

Article

Decreased fatty acid transporter FABP1 and increased isoprostanes and neuroprostanes in the human term placenta: implications for inflammation and birth weight in maternal pre-gestational obesity

Livia Belcastro^{1&}, Carolina S. Ferreira^{2&}, Marcelle A. Saraiva³, Daniela B. Mucci⁴, Antonio Murgia⁵, Carla Lai⁶, Claire Vigor⁷, Camille Oger⁸, Jean-Marie Galano⁹, Gabriela D.A. Pinto¹⁰, Julian L. Griffin¹¹, Alexandre G. Torres¹², Thierry Durand¹³, Graham J. Burton¹⁴, Fátima L.C. Sardinha^{15*}, Tatiana El-Bacha^{16*}

¹Laboratory of Nutritional Biochemistry, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (livia.belcastro@gmail.com);

²LeBioME- Bioactives, Mitochondria and Placental Metabolism Core, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (ferreira_c.s@hotmail.com);

³Laboratory of Nutritional Biochemistry, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (marcelle.a.saraiva@gmail.com);

⁴Laboratory of Nutritional Biochemistry, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (danimucci@gmail.com);

⁵Department of Biochemistry, University of Cambridge (a-murgia@hotmail.it);

⁶University of Cagliari; Department of Biochemistry, University of Cambridge (carla.lai82@gmail.com);

⁷Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, Université de Montpellier, CNRS, ENSCM, Bâtiment Balard, 1919 route de Mende, 34293 Montpellier Cedex 5 (claire.vigor@umontpellier.fr);

⁸Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, Université de Montpellier, CNRS, ENSCM, Bâtiment Balard, 1919 route de Mende, 34293 Montpellier Cedex 5 (camille.oger@umontpellier.fr);

⁹Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, Université de Montpellier, CNRS, ENSCM, Bâtiment Balard, 1919 route de Mende, 34293 Montpellier Cedex 5 (jean-marie.galano@umontpellier.fr);

¹⁰LeBioME- Bioactives, Mitochondria and Placental Metabolism Core, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (gabidap@gmail.com);

¹¹Department of Biochemistry, University of Cambridge; Department of Metabolism, Digestion and Reproduction, Imperial College London (julian.griffin@imperial.ac.uk);

¹²Laboratory of Biochemistry and Chemistry of Lipids, Department of Chemistry; LeBioME- Bioactives, Mitochondria and Placental Metabolism Core, Federal University of Rio de Janeiro (torres@iq.ufrj.br);

¹³Institut des Biomolécules Max Mousseron (IBMM), UMR 5247, Université de Montpellier, CNRS, ENSCM, Bâtiment Balard, 1919 route de Mende, 34293 Montpellier Cedex 5 (thierry.durand@umontpellier.fr);

¹⁴Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge (gjb2@cam.ac.uk);

¹⁵Laboratory of Nutritional Biochemistry, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro (sardinhaflc@nutricao.ufrj.br);

¹⁶LeBioME- Bioactives, Mitochondria and Placental Metabolism Core, Institute of Nutrition Josué de Castro, Federal University of Rio de Janeiro; Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge (tatiana@nutricao.ufrj.br).

&Equally contributed

*Correspondence: tatiana@nutricao.ufrj.br (TE) and sardinhaflc@nutricao.ufrj.br (FLCS); Tel.: +55-21-99766-7997 (TE)

Abstract: The rise in prevalence of obesity in women of reproductive age in both developed and developing countries might propagate intergenerational cycles of detrimental effects on metabolic health, contributing to substantial economic burden on society. Placental lipid metabolism might be disrupted by maternal obesity, which possibly affects the life-long health of the offspring. Here, we investigated placental lipid metabolism and handling from women with pre-gestational obesity as a sole pregnancy complication and compared to placental responses of lean women. Open profile and

targeted lipidomics were used to assess placental lipids and oxidized products of docosahexaenoic acid (DHA), neuroprostanes, and arachidonic acid (AA), isoprostanes. Placental fatty acid transporters FABP1, FABP3 and endothelial lipase protein were measured. Despite no signs of overall alterations in lipid content, increased contents of DHA, AA, DHA-derived neuroprostanes and AA-derived isoprostanes and decreased content of FABP1 protein were found in placentas from obese women. Multivariate analyses suggested that these oxidised fatty acids are associated with maternal and placental inflammation and also with birth weight. These results might shed light on the molecular mechanisms associated with altered fatty acid metabolism and lipid handling in maternal pre-gestational obesity, placing these oxidized fatty acids as novel mediators of placental function.

Keywords: maternal pre-gestational obesity; placenta; lipid metabolism; fatty acid transporter proteins; isoprostanoids; neuroprostanes; isoprostanes; docosahexaenoic acid; arachidonic acid

1. Introduction

Maternal pre-gestational obesity and excessive gestational weight gain affect short- and long-term health of both the mother and her child [1]. Gestational diabetes mellitus and pre-eclampsia are complications of pregnancy associated with gestational obesity and newborns from obese women have an increased risk of overgrowth. Obesity in Brazil [2] and worldwide [3] has reached epidemic proportions, in adults and in children. Therefore, the rise in prevalence of obesity in women of reproductive age in both developed and developing countries might propagate intergenerational cycles of detrimental effects on metabolic health, contributing to substantial economic burden on society [4-7] and highlighting the necessity of determining the mechanisms involved.

Fatty acids are essential for the accretion of body fat in the fetus, especially during the last trimester of pregnancy. Long chain polyunsaturated fatty acids, in particular docosahexaenoic acid (DHA) and arachidonic acid (AA), have specific roles in membrane composition and the development of the retina, and are the major components of the white matter of the brain. Therefore, they are paramount to proper neural and visual development and cognitive function [8-11]. *Ex vivo* placental perfusion data [12] and *in vivo* kinetics of ¹³C-fatty acid [13] show that maternal-fetal ¹³C-labelled lipid transfer is very low (1-6 %), implying that lipid metabolism and handling by the placenta are strictly controlled and that these mechanisms are possibly major players in the allocation of fatty acids to fetal organs [14,15].

The inflammatory milieu imposed by maternal obesity disrupts the cross-talk between maternal signals and the placenta, resulting in impaired placental function [16]. Uptake and metabolism of essential fatty acids, particularly DHA, is impaired in placentas from obese women [17,18]. Increased accumulation of lipids in the placenta has been described in gestational obesity which was associated with decreased oxidation of fatty acids and impaired mitochondrial function [19, 20]. Conversely, others have reported that placental total lipid content [21] and maternal-fetal transfer of ¹³C-labelled non-essential fatty acids [13] is similar between lean and obese mothers. Altogether, these observations emphasize that placental lipid handling and metabolism might be disrupted by maternal obesity and deserve further investigation to clarify the mechanisms involved.

The maternal circulation, and ultimately the maternal diet, are the sources of polyunsaturated fatty acids, as their synthesis by the fetus and the placenta is limited and insufficient to meet the high demand imposed by the growing fetus. Placental fatty acid handling relies on several proteins which are responsible for (a) the uptake of fatty acids from the maternal circulation, partly as lipoproteins and as non-esterified fatty acids and lysophospholipids bound to albumin, and (b) the numerous metabolic fates of fatty acids and also their transfer to the fetus [22]. The hydrolysis of fatty acids from triacylglycerol in lipoproteins is catalyzed by endothelial lipase and by lipoprotein lipase. The former is selective for hydrolyzing unsaturated fatty acids esterified in the *sn*-2 position of glycerol. Fatty acids are then taken up by the placenta by fatty acid translocators (FAT / CD36), fatty acid transport proteins (FATP / SLC27A) and Mfsd2a. In the cytoplasm of the syncytiotrophoblast, fatty

acid binding proteins (FABPs), which are noncatalytic binding proteins, mediate fatty acid metabolism and inflammatory processes [23].

Both the expression and the content of placental fatty acid transport proteins are altered by maternal obesity [19, 24-26]. However, how these changes affect placental fatty acid metabolism and signaling properties and fatty acid transport and availability to the fetus in maternal gestational obesity is not fully understood. Oxidative stress and inflammation have been associated with enhanced contents of oxidized fatty acids and trophoblast dysfunction in pre-eclampsia. In particular, hydroxyeicosatetraenoic acids (HETEs), products of AA oxidation catalyzed by lipoxygenases and CYP, and F₂-isoprostanes, products of non-enzymatic peroxidation of AA, were shown to be increased in placentas from pregnancies complicated by pre-eclampsia [27,28]. All these fatty acid metabolites have some degree of vasoconstrictive and pro-inflammatory effects. Possibly, oxidized fatty acids are associated with placental function and altered fatty acid metabolism in response to maternal obesity, and this deserves investigation.

Here, we characterized the major categories and classes of lipids in term placentas from pregnancies complicated by pre-gestational obesity followed by analysis of fatty acid transport proteins involved in handling of polyunsaturated fatty acids by the placenta. A novel aspect of this study was a thorough characterization of placental non-enzymatically oxidized isoprostanooids derived from AA and DHA, which as inflammatory mediators might be a mechanistic link between pre-gestational obesity and placental dysfunction. Additionally, we assessed how polyunsaturated fatty acids and their oxidized fatty acids metabolites are associated with inflammation and lipid handling by the placenta, and possibly with neonatal outcomes in maternal pre-gestational obesity.

2. Materials and Methods

2.1 Study design and participants

The current study was part of a randomized controlled trial registered in clinicaltrials.org (NCT03215784), which was designed to evaluate the effects of fish oil and probiotics supplementation throughout pregnancy on women with pre-gestational obesity. The study was conducted at the *Maternidade Escola*, between January 2015 and July 2017. This is a referral hospital, belonging to the Federal University of Rio de Janeiro, dedicated to provide pre-natal and delivery care and puerperal consultations to the local community. Women were recruited up to 13 weeks of pregnancy and the inclusion criteria were age between 19-35 y, pre-gestational body mass index (BMI) between 18.5 and 24.9 kg / m² (lean) or ≥ 30 and ≤ 40 kg / m² (obesity class 1 and class 2; 29), absence of pre-existing infectious or chronic disease, except for obesity, a single fetus, and non-smoker.

Gestational weight gain was classified accordingly [30] and gestational outcomes and newborn information (birth and length at birth and head circumference) were obtained from medical charts and classified according to the INTERGROWTH-21st Project Curves [31].

In this study, matched maternal blood, placental samples and umbilical cord blood of 12 women (6 lean and 6 obese) were used. The groups are subsequently referred to as the lean and the obese/pre-gestational obesity.

2.2 Ethics

Women were asked to read and sign a Free and Informed Consent form upon recruitment and this study was approved by the local Ethics committee of the *Maternidade Escola* and by the National Ethics committee (approval number CEP: 34611513.0.0000.5257).

2.3 Biological samples

Maternal blood was collected at the 36th gestational week and placental and umbilical cord blood samples were collected at delivery. The time between delivery and collection was up to 20-30 minutes. Umbilical cord blood was obtained by venipuncture and placental tissue was collected according to [32]. Briefly, placentas were sampled from their maternal surface after removal of 1-2 mm of the basal plate. Villous samples 1-2 cm³ were cut from 4 random sites and washed in Phosphate-buffered saline at 4° C. Smaller fragments for protein (~ 50 mg); transcripts (~ 10 mg) and lipid (50-100 mg) analyses were placed into cryovials and immediately frozen in liquid nitrogen. All samples were stored at -80° C until analysis.

2.4 Chemical and reagents

All standards that were used in the targeted isoprostanoids analyses were synthesized in house, as published [33-37]. Internal standards: C19-16-F_{1t}-PhytoP and C21-15-F_{2t}-IsoP; External standards isoprostanes 15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP and 5-*epi*-5-F_{2t}-IsoP and neuroprostanes 10-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, and 4(RS)-4-F_{4t}-NeuroP.

2.5 Fatty acid transporter proteins in placental tissue

2.5.1 Quantitative real-time PCR

Total RNA from placental tissue was extracted using TRIzol® and PureLink® RNA Mini Kit and addition of PureLink® DNase (Invitrogen™), according to the manufacturer's instructions. The concentration of RNA was determined using a NanoDrop™ spectrophotometer. cDNA synthesis was accomplished using *High-Capacity cDNA Reverse Transcription* (Applied Biosystems®). qPCR was performed using TaqMan™ Universal PCR Master Mix (Applied Biosystems®) with the following primers from TaqMan™ Thermo Fisher Scientific®: Hs00195812 (EL), Hs00155026 (FABP1) and Hs00997360 (FABP3). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene and transcripts were calculated using the threshold cycle 2- $\Delta\Delta$ CT method [38].

2.5.2 Western Blotting

Placental samples were prepared in lysis buffer containing 100 mM Tris (pH 7.5), 10 mM EDTA, 10 % SDS, 10%, 100 mM NaF, 10 μ M sodium pyrophosphate, 10 μ M Na₃VO₄. Tissues were homogenized in a IKA®RW20 instrument. After homogenization was complete, 10 % TritonX-100 was added to the samples. The Bradford method was used to determine protein concentration in the tissue lysates [39]. Placental lysates were prepared in gel loading buffer containing Laemmli buffer (Biorad) (70 mM Tris-HCl (pH 6.8), 50 mM DTT, 1 % SDS, 10 % glycerol and 0.005 % bromophenol blue) and heated at 70° C for 5 min. Samples were loaded and resolved in SDS-PAGE gels in a mini-PROTEAN® System (BioRad) and blotted onto nitrocellulose. The nitrocellulose membrane was blocked with 1 % bovine serum albumin in Tris-HCl 10 mM, 150 mM NaCl, Tween-20 50 μ L/mL and incubated overnight with the primary antibody at 4° C and 2 h at room temperature. The membrane was then incubated with HRP-conjugated secondary antibody (HRP; ABCAM®) for 1 h at room temperature.

Protein bands were analysed by enhanced chemiluminescence (Biorad) and the films were scanned in a flat-bed scanner (ImagiQuant LAS 4000) and band intensities were determined from two different exposures (within the linear detection range) with background subtraction using Image Studio Lite 5.2 software.

Primary antibodies for EL (MBS2013720) from My BioSource®, FABP1 (AB7366) and FABP3 (AB16916) from ABCAM® were used in the following dilutions, respectively: 1:1000 EL, 1:500 and 1:500. Loading control was performed with anti- β -actin antibody (Sigma-Aldich®, SAB5500001).

2.6 Cytokines in plasma and placental tissue

The quantification of the cytokines (IL-1, IL-6, IL-10 and TNF- α) in maternal plasma, umbilical cord plasma and placental tissue protein extract was performed by the Luminex xMAP (Multiple Analytic Profiling) assay [40] using 100 μ g of placental extract as described for Western Blotting analysis.

2.7 Maternal lipoprotein profile

Lipoproteins were assayed using commercial kits (Triglycerides and Monoreagent Cholesterol, Direct LDL and BIOCLIN® Enzymatic HDL Cholesterol), and absorbance measured at 500, 540 and 500 nm, respectively (SpectraMax® Plus 384 spectrophotometer). The concentration of very low density lipoprotein (VLDL-c) was estimated using the formula suggested by Friedewald, Levy and Fredrickson (1972) [41].

2.8 Placental lipid profile analysis

Lipids were extracted using a modified Folch method [42]. Briefly, 0.35 mL of cold methanol and 0.35 mL of chloroform were added to vials containing ~ 50 mg of frozen placental tissue and one metallic bead and were disrupted in a single batch (Tissue Lyser II, Qiagen; 2×2.5 min, 17 s^{-1}). Next, following addition of 0.35 mL of chloroform and 0.15 mL of ultrapure water (Chromasolv; Honeywell, Reidel-de Haën), samples were thoroughly vortexed for 30 seconds and centrifuged ($17,000 \times g$, 10 min). The lower phase containing the lipid extract was transferred to glass vials, dried under a gentle stream of nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

The dried chloroform phase was suspended in 50 μ L methanol containing the internal standards (25 deuterated lipids, representatives of phosphatidic acid, phosphatidylcholines, phosphatidylethanolamines, glycerophospholipids, phosphatidylinositols, phosphatidylserine, sphingomyelin, ceramides, triacylglycerols and fatty acids (Supplementary Table 1) and 75 μ L of a solution of isopropanol:acetonitrile:water (IPA:ACN:H₂O, 2:1:1, v/v). Quality control (QC) samples consisted of a pooled sample containing 10 μ L aliquots of each sample. All samples were analysed in positive and negative mode. For positive mode, the samples and QC were diluted 1:10 with the IPA:ACN:H₂O (2:1:1) solution.

2.8.1 Ion Mobility QTOF LC/MS lipid profile analysis of placenta samples

An Agilent 6560 Ion mobility Quadrupole Time-of-Flight (DTIM-QTOF) LC-MS system coupled with an Agilent 1290 UHPLC system was used to combine separation power and selectivity of LC, DTIM, and MS techniques. The Dual Agilent Jet Stream electrospray ionization source was operated separately in positive and negative ion mode.

The lipid extract was separated in a ACQUITY CSH C18 column (100×2.1 mm and $1.7\text{ }\mu\text{m}$ particle diameter, Waters) conditioned at $55\text{ }^{\circ}\text{C}$. The mobile phase consisted of: (A) 10 mM ammonium formate solution in 40 % ultrapure water and 60 % acetonitrile and (B) 10 mM ammonium formate solution in 90 % isopropanol, and 10 % acetonitrile (v/v). The mobile phase was pumped at a flow rate of 400 μ L/min starting at 60 % of A and 40 % of B. Solvent B was increased linearly to 43 % in 2 min, to 50 % at 2.1 min, to 54 % at 12 min and then to 99 % in 18 min. At 18.1 min solvent B was brought back to the initial conditions and remained at this percentage for 1.9 minutes. The column was re-equilibrated for 3 min at 40 % solvent B.

An Agilent tuning solution was injected before the analysis to tune the instrument in the m/z range 100-1700, and before every 10 samples to perform CCS re-calibration. During samples acquisition, an Agilent reference mix was constantly injected for mass re-calibration. The Agilent Mass Hunter LC-MS acquisition console was used for data acquisition.

2.8.1.1 Positive ion mode

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen gas at 5 L/min (set at 48 mTorr) and 375 °C was used as drying gas for solvent evaporation; sheath gas was set at 275 °C and 12 L/min flow rate. Full-scan spectra were obtained in the ranges of 50 – 1200 amu, scan time of 0.20 sec., scan width of 0.70 amu, and detector set at 2950 V.

2.8.1.2 Negative ion mode

The electrospray capillary potential was set to 60 V, and the needle at 20 kV. Nitrogen gas at 5 L/min (set at 48 mTorr) and at 375 °C was used as drying gas for solvent evaporation, sheath gas was set at 275 °C and 12 L/min flow rate. Full-scan spectra were obtained in the ranges of 50 – 1200 amu, 0.20 sec scan time, 0.70 amu scan width, and detector set at 2950 V.

2.8.1.3 Chromatogram pre-processing

Data pre-processing, including mass and CCS re-calibration and feature finding, was carried out using the packages IM-MS Reprocessor, IM-MS Browser and Mass Profiler from the MassHunter Suite (version B.08.00, Agilent Technologies, Santa Clara, USA).

The resulting data matrices were processed using a KNIME pipeline comprising both KNIME native nodes and integrated R scripts. QC-based feature filtering with missing value 50 and relative standard deviation threshold 0.2 was performed to eliminate noise/background signals and to remove features with poor repeatability. Imputation of missing values was performed using a Key-nearest neighbour (KNN) approach based on the R library impute. Feature annotation was performed based on the AccurateMassSearch node of the OpenMS library [43].

2.9 Isoprostanoids in placental tissue

Isoprostanoids in placenta were determined based on a microLC–MS/MS method [44]. Briefly, lipid extracts were obtained by the Folch method [42], which were then mixed with a mixture of internal standards, followed by alkaline hydrolysis. The metabolites were concentrated via solid phase extraction in weak-anion exchange cartridges, and then analyzed by microLC–MS/MS. Mass spectrometry analysis was performed in a AB Sciex QTRAP 5500 (Sciex Applied Biosystems) with an electrospray ionization source operated in negative mode. Detection of the fragmentation ion products from each deprotonated molecule was performed in the multiple reaction monitoring mode. Quantification of isoprostanoids was performed with the MultiQuant 3.0 software, by the ratio between each specific metabolite peak area and the internal standard peak area calibrated by curves of the metabolite standards peak area versus the internal standard peak area.

2.10 Statistical analyses

Variables' frequency distribution was assessed by standardized coefficients of skewness and kurtosis, and those with values < -2.0 or > +2.0 were characterized as having a non-normal distribution, which were presented as median and interquartile interval, and compared using the Mann-Whitney test. Groups' frequency distributions were compared using the chi-square test. Associations between continuous variables were assessed by Spearman's correlation analysis. Stepwise multiple regression analyses (backward) were used to investigate the effect of independent factors on birth weight and on mothers and placental tissue content of isoprostanoids. The criteria for the inclusion of independent variables in the multiple regression models were based on results from Spearman correlations and on biochemical soundness. In the final model, only significant variables that improved the adjustment of the model were kept (p -to-remove ≥ 0.05 ; p -to-persist < 0.05). The multiple regression models were further assessed by analysis of residual plots that were checked to determine if they were randomly distributed. Data analyses were performed with the GraphPad

Prism 7.0 (GraphPad Software, San Diego, CA, USA) and Statgraphics Centurion 18 (Statgraphics Technologies, Inc.; The Plains, VA, USA). In all analyzes, $p < 0.05$ was considered for rejection of the null hypothesis.

3. Results

3.1. General characteristics of the mothers and newborns

The general characteristics of the mothers included in this study, their gestational outcomes and newborn information are presented in Table 1. Maternal pre-gestational BMI was significantly higher in the obese group ($p < 0.05$) and the median BMI value indicates obesity class I. Additionally, gestational weight gain was on average 40 % lower in the obese mothers than in lean group. No differences were observed in all other measurements, including gestational week at delivery, delivery mode, placental efficiency (weight at birth:placental weight ratio). Newborn outcomes were also similar despite the higher pre-gestational BMI in the obese group.

Table 1: Clinical characterization of the mothers and newborns participating in the study

Mothers	Lean (n = 6)	Obese (n = 6)
Maternal age (years) ^A	26.0 (19.0 – 32.0)	26.5 (20.0 – 31.0)
Maternal pre-gestational BMI (kg / m ²) ^A	22.0 (19.0 – 24.2)	34.3 (31.2 – 37.6)*
Gestational weight gain (kg) ^A	11.6 (10.1-21.3)	6.6 (1.5-19.0)
Gestational age (weeks)	40.5 (38.0-41.0)	39.0 (38.0-41.0)
Complications of pregnancy (other than pre-gestational obesity)	none	none
Delivery mode (n ; %)	vaginal (2; 33); c/s ^B (4; 66)	vaginal (1; 17); c/s ^B (5; 83)
Newborn		
Placental weight (g) ^A	485.0 (400.0-555.0)	480.0 (480.0-575.0)
Placental efficiency (birth weight : placental weight ratio)	6.9 (6.1-10.0)	6.9 (6.4-7.4)
Birth weight (kg) ^A	3.3 (3.1-4.0)	3.4 (3.0-4.0)
Birth length (cm) ^A	49.5 (46.0-53.5)	48.5 (45.0-52.0)

^A values expressed as median (minimum-maximum); * significant different compared to lean women, $p < 0.01$, Mann-Whitney test;

^B c/s, cesarean section.

3.2. Placental lipid profile suggests alterations in long-chain polyunsaturated fatty acids abundance despite no apparent signs of inflammation and dyslipidemia in maternal pre-gestational obesity

The placental lipid profile was characterized using Ion Mobility QTOF LC/MS. Annotated species were separated into the lipid categories glycerophospholipids, sphingolipids, fatty acyls, glycerolipids and sterols [45]. In both groups, glycerophospholipids was the most abundant lipid category corresponding to 70 % of the annotated lipid signals, followed by sphingolipids (17 %), fatty acyls (8 %), glycerolipids (5 %) and sterols (< 1 %) (Figure 1A). No differences were observed in any lipid categories between the lean and obese groups. The categories of glycerophospholipids, sphingolipids and glycerolipids were divided into lipid classes and those that were the most abundant and with biological significance classes are shown in Figure 1B, represented as fold change relative to placentas from lean women. In the glycerophospholipid category, no significant differences were observed between lean and obese except for the content of lysophospholipids, which presented a tendency to a marginal ($p = 0.056$) 1.25-fold increase in placentas from the obese compared

to the lean group. Ceramides and sphingomyelins were the most abundant species in the sphingolipid category that showed similar contents between groups. Lastly, in the glycerolipid category, both groups showed similar contents of monoacylglycerol, diacylglycerol and triacylglycerol.

Looking at free fatty acid species of the fatty acyl category, some important long-chain *n*-3 polyunsaturated fatty acids varied between groups (Figure 1C) although total relative abundance did not differ between the groups (Figure 1A). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) abundance was, respectively, 1.74- and 2.20 times higher in the obese than the lean group ($p < 0.05$). Additionally, the mean value of arachidonic acid (AA) was 2.5-fold higher in the obese group, despite p -value did not reach significance cut-off. Variation in the composition of free fatty acids was restricted to essential polyunsaturated fatty acids, as contents of palmitic and stearic (saturated) and of palmitoleic and oleic (monounsaturated) fatty acids were similar in the lean and obese groups (Figure 1D).

Maternal obesity is often associated with an inflammatory milieu and dyslipidemia. Hence, the levels of IL-1 β , IL-6, IL-10 and TNF- α were measured in maternal blood, in placental tissue and in the umbilical cord blood (Supplementary Figure 1). No differences were found in any of these inflammatory markers between the lean and obese groups, except for a higher content of IL-1 β in placentas from women with pre-gestational obesity. Likewise, the content of total cholesterol, triacylglycerols and HDL, LDL and VLDL lipoproteins in maternal plasma were similar between lean and obese women (Supplementary figure 2).

These results suggest that placentas from women with pre-gestational obesity did not present signs of overall lipid accumulation but essential polyunsaturated long-chain fatty acids were increased. Furthermore, no apparent signs of inflammation nor maternal dyslipaemia were observed.

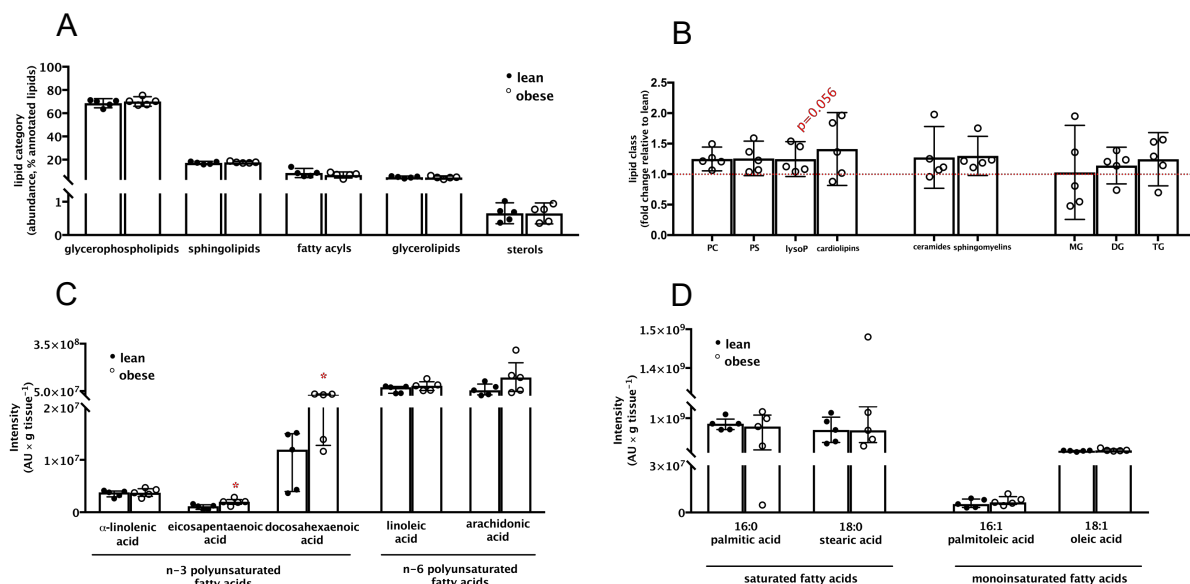


Figure 1: Placental lipid profile suggests alterations in long-chain polyunsaturated free fatty acids abundance in maternal pre-gestational obesity.

Placental lipid profile was determined by Ion Mobility QTOF LC/MS after lipid extraction by the Folch method. Ion intensities were processed using a KNIME pipeline comprising both KNIME native nodes and integrated R scripts and lipids were assigned into categories, classes and species. Placentas from lean (●) and from obese (○) women were compared according to (A) the abundance of lipid categories expressed relative to total lipid annotated; (B) the major lipid classes in each category; and (C) the annotated essential polyunsaturated and (D) saturated and monounsaturated fatty acid species.

PC: phosphatidylcholine; PS: phosphatidylethanolamine; lysoP: lysophospholipids; MG: monoacylglycerols; DG: diacylglycerols; TG: triacylglycerols.

* Significantly different from the lean group; $p < 0.05$ (Mann-Whitney test).

3.3. Fatty acid transporter protein FABP1 is decreased and negatively associated with polyunsaturated fatty acids in placentas from women with pre-gestational obesity

We next analysed the fatty acid transporter proteins that handle unsaturated fatty acids by the placenta: endothelial lipase (EL), fatty acid binding protein-1 (FABP1) and fatty acid binding protein-3 (FABP3), at the protein and mRNA levels (Figure 2A and B). Placentas from the obese group presented a significant 70 % decrease in FABP1 protein content compared with placentas from the lean group ($p < 0.05$) and a non-significant 30 % decrease in mRNA levels (Figure 2B). Endothelial Lipase and FABP3 protein and transcript levels were similar between groups (Figures 2A and 2C). Worth mentioning that median value of EL protein content in placentas from the obese group was 1.2-fold higher compared to the lean group (Figure 2A).

The associations between the changes in FABP1 and essential polyunsaturated fatty acids presented in figure 1C were assessed by Spearman rank correlations, and the ratios fatty acid (FA) to FABP1 were also assessed (Figures 2C-2G). For all *n*-3 fatty acids (Figures 2C-2E) and *n*-6 fatty acids (2F and 2G), the ratio FA:FABP1 was significantly higher in placentas from the obese group. The ratios EPA:FABP1 (Figure 2E), DHA:FABP1 (Figure 2F) and AA:FABP1 (Figure 2H) were, respectively, 5.5-, 7- and 6-fold higher in placentas from the obese compared to the lean group. Spearman correlation analyses also showed that coefficients were negative for all FA with statistically significant results for EPA ($r = -0.82$; $p = 0.02$; Figure 2E) and marginally significant for DHA ($r = -0.57$; $p = 0.09$; Figure 2F) and AA ($r = -0.60$; $p = 0.054$; Figure 2H). Taken together, these results suggest that FABP1 might play a role in placental handling of these fatty acids and that the decrease in its content might have an impact in the availability and signaling properties of polyunsaturated fatty acids, in particular EPA, DHA and AA.

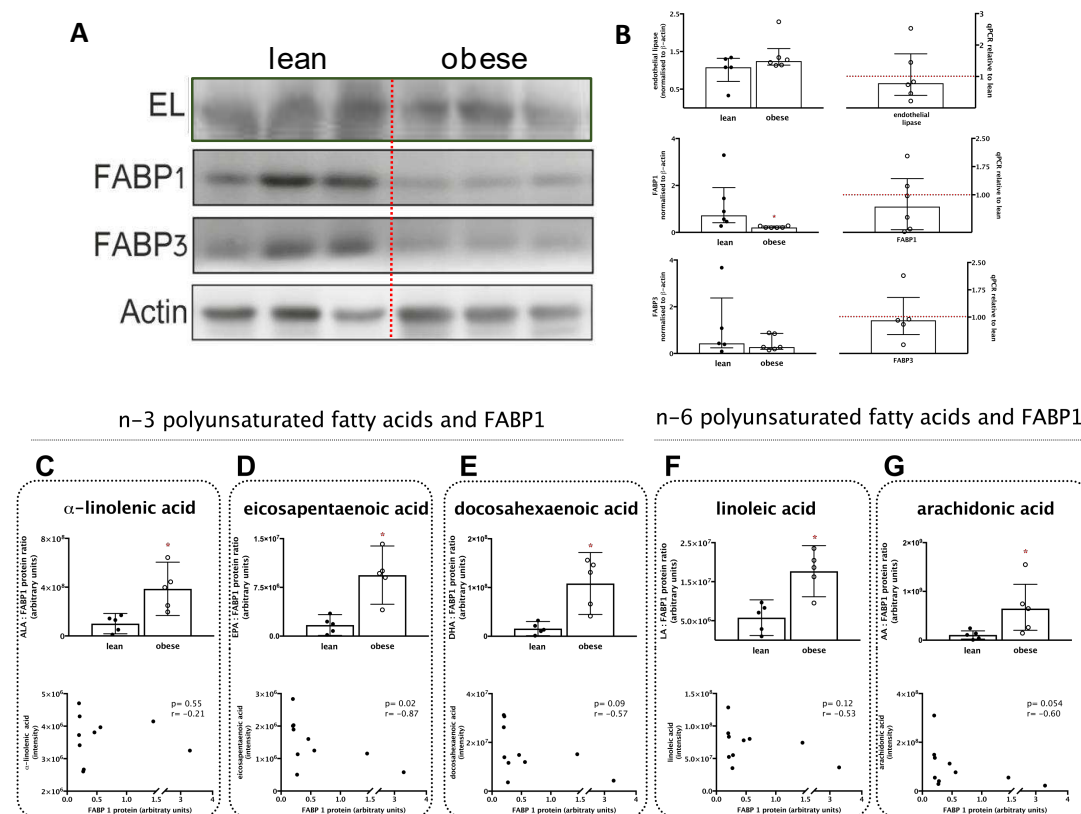


Figure 2: Fatty acid transporter protein FABP1 is decreased and negatively associates with long-chain polyunsaturated fatty acids in placentas from women with pre-gestational obesity.

Placentas from lean (●) and from obese (○) women were compared according to placental fatty acid transporter proteins endothelial lipase (A), FABP1 (B) and FABP3 (C) at the protein and transcript levels. The ratio of polyunsaturated fatty acids to FABP1 and the respective Spearman correlations were calculated for the *n*-3 fatty acids α-linolenic (D), eicosapentaenoic (E) and docosahexaenoic (F)

and for the n-6 fatty acids linoleic (G) and arachidonic (H) to investigate possible associations between alterations in FABP1 and long-chain fatty acid contents as a function of maternal pre-gestational obesity.

* Significantly different from the lean group; $p < 0.05$ (Mann-Whitney test).

3.4. Neuroprostanes and isoprostanes are increased and negatively associated with FABP1 protein in placentas from women with pre-gestational obesity

MicroLC-MS/MS targeted analysis identified seven products of non-enzymatic peroxidation of the essential polyunsaturated fatty acids DHA and AA in placental tissue. Three DHA-derived products named neuroprostanes were found, as follows: 10(R)-10-F_{4t}-NeuroP, 10(S)-10-F_{4t}-NeuroP and 4(RS)-4-F_{4t}-NeuroP- and four AA-derived products named isoprostanes were found, as follows: 15-*epi*-15-F_{2t}-IsoP, 15-F_{2t}-IsoP, 5(RS)-5-F_{2t}-IsoP and 5-F_{2t}-IsoP (Figure 3A). All neuroprostanes and isoprostanes were increased in placentas from women with pre-gestational obesity compared to lean women with significant differences ($p < 0.05$), except for 4(RS)-4-F_{4t}-NeuroP, which was marginally increase ($p = 0.07$). The overall increase of each DHA-derived neuroprostanes was close to 2-fold and of each AA-derived isoprostanes was near 1.7-fold. To investigate the combined behavior of these fatty acids derived from DHA and AA, the sums of neuroprostanes and isoprostanes were compared between groups and the same significant increase in placentas from women with pre-gestational obesity was observed (Figure 3B).

Following the same rationale used to investigate DHA and AA association with FABP1 (Figures 2F and 2H), we calculated the ratio of each neuroprostane and each isoprostane to FABP1 (Figures 3C and E) and performed correlation analyses (Figures 3D and F, respectively). Significantly higher ratios of neuroprostanes and isoprostanes to FABP1 were found in placentas from obese compared to lean women ($p < 0.05$). The neuroprostane to FABP1 ratios were nearly 6-fold higher and isoprostanes to FABP1 ratios were nearly 5-fold higher in placentas from obese women compared to lean. This magnitude of increase was similar to that observed for the free DHA and AA to FABP1 ratios (Figures 2F and 2H). Negative correlations were found between the sum of neuroprostanes and isoprostanes and FABP1, but only the latter reached statistical significance ($r = -0.42$; $p = 0.17$ for neuroprostanes, Figure 2D and $r = -0.66$; $p = 0.02$ for isoprostanes; Figure 2F). Taken together, these results suggest that the decrease in FABP1 protein observed in placentas from women with pre-gestational obesity is, to some degree, associated with the increase in neuroprostanes and isoprostanes observed in these placentas. Possibly, this association is more significant for AA-derived isoprostanes.

The metabolic sources of DHA-derived neuroprostanes, in particular, and AA-derived isoprostanes are not fully known; likely candidates are free DHA and AA, or phospholipids, mainly phosphatidylcholine, enriched in these fatty acids [46]. In an attempt to indirectly address this issue, we calculated the ratios of neuroprostanes to free DHA (Figure 3G) and DHA-enriched phosphatidylcholine species (Figure 3G and 3H) and of isoprostanes to free AA and AA-enriched phosphatidylcholine species (Figure 3I and 3J). No significant differences were observed in the ratios of neuroprostanes and isoprostanes to free FA between lean and obese groups. On the other hand, marginally lower ratios were observed for DHA-enriched phosphatidylcholine species and AA-enriched phosphatidylcholine in placentas from the obese compared to the lean group. These results suggest that DHA and AA in phosphatidylcholine might be less susceptible to non-enzymatic peroxidation in placentas from women with pre-gestational obesity. Non-esterified DHA and AA seem to similarly contribute to synthesis of neuroprostane and isoprostane, respectively, in placentas from lean and obese women.

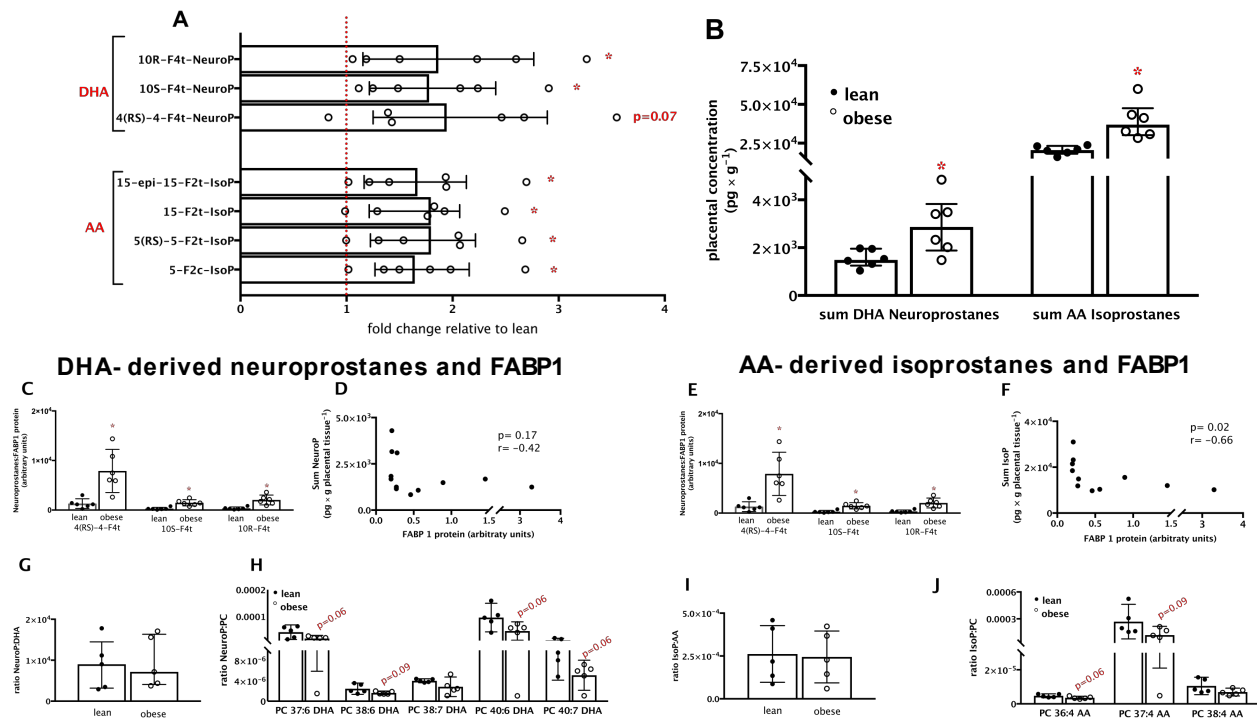


Figure 3: Neuroprostanes and isoprostanes are increased and negatively associated with FABP1 protein in placentas from women with pre-gestational obesity

Products of non-enzymatic peroxidation of the essential polyunsaturated fatty acids docosahexaenoic (DHA) and arachidonic (AA) in placental tissue were analyzed by target microLC-MS/MS. Three DHA-derived neuroprostanes (NeuroP) and four AA-derived isoprostanes (IsoP) were identified. Placentas from lean women (●) and from obese women (○) were compared to investigate differences in DHA- and AA-derived isomers in placentas from obese relative to lean women (A) and in the sum of neuroprostanes and isoprostanes (B).

The ratio of neuroprostanone isomers to FABP1 and the respective Spearman correlations (C and D) and the ratio of isoprostanes to FABP1 and the respective Spearman correlations (E and F) were calculated to investigate possible associations between alterations in FABP1 and DHA and AA non-enzymatic peroxidation as a function of maternal pre-gestational obesity. Possible differences in the sources of neuroprostanes and isoprostanes induced by maternal pre-gestational obesity were assessed by the ratios of neuroprostanes to free DHA (G) isoprostanes to free AA (I) and neuroprostanes to DHA-phosphatidylcholine species (PC; H) and isoprostanes to AA-phosphatidylcholine species (J).

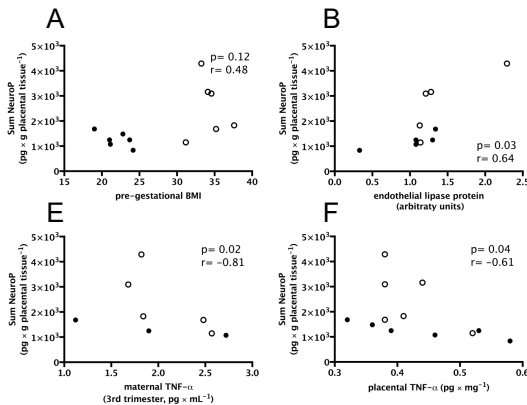
* Significantly different from the lean group; $p < 0.05$ (Mann-Whitney test).

3.4. DHA-derived neuroprostanes and AA-derived isoprostanes are positively associated with maternal pre-gestational BMI and endothelial lipase protein; and DHA-derived neuroprostanes only are negatively associated with inflammation and birth weight.

Spearman rank correlations were used to further investigate the associations of DHA-derived neuroprostanes and AA-derived isoprostanes with inflammation and placental lipid handling. Pre-gestational BMI (Figures 4A and C), endothelial lipase protein (Figures 4C and D), maternal plasma TNF- α (Figures E and G) and placental TNF- α (Figures F and H) were considered as independent variables. AA-derived isoprostanes, but not DHA-derived neuroprostanes, significantly correlated with pre-gestational BMI ($r=0.60$ and $p = 0.04$; $r = 0.48$ and $p = 0.12$, respectively). Additionally, both neuroprostanes ($r = 0.64$; $p = 0.03$) and isoprostanes ($r=0.78$; $p = 0.01$) significantly correlated with

endothelial lipase protein. Assessment of correlations between inflammation markers and oxidised fatty acids disclosed significant negative associations between DHA-derived neuroprostanes and maternal ($r = -0.81$; $p = 0.02$) and placental ($r = -0.61$; $p = 0.04$) TNF- α levels (Figures 4E and F). In contrast, no significant correlations were found between AA-derived isoprostanes and inflammation markers, except for a marginally significant negative correlation with placental TNF- α ($r = -0.51$; $p = 0.09$, Figure 4H).

DHA- derived NeuroP correlations



AA- derived IsoP correlations

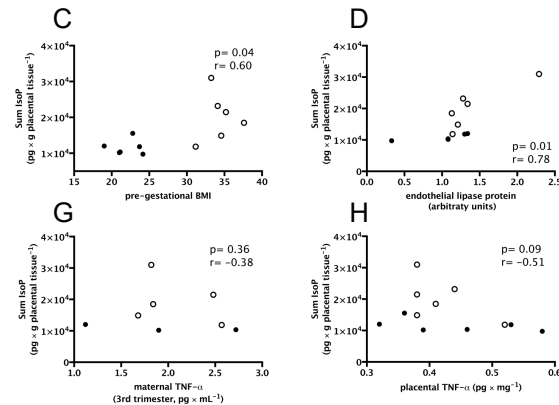


Figure 4: DHA-derived neuroprostanes are negatively correlated with inflammation markers, and AA-derived isoprostanes are positively associated with maternal pre-gestational BMI.

Spearman rank correlations of DHA-derived neuroprostanes (NeuroP; A, B, E, F) and of AA-derived isoprostanes (IsoP; C, D, G, H), with pre-gestational BMI (A and C), endothelial lipase protein (B and D), maternal plasma TNF- α (Figures E and G) and placental TNF- α (Figures F and H). Placentas from lean women (●) and from obese women (○).

Multiple regression analysis was used to investigate the predictors of placental neuroprostanes and isoprostanes and their association with birth weight (Table 2). The sum of placental DHA-derived neuroprostanes was predicted by pre-gestational BMI ($\beta = 1.64 \times 10^2$; 66 % contribution) and by placental TNF- α content, with negative β coefficient ($\beta = -5.56 \times 10^3$; 34 % contribution) (Table 2; model 1). The sole predictor of the sum of placental AA-derived isoprostanes was placental endothelial lipase protein ($\beta = 1.21 \times 10^4$; model 2). Most importantly, birth weight was significantly determined by pre-gestational BMI ($\beta = 1.26 \times 10^2$; 67 % contribution), gestational weight gain ($\beta = 7.72 \times 10^1$; 16 % contribution) and by the sum of placental neuroprostanes, with a negative β coefficient ($\beta = -4.06 \times 10^{-1}$; 17 % contribution) (Table 2, model 3).

Collectively, DHA-derived neuroprostanes might mediate a less inflammatory response and potentially prevent excessive neonatal weight gain, as opposed to AA-derived isoprostanes, which appeared related to increased maternal adiposity.

Table 2: Multiple regression models for the assessment of predictors of placental Σ neuroprostanes and Σ isoprostanes and predictors of birth weight.

Dependent variables	Independent variables	β Coefficients			Relative weight ¹	Adj. R^2	Estimated error (%) ²	P^3
		Value	SE	p-value				
Σ Neuroprostanes in placenta Model 1	Pre-gestational BMI	1.64×10^2	2.61×10^1	0.0004	66 %	93.88	20.1	0.0000
	TNF- α , placenta	-5.56×10^3	1.59×10^3	0.0101	34 %			
	Gestational weight gain	—	—	ns				
	Interleukin-6, placenta	—	—	ns				
	Endothelial lipase, protein	—	—	ns				
	FABP-1, protein	—	—	ns				
Σ Isoprostanes in placenta Model 2	Endothelial lipase, protein	1.21×10^4	1.12×10^3	0.0000	100 %	93.65	22.9	0.0000
	Pre-gestational BMI	—	—	ns				
	Gestational weight gain	—	—	ns				
	TNF- α , placenta	—	—	ns				
	Interleukin-6, placenta	—	—	ns				
Birth weight Model 3	Pre-gestational BMI	1.26×10^2	1.38×10^1	0.0001	67 %	99.07	6.83	0.0000
	Gestational weight gain	7.72×10^1	1.71×10^1	0.0040	16 %			
	Σ Neuroprostanes, placenta	-4.06×10^{-1}	1.37×10^{-1}	0.0253	17 %			
	Σ Isoprostanes, placenta	—	—	ns				
	Interleukin-6, placenta	—	—	ns				
	TNF- α , placenta	—	—	ns				
	Endothelial lipase, protein	—	—	ns				

¹Relative weight in the model= independent variable coefficient \times variable average content in samples; ²Estimated relative error of estimate= (estimated absolute error \times 100%)/average value of the dependent variable; ³Model significance. Significant associations were independent of the following variables, denoted as non-significant (ns) p-values: model 1, gestational weight gain, interleukine-6 (placenta), endothelial lipase protein (placenta) and FABP1 (placenta); model 2, pre-gestational BMI, gestational weight gain, TNF- α (placenta) and interleukine-6 (placenta); model 3, Σ of isoprostanes (placenta), interleukin-6 (placenta), TNF- α (placenta) and endothelial lipase protein (placenta).

4. Discussion

In this study, we show that despite no overall features of lipid accumulation placentas from women with pre-gestational obesity contain and increased content of polyunsaturated free fatty acids. This increase is associated with a decreased content of FABP1, a cytoplasmic protein that handles unsaturated fatty acid and it is important for determining their biological fate. Additionally, higher contents of neuroprostanes and isoprostanes, which are products of non-enzymatic oxidation of DHA and AA respectively, were observed in placentas from obese women. Correlation and multivariate analyses suggest that these oxidised fatty acids are associated with maternal and placental inflammation, and also with birth weight. To our knowledge, this is the first report to characterize DHA-derived neuroprostanes and AA-derived isoprostanes in the human placenta, and in particular to explore their role in maternal and neonatal outcomes in the context of maternal pre-gestational obesity.

The median BMI value of the obese women participating in this study was 34.3, indicating they are obesity class 1, and only one out of six women presented with a BMI of 37.6 (obesity class II). Additionally, the obese women displayed similar plasma concentrations of inflammatory markers

and lipoproteins and triacylglycerols compared to lean women. Therefore, no apparent signs of inflammation were detected and one might postulate that despite being obese they were metabolically healthy. Additionally, gestational weight gain was 40 % in obese mothers, compared to lean mothers. Although several studies have shown that maternal obesity is associated with an inflammatory and pro-oxidant milieu [47,48], there are reports of healthy obese mothers with no signs of dyslipaemia [13, 49]. On the other hand, irrespective of its severity, there are consistent data showing that several placental responses appear to be affected by maternal pre-gestational obesity [16, 50]. In this study, we draw attention to altered fatty acid handling and metabolism in placentas from obese women.

The placenta is the highly-specialized organ that interfaces between the mother and her baby. It integrates signals of maternal availability and fetal demand of nutrients and oxygen and has a central role in determining the life-long health of the offspring [51]. In the past years, the importance of placental lipid metabolism in regulating maternal-fetal transfer of fatty acids, in particular essential polyunsaturated fatty acids, has been recognized [14, 52]. In the case of maternal pre-gestational obesity, *in vivo* and *ex vivo* studies in the human placenta using ¹³C labelled fatty acids have shown that maternal pre-gestational obesity impairs maternal-fetal transfer of polyunsaturated fatty acids [17,18], although the mechanisms involved are not fully known. Indeed, in this study, we showed that placentas from women with pre-gestational obesity contain a significant increase in DHA and EPA content compared to placentas from lean women. Additionally, the median value of placental AA from obese women was nearly 2.5-fold higher than the lean group, despite these differences being non-significant statistically (Figure 1C). Increased placental lipid accumulation due to increased FA esterification and decreased β -oxidation have been shown in maternal pre-gestational obesity [20]. In our study, the changes in lipid content were mainly related to long-chain polyunsaturated fatty acids, which are critical to fetal development, in particular of the fetal brain. Additionally, the relative content of cardiolipins was 1.4-fold higher in the obese group, despite no significant difference (Figure 1B). Cardiolipin is a phospholipid mainly found in mitochondrial membranes and is a proxy of mitochondrial content [53], suggesting that mitochondrial content and possibly function was not impaired in placentas from obese women participating in this study.

Alterations in placental fatty acid transporter proteins appear to be plausible mechanisms associated with altered placental lipid handling in maternal pre-gestational obesity [54]. Indeed, in the present study we showed that FABP1 is decreased in placentas from obese women, corroborating a previous study [25]. The decrease in FABP1 in placentas from the obese group reflected in significantly higher ratios of *n*-3 and *n*-6 polyunsaturated fatty acids to FABP1 (Figure 2).

FABP1 is the liver isoform of FABP, and unlike other members in the FABP family this protein has two ligand-binding sites for fatty acids and presents higher affinity for long-chain unsaturated fatty acids. Besides fatty acids, FABP1 binds a range of hydrophobic molecules, as peroxisome proliferator-activated receptors (PPARs), prostaglandins and hydroxyl and hydroperoxyl metabolites of AA, lysophospholipids, pro-oxidants such as heme, among molecules [55]. Additionally, FABP1 has been found in the nucleus and mitochondria, in addition to the cytosol [56,57]. Therefore, due to the extensive ligand properties of FABP1 and multiple cellular localization, it is suggested that this protein has multiple roles. In this context, one might speculate that the decreased content of FABP1 protein found in placentas from women with pre-gestational obesity might have affected, in many ways, placental function.

Due to its role in trafficking fatty acids to the nucleus and also its ability to bind PPARs, the decrease in FABP1 in placentas from women with pre-gestational obesity likely altered pathways related to placental lipid handling and fatty acid metabolism, and possibly the transfer of fatty acids to the fetal circulation. There is evidence that PPARs are involved in these processes in the human placenta [58,59]. Similar contents of PPARs in placentas from obese and lean women [25] were described in parallel with increased PPAR- γ (related to fatty acid synthesis) and decreased PPAR- α (related to fatty acid oxidation) [20]. Yang et al. have proposed that regulation of PPARs is more important at the maternal-fetal interface and this fact might explain the apparent discrepancies in PPARs content in maternal pre-gestational obesity [60]. It remains to be determined if and how PPARs participate in

the cross-talk between long chain polyunsaturated fatty acids and FABP1 protein seen in the present study.

As FABP1 binds to pro-oxidant molecules and is considered a cellular antioxidant, at least in the liver [55], its decrease might render the placenta more susceptible to oxidative stress in obese women. In this context, a decreased total antioxidant capacity and increased activation of the pro-oxidant NFkB pathway has been observed in placentas from women with obesity [61]. The significant increase in DHA-derived neuroprostanes and AA-derived isoprostanes seems to corroborate with a pro-oxidant milieu in placentas from the obese group. In addition to increased concentration of neuroprostanes and isoprostanes, we found that their ratios to FABP1 were also significantly increased in placentas from women with pre-gestational obesity (Figure 3C), and that FABP1 negatively correlated with the sum of isoprostanes. These results not only support the pro-oxidant milieu due to decreased FABP1 but also suggest that FABP binds to isoprostanooids, in particular AA-derived isoprostanes, as observed for enzymatically derived fatty acid mediators.

Neuroprostanes and isoprostanes are isoprostanooids (isomers of prostaglandins), formed by the non-enzymatic peroxidation of DHA and AA, respectively. They have been described as important signaling molecules and AA-derived isoprostanes seem to act in vascular smooth muscle through tyrosine kinase and Rho kinase in human cells [62] and through prostanoid receptors in rats [63]. AA-derived isoprostanes are implicated in cardiovascular diseases acting as vasoconstrictors and considered markers of oxidative stress [46]. On the other hand, DHA-derived neuroprostanes, despite the fact they have been implicated in oxidative stress in neurodegenerative diseases [64], a considerable body of recent evidence suggests that they have anti-inflammatory properties [65,66]. Their role in placental function in pregnancies complicated by maternal obesity has not yet been described and the present study is the first to measure neuroprostanes and isoprostanes in the human term placenta.

Oxidised 9-HODE, 13-HODE, and 15-HETE, products of AA produced by cyclooxygenases activity did not affect the expression of syncytin, cyclin E and p27, which are proteins markers of trophoblast differentiation, indicating they are not implicated in trophoblast dysfunction, at least in healthy trophoblasts [67]. On the other hand, in placentas from pregnancies complicated with pre-eclampsia, it has been shown that F₂ class isoprostanes derived from AA contributed to oxidative stress and have vasoconstrictive properties [68].

The formation of isoprostanooids seems to be regulated at some extent, as it has been proposed that they are predominantly formed *in situ* from oxidation of DHA or AA esterified to phosphatidylcholine in cell membranes. Therefore, their signaling properties depend upon phospholipase A₂ activity [46]. Brien et al. found a strict correlation between placental phospholipase A₂ mRNA and free F₂-isoprostane levels in preeclampsia [68].

We did find marginally lower ratios of neuroprostanes to DHA-enriched and of isoprostanes to AA-enriched PC in placentas from obese women, suggesting that the proportion of these mediators formed *in situ* is decrease in obesity compared to the lean group. The significance of this result may be related to compensatory mechanisms in placentas from obese women, controlling membrane function and avoiding excessive release of mediators and possibly controlling neonatal birth weight. Indeed, Varastehpour showed that increased placental phospholipase A₂ activity was correlated with increased placental accumulation of lipids and adiposity in the newborn [69].

In the present study, AA-derived isoprostanes correlated with maternal pre-gestational BMI. Additionally, in the multivariate model, the sole predictor of isoprostanooids in the placenta was placental EL protein. These results could be interpreted in the light of the pro-inflammatory effect of AA-derived isoprostanes. Higher content of EL has been described in placentas from pregnancies complicated with GDM, in addition to obesity [70]. In our study, we found a non-statistical 25 % increase in EL in placentas from women with pre-gestational obesity.

In the case of neuroprostanes, multivariate models indicated that both maternal pre-gestational BMI (66 % weight in the model) and placental TNF-alpha (34 % weight in the model; with negative β coefficient), were predictors of their content in the placenta, suggesting their possible anti-inflammatory role. This association was also observed in the correlation analysis, where the sum of

neuroprostanes was negatively correlated with maternal and placental TNF- α contents. Importantly, when we evaluated the possible involvement of neuroprostanes and isoprostanes with birth weight, the sum of neuroprostanes presented a negative contribution (17 % weight in the model) and, as expected, pre-gestational BMI (64 %) and gestational weight gain (16 %) had positive associations. The sum of isoprostanes did not fit to the experimental model, using the same variables as for neuroprostanes. These results suggest that neuroprostanes present anti-inflammatory roles in placentas from women with pre-gestational obesity and by negatively affecting birth weight might attenuate the intergenerational cycles of detrimental effects on metabolic health as the risk of overweight/obesity in childhood increases gradually over the full range of maternal pre-gestational BMI [71]. Indeed, neonatal birth weight was similar between groups in the present study. Adding to the discussion of the possible mechanisms by which neuroprostanes have anti-inflammatory effects was the observation that 4-(*RS*)-4-F_{4t}-NeuroP increased mRNA levels of the enzyme heme-oxygenase, which degrades heme, in human neuroblastoma cells and in primary culture of neurons [64]. Given the fact that FABP1 is able to bind heme, a molecule with pro-oxidant properties, the increase in neuroprostanes isomers might counteract the decrease in FABP1 protein in the obese group, providing a means of anti-oxidant defense. It needs to be determined if this is the case in the term human placenta in maternal pre-gestational obesity.

The main limitation of the present study is the sample size, which impacts on the statistical power and we were not able to address sexually-dimorphic responses in placental fatty acid metabolism. This aspect would be important as a recent study showed that placentas from females appear to be more prone to store and esterify fatty acids, while placentas from males have a decreased capacity to transfer DHA to the cord blood [72].

As maternal diet is the ultimate source of DHA and AA, these results add to the body of evidence that there must be a balance between *n*-3 and *n*-6 fatty acid intake and possibly during pregnancy a higher intake of DHA is necessary, as it was suggested there is a protective effect in lipid accumulation in women with higher usual intake of DHA [73]. A recent pilot trial demonstrated that DHA supplementation starting early in pregnancy promoted higher lean mass accrual at birth and improved fetal growth [74]. The next step is to evaluate the diet of these mothers and dietary factors contributing to these placental fatty acid responses in an attempt to outline efficient dietary strategies to decrease the burden of maternal pre-gestational obesity. We hope that these results might shed light on the molecular mechanisms associated with altered fatty acid metabolism and lipid handling in maternal pre-gestational obesity and to place isoprostanooids as novel mediators of placental function.

Supplementary Materials: Table S1: Internal standards used in the Ion Mobility QTOF LC/MS lipid profile analysis. Figure S1: Cytokines in maternal plasma (3rd trimester) (A), placental tissue (B) and umbilical cord plasma (C) Refer to materials and methods for detailed methods description. Figure S2: Lipoproteins and triacylglycerol in maternal plasma, 3rd trimester

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