1. TITLE

Mitochondrial Content and Hepcidin are increased in Obese Pregnant Mothers

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3. KEYWORDS

Mitochondrial DNA, hepcidin, maternal blood, obesity, gestational diabetes mellitus, sexual dimorphism
4. ABSTRACT

Objective: Maternal obesity is characterized by systemic low-grade inflammation and oxidative stress (OxS) with the contribution of fetal sex dimorphism. We recently described increased mitochondrial content (mtDNA) in placentas of obese pregnancies. Here we quantify mtDNA and hepcidin as indexes of OxS and systemic inflammation in the obese maternal circulation.

Methods: Forty-one pregnant women were enrolled at elective cesarean section: sixteen were normal-weight [NW] and twenty-five were obese (OB). Obese women were further classified according to the presence/absence of Maternal Gestational Diabetes (GDM); [OB/GDM(-)]: n=15, [OB/GDM(+)]: n=10. mtDNA and hepcidin were evaluated in blood (Real-Time-PCR) and plasma (ELISA).

Results: mtDNA and hepcidin levels were significantly increased in OB/GDM(-) vs NW, significantly correlating with pre-gestational BMI. Male/female (M/F) ratio was equal in study groups, and overall F-carrying pregnancies showed significantly higher mtDNA and hepcidin levels than M-pregnancies both in obese and normal weight mothers.

Conclusions: Our results indicate a potential compensatory mechanism to increased obesity-related OxS and inflammation, indicated by the higher hepcidin levels found in obese mothers. Increased placental mitochondrial biogenesis, due to lipotoxic environment, may account for the greater mtDNA amount released in maternal circulation. This increase is namely related to F-carrying pregnancies suggesting a gender specific placental response.

5. WORD COUNT

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INTRODUCTION

Maternal obesity (MO) is expanding exponentially worldwide to almost epidemic proportions. It represents a significant risk factor for several pregnancy pathologies, leading to adverse pregnancy and fetal/neonatal outcomes [1], and may also affect offspring health in later life [2]. In obese pregnancies, systemic dysfunctions together with an altered intrauterine environment may contribute to these increased risks, with the possible involvement of placental/fetal sex dimorphism [3-4].

One of the most frequent complications occurring in pregnancies of obese women is Gestational Diabetes Mellitus (GDM). GDM affects approximately 7% of pregnancies, with 1.3-3.8 fold increased frequency in obese women [5]. GDM is a pregnancy complication characterized by maternal insulin resistance and systemic inflammation. Moreover, the placenta presents several alterations, leading to defective oxygen and increased nutrients availability for the fetus [6-7].

MO is characterized by energy imbalance of calories intake/consumption and by excessive macro-opposite to few micro-nutrients intake [8]. This results in high levels of intracellular fatty acids leading to increased intracellular inflammation and oxidative stress (OxS) [9].

Obesity-related inflammation and OxS possibly alter mitochondria structure and function. Mitochondria (mt) are a source of Reactive Oxygen Species (ROS), at the same time they are susceptible to ROS-mediated actions leading to DNA/protein damage and cellular senescence. Alterations of mt content and function have been reported in maternal blood and placentas of pregnancies characterized by placental insufficiency and impaired nutrient transport [10-11].

Our group recently reported increased mitochondrial DNA (mtDNA) levels in placentas of obese compared to normal-weight women [12].

Interestingly, alterations in mt function have been demonstrated to cause an excess of cellular iron deposition leading to hepcidin over-expression and further OxS-enhancing mechanism [13]. Hepcidin (Hpn) is an acute phase protein and inflammation positive regulator, also involved in iron homeostasis.
Its bioavailability is modulated by several systemic stimuli such as inflammation, anemia and iron concentration [14-15].

Here we quantified mtDNA in the obese maternal circulation, as a possible expression of OxS increase due to the high lipids overload. Moreover, in order to characterize the systemic low-grade inflammation, typical of both pregnancy and obesity, we measured hepcidin levels in maternal plasma. We also evaluated whether fetal sex was associated with different maternal levels of both biomarkers.

**MATERIAL and METHODS**

1. **Population**

This is an analytic study on a cohort of pregnant women undergoing regular antenatal visits in the Mother and Child Department of “Luigi Sacco” Hospital, Milano. The study protocol was approved by the hospital Ethical Committee and all enrolled women signed an informed consent.

Only patients with singleton spontaneous pregnancies delivering by elective Cesarean Section (CS) at term were included. Forty-one women were enrolled according to their pre-gestational Body Mass Index (BMI): 16 Normal-Weight [NW] (control group) [18≤ BMI< 25 kg/m²], 15 Normoglycemic Obese [OB/GDM(−)] and 10 Obese women with Gestational Diabetes Mellitus [OB/GDM(+)] [BMI ≥30 kg/m²].

All pregnant women had Caucasian origin. Exclusion criteria were: maternal-fetal infections, maternal drug-alcohol abuse, fetal abnormal karyotype-malformations, preeclampsia and intrauterine growth restriction.

All patients were given specific nutritional advice and recommendations on weight gain during pregnancy depending on pre-gestational BMI according to our clinical protocol.

GDM was diagnosed at 24-28 weeks of gestation by 75g oral glucose tolerance test (OGTT) [16]. GDM patients underwent several checks of glycemia and were given lifestyle guidelines and specific dietary indications, according to our clinical protocol, in order to maintain normal glycemia. No patient needed insulin therapy.
In the normal-weight group, fetuses had normal intrauterine growth and appropriate-for-gestational-age birth weight according to reference ranges for the Italian population [17]. GDM was an exclusion criteria for this group.

In all studied woman, indications for elective CS before labor were breech presentation, previous CS or maternal indications not related to fetal growth.

Data collection

We recorded maternal data (age, pre-gestational BMI, gestational weight gain), gestational age and fetal weight. Placental weight was measured after maternal the membranes trimming and umbilical cord removing, as previously described [3].

Maternal hemoglobin and hematocrit were measured at 34-36 weeks.

Maternal fasting glycemia was obtained from the first value of the OGTT performed between 24-28 weeks.

2. Sample Collection and Processing

Maternal blood sampling for mtDNA and hepcidin

Maternal venous blood was collected from a radial vein into EDTA tubes prior to CS. A whole-blood aliquot was set aside, the remaining blood centrifuged at 1500 rpm 15 minutes at room temperature to obtain plasma. Samples were stored at -80°C.

mtDNA Content

Total DNA was isolated from maternal venous blood samples using QIAamp DNA Blood Mini-Kit (Qiagen; Valencia, CA, USA) and its concentration measured by NanoDrop-ND-1000 spectrophotometer (Wilmington, DE, USA).

mtDNA content was assessed by Real-time PCR, normalizing levels of a mitochondrial gene (Cytochrome-B) to those of a single-copy nuclear gene (RNase-P) \(2^{\Delta Cq}; \Delta Cq = \text{Cytochrome-B} - \text{Rnase-}\)
30 ng of total DNA were analyzed in triplicate with TaqMan assays (MT-CYB: Hs02596867_s1 and RNase P: 4316844) by 7500 Fast Real-Time PCR (ThermoFisher-Scientific; Carlsbad, CA, USA); Cq values with standard deviation exceeding 0.25 were excluded and experiments repeated.

**Hepcidin**

Hepcidin (Hpn-25) concentration was measured in maternal venous plasma by solid-phase ELISA based on the principle of competitive binding (EIA-4705_DRG-Diagnostics; Marburg, Germany), according to manufacturer’s instructions. Plasma samples were diluted 1:2, analyzed in duplicate; the measurement interval was [2.90-12.95 ng/mL]. Hpn-25 limit of detection was 0.5 ng/mL, intra-assay coefficient of variation < 10%.

**3. Statistical Analysis**

Clinical data displayed a normal distribution (Kolmogorov-Smirnov Test) and were thus compared between groups by Student’s T-test, with applied correction when equality of variances assumption was violated (Levene’s test).

Molecular data were un-normally distributed (Kolmogorov-Smirnov Test) and thus analyzed by Mann-Withney U test.

One-way between-groups multivariate analysis of variance (MANOVA) was performed once preliminary assumptions testing noted no serious statistical violations. Maternal pre-gestational BMI and hepcidin were the dependent variables, while anemia status the categorical.

Chi-Square analyses were performed to compare anemia frequencies among groups, using Yates Continuity Correction.

Correlation between values was analyzed using bivariate Pearson Correlation.

Statistical significance was defined when p<0.05.
Statistical tests were performed using statistical package SPSS (IBM-Statistics, version 24.00, Armonk, NY, USA).

RESULTS

1. Characteristics of the Study Population

Maternal, fetal and placental data of the three analyzed groups are reported in Table 1. According to the study design, pre-gestational BMI was significantly higher in the two obese groups than in normal-weight, with no significant differences between obese subjects. Obese women gained on average less weight during pregnancy compared to normal-weight women. Maternal fasting glycemia was significantly higher in OB/GDM(+) compared to both NW and OB/GDM(-), in agreement with the study inclusion criteria.

OB/GDM(-) presented significantly lower hemoglobin levels (Hb, 10.70 ± 1.34 g/dL) compared to normal-weight (11.6 ± 1.17 g/dL; p=0.05). Anemia (Hb< 11.0 g/dL) frequency was higher in OB/GDM(+) (40%), and resulted two-fold higher (53%) in OB/GDM(-) than in NW (25%) subjects.

Gestational age, fetal weight and placental parameters did not differ between obese and normal-weight women, while in OB/GDM(+) placental weight was significantly higher and placental efficiency significantly lower compared to both NW and to OB/GDM(-).

The percentage of male and female neonates did not differ in obese and normal-weight groups.

2. mtDNA Content in Maternal Blood

mtDNA content was measured in maternal venous blood. In the statistical analyses three outlier samples from the control and one from the OB/GDM(+) group were removed.

Figure 1 presents mtDNA in maternal venous blood of the three study groups. Maternal mtDNA was significantly higher in OB/GDM(-) (68.42 ± 22.37) compared to NW (52.21 ± 14.43, p= 0.05) [Figure
1A]. In normoglycemic women (both NW and OB) mtDNA content significantly correlated with pre-gestational BMI (r=0.48, p=0.01) [Figure 1B].

mtDNA content in OB/GDM(+) (53.38 ± 5.22) was not significantly different compared to NW.

3. **Hepcidin Levels in Maternal Plasma**

Hpn levels in maternal venous plasma were significantly higher in OB/GDM(-) subjects (8.02 ± 3.28 ng/mL) compared to NW (4.67 ± 1.64 ng/mL, p = 0.05) [Figure 2A]. Maternal Hpn levels did not differ between OB/GDM(+) (4.93 ± 2.01 ng/mL) and NW women.

In normoglycemic women (both NW and OB) hepcidin significantly correlated with pre-gestational BMI (r=0.51, p=0.02) [Figure 2B]. This correlation was independent from level of Hb tested by MANOVA [F (2,18) = 2.78, p= 0.089; Wilks' Lambda = 0.76; partial eta squared = 0.24].

**mtDNA Content and Hepcidin according to Fetal Gender**

Independently from BMI categories, mothers bearing female (F) fetuses showed significantly increased levels of both mtDNA (67.12 ± 18.95) and maternal hepcidin (6.88 ± 2.96 ng/mL) compared to male (M) (49.04 ± 10.96, p=0.001; 3.95 ± 0.89 ng/mL, p=0.002 respectively) [data not shown].

In the normal-weight population of physiological pregnancies, mothers bearing female (F) fetuses presented significantly higher mtDNA levels compared to males’ (M) (F: 61.61 ± 15.15; M: 45.15 ± 9.48, p= 0.03) [Figure 3]. Similarly, when analyzed independently from GDM association, obese female-mothers showed significantly higher (69.33 ± 20.32) circulating mtDNA than male (53.48 ± 11.50, p=0.03). These differences did not reach statistical significance considering the two obese group separately [data not shown].

**DISCUSSION**
Despite worldwide increasing attention to the adverse pregnancy outcomes related to obesity, further data are needed to deeply understand molecular aspects related to its inflammatory/oxidative stress environment.

This study focuses on the evaluation of markers of OxS (mitochondrial DNA) and inflammation (Hepcidin- Hpn) in the systemic circulation of healthy normal-weight and obese mothers at elective cesarean section.

We found increased mitochondrial DNA levels in obese women compared to normal-weight. mtDNA is esteemed as a measure of mitochondrial content [10]. We previously reported that mtDNA in the maternal circulation is significantly higher in pregnancies affected by fetal growth restriction [11] that also present higher placental mtDNA [10,18]. Also in obese women we have recently found increased placental mtDNA [12], suggesting that increased maternal mtDNA is likely the result of the release of placental cell debris in the maternal circulation [19].

Obesity is a condition characterized by mild chronic inflammation and OxS [1]. At the same time, mitochondria can be critically involved in this setting since their regulation and biogenesis are influenced by several endocrine and nutritional stimuli as well as specific stress and hypoxia pathways [20-21]. The positive correlation between mtDNA and maternal hemoglobin confirmed mtDNA as a sensitive indicator of oxygen imbalances [10].

Moreover, in vitro and ex vivo studies recently reported both increased ROS production by obese mitochondria and impaired mitochondrial metabolism [13,20-21]. This suggests that the increase in mtDNA content we found in obese patients can be an adaptive mechanism to the impaired environment occurring in obese pregnancies. The positive correlation linking mtDNA with maternal BMI in OB/GDM(-) pregnancies supports this hypothesis.

In our study population, also maternal plasma hepcidin was significantly higher in OB/GDM(-) than in normal-weight women. Two other studies have previously reported higher maternal hepcidin levels in obese women [22-23]. Indeed, obesity-related inflammation may induce hepcidin biosynthesis with a consequent reduction of iron supply [15]. Garcia-Valdes et al. hypothesized that Hpn can be a
regulatory factor leading to higher risk of iron deficiency in obese pregnant women [23]. Iron deficiency can also be the result of a low-quality diet that often characterizes obese patients, possibly leading to placental impairment and pregnancy pathologies [7-9,24]. In our population, OB/GDM(-) subjects presented significantly lower hemoglobin concentration, compared to normal-weight pregnancies, confirming these associations. Nonetheless, anemia was not related to the increase in hepcidin, while this was positively associated with maternal BMI.

Recently, a cause-effect relationship involving mitochondria and hepcidin has been reported in vitro proposing that mitochondrial alterations, caused by excessive OxS, lead to too much cellular iron deposition and consequently to hepcidin over-expression, and that conversely this increased Hpn levels may result in disturbance of mt function [13]. The concordance between circulating mtDNA and hepcidin levels we found in our study population supports this hypothesis.

**Obese Pregnancies with GDM**

When analyzing obese groups, we found that women with Gestational Diabetes Mellitus (GDM) presented a different trend compared to obese without GDM. GDM is characterized by systemic insulin resistance and has been associated with impaired placental features [6]. Consistent with previous data [3], in our study OB/GDM(+) presented significantly higher placental weight and lower placental efficiency than both OB/GDM(-) and NW.

Insulin resistance has been associated with inadequate hepcidin levels in diabetes [25]. This suggests that the presence of insulin resistance might drive Hpn biogenesis towards an opposite direction than the inflammation stimuli.

On the other side, metabolic status affects mitochondria. Several studies analyzed mt dysfunctions in the context of obesity and glucose intolerance, both in humans and in animals [25-27]. Contrasting data have been reported suggesting that mitochondrial alterations are not a stable feature of insulin resistance in all circumstances. Nevertheless, mtDNA copy number in obese human subjects has been negatively related to insulin resistance [27]. Previous data also show a negative relationship between
insulin resistance and both hepcidin and mitochondrial levels. This suggests that when gestational diabetes and insulin resistance occur in obese women, an opposite driving force may act on hepcidin concentration and mitochondrial biogenesis, leading to the decrease in Hpn and mtDNA levels compared to obese normoglycemic mothers. Moreover, our clinical protocol for diabetic women provides habitual checks of glycemia values and specific dietary indications for glycemic control. Indeed, OB/GDM(+) women presented lower gestational weight gain compared to OB/GDM(-) and to NW, and their hemoglobin levels were similar to controls and higher compared to OB/GDM(-). This may occur in relation to a more balanced intake of micro/macro nutrients [28], which may also contribute to lower hepcidin and mtDNA levels.

Sexual Dimorphism

Here we report for the first time significantly increased levels of maternal hepcidin and circulating mtDNA in female- compared to male-carrying pregnancies. While Hpn differences were evident only comparing all the female to male mothers of the study population, statistical comparison according to BMI revealed significantly higher mtDNA levels in females-carrying physiological/normal-weight pregnancies than in males-. Indeed, in females’ mothers mtDNA was significantly higher in obese pregnancies compared to normal-weight, with a lack of statistical significance- probably related to the small sample size- when the two obese subgroups are analyzed separately. Importantly, no significant differences of fetal sex frequencies within the study groups were found, suggesting no involvement of fetal gender distribution.

Sexual dimorphism is well recognized in placental formation and function [4]. Gonadal steroids may play a key role in this context. Indeed, estrogens coordinate and integrate cellular metabolism and mitochondrial activities by regulating mitochondrial biogenesis and function [29]. We thus might speculate that female placentas have higher mitochondrial content compared to males’, thus resulting in increased circulating mtDNA levels in the mother.
Another potential explanation is that placental shedding may be higher in female obese pregnancies, as recorded for placental metabolic pathways, morphology and efficiency in few murine/human models [3,30]. Indeed, sexual dimorphism is increasingly reported as a conditioning parameter in the intrauterine response to maternal inflammation/stress, with females more inclined to respond to insults [3,4]. No reports of sex-differences in inflammation markers come from recent literature. Hepcidin higher levels in female-carrying pregnancies may be linked to the placental fraction of Hpn production, but focused analyses are needed to verify this hypothesis.

**Limitations**

A possible limit of this study lies on the origin of mtDNA in the maternal blood in different BMI groups. Among blood cells, white cells, erythroblasts and platelets can contribute to the amount of measured mtDNA. Here, we measured mtDNA content by normalizing levels of mitochondrial Cyt-B gene to the nuclear RNase-P. As platelets don’t carry nuclear DNA, this might represent a possible bias in case of different platelets distribution within these groups. Indeed, blood cell content might be affected by pre-gestational BMI. However, maternal hematocrit was not different between analyzed groups, suggesting no differences in blood cells content and thus a not different contribution to mtDNA among BMI groups.

Moreover, a larger study population would be needed to confirm possible differences in the mitochondrial machinery between female and male fetuses undergoing the insult of the obese intrauterine environment.

**Conclusions**

Our data provide new insight on the relation between maternal mtDNA and hepcidin and excessive maternal BMI or insulin resistance. This may conduce to establish new promising biomarkers and possible life-care intervention, ameliorating both the inflammatory and oxidative obesity-related milieu, aimed at reducing the obesity-related risks in pregnancy.
7. ACKNOWLEDGEMENTS

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8. DECLARATION OF INTEREST STATEMENT

The authors report no conflict of interest.
9. REFERENCES


Table 1: Maternal and delivery data in the study population. Data are presented as mean ± standard deviation. Comparisons were made between both Obese groups vs Normal Weight [★p≤ 0.05, ★★★p≤ 0.01; ★★★★p≤ 0.001] and between OB/GDM(+) vs OB/GDM(-) [§p≤ 0.05, §§p≤ 0.01]. NW: Normal Weight; OB/GDM(-): Obese without GDM; OB/GDM(+): Obese with GDM; BMI: Body Mass Index; GDM: Gestational Diabetes Mellitus; GWG: Gestational Weight Gain; Maternal Fasting Glycemia: referred to the first value of the OGTT; Placental Efficiency: fetal/placental weight ratio.

<table>
<thead>
<tr>
<th></th>
<th>NW n= 16</th>
<th>OB / GDM (-) n= 15</th>
<th>OB / GDM (+) n= 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age [yrs]</td>
<td>35.4 ± 5.2</td>
<td>33.7 ± 6.0</td>
<td>35.5 ± 4.4</td>
</tr>
<tr>
<td>Maternal Pre-Gestational BMI [kg/m²]</td>
<td>21.6 ± 1.8</td>
<td>35.4 ± 4.8 ★★★</td>
<td>35.6 ± 4.6 ★★★</td>
</tr>
<tr>
<td>Maternal GWG [Kg]</td>
<td>12.0 ± 4.1</td>
<td>9.5 ± 5.3</td>
<td>8.1 ± 5.3</td>
</tr>
<tr>
<td>Maternal Venous Hemoglobin [g/dL]</td>
<td>11.6 ± 1.2</td>
<td>10.7 ± 1.3 ★</td>
<td>11.3 ± 0.8</td>
</tr>
<tr>
<td>Maternal Venous Hematocrit [%]</td>
<td>34.2 ± 3.4</td>
<td>32.6 ± 3.5</td>
<td>34.0 ± 2.6</td>
</tr>
<tr>
<td>Maternal Fasting Glycemia [mg/dL]</td>
<td>78.4 ± 8.0</td>
<td>79.0 ± 7.3</td>
<td>93.9 ± 12.2 ★★ §§</td>
</tr>
<tr>
<td>Gestational Age [wks]</td>
<td>39.1 ± 0.3</td>
<td>39.1 ± 0.3</td>
<td>39.1 ± 0.2</td>
</tr>
<tr>
<td>Fetal Weight [g]</td>
<td>3420.0 ± 287.9</td>
<td>3320.7 ± 421.8</td>
<td>3371.0 ± 409.0</td>
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<tr>
<td>Placental Weight [g]</td>
<td>460.7 ± 97.1</td>
<td>471.6 ± 72.7</td>
<td>554.0 ± 70.6 ★ §§</td>
</tr>
<tr>
<td>Placental Efficiency</td>
<td>7.9 ± 1.7</td>
<td>7.1 ± 1.1</td>
<td>6.1 ± 1.7 ★★ §§</td>
</tr>
</tbody>
</table>
FIGURE 1 (A) and (B)
FIGURE 2 (A) and (B)
FIGURE 3

Box plots showing Blood mtDNA Content for NW and All Obese groups. The plots indicate significant differences marked with asterisks (*).
11. FIGURE CAPTIONS

Figure 1: Maternal Blood mtDNA Content. (A) mtDNA levels in OB/GDM(-) [n= 15] and OB/GDM(+) [n= 9] vs NW [n= 14] [★ p≤ 0.05] pregnant women at delivery. Data are shown as box plots, indicating the median and the 25th and 75th percentiles; (B): Relationship between mtDNA Content and Maternal Pre-Gestational BMI in OB/GDM(-) [◆; n=15], OB/GDM(+) [◆; n=9] and NW [○; n=14]. Pearson Correlation data referred only to the Normoglycemic Population [r= +0.48, ★★p≤ 0.01]

Figure 2: Maternal Plasma Hepcidin. (A) Hepcidin levels in OB/GDM(-) [n= 11] and OB/GDM(+) [n= 5] vs NW [n= 10] [★★ p≤ 0.01] pregnant women at delivery. Data are shown as box plots, indicating the median and the 25th and 75th percentiles; (B): Relationship between Hepcidin Levels and Maternal Pre-Gestational BMI in OB/GDM(-) [◆; n=11], OB/GDM(+) [◆; n=5] and NW [○; n=10]. Pearson Correlation data referred only to the Normoglycemic Population [r= +0.51, ★p≤ 0.05]

Figure 3: Maternal Blood mtDNA Content according to Fetal Gender. Comparisons between females’ (white) or males’ (grey) mothers in Normal-Weight and All Obese groups [★ p≤ 0.05 M vs F]