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Clinical Research

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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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 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 (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 (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.

Supplementary information The online version of this article (<https://doi.org/10.1038/s41366-018-0291-y>) contains supplementary material, which is available to authorized users.

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Introduction

Obesity is a chronic pathological condition characterized by the excessive accumulation of adipose tissue often complicated by one of the core components of the metabolic

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

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

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

88 identify changes in lipid profile that might underpin, and
 89 serve as early biomarkers of, the disease risk resulting from
 90 maternal obesity.

91 Materials and methods

92 Study population and sample collection

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

128 Dietary assessment

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

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

Metabolite determinations

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

Lipidomic analysis

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

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

Quantification of plasma ceramides

We used the method described by Croyal et al. [23], to quantify different species of ceramides. A pool of standard solutions, including 10 species of ceramides (Cer), was prepared and then serially diluted in methanol (Sigma Aldrich, Saint-Quentin Fallavier, France), to obtain seven standard solutions at 1, 5, 10, 50, 100, 250, and 500 nM. Standard solutions and plasma samples (25 μ L), were extracted as described in the previous section after the addition of Cer (d18:1/17:0) as internal standard. UPLC–MS/MS analyses were performed on a Xevo[®] triple–quadrupole mass spectrometer with an ESI interface and an Acquity H-Class[®] UPLCTM device (Waters Corporation). Data acquisition and analyses were performed with MassLynx[®] and TargetLynx[®] software, respectively (version 4.1, Waters Corporation, Milford, MA, USA). Compounds were separated on an Acquity[®] BEH C₁₈ column (2.1 \times 50 mm, 1.7 μ m, Waters). Ceramides and internal standards were detected by the mass spectrometer with the ESI interface operating in the positive ion mode. The multiple reaction mode (MRM) was applied as described in Supplemental Table S2. Chromatographic peak area ratios between ceramides and their internal standard constituted the detector responses. Standard solutions were used to plot calibration curves and a linear regression model (1/ x weighting) was used for quantification. The intra-assay and inter-assay variabilities of the analytical method were assessed over three distinct experiments (four concentration levels, six replicates by level), and were below 13.6%. Recoveries were assessed with internal standard and were >91%.

Statistical analysis

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

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

Variable	Maternal body weight characteristics			One-way ANOVA <i>p</i> -value
	Normal weight (<i>n</i> = 18)	Overweight (<i>n</i> = 18)	Obese (<i>n</i> = 11)	
<i>Maternal characteristics</i>				
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
<i>Infant characteristics at birth</i>				
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	
<i>Child characteristics and metabolite profile in serum at 4 years</i>				
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 <i>z</i> -score	−0.17 (−0.54 to 0.19)	0.49 (−0.14 to 1.12)	0.13 (−0.84 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 ± 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 gamma-glutamyl-transferase

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

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

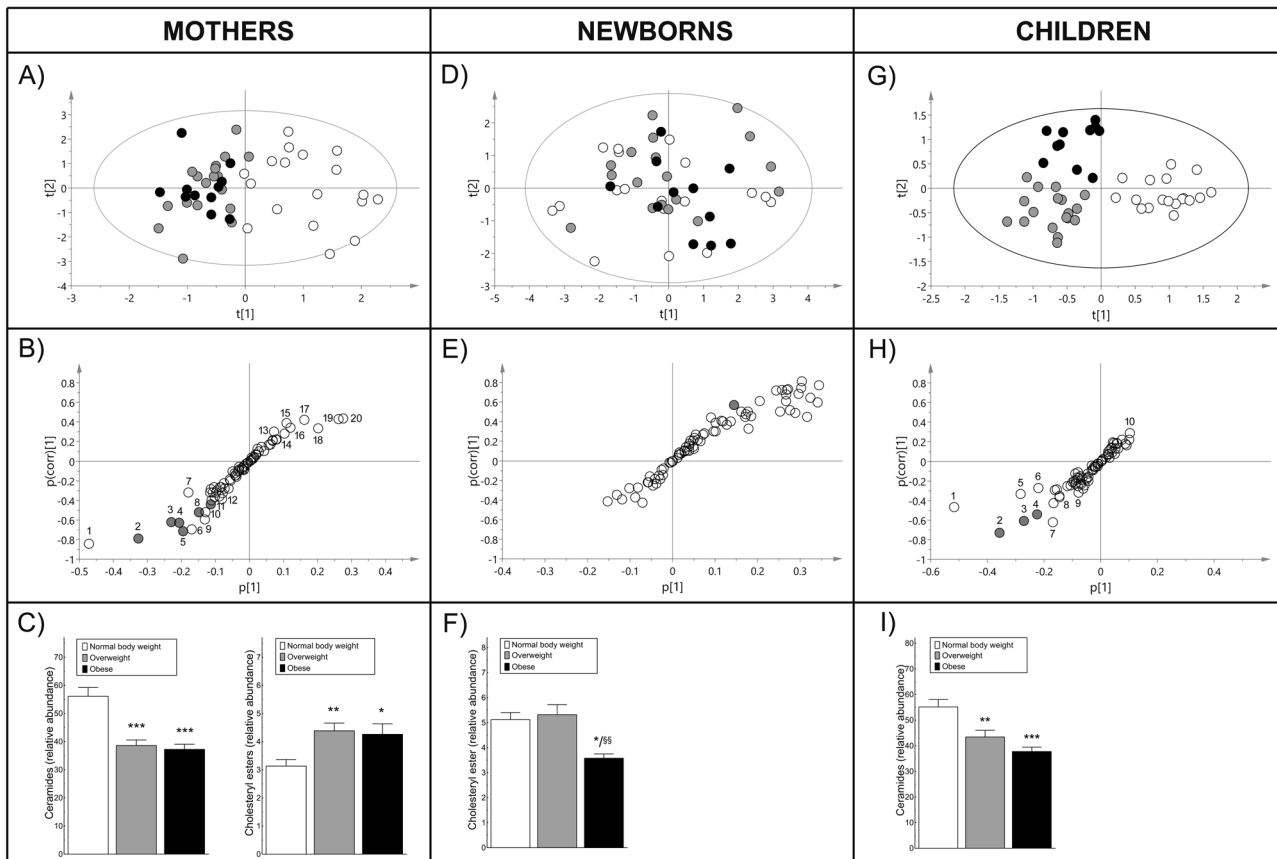


Fig. 1 Global differences in plasma lipidome between mothers and between children of normal body weight (NBW), overweight (OW) or obese (OB) mothers. Scores plots of the OPLS-DA models of lipidomic profile in mothers (a) and their newborns (d) and children (g). Note the clear segregation pattern among 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

(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 (18:1/22:0). 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

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

Results

267

Clinical characteristics of the study population

268

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

269
 270
 271
 272
 273
 274

Table 2 . Maternal lipidomic profile in plasma according to body mass index

Type of Lipid	Maternal body weight characteristics			One-way ANOVA <i>p</i> -value
	Normal weight (<i>n</i> = 18)	Overweight (<i>n</i> = 18)	Obese (<i>n</i> = 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 ± 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 ± 0.04**	0.95 ± 0.05*	0.0004
Cer (d18:1/20:0)	1.96 ± 0.10	1.27 ± 0.05***	1.35 ± 0.07***	<0.0001
Cer (d18:1/22:0)	9.42 ± 0.39	7.75 ± 0.30**	7.81 ± 0.31*	0.0037
Cer (d18:1/23:0)	6.35 ± 0.38	4.46 ± 0.25***	4.56 ± 0.25**	0.0001
Cer (d18:1/24:0)	20.61 ± 1.86	11.32 ± 0.66***	11.19 ± 0.67***	<0.0001
Cer (d18:1/24:1)	14.97 ± 0.95	11.59 ± 0.93*	10.00 ± 0.77**	0.0031
PE (38:4)	5.25 ± 0.42	6.51 ± 0.45	7.41 ± 0.78*	0.0321
CE (18:2)	1.25 ± 0.14	2.08 ± 0.25*	2.09 ± 0.30*	0.0119
DG (34:1)	0.72 ± 0.06	1.00 ± 0.08*	1.00 ± 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 one-way 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
276 offspring of overweight and obese mothers presented higher
277 plasma concentrations of AST and ALT compared with
278 children of mothers of normal body weight. Nevertheless,
279 the plasma concentrations of these metabolites were below
280 the cut-off reference values of hepatic steatosis. Apart from
281 these differences, the plasma metabolite profile of all chil-
282 dren at 4 years was very similar.

283 Maternal lipidome fingerprints

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

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

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

Table 3 Impact of maternal obesity on the serum lipidomic profile of the offspring

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

Data are expressed as mean ± 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

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

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

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

Quantification of plasma ceramides

362 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

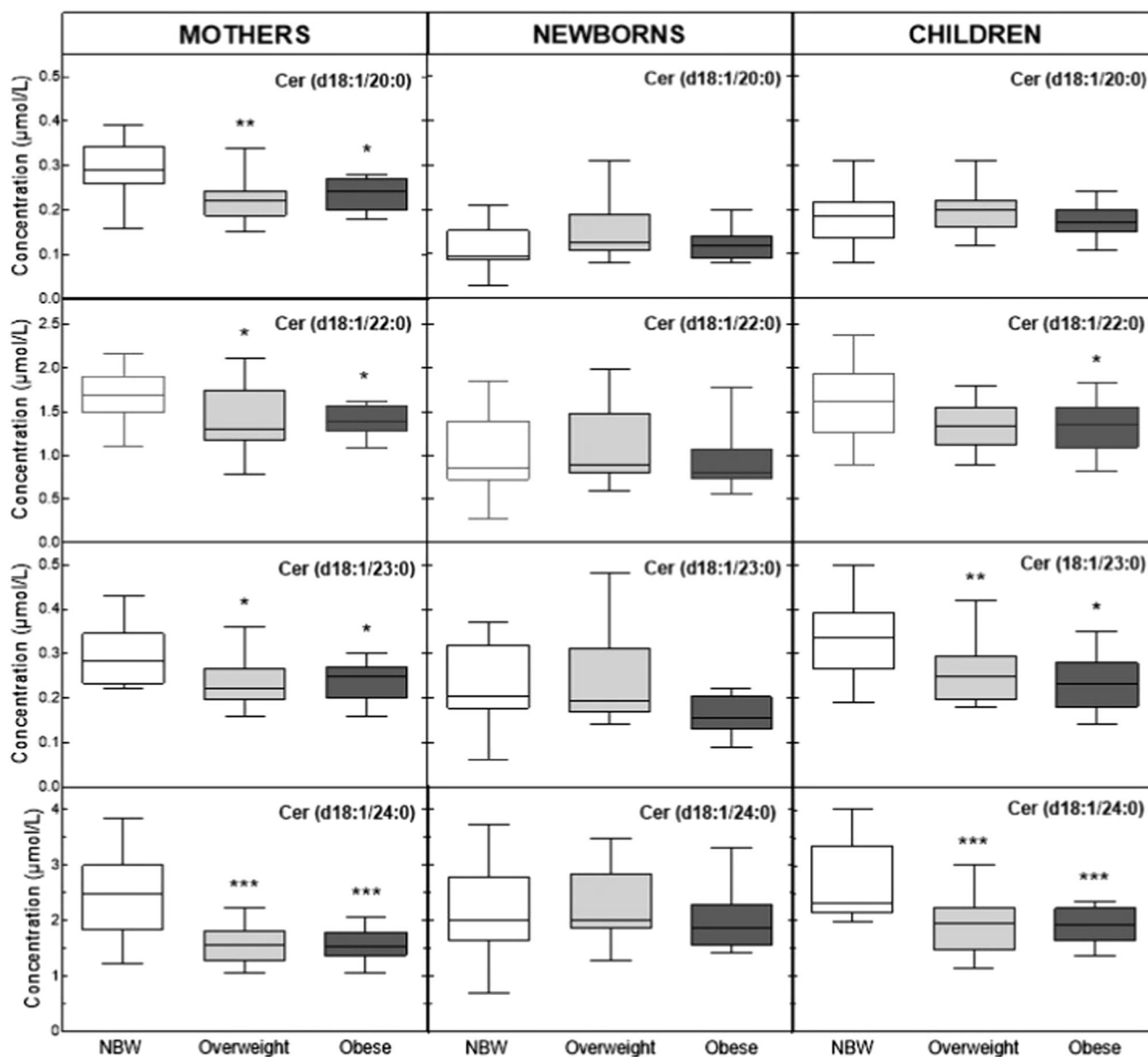


Fig. 2 Plasma concentration of ceramides in mothers and their offspring. Graph represents the boxes and whisker plots corresponding to the concentrations of ceramides in plasma of mothers and their children at birth and at 4 years old. Ceramides were quantified by UPLC–MS/MS using Cer (18:1/17:0) as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

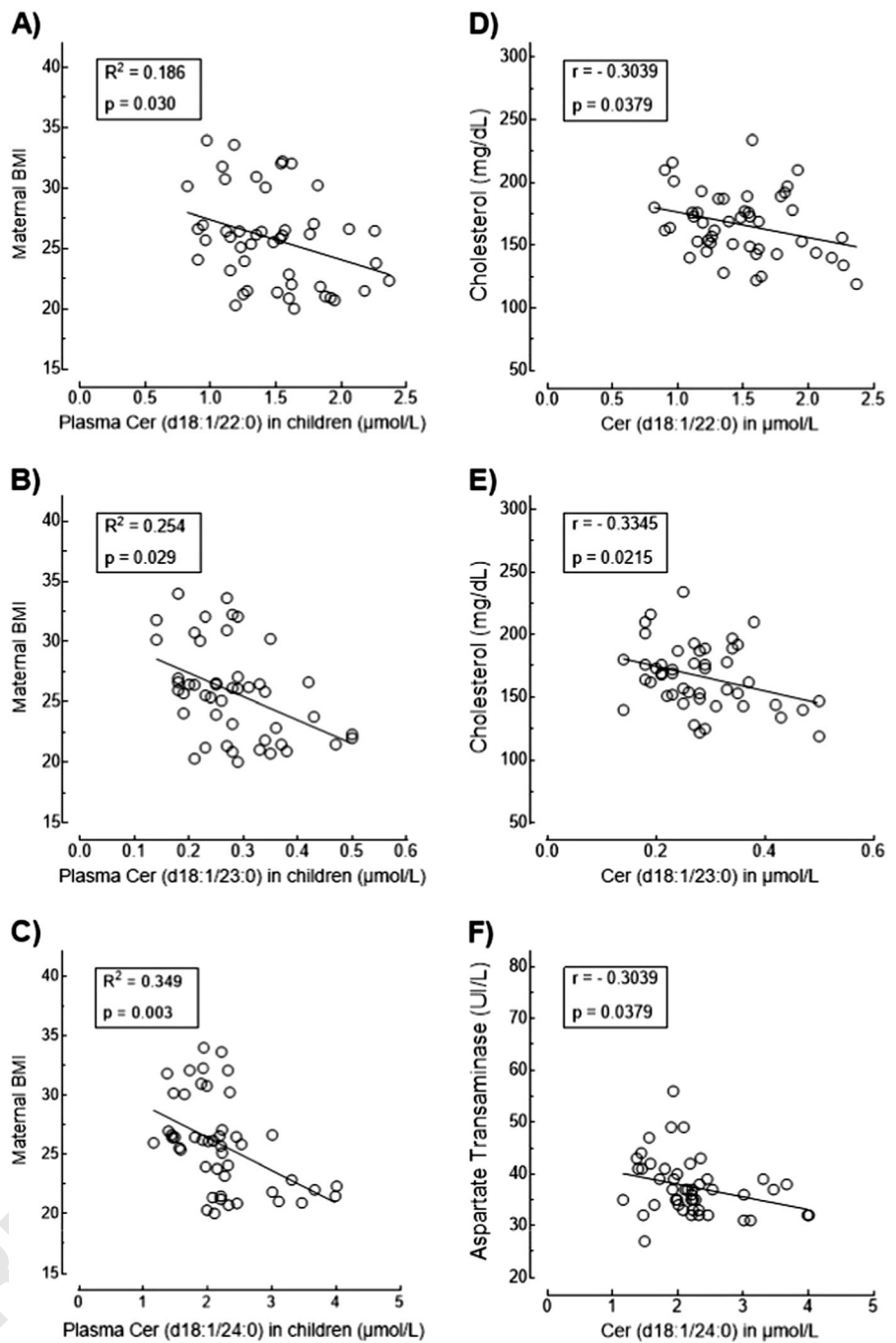
indicate the medians. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, versus their corresponding normal body weight counterparts (normal body weight mothers or children of mothers with normal body weight), as determined by one-way ANCOVA followed by Dunnett's multiple comparison test using maternal age,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

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

399 To determine whether the BMI of the mother could
 400 predict ceramide plasma levels in her 4-year-old children,

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

Fig. 3 Predictive value of maternal BMI for plasma ceramide concentrations in 4-year-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 *p* values are shown



412 plasma levels of Cer (18:1/22:0), when data were adjusted
 413 for children fasting length and children zBMI [$F(3,43) =$
 414 3.271 , $p = 0.030$, $R^2 = 0.186$; $p = 0.016$ for maternal BMI,
 415 Fig. 3a].

416 We further examined the relationship, in children,
 417 between plasma ceramides and other disease risk factors
 418 linked to obesity. Using Pearson's correlation analysis, we
 419 found a negative association between the levels of chole-
 420 sterol and those of Cer (d18:1/22:0) and Cer (d18:1/23:0), as
 421 well as between the levels of AST and those of Cer (d18:1/

24:0) (Fig. 3d-f). The concentrations of LDL were also
 negatively correlated with the plasma levels of, respec-
 tively, Cer 23:0 and Cer 24:0. Similarly, there was a
 negative correlation between zBMI and the circulating
 levels of Cer 22:0 and Cer 24:0. However, none of the
 associations persisted when the data were submitted to
 multiple linear regression analysis using sex and fasting
 length as adjusting variables. This might be explained by
 the fact that children do not show yet any sign of metabolic

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

433 Dietary intake

434 To exclude the possibility that the differences in the lipi-
435 domic profile between children could result from different
436 familial dietary habits, their dietary intake was evaluated.
437 As shown in Supplemental Table S5, no significant differ-
438 ences in mean daily energy and macronutrient intakes were
439 observed among the different groups of children.

440 Discussion

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

472 The development of the fetus depends on the provision
473 of nutrients from the maternal circulation. This leads
474 intuitively to think, that there should be a perfect match in
475 the metabolite composition of the maternal and infant
476 plasma at delivery. It may therefore seem paradoxical that
477 there is a correlation between the plasma concentrations of
478 ceramides of mothers and their 4-year-old children, but not

between the circulating levels of ceramides of mothers and
babies at birth. However, previous studies have consistently
shown that the fatty acid composition of umbilical cord
plasma is different from that of maternal plasma [29, 30]
likely because of the high rate of fatty acid metabolism
within the placenta [31].

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

532 adverse neuropsychiatric problems including attention-deficit/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

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

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

581 In conclusion, this study shows that maternal obesity is 582 associated with concomitant changes in the circulating 583 levels of ceramides in mother and their 4-year-old offspring. 584 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

612 Compliance with ethical standards

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

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