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

Omar Elfeky [a], Sherri Longo [b], Andrew Lai [a], Gregory E. Rice [a, b], Carlos Salomon [a, b, *]

[a] Exosome Biology Laboratory, Centre for Clinical Diagnostics, UQ Centre for Clinical Research, Royal Brisbane and Women's Hospital, Faculty of Medicine and Biomedical Sciences, The University of Queensland, Australia
[b] Department of Obstetrics and Gynecology, Ochsner Baptist Hospital, New Orleans, LA, USA

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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.



## 1. 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).

* Corresponding author. Exosome Biology Laboratroy, Centre for Clincal Diagnostics, UQ Centre for Clinical Research Faculty of Health Sciences, University of Queensland, Building 71/918, Royal Brisbane Hospital, Herston, QLD, 4029, Brisbane, Australia.
*E-mail address:* c.salomongallo@uq.edu.au (C. Salomon).

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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-$\alpha$ 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.

## 2. Methods

### 2.1. 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 (2000 g × 10 min at Room temperature) and 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 Code of Federal Regulation (CFR) part 11 compliant electronic laboratory notebook (Lab Archives, Carlsbad, CA 92008, USA). The schematic in Fig. S1 summarizes the experimental design used in this study.

### 2.2. Isolation of exosomes from maternal circulation

Exosomes were isolated from plasma (1 ml) as previously described [13,14,18] (Fig. S2). The 100,000 g pellet was resuspended in 500 μl PBS and stored −80 °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].

### 2.3. 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 °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 s each were captured for each sample and analyzed. The