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Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation

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Although there is significant interest in elucidating the role of placenta-derived exosomes (PdEs) during pregnancy, the exosomal profile in pregnancies complicated by gestational diabetes mellitus (GDM) remains to be established. The aim of this study was to compare the gestational-age profile of PdEs in maternal plasma of GDM with normal pregnancies and to determine the effect of exosomes on cytokine release from human umbilical vein endothelial cells. A prospective cohort of patients was sampled at three time points during pregnancy for each patient (i.e., 11–14, 22–24, and 32–36 weeks' gestation). A retrospective stratified study design was used to quantify exosomes present in maternal plasma of normal (n = 13) and GDM (n = 7) pregnancies. Gestational age and pregnancy status were identified as significant factors contributing to variation in plasma exosome concentration (ANOVA, P < 0.05). Post hoc analyses established that PdE concentration increased during gestation in both normal and GDM pregnancies; however, the increase was significantly greater in GDM (~2.2-fold, ~1.5-fold, and ~1.8-fold greater at each gestational age compared with normal pregnancies). Exosomes isolated from GDM pregnancies significantly increased the release of proinflammatory cytokines from endothelial cells. Although the role of exosomes during GDM remains to be fully elucidated, exosome profiles may be of diagnostic utility for screening asymptomatic populations.

Gestational diabetes mellitus (GDM) is defined as glucose intolerance with onset or first recognition during pregnancy (1). Currently, GDM affects ~15% of all pregnancies worldwide and its incidence is increasing in parallel with the global increase in obesity and type 2 diabetes (2). Indeed, the incidence of GDM worldwide is predicted to reach 18% when the new International Association of the Diabetes and Pregnancy Study Groups (IADPSG) criteria are adopted (3). GDM has been associated not only with acute increased risk for complications of pregnancy but also long-term disease risks for both mother and baby.

Current management guidelines recommend "universal screening" for GDM at 24–28 weeks of gestation by oral glucose tolerance tests (OGTTs) (4). The recommended interventions of diet, oral hypoglycemic agents, and insulin administration have a good impact in perinatal morbidity (5). When GDM is diagnosed in the late second or early third trimester of pregnancy, the "pathology" is most likely well established and the possibility to reverse or limit potential adverse effects on perinatal outcomes may be limited (6,7). If an effective first trimester screening test was available, the damage accumulated during the clinically occult phase (i.e., up to 24–28 weeks) may be averted by early intervention.

During normal pregnancy, diabetogenic autacoids are released by the placenta and induce insulin resistance and hyperinsulinemia (8). GDM develops in women when insulin release fails to compensate for pregnancy-induced

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insulin resistance. The progressive physiological changes that occur during pregnancy are essential to support and protect the developing fetus and also to prepare the mother for parturition (9). During human pregnancy, the placenta plays a pivotal role in mediating maternal physiological changes and fetal development. Interestingly, insulin resistance during pregnancy has been attributed to the release of placental hormones. Changes in placental hormones, however, do not directly correlate with changes in maternal insulin resistance (8). Recent studies, however, highlight the utility of tissue-specific nanovesicles (i.e., exosomes) for the diagnosis of disease, onset, and treatment monitoring (10,11).

Exosomes are small (~40–120 nm diameter) membrane-bound vesicles that are released after the exocytotic fusion of multivesicular bodies with the cell membrane. They are characterized by *1*) a spherical or cup-shaped form, *2*) a buoyant density of ~1.12–1.19 g/mL, *3*) endosomal origin, and *4*) the enrichment of late endosomal membrane markers, including Tsg101, CD63, CD9, and CD81 (12,13).

Previously, we established that exosomes are released in response to different oxygen tensions from primary placental (trophoblast) cells in vitro. These cells are the most abundant cell type of the human placenta and fuse to give rise to the syncytiotrophoblast, which senses and regulates oxygen and nutritional exchange between the mother and fetus during gestation (14,15). Moreover, we established that the plasma concentration of exosomes is greater in normal pregnant women than that observed in nonpregnant women (11). Interestingly, the placenta releases exosomes into maternal circulation at as early as 6 weeks of pregnancy (10).

Hypoxia, hyperglycemia, and hyperinsulinemia are risk factors for GDM and may adversely affect placentation and development of the maternal-fetal vascular exchange. The placental exosome profile across gestation (i.e., early, mid, and late gestation) in women who develop GDM has not been established. Thus, the aim of this study was to test the hypotheses that *1*) the concentration of placenta-derived exosomes (PdEs) in maternal plasma is greater in women who develop GDM than in normoglycemic pregnant women and *2*) exosomes isolated from pregnant women promote the release of proinflammatory cytokines from human umbilical vein endothelial cells (HUVECs); an effect is significantly greater when using exosomes isolated from GDM pregnancies. We propose that changes in the exosome concentration, composition, and/or bioactivity (i.e., interaction with maternal cells) are early biomarkers of GDM in presymptomatic women.

RESEARCH DESIGN AND METHODS

Study Group and Samples

A time-series experimental design was used to established pregnancy-associated change in exosome concentration and bioactivity in maternal blood obtained from normal and GDM pregnancies. Women (n = 500) were recruited between January 2008 and December 2010 with informed, written consent by research midwives from the Hospital Parroquial de San Bernardo, Santiago, Chile. Serial blood samples (BD Vacutainer PLUS Tubes, EDTA) at 11–14 (early), 22–24 (mid), and 32–36 (late) weeks were collected. Uterine artery Dopplers, clinical variables, and pregnancy outcomes were recorded. A retrospectively stratified study was designed involving normal healthy pregnant women (n = 13) and patients with GDM (n = 7), matched for age, gestational age, parity, and BMI. Patients between 24 and 28 weeks of gestation with fasting plasma glucose level >7.0 mmol/L (126 mg/dL) or >140 mg/dL at 2 h after an oral glucose load (75 g) were diagnosed as having GDM according to the World Health Organization criteria 1999 (16), which was used in Chile at the time of the study. Only patients diagnosed with GDM treated with diet (1,500 kcal/day and 200 g of carbohydrates as maximum per day) and samples collected at three time points during pregnancy were included in this study. Human plasma samples were obtained in accordance with the Declaration of Helsinki and approved by the ethics committee of The University of Queensland. The Human Research Ethics Committees of the Royal Brisbane and Women's Hospital and The University of Queensland (HREC/09/QRBW/14) approved tissue collection for the isolation of endothelial cells. Doppler ultrasound examinations of the umbilical artery (left and right arteries) were performed across normal and GDM pregnancies conducted with an Aplio Toshiba ultrasound. This sonogram provides a measure of the changing velocity throughout the cardiac cycle and the distribution of velocities in the sample volume (or gate) using B-mode image and color image frozen. Pulsatility index (PI) is based on the maximum Doppler shift waveform, and it measures the mean height of the waveform. Generally, a low pulsatility waveform is indicative of low distal resistance, and high pulsatility waveforms occur in high-resistance vascular beds. All evaluations were conducted by trained physicians according to international recommendations (17). Plasma samples were stored at −80°C until analyses. All experimental procedures were conducted within an ISO17025-accredited (National Association of Testing Authorities, Australia) research facility. All data were recorded within a 21 CRF part 11–compliant electronic laboratory notebook (Irisnote, Redwood City, CA).

Isolation of Exosomes From Maternal Circulation

Exosomes were isolated from plasma (1 mL) as previously described (10,11) with modifications. In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at 2,000*g* for 30 min at 4°C (high-speed microcentrifuge, fixed rotor angle of 90°, Sorvall; Thermo Fisher Scientific, Asheville, NC). The 2,000*g* supernatant fluid was then centrifuged at 12,000*g* for 45 min at 4°C (high speed microcentrifuge, fixed rotor angle of 90°, Sorvall). The resultant supernatant fluid (2 mL) was transferred to an ultracentrifuge tube (10 mL, Beckman) and centrifuged at 100,000*g* for 2 h (T-8100, fixed angle ultracentrifuge

rotor, Sorvall). The pellet was suspended in PBS (10 mL) and filtered through a 0.22-μm filter (Steritop; Millipore, Billerica, MA) and then centrifuged at 100,000*g* for 2 h. The 100,000*g* pellet was resuspended in 500 μL PBS and stored −80°C until exosome purification.

Exosome-Enriched Fractions

The extracellular vesicle (EV) pellet (i.e., 100,000*g* pellet) was resuspended in PBS (500 μL), layered on the top of a discontinuous iodixanol gradient containing 40% (weight for volume [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 the top to the bottom (with increasing density), diluted with PBS, and centrifuged at 100,000*g* for 2 h at 4°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 1.122–1.156 g/mL) were combined in a single tube and further characterized by the size distribution, presence of endosomal marker CD63, and morphologically using the nanoparticle tracking analysis, Western blot, and electron microscopy, respectively.

Quantification of Total Exosomes and PdEs

The concentrations of total and placental exosomes in maternal circulation were quantified using a CD63 and placental alkaline phosphatase (PLAP) ELISA as previously described (10,11). PLAP is syncytiotrophoblast-specific marker; therefore, exosomes derived from placental origin are positive for PLAP.

Endothelial Cell Isolation

Umbilical cords were collected immediately after delivery from three full-term normal pregnancies from the Royal Brisbane and Women's Hospital. The investigation conforms to the principles outlined in the Declaration of Helsinki and institutional ethics regulations. HUVECs were used to determine the bioactivity of exosomes isolated from maternal plasma. HUVEC primary cultures (37°C, 5% CO_2) were isolated by enzymatic digestion using Collagenase Type II (Gibco Life Technologies, Carlsbad, CA) as previously described (11,18). Cells were cultured in primary culture medium containing 2% exosome-depleted FBS (culture media was depleted of the contaminating exosomes using the same protocol for exosome isolation described previously, and exosome-free culture media medium was confirmed by electron microscopy) for 24 h before experiments.

Cytokine Array

To assess the effect of exosomes on endothelial cells, HUVECs were cultured in 96-well culture plates (Corning Life Sciences, Tewksbury, MA) according to the manufacturer's instructions and visualized using a real-time cell imaging system (IncuCyte live-cell; ESSEN BioScience, Ann Arbor, MI). Before the experiments, HUVECs were cultured in primary culture medium supplemented with 0.2% exosome-depleted FBS in 96-well culture plates (Corning Life Sciences) according to the manufacturer's instructions for 18–24 h. Cells were imaged every hour to monitor treatment-induced cell confluence and morphologic changes. Exosomes (100 μg protein/mL) were then incubated on HUVECs in medium containing 5 mmol/L D-glucose under an atmosphere of 8% O_2. Cytokine release (defined as the accumulation of immunoreactive cytokine in cell-conditioned medium) was quantified using protein solution arrays (BioPlex 200). Data were expressed as cytokine pg/10^5 cells/24 h. Exosomes were subjected to heat inactivation (30 min at 65°C) or sonication for 30 min (sonication bath) before the incubation on HUVECs.

Statistical Analysis and Power

Data are presented as mean ± SEM, with n = 13 (normal = control subjects) and n = 7 (GDM = case subjects) different patients per group (i.e., early, mid, and late gestation; total samples for GDM group = 21 and for the normal group = 39). The effects of gestational age on plasma exosome number, exosomal protein, and PLAP concentrations were assessed using repeated-measures ANOVA, with the variance partitioned between gestational age and condition; thus, gestational age was treated as an independent factor. Statistical differences between groups were identified by post hoc analysis Dunnett tests to compare each treatment to the control group where the data distribution approximates normality or by Mann-Whitney U test for distribution-independent data analysis. For two-group analyses, Student t tests were used to assess statistical difference. Linear regression analysis and Spearman rank correlation was used to assess the relationship between two quantitative variables (i.e., between PI, placental weight, and glucose level with exosomes). Statistical significance was defined as at least $P < 0.05$. Statistical analyses were performed using commercially available packages (Stata 11 [StataCorp, College Station, TX] and Prism 6 [GraphPad, La Jolla, CA]).

With respect to total exosome number, the experimental designs described above, at a significance level of $\alpha = 0.01$, with size effect of 2.15 and control-to-GDM sample ratio of 1.8 achieves a power of 0.92. With respect to total PdEs, the experimental designs described above, at a significance level of $\alpha = 0.05$, with size effect of 1.4 and control-to-GDM sample ratio of 1.8 achieves a power of 0.83.

A multivariate modeling approach was used to develop a proof-of-principle classification model for GDM. A logist boost regression analysis was used for modeling (Weka, version 3.6) (19). The implemented classification algorithm reported a predicted posterior probability value (PPV; i.e., the likelihood that a sample came from a woman with GDM) for each patient sample. PPV values were used to generate the receiver operator characteristic (ROC)

curve, and the area under the curve was calculated within the Weka analysis package.

RESULTS

Patients

A total of 20 women with either normal glucose tolerance (control subjects n = 13, 39 samples across pregnancy) or GDM (n = 7, 21 samples across pregnancy) were included in the study and were matched for age, weight, BMI, and gestational age (Table 1). All women included in this study were nonsmokers; had singleton, normotensive pregnancies; and were without intrauterine infection or any other medical or obstetric complications except GDM. PI (mean between right and left uterine artery) progressively declined across gestation in both normal and GDM pregnancies, without differences in the two groups matched for gestational age ($P > 0.05$). This reflects a normal uteroplacental circulation in the group with GDM. Birth weights and placental weights were similar in the two groups ($P > 0.05$).

Exosome Isolation and Characterization

Exosomes were isolated using the gold-standard methods (20,21) and purified by a density gradient. Nanoparticle tracking analysis showed that particles between 50 and 150 nm were obtained (Fig. 1*A–C* and Table 2). Morphological analysis identified cup-shape characteristics of exosome vesicles (Fig. 1*D*) and positive expression for CD63 (Fig. 1*E*). A complete size distribution analysis and enrichment of TSG101 (markers involved in the biogenesis of exosomes vesicles) of all the fractions obtained after exosome purification is presented in Supplementary Fig. 1. No significant differences between the nanoparticle tracking analysis characteristics of exosomes isolated from normal and GDM across gestation were identified (Table 2).

Gestational-Age Variation in Exosome Concentration in GDM

To determine the effect of GDM on exosome concentration during gestation, pooled exosome-containing fractions (fractions 4–6) were further quantified by measuring the total number of exosome vesicles. The number of exosomes present in maternal plasma obtained from normal and GDM pregnancies averaged $1.34 \times 10^{12} \pm 2.75 \times 10^{11}$ and $2.89 \times 10^{12} \pm 5.33 \times 10^{11}$ vesicles/mL plasma, respectively (Fig. 2*A*). Exosome concentration progressively increased throughout gestation in both normal and GDM (Fig. 2*B*), with large interindividual variations (Supplementary Fig. 2). The gestational-age variation in plasma exosome number was analyzed by two-way ANOVA with the variance partitioned between gestational age, subject, and pregnancy condition (normal or GDM). A significant effect of gestational age and GDM was identified ($P < 0.005$). A

Table 1—Clinical characteristics of patients and newborns

	Normal (n = 13)	GDM (n = 7)
Maternal variables		
Age (years)	24 ± 1.6 (18–36)	25.53 ± 7.1 (20–30)
Weight (kg)	65 ± 4.3 (54–108)	60.44 ± 10.92 (55–70)
Height (cm)	158 ± 2.2 (149–173)	155.6 ± 5.35 (150–163)
BMI (kg/m^2)	25.9 ± 1.2 (21–36)	26.86 ± 5.4 (22–30)
Glycemia basal at early gestation (mg/dL)	73 ± 3.1 (50–90)	76 ± 5.0 (70–84)
Glycemia basal at midgestation (mg/dL)	70 ± 2.6 (57–90)	83 ± 3.8 (66–98)
OGTT at midgestation (mg/dL)	95 ± 5.3 (61–124)	163 ± 10.5* (142–213)
Early gestation (weeks)	12 ± 2.3 (11–14)	12.1 ± 1.9 (11–14)
Midgestation (weeks)	26 ± 3.0 (22–28)	27.2 ± 2.6 (22–28)
Late gestation (weeks)	35 ± 3.6 (30–38)	35 ± 3.8 (30–38)
Gestational age at delivery (weeks)	39 ± 1.9 (38–40)	39 ± 1.8 (38–40)
Systolic blood pressure (mmHg)		
Early gestation	107 ± 3.0 (90–120)	111 ± 3.4 (100–120)
Midgestation	106 ± 3.3 (90–130)	110 ± 3.1 (100–120)
Late gestation	111 ± 2.2 (100–120)	108 ± 4.1 (100–120)
Diastolic blood pressure (mmHg)		
Early gestation	65 ± 2.4 (50–80)	67 ± 3.5 (60–80)
Midgestation	65 ± 2.1 (50–90)	68 ± 2.6 (60–80)
Late gestation	68 ± 2.9 (50–70)	60 ± 3.6 (50–70)
Uterine artery Doppler (PI right and left uterine artery)		
Early gestation	1.69 ± 0.16 (0.82–2.7)	1.23 ± 0.14 (0.60–1.8)
Midgestation	0.87 ± 0.06 (0.56–1.2)	0.75 ± 0.05 (0.55–0.98)
Late gestation	0.60 ± 0.03 (0.39–0.83)	0.47 ± 0.05 (0.27–0.62)
Newborn variables		
Placental weight (g)	629 ± 24 (501–731)	650 ± 30 (535–730)
Fetal weight (g)	3,449 ± 141 (2,660–4,175)	3,369 ± 223 (2,340–3,800)
Fetal sex (male/female)	4/4	6/7

Data are presented as mean ± SEM (range). All pregnancies were singleton, normotensive, nonsmoking, not alcohol or drug consuming, and without intrauterine infection or any other medical or obstetrical complications except GDM. OGTT glucose measure was 2-h postglucose challenge (75 g). *$P < 0.05$ vs. normal.

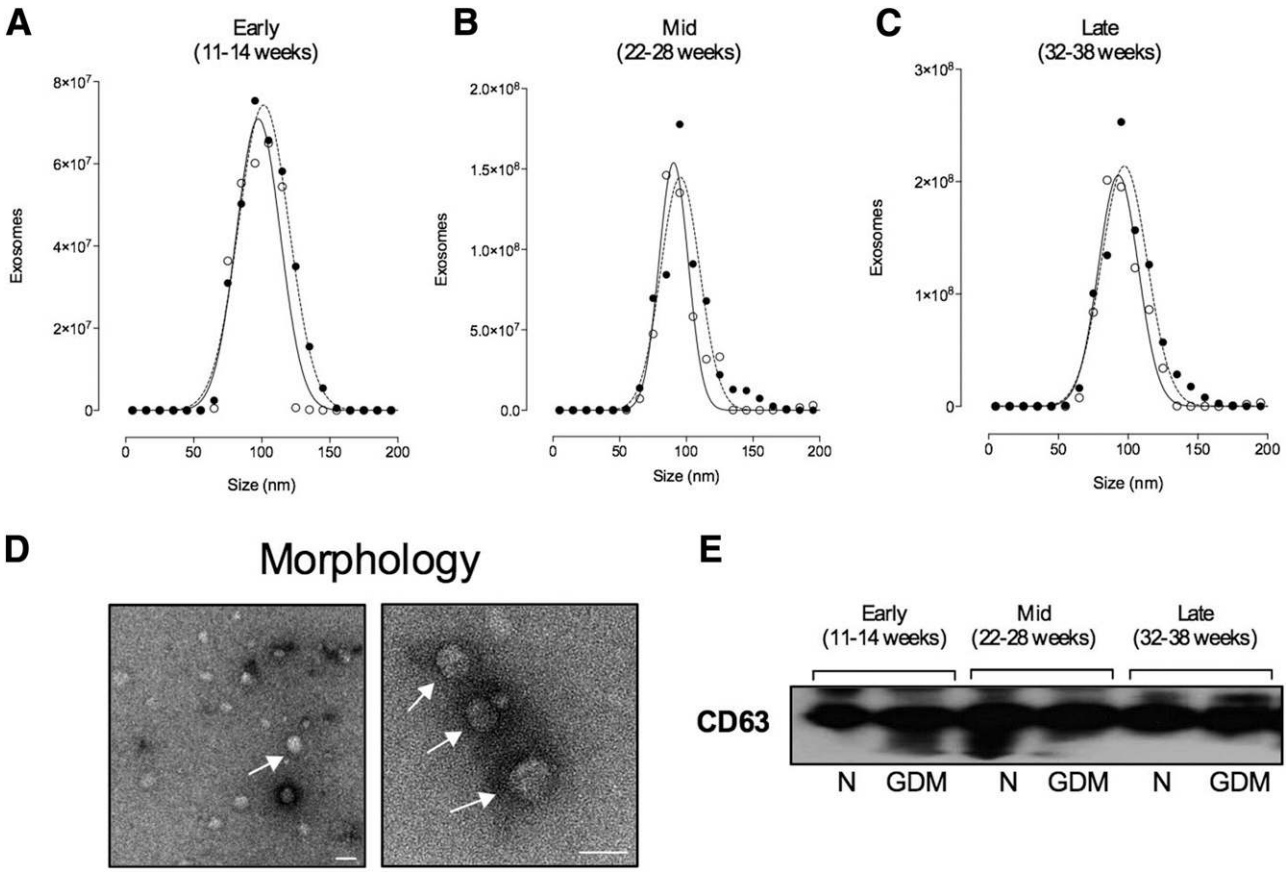

Figure 1—Characterization of exosomes from GDM pregnancies. Exosomes were isolated from maternal plasma during early (i.e., 11–14 weeks), mid (i.e., 22–28 weeks), and late (i.e., 32–38 weeks) gestation by differential and buoyant density centrifugation from normal (white circles) and GDM (black circles) pregnancies. *A–C*: Representative exosome enriched fraction size distribution isolated from maternal circulation across the pregnancy using a NanoSight NS500 instrument (NanoSight, Amesbury, U.K.). *D*: Representative electron micrograph of exosome fractions (pooled enriched exosome population from fractions 4–8). *E*: Representative Western blot for exosome enriched marker CD63. In *D*, scale bar = 100 nm and arrows showing the exosomes. N, normal.

post hoc multiple range test was used to identify statistically significant ($P < 0.05$) differences between group means. The exosome concentration was significantly higher in GDM compared with normal pregnancies matched by gestational age (i.e., early, mid, and late gestation). The total exosome numbers in early pregnancy (i.e., 11–14 weeks) was $1.27 \times 10^{12} \pm 4.97 \times 10^{11}$ and $2.77 \times 10^{11} \pm 6.20 \times 10^{10}$ vesicles/mL plasma for GDM and normal, respectively. At midgestation (i.e., 22–28 weeks), the total exosome number was $2.85 \times 10^{12} \pm 7.78 \times 10^{11}$ and $1.37 \times 10^{12} \pm 4.14 \times 10^{11}$ vesicles/mL plasma for GDM and normal, respectively. Finally, at late gestation (i.e., 32–38 weeks), the total exosome number was $4.55 \times 10^{12} \pm 1.04 \times 10^{12}$ and $2.38 \times 10^{12} \pm 5.99 \times 10^{11}$ vesicles/mL plasma for GDM and normal, respectively. No significant effects of fetal sex, maternal BMI, maternal age, maternal weight, and maternal height on exosome number or exosomes were identified.

Variation in Placental Exosome Concentration in GDM

To determine the number of PdEs in maternal plasma, exosomal PLAP concentration was quantified. Exosomal PLAP concentration was significantly greater ($P < 0.05$) in plasma obtained from GDM than normal pregnancies and averaged 276 ± 33 and 191 ± 18, respectively (Fig. 2*C*). In GDM, the number of PdEs (as indicated by PLAP ELISA) increased with gestational age (Fig. 2*D*), with large interindividual variations (Supplementary Fig. 3). A post hoc multiple range test was used to identify statistically significant ($P < 0.05$) differences between gestational age

Table 2—Vesicle size distribution during pregnancy

Gestational age	Normal (nm)	GDM (nm)
Early (11–14 weeks)	103 ± 41	108 ± 45
Mid (22–28 weeks)	107 ± 48	100 ± 51
Late (32–28 weeks)	110 ± 51	107 ± 53

The size distribution of exosome preparations were analyzed using a NanoSight NS500 system according to the manufacturer's instructions (see RESEARCH DESIGN AND METHODS). Data are presented as mean ± SD. All preparations were analyzed in duplicate, and five videos were recorded for normal and GDM pregnancy.

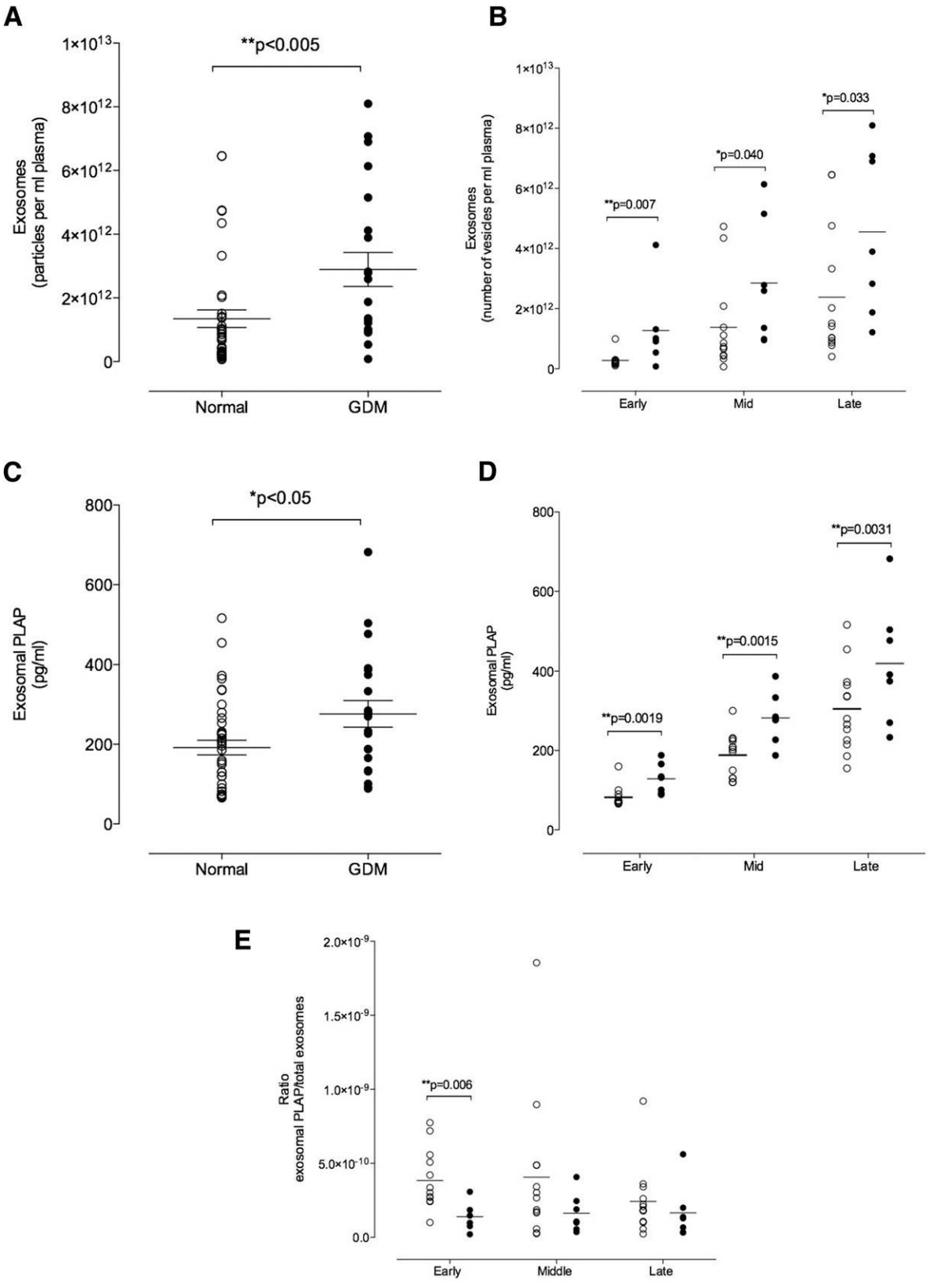

Figure 2—Exosome profiling across GDM pregnancies. Enriched exosome populations were quantified in peripheral plasma of women with normal (white circles) and GDM (black circles) pregnancies across gestation. *A*: Total exosome number presented as the average across early, mid, and late gestation. *B*: Gestational-age variation in plasma exosome number across pregnancy. *C*: PdE variation is presented as the average across early, mid, and late gestation. *D*: Gestational-age variation in PdEs across pregnancy. *E*: Contribution of PdEs to the total exosomal population across early, mid, and late gestation. Data are presented as aligned dot plot, and values are mean ± SEM.

and GDM. At early gestation (i.e., 11–14 weeks, before GDM was diagnosed), the concentration of PLAP in exosomes was ~1.6-fold greater in GDM compared with normal (81 ± 7 vs. 128 ± 14 pg/mL for normal and GDM, respectively). At midgestation (i.e., 22–28 weeks, gestational period when GDM was diagnosed), the concentration of PLAP in exosomes was ~1.5-fold higher in GDM compared with normal (188 ± 14 vs. 282 ± 24 pg/mL for normal and GDM, respectively). At late gestation (i.e., 32–38 weeks), the concentration of PLAP in exosomes was ~1.3-fold higher in GDM compared with normal (304 ± 29 vs. 418 ± 57 pg/mL for normal and GDM, respectively). No significant effects of fetal sex, maternal BMI, maternal age, maternal weight, and maternal height on exosome number or exosomal PLAP were identified.

To estimate changes in the relative contribution of placental exosomes to total exosomes present in maternal plasma, PLAP content per exosome (PLAP ratio) was determined. Both total number of exosomes and exosomal PLAP increased throughout normal and GDM pregnancies. Interestingly, fold changes were similar across gestation for GDM and similar during first and second trimester in normal pregnancies. In addition, PLAP ratio increased independent of exosomal PLAP during third trimester for normal pregnancy (Fig. 2*E*). PLAP ratio was lower in GDM compared with normal at early (63 ± 14%), mid (59 ± 19%), and late (31 ± 11%) gestation; however, statistical significance was only archived at early gestation ($P = 0.006$) (Fig. 2*E*).

Exosomal PLAP and Physiological Correlates

To establish whether or not changes in the uteroplacental circulation are associated with exosome concentrations across gestation, the association between PI, placental weight, and exosomes was determined. Regression analysis identified a negative association between PI and total exosomes and PdEs in both normal and GDM, with no significant differences between the groups, where ~11 and ~37% of the variance in total exosome numbers and PdEs is accounted for by variation in PI, respectively (Fig. 3*A* and *B*). Regression analysis established a negative association between total exosomes at late gestation and placental weight (Fig. 3*C*), where ~34% of the variance in total exosome number is accounted for by variation in placental weight. Finally, GDM displayed a positive association between placental weight and PdE concentration at late gestation, where ~31% of the variance in PdE concentration can be explained by variation in placental weight at late gestation (i.e., 32–28 weeks). No significant association was identified between placental weight and PdEs at late gestation in normal pregnancies with just ~4% of the variance shared between placental weight and PdE concentration at late gestation (Fig. 3*D*). Finally, the association between glucose concentration and exosome concentration into maternal circulation was evaluated (Supplementary Fig. 5). No significant correlation ($P > 0.05$) was identified between glycemia basal and total exosomes (Supplementary Fig. 5*A*) or PdEs (Supplementary Fig. 5*B*). Similarly, we did not find a statistical correlation between OGTT values and total exosomes (Supplementary Fig. 5*C*) or PdEs (Supplementary Fig. 5*D*).

ROC Curve Analysis

The sensitivity and specificity of exosome number as a predictor for GDM was also examined by performing ROC curve analysis. The number of observations (i.e., 7 pregnant women with GDM and 13 normal pregnant women) in the current study limit the generation of robust estimates of sensitivity and specificity. We, however, as a proof-of-principle exercise, generated a multivariate index assay based on exosome number (placental and total) and gestational age (trimester). The model delivers an area under the curve of >0.95 and, at a cutoff value of 0.49 (predicted PPV), has a sensitivity of 85.7% and specificity of 89.7%. The model was cross-validated (twofold) and, as anticipated (given the small number of observations), degraded slightly (AUC = 0.78; sensitivity = 0.89; specificity = 0.68) (Supplementary Fig. 6).

Effect of Exosomes on Cytokine Release From Endothelial Cells

GDM is a syndrome associated with a proinflammatory state. Thus, we determined the effect of exosomes isolated from maternal plasma during gestation obtained from normal and GDM on cytokine release from endothelial cells. Exosomes increased ($P < 0.05$) the release of all cytokines from HUVECs compared with control (without exosomes), with the exception of IL-2 and IL-10 ($P > 0.05$) (Fig. 4). Exosomes from early, mid, and late gestation obtained from normal pregnancy significantly increased (~1.8-fold) the release of GM-CSF, IL-4, IL-6, IL-8, IFN-γ, and TNF-α, without significant differences between gestational age. The effect of exosomes on cytokine release from HUVECs was significantly higher (~3.3-fold) using exosomes isolated from GDM pregnancies. A post hoc multiple range test was used to identify statistically significant ($P < 0.05$) differences between pairwise comparisons (i.e., normal vs. GDM matched by gestational age).

Heat treatment (65°C for 30 min) did not significantly affect exosome-induced cytokine release from endothelial cells. In contrast, sonication completely abolished the effect of exosomes (isolated from plasma and cell-conditioned media) on endothelial cells (Supplementary Fig. 4*A*).

The internalization of exosomes labeled with PKH67 (green) in endothelial cells was visualized and quantified using fluorescence microscopy and real-time cell imaging (IncuCyte), respectively (Supplementary Fig. 4*B*). Exosome uptake by HUVECs was observed in a time-dependent manner with the maximum at 24 h. For all conditions studied, sonication abolished the uptake of fluorescent exosomes compared with exosomes without

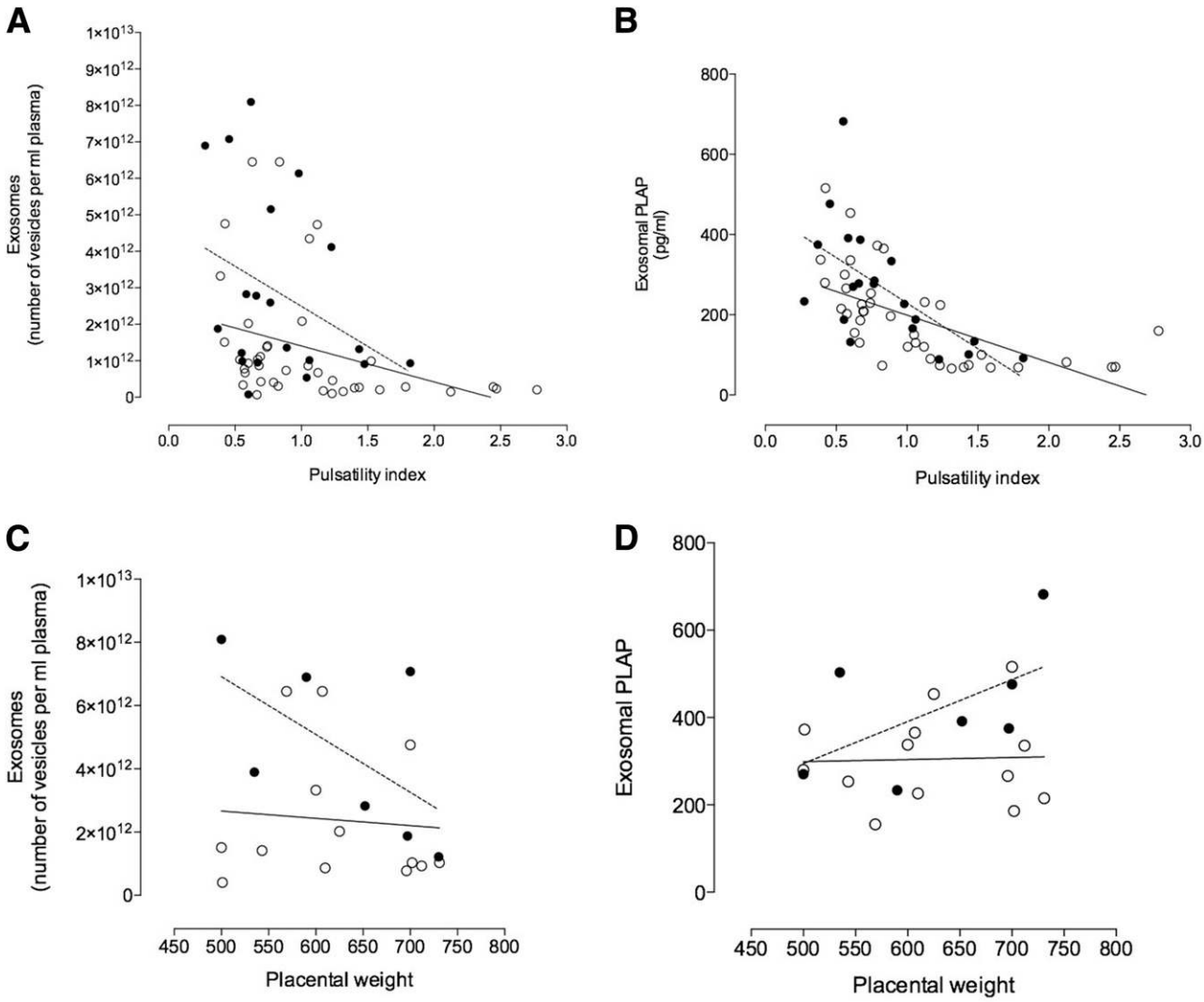

Figure 3—Relation between exosomes and placental features. We used linear regression to evaluate the relationship between exosomes, arterial PI, and placental weight in normal (white circles) and GDM (black circles) pregnancies. *A*: Relationship between total exosomes and PI. *B*: Relationship between exosomal PLAP and PI. *C*: Relationship between total exosomes at late gestation (i.e., 32–38 weeks) and placental weight at delivery. *D*: Relationship between PdEs ($PLAP^+$) at late gestation (i.e., 32–38 weeks) and placental weight at delivery. In *A* and *B*, linear regression for normal (solid line) and GDM (dashed line). In *A*, linear regression analysis for normal ($P = 0.0336$) and GDM ($P = 0.1259$). In *B*, linear regression analysis for normal ($P < 0.0001$) and GDM ($P = 0.0037$). In *C*, linear regression analysis for normal ($P = 0.7815$) and GDM ($P = 0.1668$). In *D*, linear regression analysis for normal ($P = 0.9064$) and GDM ($P = 0.1902$).

sonication. Exosome uptake is presented as fluorescence per cell confluence normalized to maximum uptake of 100%. Heat inactivation did not affect the exosome uptake by HUVECs.

DISCUSSION

The aim of this study was to characterize changes in the concentration and bioactivity of exosomes present in maternal peripheral plasma in pregnancies complicated with GDM. The data obtained in this study established that GDM pregnancies are associated with a twofold increase in the concentration of exosomes in maternal plasma compared with normal pregnancy. In a longitudinal study, the plasma concentration of total and PdEs increased during pregnancy, both normal and GDM; however, the concentration of exosomes was higher in GDM compared with the normal pregnancy matched by gestational age. The study further established that exosomes present in maternal plasma from GDM are bioactive and modulate the proinflammatory cytokines released from endothelial cells. These results suggest that in early pregnancy (i.e., 11–14 weeks), presymptomatic women who subsequently develop GDM (diagnosed between 24 and 28 weeks) can be identified by their plasma exosome profile (i.e., concentration and/or bioactivity). Moreover, PdEs may contribute to the proinflammatory state associated with pregnancy, a phenomenon enhanced under diabetes conditions.

In the past decade, EVs have been established as an important mediator of cell-to-cell communication regulating biological process on target cells. EVs are classified

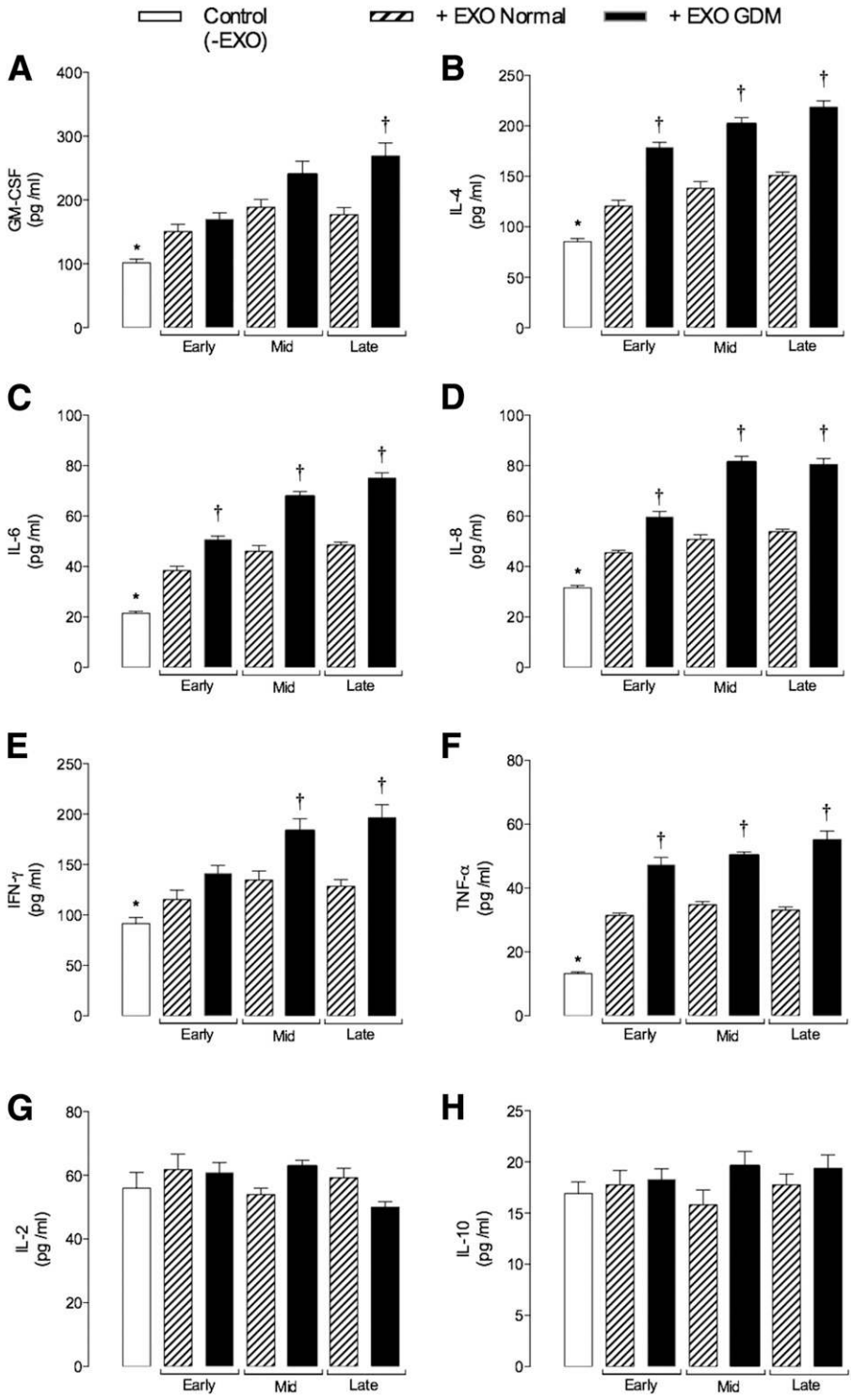

Figure 4—Induction of cytokine release from endothelial cells by exosomes. The effect of exosomes (100 μg/mL) isolated from plasma obtained from normal (EXO Normal) and GDM (EXO GDM) pregnancies across gestation on the release of GM-CSF (*A*), IL-4 (*B*), IL-6 (*C*), IL-8 (*D*), IFN-γ (*E*), TNF-α (*F*), IL-2 (*G*), and IL-10 (*H*) from HUVECs is presented as mean ± SEM (n = 6). Data were subject to two-way ANOVA with the variance partitioned between exosome source (i.e., normal or GDM) and gestational age (i.e., early, mid, or late gestation). Values are mean ± SEM. *$P < 0.05$ vs. all conditions; †$P < 0.05$ vs. corresponding values with EXO normal.

according of their size and cellular origin as microvesicles (~50–1,000 nm and generated by budding from the plasma membrane) and exosomes (~40–130 nm and generated via an endosomal exocytotic pathway). Several studies have demonstrated the presence of EVs of placental origin in maternal circulation during pregnancy

(10,11,22–26); however, these studies used disparate procedures to isolate EVs.

In this study, we used a well-established and validated method to obtain an enriched exosome fraction using buoyant density centrifugation, thus minimizing the contribution from other EVs. The total number of exosomes present in maternal plasma was approximately twofold greater in women who subsequently developed GDM than women who experienced a normoglycemic pregnancy.

Previously, we and others have established that normal pregnancy is associated with a higher concentration of exosomes compared with nonpregnant women and that the concentration of placental exosomes increases during pregnancy (11,24). Moreover, PdEs are present in maternal plasma from 6 weeks of pregnancy and increase during the first trimester of pregnancy (i.e., from 6 to 12 weeks) (10). The data obtained in this study further establish that during early pregnancy (i.e., 11–14 weeks), women who subsequently develop GDM have consistently higher concentrations of exosomes and PdEs than women who have a normoglycemic pregnancy. The contribution of placental exosomes to the total exosome concentration (as determined by exosomal PLAP/total exosomes), however, was lower in GDM pregnancies than in normal pregnancy. Interestingly, even though both PdEs and total exosomes are higher in GDM, the ratio of exosomal PLAP to total exosomes is diminished in GDM compared with normal pregnancy. These data may represent changes in the number of exosomes released from the placenta, increased release of exosomes from nonplacental sources, or a combination of both of them. The data obtained herein do not allow discrimination between these possible scenarios.

Maternal metabolic and immune status may alter placental metabolism and function at an early stage. Maternal inflammation (quantified by leukocyte number at the beginning of gestation) has been associated with a higher susceptibility to develop GDM (27), suggesting that an inflammatory environment modulates the maternal glucose metabolism, and this phenomenon might be associated with changes in the bioactivity of both placental and nonplacental exosomes. The effect of proinflammatory cytokines on the exosome release from placental cells has not been established. Moreover, future studies are required to establish the effect of placental exosomes on the exosome releases from nonplacental sources. Consistent with this hypothesis, we recently established that first trimester trohoblast cells increase the exosomes released in response to high glucose concentration, and the exosomes released under these conditions increased the release of proinflammatory cytokines from endothelial cells (28).

Early diagnosis of GDM by identifying women in the first trimester of pregnancy who develop the pathology later in gestation may decrease the long-term effect on mother and fetus (29). Moreover, when GDM is diagnosed in the late second or early third trimester of pregnancy, the disease is most likely well established and the possibility to reverse or limit potential adverse effects on perinatal outcomes may be limited. This complication of pregnancy not only causes acute adverse pregnancy outcomes for mother and infant but also increases the lifetime risk of the infant developing metabolic syndromes (including obesity and type 2 diabetes) and type 2 diabetes in the mother. Moreover, a female born from a GDM pregnancy has a higher chance of developing GDM during her pregnancy, thus creating a recurring disease cycle. If we can identify women during the first trimester of pregnancy who are at higher risk of developing GDM, then an opportunity is created for treatment to improve pregnancy outcome and reduce the incidence and/or severity of this complication. Thus, the intergenerational recurring disease cycle will be prevented. Interestingly, fasting plasma glucose concentration and HbA_{1c} were used to screen early pregnant (<24 weeks of pregnancy) women for GDM (30). The authors reported that although early pregnancy screening doubled the clinical diagnosis of GDM, it was able to identify women with more severe hyperglycemia (necessitating pharmacotherapy), including those with a low BMI. A larger trial is required to further validate the utility of this approach for population screening and animal studies are needed to provide additional in vivo data. In this regard, microRNA (miRNA)-145 expression in urinary exosomes was greater in patients with type 1 diabetes compared to the control subjects. Exosomal miRNA-145 expression was also greater in an animal model of early experimental diabetic nephropathy (31).

To our knowledge, this is the first study to identify gestation-associated changes in the exosome concentration in women with GDM. That exosome concentration is greater in women who subsequently develop GDM may afford new opportunities for the prediction of pregnancy-related diseases. Moreover, exosomal biomarkers could have a number of advantages over previously used biomarkers, including capacity to separate from high-abundance proteins that have confounded all previous blood-based proteomics, stability on storage, protection from degradation (e.g., miRNA), and reflection of cell phenotype and conditioning. Indeed, it has become clear that "cell-free miRNA and RNA" in plasma (that are currently used in prenatal diagnosis) are contained within the exosome. Recently, it has been established that small RNA (including miRNAs) contained within exosomes are protected against RNase treatment (32). Interestingly, unique and common miRNA between plasma and PdEs was reported.

Under normal conditions, the number of PdEs is positively associated with placental weight at the third trimester of pregnancy (11). These data are consistent with the hypothesis that placental mass is a significant factor in determining the concentration of PdEs. Interestingly, placental mass increases significantly in GDM compared with normal pregnancy (33). We did not observe a

significant difference in placental weight between normal and GDM, probably because patients with GDM treated with diet instead of insulin were studied; however, it could also be because a population with a similar BMI was evaluated (BMI 26.8 kg/m^2 in subjects with GDM vs. 25.9 kg/m^2 in control subjects). Interestingly, at late gestation (i.e., 32–33 weeks), total exosome concentration was negatively associated with placental weight at delivery, whereas PdE concentration was positively associated. Both placental and nonplacental-originated exosomes are elevated in GDM pregnancies; however, the potential role of these nanovesicles during GDM pregnancies have not yet been established.

Several reports have shown the effect of PdEs on maternal immune modulation during pregnancy activated NK cell receptor NKG2D (34) and express the proapoptotic molecules Fas and TRAIL (35). Exosomes carry a wide range of signaling molecules, and their roles in physiology and pathophysiology during pregnancy remain to be fully elucidated. Recently, dysregulation of exosomal miRNA in patients with diabetes has been reported. Changes in glycemic control were associated with exosomal miRNA profiling involving the regulation of the adiponectin pathway (36). Despite these interesting results, a commercial kit was used to isolate EVs. This study cannot distinguish between exosomes and other EVs.

Hyperglycemia-induced oxidative stress makes an important contribution to the etiology of GDM, with consequences for both mother and baby (37). In support of an etiological role of hypoglycemia and attendant oxidative stress in poor pregnancy outcome, the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study reported a strong and continuous association between maternal glucose concentrations and pregnancy outcome; it confirmed a relationship between birth weight and maternal hyperglycemia (5). Normal pregnancy is a proinflammatory state, associated with high concentrations of proinflammatory cytokines, a phenomenon that is even higher in GDM. Exosomes isolated from normal and GDM across gestation are bioactive with the capacity to be internalized by endothelial cells and increase the cytokine release. Other studies have provided evidence that high glucose significantly increased the concentrations of TNF-α and IL-6 in the culture supernatants of HUVECs (38) and induced the adhesion of monocytes to endothelial cells (39). Interestingly, release of the anti-inflammatory cytokine IL-4 from endothelial cells was also increased in the presence of exosomes, suggesting a dual effect of exosomes in the regulation of the proinflammatory response. Moreover, imbalance between circulating pro- and anti-inflammatory cytokines in patients with GDM has been previously reported (40). Although the mechanisms by which exosomes modulate cytokine release from endothelial cells remain to be elucidated, these nanovesicles have a payload of receptors, proteins, and/or oligonucleotides that have been specifically preconditioned by the GDM placenta to be delivered to maternal response systems. The extent and impact of PdEs on maternal physiology, however, remain to be elucidated.

In summary, the data presented in this study have established that there is a differential release of exosomes from the placenta and other maternal tissues obtained from women with GDM and from normal pregnant women, and dysregulation of exosome effects and/or function on endothelial cells may be implicated in the proinflammatory state of GDM. Cytokine release from endothelial cells in response to exosomes is greatly affected in GDM, but future studies are warranted to elucidate fully the function of total and PdEs on the maternal metabolic adaptation in normal and GDM pregnancies.

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