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Influence of maternal BMI on the exosomal profile during gestation and their role on maternal systemic inflammation

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Abstract

Recent studies report that 35% of women are either overweight or obese at reproductive age. The placenta continuously releases exosomes across gestation and their concentration is higher in pregnancy complications. While there is considerable interest in elucidating the role of exosomes during gestation, important questions remain to be answered: *i*) Does maternal BMI affect the exosomal profile across gestation? and *ii*) What is the contribution of placenta-derived exosomes to the total number of exosomes present in maternal plasma across gestation?. Plasma samples were classified according to the maternal BMI into three groups (n=15 per group): Lean, overweight, and obese. Total exosomes and specific placenta-derived exosomes were determined by Nanoparticle Tracking Analysis (NanoSight™) using quantum dots coupled with CD63 or PLAP antibodies. The effect of exosomes on cytokine (IL-6, IL-8, IL-10 and TNF-α) release from endothelial cells was established by cytokine array analysis (Bioplex-200). The total number of exosomes present in maternal circulation was strongly correlated with maternal BMI. Between ~12% and ~25% of circulating exosomes in maternal blood are of placental origin during gestation, and the contribution of placental exosomes to the total exosomal population decreases with higher maternal BMI across gestation. Exosomes increase IL-6, IL-8 and TNF-α release from endothelial cells, an effect even higher when exosomes were isolated from obese women compared to lean and overweight. This study established that maternal BMI is a factor that explains a significant component of the variation in the exosomes data. Exosomes may contribute to the maternal systemic inflammation during pregnancy.

Background

Obesity is one of the largest and most serious health issues we face today [1]. The Centers for Disease Control and Prevention has reported that in the 2011-2012 period over 35% of adults 20 years and over were considered obese, and 69% were considered either obese or overweight. In the USA, about 1 in 3 women of reproductive age is obese and the numbers are steadily increasing [2]. This poses a serious problem as studies have shown that obesity is linked to complications for pregnant women and their babies, including metabolic syndrome [3]. For the women, obesity may result in induced preterm delivery, gestational diabetes, miscarriages, and preeclampsia, while for the babies obesity in the mother may result in complications such as fetal death and birth defects [4-7].

Maternal health and microenvironment have direct and significant impacts on the fetus during development as well as an impact on subsequent adult health. The maternal microenvironment is influenced by a number of factors, with the placenta being a unique contributor. Interestingly, women with gestational diabetes have a higher probability of having a large placenta, a phenomenon even higher in obese women [8]. Moreover, placental efficiency (ratio of fetal to placental weight) is lower in overweight and obese women compared to lean women [9]. These data suggest that maternal metabolic status affects placental function and may modify the release of placental factors into maternal circulation. The placenta releases a wide range of molecules, including hormones, cytokines, and extracellular vesicles (EVs).

Recently, much attention has focused on the role of placenta-derived EVs during gestation [10] and specifically, on exosomes [11]. Exosomes are membrane-bound nanovesicles of around 100 nm diameter that transport molecular signals (consisting

of proteins, bioactive lipids, and RNAs) between cells; they are released from a wide range of cells, including the human placenta. Exosomes are of endosomal origin and formed by the inward budding of multivesicular bodies (MVB) and are released to the extracellular environment by the fusion of MVB with the plasmatic membrane at the end of the endocytic-recycling pathway. As such, they are enriched with late endosomal membrane markers, including, Tsg101 and enriched in members of the tetraspanin family such as CD63, CD9, and CD81 [12].

Exosomal signaling represents an integral pathway mediating intercellular communication. During pregnancy, the placenta releases exosomes into the maternal circulation from as early as 6 weeks of gestation [13] and the concentration of placenta-derived exosomes during third trimester is positively correlated with placental weight at delivery under normal gestation [14]. Interestingly, the release of exosomes from trophoblast cells at early gestation (i.e. ~10 weeks) is regulated by the microenvironment milieu, including oxygen tension and glucose concentration [15-17]. Recent studies highlight the putative utility of exosomes for the diagnosis of disease and the onset of complication of pregnancies. For example, the gestational profile of exosomes concentration in plasma is different in gestational diabetes compared to normal pregnancy [18]. Taken together, these results support the hypothesis that placenta-derived exosomes are regulated by environmental factors, and may play a role in feto-maternal communication under both normal and pathological conditions.

Maternal obesity is associated with endothelial cell dysfunction [19]. Endothelial cell dysfunction can be related to obesity through factors such as hormones, fat-derived metabolic products, as well as cytokines. These adipocyte-derived products can have

an impact on vascular function as well as inducing insulin resistance. Free fatty acids have been associated with impaired vascular reactivity, an indicator of endothelial dysfunction [20]. TNF-α is another factor that may play a role in endothelial cell dysfunction; however, the mechanism is still unclear [21]. Many studies focusing on cytokines IL-1 and IL-6 have related them to endothelial dysfunction as well as subclinical inflammation [22]. For instance, IL-6 stimulates the production of C-reactive protein (CRP) in the liver, which leads to inflammation and impacts the vascular wall. Steinberg *et al.* showed that subjects with Type 2 DM has the same degree of impairment in vascular reactivity and blood flow as compared with obese subjects with normal glucose tolerance and insulin resistance [23]. Interestingly, we have previously described that exosomes present in maternal circulation regulates the function of endothelial cells including cell migration [14] and secretion of cytokines [18], however, the impact of maternal BMI on the effect of exosomes on endothelial cells has not been established.

There is now increasing evidence that maternal BMI alters the placental function [24] and that pregnancy is associated with maternal systemic inflammation, a state even higher in obese women [24]. There are no studies, however, that have defined the relationship between maternal BMI and placenta-derived exosome concentration during gestation. Thus, the aim of this study was to establish the relationship between maternal Body Mass Index (BMI) and exosomes present in maternal circulation during gestation. Moreover, we established the contribution of placental exosomes to the total exosomes concentration present in maternal circulation during gestation and the effect of exosomes on cytokines released from endothelial cells. The data of this study established that maternal BMI is a factor that explains a significant component

of the variation in the total exosomes and placenta-derived exosomes concentration present in maternal circulation during gestation.

Methods

Study group and samples

A time-series study design was used to establish the relationship between maternal BMI and exosome concentration during pregnancy. Women were recruited between January 2013 and December 2013 with informed written consent, at the Ochsner Baptist Medical Center (New Orleans, USA). Blood samples (BD Vacutainer® PLUS Tubes EDTA) were obtained from pregnant women at different times of gestation (10-38 weeks) and classified according to maternal BMI into lean (n=15, BMI 18.5-24.9 Kg/m^2), overweight (OW, n=15, BMI 25-29.9 Kg/m^2), and obese (n=15, BMI >30 Kg/m^2) at the moment of sample collection. Gestational age was calculated from the first day of the last menstrual period. All pregnant women included in this study were normotensive and without intrauterine infection or any other medical or obstetric complications. Plasma samples were obtained in accordance with the declaration of Helsinki and approved by the Ethics Committee of The University of Queensland and the Ochsner Medical Center (New Orleans, USA). Plasma was separated from whole blood by centrifugation (2000g x 10 min at Room temperature) and stored at -80^{o}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 Code of Federal Regulation (CFR) part 11 compliant electronic laboratory notebook (Lab

Archives, Carlsbad, CA 92008, USA). The schematic in Figure S1 summarizes the experimental design used in this study

Isolation of exosomes from maternal circulation

Exosomes were isolated from plasma (1 ml) as previously described [13, 14, 18] (Figure S2). The 100,000 g pellet was resuspended in 500 µl PBS and stored -80^{o}C until exosome purification using a discontinuous iodixanol gradient (Supplemental Material and Methods). We have previously confirmed the stability of exosomes after a freeze and thaw cycles using fresh and frozen samples [13].

Quantification of total exosomes and placenta-derived exosomes by Nanoparticle Tracking Analysis (NTA)

The concentration of total and placenta-derived exosomes in maternal plasma was quantified using CD63 and Placental Alkaline Phosphatase (PLAP) by immunofluorescent NTA. PLAP is a syncytiotrophoblast-specific marker, therefore, exosomes derived from placental origin are positive for PLAP [14]. Qdots (Qdot® nanocrystals or R-PE) were conjugated to anti-CD63, anti-PLAP 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 [25]. Exosomes were diluted in PBS and incubated with FcR blocking reagent (10 µl, 10 min at 4^{0}C) (MACS Miltenyi Biotec), followed by incubation with anti-CD63-Qdot605 or anti-PLAP-Qdot605 or IgG1-Qdot605 (10 µl, 1:100) for 30 min in the dark at room temperature. Samples were then diluted to 500 µl 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 sec 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 (positive control; Supplemental Material and Methods) and exosomes isolated from plasma obtained from non-pregnant women (negative control).

Endothelial cells isolation

Primary human umbilical vein endothelial cells (HUVEC) were isolated by enzymatic digestion as previously described [17] and used as an *in vitro* model to determine the effect of BMI on the internalization of exosomes and cytokine release (Supplemental Material and Methods). HUVEC were isolated from placenta obtained from term pregnancies (>37 weeks). To discard the effect of maternal BMI on the response of HUVEC to exosomes, only HUVEC from placenta obtained from lean women (BMI 18.5-24.9 Kg/m^2) were used in this study.

Effect of BMI on exosome-induced cytokine release from endothelial cells

To determine the effect of BMI on exosome-induced cytokine release, exosomes (100 µg protein/ml which is equivalent to 5 x 10^8 vesicles per ml) were incubated with primary human umbilical vein endothelial cells (HUVECs) in medium containing 5mM D-glucose. The experiments were performed at an atmospheric pressure of 8% O_2 to mimic the physiological conditions (oxygen tension in human blood is normally between 10% to 13%[26]). The association between total number of exosomes and protein concentration was determined by correlation analysis (Pearson r = 0.99; R2 =

0.98; ***p value = 0.0010). Cytokine release (defined as the accumulation of immuno-reactive cytokine in cell conditioned medium) was quantified using protein solution arrays. Data are expressed as cytokine pg /10^3 cells/24h and normalized to the level of cytokines in cell-conditioned media without exosomes (control).

Internalization of Exosomes

The internalization of exosomes by endothelial cells was assessed as previously described [27] using fluorescently labeled (PKH67 green, Sigma-Aldrich) exosomes. A live-cell imaging system (The Incucyte FLR fluorescent) was used for continuous tracking of exosome internalisation in endothelial cells (Supplemental Material and Methods).

Statistical analysis

The relationship between maternal BMI and exosome concentration present in plasma was assessed using 2-way ANOVA, with the variance partitioned between gestational age and maternal BMI, thus, maternal BMI was treated as an independent factor. In this study, we did not find a statistically significant association between gestational age and maternal BMI ($p>0.05$; Supplemental material Figure S3), therefore, multiple regression analyses of 3 continuous variables (i.e. dependent variable: Exosomes; independent variables: gestational age and maternal BMI) was also used. Statistical significance was defined as at least $p < 0.05$. Statistical analyses were performed using commercially available packages (Stata 11, StatCorp, College Station, Texas USA and Prism 6, GraphPad Inc, La Jolla, CA 92037 USA).

Results

Exosome isolation and characterization.

The characteristics of exosomes isolated and purified using a well-established and validated method are presented in Supplemental Figure S2. Nanoparticle tracking analysis identified particles with sizes of ~100 nm (Figure 1A) without significant differences between Lean, OW, and obese women. Morphological analysis identified circulate shape characteristics of exosomes (Figure 1B) and enrichment of the CD63 protein abundance (Figure 1C). The total number of particles was quantified under light scatter mode and the vesicles positive for CD63 (enriched marker associated with exosomes) and for PLAP (placental origin) were quantified under fluorescence mode using nanocrystals (Qdot) coupled with CD63 or PLAP, respectively. No effect on the size distribution of exosomes present in maternal circulation was identified in light scattering mode or fluorescence mode (*i.e.* $CD63^+$ or $PLAP^+$), showing that the exosome-Qdot binding did not affect the vesicles characteristics. A similar number of particles were identified in light scatter in the absence and in the presence of Qdot-IgG (~90% *i.e.* nonspecific binding of ~10%). The percent of vesicles positive for CD63 in the total vesicle population (defined as total vesicles in light scatter mode) was 88 ± 8.9 % (Figure 1 D), indicating that the majority of the isolated vesicles are positive for CD63. The specificity of Qdot-PLAP in binding only vesicles PLAP positive was evaluated using exosomes isolated from syncytiotrophoblast (ST) cells and exosomes isolated from non-pregnant women (Figure 1E and F). No significant differences were obtained between the quantification exosomes from ST in light scatter and fluorescence mode (Qdot-PLAP), indicating that the binding was over 90%. On the other hand, particles positive for PLAP were not found in exosomes isolated from plasma obtained from non-pregnant women (<5% = unspecific binding).

Relationship between maternal BMI and exosomes.

This study sample consisted of 45 pregnant women. These women were categorized according to their BMI into lean (reference group), OW, and obese. Clinical characteristics of the patients are present in Table 1.

Pooled exosome-containing fractions (*i.e.* fractions 5 to 8; Supplemental material Figure S2) were further characterized by determining the total number of exosomes and PLAP exosomes present in maternal circulation across gestation (Figure 2). The relationship between maternal BMI and gestational age variation in plasma exosome number and placental exosomes were analyzed by two-way ANOVA with the variance partitioned between gestational age and BMI. Significant effects of gestational age and maternal BMI were identified ($p<0.005$) (Figure 2). Linear regression analysis showed that both total exosomes and placental exosomes increased progressively across gestation from 10 to 38 weeks, and this was independent of the maternal BMI. (Figure 2A and B). The slopes of the regression lines (± SD) for exosome concentration per gestational age data for lean and OW women were $4.7 \times 10^7 \pm 3.3 \times 10^6$ and $4.2 \times 10^7 \pm 9.2 \times 10^6$, respectively; and significantly different ($p <0.001$) from the slope for obese women ($6.3 \times 10^7 \pm 1.4 \times 10^7$). The slopes of the regression lines (± SD) for placental exosomes concentration x gestational age data for lean, OW women were $9.2 \times 10^6 \pm 5.7 \times 10^5$ and $7.2 \times 10^6 \pm 1.5 \times 10^6$, respectively; and significantly different ($p <0.001$) than the slope for obese women ($1.3 \times 10^7 \pm 2.9 \times 10^6$). The relationship between maternal BMI and exosomes present in maternal circulation is presented in Figure 3. Interestingly, the total number of exosomes present in maternal circulation was strongly correlated with maternal BMI (Figure 3A). Placenta-derived exosomes were positively correlated with maternal BMI, but without a statistical significant difference ($p=0.135$) (Figure 3B).

Contribution of placental-derived exosomes to total exosomes present in maternal plasma across gestation.

Linear regression analysis showed that the percentage of placental exosomes present in maternal circulation negatively correlated with maternal BMI (Figure 4A). We then determined the variation in the contribution of placenta-derived exosomes in maternal circulation during gestation (Figure 4B). The contribution of placenta-derived exosomes increases with gestational age ($p<0.05$), however, we did not find a correlation between placenta-derived exosomes in maternal circulation with gestational age in OW and obese women. No significant relationship between exosomes (total or placenta-derived) and fetal sex has been found.

Effect of exosomes on cytokine release from endothelial cells

Exosomes increase IL-6, IL-8, and TNF-α release from endothelial cells in all the conditions studied and were significantly higher when exosomes were isolated from obese women (Figure 5). Interestingly, a positive correlation ($p<0.05$) was found between cytokine release and gestational age (at the moment of sample collection) for IL-6 (lean and obese) and TNF-α (lean and obese). Interestingly, a negative correlation was found between IL-6 and gestational age using exosomes from obese pregnant women (Figure 5A, C, E). No effect of exosomes (lean, OW or obese) on IL-10 release was identified (Figure 5G and H). The effect of exosomes (average combined gestational age) on IL-6 release was 2.0 ± 0.4, 1.6 ± 0.7, and 3.3 ± 1.3 fold higher compared to the control (without exosomes) for lean, OW, and obese, respectively (Figure 5B), on TNF-α release was 2.3 ± 0.5, 3.8 ± 0.9, and 4.4 ± 1.0 fold higher compared to the control (without exosomes) for lean, OW, and obese, respectively (Figure 5D) and on IL-8 release, was 1.3 ± 0.3, 1.8 ± 0.3, and 2.8 ± 0.3

fold higher when compared to the control (without exosomes) for lean, OW, and obese, respectively (Figure 5F).

Finally, the internalization of exosomes labeled with PKH67 (green) in endothelial cells was visualized and quantified using fluorescence microscopy and real time cell imaging (The IncuCyte,), respectively (Figure S4). Exosome uptake by endothelial cells was observed in a time-dependent manner with the maximum at 24 h. The rate of exosome uptake was compared using the half-maximal stimulatory time (ST_{50}). We did not find differences in the ST_{50} for the exosomes internalization between exosomes isolated from lean, OW, and obese women.

Discussion

The field of exosomes-mediated cell-to-cell communication is a burgeoning field and may provide unique insights into the aetiology of disease, early detection, and treatment monitoring. Excessive weight and obesity are recognized as important public health issues worldwide; recent findings show that 35% of women aged 25-35 years of age (reproductive age) are overweight or obese [28]. In fact, excess weight and obesity are the most powerful drivers for the onset and development of complication in pregnancies with both short and long term consequences for both the mother and child [29]. In the presented study, we investigated the effect of maternal BMI on the exosomal profile during gestation. The concentration of total exosomes and placental exosomes present in maternal circulation were different in obese women

compared to lean or OW women. Interestingly, obese women present a higher concentration of total exosomes and placental exosomes in maternal circulation across gestation. Moreover, in this study we have partially answered the question about the contribution of placental exosomes to the total exosomal population in maternal circulation during pregnancy. This study established that the contribution of placental exosomes (expressed as percentage of exosomes positive for PLAP compared to total exosomes) did not change significantly across gestation. Placental-derived exosomes present in maternal circulation were ~12% at early gestation (i.e. ~10-12 weeks) and increased during pregnancy until ~20% at third trimester (i.e. >32 weeks). Finally, exosomes present in maternal circulation during gestation may contribute to the maternal systemic inflammation during pregnancy, an event of significantly higher incidence in obese women.

Exosomes are well thought of as a “fingerprint” of their cell origin and their metabolic status. In other words, isolation and characterisation of placental exosomes present in maternal circulation can be considered as a non-invasive biopsy of placental cells. Several studies have demonstrated that maternal BMI affects pregnancy outcomes with long-term consequences for the offspring [30-32]. To our knowledge, this is the first study to identify maternal BMI-associated changes in the exosomes concentration across gestation.

In this study, we used a well-established and validated method to obtain an enriched exosome fraction using buoyant density centrifugation to minimize the contribution from other extracellular vesicles. Total exosomes and placental exosomes increase progressively with the pregnancy progression (Figure 2), an effect higher in obese

pregnancies. Interestingly, we have previously described an association between placental weight and exosomal PLAP (an indirect measurement of placental exosomes) at third trimester of pregnancy [14]. In this study, we did not record the placental weight, therefore, the relationship between maternal BMI, placental weight or placental efficiency and exosomes concentration requires future studies. However, no interaction between maternal BMI and placental weight has been previously described [9].

Supporting our results, we have previously showed that the concentration of placental exosomes in maternal circulation increases across gestation [14]. We quantified the total number of exosomes and placenta-derived exosomes using the method described by Dragovic *et al*., using fluorescence nanoparticle tracking analysis [25], that gave us the opportunity to measure individual vesicles and determine the contribution of exosomes from placental origin to the total exosome population.

Higher levels of total and placental exosomes were reported in this study in obese women compared to lean and overweight women, which may be due to obesity being associated with pro-inflammatory state causing a higher secretion of exosomes. Interestingly, the capacity of exosomes present in maternal circulation across gestation to regulate the pro-inflammatory cytokine secretion from endothelial cells was significantly higher using exosomes from obese and OW women when compared to lean women. These outcomes are consistent with previous studies in which levels of IL-6 and TNF-α increase across gestation [33]. IL-6 is a pro-inflammatory cytokine secreted pre-dominantly by adipocytes, macrophages, skeletal muscle, endothelial cells and fibroblasts. IL-6 has been associated with obesity and affecting glucose metabolism [34, 35]. During gestation, IL-6 regulates embryo implantation and placental development; enhances the secretion of human chorionic gonadotropin

(HCG) from trophoblast cells HCG and mediating inflammation and induces insulin resistance [36-41]. High levels of TNF-α are associated with maternal systemic inflammation and obesity [42]. During gestation, higher levels of TNF-α in maternal plasma lead to complications of pregnancies including gestational diabetes [43]. Interestingly, our data established that the effect of exosomes on the secretion of TNF-α was higher at late gestation (Figure 5C). TNF-α has been shown to impact parturition [44], however, the effect of exosomes on signal of parturition has not been established yet. While the mechanisms controlling the concentration of cytokines in maternal circulation during gestation remain unclear, we suggest that exosomes may have a role regulating the concentration of cytokines (e.g. IL-6 and TNF-α) during gestation.

Maternal BMI is a risk factor for the development of gestational diabetes mellitus (GDM) [45], a phenomenon associated with maternal systemic inflammation [24]. Interestingly, we have recently reported that the concentration of placental exosomes in GDM pregnancies is higher when compared to normal pregnancies [18]. Moreover, exosomes isolated from GDM pregnancies increase the pro-inflammatory cytokines release (*e.g* TNF-α) from endothelial cells compared to exosomes isolated from normal pregnancy [18]. Recently, Aye *et al*., reported elevated levels of pro-inflammatory cytokines in maternal circulation and activation of placental p38-MAPK and STAT3 pathways with increasing maternal BMI [24]. These findings suggest that increased maternal BMI is associated with maternal pro-inflammatory state affecting both maternal tissues and placenta, which may be due to elevated exosomes (total and placental) in maternal circulation.

In this study, we demonstrated that maternal BMI is a factor that explains for >20% of the observed variation in plasma exosomes concentration (Figure 3). These observations give rise to the question: how does maternal BMI increase the exosome concentration in maternal circulation? Exosomes are a subtype of EVs with specific biogenesis and secretion mechanisms that are not fully understood. Exosomes are a product of endosomal trafficking in which intraluminal vesicles (ILVs) are incorporated into multivesicular bodies (MVB) and then released via exocytosis as exosomes to the microenvironment milieu by the transport and fusion of MVB with the plasmatic membrane. Recent studies have shown that the endosomal-sorting complex required for transport (ESCRT) and TSG101 protein are required for the exosome secretion from HeLa cells [46]. The expression of these proteins in placental cells obtained from women with different metabolic status (e.g. obese and overweight) has not been established yet. Interestingly, the RAB family of small GTPase proteins have been implicated in the intracellular vesicular tracking [47]. RAB proteins are expressed in the human placenta [48], however, the expression of these proteins in placentas obtained from obese or overweight women has not been studied. Interestingly, RAB protein has been implicated in lipid storage and insulin-regulated GLUT-4 trafficking in adipose tissue. Hypoxia and high glucose are other factors that increase the exosome release from placental cells [15-17]. Recently, Wang *et al*., demonstrated that hypoxia increases the expression of RAB22A involved the activation of hypoxia-inducible factors (HIFs), which results in an increase in the secretion of microvesicles from breast cancer cells [49]. Thus, RAB proteins are involved in the secretion of exosomes from placental cells to maternal circulation in response to increasing maternal BMI requires further investigation.

In this study, the levels of circulating exosomes in maternal plasma were significantly higher in obese women compared to normal or overweight women. We suggest that maternal BMI modulates the exosome secretion from placental cells and maternal sources, however, we cannot ignore that maternal BMI modifies the clearance or half-life of circulating exosomes. Recently, it has been proposed that macrophages play a crucial role in the clearance of exosomes from the body [50]. Imai *et al.*, demonstrated that the clearance of exosomes is significantly lower in macrophage-depleted mice compared to mice control. The placenta-derived exosomes clearance has not been established, however, the levels of placental miRNA, which are secreted from placental cells into exosomes, decrease dramatically after delivery [51].

Conclusions

In this study we have established that *i*) maternal BMI modulated the exosome concentration in maternal circulation across gestation; *ii*) 12% to 25% of the circulating exosomes in maternal plasma during gestation are from placental origin; and *iii*) exosomes isolated from maternal circulation increase the pro-inflammatory cytokines releases from endothelial cells, and strongly positively correlated with maternal BMI. Take all together, we suggest that exosomes may have a role in the chronic metabolic inflammatory state associated with obesity.

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635 **Table 1.** Clinical characteristics of patients and newborns
636

Maternal Variables				
	Lean (n=15)	OW (n=15)	Obese (n=15)	ANOVA
Maternal age (years)	29 ± 3.9 (23-36)	30 ± 5.3 (20-37)	29 ± 6.4 (20-41)	0.1251
Height (cm)	163 ± 0.6 (155-172)	161 ± 0.7† (154-175)	165 ± 0.7* (152-175)	<0.001
Weight (Kg)	60 ± 6.3 (50-73)	71 ± 9.3† (64-90)	92 ± 10* (72-110)	<0.001
BMI (Kg/m^2)	22 ± 1.6 (19-24)	28 ± 1.3† (25-29)	34 ± 3.8* (30-42)	<0.001
Gestational age (weeks)	22 ± 3.5 (11-36)	23 ± 2.6 (12-38)	24 ± 2.8 (10-38)	0.2524
Gestational age at delivery (weeks)	39 ± 1.2 (37-41)	38 ± 0.9 (38-40)	39 ± 1.1 (38-40)	0.0656
Type of delivery (% caesarean/ %vaginal)	4/11	5/10	7/8	(-)
Newborn variables				
Fetal weight (g)	3307 ± 424 (2892-4167)	3261 ± 408 (2756-3714)	3517 ± 524 (2972-4217)	0.3463
Fetal sex (male/female)	9/6	10/5	10/5	(-)

637
638
639 Data are presented as mean ± SD (range). Groups were classified according to the maternal
640 BMI in lean, overweight (OW), and obese. Maternal age, height, weight, BMI and gestational
641 age are presented at the time of sample collection. This study was designed to include only
642 normal pregnancies. Maternal BMI was calculated as weight (kg) divided by height (m^2) at
643 the time of sample collection. *$p < 0.05$ versus OW or lean; †$p < 0.05$ versus lean. (-) Not
644 applicable.

645

646 **Figures legends**
647
648
649 **Figure 1. Characterisation of exosomes present in maternal circulation.** Plasma
650 samples were obtained from women with different metabolic states and classified into
651 lean, overweight (OW), and obese. Exosomes were isolated from plasma as was
652 indicated in Methods and characterised using nanoparticle tracking analysis, electron
653 microscopy, and western blot. (A) Representative size distribution of exosomes in
654 light scatter mode. (B) Representative electron micrograph of exosomes. (C)
655 Representative Western blot for exosome enriched marker CD63. (D) NTA
656 Comparison between light scatter mode and fluorescence mode (Qdot-CD63) in
657 exosomes isolated from maternal plasma. (E) NTA Comparison between light scatter
658 mode and fluorescence mode (Qdot-PLAP) in exosomes isolated from
659 Syncytiotrophoblast cells and (F) from non-pregnant women (see supplemental
660 material). In B, Scale bar 100 nm and arrows indicate the exosomes.
661
662
663 **Figure 2. Relationship between maternal BMI and exosome concentration across**
664 **gestation.** Enriched exosome populations were quantified using nanoparticle tracking
665 analysis in fluorescence mode in peripheral plasma of lean, overweight (OW), and
666 obese women across gestation. (A) Linear regression analysis between total exosomes
667 number presented as total vesicles $CD63^+$ per l ml of plasma across gestation. (B)
668 Linear regression analysis between placenta-derived exosomes number presented as
669 total vesicles $PLAP^+$ per l de plasma across gestation.
670
671 **Figure 3. Relationship between number of exosomes and maternal BMI.** We used
672 multivariate linear regression analysis to evaluate the relationship between exosomes
673 and maternal BMI. (A) Relationship between total exosomes (total vesicles $CD63^+$)
674 and maternal BMI. (B) Relationship between placenta-derived exosomes (vesicles
675 $PLAP^+$) and maternal BMI. In A and B, Lineal correlation (-) and 95% confidence
676 interval (--).
677
678 **Figure 4. Contribution of placental-derived exosomes into maternal circulation**
679 **across gestation.** The ratio of placental exosomes and total exosomes present in
680 maternal circulation across gestation was quantified using nanoparticle tracking
681 analysis in fluorescence mode and presented as percentage (%) of exosomes PLAP+
682 to total exosomes CD63+. (A) linear regression analysis between percentage of
683 placenta-derived exosomes and maternal BMI. (B) Contribution of placenta-derived
684 exosomes across gestation.
685
686 **Figure 5. Induction of cytokine release from endothelial cells by exosomes.** Effect
687 of exosomes (100 μg/ml) isolated from plasma obtained from lean, overweight (OW),
688 and obese women across gestation on the release of IL-6 (A and B), TNF-α (C and
689 D), IL-8 (E and F), and IL-10 (G and H) from endothelial cells. In A, C, E, and G the
690 data is presented as XY graph where each point is defined as the fold change on
691 cytokine release in the presence of exosomes compared to control (without exosomes)
692 and the gestational age at the moment to collect the sample in which the exosomes
693 were isolated. Red line represents no difference between exosomes and the control.
694 Lines represents liner correlation for lean, OW (-), and obese (--) exosomes. In B, D,

695 F, and H data is present as the fold change in average on the effect of exosomes on
696 cytokines release across gestation.
697
698

699

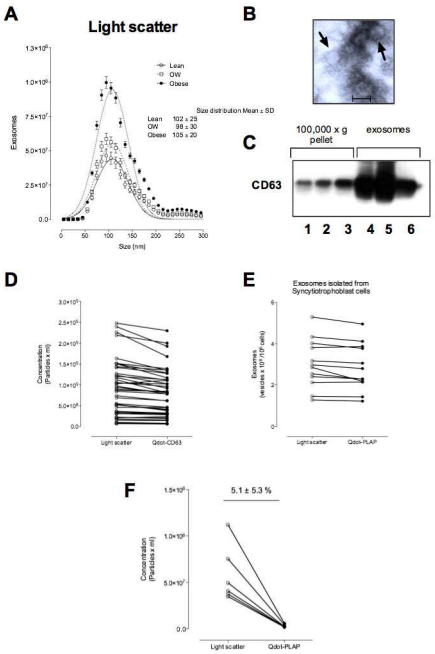

Figure 1

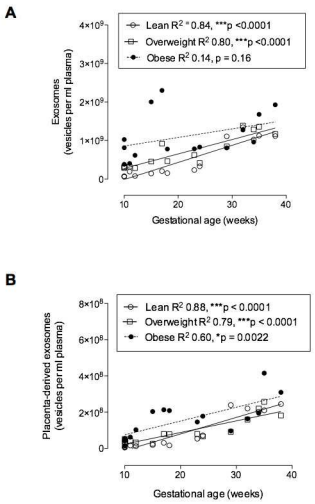

Figure 2

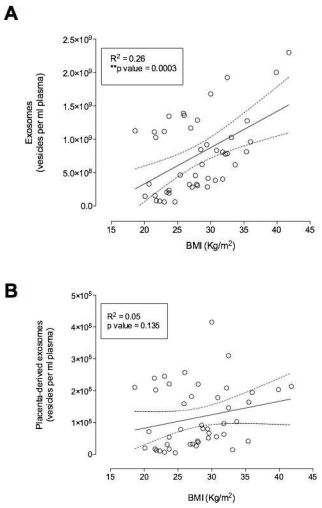

Figure 3

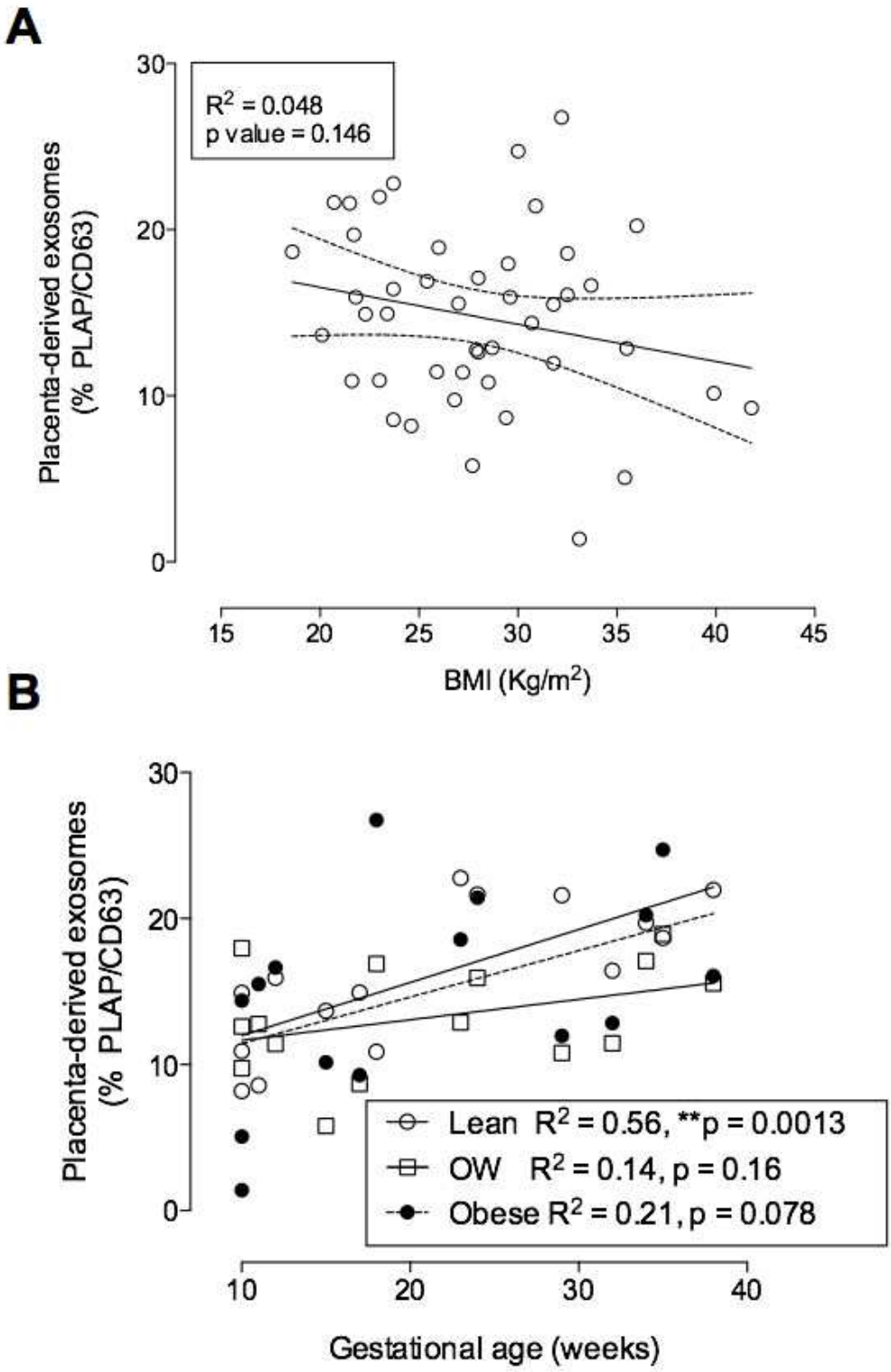
A
R^2 = 0.048
p value = 0.146
Placenta-derived exosomes
(% PLAP/CD63)
30
20
10
0
15
20
25
30
35
40
45
BMI (Kg/m^2)
B
Placenta-derived exosomes
(% PLAP/CD63)
30
20
10
0
Lean R^2 = 0.56, **p = 0.0013
OW R^2 = 0.14, p = 0.16
Obese R^2 = 0.21, p = 0.078
10
20
30
40
Gestational age (weeks)

Figure 4

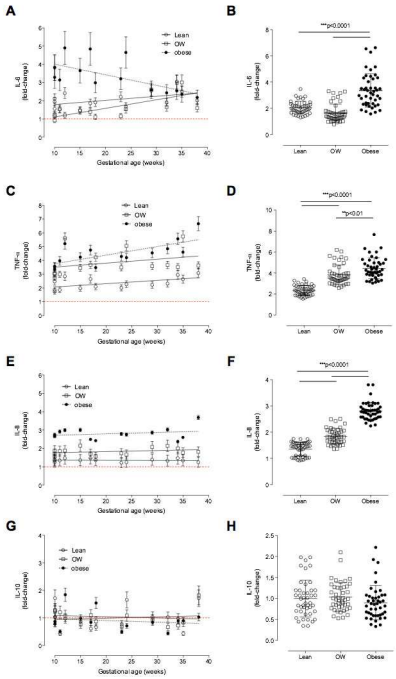

Figure 5

Highlights

- An association between maternal BMI and exosomes has been established.
- Total and placental exosomes increase with higher maternal BMI.
- ~20% of circulating exosomes in the mother are from placental origin.
- Exosomes from obese pregnant women induce cytokine release.

Conflict of Interest Statement

The authors declare that they have no conflict of interests.