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Abstract: Introduction: Fetal growth restriction (FGR) is a common complication of pregnancy. Placenta-derived exosomes may represent an additional pathway by which the placenta communicates with the maternal system to induce maternal vascular adaptations to pregnancy and it may be affected during FGR. The objective of this study was to quantify the concentration of total and placenta-derived exosomes in maternal and fetal circulation in small fetuses classified as FGR or small for gestational age (SGA).
Methods: Prospective cohort study in singleton term gestations including 10 normally grown fetuses and 20 small fetuses, sub-classified into SGA and FGR accordingly to birth weight (BW) percentile and fetoplacental Doppler. Exosomes were isolated from maternal and fetal plasma and characterized by morphology, enrichment of exosomal proteins, and size distribution by electron microscopy, western blot, and nanoparticle tracking analysis, respectively. Total and specific placenta-derived exosomes were determined using quantum dots coupled with CD63+ve and placental-type alkaline phosphatase (PLAP)+ve antibodies, respectively.
Results: Maternal concentrations of CD63+ve and PLAP+ve exosomes were similar between the groups (all $p>0.05$). However, there was a significant positive correlation between the ratio of placental-derived to total exosomes (PLAP+ve ratio) and BW percentile, [rho=0.77 (95% CI: 0.57 to 0.89); $p=0.0001$]. The contribution of placental exosomes to the total exosome concentration in maternal circulation showed a significant decrease among cases, with lower PLAP+ve ratios in FGR compared to controls and SGA cases. Likewise, the contribution of placental exosomes to the total exosome in the fetal circulation was significantly lower in cases than in controls (both $p<0.001$).
Discussion: Quantification of placental exosomes in maternal and fetal plasma reflects fetal growth and it may be a useful indicator of placental function. Future studies with a large sample size are required to confirm these results.

Placental exosomes profile in maternal and fetal circulation in intrauterine growth restriction - Liquid biopsies to monitoring fetal growth

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Discussion: Quantification of placental exosomes in maternal and fetal plasma reflects fetal growth and it may be a useful indicator of placental function. Future studies with a large sample size are required to confirm these results.

Key words: Extracellular vesicles, intrauterine growth, non-invasive diagnosis, placenta, and pregnancy.

Highlights

- The maternal PLAP^{+ve} ratio is an indicator of the contribution of placental-derived to the total circulating exosomes.
- The maternal PLAP^{+ve} ratio is a marker of fetal growth and placental function.
- Compared to controls, the maternal PLAP^{+ve} ratio was ~14% lower in SGA and until ~23% lower in patients with FGR.
- The fetal blood PLAP^{+ve} ratio has a similar behavior to the maternal PLAP^{+ve} ratio.

Introduction

Fetal growth restriction (FGR) affects 7-10% of all pregnancies and is defined as the failure to achieve the genetic growth potential [1,2]. Growth restricted fetuses have a 5 to 10-fold risk of dying *in-utero*, and a higher risk of perinatal morbidity and mortality [3]. Prenatal identification of FGR has been shown to significantly reduce perinatal morbidity and mortality, by 4 to 5-fold [4]. However, accurate identification of sub-optimal fetal growth *in-utero* remains to be an unsolved problem [5]. Detection of fetal smallness has proven elusive, with high-quality ultrasound programs detecting not more than 50% of the cases [6]. Current understanding of the clinical classification of small fetuses encompasses two different forms: Fetal growth restriction (FGR) and small for gestational age (SGA) fetuses. While the former has been consistently associated with higher risk of perinatal morbidity and mortality the latter is considered a physiological variable of the average population with near normal perinatal outcome [2]. Recent studies have shown evidence of intrauterine programming in SGA fetuses that is expressed later in life in the form of neurologic impairment [7], cardiovascular disease [8,9] and metabolic syndrome [10], challenging the concept of "constitutional" small fetuses.

Recently, there has been great interest on the field of extracellular vesicles (EVs). EVs can be classified according to their size and origin as exosomes and microvesicles [11]. Exosomes are membrane-bound vesicles with a diameter of ~40-120 nm, enriched with endosomal protein markers (e.g. TSG101, CD9, CD63 and CD81) [12], that are actively released from all types of cells into the extracellular environment upon exocytic fusion of multivesicular endosomes with the cell membrane. Several investigators have reported that exosomes are key players for intercellular communication and carry diverse molecular components such as proteins [13], lipids [14], miRNA [15], and membrane receptors. Exosomes are transferred to adjacent and/or distal cells in the extracellular space under physiological and pathological conditions [16], making them a novel mechanism of paracrine and autocrine regulation, which have even been used as natural nanocarriers to deliver therapeutic agents to injured cells [17].

The discovery of circulating fetal genetic material in maternal plasma has accelerated the generation of new potential tools for non-invasive prenatal diagnosis. Placenta-derived exosomes are unique compared to other exosomes due to the presence of specific proteins like the placental-type alkaline phosphatase (PLAP) [18–21] and HLA-G [22], as well as miRNAs such as those within the chromosome 19 miRNA cluster [23–25]. It has been shown that

exosomes are released from the placenta into the maternal circulation from as early as ~7 weeks of gestation, and the concentration of placenta-derived exosomes increases throughout gestation [26]. This release is tightly regulated by a number of factors, such as oxygen tension and glucose concentration [13,27,28]. Interestingly, exosome release correlates with placental mass and perfusion in normal pregnancy [20]. Placenta-derived exosomes may represent an additional pathway by which the placenta communicates with the maternal system to induce maternal vascular adaptations to pregnancy and development of maternal-fetal vascular exchange [29,30]. Recent studies also support a role for exosomes in embryo implantation [31], normal placental development, as well as maternal immunotolerance [32–34]. Previous studies have associated changes in the release of placental and non-placental exosomes concentration in maternal plasma, composition and bioactivity with complications of pregnancy, including gestational diabetes [35] and preeclampsia [34,36]. However, the precise function and significance of placental exosomes during pregnancy remain to be elucidated.

Attempts to further our understanding of the role of placental exosomes during gestation have been limited by the use of several isolation methodologies and analysis of potentially diverse vesicle subpopulations [12]. Hence, it is imperative to apply well-characterized and validated isolation methods to this field to determine the precise role of exosomes in the context of pregnancies, and potentially, the clinical utility of these exosomes. Here, we used a well-validated method to enrich exosomes compared to other EVs. In this study we demonstrated the presence of placental exosomes in maternal and fetal circulation using western blot, then we quantified the number of circulating placental and non-placental exosomes using quantum dots coupled with $CD63^{+ve}$ or $PLAP^{+ve}$, and a commercial ELISA for PLAP, in maternal and fetal circulation in pregnancies complicated by sub-optimal fetal growth. The specificity of the methods used in this study were validated using placental tissue (positive control for PLAP) and exosomes isolated from non-pregnant women (negative control for PLAP).

Methods

Study groups and data collection

This was a prospective cohort study, including singleton gestations that were enrolled at third trimester between December 2014 and June 2016 and delivered at Department of Maternal-Fetal Medicine in BCNatal, Barcelona, Spain. All pregnant women included in this study

were normotensive and without intrauterine infection or any other medical or obstetric complications who delivered at term (> 37 weeks). Pregnancies with a fetal congenital or chromosomal anomaly were ineligible to participate. Study groups included: (1) pregnancies with a normally grown fetuses who delivered appropriately for gestational age neonates (n=10) and (2) pregnancies complicated with sub-optimal fetal growth who delivered neonates with a BW <10th centile by local reference customized standards (n=20) [37]. Additionally, small fetuses were sub-classified according to clinical severity into small for gestational age (SGA) if the BW percentile was between the 3rd and the 9th centile and the fetoplacental Doppler was normal (n=10) and as FGR to those fetuses with a BW of less than the 3rd centile and/or either abnormal cerebroplacental ratio (CPR) (< 5th centile) and/or the uterine artery Doppler pulsatility index (UtA-PI) was ≥ the 95th centile (n=10) [2]. Gestational age in all pregnancies was calculated on the basis of the measurement of ultrasonographic fetal crown-rump length at 11-13 weeks. Plasma samples were obtained in accordance with the declaration of Helsinki, the institutional ethics committee approved the study protocol (IRB 2014/7154) and all patients provided written informed consent. Maternal baseline characteristics including demographic characteristics, obstetric, and medical histories were recorded at the time of delivery and the data were entered into our database. The placenta was weighed using an electronic scale; the length of the surface was measured as the longest diameter on the maternal side; the breadth as the longest diameter at right angles to the length. Placental efficiency was defined by birth weight: placental weight ratio (BW: PW ratio).

Feto-placental ultrasound

Transabdominal ultrasound was performed at enrollment with 6-4-MHz probes (Siemens Sonoline Antares, Siemens Medical Systems, Malvern, PA, USA) and a Voluson 730 Expert Machine (GE Medical systems, Zipf, Austria) including fetal biometry and feto-placental Doppler. The Doppler examination included: Umbilical artery (UA)-PI calculated from a free-floating portion of the umbilical cord, at insonation angles of <30° [38]. Middle cerebral artery (MCA) flow velocity waveforms were recorded at 1 – 2 cm from the circle of Willis, during the absence of fetal movements, at insonation angles of less than 30° [39]. Cerebroplacental ratio (CPR) was calculated as the ratio of MCA-PI to UA-PI [39]. Mean UtA-PI was calculated as the average PI of the right and left arteries [40].

Collection of maternal and fetal samples

Maternal plasma samples and umbilical fetal blood samples (BD Vacutainer® PLUS Tubes EDTA) were collected at the time of delivery. Plasma was separated from whole blood by centrifugation (2000 g 10 min at room temperature) and stored at -80° C at Hospital Clinic-IDIBAPS Biobank until analyses. All experimental procedures were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility.

Isolation of exosomes from maternal and fetal samples

Exosomes were isolated from plasma (1 ml) as previously described [21]. In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at 800 x g for 10 min and 2,000 x g for 30 min at 4 ^{0}C (Sorvall®, high speed microcentrifuge, fixed rotor angle: 90^0, Thermo Fisher Scientific Ins., Asheville, NC, USA,). The 2,000 x g supernatant fluid was then centrifuged at 12,000 x g for 45 min at 4 ^{0}C (Sorvall, high speed microcentrifuge, fixed rotor angle: 90^0). The resultant supernatant fluid (2 ml) was transferred to an ultracentrifuge tube (Beckman, 10 ml) and centrifuged at 100,000 x g for 2 h (Sorvall, T-8100, fixed angle ultracentrifuge rotor). The pellet was suspended in PBS (10 ml) and filtered through a 0.22 μm filter (Steritop^{TM}, Millipore, Billerica, MA, USA) and then centrifuged at 100,000 x g for 2 h. The 100,000 g pellet was resuspended in 500 μl PBS and stored -80^{o}C until exosome purification. The pellet (500 µl) was layered on the top of a discontinuous iodixanol gradient containing 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) iodixanol (solutions were made by diluting a stock solution of OptiPrep™ (60% (w/v) aqueous iodixanol from Sigma-Aldrich) and centrifuged at 100,000 g for 20 h. Fractions were collected manually from top to bottom (with increasing density), diluted with PBS and centrifuged at 100,000 g for 2h at 4^o C. Finally, the pellet containing the enriched exosome population was resuspended in 50 µl PBS. The density of each fraction was measured in a control OptiPrep™ gradient tube by determining the absorbance at 244 nm. Exosome-containing fractions (density 1.12 to 1.188 g/ml) were combined in a single tube and stored to -80 ^{0}C. We have previously confirmed the stability of exosomes after freeze and thaw cycles using fresh and frozen samples [20,41,42]. Exosomes were characterized by size distribution, abundance of proteins associated with exosomes (*i.e*. CD63, sc15363; Flotillin-1, sc25506; and TSG101, EPR7130), placenta-derived exosomes (PLAP, ab96588) and a negative control for Grp94 (20292T)), and morphology accordantly to the recommendation of the international society of extracellular

vesicles [21,35] using Nanoparticle Tracking Analysis (NTA), Western blot analysis and electron microscopy, respectively.

Quantification of total exosomes and placenta-derived exosomes

Using immunofluorescent NTA in fluorescence mode, the concentration of total and placenta-derived exosomes in maternal plasma was quantified using quantum dots coupled with CD63 or PLAP as we previously described [21]. PLAP is a syncytiotrophoblast-specific marker; therefore, exosomes derived from placental origin are positive for PLAP [20]. Qdots (Qdot® nanocrystals or R-PE) were conjugated to anti-CD63, anti-PLAP (MA1-20245, clone H17E2, ThermoFisher) or IgG1 isotype control antibody (IgG1 sc-34665, Santa Cruz Biotechnology) with a SiteClick Qdot 605 Antibody Conjugation Kit (Life Technologies) according to the manufacturer's instructions as previously described [21]. Exosomes were diluted in PBS and incubated with FcR blocking reagent (10 ml, 10 min at 4 C) (MACS Miltenyi Biotec), followed by incubation with anti-CD63-Qdot605 or anti-PLAP-Qdot605 or IgG1-Qdot605 (10 ml, 1:100) for 30 min in the dark at room temperature. Samples were then diluted to 500 ml with PBS and analyzed using the NanoSight NS500 instrument and NTA software. Samples were analyzed using fluorescence mode (i.e. camera level 9, shutter speed 11.25 ms and slider gain 250). Five videos x 60 s each were captured for each sample and analyzed. The specificity of the Qdot-PLAP in binding only exosomes from the placenta was measured using exosomes isolated from first trimester trophoblast cells and exosomes isolated from plasma obtained from non-pregnant women (negative control). The quantification of PLAP in the exosomal fraction indicates the relative concentration of placental-derived exosomes ($PLAP^+$ exosomes) in maternal and fetal circulation. Finally, the concentrations of PLAP-associated exosomes were quantified using a commercial Placental Alkaline Phosphatase (PLAP) ELISA kits (MyBiosource; Cat# MBS289869) as we previously described [20].

Statistical analysis

Distributions were examined for normality using the Kolmogorov–Smirnov test. When there was normality of continuous variables, the one-way ANOVA test and unpaired t-tests were used to compare differences. When data were far from normality, the Kruskal–Wallis one-way analysis of variance and Mann–Whitney U-test were performed. To assess the categorical variables, proportions were compared with Fisher's exact test or the chi-square

test. Categorical data are presented as n (%) and continuous data as median [interquartile range (IQR)]. The Jonckheere–Terpstra test was used to compare continuous variables among multiple-ordered groups. Statistical analysis was performed using STATA 14 (Stata Corp LP, 2015, College Station, Texas), and a p-value <0.05 was considered to be statistically significant.

Results

Clinical characteristics of the study population

Table 1 shows the clinical characteristics, perinatal outcomes and morphometric characteristics of the placenta in normal patients who delivered at term as well as those who had small for gestational age and growth-restricted fetuses. There were no significant differences in respect to maternal age, BMI, smoking, ethnicity, fetal gender or nulliparity. Although, smoking during pregnancy was only identified in FGR group. At ultrasound evaluation, as expected, fetuses with sub-optimal fetal growth had significantly lower estimated fetal weight percentile and also worst values in the fetoplacental Doppler parameters (Table 1). Although there was no significant difference in the gestational age at delivery between cases and controls, birth weight centile, placental weight and breadth was significantly lower in cases with sub-optimal fetal growth. The rate of obstetric interventions such as induction of labor and cesarean section were significantly higher in the group of patients with FGR compare to controls (Table 1).

Characterization of total and placental-derived exosomes

Exosomes were isolated from maternal and fetal plasma by differential and buoyant density centrifugation (Supplemental material Figure S1). The distribution of particles and size distribution of total (CD63+) and placenta-derived exosomes ($PLAP^{+ve}$) across the fractions in maternal and fetal plasma is presented in Supplemental material Figure S2). In maternal plasma, the size distribution was 87 ± 23 nm, 82 ± 18 nm and 108 ± 37 nm for controls, SGA, and FGR groups, respectively (Figure 1C). In fetal plasma, the size distribution was 90 ± 17 nm, 85 ± 17 nm and 81 ± 15 nm for controls, SGA and FGR groups, respectively (Figure 1D). No significant differences were observed in the size distribution of exosomes between groups and/or maternal and fetal plasma. Exosome enriched fractions from maternal and fetal plasma contained vesicles of around 100 nm diameter identified by electron microscopy (Figure 1C and 1D). Exosomes were positive for CD63, Flotillin-1, and TSG101 (Figure 1A), which are proteins associated with exosomes. Interestingly, the enrichment of exosomes was negative for Grp94 (Figure 1A), which is an endoplasmic reticulum marker and demonstrated the purity of the exosome isolation. The abundance of these proteins was not significantly affected between controls, SGA and FGR groups. Therefore, these results confirmed the reproducibility of our method to enrich a specific type of EVs defined by enrichment of

proteins associated with exosomes (i.e. CD63, Flotillin-1, and TSG101) and lack of Grp94 (negative control); size distribution of ~100 nm (within of the size of exosomes) and spherical morphology.

The presence of exosomes from placental origin in maternal plasma was confirmed by the presence of PLAP by Western blot (Figure 1B e). Interestingly, exosomes isolated from fetal plasma were positive for PLAP (Figure 1B e). Therefore, our next step was quantified the number of total exosomes and placenta-derived exosomes in maternal and fetal circulation. We did not find PLAP protein in exosomes isolated from non-pregnant women (Supplemental material Figure S3).

Exosomal profile in maternal blood

The total number of exosomes (Qdot-CD63^{+ve}) and placenta-derived exosomes (Qdot-PLAP^{+ve}) in the maternal circulation was not affected by maternal age, parity or pre-gestational maternal BMI (Supplemental material *Table S2*). There were no correlations between maternal plasma exosomes, birth weight or placental characteristics. The concentration of total exosomes (*CD63^{+ve}*), Placenta-derived exosomes (CD63^{+ve} and PLAP^{+ve}), non-placenta-derived exosomes (CD63^{+ve} and PLAP^{-ve}), and the contribution of placental exosomes to the total exosomal concentration (CD63^{+ve} and PLAP^{+ve} / CD63^{+ve} and PLAP^{-ve} x 100) in cases and controls are presented in Figure 2. The total number of circulating exosomes was significantly differences (ANOVA $p<0.05$) across the groups studied (Figure 2A). There were no significant differences in the maternal plasma concentration of placenta-derived exosomes between cases (SGA and FGR) and controls (Figure 2B). However, the contribution of non-placental and placental exosomes was significantly different (ANOVA ***$p<0.0001$) (Figure 2 C and D). Interestingly, the levels of exosomes from non-placental origin were significantly lower in SGA compared to FGR and controls (Bonferroni's multiple comparisons test **$p<0.001$). Similarly, the levels of placenta-derived exosomes were significantly lower in SGA and FGR compared to controls (Bonferroni's multiple comparisons test **$p<0.001$). Interestingly, when cases with sub-optimal fetal growth were sub-classified according to the severity of the cases in small for gestational age (normal feto-placental Doppler and birth weight percentile between 9 and 3) and fetal growth restriction (Abnormal fetoplacental Doppler and/or birth weight percentile below the third percentile), the contribution of placental exosomes to the total exosome

showed a significant decrease among cases, with lower PLAP^{+ve} ratios in FGR compared to controls and SGA cases (Figure 2). Maternal PLAP^{+ve} ratio has a significant positive correlation with birth weight percentile [Spearman correlation=0.77 (95% CI: 0.57 to 0.89); p=0.0001], but no significant correlation with placental characteristics or fetoplacental Doppler (Supplemental material *Table S1*). The individual values of total and placenta-derived exosomes present in maternal circulation are presented in Supplemental material table S2. The presence of PLAP in exosomes isolated from maternal circulation was confirmed using a commercial ELISA kit (Supplemental material Figure S4A). We did not find a significant difference in the PLAP-associated exosomes across the studied groups.

Exosomal profile in fetal blood

The total number of exosomes (Qdot-CD63^{+ve}) and placenta-derived exosomes (Qdot-PLAP^{+ve}) in the fetal circulation was not affected by parity or fetal gender. There were no significant differences in the total and placenta-derived exosomes among the study groups (Figure 3A and B). Interestingly, the contribution of non-placental and placental-derived exosomes to the total circulating exosomes significantly different among the groups, with a significant decrease among cases (Figures 3 C and D). Similarly to the profile in maternal plasma, we identified significant changes in the contribution of non-placental and placental exosomes to the total concentration of circulating exosomes between the study groups (ANOVA, $p<0.0001$). Post hoc multiple comparison test (Bonferroni) showed statistical difference (**$p<0.005$) between SGA or FGR compared to controls (Figure 3 C and D). The contribution of non-placental to the total exosomes was higher in SGA and FGR compared to controls. On the other hand, the contribution of placental-derived to the total exosomes was lower in SGA and FGR compared to controls. No significantly different between SGA and FGR for non-placenta and placenta-derived exosomes were identified. No significant effects of fetal gender, maternal characteristics or fetoplacental Doppler on CD63^{+ve} or PLAP^{+ve} exosomes in fetal blood were identified (Supplemental material *Table S1*). However, when the contribution of placenta-derived exosomes was determined in fetal blood plasma, strong and significant positives correlations with birth weight percentile, placental weight and breadth were obtained (r=0.91; 0,71 and 0,80, respectively; all $p<0.05$) (Supplemental material *Table S1)*. The individual values of total and placenta-derived exosomes present in fetal circulation are presented in Supplemental material table S3. The presence of PLAP in exosomes isolated from fetal circulation was confirmed using a commercial ELISA kit

(Supplemental material Figure S4B). We did not find a significant difference in the PLAP-associated exosomes across the studied groups. Placental tissue and exosomes isolated from non-pregnant women were used as positive and negative control, respectively (Supplemental material Figure S4C).

Comparison analysis on the circulating exosomes between maternal and fetal circulation

To compare the number of total, placenta-derived and contribution of non-placental and placental exosomes between maternal and fetal circulation, a two-way ANOVA with variance partitioned between pregnancy condition (i.e. controls, SGA and FGR) and circulation (i.e. maternal or fetal) was used (Figure 4). The concentration of exosomes including total, placenta-derived and the proportion of non-placenta and placental origin compared to the total exosomes was significantly different between maternal and fetal circulation. Post hoc multiple comparison test (Bonferroni) showed statistical difference (**$p<0.005$) in the SGA group in both total and placenta derived exosomes (Figure 4A and B). Interestingly, significantly difference between maternal and fetal circulation on the contribution of non-placental (Figure 4C) and placenta-derived (Figure 4D) exosomes to the total exosomes were identified for each group (i.e. controls, SGA and FGR).

Finally, correlation analysis on the total, placenta-derived, and proportion of non-placenta and placental origin compared to the total circulating exosomes between fetal and maternal plasma was performed (Supplemental material Figure S5). Interestingly, a significant positive correlation between the contribution of placenta-derived exosomes to the total exosomes present between fetal and maternal circulation was identified (Figure 5C).

Discussion

Principal findings of the study

The principal findings of this study are: 1) The contribution of placental exosomes ($CD63^{+ve}$ and $PLAP^{+ve}$) to the total circulating exosomes ($CD63^{+ve}$) is an indicator of fetal growth (significant and positive correlation with neonatal and placental weight); 2) The proportion of circulating placental exosomes compared to the total exosomes was significantly reduced in small fetuses as compared to controls, and displays a clear trend according to the severity of the disease (more reduced in cases with abnormal Doppler or severe growth restriction) and 3)

A positive correlation between maternal and fetal circulation on the contribution of placental exosomes to the total circulation exosomes was identified, suggesting a potential mechanism of regulation to maintain the proportion of exosomes between these circulation.

In this study, we isolate exosomes using a density gradient and every fraction was analysed by nanoparticle tracking analysis in fluorescence mode using quantum dots coupled with CD63 or PLAP and classified by size in <35 nm, 35-150 nm, and >150 nm. As we showed in the supplemental figure S2, between fractions 4 to 8 (*i.e*. density of 1.12 to 1.18g/ml) contained a greater number of vesicles with a majority of 50–150 nm in diameter, compared to the other fractions. Therefore, floatation into iodixanol gradients allows the enrichment of exosomes with a high yield of vesicles $CD63^{+ve}$. We would like to highlight that this is the first study reporting the individual number of vesicles $CD63^{+ve}$ and $PLAP^{+ve}$ in every fraction of an iodixanol gradient.

Using NTA in fluorescence mode, we establish the presence of exosomes (total and placental) in maternal and fetal circulation, and only individual vesicles were quantified. In addition, the presence of PLAP in exosomes isolated from maternal and fetal plasma was confirmed using a commercial ELISA kit. In order to quantify the number of placental-derived exosomes, we used placental-type alkaline phosphatase ($PLAP^{+ve}$), a plasma membrane enzyme isoform specifically produced by the syncytiotrophoblast [18,43]. $PLAP^{+ve}$ exosomes are specific for pregnancy and are not found in the circulation of non-pregnant women [19,20]. Herein, we are reporting that the contribution of placental exosomes (expressed as percentage of exosomes positive for $PLAP^{+ve}$ compared to total exosomes $CD63^{+ve}$) in the maternal circulation was ~14% lower in SGA and until ~23% lower in patients with FGR and it may be used as a marker for placental insufficiency and fetal growth. This is a small study, so the results are preliminary; much larger numbers would be required for confirmation and assessment of any clinical utility. If these results are confirmed, we believe that it would aid our understanding of whether placental insufficiency, affects the concentrations of exosomes in the maternal and fetal circulation, and whether reduced ratio $PLAP^{+ve}$ /$CD63^{+ve}$ observed in FGR (more severe cases with abnormal fetoplacental Doppler) is indeed a reflection of failed placental adaptation.

The role of exosomes in human gestation

Within the maternal circulation, the exosome population is secreted from multiple cell types, such as erythrocytes [44], endothelial cells [45], lymphocytes and dendritic cells, in addition

to the placenta during gestation [41]. During pregnancy, exosomes are involved in cell-to-cell communication between the placenta and maternal immune system [12]. Placenta derived exosomes suppress maternal T-cell signaling, which is thought to promote maternal immunotolerance towards the fetal allograft [19]. This local immune privilege at the feto-maternal interface has been attributed to the expression of placental exosome-associated functional Fas ligand (FasL), programmed death ligand 1 (PD-L1) and TNF-related apoptosis inducing ligand (TRAIL), which all induce maternal T-cell anergy and death [19, 32, 46–48]. The expression of NKG2D receptor ligands, UL-16 binding proteins (ULBP) and MHC class I chain-related (MIC) proteins on placental exosomes have been shown to down regulate NK cell activity and suppress maternal cytotoxic activity [34], thereby promoting fetal allograft survival. In normal pregnancies, placental exosomes have been reported to interact with and modulate the function of maternal endothelium to promote trophoblast migration, angiogenesis and spiral artery remodeling [42]. However, under pro-inflammatory (i.e. hypoxia and obesity) or pathological conditions such as gestational diabetes, the number of circulating exosomes is higher and promoting the release of pro-inflammatory cytokines from endothelial cells [20,21, 35,42]. This suggests that the content and effects of exosomes depends on the pathological and physiological status of the pregnant woman. Under normal conditions placental exosomes may play a role in maternal immunosuppression and fetal survival. However, the contribution of placental exosomes in pathological pregnancies remains to be elucidated. Therefore, this field of research holds great promise for the development of exosome-based predictive and prognostic markers of pathological pregnancies and in the future to monitoring placental function in real time. It has been reported that EVs in the maternal circulation may also exert a pro-inflammatory effect [49,50], contributing to the development of systemic inflammation and pathological pregnancies [51,52].

Exosomes in fetal circulation

This is the first study, reporting the presence of exosomes positive for PLAP in fetal circulation in human subjects, and showing its relation with fetal growth. The presence of PLAP in exosomes isolated from fetal circulation was evaluated using different methods, including western blot, nanoparticle tracking analysis, and ELISA. These methods are antibodies based which might be one of the limitations of this study. However, we used three different antibodies for PLAP. The antibody PLAP, ab96588 was used for Western blot and

the antibody anti-PLAP MA1-20245, clone H17E2, ThermoFisher was used for the quantification of placental exosomes by fluorescence nanoparticle tracking analysis. The antibody from Abcam has been validated for western for the company and the antibody anti-PLAP MA1-20245, clone H17E2, ThermoFisher has been validated for flow cytometer which uses the same principle of nanoparticle tracking analysis. Moreover, the clone H17E2 has been previously showed to be non-cross reactive to both human liver and intestinal alkaline phosphatases [53]. We used a commercial ELISA kit for PLAP to validate the presence of exosomes positive for PLAP in maternal and fetal circulation. For each analysis, we used exosomes isolated from non-pregnant women as negative control for PLAP as we previously described [21].

PLAP exosomes in umbilical cord blood and serum in pregnant sheep has been previously reported, and interestingly, exosome-associated miRNAs identified in the maternal and fetal circulation were differently expressed [54]. Furthermore, the authors described that pathway analysis predicted that exosome-associated miRNAs in the maternal circulation were related to cellular growth and proliferation as well as organ development pathways, while exosome-associated miRNAs in fetal blood were involved in embryonic development [54]. Several studies have detected exosomes isolated from mesenchymal stem cells obtained from fetal blood, and have also discussed their role in different clinical implications including decreased liver fibrosis [55], inflammation [56] and response to chemotherapy [57]. Interestingly, it has been recently reported that proteomic analysis of total exosomes isolated from umbilical cord blood of neonates that were born from mothers with preeclampsia was significantly different compared to those born from normal healthy pregnancies [58].

The placental exosomes are released into the maternal circulation from the syncytiotroblasts and extravillous trophoblast cells. Placental exosomes are characterized by the presence of placental alkaline phosphatase enzyme (PLAP), which is an integral membrane protein unique to trophoblastic cells [12]. Thus, the presence of $PLAP^{+ve}$ exosomes in fetal circulation refers to a mechanism by which the exosomes originating from the basal membrane of the trophoblast permeates through the mesenchymal cells and fetal endothelium and enters the fetal circulation. Placenta is a specialized barrier between the mother and fetus, which is the major route for transfer of nutrients, gases and ions to the fetus and has predominant role in fetal growth and development. Even maternal cells can cross placenta and reach fetal circulation and can lodge in fetal organs, which is called microchimerism [59]. The transmigration of the maternal cells to the fetal side is by interaction of Vascular endothelial

growth factor (VEGF A) and Vascular endothelial growth factor receptor 1 (VEGFR-1), with the help of cell surface integrins [60]. VEGF A has been described as the vascular permeability factor. Vessel fenestrations induced by VEGF A in the form of caveolae or assembly of caveolae to form trans-endothelial pores help in the passage of small molecules. However, larger proteins and cells depend on VEGF-cadherin based loosening of tight junctions and passage between endothelial cells [61]. Nevertheless, exosomes originating from the endosomal pathway and released into the extracellular space can enter the systemic circulation and taken up by different cells [62,63]. Thus, exosomes could be permeating through the endothelial barrier by any of the above means to enter the systemic circulation. Even the blood-brain barrier comprising of specialized endothelial cells with multimolecular complex tight junctions is permeable to exosomes and can be specifically used to deliver cargo to the neuronal cells [64,65].

It has been demonstrated that tumour exosomes are capable of modulating extracellular matrix (ECM) by degrading collagens, laminins and fibronectins helping pre-metastatic niche formation in tumour [66,67]. Exosomes can bind to selective targets in the extracellular matrix based on their adhesion molecule profile. The proteases especially the matrix degrading enzymes like matrix metalloproteases which are enriched in these vesicles can help them to permeate through the mesenchyme [66]. The ECM modulatory properties coupled with the smaller size, which helps them to permeate the endothelial barrier, could possibly explain the mechanism by which basal trophoblast membrane derived exosome traffic across the mesenchyme and fetal endothelium and enter the fetal circulation.

The potential value and expression of miRNAs of placental-derived exosomes in the fetal circulation may be an interesting source of knowledge to elucidate fetal response to placenta dysfunction. In this study, we have shown that placental-derived exosomes can be isolated in the fetal circulation, and that its concentration is influenced by placental weight and breadth. In addition, there was also a strong, positive correlation with birth weight; altogether pointing circulating placental-derived exosomes and their content as a valuable tool for better understanding the complex maternal and fetal circulation crosstalk.

Comparison of these findings with previous studies

The concentrations and contents of exosomes are thought to depend on their cells of origin and the stimuli, which trigger their production. We have also previously reported that the extracellular milieu (including oxygen tension and glucose concentration) regulates the number and protein content of placenta-derived exosomes [13,27], with greater release of

placental-derived exosomes under hypoxic conditions *in-vitro* [13] and that placental cell-derived exosomes regulate endothelial cell migration and vascular tube formation [27]. In this study, we are also confirming our previous observation that placental and neonatal weight have a strong positive correlation with placental cell-derived exosomes at third trimester of pregnancy [20]. A recent study has reported maternal concentrations of placenta-derived exosomes in normal pregnancies, and those complicated with early- and late-onset preeclampsia [68]. The authors described that the total number of exosomes increased in early- and late-onset preeclampsia compared to controls. However, the PLAP ratio had a contradictory direction, being significantly higher in early onset preeclampsia and reduced in late-onset preeclampsia, suggesting that exosomes may be involved in the physiopathology of preeclampsia [68]. Although, the authors confirmed that exosomes increases with the gestational age in normal gestations, there was no report regarding fetal growth in this study [68].

It is possible that aberrant exosomal signaling by placental cells is a key event in complications of pregnancy associated with poor placentation and impaired infiltration of spiral arteries such as preeclampsia, fetal growth restriction and preterm birth. We have previously reported that the maternal plasma concentrations of placenta-derived exosomes are different in obese women compared to lean or overweight women [21]. However, the population included in this study is in the majority from the Mediterranean area, which has a lower prevalence of obesity compared to other western countries.

Differences between SGA and FGR

Although the dichotomous view of suboptimal fetal growth as a SGA or FGR is overly naive, the contributions of placental insufficiency differ between both conditions, ultimately resulting in a diverse spectrum of clinical presentations. The release of exosomes to the maternal circulation is an active process, with purpose and regulated by tissue physiology and cellular function [47]. In contrast, the release of syncytiotrophoblast apoptotic debris appears to be a stimulus for a systemic inflammatory response [69]. Thus, a reduced secretion of placental derived exosomes may reduce maternal-fetal tolerance, predisposing to placental damage and apoptosis, which in turns lead to systemic maternal inflammation.

Herein, we quantified the number of placental exosomes present in maternal and fetal circulation (i.e. individual exosomes positive PLAP were tracked) and established the contribution of placental exosomes to total exosomes in maternal and fetal circulation in pregnancies with different degrees of fetal growth. All subgroups of small fetuses were

460 associated with significant alteration in the placental-derived exosomes profile, but there was
461 a gradation according to the severity of the disease. The presence of significant differences in
462 mother and SGA fetuses supports the view that at least a large proportion of these fetuses are
463 not "constitutionally" small, indicating some degree of placental dysfunction and challenging
464 the concept of constitutionally small. This study provides proof-of-concept that quantification
465 of placenta-derived exosomes may facilitate the early identification of suboptimal fetal
466 growth. We believe that the analysis of placenta-derived exosomes in maternal blood may
467 represent in the future a clinically useful, non-invasive test for placental function and/or
468 dysfunction.

469 ***Future directions in the characterization of placental-derived exosomes***

470 Exosomes carry a wide range of molecules including RNAs, proteins and DNA. The EVs
471 field is growing rapidly mainly because these vesicles protect their content and deliver a
472 specific message to target cells. Therefore, the next step of this study would be to characterize
473 the bioactive molecular contents (mRNA, miRNA, proteins, lipids, and metabolites) of
474 placenta-derived exosomes in order to elucidate their functions among targeted cells. To
475 elucidate the role of placental exosomes under normal and pathological conditions, the
476 specific isolation of circulating placental exosomes from maternal/fetal circulation is required
477 [70]. In order to determine the role of exosomes originating from different sources present in
478 maternal and fetal circulation, further studies are required. As we discussed, the presence of
479 exosomal PLAP in fetal circulation was determined using antibodies based methods.
480 Therefore, we suggest that additional experiments using different methodologies might be
481 necessary to elucidate the mechanisms of tracking of exosomes from maternal to fetal
482 circulation. Finally, we suggest that this burgeoning field will provide unique insights into the
483 etiology of disease, early detection, and treatment monitoring.

484

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Tables and Figure legends

Table 1. Baseline, pregnancy and perinatal characteristics of the study population subdivided into uncomplicated pregnancies with normal birth weight (controls) and small fetuses.

Characteristics	Uncomplicated pregnancies (N=10) Median (IQR) or n (%)	Small for Gestational Age (N=10) Median (IQR) or n (%)	Fetal Growth Restriction (N=10) Median (IQR) or n (%)	p value
Maternal baseline characteristics				
Age (years)	33 (31 – 34)	31.5 (25 – 35)	33 (26 – 35)	0.91
BMI (kg/m^2)	23.7 (21.7 – 24.6)	21.6 (19.9 – 23.1)	21.6 (20.4 – 23.7)	0.67
Ethnicity				0.31
White	6 (60)	8 (80)	5 (50)	
Latin	2 (20)	0	4 (40)	
Others	2 (20)	2 (20)	1 (10)	
Nulliparous	5 (50)	5 (50)	7 (70)	0.58
Smoking during pregnancy	0	0	2 (20)	0.34
Feto-placental ultrasound				
Gestational age at ultrasound (weeks)	33.3 (33.1 – 35.5)	33.7 (32 – 35.3)	33.4 (32.4 – 37.3)	0.95
EFW percentile	53 (39 – 82)	5 (4 – 7)	4 (2 – 9)	0.003
Umbilical artery PI	0.67 (0.63 – 0.72)	1.08 (1 – 1.22)	1.05 (0.89 – 1.14)	0.42
Middle cerebral artery PI	1.76 (1.2 – 2.32)	1.63 (1.32 – 1.9)	1.45 (1.22 – 1.66)	0.87
IP middle cerebral artery <5th centile	0	0	2 (20)	0.14
Cerebro-placental ratio	2.67 (1.67 – 3.68)	1.55 (1.9 – 1.99)	1.45 (1.19 – 1.79)	0.33
Cerebro-placental ratio <5th centile	0	0	3 (30)	0.39
Mean Uterine artery PI	0.79 (0.66 – 0.87)	0.85 (0.73 – 0.97)	0.8 (0.63 – 0.96)	0.79
Mean Uterine artery PI >95th centile	0	0	3 (30)	0.49
Perinatal outcomes				
Gestational age at delivery (weeks)	40.1 (39.2 – 40.6)	39.4 (38.1 – 40)	38.7 (37.6 – 40.1)	0.18
Induction of labor	3 (30)	5 (50)	10 (100)	0.004
Cesarean section	1 (10)	1 (10)	0	0.58
Male fetal gender	4 (40)	3 (30)	3 (30)	0.86
Birth weight (grams)	3372 (3080 – 3500)	2765 (2580 – 2850)	2348 (2328 – 2588)	0.0001
Birth weight percentile	45 (32 – 52)	6 (5 – 7)	1 (1 – 2)	0.0001
Placental weight (grams)	584 (440 – 610)	397 (345 – 428)	392 (365 – 455)	0.06
Placental breadth (cms)	17 (15.5 – 18)	12.5 (11.5 – 14.5)	14 (12 – 14.7)	0.1
Birth weight/Placental weight	6.15 (5.5 – 7.9)	7 (6.6 – 7.5)	6.1 (5.5 – 6.4)	0.14

BMI: Body mass index; EFW: Estimated fetal weight and PI: Pulsatility index.

Figure 1. Characterization of exosome isolated from maternal and fetal circulation. Exosomes were isolated from maternal plasma and fetal plasma (*i.e*. cord blood) by differential and buoyant density centrifugation from controls (appropriate for gestational age), small for gestational age (SGA) and fetal growth restriction (FGR) pregnancies. (A) Representative Western blot (Top) and corresponding SDS-gel stained with with SimplyBlue SafeStain (bottom) for exosome-enriched marker TSG101, flotilin-1, CD63 and negative marker Grp94. The presence of exosomes from placental origin was evaluated using Western for PLAP. Exosomes were positive for CD63, Flotilin-1, and TSG101, which are proteins associated with exosomes and negative for Grp94, which is an endoplasmic reticulum marker demonstrating the purity of the exosome isolation. (B and C) Representative size distribution and electron micrograph (insert) of exosomes isolated from maternal and fetal plasma, respectively. In A, 1: controls, 2: SGA, 3: FGR, and 4: cell lysate. In B and C, scale bar 100 nm.

Figure 2. Profile of exosomes in maternal plasma. Quantification of total, placenta-derived exosomes and their contribution to the total circulating exosomes in maternal plasma. Small fetuses were subdivided in small for gestational age (SGA) and fetal growth restriction (FGR), according to the severity of the case. (A) Total exosomes ($CD63^{+ve}$). (B) Placenta-derived exosomes ($CD63^{+ve}$ and $PLAP^{+ve}$). (C) The contribution of non-placental exosomes to the total exosomal concentration (%). (D) The contribution of placental exosomes to the total exosomal concentration ($CD63^{+ve}$ and $PLAP^{+ve}$ / $CD63^{+ve}$ and $PLAP^{-ve}$ x 100) (%) in cases and controls. In C and D, *** $p<0.0001$ and **** $p<0.00001$.

752 **Figure 3. Profile of exosomes in fetal plasma.** Quantification of total, placenta-derived
753 exosomes and their contribution to the total circulating exosomes in fetal plasma. Small
754 fetuses were subdivided in small for gestational age (SGA) and fetal growth restriction
755 (FGR), according to the severity of the case. (A) Total exosomes (*CD63^{+ve}*). (B) Placenta-
756 derived exosomes (CD63^{+ve} and PLAP^{+ve}). (C) The contribution of non-placental exosomes to
757 the total exosomal concentration (%). (D) The contribution of placental exosomes to the total
758 exosomal concentration (CD63^{+ve} and PLAP^{+ve} / CD63^{+ve} and PLAP^{-ve} x 100) (%) in cases and
759 controls. In C and D, *** $p<0.0001$ and **** $p<0.00001$.
760
761 **Figure 4. Comparison of the profile of exosomes between maternal and fetal plasma.** The
762 populations of circulating exosomes present in maternal and fetal circulation was analysed.
763 (A) Total exosomes CD63^{+ve} (fold changes). (B) Placental exosomes (CD63^{+ve} & PLAP^{+ve}) –
764 (C) Contribution of non-placental exosomes. (D Contribution of placental exosomes to the
765 total exosomal concentration. Data were normalised to values in maternal circulation
766 observed in controls and expressed as fold changes. In A, B and C, * $p<0.05$ and ***
767 $p<0.0001$.
768
769 **Figure S1. Correlations between maternal and fetal plasma.** Spearman correlation
770 between: A. Total exosomes CD63^{+ve} per ml plasma between maternal and fetal circulation.
771 B. Placental exosomes PLAP^{+ve} per ml plasma between maternal and fetal circulation. C. The
772 contribution of placental to total exosomes between maternal and fetal circulation. D. The
773 contribution of non-placental to total exosomes between maternal and fetal circulation.
774

775 **Supplemental material**

776 **Table S1**. Spearman correlation coefficient between maternal and fetal blood plasma concentrations of Qdot-CD63^{+ve} and placental alkaline
777 phosphatase (PLAP^{+ve}) exosomes and clinical parameters.
778

	Maternal Qdot-CD63^{+ve} Spearman correlation (95% CI); p value	**Maternal Qdot-PLAP^{+ve} Spearman correlation (95% CI); p value**	**Maternal PLAP^{+ve} ratio Spearman correlation (95% CI); p value**	**Fetal Qdot CD63^{+ve} Spearman correlation (95% CI); p value**	**Fetal Qdot PLAP^{+ve} Spearman correlation (95% CI); p value**	**Fetal PLAP^{+ve} ratio Spearman correlation (95% CI); p value**
Maternal pre-gestational BMI (kg/m^2)	0.06 (-0.31 to 0.42) p=0.75	0.07 (-0.30 to 0.43) p=0.71	0.1 (-0.27 to 0.45) p=0.6	0.11 (-0.27 to 0.46) p=0.57	0.19 (-0.19 to 0.52) p=0.33	**0.42 (0.06 to 0.68) p=0.02**
Uterine artery Doppler mean PI (z-scores)	0.04 (-0.35 to 0.41) p=0.85	-0.01 (-0.39 to 0.37) p=0.96	-0.18 (-0.53 to 0.21) p=0.36	-0.26 (-0.58 to 0.14) p=0.20	-0.28 (-0.60 to 0.11) p=0.15	-0.03 (-0.41 to 0.35) p=0.88
Umbilical artery Doppler PI (z-scores)	-0.02 (-0.39 to 0.35) p=0.91	-0.08 (-0.44 to 0.30) p=0.69	-0.24 (-0.56 to 0.15) p=0.22	0.19 (-0.20 to 0.52) p=0.34	0.15 (-0.23 to 0.50) p=0.43	-0.23 (-0.56 to 0.15) p=0.23
Cerebroplacental ratio	-0.09 (-0.52 to 0.38) p=0.72	-0.02 (-0.47 to 0.44) p=0.93	0.21 (-0.27 to 0.60) p=0.40	-0.19 (-0.59 to 0.29) p=0.43	-0.06 (-0.50 to 0.41) p=0.82	0.44 (-0.02 to 0.75) p=0.06
Birth weight percentile	-0.09 (-0.44 to 0.28) p=0.62	0.03 (-0.34 to 0.38) p=0.88	**0.77 (0.57 to 0.89) p=0.0001**	-0.29 (-0.59 to 0.08) p=0.12	-0.15 (-0.48 to 0.22) p=0.43	**0.91 (0.81 to 0.96) p=0.0001**
Placental weight (g)	-0.49 (-0.82 to 0.09) p=0.09	-0.39 (-0.77 to 0.20) p=0.19	0.12 (-0.46 to 0.63) p=0.69	0.20 (-0.39 to 0.68) p=0.50	0.32 (-0.28 to 0.74) p=0.29	**0.71 (0.27 to 0.91) p=0.01**
Placental breadth (cm)	-0.73 (-0.92 to -0.27) p=0.01	-0.67 (-0.90 to -0.15) p=0.02	0.46 (-0.15 to 0.82) p=0.13	0.13 (-0.48 to 0.66) p=0.68	0.33 (-0.30 to 0.76) p=0.29	**0.80 (0.41 to 0.94) p=0.001**
Birth weight/Placental weight	0.02 (-0.54 to 0.57) p=0.94	0.01 (-0.54 to 0.56) p=0.97	0.49 (-0.09 to 0.82) p=0.09	-0.15 (-0.65 to 0.43) p=0.62	-0.11 (-0.62 to 0.47) p=0.72	0.13 (-0.45 to 0.64) p=0.67

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Figure 1

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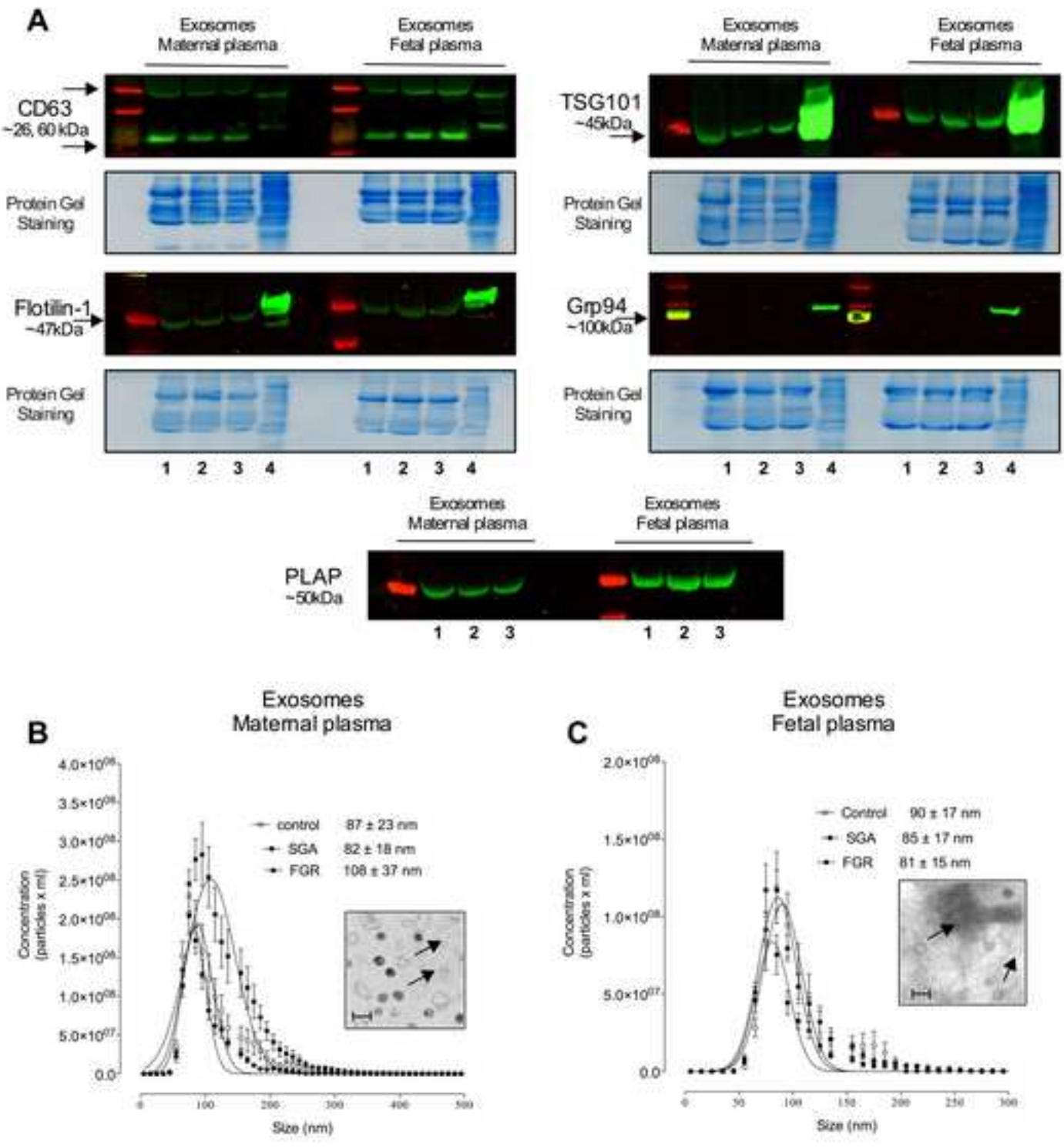

Figure 1

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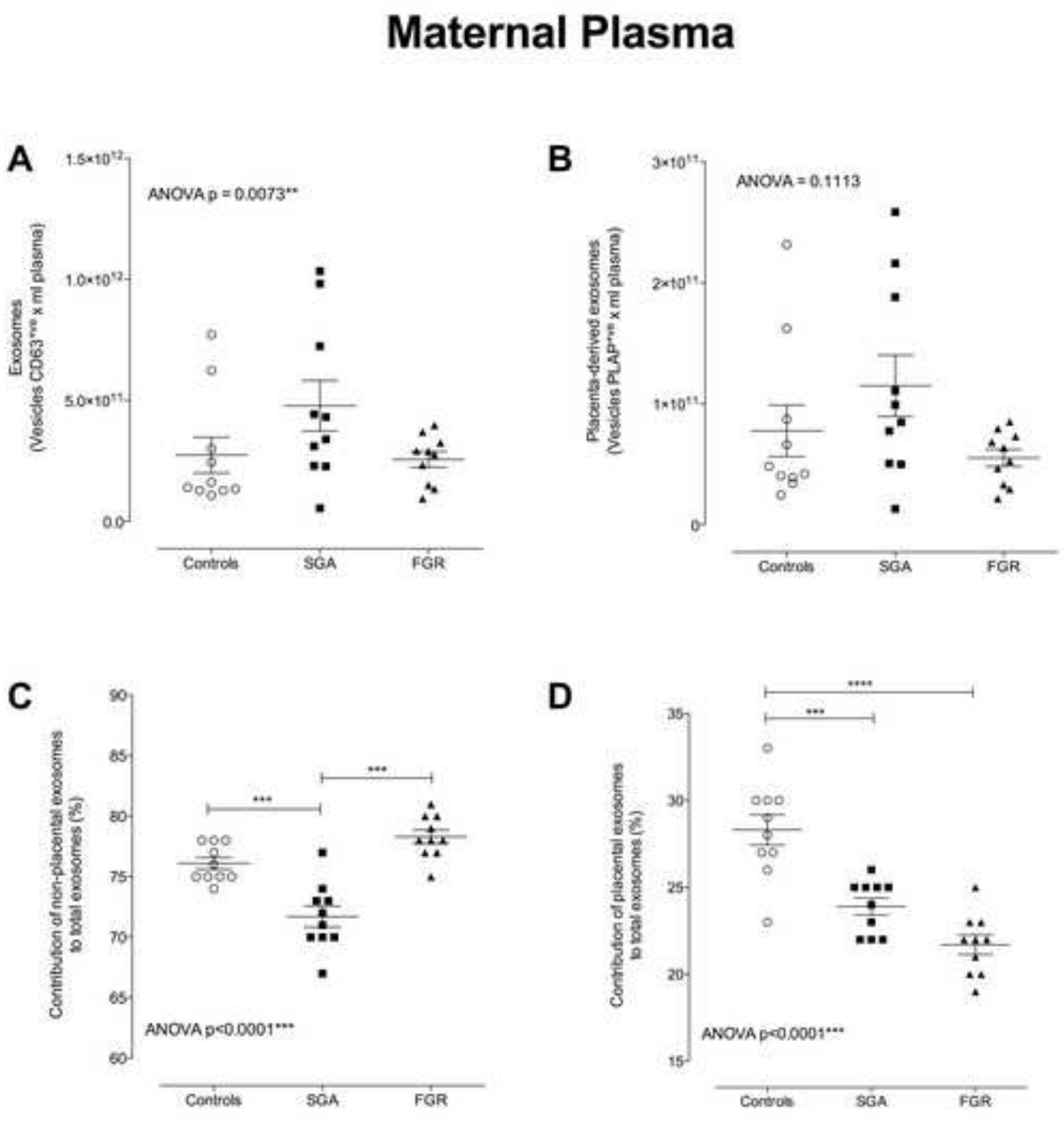

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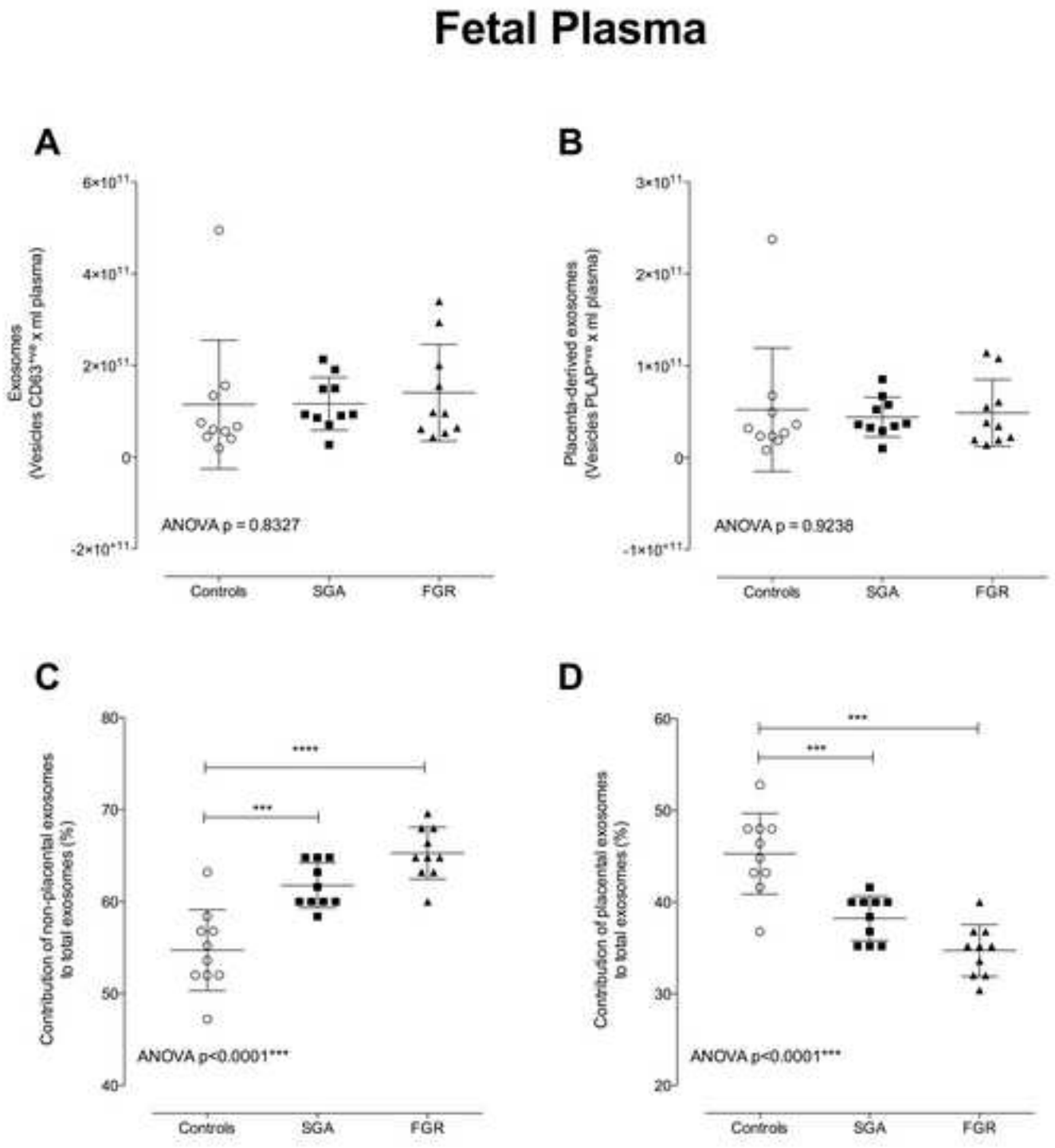

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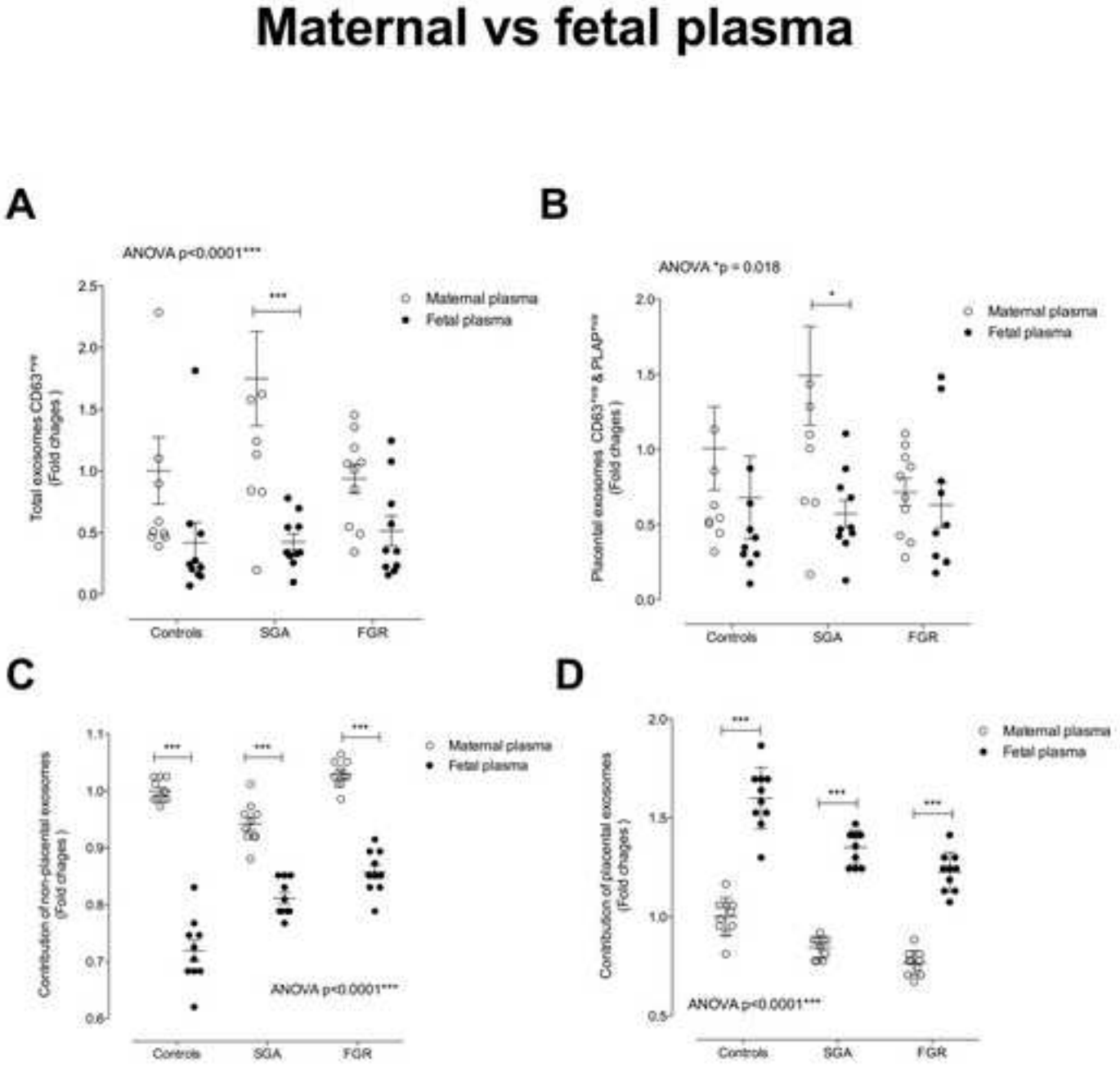

Figure 4

Supplementary File

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