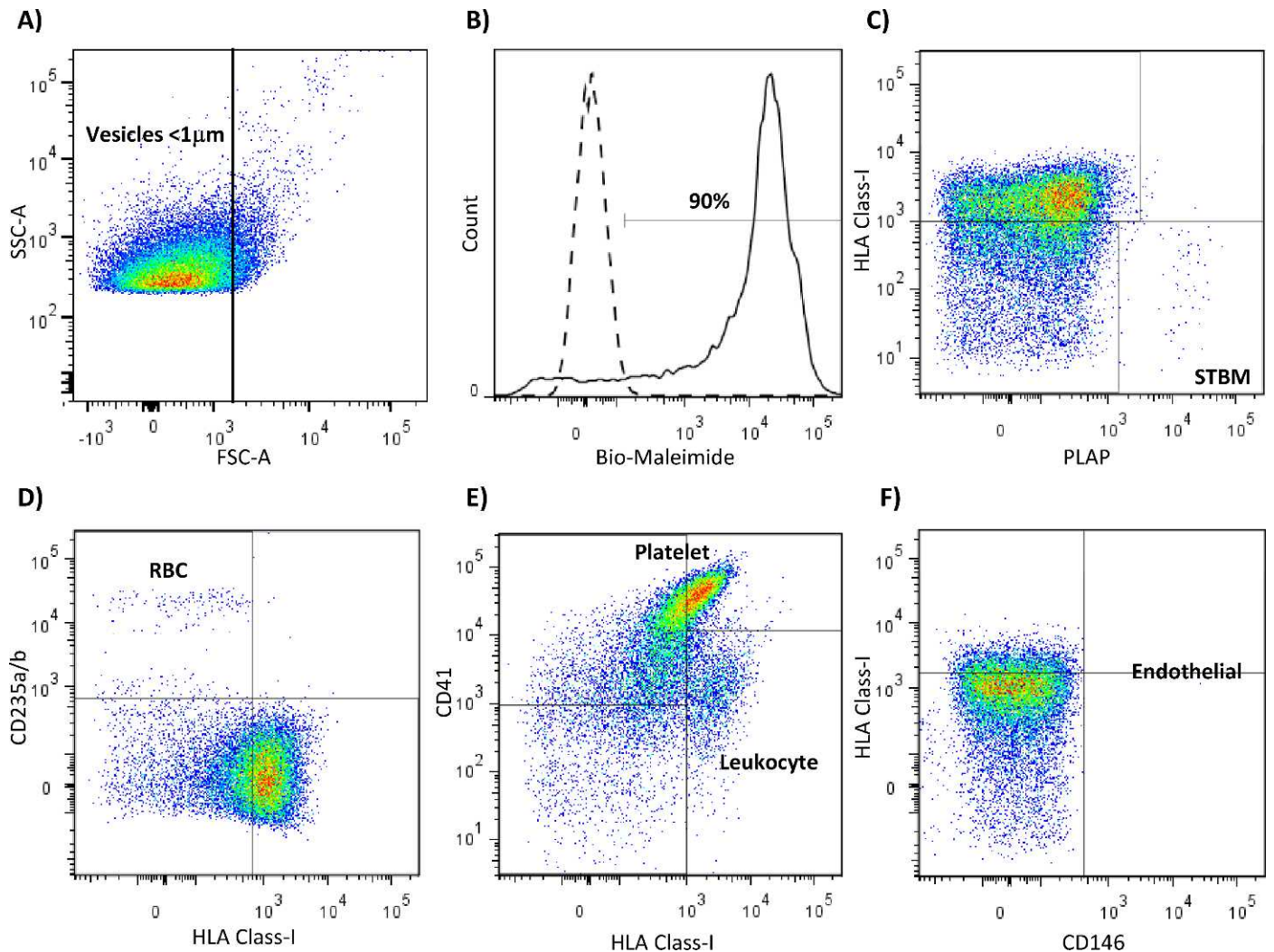


FIG. 2. Flow cytometry gating strategy to phenotype PFP EVs. EVs displayed on FSC versus SSC plot with 1- μm cutoff gate (A), <1- μm EVs displayed and gated on Bio-Maleimide histogram (B). Bio-Maleimide-positive EVs then displayed on two-parameter plots and phenotype identified: pSTBM, NDOG2⁺/W6/32⁻ (C); RBC, CD235a/b⁺/W6/32⁻ (D); platelet, CD41⁺/W6/32⁻ or CD41⁺/W6/32⁺, leukocyte, W6/32⁺/CD41⁻ (E); and endothelial, CD146⁺/W6/32⁻ (F).

identified a population of EVs that were Bio-Maleimide positive, but did not label with any of the markers of interest; these were termed orphan EVs and accounted for 24%–39% of the population (Fig. 4, A and B). There were no significant differences in either the total count or the percentage of orphan EVs in the three patient categories that were studied.

Flow Cytometry and NTA: Total EV Counts Measured in PFP from NonP, NormP, and PE Women

The NanoSight LM10 instrument used in this study measures vesicle size and concentration based on light scatter, and can analyze vesicles much smaller than those detected by flow cytometry. However, the technology is limited in that it cannot measure fluorescent antibody labeling to discriminate between cellular vesicles and other nanoparticles. For this reason, Bio-Maleimide-positive and -negative vesicles were measured both by flow cytometry and NTA in order to compare the two methodologies.

There were no differences in PFP total EV counts as measured by flow cytometry between NonP, NormP, and PE women (Fig. 5A). NTA detected more than two orders of magnitude more EVs than did flow cytometry; moreover,

NormP and PE PFP had significantly more EVs than NonP PFP (Fig. 5A), but there was no difference between NormP and PE PFP (Fig. 5A). PFP EV size was measured using NTA and compared in NonP, NormP, and PE women (Fig. 5B). Mean EV size was found to be larger in PFP from NonP women compared to NormP and PE women (Fig. 5C). There was no difference in EV size between PFP from NormP and PE women (Fig. 5C).

DISCUSSION

Multicolor flow cytometry was developed to phenotype circulating STBMs in relation to other EVs of the vascular compartment in PFP from NonP, NormP, and PE women. To our knowledge, this is the first study to apply multicolor flow cytometry using directly conjugated antibodies to phenotype circulating STBMs and other EVs in pregnancy. Different EV populations have been analyzed in relation to each other, rather than in isolation as in previous studies [28, 31, 45]. Importantly, we show that not all cellular markers are specific for one cell type and, used in isolation, cannot identify specific cells as the source of particular PFP EVs. For example, antibodies toward CD61 are commonly used to identify

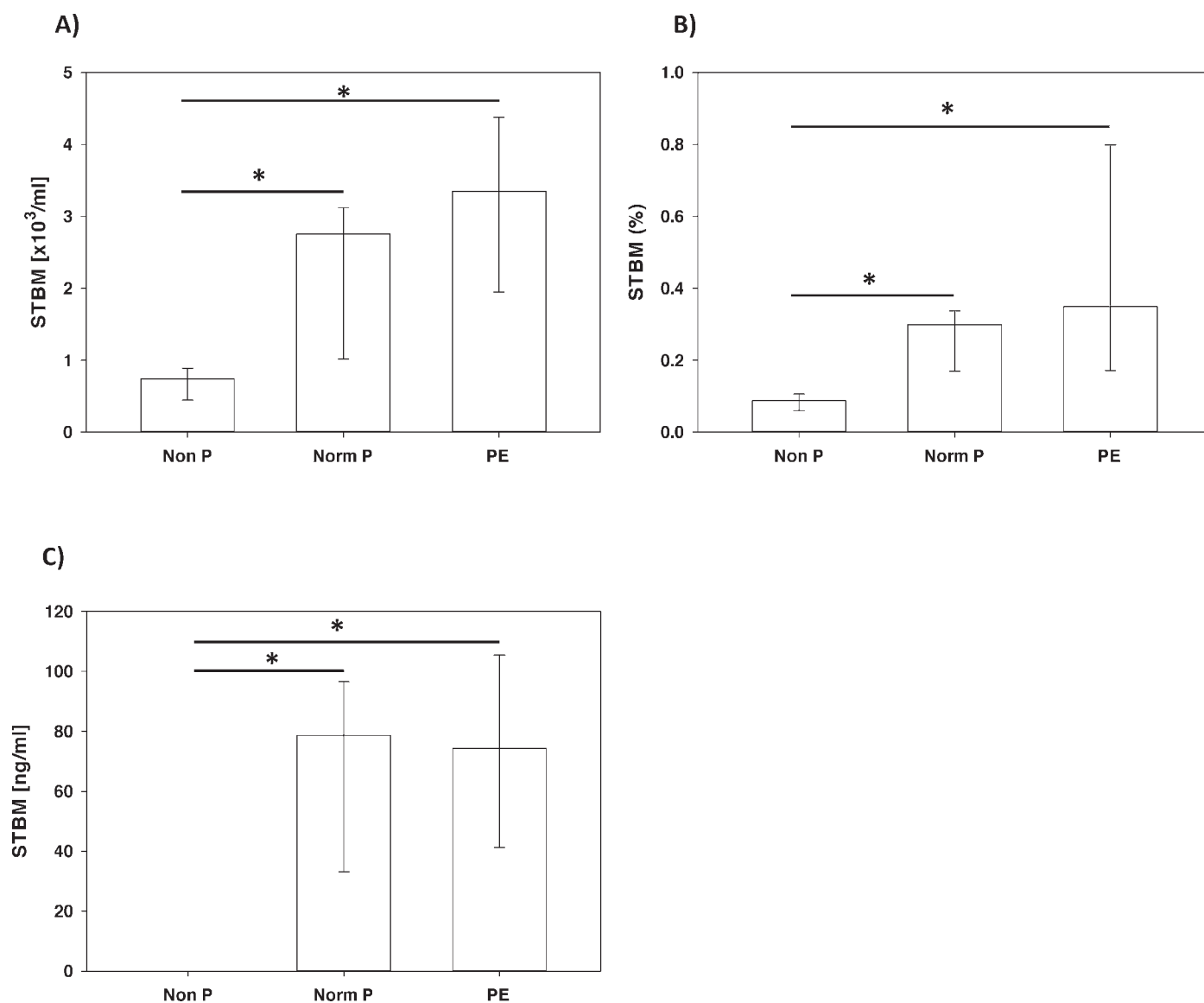


FIG. 3. Analysis of STBMs in PFP from NonP, NormP, and PE women measured by flow cytometry and ELISA. **A)** Flow cytometry total STBM counts. **B)** Flow cytometry percentage positive STBMs. **C)** ELISA STBM levels. Bars represent median with the interquartile range. * $P < 0.05$ ($n = 10$ per group).

platelets, but we show that CD61 is also expressed on pSTBMs and on HUVECs and their EVs.

Many researchers assume that EVs express the cell surface antigens that characterize their parent cells. We show that, while this is true of most platelet and RBC EVs, the level of antigen expression is significantly less. Our findings are consistent with previous reports examining HUVECs and HUVEC EVs, whereby antigen expression on EVs varied in comparison to HUVECs [37, 38]. To examine the expression of leukocyte markers, we used cultured primary blood lymphocytes, monocytes, and granulocytes, whereas previous reports describe the use of cell lines [46–51], which may not be comparable to primary cells. Of the antigens studied, HLA class I was the most abundant on monocyte and lymphocyte EVs, whereas it was virtually absent from granulocyte EVs. Previous studies [52–54] have reported that most in vitro-derived granulocyte EVs express CD66b, contrary to our findings. This discrepancy may be partly due to differences in: 1) the protocol used to generate the granulocyte EVs, 2) methodology used for the isolation and labeling of the EVs, and 3) the flow cytometer instrumentation and gating analysis.

There are two possible explanations for the significant reduction in the percentage of positively labelled EVs as compared to labeling of the parent cell. First, the antigens present on the surface of the parent cell may be selectively excluded from the surface of the EVs, or, second, the antigen copy number present on the surface of some EVs is too low to be detected by flow cytometry. Therefore, previous studies may be missing EVs from their analyses.

Circulating EVs derived from STBMs, platelets, RBCs, endothelium, and leukocytes were analyzed in PE women matched to NormP and NonP women. We used PFP samples from late-onset PE women (mean gestation, 37.2 wk) matched appropriately to NormP and NonP women, due to their availability in the Oxford Pregnancy Biobank. There were no differences in total Bio-Maleimide EV numbers between groups. The smallest population of EVs was that derived from STBMs. The numbers and percentages of STBMs in plasmas from NormP and PE women were not significantly different, as has been previously reported [10]. The STBM ELISA showed a similar pattern, with significantly more STBMs in NormP and PE women than in NonP women. Overall, these data are

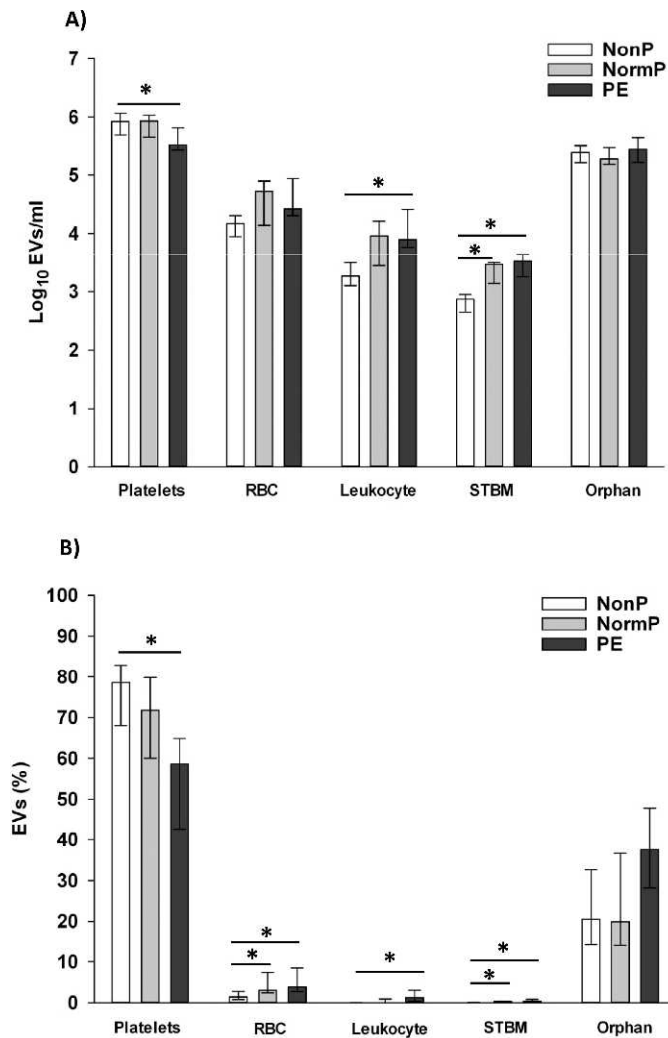


FIG. 4. Flow cytometry analysis of EVs in PFP from NonP, NormP, and PE women. **A)** Total EV counts. **B)** Percentage of EVs. Bars represent median with the interquartile range. * $P < 0.05$ ($n = 10$ per group).

consistent with two previous studies, both of which used flow cytometry and indirect labeling [28, 33]. There is, however, wide variation in the reported total numbers of circulating STBMs, of which at least some can be attributed to methodological differences (different antibodies and indirect staining protocols). Orozco et al. [33] used an anti-PLAP antibody, whereas Lok et al. [27, 28] used ED822, an antibody that recognizes an unknown epitope expressed on the apical surface of syncytiotrophoblasts. To our knowledge, its specificity and cross reactivity with other circulating cells types has not been documented. Anti-PLAP antibodies may also have their own limitations, as we recently showed that PLAP expression is decreased on STBMs prepared from PE placentas compared to those from normal placentas [14], which may lead to an underestimation of the number of STBMs in PE plasma. We are now investigating other markers that may be expressed on STBMs that may be more reliable. The multicolor labeling method we have developed using pSTBMs as a model will also allow us to compare the phenotypes of pSTBMs and those in the maternal circulation using a wide range of markers. Although we have begun to study the labeling of pSTBMs with other markers, such as CD61 and CD41, we have not yet made a formal comparison between pSTBMs and those in the

maternal circulation. This will be done in the future, but was not the purpose of this study.

There are also major inconsistencies in the reported numbers of other circulating EVs shed from blood (platelets, RBCs, T and B lymphocytes, monocytes, and granulocytes) or endothelial cells in NormP and PE [27, 28, 31–33, 45, 55–65]. Some studies suggest changes in NormP compared to NonP [31, 32, 45, 55, 57], and in PE compared to NormP [28, 31, 32, 45, 55, 57–59, 62–65], whereas others report no change [27, 28, 31, 32, 45, 55, 57, 58]. These inconsistencies may reflect preanalytical methodological differences that are thought to affect quantification of EVs in plasma [36, 66–72].

Our results show that the majority of circulating EVs were derived from platelets, and both number and the percentages were significantly decreased in PE compared to NonP controls. We are aware that our analysis of platelet EVs using the marker CD41 may also incorporate the analysis of STBMs (as CD41 showed some cross-reactivity with pSTBMs); however, as this population makes up $<1\%$ of the total EVs in plasma, this would have a negligible effect on the platelet data. RBC-derived EVs made up less than 10% of the total population. The percentage of RBC EVs in NormP and PE were increased compared to NonP. Osmotic fragility of RBCs increases during NormP compared to NonP [73], hence increasing susceptibility to fragmentation and production of RBC EVs. Oxidative stress may have the same effect [74–76]. We used a pan-leukocyte marker, and can only speculate on total leukocytes, not individual populations that contributed to the observed increase in numbers and percentages in PE compared to NonP. At present, it is unclear if the observed elevation in leukocyte-derived EVs in PE is a direct consequence of leukocyte cell activation and whether these leukocyte EVs may play a role in inducing endothelial dysfunction.

Our endothelial cell marker, CD146, was chosen because it did not cross-react with pSTBMs. In previous studies, a variety of markers (singular or dual) for plasma endothelial EVs have been used, including E-selectin (CD62E) [27, 59, 65], CD144 [55], CD62E⁺/CD144⁺ [32], PECAM (CD31) [64], CD31⁺/CD41⁻ [55], CD31⁺/CD42⁻ [58, 65], CD51 [31], CD51⁺/CD41⁻ [57], and CD105 [65]. The specificities of some are questionable. For example, CD31 is also found on the surface of platelets, monocytes, and granulocytes, and CD51 is expressed on activated T cells, platelets, and granulocytes. We show that CD105 is highly expressed on STBMs, and cannot be used on its own to define populations of endothelial EVs [14]. Our inability to detect endothelial EVs in any of our samples is difficult to explain. The same EV marker has been used previously to investigate other disease states, with elevated numbers compared to control samples [25, 77, 78]. One previous study showed that increased endothelial EVs were detected in plasma when using anti-CD146 in combination with anti-CD105 compared to using each antibody alone. In the current study, it was not feasible to have a third panel using anti-CD146 and anti-CD105 in combination. Future experiments will be needed to address this.

This was not only the first study to use multicolor flow cytometry to investigate STBMs in relation to other EVs in plasmas from NonP, NormP, and PE, but also to use NTA in parallel. NTA has shown that flow cytometry detects only a minority of all cell-derived EVs in biological samples [14]. NTA revealed changes in total EV number between groups, which were not apparent by flow cytometry. Total EV counts were increased in NormP and PE relative to NonP, and the EV size was smaller. PE is associated with an exacerbated systemic inflammatory response, which would be expected to activate intravascular cells, causing them to shed more EVs. However,

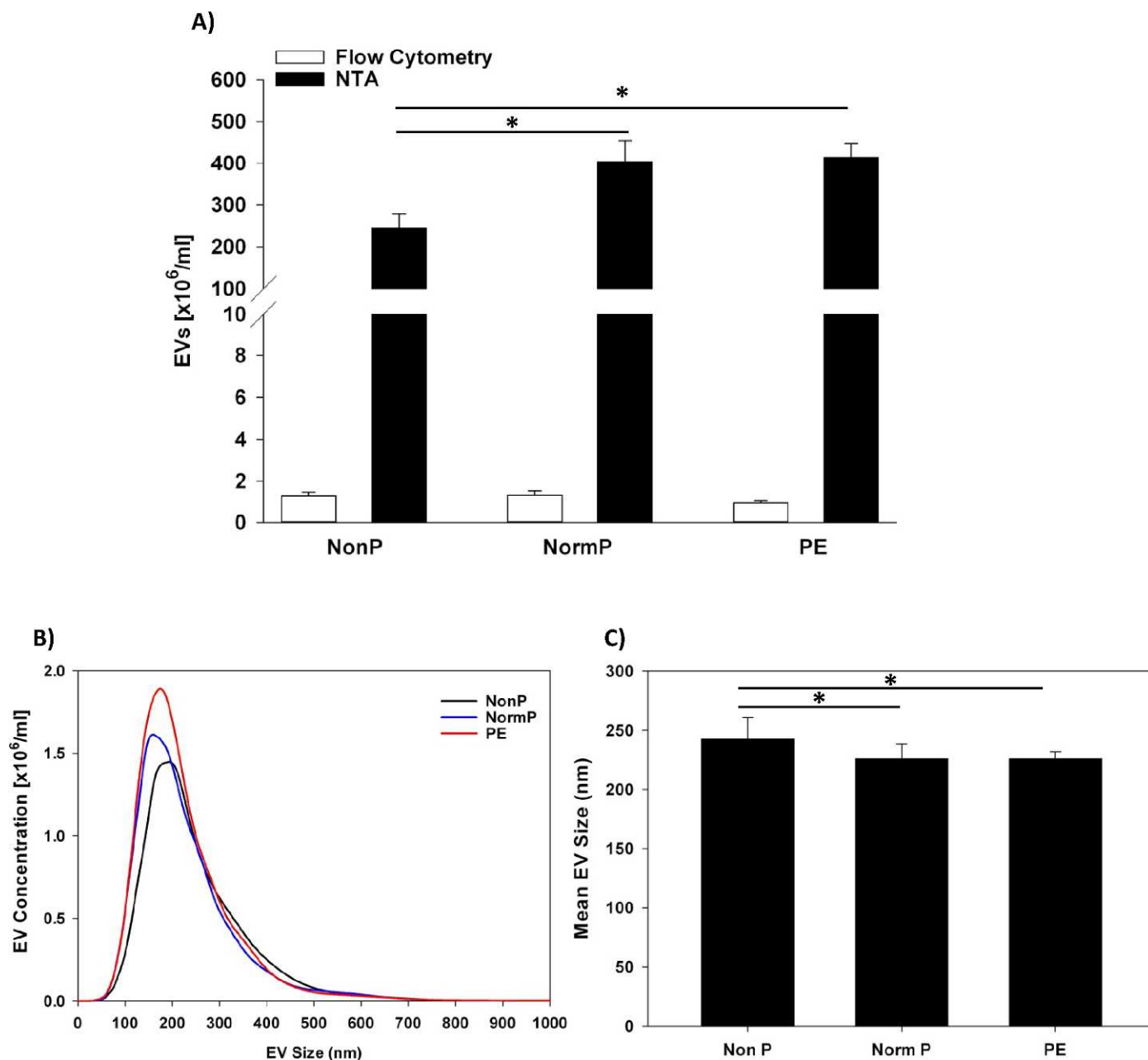


FIG. 5. Measurement of total PFP EVs in NonP, NormP, and PE women using flow cytometry and NTA. **A)** EV counts measured by flow cytometry and NTA. Bars represent mean \pm SE. **B)** NTA EV size versus concentration profile. **C)** NTA mean EV size measurements. Bars represent mean \pm SE. * $P < 0.05$ ($n = 10$ per group).

total counts were comparable in NormP and PE. The rate at which EVs are cleared from the circulation is not known, so total counts are not necessarily informative. There is scope to develop NTA using the NanoSight NS500 instrument, which has a fluorescence capability and could therefore be used to phenotype EVs using fluorescent antibodies [34, 79].

This study demonstrates the difficulties and limitations of *ex vivo* flow cytometric analysis of plasma EVs, both in general and in specific relation to pregnancy. We show that problems with antibody specificities have been underestimated in previous studies. Smaller EVs, only detected by NTA, are two orders of magnitude more numerous than those characterized by flow cytometry. We studied EVs, including those of syncytiotrophoblast origin, in plasmas from NonP, NormP, and PE. The differences between NonP and NormP women were

greater than those between NormP and PE women, which were not significant. This is probably because we studied the less severe late-onset disease, which is harder to distinguish from the background of normal pregnancies, which increases with gestation. In general, NormP and PE were associated with more circulating EVs than NonP, except for platelet EVs, which were reduced, perhaps explicable in that, during pregnancy, platelet counts, which were not available to us, tend to be lower. Furthermore, circulating EVs were smaller during pregnancy. Finally, the second most abundant EV population after platelet EVs was that of unidentified orphan EVs. The origin of these EVs is still likely to be the different cell types investigated in this study (i.e., STBM, platelet, RBC, leukocyte, and endothelial); however, the antigen copy number on the surface of the EVs might be too low to be detected by

flow cytometry. Further investigation may require different markers to identify these EVs.

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