

Maternal obesity leads to long-term altered levels of plasma ceramides in the offspring as revealed by a longitudinal lipidomic study in children

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ARTICLE

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2 Clinical Research



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- 4 ceramides in the offspring as revealed by a longitudinal lipidomic
- s study in children
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11 Abstract

Background/objectives Maternal obesity is associated with increased risk of obesity and other symptoms of the metabolic syndrome in the offspring. Nevertheless, the molecular mechanisms and cellular factors underlying this enhanced disease susceptibility remain to be determined. Here, we aimed at identifying changes in plasma lipids in offspring of obese mothers that might underpin, and serve as early biomarkers of, their enhanced metabolic disease risk.

- Subjects/methods We performed a longitudinal lipidomic profiling in plasma samples from normal weight, overweight, and obese pregnant women and their children that participated in the Prenatal Omega-3 Fatty Acid Supplementation, Growth, and Development trial conducted in Mexico. At recruitment women were aged between 18 and 35 years and in week 18–22
- of pregnancy. Blood samples were collected at term delivery by venipuncture from mothers and from the umbilical cord of
- their newborns and from the same infants at 4 years old under non-fasting conditions. Lipidomic profiling was done using
- 21 ultra-performance liquid chromatography high-resolution mass spectrometry.
- **Results** Analysis of the lipidomic data showed that overweight and obese mothers exhibited a significant reduction in the total abundance of ceramides (Cer) in plasma, mainly of Cer (d18:1/20:0), Cer (d18:1/22:0), Cer (d18:1/23:0), and Cer
- 24 (d18:1/24:0), compared with mothers of normal body weight. This reduction was confirmed by the direct quantification of
- these and other ceramide species. Similar quantitative differences in the plasma concentration of Cer (d18:1/22:0), Cer
- 26 (d18:1/23:0), and Cer (d18:1/24:0), were also found between 4-year-old children of overweight and obese mothers compared
- with children of mothers of normal body weight. Noteworthy, children exhibited equal daily amounts of energy and food
- intake independently of the BMI of their mothers.
- **Conclusions** Maternal obesity results in long-lasting changes in plasma ceramides in the offspring suggesting that these lipids might be used as early predictors of metabolic disease risk due to maternal obesity.

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Introduction

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Obesity is a chronic pathological condition characterized by32the excessive accumulation of adipose tissue often com-33plicated by one of the core components of the metabolic34

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syndrome. Obesity is also a primary risk factor for type 2 35 diabetes and coronary artery disease and has become a 36 global public health burden affecting all segments of the 37 population [1]. According to the World Health Organiza-38 tion, 41 million children under the age of 5 years are 39 overweight or obese, and 650 million adults are obese, 40 among which 15% are women [2]. The prevalence of 41 obesity in pregnant women is as high as 30% [3, 4] and 42 ~40% of women gain an excessive amount of weight during 43 pregnancy in western countries [3, 5]. This is of special 44 concern because maternal obesity or excessive body weight 45 gain during pregnancy leads to an increased risk of devel-46 oping metabolic syndrome and cardiovascular diseases in 47 the offspring, via a developmental programming process not 48 only in the first but also in the second generation, thus 49 initiating a vicious cycle to perpetuate chronic degenerative 50 diseases [6-9]. However, there is little information about 51 the mechanistic link between maternal obesity and the long-52 term adverse metabolic outcomes in the offspring. In 53 addition, it remains to be clearly established whether the 54 increased disease risk exhibited by children born to obese 55 mothers is due to their exposure to an imbalanced nutri-56 tional environment during fetal development or results from 57 genetic factors and/or deleterious familial life style habits. 58

Lipotoxicity is a hallmark of several of the pathological 59 60 conditions associated with obesity as heart attacks, strokes peripheral vascular diseases [10], and non-alcoholic fatty 61 liver disease [11, 12]. The accumulation of fatty acids in 62 skeletal muscle leads also to inhibition of insulin receptor 63 signaling, increased production of inflammatory and stress 64 factors, as well as impaired mitochondrial β -oxidation [13, 65 14]. Overall, these lipid-mediated changes contribute to 66 diabetes and other chronic degenerative diseases associated 67 with obesity. 68

Given the key role of altered lipid metabolism in the 69 etiology of obesity-related disorders, the quantification of 70 blood lipids is routinely used in the clinic to estimate 71 metabolic and cardiovascular disease risk, and has also been 72 employed to determine the susceptibility of children born to 73 obese mothers to developing the metabolic syndrome [15, 74 16]. However, these latter analysis have been restricted to a 75 small number of circulating lipids, namely triacylglycerol 76 (TG), total cholesterol and low-density lipoprotein choles-77 78 terol and high-density lipoprotein cholesterol (LDL-C, HDL-C), thus providing a very narrow overview of the 79 alterations occurring in the circulating levels of the many 80 hundreds of individual lipid species that constitute the blood 81 lipidome. Actually, increasing evidence suggests that 82 sphingolipids and other less abundant circulating bioactive 83 lipids play an important role in the etiology of several 84 obesity-related diseases [17, 18]. Here, we performed a 85 longitudinal lipidomic analysis in plasma samples of chil-86 dren born to lean, overweight, or obese mothers in order to 87

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identify changes in lipid profile that might underpin, and88serve as early biomarkers of, the disease risk resulting from89maternal obesity.90

Materials and methods

Study population and sample collection

This study was conducted in plasma samples that were 93 obtained from pregnant women and their children who gave 94 their informed consent to participate in the Prenatal Omega-95 3 Fatty Acid Supplementation, Growth, and Development 96 (POSGRAD) trial registered at the Instituto Nacional de 97 Salud Pública (INSP) in Mexico (#CI-011) and at clin-98 icaltrials.gov (NCT00646360). The design of the trial has 99 been described in detail elsewhere [19]. Eligible women 100 were aged between 18 and 35 years old and between 18 and 101 22 weeks of pregnancy. Blood samples were collected from 102 mothers and from the umbilical cord blood of the babies by 103 venipuncture at term delivery, as well as from the same 104 infants at 4 years old under non-fasting conditions. Since 105 the original objective of the study was to evaluate the 106 impact of maternal docosahexaenoic acid (DHA) supple-107 mentation during pregnancy on the offspring's cognitive 108 development, maternal blood samples during pregnancy 109 were not collected. After collection, blood samples were 110 placed into a tube containing EDTA, and blood cells were 111 separated from plasma which was frozen and stored at 112 -70 °C until analysis. The original study included 973 113 women who received capsules containing either DHA (n =114 485) or olive oil as placebo (n = 488) and that delivered 973 115 live infants. Among these children, 524 were followed-up 116 during 4 years of whom 341 were excluded because of the 117 lack of complete information or enough amount of plasma 118 sample to give a final number of 91 children in the DHA 119 group and 92 in the placebo group. (Supplemental Fig. 1). 120 For this study, we used 47 mother-infant dyads from the 121 placebo group with each dyad having three plasma samples 122 corresponding to the maternal and new-born samples taken 123 at delivery and the samples of the children at 4 years. 124 Mothers were categorized by standard convention as normal 125 body weight $(18.5 \le BMI < 25)$, overweight $(25 \le BMI < 25)$ 126 30), or obese (BMI \ge 30). 127

Dietary assessment

The daily intake of energy of the offspring at 4 years was estimated using a food frequency questionnaire of 108 food items adapted to the Mexican population as described by Hernández-Avila et al. [20] and Romieu et al. [21]. Daily nutrient intakes were obtained by multiplying the portion size of each food item reported in the questionnaire by the 134

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weight corresponding to its frequency of consumption and 135 by its nutrient value obtained from the food composition 136 tables of the US Department of Agriculture (https://ndb.nal. 137 usda.gov/ndb/) or other sources for specific Mexican foods 138 and portion sizes (http://www.myfitnesspal.com/es/food/ca 139 lorie-chart-nutrition-facts). Total daily calorie intake was 140 calculated by adding the results corresponding to all food 141 items. We also calculated the daily intake of each group of 142 food and its contribution to total energy intake. 143

Metabolite determinations 144

Plasma from children was assayed for insulin, glucose, TG, 145 total cholesterol, HDL-C, LDL-C, aspartate aminotransfer-146 (AST). alanine aminotransferase 147 ase (ALT). gammaglutamyl-transferase (GGT), creatinine, and uric 148 acid using commercially available reagents as previously 149 described [22].

Lipidomic analysis 151

The lipidomic analysis was performed by ultra-performance 152 liquid chromatography-tandem mass spectrometry (UPLC-153 MS/MS) as described in our previous publications [23, 24]. 154 A mixture of seven endogenous standards (Supplemental 155 156 Table S1) was added to 25 µL of each plasma sample and to 25 µL of a quality control pool (QC) that was constituted by 157 mixing an equal amount of each plasma sample. Lipids 158 were extracted by the Bligh and Dver method [25]. Lipid 159 separation was achieved on an Acquity[®] H-Class UPLC 160 system (Waters Corporation, Milford, USA) equipped of an 161 Acquity[®] charge surface hybrid (CSH C₁₈) column (2.1 162 mm \times 100 mm, 1.7 µm; Waters, France) held at 55 °C. 163 Detection of lipids was performed by a Synapt G2 HDMS, 164 Q-TOF mass spectrometer equipped with a Z-Spray inter-165 face for electrospray ionization (ESI) (Waters Corporation). 166 The resolution mode was used to scan ions with mass-to-167 charge ratios (m/z) from 50 to 1200 at a mass resolution of 168 25,000 full width half maximum (FWHM) for both positive 169 (ESI+) and negative (ESI-) ionization modes. Data were 170 collected in the centroid mode at a rate of four spectra 171 per second. A solution of leucine enkephalin (2 µg/mL in an 172 acetonitrile:water), containing 0.1% of formic acid, was 173 174 infused at a constant flow of 10 µL/min in the lockspray channel to correct the measured m/z ratio during experi-175 ments. Mass correction was applied throughout the batch at 176 m/z 556.2771 and 554.2616 for ESI+ and ESI-, respec-177 tively. To evaluate the performance of the analytical system 178 and control for inter run changes in sensitivity of the mass 179 spectrometer, QC samples were included in each run. All 180 samples were analyzed in a random order within a period no 181 longer than one week between the first and the last run. 182

Data acquisition and processing of mass spectrometry 183 data, including peak detection, integration, alignment, and 184 normalization, were achieved using MassLynx[®] and 185 MakerLynx[®] software (version 4.1, Waters Corporation, 186 Milford, MA, USA). Lipid markers were selected from the 187 detected features using an in-house lipidomic database of 188 180 lipid species built by the use of lipid standards, the 189 exact mass measured, the elemental compositions with a 190 mass error below 5 ppm, the retention times, and the frag-191 mentation patterns as described in other studies [26, 27]. 192 The relative standard deviation (RSD, %) was calculated for 193 peak areas to highlight the repeatability of the analytical 194 process. Finally, selected lipid markers having a RSD value 195 below 30% were retained for multivariate analysis (30). 196 Q3

Quantification of plasma ceramides

We used the method described by Croval et al. [23], to 198 quantify different species of ceramides. A pool of standard 199 solutions, including 10 species of ceramides (Cer), was 200 prepared and then serially diluted in methanol (Sigma 201 Aldrich, Saint-Quentin Fallavier, France), to obtain seven 202 standard solutions at 1, 5, 10, 50, 100, 250, and 500 nM. 203 Standard solutions and plasma samples (25 µL), were 204 extracted as described in the previous section after the 205 addition of Cer (d18:1/17:0) as internal standard. UPLC-206 MS/MS analyses were performed on a Xevo® triple-quad-207 rupole mass spectrometer with an ESI interface and an 208 Acquity H-Class[®] UPLCTM device (Waters Corporation). 209 Data acquisition and analyses were performed with Mas-210 sLynx[®] and TargetLynx[®] software, respectively (version 211 4.1, Waters Corporation, Milford, MA, USA). Compounds 212 were separated on an Acquity[®] BEH C_{18} column (2.1 × 50 213 mm, 1.7 µm, Waters). Ceramides and internal standards 214 were detected by the mass spectrometer with the ESI 215 interface operating in the positive ion mode. The multiple 216 reaction mode (MRM) was applied as described in Sup-217 plemental Table S2. Chromatographic peak area ratios 218 between ceramides and their internal standard constituted 219 the detector responses. Standard solutions were used to plot 220 calibration curves and a linear regression model (1/x)221 weighting) was used for quantification. The intra-assay and 222 inter-assay variabilities of the analytical method were 223 assessed over three distinct experiments (four concentration 224 levels, six replicates by level), and were below 13.6%. 225 Recoveries were assessed with internal standard and were 226 >91%. 227

Statistical analysis

UPLC-MS/MS spectral data were first normalized by 229 dividing the intensity of each specific signal, corresponding 230 to a putative marker, by the sum of the intensities of all 231

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Table 1 Clinical characteristics of mothers and their offspring at birth and at 4 years

| Variable | Maternal body weight ch | One-way ANOVA p-value | | | |
|---------------------------------|------------------------------|--|-------------------------------|----------|--|
| | Normal weight $(n = 18)$ | nal weight $(n = 18)$ Overweight $(n = 18)$ Obese $(n = 11)$ | | _ | |
| Maternal characteristics | | | | | |
| Height (m) | 1.56 (1.54–1.58) | 1.53 (1.50-1.56) | 1.51 (1.47–1.55)* | 0.0412 | |
| BMI (kg/m ²) | 21.86 (21.24-22.48) | 26.20 (25.93-26.46)*** | 31.62 (30.72-32.52) ***/ | < 0.0001 | |
| Pregnancy weight gain (kg) | 13.78 (12.83-14.72) | 14.17 (13.17–15.16) | 13.36 (12.35–14.37) | 0.6979 | |
| Age (years) | 24.59 (22.43-26.75) | 25.56 (24.36-28.37) | 29.75 (27.18-32.27)** | 0.0113 | |
| Time of delivery | 13h18 (9h37-16h18) | 13h50 (11h10-16h40) | 11h35 (8h42-13h47) | 0.5245 | |
| Infant characteristics at birth | h | | | | |
| Gestational age (weeks) | 38.61 (37.68-39.54) | 39.40 (38.88-39.91) | 38.30 (36.84–39.75) | 0.1904 | |
| Weight | 3.38 (3.15-3.62) | 3.40 (3.07-3.74) | 3.26 (2.83-3.70) | 0.8149 | |
| Boys | 10 | 9 | 7 | | |
| Girls | 8 | 9 | 4 | | |
| Child characteristics and me | etabolite profile in serum a | t 4 years | | | |
| Body weight (kg) | 15.63 (14.90–16.37) | 17.41 (15.55–19.27) | 16.78 (14.48–19.08) | 0.2074 | |
| Height (cm) | 101.8 (99. 75-103.90) | 103.30 (100.30-106.20) | 103.2 (100.00-106.30) | 0.6612 | |
| BMI z-score | -0.17 (-0.54 to 0.19) | 0.49 (-0.14 to 1.12) | 0.13 (-084 to 1.09) | 0.2282 | |
| Fasting length (h) | 3.6 (3.0-4.13) | 2.79 (2.32-3.26) | 3.97 (3.18–4.76) [§] | 0.0153 | |
| Insulin (mUI/mL) | 8.03 (4.12-11.95) | 5.60 (3.87-7.34) | 7.85 (2.65–13.05) | 0.3685 | |
| Glucose (mg/dL) | 93.61 (89.02-98.20) | 92.11 (88.12–96.10) | 94.09 (84.94–103.20) | 0,3403 | |
| Creatinine (mg/dL) | 0.33 (0.29–0.37) | 0.36 (0.32-0.40) | 0.33 (0.29–0.37) | 0.4122 | |
| Uric acid (mg/dL) | 4.01 (3.67-4.37) | 3.98 (3.64-4.34) | 4.15 (3.85-4.46) | 0.7919 | |
| Triglycerides (mg/dL) | 95.17 (74.33-116.00) | 123.50 (93.19–153.80) | 141.00 (88.96–193.00) | 0.1170 | |
| Cholesterol (mg/dL) | 155.50 (143.30–167.70) | 176.60 (164.10-189.30)* | 168.60 (152.70–184.50) | 0.0430 | |
| HDL-C (mg/dL) | 53.28 (47.79–58.77) | 54.67 (50.23-59.10) | 53.36 (45.07-61.66) | 0.9132 | |
| LDL-C (mg/dL) | 83.22 (71.89–94.56) | 99.06 (87.83-110.30) | 89.70 (79.80–99.60) | 0.0905 | |
| AST (UI/L) | 34.50 (33.17-35.83) | 38.67 (36.02–41.31)* | 40.45 (35.64-45.27)** | 0.0070 | |
| ALT (UI/L) | 16.17 (14.34–18.00) | 21.06 (17.44–24.67) | 24.18 (14.55-33.81)* | 0.0464 | |
| GGT (UI/L) | 9.66 (8.49-10.85) | 10.94 (9.54–12.35) | 9.70 (8.69–10.71) | 0.2310 | |

Children are grouped according to the body mass index of their mothers. Fasting length refers to the interval of time between the last meal and the time at which blood samples were collected. Values correspond to the mean \pm SEM. *p < 0.05; **p < 0.01; ***p < 0.001 compared with mothers of normal body weight or with children born to mothers of normal body weight; ^{§§§}p < 0.001 compared with overweight mothers (as determined by one-way ANOVA followed by Tukey's multiple comparison test)

HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, AST aspartate aminotransferase, ALT alanine aminotransferase, GGT gammaglutamyl-transferase

signals detected in the same plasma sample. Thereafter, 232 normalized data were submitted to unsupervised "principal 233 component analysis" (PCA), followed by the construction 234 235 of orthogonal partial least-squares discriminant (OPLS-DA) models using SIMCA-P+ software (Umetrics, Umea, 236 Sweden). For the construction of the S-Plots corresponding 237 to the OPLS-DA analysis, both overweight and obese 238 groups were merged and compared with the normal body 239 weight group. PCA analysis determines the global spectral 240 differences between two or more experimental groups and 241 allows one to establish an initial hypothesis about the 242 variables that drive the differences among the groups. 243 OPLS-DA analysis permits to verify the hypothesis and to 244

get information about the variables in which samples are 245 distinguished. Corresponding OPLS models S-Plots permits 246 a visualization of the most discriminant features between 247 dyslipidemic status or origins. We therefore used PCA to 248 identify the global lipidomic differences between the groups 249 and OPLS-DA to reveal specific lipidomic changes and 250 improve the separation between the different lipid classes. 251 Models validity was appraised using permutation tests and 252 CV-ANOVA. Data related to the clinical characteristics of 253 mothers and offspring and the results of the direct quanti-254 fication of ceramides, were checked for normality before 255 statistical processing using the D'Agostino & Pearson 256 omnibus normality test. Data are presented as mean ± SEM 257

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Fig. 1 Global differences in plasma lipidome between mothers and between children of normal body weight (NBW), overweight (OW) or obese (OB) mothers. Scores plots of the OPLS-DA models of lipidomic profile in mothers (a) and their newborns (d) and children (g). Note the clear segregation pattern among the data from the NBW (open circles), OW (gray circles), and OB (black circles) groups of mothers and children but not among the data of newborns. Representative OPLS-DA loading S-plot showing the distribution patterns of specific lipids contributing to the differences between NBW, OW, and OB mothers (b) and between their newborns (e) and children (h) on the score plots. Note that though some lipids show a discriminative pattern in newborns, these differences are not statistically significant as indicated by the results of the multivariate model ($R^2(X) = 0.197$, R^2 (Y) = 0.225, and $Q^2(Y) = -0.165$, p > 0.05. Gray circles in (b) correspond to the following ceramide species: 2 = Cer (d18:1/24:0); 3 =Cer (d18:1/24:1); 4 = Cer (d18:1/23:0); 5 = Cer (d18:1/20:0); 8 = Cer(d18:1/22:0); 11 = Cer (d18:1/18:0). Other discriminating lipids are: 1 = PC (36:2); 6 = SM (20:0); 7 = FA (18:3); 9 = SM (22:0); 10 = LPC

258 (standard error to the mean). Statistical differences between groups were assessed by one-way ANOVA or one-way 259 analysis of covariance (ANCOVA) followed by Tukey's or 260 Dunnett's multiple comparison tests. Correlations were 261 performed using multiple regression analysis, Pearson's 262 correlation coefficient, and Partial correlation. Statistical 263 analyses were performed with GraphPad Prism software 264 (version 6.0, GraphPad Software Inc., La Jolla, CA, USA), 265 and IBM SPSS. Statistical significance was set at p < 0.05. 266

(20:5); 12 = SM (24:1); 13 = PC (36:6); 14 = PE (38:4); 15 = DG (34:1): 16 = SM (16:0): 17 = CE (18:2): 18 = FA (18:1): 19 = FA (22:5); 20 = FA (20:3). Gray circles in (h) correspond to the following ceramide species 2 = Cer (18:1/24:0); 3 = Cer (18:1/23:0); Cer (18:1/24:0); 3 = Cer (18:1/24:0); Cer (18:1/24:0); 3 = Cer (18:1/2422:0). Other discriminating lipids are: 1 = FA (16:0); 5 = FA (16:1); 6= FA (20:3); 7 = SM (24:0); 8 = PC (36:2e); 9 = LPC (18:0e); 10 =SM (24:1); 11 = TG (50:3). Relative abundance of total plasma ceramides and cholesteryl esters in mothers (c), of total cholesteryl esters in newborns (f) and of total ceramides in children (i). In all cases, relative abundance was calculated by normalizing the sum of the intensities of all signals of the corresponding lipid to the sum of the intensity signals of all the variables detected in the quality control samples. ***p < 0.001; **p < 0.01; *p < 0.05 compared with mothers of normal body weight or with children/newborns of normal body weight mothers. ${}^{\$\$}p < 0.01$ compared with newborns of normal body weight mothers. All statistical differences were assessed by one-way ANOVA followed by Tukey's multiple comparisons test

Results

Clinical characteristics of the study population

The maternal and childhood characteristics of the different 269 groups of patients are presented in Table 1. In spite of the 270 differences in BMI, mothers exhibited the same body-271 weight gain during pregnancy. Moreover, the anthropometric characteristics of the children, both at birth and at 4 years, were within normal reference values independently of 274

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p-value

| Table 2 Maternal lipidomic profile in plasma according to body mass index | Type of Lipid | Maternal body weight characteristics | | | One-way ANOVA |
|---|------------------|--------------------------------------|------------------------|-----------------------|---------------|
| | | Normal weight $(n = 18)$ | Overweight $(n = 18)$ | Obese $(n = 11)$ | |
| | LPC (18:0) | 134.41 ± 4.02 | 127.27 ± 4.35 | 114.45 ± 5.31* | 0.0335 |
| | SM (d18:1//16:0) | 261.50 ± 7.80 | $334.80 \pm 28.70^*$ | 264.50 ± 9.02 | 0.0227 |
| | SM (d18:1/20:0) | 135.70 ± 3.03 | 114.67 ± 3.12*** | 112.28 ± 3.92*** | < 0.0001 |
| | Cer (d18:1/18:0) | 1.32 ± 0.10 | $0.87 \pm 0.04^{**}$ | $0.95 \pm 0.05*$ | 0.0004 |
| | Cer (d18:1/20:0) | 1.96 ± 0.10 | $1.27 \pm 0.05^{***}$ | $1.35 \pm 0.07^{***}$ | < 0.0001 |
| | Cer (d18:1/22:0) | 9.42 ± 0.39 | $7.75 \pm 0.30^{**}$ | $7.81 \pm 0.31*$ | 0.0037 |
| | Cer (d18:1/23:0) | 6.35 ± 0.38 | $4.46 \pm 0.25^{***}$ | $4.56 \pm 0.25 **$ | 0.0001 |
| | Cer (d18:1/24:0) | 20.61 ± 1.86 | $11.32 \pm 0.66^{***}$ | $11.19 \pm 0.67 ***$ | < 0.0001 |
| | Cer (d18:1/24:1) | 14.97 ± 0.95 | $11.59 \pm 0.93^*$ | $10.00 \pm 0.77 **$ | 0.0031 |
| | PE (38:4) | 5.25 ± 0.42 | 6.51 ± 0.45 | $7.41 \pm 0.78*$ | 0.0321 |
| | CE (18:2) | 1.25 ± 0.14 | $2.08 \pm 0.25^{*}$ | $2.09 \pm 0.30^{*}$ | 0.0119 |
| | DG (34:1) | 0.72 ± 0.06 | $1.00 \pm 0.08*$ | $1.00 \pm 0.11^*$ | 0.0061 |

Data are expressed as mean ± S.E.M. and correspond to relative abundance of each lipid as determined by normalizing the intensity of its specific signal to the sum of the intensity signals of all the variables detected in the quality control samples. *p < 0.05; **p < 0.01; ***p < 0.001 compared to the values of mothers of normal body weight. p < 0.05 compared with overweight mothers. All comparisons were made using oneway ANOVA analysis followed by Tukey's multiple comparisons test

LPC lysoglycerophosphatidylcholine SM sphingomyelin, Cer ceramide, PC glycerophosphatidylcholine, PE glycerophosphatidylethanolamine, CE cholesteryl ester, DG diglyceride

275 the BMI of their mothers. However, at 4 years old, the offspring of overweight and obese mothers presented higher 276 plasma concentrations of AST and ALT compared with 277 children of mothers of normal body weight. Nevertheless, 278 the plasma concentrations of these metabolites were below 279 the cut-off reference values of hepatic steatosis. Apart from 280 these differences, the plasma metabolite profile of all chil-281 dren at 4 years was very similar. 282

Maternal lipidome fingerprints 283

The automatic processing of the raw mass spectrometry data 284 indicated the presence of 2356 variables in all plasma 285 samples. From these, 158 lipid markers were identified and 286 selected with the use of our in-house lipidomic database 287 288 (Supplemental Table S3). Thereafter, all features related to an isotopic overlapping signal were excluded and features 289 corresponding to a single marker detected in several adducts 290 291 were summed. We took into account only those lipids that were present in all groups and in every patient of each 292 group. As a result of this cleaning process, 68 lipid markers, 293 exhibiting a RSD value below 30% in QC samples (n = 12), 294 were used for analysis (Supplemental Table S4). 295

As expected, PCA of the data showed that the lipid 296 fingerprints of obese and overweight mothers were clustered 297 closely and separated from lean mother's fingerprints 298 (Supplemental Fig. 2). The first component explained 299 19.7% of the variance and the second one 13.1%. 300

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Supervised OPLS-DA statistical models were subsequently 301 performed in order to determine the relative contribution of 302 the various classes of lipids to the differences in the lipi-303 domic profile between the maternal groups and to identify 304 putative biomarkers (Fig. 1a). The performance character-305 istics associated to the resulting multivariate model were R^2 306 $(X) = 0.285, R^2(Y) = 0.719$, and $Q^2(Y) = 0.420$ (p-value of 307 CV-ANOVA = 0.0001). Using as discrimination criterion 308 the lipids located in the upper right and lower left squares of 309 the OPLS-DA-associated S-plot (Fig. 1b), we noticed that 310 glycerophosphatidylchonines (PC), ceramides and free fatty 311 acids (FA) were the lipid class contributing the most to the 312 differences between the groups. Quantitative examination of 313 the data followed by one-way ANOVA further showed that 314 these differences were mainly associated with a decrease in 315 the total abundance of Cer $(F_{(2,44)} = 17.34, p < 0.0001),$ 316 along with an increase in cholestery esters $(F_{(2 41)} = 6.230,$ 317 p = 0.043), in obese and overweight mothers compared 318 with mothers of normal body weight (Fig. 1c). This was 319 confirmed by the analysis of the relative abundance of each 320 individual lipid (Table 2). Indeed, the plasma concentrations 321 of six out of the seven species of ceramides that could be 322 identified were decreased in overweight and obese mothers 323 compared with their lean counterparts. Lysoglyceropho-324 sphatidylcholine (LPC) 18:0, sphingomyelin (SM) d18:1/ 325 20:0, glycerophosphatidylethanolamine (PE) 38:4; choles-326 teryl ester (CE) (18:2), and diglycerides (DG) (34:1) 327 exhibited also a discriminative profile (Table 2). 328

Table 3 Impact of maternalobesity on the serum lipidomicprofile of the offspring

| Type of Lipid | Maternal body v | One-way ANOVA p-value | | |
|------------------|--------------------------|------------------------|---------------------------------|----------|
| | Normal weight $(n = 18)$ | Overweight $(n = 18)$ | Obese $(n = 11)$ | |
| Newborns | | | | |
| SM (d18:1/22:0) | 121.52 ± 2.04 | 130.79 ± * | $149.28 \pm 3.42^{***/^{\$\$}}$ | < 0.0001 |
| Cer (d18:1/18:0) | 0.43 ± 0.03 | $0.53 \pm 0.03*$ | 0.40 ± 0.02 | 0.0201 |
| PC (40:4) | 22.43 ± 1.51 | 25.32 ± 1.75 | $32.39 \pm 1.28^{***/\$}$ | 0.0009 |
| Children | | | | |
| Cer (d18:1/22:0) | 9.41 ± 0.52 | $7.59 \pm 0.44*$ | $6.79 \pm 0.35^{**}$ | 0.0020 |
| Cer (d18:1/23:0) | 7.01 ± 0.47 | $5.21 \pm 0.32^{**}$ | $4.67 \pm 0.25^{**}$ | 0.0005 |
| Cer (d18:1/24:0) | 24.65 ± 1.57 | $15.48 \pm 1.15^{***}$ | $14.29 \pm 0.65^{***}$ | < 0.0001 |
| PC (36:2e) | 18.44 ± 0.82 | $15.32 \pm 0.80^{*}$ | 15.39 ± 0.94 | 0.0180 |

Data are expressed as mean \pm SEM and correspond to relative abundance of each lipid as determined by normalizing the intensity of its specific signal to the sum of the intensity signals of all the variables detected in the quality control samples. *p < 0.05; **p < 0.01; ***p < 0.001 compared to the respective values obtained in serum from babies or children born to mothers of normal body weight using one-way ANOVA analysis followed by Tukey's multiple comparisons test

SM sphingomyelin, *Cer* ceramide, *PC* glycerophosphatidylcholine, *TG* triglyceride, *LPC* lysoglycerophosphatidylcholine, *DG* diglyceride

329 Lipidomic fingerprints of the offspring

PCA analysis of the data showed that there were no dif-330 ferences by maternal BMI status in the clustering of the 331 lipidomic fingerprints or the total abundance of the different 332 classes of lipids among the offspring at birth (Supplemental 333 Fig. 2), and, consequently, the performance characteristics 334 associated to the resulting multivariate model (Fig. 1d, e), 335 were not statistically significant $(R^2(X) = 0.197, R^2(Y) =$ 336 0.225, and $Q^{2}(Y) = -0.165$, *p*-value of CV-ANOVA > 337 0.05). However, newborn babies of obese mothers had 338 lower total concentration of CEs ($F(_{2,42}) = 5.296$, p =339 0.0089, Fig. 1f), together with higher plasma concentrations 340 of SM (d18:1/22:0) and of PC (40:4) when compared to the 341 offspring of normal weight mothers (Table 3). Maternal 342 overweight, but not maternal obesity, was associated with 343 enhanced plasma levels of Cer (d18:1/18:0), in newborn 344 babies (Table 3). 345

We observed a segregation of the lipidomic profile of 346 347 children at 4 years by maternal BMI status by PCA analysis with 15.9% of the variance explained by component 1 and 348 15.2% explained by component 2 (Supplemental Fig. 2). As 349 350 for mothers, ceramides were the lipids that contributed the most to these differences as indicated by the results of the 351 supervised OPLS statistical analysis ($R^2(X) = 0.374$, $R^2(Y)$) 352 = 0.876, and $Q^2(Y) = 0.584$, p-value of CV-ANOVA < 353 0.0001, Fig. 1g, h), and the quantification of the total 354 abundance of each lipid class ($F_{(2,44)} = 10.39$, p = 0.0002, 355 Fig. 1i). Examination of the plasma levels of each indivi-356 dual lipid further showed that in addition to ceramides 357 (d18:1/22:0), (d18:1/23:0), and (d18:1/24:0), children of 358 overweight and obese mothers exhibited differences in the 359

circulating levels of PC (36:2e) compared with children 360 born to mothers of normal body weight (Table 3). 361

Quantification of plasma ceramides

The concomitant changes in the plasma levels of ceramides 363 exhibited by overweight and obese mothers and their 4-364 year-old children, indicated that ceramides might be a 365 strong marker of the metabolic disease risk induced in the 366 offspring by maternal obesity. To add further support to this 367 hypothesis and validate the lipidomic data, we performed a 368 direct quantification of 10 different ceramides species in all 369 plasma samples using Cer (d18:1/17:0) as internal standard. 370 Data issued from this study were submitted to one-way 371 ANCOVA, using maternal age, and time of delivery as 372 adjusting factors for mothers and fasting length, sex, and 373 zBMI for children. The results of this study confirmed that 374 both maternal overweight and maternal obesity are asso-375 ciated with significant changes in the circulating levels of 376 ceramides in the offspring. Actually, the plasma con-377 centrations of Cer (d18:1/22:0), Cer (d18:1/23:0), and Cer 378 (d18:1/24:0) were all reduced by 20-35% among over-379 weight and obese mothers and their 4-year-old infants when 380 compared to lean mothers and their children (Fig. 2). Of 381 note, the concentration of Cer (d18:1/20:0) was also 382 decreased in obese and overweight mothers but not in their 383 children. The results of the analysis of covariance model for 384 mother's data were as follows: Cer 20:0 $F_{(2,42)} = 6.187$, p =385 0.004; Cer 22:0 $F_{(2,41)} = 4.260$, p = 0.021; cer 23:0 $F_{(2,40)}$ 386 = 3.339, p = 0.0465; cer 24:0 $F_{(2,42)} = 13.10$, p < 0.0001. 387 And those for children's data: Cer 22:0 $F_{(2,39)} = 3.888$, p =388 0.044; Cer 23:0 $F_{(2,41)} = 6.475$, p = 0.004; Cer 24:0 $F_{(2,41)}$ 389



Fig. 2 Plasma concentration of ceramides in mothers and their offspring. Graph represents the boxes and whisker plots corresponding to the concentrations of ceramides in plasma of mothers and their children at birth and at 4years old. Ceramides were quantified by UPLC– MS/MS using Cer (18:1/17:0) as internal standard. The boxes represent the 5th and 95th percentiles and horizontal lines within the boxes

indicate the medians. *p < 0.05; **p < 0.01; ***p < 0.001, versus their corresponding normal body weight counterparts (normal body weight mothers or children of mothers with normal body weight), as determined by one-way ANCOVA followed by Dunnett's multiple comparison test using maternal age, time of delivery, children fasting length, sex, and zBMI as covariates

= 9.37, p < 0.0001. Partial correlation analysis of the data 390 391 further showed that maternal plasma concentrations of Cer (d18:1/22:0) and Cer (d18:1/24:0) are also highly correlated 392 with the offspring concentrations at age 4 but not at birth 393 after adjusting for maternal BMI, and maternal age and 394 children fasting length, sex, and zBMI [r = 0.318, p =395 0.043 for the correlation between maternal and children Cer 396 (d18:1/22:0), and r = 0.554, p < 0.0001 for the correlation 397 between maternal and children Cer (d18:1/24:0)]. 398

To determine whether the BMI of the mother could predict ceramide plasma levels in her 4-year-old children, data were analyzed by multiple regression analysis using a 401 model in which the aforementioned variables were included 402 as confounding factors. As illustrated in Fig. 3b and c, there 403 was a statistically significant negative association between 404 maternal BMI and the plasma levels of Cer (d18:1/23:0) [F 405 $(5.41) = 2.796, p = 0.029, R^2 = 0.254; p = 0.003$ for maternal 406 BMI], and the plasma levels of Cer (d18/1:24:0 F(5,41) =407 4.399, p = 0.003, $R^2 = 0.349$; p < 0.001 for maternal BMI] 408 in children after adjusting for maternal age, children sex, 409 children zBMI, and children fasting length. We observed 410 also a negative association between maternal BMI and 411

Fig. 3 Predictive value of maternal BMI for plasma ceramide concentrations in 4year-old offspring and correlations between disease risk factor associated with obesity and plasma ceramide concentrations in children. Multiple regression analysis of the BMI of mothers versus the plasma concentrations of Cer (d18:1/22:0) (a). Cer (d18:1/ 23:0) (b), and Cer (d18:1/24:0) (c) in their children. Multiple correlation analysis in (a) was adjusted for children fasting length and children zBMI whereas maternal age, children sex, children zBMI, and children fasting length were used as confounding factors for the multiple linear regression analysis illustrated in (b) and (c). Square inserts in the figures correspond to coefficients of determination and the p values of the regressions models. (d), (e) and (c) illustrate Pearson's correlation coefficient analysis of the plasma concentrations of cholesterol versus those of Cer (d18:1/22:0) and Cer (d18:1/ 23:0), and between the circulating levels of AST and Cer (d18:1/22:4) in children. Correlation coefficients (r) and pvalues are shown



plasma levels of Cer (18:1/22:0), when data were adjusted for children fasting length and children zBMI [F(3,43) =3.271, p = 0.030, $R^2 = 0.186$; p = 0.016 for maternal BMI, Fig. 3a].

We further examined the relationship, in children, between plasma ceramides and other disease risk factors linked to obesity. Using Pearson's correlation analysis, we found a negative association between the levels of cholesterol and those of Cer (d18:1/22:0) and Cer (d18:1/23:0), as well as between the levels of AST and those of Cer (d18:1/ 24:0) (Fig. 3d-f). The concentrations of LDL were also 422 negatively correlated with the plasma levels of, respec-423 tively, Cer 23:0 and Cer 24:0. Similarly, there was a 424 negative correlation between zBMI and the circulating 425 levels of Cer 22:0 and Cer 24:0. However, none of the 426 associations persisted when the data were submitted to 427 multiple linear regression analysis using sex and fasting 428 length as adjusting variables. This might be explained by 429 the fact that children do not show yet any sign of metabolic 430

dysfunction and, therefore, the circulating levels of theirmetabolites fall within the normal range.

433 Dietary intake

To exclude the possibility that the differences in the lipidomic profile between children could result from different familial dietary habits, their dietary intake was evaluated. As shown in Supplemental Table S5, no significant differences in mean daily energy and macronutrient intakes were observed among the different groups of children.

440 **Discussion**

A large number of observational studies indicate that 441 maternal obesity is associated with several adverse health 442 outcomes in offspring including increased BMI and fat 443 mass and enhanced incidence of cardiovascular diseases, 444 type 2 diabetes, and stroke at adulthood. Further studies 445 aimed at identifying the underlying mechanisms of this 446 disease susceptibility, have shown that children of over-447 weight/obese mothers have high circulating levels of tri-448 glycerides, insulin, C-peptide, and IL-6 together with low 449 levels of HDL-C and apolipoprotein A-I [15, 16, 28]. Here, 450 we performed a lipidomic analysis and show that obese and 451 overweight pregnant mothers exhibit an altered lipidomic 452 profile in plasma and that the same alterations are present in 453 their offspring at age 4 but not at birth. Specifically, we 454 observed a concomitant decrease in the total relative 455 abundance of ceramides in obese and overweight mothers 456 and their children in comparison to mothers of normal body 457 weight and their offspring. The quantification of several 458 species of ceramides subsequently showed a specific 459 decrease in the concentration of Cer (d18:1/22:0), Cer 460 (d18:1/23:0), and Cer (d18:1/24:0). Interestingly, there were 461 no differences neither in the circulating levels of usual 462 markers of metabolic dysfunction (glucose, insulin, trigly-463 cerides, etc.), nor in zBMI among the children of the three 464 groups. Moreover, children exhibited equal daily amounts 465 of energy and food intake independently of the BMI of their 466 mothers. Taken together, these observations suggest that the 467 nutritional status of the mother during pregnancy affects in 468 469 the long term the lipidomic profile of the children and point out the potential role of ceramides as early predictors of 470 disease risk associated with maternal obesity. 471

The development of the fetus depends on the provision of nutrients from the maternal circulation. This leads intuitively to think, that there should be a perfect match in the metabolite composition of the maternal and infant plasma at delivery. It may therefore seem paradoxical that there is a correlation between the plasma concentrations of ceramides of mothers and their 4-year-old children, but not between the circulating levels of ceramides of mothers and 479 babies at birth. However, previous studies have consistently 480 shown that the fatty acid composition of umbilical cord 481 plasma is different from that of maternal plasma [29, 30] 482 likely because of the high rate of fatty acid metabolism 483 within the placenta [31]. 484

It has been reported that ceramides are increased in obese 485 humans and are positively related with insulin resistance 486 [32–35]. These results are at odds with the herein presented 487 observations in overweight and obese mothers and raise the 488 question of the physiopathological relevance of the altera-489 tions in the concentration of ceramides detected in their 490 children. However, other data from the literature could 491 explain our seemingly contradictory results. First, a close 492 inspection of the clinical phenotype of the patients in which 493 enhanced ceramide levels have been linked with obesity, 494 shows that these patients are already insulin resistant [32-495 35]. In fact, several studies have failed to observe differ-496 ences in the concentration of ceramides between lean and 497 obese non-diabetic patients [36-39], indicating that an 498 increase in ceramide content is a hall mark of insulin 499 resistance rather than of obesity per se. Second, to date, all 500 the studies linking ceramides to obesity have been per-501 formed in biological samples from men or non-pregnant 502 women. This makes it difficult to establish a direct com-503 parison with the present data because of the major adjust-504 ments in lipid metabolism occurring during gestation which 505 include dynamic changes in the concentration of ceramides 506 in blood [40]. For instance, it has been shown that the 507 plasmatic levels of glycerophosphatidylcholine (PC), 508 decrease during the second trimester of pregnancy while 509 those of LPC, lysophosphatidylethanolamine (LPE), and 510 lipopolysaccharides (LPS) increase during the same period 511 (Luan et al., 2014). Similarly, plasma levels of several 512 ceramide species have been reported to increase across 513 gestation [40]. Indeed, to the best of our knowledge, this is 514 the first report about the effects of obesity on the circulating 515 levels of ceramides during pregnancy. Third, although the 516 enhanced concentration of ceramides in blood followed by 517 their accumulation in tissues has been proposed to have a 518 key role in obesity-related metabolic disorders, ceramides 519 are also down regulated in several pathological conditions. 520 In particular, a significant decrease in the concentration of 521 ceramides has been reported in the amniotic fluid of women 522 with fetal Down syndrome [41]. Similarly, preeclamptic 523 patients exhibit a reduction of plasma Cer (d18:1/24:0) 524 during the third trimester of pregnancy when compared to 525 match-gestational control women [40]. An inverse rela-526 tionship between the serum levels of Cer (d18:1/24:1) and 527 the occurrence of ischemic heart disease [42], or the pro-528 gression of hepatic cirrhosis [43], has also been reported. 529 Finally, in addition to be at high risk of developing meta-530 bolic disorders, offspring of obese mothers exhibit also 531

adverse neuropsychiatric problems including attention-def-532 icit/hyperactivity disorder (ADHD) [44, 45]. In this respect, 533 it is worth mentioning that children with ADHD have 534 decreased serum levels of ceramides [46]. The herein 535 reported alterations in the concentration of ceramides in 536 children of obese or overweight mothers may therefore 537 constitute an early marker of both cognitive disability and 538 metabolic disease risk. 539

The de novo ceramide synthesis involves the condensa-540 tion of serine and palmitoyl-CoA to form 3-ketosphinganine 541 followed by the sequential formation of sphinganine, 542 dihydroceramide, and ceramide. The N-acylation of sphin-543 ganine to produce dihydroceramide is catalyzed by a family 544 of six enzymes, the ceramide synthases (CerS), which differ 545 in their specificity toward the acyl CoA chain length used 546 for N-acylation leading to the synthesis of ceramides of 547 different length chain [47, 48]. This is of importance 548 because accumulating evidence indicates that the physio-549 550 logical and pathological effects of ceramides vary according to their acyl chain length [49-51]. In the present study we 551 observed that obese and overweight mothers and their 552 children exhibit decreased circulating levels of ceramides 553 carrying C22:0, C23:0, and C24:0 saturated fatty acyl 554 chains which are produced by ceramide synthase 2 [47, 52]. 555 This indicates that maternal obesity is associated with 556 selective changes in ceramide metabolism. 557

To the best of our knowledge, this is the first report 558 describing the impact of maternal obesity on the offspring 559 lipidome. An additional strength of the study is the simul-560 taneous analysis of the mother-child dyad lipid profile at 561 delivery and its longitudinal nature. Few studies have 562 reported data on children blood metabolites collected from 563 birth into childhood. This approach enabled us to identify 564 potential early markers of disease risk and to get a more 565 comprehensive understanding of the metabolic disturbances 566 associated with maternal obesity that can be transmitted to 567 the offspring. We are nevertheless aware that our study has 568 several limitations that should be addressed in future work. 569 First, our results were obtained on a limited number of 570 patients and, therefore, need to be confirmed in larger 571 572 cohorts. Second, though children exhibited similar amounts of total calorie intake, we cannot exclude the possibility that 573 the observed lipidomic differences are secondary to genetic 574 575 factors or to other life style behaviors or socio-economic factors like physical activity, snacking, and family eco-576 nomic income. Finally, all people included in the study 577 were living in the same geographic area. Consequently, it 578 remains to be determined whether the herein reported 579 results can be generalized to other populations. 580

In conclusion, this study shows that maternal obesity is associated with concomitant changes in the circulating levels of ceramides in mother and their 4-year-old offspring. Given the involvement of ceramides in the pathogenesis of obesity-related pathologies, this finding points out the 585 potential role of ceramides as early predictors of disease risk 586 due to maternal obesity. 587

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Author contributions LFL-A and MC performed the lipidomic ana-600 lysis and contributed to data analysis and to the writing of the 601 manuscript; VF-R contributed to data analysis; FH and LAM per-602 formed the metabolite determinations in plasma; AB-V designed the 603 data collection instruments, coordinated, and supervised data collec-604 tion during the age-4 follow-up and provided the plasma samples; UR 605 and IR conducted the original clinical trial; MK and KO supervised the 606 lipidomic analysis and provided direction for data analysis; RM-C 607 provided direction to the original idea; FB-J designed and coordinated 608 the study, analyzed the data, wrote the manuscript and had primary 609 responsibility for the final content. All authors were involved in editing 610 the manuscript and had final approval for the submitted version. 611

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of 613 interest. 614

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