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## **Sex-specific responses in placental fatty acid oxidation, esterification and transfer capacity to maternal obesity**

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**Running title:** Placental lipid metabolism in maternal obesity.

## **Abbreviations**

ARA, arachidonic acid; BM, basal plasma membrane; CE, cholesteryl ester; CPT, carnitine palmitoyl transferase, DGAT, diacylglycerol acyltransferase; DHA, docosahexaenoic acid; FA, fatty acid; FABP, fatty acid binding protein; FAT/CD36, fatty acid translocase; FATP, fatty acid transport protein; GC-MS, gas chromatography–mass spectrometry; LC-MS/MS, liquid chromatography–mass spectrometry tandem; LCPUFA, long chain polyunsaturated fatty acid; Lyso-PC, lysophosphatidylcholine; MUFA, monounsaturated fatty acid, OA, oleic acid; OCTN2, organic cation transporter 2; PC, phosphatidylcholine; PL, phospholipid; POA, palmitoleic acid; MFSD2a, Major facilitator superfamily domain-containing protein 2; MVM, microvillous plasma membrane; NEFA, nonesterified fatty acid; SCD1, stearoyl CoA desaturase 1; SFA, saturated fatty acid; TG, triglyceride; WB, western blot.

## **ABSTRACT**

Fatty acid metabolism and oxidation capacity in the placenta, which likely affects the rate and composition of lipid delivered to the fetus remains poorly understood. Long chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), are critical for fetal growth and brain development. We determined the impact of maternal obesity on placental fatty acid oxidation, esterification and transport capacity by measuring PhosphatidylCholine (PC) and LysoPhosphatidylCholine (LPC) containing DHA by mass spectrometry in mother-placenta-baby triads as well as placental free carnitine and acylcarnitine metabolites in women with normal and obese pre-pregnancy BMI. Placental protein expression of enzymes involved in beta-oxidation and esterification pathways, MFSD2a (lysophosphatidylcholine transporter) and OCTN2 (carnitine transporter) expression in syncytiotrophoblast microvillous (MVM) and basal (BM) membranes were determined by Western Blot. Maternal obesity was associated with decreased umbilical cord plasma DHA in LPC and PC fractions in male, but not female, fetuses. Basal membrane MFSD2a protein expression was increased in placenta of males of obese mothers. In female placentas, despite an increased MVM OCTN2 expression, maternal obesity was associated with a reduced MUFA-carnitine levels and increased esterification enzymes. We speculate that lower DHA-PL in fetal circulation of male offspring of obese mothers, despite a significant increase in transporter expression for LPC-DHA, may lead to low DHA needed for brain development contributing to neurological consequences that are more prevalent in male children. Female placentas likely have reduced beta-oxidation capacity and appear to store FA through greater placental esterification, suggesting impaired placenta function and lipid transfer in female placentas of obese mothers.

**Keywords:** Maternal-fetal exchange, human pregnancy, fetus, LPC-DHA, MFSD2a, metabolic disorders

## 1. Introduction

As a result of the rapidly increasing prevalence of high body mass index (BMI) world wide, overweight and obesity in pregnancy has emerged as a major public health problem. For example, almost 2/3 of American women now enter pregnancy either overweight or obese (1-3), which is associated with a range of short- and long-term adverse maternal and fetal consequences. Obese women are more susceptible to develop hypertension, gestational diabetes, respiratory complications and thromboembolic events [1, 2]. They are more likely to deliver large for gestational age (LGA) infants or infants with greater adiposity and these children have greater risk for type 2 diabetes mellitus and cardiovascular disease later in life.

Obese pregnant women typically have elevated circulating plasma lipid levels [3] and circulating levels of nonesterified fatty acid (NEFA) correlate positively with maternal BMI [4]. Nonetheless, recent studies have reported no changes in TG and NEFA profiles in obese women at term but rather increased TG levels in placenta [5, 6]. Docosahexaenoic acid (DHA, C22:6n-3) and arachidonic acid (ARA, C20:4n-6) are bioactive long chain polyunsaturated fatty acids (LCPUFA) influencing membrane structure and function, cell signaling and lipid mediator production. In addition, LCPUFAs are crucial for central nervous system development and human brains have accelerated growth with increasing cerebral content of DHA and ARA across the last trimester of pregnancy and early postnatal months [7]. These LCPUFAs are considered essential in fetal life due to low or absent delta 5 and 6 desaturase activity in the placenta and fetus. Therefore, all LCPUFAs are of maternal origin and must be transported across the placenta. Altered lipid availability in utero such as decreased n-3 LCPUFA during fetal development has been associated with specific adverse neuronal infant outcomes [8] where males are more likely to develop attention deficit/hyperactivity disorder or earlier-onset schizophrenia [9]. Pre-gravid maternal BMI has been associated with lower LCPUFA concentrations in fetal erythrocyte phospholipid fraction in pregnancies complicated by gestational diabetes mellitus (GDM) [10].

Mitochondria are the main energy producers in the cell through oxidative phosphorylation and placental mitochondrial biogenesis and activity increase throughout the pregnancy to sustain the metabolic activity of the placenta. Altered mitochondrial structure and function negatively impacts placental and fetal growth [11]. Carnitine facilitates the transport of long-chain fatty acids (LCFA) across the inner mitochondrial membrane and is critical for mitochondrial  $\beta$ -oxidation [12]. In addition, carnitine allows for the transfer of  $\beta$ -oxidation metabolites produced

in the peroxisome to the mitochondria [13]. Long-chain fatty acids must be esterified to carnitine via the carnitine palmitoyl transferase (CPT1) enzyme which is the rate limiting step in LCFA  $\beta$ -oxidation. In humans, 75% of carnitine is supplied by the food and 25% is synthesized endogenously from two essential amino acids, lysine and methionine [14]. This occurs mainly in kidney, liver and brain [15] and has been demonstrated in the placenta as well [16]. Several studies suggest that fetal carnitine is derived from the mother via transplacental transfer of carnitine [17], which is supported by the observation that the carnitine transporter OCTN2 (organic cation transporter 2, SCL22A5), is expressed and localized in both the microvillous (MVM) and basal (BM) plasma membranes of the syncytiotrophoblast of the human placenta [18]. Decreased free carnitine has been reported in placentas of obese women [19]. Obesity is also associated with a decrease in the number and activity of mitochondria in human placental cells [20].

Male and female fetuses respond differently to adverse intrauterine environments such as maternal obesity leading to different risk profiles for developing metabolic disease later in life [21]. There is also growing evidence that the female and male placental functions are functionally different and placental responses to, for example, maternal obesity depends on fetal sex. Indeed, studies in rodents suggest that placental development is adaptable and responds to altered maternal nutrition in a fetal sex dependent manner [22]. In healthy human pregnancies, sex differences in placental size and gene expression are present throughout the gestation [23], which has been proposed to play an important role in determining sex differences in intrauterine growth [24] that are apparent from the early stages of development [25]. Maternal obesity has also been reported to increase the uptake of oleic acid, the most abundant circulating monounsaturated fatty acid (MUFA), in trophoblast cells isolated from placentas of female fetuses and decreases the uptake of MUFA in placentas of males [26]. A recent study reported sexual dimorphism in mitochondrial respiration by studying glutamine, fatty acid and glucose as fuels, in pregnancies complicated by obesity and GDM [27]. The authors demonstrated an increased dependency on glucose and fatty acid for baseline respiration and a decreased flexibility for using both fuels in placenta of obese women with GDM only in male infants [27].

Fatty acid metabolism and oxidation capacity in the placenta, which likely affects the rate and composition of fatty acid delivered to the fetus remains poorly understood, particularly in pregnancy complicated by obesity without diabetes. We hypothesized that maternal obesity

impacts placental lipid metabolism, fatty acid oxidation and transport capacity with differences due to the sex of the fetus. To test this hypothesis, we collected term male and female placentas from obese and normal BMI women who were otherwise healthy and studied the protein expression of specific enzymes involved in FA oxidation and esterification and lipid transporters, FA content in four lipid classes, acyl-carnitine metabolites and free-carnitine in placental tissue and FA and phospholipid content of maternal and umbilical cord plasma.

## **2. Materials and methods**

### ***2.1. Study subjects for plasma and placenta collection***

Pregnant women were enrolled to donate their blood, placenta and umbilical cord blood to a data/bio-sample repository for use in multiple research studies under a protocol approved by the Institutional Review Board at University of Colorado, Denver (COMIRB 14-1073). All participants gave informed written consent for sample collection and the use of their protected health information. Inclusion criteria for the repository included information on pre-pregnancy/early pregnancy BMI, ultrasound confirmation of gestational age at 14-18 weeks, singleton pregnancy and maternal age 18-45 years. Exclusion criteria included concurrent inflammatory or vascular disease, current use of tobacco, street drugs, or medications, fetal malformations, history of pregnancy loss or pre-term delivery. For the current study, control women were selected if their pre-pregnancy or first trimester BMI was less than 25 kg/m<sup>2</sup> while obese women were defined based on having a pre-pregnancy/early pregnancy BMI greater than 30 kg/m<sup>2</sup>. Women with diagnosis of any form of diabetes, including gestational mellitus diabetes (GDM), were excluded. Maternal, umbilical cord vein and artery blood (16 obese and 16 normal BMI women) was rapidly centrifuged and the plasma were stored at -80°C. In association with the blood collection, all the placentas of these 32 women were collected at delivery. For protein expression studies, we increased the number of male and female placentas to 21 in each group in order to achieve power for analysis in each group and processed as described below. Maternal and infant characteristics for 32 subjects with complete sampling (triad of maternal and cord blood and placenta) and for the total 42 participants who donated placentas in the current study are summarized in **Table 1**.

### ***2.2. Placental homogenates***

Placentas were obtained within 30 min after delivery. After removing decidua basalis and chorionic plate, approximately 100 g of villous tissue was dissected and rinsed in cold physiological saline. The villous tissue was transferred to ice-cold buffer D (250 mM sucrose, 10 mM hepes, pH 7.4) containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO) and homogenized on ice. Placental homogenates (PH) were rapidly frozen in liquid nitrogen and stored at -80°C until further processing for isolation of plasma membranes.

### ***2.3. Isolation of microvillous and basal plasma membranes***

Syncytiotrophoblast microvillous membrane (MVM) and basal plasma membrane (BM) were isolated according to an established protocol [28, 29]. In brief, thawed PH underwent differential centrifugation to isolate the apical MVM by  $MgCl_2$  precipitation and fetal facing BM by sucrose gradient centrifugation. The MVM and BM were re-suspended in buffer D containing protease and phosphatase inhibitors, frozen in liquid nitrogen, and stored at  $-80^{\circ}C$ . The ratio of MVM/homogenate of alkaline phosphatase activity, a marker of MVM enrichment [28], was  $17.7 \pm 1.9$ -fold in obese group ( $n=16$ ) which was not significantly different from  $17.5 \pm 1.3$ -fold in the control group ( $n=16$ ) ( $p>0.05$ ). Likewise, MVM enrichment of alkaline phosphatase was not significantly different between females and in males in either group (data not shown). BM enrichment was determined by BM/homogenate ratio of VDAC1 protein expression by Western blot. VDAC1 is exclusively localized to the placental BM [30, 31]. Enrichment of VDAC1 in BM fractions was not significantly different between ( $34.9 \pm 3.7$ -fold) and obese groups ( $37.1 \pm 3.3$ -fold,  $p>0.05$ ) or between female and male in either group (not shown).

#### **2.4. Western Blotting**

Western blot was performed as previously described [32] using a pre-cast gel systems (Bio-Rad). Total protein content of placental homogenates or isolated membranes (MVM and BM) was determined using BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, MA, USA). Twenty  $\mu g$  total protein was loaded and separated on Bis-Tris gels (4-20%). After electrophoresis and transfer onto a PVDF membrane (Bio-Rad Laboratories Inc.) the membrane was stained for total protein using Amido Black Stain (Sigma-Aldrich). Primary antibody incubation (additional detail on primary antibodies is provided in **Table A1** in supplementary data) was carried out overnight at  $4^{\circ}C$ , and secondary antibody (peroxidase labeled anti-Rabbit IgG, diluted 1:3000, Cell Signaling Technology (Danvers, MA)) for 1 h at room temperature. Immunolabeling was made visible with SuperSignal West Pico Plus detection solution (Thermo Scientific) in a G:Box ChemiXL1.4 (SynGene, Cambridge, UK). Densitometry analysis of target protein bands was performed with GeneTools (SynGene) and target protein expression was adjusted for Amido Black staining to account for any variation in protein loading and transfer.

#### **2.5. Analysis of fatty acids in plasma and placental tissue**

Thirty  $\mu L$  of plasma or placental homogenate were used to quantify FAs in four lipid classes according to the method previously described [33, 34]. Briefly, four lipid classes were extracted

from total lipid pool (cholesterol esters (CE), triglycerides (TG), NEFA and phospholipids (PL)) by solid phase extraction on NH<sub>2</sub> cartridges (Phenomenex, CA, USA). Lipids were hydrolyzed with sodium hydroxide (1N) and free fatty acids were extracted and derivatized to pentafluorobenzyl FA esters. Quantification of FAs was conducted under negative ion chemical ionization (NICI) mode using an Agilent 7890B gas chromatograph coupled to an Agilent 5977B single quadrupole mass spectrometer (Agilent Technologies, CA, USA). Injections are executed with an Agilent 7693A Autoinjector into a split-splitless liner setup to a non-polar capillary GC column (HP-5, 5% Phenyl-methylpolysiloxane). Chromatographic analysis is completed using Agilent's MassHunter Qualitative Analysis (Version B.08.00) integration and quantification performed with Quantitative Analysis (Version B.09.00) software. Internal standard mixture was added to each sample to determine the absolute concentration of each FA detected in these samples following calibration curves determined for all major FAs. The list of different studied fatty acids with common names and formula is provided in **Table A7** in supplementary data.

## ***2.6. Acylcarnitine metabolite and free carnitine quantification in placental tissue by LC-MS/MS***

The quantification method of acylcarnitine metabolites was adapted from the report of Ghoshal and coworkers [35]. Briefly, placental homogenates were thawed at room temperature and vortexed for 5s. Cambridge Isotope internal standard (50 µl diluted in 1 ml LC/MS grade methanol (#NSK-B-1, CIL, MA, USA)) was added to 50 µl placental homogenate., then 50 µl custom C12/C18 internal standard were added to the samples (d<sub>3</sub>-C12 and d<sub>3</sub>-C18 internal standards were reconstituted in methanol and diluted 2:2500 with methanol to produce a working concentration of 0.2269 and 0.18755 nMol/mL respectively). Samples were entirely dried in a centrifugal evaporator at 55 °C then reconstituted with 100 µl of 50:50 HPLC buffer A:B solution and vortexed for 5 min. HPLC Buffer A was constituted by LC/MS grade water with 0.1% formic acid and HPLC Buffer B with 50:50 Acetonitrile:Isopropanol with 0.1% formic acid. Finally, extracted samples were centrifuged at 14,000 RPM at 4 °C for 10 min and supernatants were run into a LC/ESI-MS system equipped with a chromatographic column Zorbax SB-C18 2.1x100mm 1.8 micron and a guard column Zorbax SB-C18 2.1x5mm 1.8 micron (Agilent Technologies, CA, USA).

## ***2.7. Analysis of phosphatidylcholine and lysophosphatidylcholine species in plasma and placental tissue by LC-MS/MS***

Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) species were extracted and quantified in placental tissue and maternal and cord plasma samples [maternal vein (MV), umbilical vein (UV) and artery (UA)] by LC-MS/MS in the Mass Spectrometry Lipidomics Core facility at the University of Colorado as previously described [36]. Briefly, 1  $\mu$ l of an internal standard mixture of phospholipids (SPLASH Lipidomix, Avanti Polar Lipids, Inc.) was added to 50  $\mu$ l of each plasma and placental homogenate sample and lipids were extracted according to the method of Bligh and Dyer [37].

LC-MS/MS analysis was carried out on two Shimadzu UFLCXR 50326 LC-20AD pumps (Kyoto, Japan) and a Leap Technologies PAL HTC-xt Sample Handler (Carrboro, North Carolina) were coupled to an AB Sciex Triple Quad QTRAP 5500 ESI-LC-MS/MS mass spectrometer (Framingham, MA) for all experiments. The mobile phase consisted of (A) 30/40 hexane/isopropanol v/v and (B) 30/40/7 hexane/isopropanol/water with 5 mM ammonium acetate with a flow rate of 200  $\mu$ l/min. The stationary phase was a normal phase silica column (Supelco Ascentis 5 $\mu$  Si, 100 $\text{\AA}$  pore size, 150x2.1mm). Mass spectrometric analyses were performed in the negative ion mode for precursors of m/z 327 for DHA and m/z 288 for the internal standards and positive ion precursors of m/z 184 scanning from m/z 450-900. Results were analyzed using MultiQuant software (SCIEX) and all detected PC species containing DHA are reported as the ratio between the integrated area of each analyte and the integrated area of the corresponding internal standard for PC class (PC-C15:0/[ $^2\text{H}_7$ ]18:1). The internal standard LPC C18:1/[ $^2\text{H}_7$ ] was used for determining the LPC ratios.

## ***2.8. Statistical analyses***

Data are presented as mean  $\pm$  SEM. All statistical tests were performed using GraphPad Prism, version 6.07. All variables were compared between two independent groups, obese vs control (normal BMI) within each fetal sex by Mann-Whitney U test based on the satisfaction of assumptions of normality as tested by Shapiro-Wilk test. Only the “BMI” variable was compared in each sex group using a t-test based on the normal distribution of the data. Pearson’s correlation analysis was used to assess correlations between maternal BMI and protein

expression of transporters in syncytiotrophoblast plasma membranes or with DHA-PL content in umbilical cord venous (UV) plasma for each fetal sex. A *P*-value < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Study participants

Selected clinical characteristics of mothers, placentas and infants included in the current study are reported in **Table 1**. Maternal BMI in the obese group were greater than control samples by design. Maternal age at delivery, gestational age, placenta and birth weights were not significantly different between the obese vs control groups for either sex.

**Table 1:** Clinical characteristics of study subjects.

Variable	Female		Male	
	Control	Obese	Control	Obese
<b><i>Plasma and placenta of triads (mother-placenta-fetus) used for FA and carnitine metabolite study</i></b>				
<b>N</b>	9	11	7	5
<b>Maternal BMI (kg/m<sup>2</sup>)</b>	22.6 ± 2.5	38.2 ± 1.2***	23.5 ± 0.8	33.3 ± 1.3**
<b>Age at delivery (years)</b>	31.0 ± 1.4	30.0 ± 1.5	31.9 ± 2.4	28.0 ± 3.0
<b>Gestational age (weeks)</b>	39.3 ± 0.5	39.2 ± 0.1	39.3 ± 0.5	39.6 ± 0.5
<b>Ethnicity</b>	7 WnH/ 1 WH/ 1 Asian-nH	6 WnH/2 WH/2 BnH/ 1 Asian-nH	6 WnH/1 WH	1 WnH/4 WH
<b>Delivery mode (C/V)</b>	7/2	11/0	5/2	10/0
<b>Placental weight (g)</b>	658.5 ± 36.5	732.5 ± 59.6	643.2 ± 48.9	646.9 ± 127.8
<b>Birth weight (g)</b>	3401.1 ± 36.5	3559.1 ± 147.5	3429.9 ± 94.6	3718.4 ± 559.2
<b><i>Placental tissue used for western blotting study</i></b>				
<b>N</b>	11	11	10	10
<b>Maternal BMI (kg/m<sup>2</sup>)</b>	22.6 ± 0.7	38.2 ± 1.2***	22.7 ± 0.7	36.6 ± 1.5 ***
<b>Age at delivery (years)</b>	29.8 ± 1.4	30.0 ± 1.5	32.5 ± 1.7	29.6 ± 1.8
<b>Gestational age (weeks)</b>	39.3 ± 0.4	39.2 ± 0.1	39.2 ± 0.3	39.4 ± 0.3
<b>Ethnicity</b>	8 WnH/2 WH/ 1 Asian-nH	6 WnH/2 WH/ 2 BnH/ 1 Asian-nH	9 WnH/1 WH	3 WnH/4 WH/ 1 BnH/1 Asian- nH/1 BH
<b>Delivery mode (C/V)</b>	9/2	11/0	8/2	10/0
<b>Placental weight (g)</b>	628.5 ± 36.5	732.5 ± 59.6	654.3 ± 36.8	660.1 ± 75.9
<b>Birth weight (g)</b>	3297.3 ± 143.1	3559.1 ± 147.5	3387.9 ± 71.7	3696.4 ± 273.6

Data are presented as mean ± SEM. \*\*P-value < 0.005 (t-test) ob vs Ctr, \*\*\*P-value < 0.001, Wnh=White non Hispanic, WH=White Hispanic, Asian-nH=Asian non Hispanic, BnH=Black non Hispanic, BH=Black Hispanic, C = Caesarean section, V = vaginal delivery.

### ***3.2. Impact of maternal obesity on fatty acid profiles vary with fetal sex.***

The majority of women were delivered by elective caesarian-section except for 4 “control” women who delivered by spontaneous vaginal delivery (Table 1). Previous studies have indicated that the delivery mode, in particular the vaginal mode with extended labor may influence the plasma fatty acid profile [38], however the portion of vaginal deliveries in the current study was low and therefore is it unlikely to impact the outcomes of the study.

We found interesting differences in FA composition in normal BMI and obese mothers depending on the sex of their baby (summarized in **Table 2**). Maternal plasma oleic acid (OA, C18:1n-9), the most abundant circulating monounsaturated fatty acid (MUFA) showed a trend toward an increase in the TG ( $p=0.067$ ) and CE ( $p=0.062$ ) fractions in obese mothers as compared to control with female babies, but not when the fetus was male. In contrast, maternal obesity was associated with lower maternal plasma OA in the PL fraction in male but not in female fetuses.

**Table 2:** Maternal plasma fatty acid concentrations in lipid classes in normal BMI (Ctr) and obese (Ob) women.

Maternal plasma				
FA ( $\mu\text{mol/L}$ )	Female		Male	
	Ctr (n=9)	Ob (n=11)	Ctr (n=7)	Ob (n=5)
<i>Cholesterol ester</i>				
<b>C18:0</b>	23.2 $\pm$ 3.7	30.7 $\pm$ 10.7	53.0 $\pm$ 17.0	15.4 $\pm$ 4.6
<b>C18:1n-9</b>	583.8 $\pm$ 138.3	1152.1 $\pm$ 201.6	844.6 $\pm$ 112.8	570.5 $\pm$ 88.3
<b>C18:2n-6</b>	420.1 $\pm$ 173.6	1086.6 $\pm$ 142.3*	665.4 $\pm$ 199.1	515.7 $\pm$ 181.6
<b>C22:6n-3</b>	9.5 $\pm$ 2.2	9.8 $\pm$ 1.5	13.6 $\pm$ 1.5	10.4 $\pm$ 4.7
<b>Total</b>	1434.2 $\pm$ 311.5	2882.8 $\pm$ 386.1*	2240.1 $\pm$ 289.6	1491.7 $\pm$ 375.8
<i>Triglycerides</i>				
<b>C16:0</b>	899.4 $\pm$ 202.7	1184.1 $\pm$ 132.9	1155.9 $\pm$ 128.4	1229.5 $\pm$ 307.1
<b>C18:1n-9</b>	1866.2 $\pm$ 700.3	3103.8 $\pm$ 614.7	1722.0 $\pm$ 251.0	1327.7 $\pm$ 123.3
<b>C18:2n-6</b>	481.1 $\pm$ 197.9	711.6 $\pm$ 89.9	363.0 $\pm$ 107.1	335.4 $\pm$ 135.2
<b>C22:6n-3</b>	11.3 $\pm$ 2.8	14.1 $\pm$ 5.0	18.1 $\pm$ 4.8	15.5 $\pm$ 8.6
<b>Total</b>	3840.1 $\pm$ 1164.7	5448.8 $\pm$ 837.7	3835.5 $\pm$ 347.5	3039.4 $\pm$ 387.4
<i>Non esterified fatty acids</i>				
<b>C16:0</b>	109.6 $\pm$ 40.7	175.8 $\pm$ 34.3	104.4 $\pm$ 44.3	102.1 $\pm$ 26.0
<b>C16:1n-7</b>	8.1 $\pm$ 2.8	4.0 $\pm$ 0.7	6.8 $\pm$ 1.7	8.1 $\pm$ 4.9
<b>C18:1n-9</b>	165.5 $\pm$ 44.1	118.6 $\pm$ 13.6	151.7 $\pm$ 37.5	148.9 $\pm$ 74.9
<b>C22:4n-6</b>	0.6 $\pm$ 0.2	1.1 $\pm$ 0.1*	1.3 $\pm$ 0.2	0.6 $\pm$ 0.1*
<b>Total</b>	496.8 $\pm$ 126.8	520.7 $\pm$ 69.6	387.1 $\pm$ 75.3	414.1 $\pm$ 84.9
<i>Phospholipids</i>				
<b>C14:0</b>	31.8 $\pm$ 3.7	10.4 $\pm$ 2.8**	40.4 $\pm$ 6.5	6.3 $\pm$ 1.3**
<b>C16:0</b>	1158.7 $\pm$ 84.0	1180.3 $\pm$ 83.8	1317.7 $\pm$ 87.8	933.9 $\pm$ 74.8*
<b>C16:1n-7</b>	15.7 $\pm$ 7.6	5.8 $\pm$ 0.8	18.1 $\pm$ 5.8	4.1 $\pm$ 1.9*
<b>C18:1n-9</b>	418.5 $\pm$ 46.5	394.5 $\pm$ 39.3	563.1 $\pm$ 37.3	263.3 $\pm$ 39.1**
<b>C20:4n-6</b>	518.9 $\pm$ 49.5	608.1 $\pm$ 65.1	633.5 $\pm$ 54.1	410.8 $\pm$ 44.6*
<b>C20:5n-3</b>	17.3 $\pm$ 6.6	12.4 $\pm$ 3.3	25.3 $\pm$ 4.6	11.3 $\pm$ 2.3*
<b>C22:6n-3</b>	144.7 $\pm$ 21.5	103.4 $\pm$ 9.8	136.4 $\pm$ 15.3	87.0 $\pm$ 12.3
<b>Total</b>	3571.8 $\pm$ 184.5	3890.4 $\pm$ 209.9	4102.0 $\pm$ 298.6	3006.3 $\pm$ 202.4*

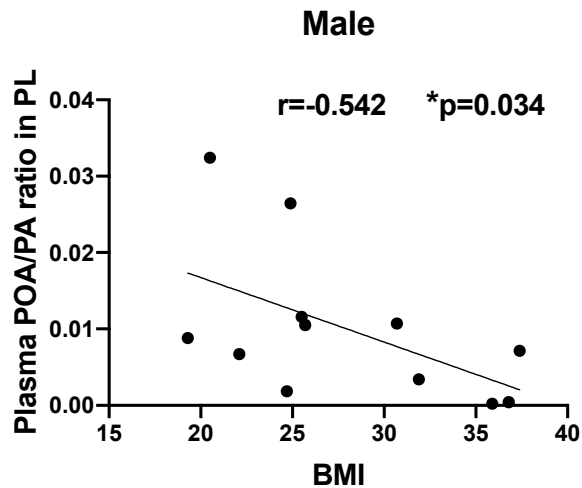
Mann Whitney test between Obese and Ctr groups within each fetal sex,

Mean  $\pm$  SEM, \*  $p < 0.05$ , \*\*  $p < 0.005$

The trend to raised OA suggest a greater availability of this MUFA for placental transfer to female fetuses if the mother is obese. Similarly, maternal plasma linoleic acid (LA, C18:2n-6), an essential fatty acid involved in the n-6 LCPUFA pathway, in the CE fraction was found to be significantly higher in obese mothers with female babies, whereas maternal obesity had no effect on maternal LA levels in women carrying a male fetus. As a result, total FA was notably

increased in CE fraction and showed a trend to be higher in TG in obese mothers with female babies.

Obese mothers had markedly decreased plasma myristic acid (MA, C14:0), independent of fetal sex, and maternal obesity was associated with a decrease in maternal plasma PL palmitic acid (PA, C16:0) only if the fetus was male. Moreover, maternal palmitoleic acid (POA, C16:1n-7) in the PL fraction were markedly lower in obese women independent of fetal sex, however this decrease was significant only in women with male infants. Maternal ARA (C20:4n-6, LCPUFA) plasma concentrations were decreased in PL fraction in response to maternal obesity in women carrying male infants. Plasma adrenic acid (docosatetraenoic acid, C22:4n-6) concentrations were increased in the NEFA fraction in obese women with a female fetus whereas maternal plasma levels were decreased in NEFA fraction in male babies. Finally, maternal EPA (eicosapentaenoic acid, C20:5n-3) concentrations were lower in the PL fraction in obese women with a male, but not female, fetus. Interestingly, the total PL content was decreased in obese mothers carrying a male baby. The POA/PA ratio in PL was negatively correlated with maternal BMI in women with male fetuses (**Fig. 1**). The entire FA profile of obese and control mothers separated by neonatal sex is presented in **Supplemental Table A2**.



**Fig. 1:** Maternal plasma POA/PA ratio in PL and BMI of women with male infants. Palmitoleic acid/Palmitic acid (POA/PA ratio) in maternal circulating phospholipids, reflecting the SCD1 enzyme activity, is negatively correlated with maternal BMI in women with male fetuses only.

We found also significant changes in the plasma of umbilical vein and artery between obese and normal BMI mothers depending of fetal sex (**Table 3**).

**Table 3:** Fatty acid concentrations in lipid classes in umbilical cord plasma in obese (ob) women compared to normal BMI (Ctr) women.

Plasma	Umbilical Cord Vein				Umbilical Cord Artery			
	Female		Male		Female		Male	
FA ( $\mu\text{mol/L}$ )	Ctr (n=9)	Ob (n=11)	Ctr (n=7)	Ob (n=5)	Ctr (n=9)	Ob (n=11)	Ctr (n=7)	Ob (n=5)
<i>Cholesterol ester</i>								
<b>C18:0</b>	40.0 $\pm$ 12.9	21.8 $\pm$ 3.7	43.8 $\pm$ 11.5	15.4 $\pm$ 4.4*	28.3 $\pm$ 6.4	21.7 $\pm$ 5.9	40.2 $\pm$ 5.0	36.9 $\pm$ 15.9
<b>C18:1n-9</b>	197.4 $\pm$ 36.6	282.6 $\pm$ 49.3	248.3 $\pm$ 56.3	147.3 $\pm$ 52.6	295.6 $\pm$ 63.2	244.6 $\pm$ 49.9	259.3 $\pm$ 58.0	129.3 $\pm$ 38.1
<b>C18:2n-6</b>	51.8 $\pm$ 14.4	80.7 $\pm$ 11.1	84.4 $\pm$ 28.5	36.1 $\pm$ 15.2	234.5 $\pm$ 116.1	85.7 $\pm$ 12.0	60.0 $\pm$ 14.6	29.8 $\pm$ 11.7
<b>C22:6n-3</b>	4.5 $\pm$ 1.1	4.2 $\pm$ 0.7	5.7 $\pm$ 0.6	2.8 $\pm$ 1.4	4.8 $\pm$ 1.0	4.1 $\pm$ 0.7	7.2 $\pm$ 1.0	2.5 $\pm$ 1.2*
<b>Total</b>	568.9 $\pm$ 89.1	618.1 $\pm$ 86.5	748.4 $\pm$ 94.7	431.5 $\pm$ 89.1*	871.4 $\pm$ 188.3	612.0 $\pm$ 86.5	687.8 $\pm$ 92.7	442.2 $\pm$ 124.7
<i>Triglycerides</i>								
<b>C16:0</b>	88.3 $\pm$ 16.7	125.1 $\pm$ 15.2	122.1 $\pm$ 21.3	204.1 $\pm$ 71.9	103.4 $\pm$ 19.5	136.4 $\pm$ 19.8	147.2 $\pm$ 55.6	196.3 $\pm$ 63.1
<b>C18:1n-9</b>	179.1 $\pm$ 52.1	216.1 $\pm$ 27.3	183.8 $\pm$ 26.1	216.6 $\pm$ 40.3	379.3 $\pm$ 139.5	271.3 $\pm$ 56.4	176.1 $\pm$ 21.3	201.5 $\pm$ 55.1
<b>C18:2n-6</b>	42.4 $\pm$ 17.1	42.1 $\pm$ 6.0	47.6 $\pm$ 6.8	49.0 $\pm$ 19.3	38.2 $\pm$ 7.7	41.3 $\pm$ 9.2	45.0 $\pm$ 7.7	29.9 $\pm$ 12.6
<b>C22:6n-3</b>	7.8 $\pm$ 1.4	10.5 $\pm$ 3.2	16.6 $\pm$ 2.2	9.6 $\pm$ 5.3	6.3 $\pm$ 1.3	9.4 $\pm$ 2.7	14.4 $\pm$ 2.7	7.0 $\pm$ 3.3
<b>Total</b>	405.4 $\pm$ 87.3	492.7 $\pm$ 39.9	502.5 $\pm$ 65.8	601.7 $\pm$ 127.4	653.2 $\pm$ 182.9	565.3 $\pm$ 82.9	519.5 $\pm$ 93.8	558.5 $\pm$ 144.6
<i>Non esterified fatty acids</i>								
<b>C16:0</b>	100.7 $\pm$ 23.7	54.7 $\pm$ 17.4	76.5 $\pm$ 44.0	114.0 $\pm$ 19.6	86.0 $\pm$ 44.2	122.7 $\pm$ 19.0*	178.5 $\pm$ 85.6	113.5 $\pm$ 49.7
<b>C16:1n-7</b>	2.6 $\pm$ 0.6	1.1 $\pm$ 0.4*	1.5 $\pm$ 0.1	4.2 $\pm$ 1.2**	3.0 $\pm$ 0.6	1.0 $\pm$ 0.3*	1.2 $\pm$ 0.2	2.9 $\pm$ 1.1
<b>C18:1n-9</b>	25.6 $\pm$ 5.0	15.3 $\pm$ 1.8*	29.7 $\pm$ 9.3	15.8 $\pm$ 2.8	38.5 $\pm$ 13.3	11.5 $\pm$ 3.0*	14.8 $\pm$ 5.1	20.0 $\pm$ 8.3
<b>C22:4n-6</b>	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	0.9 $\pm$ 0.1	0.9 $\pm$ 0.2	1.2 $\pm$ 0.7	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2	1.1 $\pm$ 0.52
<b>Total</b>	319.6 $\pm$ 56.1	164.1 $\pm$ 24.8*	253.1 $\pm$ 91.0	334.4 $\pm$ 117.6	271.4 $\pm$ 57.5	286.1 $\pm$ 48.8	348.1 $\pm$ 82.1	308.6 $\pm$ 96.5
<i>Phospholipids</i>								
<b>C14:0</b>	7.6 $\pm$ 2.2	10.4 $\pm$ 2.8	16.6 $\pm$ 5.8	6.3 $\pm$ 1.3	8.7 $\pm$ 3.0	9.6 $\pm$ 2.1	19.3 $\pm$ 5.2	7.0 $\pm$ 0.3*
<b>C16:0</b>	307.3 $\pm$ 30.1	266.9 $\pm$ 33.5	384.8 $\pm$ 61.1	252.9 $\pm$ 33.7	272.9 $\pm$ 29.6	286.5 $\pm$ 33.7	341.0 $\pm$ 62.0	377.7 $\pm$ 46.9
<b>C16:1n-7</b>	6.9 $\pm$ 2.3	3.4 $\pm$ 0.6	4.7 $\pm$ 0.9	4.3 $\pm$ 0.8	6.3 $\pm$ 2.2	4.0 $\pm$ 0.6	4.8 $\pm$ 1.2	3.6 $\pm$ 0.4
<b>C18:1n-9</b>	96.5 $\pm$ 18.0	61.1 $\pm$ 9.7	94.1 $\pm$ 22.3	50.3 $\pm$ 6.1	87.4 $\pm$ 13.8	69.2 $\pm$ 12.0	90.3 $\pm$ 22.1	68.1 $\pm$ 14.1
<b>C20:4n-6</b>	293.9 $\pm$ 27.6	232.3 $\pm$ 13.4	332.6 $\pm$ 45.3	169.3 $\pm$ 25.9*	268.1 $\pm$ 32.1	243.1 $\pm$ 15.9	284.8 $\pm$ 39.3	177.6 $\pm$ 16.6*
<b>C20:5n-3</b>	5.2 $\pm$ 1.5	3.9 $\pm$ 0.7	4.5 $\pm$ 1.6	2.0 $\pm$ 0.9	4.4 $\pm$ 1.3	3.5 $\pm$ 0.7	2.8 $\pm$ 0.9	1.6 $\pm$ 0.5
<b>C22:6n-3</b>	46.2 $\pm$ 4.3	38.3 $\pm$ 3.6	60.2 $\pm$ 5.1	28.0 $\pm$ 5.5**	43.1 $\pm$ 5.7	38.7 $\pm$ 3.7	51.7 $\pm$ 5.5	29.9 $\pm$ 3.8*
<b>Total</b>	1121.0 $\pm$ 105.1	931.5 $\pm$ 92.8	1322.3 $\pm$ 185.9	806.0 $\pm$ 141.6	1015.3 $\pm$ 98.8	998.2 $\pm$ 92.2	1179.5 $\pm$ 181.2	921.2 $\pm$ 105.7

Mann Whitney test between Obese and Ctr groups within each fetal sex, Mean  $\pm$  SEM, \* p<0.05, \*\* p< 0.005

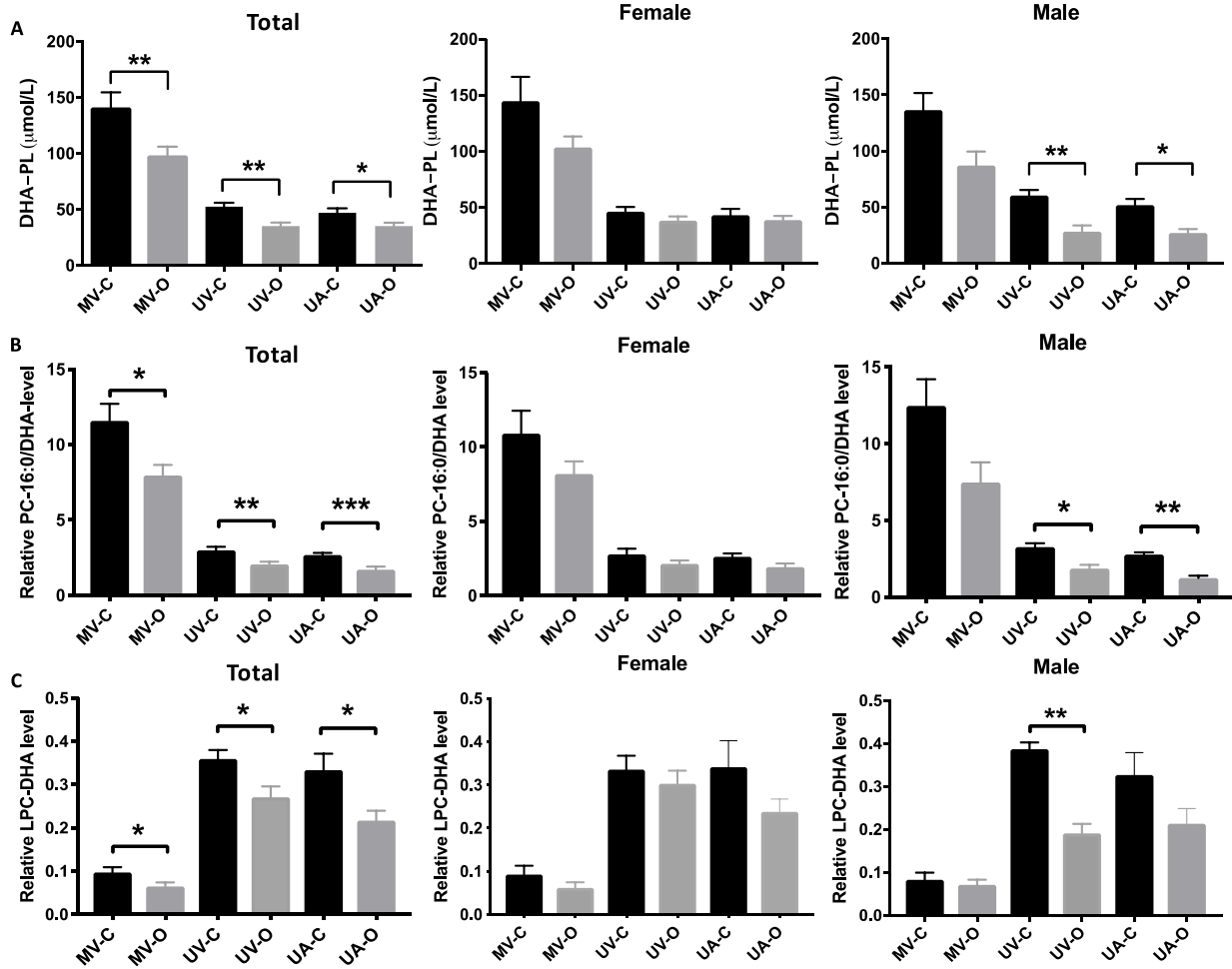
In umbilical vein, total FA content and in particular stearic acid (SA, C18:0) levels were significantly decreased in CE fraction in male fetuses in response to maternal obesity. Total FA content in NEFA was decreased with oleic acid (C18:1n-9) levels lower in both umbilical vein and artery in female infants of obese mothers. Interestingly, PA (C16:0) concentrations were higher in the NEFA fraction in umbilical artery but not in vein, in female neonates of obese mothers suggesting fetal PA synthesis. Again, in female fetuses, POA (C16:1n-7) concentrations in the NEFA fraction were decreased in both umbilical vein and artery plasma if the mother was obese. Conversely, in male fetuses, both plasma umbilical vein and artery POA levels in the NEFA fraction were markedly higher in obese women, albeit significant only for the umbilical vein.

Both the umbilical venous (V) and arterial (A) plasma DHA (C22:6n-3) and ARA (C20:4n-6) concentrations in the phospholipid fraction were markedly reduced in male fetuses of obese mothers with no umbilical V-A difference (data not shown), consistent with decreased placental

delivery resulting in LCPUFA deficiency in male infants. The complete FA composition in four lipid classes in umbilical cord artery and vein plasma of male and female neonates from obese and control women are presented in **Supplemental Table A3**. Direct comparison between NEFA levels in plasma in umbilical cord vein and artery did not reach statistical significance in either fetal sex group likely because we were not powered to determine these differences (data not shown).

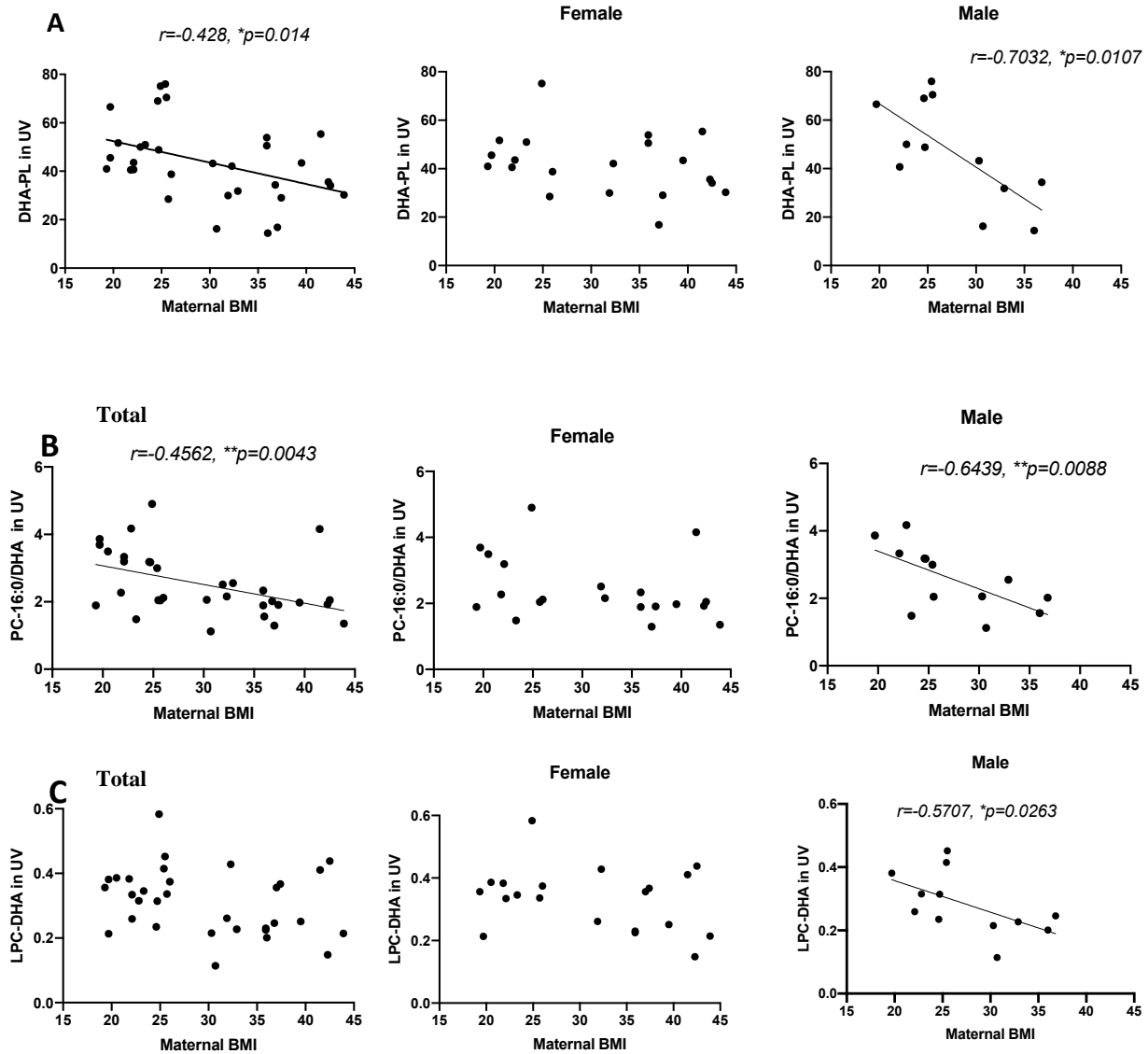
### ***3.2. DHA in phosphatidylcholine fraction is reduced in obese women carrying male fetuses.***

When analyzing all samples, without accounting for fetal sex, DHA concentrations in the PL fraction (DHA-PL) were decreased in maternal, and both umbilical venous and arterial plasma in association to maternal obesity. The observed decreased in DHA-PL in the umbilical circulation was almost entirely due to changes in male fetuses (**Fig. 2A**). We quantified targeted phosphatidylcholine (PC) species and lysophosphatidylcholine (LPC) containing DHA. We found decreased levels of PC containing C16:0 (PA) and DHA (PC-C16:0/DHA species) in umbilical plasma of obese mothers carrying a male, but not in pregnancies with a female fetus (**Fig. 2B**). Similarly, LPC-DHA levels were lower in umbilical vein in male fetuses when the mothers were obese (**Fig. 2C**).



**Fig 2:** DHA levels in different subclasses of PL in maternal and fetal plasma in obese and normal BMI women. (A) Plasma DHA-PL concentrations in all subjects (Total) in female, and in male groups, (B) plasma relative PC-16:0/DHA levels and (C) plasma relative LPC-DHA levels regarding the fetal sex. C=control, O=obese, MV=maternal vein, UV=umbilical cord vein, UA=umbilical cord artery. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ .

DHA-PL, PC-C16:0/DHA and LPC-DHA contents in umbilical venous plasma of males were all significantly negatively correlated with maternal BMI (Fig. 3A, B and C).



**Fig 3:** Correlations between umbilical vein DHA levels in different lipid subclasses and maternal BMI. Umbilical vein (UV) DHA-PL in total subjects, in female and in male groups (A), PC-16:0/DHA (B) and LPC-DHA (C) and maternal BMI were negatively correlated to maternal BMI in women with male, but not female, fetuses.

### 3.3. Placental fatty acid concentrations

Fatty acid concentrations in lipid classes in placenta from obese women and controls demonstrated a sex dependence (Table 4). Decreased saturated lauric (C12:0) and palmitic (C16:0) acids were observed in cholesterol ester fraction of female placentas while myristic

(C14:0) and stearic (C18:0) acids in CE were increased in male placentas if the mother was obese. Total placental CE content was decreased in obese women with female fetuses only. Palmitoleic acid (C16:1n-7, POA) level was decreased in the NEFA fraction in both female and male placentas of obese mothers while the oleic acid (C18:1n-9, OA) level was significantly decreased in male placentas if the mother was obese.

**Table 4:** Placental FA concentrations in lipid classes in obese (ob) women compared to normal BMI (Ctr) women.

FA ( $\mu\text{mol/g protein}$ )	Placenta			
	Female		Male	
	Ctr (n=9)	Ob (n=11)	Ctr (n=7)	Ob (n=5)
<i>Cholesterol ester</i>				
<b>C12:0</b>	0.6 $\pm$ 0.1	0.3 $\pm$ 0.1*	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1
<b>C14:0</b>	2.4 $\pm$ 0.6	1.0 $\pm$ 0.3	1.1 $\pm$ 0.2	2.3 $\pm$ 0.3*
<b>C16:0</b>	41.6 $\pm$ 10.1	13.7 $\pm$ 4.2*	18.3 $\pm$ 4.2	29.9 $\pm$ 5.3
<b>C18:0</b>	54.5 $\pm$ 16.9	26.2 $\pm$ 6.7	19.8 $\pm$ 5.6	47.3 $\pm$ 11.4*
<b>C18:1n-9</b>	23.2 $\pm$ 7.4	11.2 $\pm$ 2.7	28.8 $\pm$ 9.6	15.1 $\pm$ 5.3
<b>C20:4n-6</b>	7.9 $\pm$ 1.3	5.5 $\pm$ 0.6	6.7 $\pm$ 1.0	5.3 $\pm$ 1.0
<b>C20:5n-3</b>	0.4 $\pm$ 0.2	0.1 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1
<b>C22:6n-3</b>	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1	0.7 $\pm$ 0.3	0.2 $\pm$ 0.1
<b>Total</b>	202.0 $\pm$ 47.1	87.9 $\pm$ 10.78*	127.6 $\pm$ 20.6	144.0 $\pm$ 32.0
<i>Triglycerides</i>				
<b>C18:1n-9</b>	45.6 $\pm$ 9.4	32.6 $\pm$ 7.5	35.0 $\pm$ 7.2	32.9 $\pm$ 12.3
<b>C20:4n-6</b>	26.1 $\pm$ 5.0	20.2 $\pm$ 1.6	25.4 $\pm$ 5.0	22.2 $\pm$ 2.6
<b>C20:5n-3</b>	1.0 $\pm$ 0.4	0.5 $\pm$ 0.1	0.8 $\pm$ 0.2	0.6 $\pm$ 0.2
<b>C22:6n-3</b>	6.2 $\pm$ 1.6	4.6 $\pm$ 0.8	8.7 $\pm$ 2.7	4.1 $\pm$ 0.8
<b>Total</b>	241.0 $\pm$ 29.1	175.4 $\pm$ 12.6	225.4 $\pm$ 43.9	183.7 $\pm$ 34.2
<i>Non esterified fatty acids</i>				
<b>C16:1n-7</b>	1.2 $\pm$ 0.2	0.7 $\pm$ 0.1*	1.1 $\pm$ 0.2	0.3 $\pm$ 0.03*
<b>C18:1n-9</b>	11.8 $\pm$ 2.4	4.9 $\pm$ 1.0*	6.9 $\pm$ 1.7	4.9 $\pm$ 1.0
<b>C20:4n-6</b>	70.7 $\pm$ 16.3	42.2 $\pm$ 3.0	57.4 $\pm$ 17.4	30.9 $\pm$ 3.7*
<b>C20:5n-3</b>	1.4 $\pm$ 0.3	0.9 $\pm$ 0.2	1.1 $\pm$ 0.2	0.5 $\pm$ 0.1*
<b>C22:6n-3</b>	4.6 $\pm$ 0.9	3.5 $\pm$ 0.4	4.9 $\pm$ 1.7	1.8 $\pm$ 0.2*
<b>Total</b>	784.4 $\pm$ 240.4	647.0 $\pm$ 139.9	533.7 $\pm$ 158.0	668.5 $\pm$ 124.2
<i>Phospholipids</i>				
<b>C20:4n-6</b>	79.2 $\pm$ 35.8	102.4 $\pm$ 47.8	36.0 $\pm$ 10.0	17.6 $\pm$ 5.7
<b>C20:5n-3</b>	0.5 $\pm$ 0.2	0.2 $\pm$ 0.1	0.1 $\pm$ 0.03	0.1 $\pm$ 0.1
<b>C22:6n-3</b>	7.4 $\pm$ 4.7	8.0 $\pm$ 4.1	2.4 $\pm$ 0.6	1.0 $\pm$ 0.3
<b>Total</b>	900.5.0 $\pm$ 186.8	711.0 $\pm$ 137.8	745.0 $\pm$ 89.5	737.2 $\pm$ 60.2

Mann Whitney test between Obese and Ctr groups within each fetal sex, Mean  $\pm$  SEM, \* p<0.05, \*\* p< 0.005

Placental levels of ARA (C20:4n-6), EPA (C20:5n-3) and DHA (C22:6n-3) in the NEFA fraction were all lower in male placentas (ARA -46%, p=0.048, EPA -58%, p=0.018 and DHA -63%,

p=0.048) in obese mothers suggesting a reduced source of these essential LCPUFAs for transfer to the male fetus. The placental fatty acid composition in the TG and PL fractions was unaffected by maternal obesity in both females and males. Complete placental FA composition is reported in **Supplemental Tables A4**.

### ***3.4. Effects of fetal sex on placental lipid transporter expression in maternal obesity.***

We determined the expression of the LPC-DHA transporter MFSD2a in MVM and BM from control and obese pregnancies. MFSD2a protein expression in BM was up-regulated in placentas of obese mothers but the difference was significant only in the placenta of males (**Fig 4A-B**). We also found a significant positive correlation between BM MFSD2a protein expression and maternal BMI only in women carrying a male fetus (**Fig. 4C**).

Protein expression of FA transporter proteins FATP1, 2, 4, 6, FAT/CD36, FABPm, PCTp were not different in MVM or BM isolated from placentas of obese mothers compared to controls (**Supplemental Table A6**). We found no effect of fetal sex on expression of these lipid transporters.

### ***3.5. Reduced free carnitine and acyl-carnitine concentrations in female placentas of obese women.***

Maternal obesity was associated with up-regulated protein expression of carnitine transporter (OCTN2) in MVM in female, but not male, placentas (**Fig. 5A-B**). We found a positive correlation between MVM OCTN2 protein expression and maternal BMI in female placentas (**Fig. 5C**).

We also found that maternal obesity was associated with decreased placental free carnitine and FA acyl-carnitine metabolites esterified with MUFA (C16:1n-7, POA and C18:1n-9, OA) in female fetuses (**Table 5**).

**Table 5:** Placental free carnitine and acyl-carnitine metabolite concentrations in obese (ob) women compared to normal BMI (Ctr) women.

(mmol/g protein)	Placenta			
	Female		Male	
<i>Carnitine metabolites</i>	Ctr (n=9)	Ob (n=11)	Ctr (n=7)	Ob (n=5)
<b>Free carnitine</b>	1.0 ± 0.1	0.8 ± 0.1**	0.9 ± 0.2	0.9 ± 0.04
<b>Decenoyl C10:1</b>	0.09 ± 0.02	0.03 ± 0.01**	0.03 ± 0.01	0.07 ± 0.01*
<b>Lauroyl C12:1</b>	2.0 ± 0.5	0.7 ± 0.1**	1.2 ± 0.3	1.0 ± 0.2
<b>Myristoleyl C14:1</b>	9.6 ± 2.3	3.0 ± 0.6*	4.7 ± 0.9	5.0 ± 1.3
<b>Palmitoleyl C16:1</b>	2.8E-07 ± 4.7E-08	1.4E-07 ± 2.2E-08*	0.2E-07 ± 0.1E-07	0.2E-07 ± 0.1E-07
<b>Oleyl C18:1</b>	1.1E-06 ± 1.7E-07	6.7E-07 ± 1.2E-07*	0.1E-06 ± 0.1E-07	0.7E-07 ± 0.1E-07
<b>C14:1OH</b>	0.5 ± 0.1	0.2 ± 0.04*	0.3 ± 0.1	0.3 ± 0.04
<b>C6DC</b>	0.3 ± 0.03	0.2 ± 0.02*	0.2 ± 0.03	0.3 ± 0.06

Mann Whitney test between Obese and Ctr groups within each fetal sex, Mean ± SEM, \* p<0.05, \*\* p<0.005

Table only lists carnitine metabolites that were significantly different in maternal obesity

Furthermore, total POA and POA-carnitine concentrations were positively correlated in female placentas of obese mothers ( $r=0.726$ ,  $p=0.017$ ). Maternal obesity did not affect placental concentrations of short chain fatty acid-carnitine metabolites or carnitine esterified with saturated fatty acid in either fetal sex (**Supplemental Table A5**). We found no change in expression of placental carnitine palmitoyl transferase 1 (CPT1a and b) and 2 (CPT2) in obese vs control mothers in either female and male fetuses (**Fig. 6A-B**).

### 3.8. Up-regulation of esterification enzymes in female placenta of obese women.

Protein expression of diacylglycerol-o-acyltransferase 2 (DGAT2), a key enzyme for esterification of FA into TG, was up-regulated only in female placentas of obese mothers (**Fig. 7A-B**) suggesting an increased FA esterification to TG. We found no changes in protein expression of placental DGAT1 and Stearoyl-CoA Desaturase 1 (SCD1) in maternal obesity (**Supplemental Table A6**).

#### 4. Discussion

To the best of our knowledge, this is the first study to investigate protein expression of specific enzymes involved in FA oxidation and esterification and lipid transporters, FA content in four lipid classes, acyl-carnitine metabolites and free-carnitine in placental tissue and FA and phosphatidylcholine (PC, LPC) content of maternal and umbilical cord plasma in maternal obesity. We found a strikingly different effect of maternal obesity on placental lipid metabolism that was impacted by fetal sex. Obese mothers with male fetuses have lower circulating DHA in the PL fraction and evidence of increased placental DHA transfer capacity, represented by higher MFSD2a expression in the BM. Moreover, males of obese mothers have lower circulating DHA in the phospholipid fraction in umbilical cord venous plasma at birth. These data suggest a decreased availability of DHA, a LCPUFA critical for brain development, in the male fetus of obese mothers and this, despite up-regulation of the specific lysophosphatidylcholine-DHA, MFSD2a transporter in the BM. Conversely, female fetuses have reduced placental free carnitine and MUFA-carnitines even though the carnitine uptake transporter (OCTN2) was higher in the MVM of female placentas. Maternal obesity was also associated with higher expression of esterification enzyme DGAT2 in female placentas.

The impact of maternal obesity on lipid profiles in the umbilical cord plasma and the expression of placental lipid transporters, particularly for DHA, and whether lipid status is influenced by fetal sex, remains poorly understood. Male infants are born with a higher birth weight compared to females in both normal BMI pregnancies and pregnancies complicated by maternal obesity. In our small cohort, albeit a clear trend towards fetal overgrowth in both female and male infants of obese mothers, these differences did not reach statistical significance. We also found no significant increase in circulating NEFA in obese mothers regardless the fetal sex as well as no changes in FA levels in the TG fraction. Our findings are consistent with the recent study of Gazquez and coworkers showing no hyperlipidemia in obese women at term [39] and with other studies showing no maternal NEFA and TG changes in obese mothers without diabetes compared to controls [5, 6]. However, we found increased total FA in the cholesterol ester fraction and a trend to higher total FA content in the TG fraction only in obese women carrying female babies suggesting a fetal sex effect on the circulating maternal plasma lipids.

We found decreased DHA in the phospholipid fraction in maternal circulation of obese women compared to controls, which may be related to lower dietary intake of n-3 LCPUFA and higher consumption of saturated FAs in obese pregnant women [40]. LCPUFA levels are also influenced by genetically determined capacity to metabolize polyunsaturated fatty acids [41] through delta-5 and 6 desaturases, encoded respectively by FADS1 and FADS2 genes which are the rate-limiting enzymes for PUFA conversion from essential fatty acid (EFA) and are recognized as main determinants of LCPUFA levels [42]. The observation that plasma EFA concentrations were unchanged in obese women compared to controls in our study, whereas maternal obesity was associated with low circulating DHA and EPA (C20:5n-3, LCPUFA) is consistent with the possibility that desaturase enzyme activity is reduced in obese mothers. DHA is readily incorporated in PL, in particular PC in circulating maternal lipoprotein (HDL, LDL, VLDL). In the current study, reduced levels DHA in circulating PC species in obese mothers suggest modifications in PC profiles perhaps due to poor dietary intake of n-3 LCPUFA and/or choline. It has been previously reported that pregnancy alters choline dynamics reducing the CDP-choline pathway which is critical for PC synthesis containing LCPUFA, particularly DHA [43]. A limitation is the lack of dietary information of women included in our study.

We observed lower umbilical vein DHA-PL concentrations in maternal obesity suggesting a decrease in placental DHA transport capacity. These results are in agreement with reduced materno-fetal transfer of LA and DHA observed in obese pregnant women compared to normal weight women in a recent study by Gazquez et al. [44]. We found lower DHA-PL and PC C16:0/DHA species only in male fetuses. Similarly, LPC-DHA was reduced in umbilical vein and in placenta in male infants of obese mothers while protein expression of MFSD2a, a lysophosphatidylcholine transporter, was upregulated in the basal membrane (BM) of these placentas. This data suggests a compensatory mechanism to increase the transfer of LPC-DHA to the fetus that was not sufficient to maintain normal LPC-DHA in the male fetus. This appears to be a specific response since no changes were found in FATPs, FABPm or CD36/FAT protein expression in the MVM or BM in placenta of either sex in association to maternal obesity. This specific response is consistent with our previous study in healthy women showing a strong correlation between MFSD2a expression in BM of the placenta and the LPC-DHA levels in umbilical cord venous plasma [45]. The lower circulating DHA-PL, PC-C16:0/DHA and LPC-DHA in the umbilical circulation of male fetuses of obese mothers could be due to rapid DHA

uptake in fetal tissues, including across the blood brain barrier, however this would result in lower LPC-DHA levels predominantly in the umbilical artery, which was not the case.

A recent study showed males born to mothers supplemented with essential fatty acids including DHA had significantly larger total brain volume when compared to the placebo group, suggesting a direct link between DHA availability and brain growth in male fetuses [7]. Moreover, the authors showed only in males, a positive effect of the EFA supplementation on corpus callosum size [7], brain region which has been related to significant changes in the Autism Spectrum Disorders including Attention Deficit Hyperactive Disorder that affects boys more frequently than girls [46]. Although the effect of decreased DHA supply on brain growth and development remains to be fully established, we speculate that limitations in DHA availability in male fetuses of obese mothers may negatively influence the development of the brain, programming early life neurodevelopmental disorders. Male fetuses grow more rapidly from early pregnancy and may invest in brain growth rather than placental growth [47]. Previous data suggest that male placentas are more efficient and rapidly transfer nutrients to the developing fetus and have less reserve capacity [48]. Therefore, males in utero may be more dependent on their mothers' dietary intake of DHA and transport across the placenta, leading to higher risk of nutrient transfer deficiency in women with reduced maternal DHA supply, which was the case in the obese mothers of this study. Increasing MFSD2a expression in BM may represent an adaptive regulatory mechanism to enhance DHA supply to the fetus in maternal obesity but the increased transporter expression was not adequate to maintain fetal levels in the males of obese mothers in this cohort.

We found a trend for enhanced oleic acid (OA, C18:1n-9) content in maternal circulating TG and PL suggesting greater OA availability for transfer but only in the obese mothers carrying female infants. However, we found decreased placental OA in the NEFA fraction in female placentas of obese mothers compared to controls, suggesting decreased placental OA uptake from the maternal circulation with unchanged MVM FATP expression. Despite lower OA-NEFA concentrations in female placentas in maternal obesity, placental OA in TG fraction were not different in obese mothers. DGAT2 is primarily responsible for incorporating OA into TG and placental DGAT2 protein expression was increased in female placentas in maternal obesity, which may explain the increase in OA incorporation into TG and could contribute to lower OA as NEFA in these placentas.

The maternal plasma concentrations of palmitoleic acid (POA, C16:1n-7), an anti-inflammatory signaling fatty acid, in the PL fraction were decreased by 77% in obese women with male babies. Moreover, the circulating concentration of palmitic acid (PA, C16:0), the precursor of POA, were decreased by 31% in the PL fraction, resulting to a trend to lower POA/PA ratio in PL in obese women carrying a male infant. This ratio reflects the activity of SCD1 (Stearoyl-CoA desaturase 1), which catalyzes the synthesis of monounsaturated fatty acids, mainly oleic and palmitoleic acids, in the liver. Although SCD1 activity can be induced by high fat intake [49], we found a negative correlation between POA/PA ratio in maternal circulation and the pre-pregnancy BMI of women with male infants only, indicating an effect of fetal sex on SCD1 activity in pregnancies complicated by maternal obesity. The levels of POA in serum cholesterol ester reflect the hepatic lipid pool of carbon flux from carbohydrates to fatty acids [50], however we found no difference in CE POA levels in association to maternal obesity.

Placental non-esterified POA concentrations were decreased in maternal obesity, independent of fetal sex, an indication of lower placental POA uptake and/or synthesis. However, placental SCD1 expression was unaffected by maternal obesity, consistent with our previous data in primary human trophoblast cells isolated from placentas obtained from pregnancies of obese women [51]. Thus, lower placental POA levels in maternal obesity are likely to be due to decreased POA uptake from the maternal circulation by the syncytiotrophoblast. In general agreement with placental levels, umbilical vein POA concentrations were lower in maternal obesity in female fetuses. In contrast, maternal obesity was associated with increased umbilical vein POA levels in male fetuses. Because expression of fatty acid transporters was unchanged in the basal membrane of male placentas from obese mothers, the higher POA levels in circulation suggest a higher POA synthesis in the male fetal liver, possibly due to an activation of the SCD1. Higher POA content in male fetal circulation at birth may be associated with the risk of developing metabolic syndrome since higher POA concentration in plasma of obese children was associated with abdominal adiposity [52].

Maternal obesity was associated with decreased placental free carnitine in female fetuses, in general agreement with previous reports of lower serum total carnitine levels (free + esters) in obese women [53]. Protein expression of OCTN2 in MVM was increased in maternal obesity suggesting that the lower free carnitine is unlikely to be due to decreased capacity to transport carnitine across the maternal-facing plasma membrane of the syncytiotrophoblast. Therefore

lower maternal carnitine concentrations [53] and decreased activity of placental enzymes involved in the *de novo* synthesis of carnitine, in particular gamma-butyrobetaine deoxygenase (gamma-BBD) [16], may contribute to lower carnitine content in female placentas of obese women. Moreover, a higher use of carnitine for acyl-CoA transport into the mitochondria to provide the necessary substrates for production of energy [54], may also contribute to our findings. However, the placental protein expression of CPT1 (a or b isoforms) and CPT2 in female placentas was unaffected by maternal obesity. These findings are consistent with our previous observations in cultured primary human trophoblast cells [51] showing no effect of maternal obesity on CPT1a protein expression [55]. Levels of placental MUFA-carnitine metabolites were also reduced in female fetuses in maternal obesity, suggesting lower uptake of MUFA, increased beta-oxidation or lack of adequate supply of carnitine. Low placental carnitine and low MUFA-carnitine levels may lead to lower carnitine transfer to the fetus.

Taken together, these findings suggest a down-regulation of placental mitochondrial function only in female placentas of obese women. These results are consistent with Muralimanoharan *et al.* showing an activation of NFκB1, which induces expression of miR-210 and mitochondrial dysfunction in trophoblasts of female placentas of obese mothers potentially contributing to programming female fetuses of obese mothers for metabolic diseases in later life [56]. Further investigations are needed to clarify the role of low carnitine in female placentas in pregnancies complicated by obesity.

## **5. Conclusion**

We demonstrate marked fetal sex-specific responses in placental fatty acid oxidation, esterification and transfer capacity to maternal obesity summarized in Figure 8. Maternal obesity was associated with lower circulating DHA, DHA-PL, PC-C16:0/DHA and LPC-DHA in the umbilical circulation of male, but not female, fetuses, likely due to decreased placental DHA transfer. Reduced delivery of these critical fatty acids may negatively affect fetal brain development in males of obese mothers. We have also found that maternal obesity is associated with reduced placental availability of substrates for beta-oxidation (free carnitine and MUFA-carnitine) only in females, despite increased expression of MVM carnitine transporters. Reduced beta-oxidation and higher fatty acid esterification enzyme activity in female placentas may contribute to impaired lipid metabolism in female offspring of obese mothers. This current report

demonstrates the critical importance of evaluating placental functional responses to maternal metabolic disease in male and female separately in order to better understand the role of the placenta in determining lifelong health and risk for disease.

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## **8. CRediT Author statement**

**Theresa L. Powell:** Conceptualization, Writing-Review and Editing. **Kelsey Barner:** Validation, Lana Madi: Validation. **Michael Armstrong and Jonathan Manke:** methodology. **Charis Uhlson:** Validation, **Thomas Jansson:** Writing-Review and Editing. **Veronique Ferchaud-Roucher:** Conceptualization, Investigation, Writing-Review and Editing.

## **9. Declaration of competing interest**

The authors declare no competing financial interests.

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