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Impact of Maternal Weight Gain on the Newborn Metabolome

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Abstract: Pre-pregnancy obesity and excessive gestational weight gain (GWG) appear to affect birth weight and the offspring's risk for obesity and disease later in life. However, the identification of the mediators between this relationship, could be of clinical interest, taking into account that there are other confounding factors, such as genetics and other shared influences. The aim of this study was to evaluate the metabolomic profile of infants at birth (cord blood) and 6 and 12 months after birth to identify offspring metabolites associated to maternal GWG. Nuclear Magnetic Resonance (NMR) metabolic profiles have been measured in 154 plasma samples from newborn (82 cord blood samples) and from 46 and 26 of them at 6 months and 12 months of age, respectively. The levels of relative abundance of 73 metabolomic parameters have been determined in all the samples. We performed univariate and machine-learning analysis of the association between metabolic levels and maternal weight gain adjusted for mother age, Body Mass Index (BMI), diabetes, and diet adherence and for infant sex. Overall, our results show differences, both at univariate level and in the machine-learning models, in the offspring according to tertiles of maternal weight gain. Some of these differences are resolved at 6 and 12 months of age whereas some other remain. Lactate and leucine are the metabolites with the strongest and longest association with maternal weight gain during pregnancy. Leucine, as well as other significant metabolites, have been associated in the past with metabolic wellness in both general and obese population. Our results suggest that metabolic changes associated to excessive GWG are present in children since early life.

Keywords: metabolomics; gestational weight gain; offspring; newborn; umbilical cord

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1. Introduction

In 1989 Barker et al., hypothesized that an unfavourable environment in utero could cause programmed adaptations in fetal development that would persist into extrauterine life with a phenotype more susceptible to cardiovascular disease [1]. An example of an unfavourable intrauterine milieu is fetal overnutrition, defined as fetal exposure to excess of maternal fuels due to maternal obesity, gestational diabetes (GD), or excessive gestational weight gain (GWG). Many epidemiological, clinical, and animal model research strongly suggests that mother prenatal obesity and high-fat dietary intake are associated with cardiometabolic morbidity in the progeny, including obesity, hypertension, hyperglycaemia and insulin resistance, hyperlipidaemia and non-alcoholic fatty liver disease [2,3].

Many studies support the possibility that cord plasma metabolite profile (resultant of fetal response to in utero exposure) can serve as early-life risk biomarkers for metabolic disease in the long term. Hence, the growing interest in investigating the metabo-

lomic profile in cord blood of mother-child pairs studies with inconsistent results.

Perng et al., reported that metabolites in energy production and DNA/RNA turnover pathways and branched-chain amino acids (BCAAs) in cord blood were associated with larger neonatal size (a known risk factor for poor cardiovascular health later in life) and that BCAAs and androgen hormone pattern were higher in obese vs. lean school-age children (6–10 years). Furthermore, higher factor scores for these patterns were correlated with continuous measures of overall and central adiposity and other cardiometabolic biomarkers like higher HOMA-IR [4,5]. Kadakia et al., found that cord blood BCAAs and ketones body metabolites were positively correlated with newborn adiposity [6]. Cao et al., linked cord plasma metabolites with 946 children's longitudinal body mass index (BMI) trajectories from birth up to age 18 years, categorized into 3 patterns: early onset overweight and obesity (early-OWO), late onset OWO (late-OWO) and normal weight trajectory (NW). Their results suggested that cord blood metabolome was most useful in identifying children at risk of early-OWO, with 22 triacylglycerols and diacylglycerols negatively associated with early-OWO, and 5 cholesterol esters positively associated [7].

Again, Perng et al., did not find any significant associations of cord blood metabolite patterns and maternal characteristics except for caesarean delivery and some metabolites of energy production and cell proliferation pathways, associated with larger size at birth. On the other hand, they observed that the BCAAs score was higher in cord plasma of overweight women before pregnancy [5]. Lowe et al., found an association of maternal BMI with cord plasma levels of BCAAs and their metabolic bioproducts and phenylalanine[8]. Francis et al., noted differences in 52 metabolites between exposed and non-exposed to fetal overnutrition with small amount of variation between maternal obesity only, GD only and both [9].

However, while obesity and GD are widely studied as conditions for fetal overnutrition, few metabolomic studies have focused on GWG. To gain a better understanding of the latter's influence we performed a longitudinal study in obese and lean pregnant women classified by tertiles of GWG. The aim of our study was to identify differences in the offspring metabolite profiles, from newborn (cord blood) to 12 months age, with respect to maternal GWG.

2. Materials and Methods

2.1. Study Population: Clinical and Biochemical Measurements

The research work hypothesizes that maternal-dependent factors, especially GWG, lead to metabolomic changes in the newborn and during the first year of life.

For this purpose, a prospective observational cohort study was designed, beginning at birth and followed up to 12 months of life. Eighty-three mother-newborn pairs were divided into three groups according to tertiles of weight gain to analyse the impact of the mother's gestational weight gain. To participate in the study, the following inclusion and exclusion criteria must be fulfilled: Newborns were born at full term (over 37 weeks gestational age at birth) ascertained according to the Ballard method [10], by normal delivery or caesarean section, at the Hospital General Universitario of Valencia, Spain. Preterm newborns or newborn with any perinatal pathology were excluded. Mothers included were normal weight, obese or obese with gestational diabetes but mothers with gestational diabetes treated with insulin or any other illness.

All participating mothers received written information about the study and signed an informed consent to participate. The study was approved by the hospital's review board and was carried out in accordance with the Declaration of Helsinki. Both the samples (plasma from umbilical cord of the newborns and plasma from peripheral veins from the mothers), as well as the collected data, were stored according to the directives dictated by the law of Biomedical Investigation of 2007 (Law 14/2007) and all applicable rules.

(A) From mothers, age, height, weight measurements prior to gestation as recorded in their pregnancy booklet by the midwife, and weight at the end of gestation was

collected. GWG was calculated and its adequacy to the 2009 recommendations of the Institute of Medicine (IOM) was addressed [11]. Regarding the evolution of the pregnancy, the presence or absence of gestational diabetes, the degree of maternal adherence to the Mediterranean diet style (AMD) using the validated questionnaire of Trichopoulos A et al. [12] and the smoking habit were recorded. Finally, the type of delivery was also recorded (vaginal or caesarean section).

(B) From all newborns, the following information was recorded:
- Somatometry (weight, length, head circumference and ponderal index and percentiles according to Intergrowth-21st), performed by trained nurses in the maternity ward. Weight was measured with ADE scale model M112600 (ADE GmbH & Co, Hamburg, Germany). Length was measured in the supine position using a neonatometer. Head circumference was measured with a tape measure at the maximum circumference. The ponderal index, also known as a corpulence index or Rohrer's index, was calculated with the following formula PI = weight/length^3 × 100. Newborns were classified as small for gestational age (SGA), appropriate for gestational age (AGA) or large for gestational age (LGA) [13].
- Blood pressure (BP) by oscillometric method, taking 3 measurements with System 7100 Non-invasive Blood Pressure AMI (Advanced Medical Instruments Inc., Broken Arrow, OK, USA) and heart rate (HR).
- Type of feeding (breastfeeding or infant formula feeding); as well as the need for supplementation with artificial formula during the first days of life in those who were breastfed.
- Umbilical cord blood samples were obtained from the clamped umbilical cord immediately after delivery for metabolomic studies.

(C) Blood sample was collected at 6 months of age to make metabolomic study.

(D) In infants, at 12 months of life, weight, length, head circumference and BMI were recorded and its percentiles according to the WHO 2006/2007 curves were calculated [14]. The type of feeding at 12 months was recorded. Finally, blood sample was collected to make metabolites and biochemical studies.

2.2. NMR Metabolomics

Umbilical venous cord blood was collected in EDTA-tubes, centrifuged to yield plasma, stored at −80 °C and thawed before use. For Nuclear Magnetic Resonance (NMR) analysis, 500 µl of plasma was mixed with 50 µl of D2O (as a field lock). A total of 500 µl of the mixture of each sample was then individually transferred into a 5 mm high quality NMR tube. A single pulse presaturation NMR spectrum in all samples was acquired, with 256 transients collected into 65 k data points for all experiments. NMR is a reproducible and accessible technique that has been applied successfully to the metabolic profiling of a variety of samples [15–17]. Spectra were processed using MestReNova 8.1 (Mestrelab Research S.L., A Coruña, Spain). Spectra were chemical shift referenced on the alanine CH3 doublet signal at 1.475 ppm, normalized to the total aliphatic area, lipid excluded, and transferred to MATLAB (MathWorks, Natick, MA, USA, 2012). We analysed the chemical shift region including resonances 0.50–4.70 ppm (the aliphatic region) and 5.20–10.00 ppm (the aromatic region). Resonances were annotated by literature data and Chenomx resonances database (Chenomx NMR 7.6). NMR peaks were quantified using semi-automated in-house MATLAB peak-fitting routines. The final metabolite relative spectral abundance was calculated. Machine learning and regression models were calculated in R 4.1 with the package 'mdatools'. Finally, each metabolic feature was normalized to the standard deviation in all the samples of the analysis group (birth, 6 months, or 12 months) to obtain z-scores.

Partial least-squares discriminant analysis (PLS-DA) was applied to maximize the separation between samples and to identify discriminant patterns. [18] The PLSDA

models were for child sex and weight at birth and for maternal obesity, adherence to the Mediterranean diet and diabetes by calculating a linear regression model with these variables for each metabolic feature and using the residues for the PLS-DA analysis. A permutation test was performed to check the potential overfitting of the PLS-DA models. The chemometric models were cross-validated with 10-fold Venetian blind cross-validation. In each replicate, 10% of the data were left out of the training calculations and used as a test dataset. Variable importance in projections (VIP) coefficients were calculated for all the models to select spectral features contributing the most to the models. Spectral features with high VIP coefficients contribute more to class separation during analysis, while those with very small VIP coefficients provide little contribution to classification. A Metabolite Set Enrichment Analysis (MSEA) over metabolites with VIPs scores higher than 1 and *p*-values below 0.05 overall the analysis was performed with MetaboAnalyst and the Small Molecule Pathway Database (SMPDB). MSEA is conceptually similar to Gene Set Enrichment Analysis. A collection of predefined metabolite sets is used by MSEA algorithms to rank the lists of metabolites obtained in the analysis. This prior knowledge about metabolite sets allows us to identify significant and coordinated changes in metabolic networks and obtain biological insight.

2.3. Statistical Analysis

Demographics and Clinical Data Comparison

The IBM SPSS Statistics v.26 statistical program was used for data analysis. First, the database was segmented according to the 3 study groups, and the Kolmogorov-Smirnov normality test was performed to verify that the population sample conformed to a normal distribution for the quantitative variables in the 3 groups. When a normal distribution of the variable was not observed in any of the groups, the means of the variable in the 3 groups were compared with the nonparametric Kruskal-Wallis test. In case the variable had a normal distribution in the 3 groups, the means of the variable in the 3 groups were compared with ANOVA. The data of the quantitative variables were summarized in their simple statistics (mean ± standard deviation). Differences between qualitative variables were analysed using the Chi-square test and the data were expressed as percentages.

3. Results

The sample was divided according to tertiles mother's absolute GWG. Maternal characteristics were comparable among the three groups except for BMI prior to gestation which was higher in the group in the first tertile of GWG ($p = 0.004$) and GWG adequacy according to Institute of Medicine recommendations [11], higher in the first group as expected. Table 1 shows the clinical differences of the mothers.

Table 1. Characteristics of mothers, according to absolute maternal GWG tertiles. Data are presented as either mean ± SD or no. (%). ǂ Analysis of variance (ANOVA). ¥ The Kruskal Wallis test. • chi-squared test. Data with significant *p*-value are indicated:** for $p < 0.01$ and *** for $p < 0.001$.

Mother's Variables	1st Tertile GWG (N = 28)	2nd Tertile GWG (N = 27)	3rd Tertile GWG (N = 28)	*p* Value
Age (years) ¥	31.5 (±8.48)	34.11 (±5.17)	33.18 (±5.41)	0.429
Maternal obesity	Yes 75% No 25%	Yes 48.1% No 51.9%	Yes 57.1% No 42.9%	0.116
BMI prior to pregnancy (Kg/m²) ¥	33.61 (±7.31)	28.34 (±7.07)	28.07 (±6.03)	0.004 **
GWG (Kg) ǂ	2.33 (±4.31)	10.11 (±1.12)	16.78 (±3.91)	<0.001 ***
GWG adequacy according to IOM recommendations •	Yes 100% No 0%	Yes 66.7% No 33.3%	Yes 21.4% No 78.6%	<0.001 ***
AMD score ¥	6.96 (±2)	7.76 (±1.7)	7.46 (±1.6)	0.396

Degree of AMD •	High 12.5% Medium 58.3% Low 29.2%	High 23.5% Medium 70.6% Low 5.9%	High 8.3% Medium 75% Low 16.7%	0.266
Diabetes during pregnancy	Yes 35.7% No 64.3%	Yes 11.1% No 88.9%	Yes 17.9% No 82.1%	0.072
Smoking habit •	Yes 14.3% No 85.7%	Yes 18.5% No 81.5%	Yes 21.4% No 78.6%	0.78
Type of delivery •	Vaginal 50% Cesarean 50%	Vaginal 44.4% Cesarean 55.6%	Vaginal 60.7% Cesarean 39.3%	0.469

There were no clinical differences in offspring at birth according to tertiles of maternal GWG, as shown in Table 2.

Table 2. Characteristics of children at birth, according to absolute maternal GWG tertiles. Data are presented as either mean ± SD or no. (%). ǂ Analysis of variance (ANOVA). ¥ The Kruskal Wallis test. • chi-squared test. Data with significant *p*-value are indicated:

Newborn's Variables	1st Tertile GWG (N = 28)	2nd Tertile GWG (N = 27)	3rd Tertile GWG (N = 28)	*p* Value
Birth weight (BW) (g) ǂ	3363 (±552)	3311 (±557)	3454 (±449)	0.593
Weight percentile ǂ	53.45 (±31.14)	52.4 (±31.1)	58 (±30.4)	0.773
BW Classification •	SGA 7.1% AGA 75% LGA 17.9%	SGA 11.1% AGA 70.4% LGA 18.5%	SGA 10.7% AGA 60.7% LGA 28.6%	0.8
Length (cm) ǂ	49.37 (±2.08)	48.6(±1.94)	49.67 (±2.13)	0.149
Length percentile ǂ	48.81 (±28.2)	39.78 (±25.71)	53.33 (±31.8)	0.21
Head circumference (HC) (cm) ¥	34.46 (±1.52)	34.38 (±1.57)	34.97 (±1.41)	0.351
HC percentile¥	62.8 (±27.01)	64 (±28.79)	71.68 (±27.67)	0.323
ponderal index ¥	2.77 (±0.267)	2.85 (±0.24)	2.81 (±0.31)	0.406
Systolic blood pressure (mmHg) ǂ	78.63 (±87)	81.5 (±12.5)	78.89 (±12.66)	0.605
Diastolic blood pressure (mmHg) ǂ	50.66 (±10)	49.79 (±9.63)	49.5 (±9.45)	0.899
Heart rate (bpm) ǂ	126.81 (±14.69)	129.65 (±12.65)	132.69 (±16.93)	0.366
Type of feeding •	breast 67.9% formula 32.1%	breast 88.9% formula 11.1%	breast 75% formula 25%	0.169
Formula Supplementation •	Yes 31.6% No 68.4%	Yes 29.2% No 70.8%	Yes 19% No 81%	0.62

Table 3 shows the characteristics of children at 12 months of life according to tertiles of absolute maternal GWG. There were no clinical differences at 12 months of age except for length ($p = 0.039$). There were also no statistically significant differences in children blood tests at 12 months of age.

Table 3. Characteristics of children at 12 months of life according to tertiles of absolute maternal GWG. Data are presented as either mean ± SD. ǂ Analysis of variance (ANOVA). ¥ The Kruskal Wallis test. Data with significant *p*-value are indicated: * for $p < 0,05$,

	1st Tertile GWG	2nd Tertile GWG	3rd Tertile GWG	*p* Value
12 Months Somatometry	(N = 27)	(N = 24)	(N = 25)	
Weight (g) ǂ	10,509 (±1516)	9736 (±1193)	10,293 (±1242)	0.114
Weight percentile ¥	71.14 (±28.75)	58.54 (±29.43)	61.92 (±27.87)	0.214
Length (cm) ǂ	76.74 (±3.4)	74.83 (±2.64)	76.64 (±2.4)	0.039 *
Length percentile ¥	60.4 (±32)	48.79 (±28.72)	60.08 (±26.68)	0.128
Head circumference (HC) (cm) ¥	46.38 (±1.32)	45.81 (±1.15)	46.80 (±1.69)	0.094
HC percentile ¥	64.64 (±27.89)	57.83 (±24.94)	68.2 (±29.1)	0.216
BMI (Kg/m²) ǂ	17.65 (±1.53)	17.29 (±1.76)	17.38 (±1.69)	0.722
BMI percentile ¥	67.86 (±29.172.63)	6(±28.24)	60.07(±29.59)	0.516
12 Months blood test	(N = 12)	(N = 8)	(N = 12)	
Leptin (pg/ml) ¥	452 (±613)	691 (±380)	513 (±626)	0.378
Total cholesterol (mg/dl) ǂ	160.6 (±32)	158 (±38.2)	152.75 (±25.64)	0.827
HDL cholesterol (mg/dl) ǂ	40.5 (±9.4)	44.75 (±10.91)	43.16 (±10.66)	0.64
LDL cholesterol (mg/dl) ǂ	95.75(±34.6)	100.2 (±33.8)	96.5 (±20.5)	0.94
Triglycerides (mg/dl) ǂ	183 (±90)	173 (±90.7)	119 (±45)	0.115
HOMA index ¥	0.955 (±0.77)	1.99 (±1.58)	1.34 (±1.15)	0.126
Uric acid (mg/dl) ǂ	3.15 (±0.64)	3.22 (±0.49)	3.05 (±0.669)	0.822

We quantified 43 metabolic spectral features in blood serum samples from 82 samples in newborns (0 months), 46 samples at 6 months of age and 26 samples at 12 months. We analysed data by age exploring the associations between metabolic profiles and maternal weight at the three different timepoints adjusting for sex, weight at birth, and lactation of the newborn as well as for maternal obesity, maternal diabetes, and adherence to the Mediterranean diet. Adjusted results were calculated on the subset with all covariates measured, which included 56 samples at 0 months, 42 at 6 months and 26 at 12 months.

The adjusted linear regression between metabolite levels and maternal GWG was significant for different metabolites at different ages (Figure 1). Interestingly, the significant associations at birth were strongly dominated by short-chain fatty acids and this influence decreased at 6 months of age and disappears at 12 months of age.

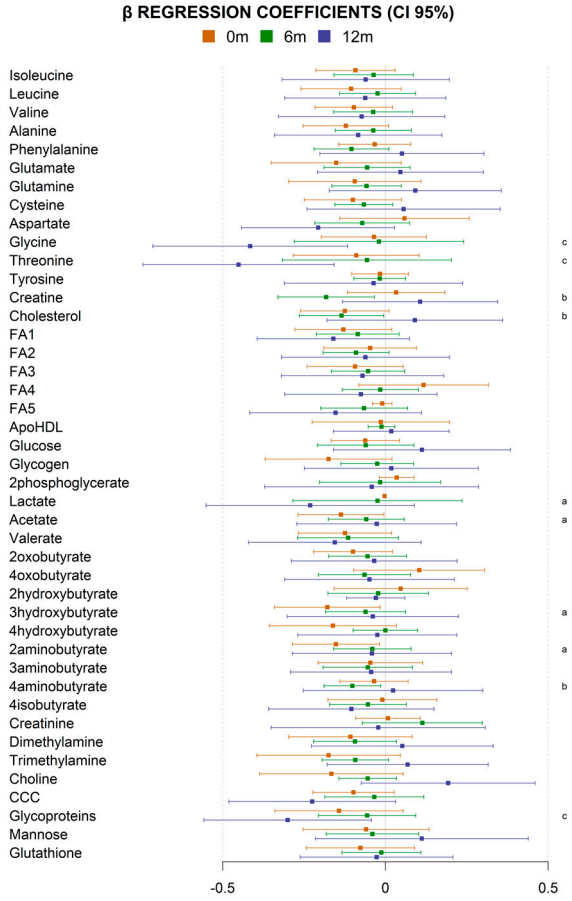

Figure 1. Linear association between maternal weight gain and metabolite levels. Linear regression beta coefficients and 95% confidence intervals (CI) for the association at birth (orange), 6 months of age (green), and 12 months of age (blue) between maternal weight gain and individual child serum metabolites adjusted for child sex, child weight at birth, maternal obesity, maternal diabetes, and maternal adherence to the Mediterranean diet. Each square represents the beta coefficient for a single metabolite, and the horizontal line indicates the 95% CI. Positive beta coefficients indicate a positive association with the outcome, while negative beta coefficients indicate a negative association. Metabolites with a statistically significant association (adjusted p value < 0.05) are marked with an "a" (birth), "b" (6 months of age), and/or "c" (12 months of age). Label keys: CCC, choline-containing compounds; FA1, CH3 groups in fatty acids; FA2, beta CH2 groups in all, saturated and unsaturated, fatty acids; FA3, =CH_2-CH_2-CH_2-CH= in fatty acids; FA4, =CH-CH2-CH= in fatty acids; FA5, all -CH=CH- in fatty acids; ApoHDL, apolipoproteins in HDL lipoparticles.

To further explore the metabolic impact of maternal weight gain on offspring metabolism and identify non-linear relationships that may not be detected by linear regression, we split children into groups according to maternal weight gain tertiles and used partial least-squares discriminant analysis (PLS-DA) to maximize the separation between samples and to identify discriminant patterns. We adjusted the analysis for the same confounders as the linear regression analysis by calculating a linear regression model with confounders for each metabolic feature and using the residues for the PLS-DA analysis. A permutation test was performed to check for over fitting of the PLS-DA models. The multivariate chemometric models were cross-validated with 10-fold Venetian blind cross-validation. In each run, 10% of the data were left out of the training and used to test the model. Spectral regions with high variable importance in projections (VIP) coefficients obtained during PLS-DA are more critical in providing class separation during analysis. In contrast, those with very small VIP coefficients contribute less to classification. We applied the complete PLS-DA analysis to the data at birth (Figure 2A,B), at 6 months of age (Figure 3A,B), and at 12 months of age (Figure 4A,B).

The scores plot of the PLS-DA analysis of the metabolome at birth (Figure 2A) shows some moderate discrimination and a continuous trend from tertile 1 to tertile 3 of maternal weight gain.

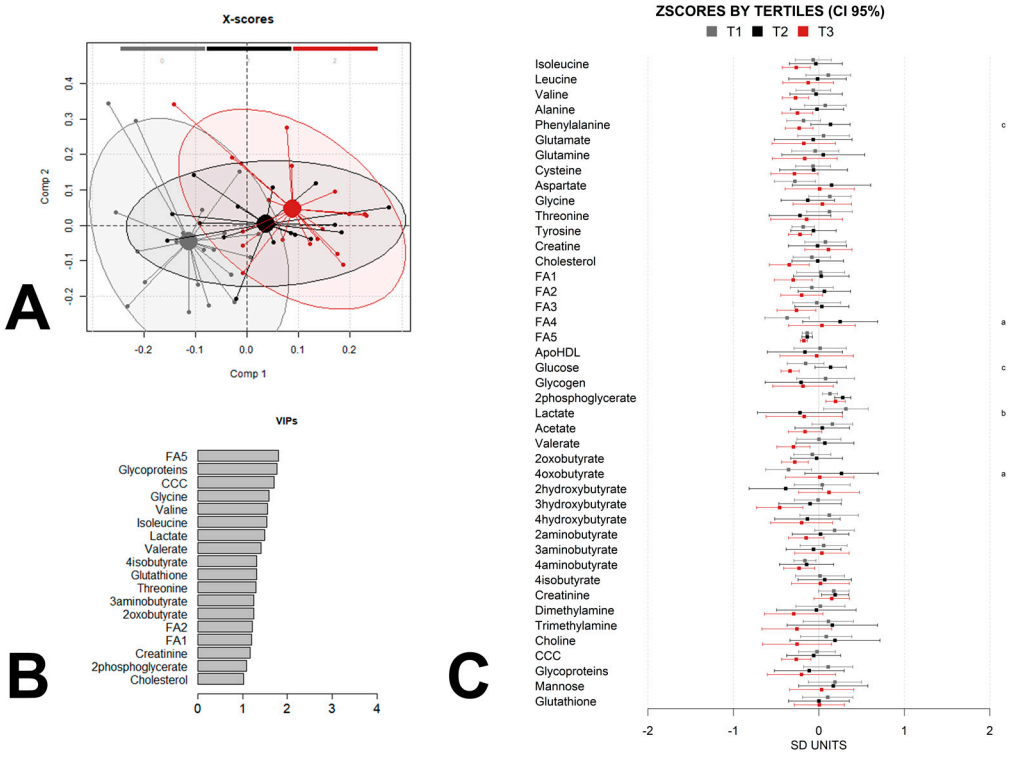

Figure 2. PLSDA analysis and z-scores for newborn metabolome at birth by maternal weight gain tertiles. (**A**) Scores plot with 95% confidence ellipse of the PLS-DA analysis of the metabolome, adjusted for child sex, child weight at birth, maternal obesity, maternal diabetes, and maternal adherence to the Mediterranean diet for the discrimination between tertiles (tertile 1(T1) grey, tertile 2 (T2) black, tertile 3 (T3) red). Cross-validation parameters: RMSECV 0.304, R2CV: 0.582; ROC Curve AUC: 0.96. (**B**) Metabolites with PLS-DA VIP score higher than 1 for the same PLS-DA model of the metabolome. (**C**) Z-score and 95% confidence interval of individual metabolites in offspring grouped by tertiles. Metabolites with pairwise statistically significant differences between tertiles (adjusted p-value < 0.05) are marked with an "a" (tertile 1 vs tertile2), "b" (tertile 1 vs tertile 3), and/or "c" (tertile 2 vs tertile 3). Label keys are the same as those in Figure 1.

These trends disappear at months 6 (Figure 3A) and 12 (Figure 4A) of age, suggesting the influence of other factors. However, the metabolome at 6 months of age still shows some differences between the middle tertile and the extreme tertiles. These differences disappear again at month 12 of age in favour of specific metabolic profile for tertile 3 of larger maternal weight gain. The VIPS scores (Figures 2B, 3B and 4B) also show that the contribution to the models of the different metabolites is different at birth, 6 and 12 months of age. Despite these variations, some metabolites are present in all the sets of VIPs scores larger than 1. Among them, glycoprotein fragments, fatty acids (and choline-containing compounds are among the top contributors in all the models. Additionally, branched chain amino acids like isoleucine and leucine, butyrate derivatives and lactate are also present in most of the sets.

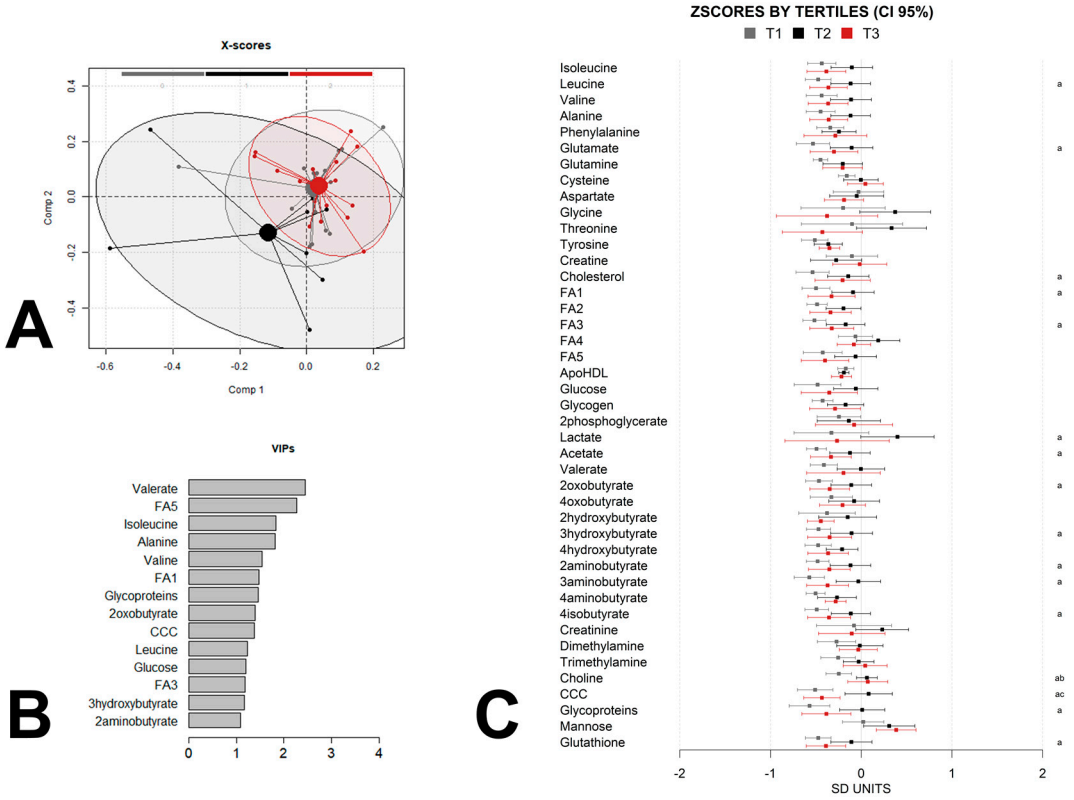

Figure 3. PLSDA analysis and z-scores for newborn metabolome at 6 months of age by maternal weight gain tertiles. (**A**) Scores plot with 95% confidence ellipse of the PLS-DA analysis of the metabolome, adjusted for child sex, child weight at birth, maternal obesity, maternal diabetes, and maternal adherence to the Mediterranean diet for the discrimination between tertiles (tertile 1 (T1) grey, tertile 2 (T2) black, tertile 3 (T3) red). Cross-validation parameters: RMSECV 0.304, R2CV: 0.582; ROC Curve AUC: 0.96. (**B**) Metabolites with PLS-DA VIP score higher than 1 for the same PLS-DA model of the metabolome. (**C**) Z-score and 95% confidence interval of individual metabolites in offspring grouped by tertiles. Metabolites with pairwise statistically significant differences between tertiles (adjusted p-value < 0.05) are marked with an "a" (tertile 1 vs tertile 2), "b" (tertile 1 vs tertile 3), and/or "c" (tertile 2 vs tertile 3). Label keys are the same as those in Figure 1.

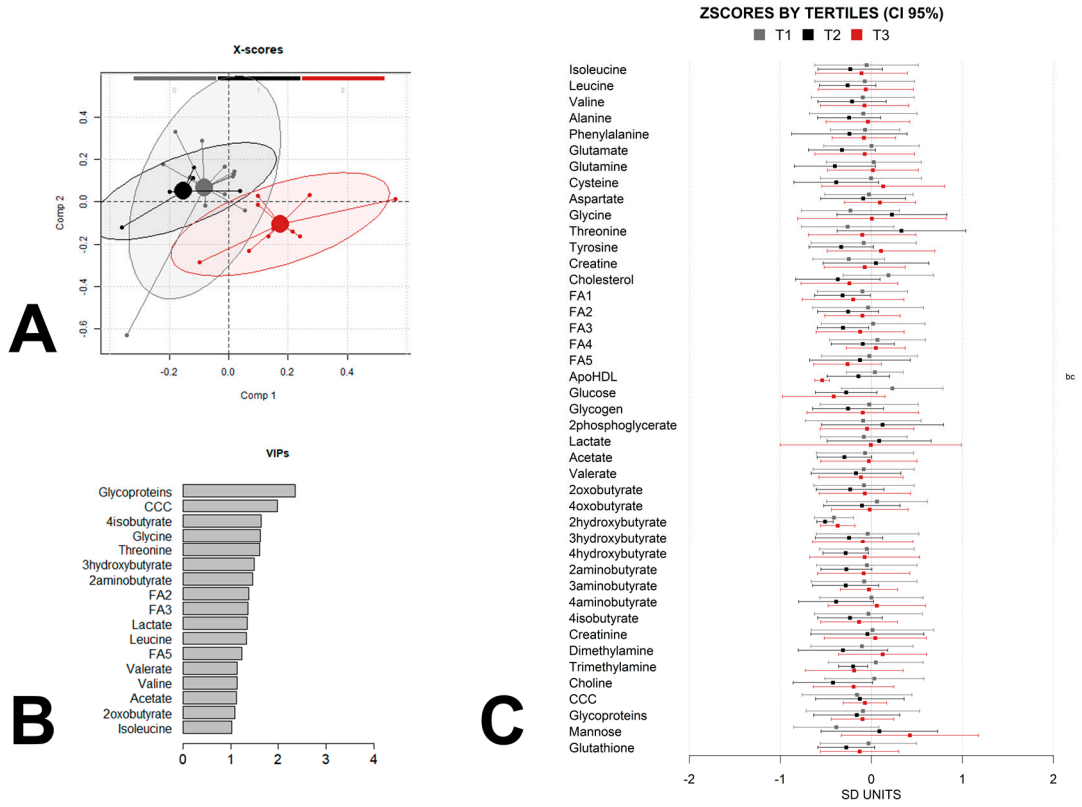

Figure 4. PLSDA analysis and z-scores for child metabolome at 12 months of age by maternal weight gain tertiles. (**A**) Scores plot with 95% confidence ellipse of the PLS-DA analysis of the metabolome, adjusted for child sex, child weight at birth, maternal obesity, maternal diabetes, and maternal adherence to the Mediterranean diet for the discrimination between tertiles (tertile 1 (T1) grey, tertile 2 (T2) black, tertile 3 (T3) red). Cross-validation parameters: RMSECV 0.304, R2CV: 0.582; ROC Curve AUC: 0.96. (**B**) Metabolites with PLS-DA VIP score higher than 1 for the same PLS-DA model of the metabolome. (**C**) Z-score and 95% confidence interval of individual metabolites in offspring grouped by tertiles. Metabolites with pairwise statistically significant differences between tertiles (adjusted p-value < 0.05) are marked with an "b" (tertile 1 vs tertile 3), and/or "c" (tertile 2 vs tertile 3). Label keys are the same as those in Figure 1.

In addition to the VIPs contribution, we calculated z-scores 95% confidence intervals for each group and built logistic regression models for pairwise group comparisons between tertile groups also adjusted by the same confounders as the linear regression models for a simpler visualization of differences between groups (Figures 2C, 3C and 4C). The analysis by tertiles revealed that, many of the highest contributors to the PLS-DA models at the 3 time-points did not exhibit statistically significant pairwise associations between any of the three tertile groups. According to the mean differences analysis, influence of maternal weight gain on offspring metabolome is largest at 6 months of age with 16 significant differences between tertile 1 and 2 and 2 differences between tertile 1 and 3 compared with at birth with a total of 5 statistically significant differences and at month 12 with only 2 statistically significant differences, although the sample size is rather low at this time point and may preclude achieving statistical significance. At all ages, most of the statistically significant associations correspond to comparisons between tertile 1 and tertile 2. Interestingly, a few of these statistically significant associations expand from birth to 6 months of age and include fatty acids, branched-chain amino acids and choline-containing compounds.

Our metabolite set enrichment analysis (Figure 5) suggests that studying the hypoxic-like metabolism, ketone bodies production, and ammonia-related metabolic pathways may help in understanding this connection.

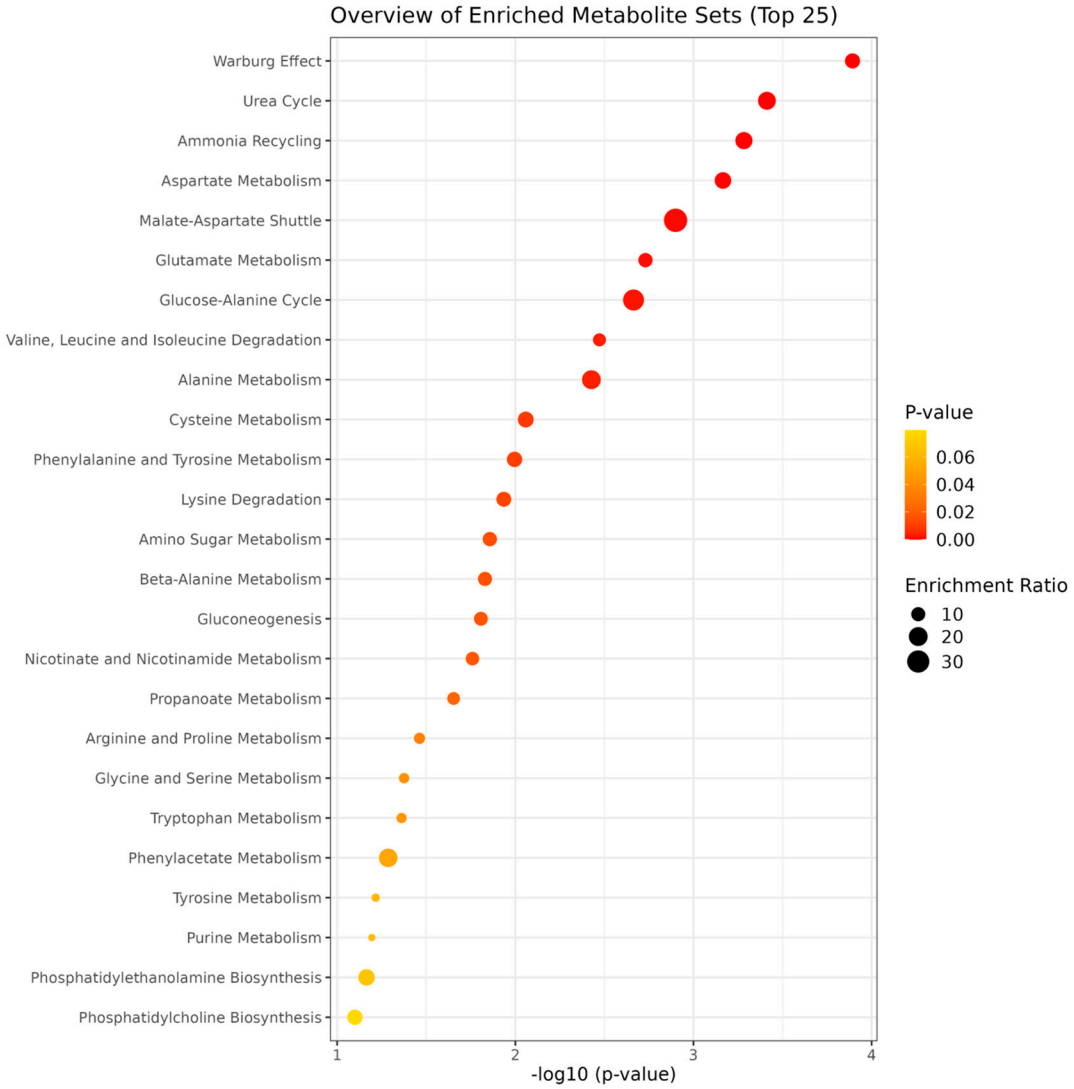

Figure 5. Metabolite set enrichment analysis of the association between maternal weight gain and offspring metabolome. The metabolite set enrichment analysis of metabolites with PLS-DA scores higher than 1 and adjusted p values < 0.05 at birth, 6 months of age, or 12 months of age. Metabolic pathways whose name is indicated are significant (p-value lower than 0.05 after the adjustment using the Holm-Bonferroni method and False Discovery Rate) and have a pathway impact value, calculated from pathway topology analysis, over 0. The pathways are represented as circles. The circle colour indicates the significance level, from highest (red) to lowest (white) in the enrichment analysis. The circle size is proportional to the impact value of each road from the topology analysis.

4. Discussion

Maternal GWG during pregnancy is an important determinant of fetal growth and development and can have a significant impact on the metabolic health of the offspring later in life. Our study aimed to explore the metabolomic profiles of children at birth, at 6 and at 12 months of age and to analyse them with respect to the maternal GWG. Although previous studies have explored metabolomic profiles of the offspring and their association with maternal metabolic states, this is the first time that the profiles are analysed with respect to maternal GWG using both regression models and multivariate PLS-DA models adjusted for confounders. Adjusting for confounders both in the mothers and children allows for more accurate detection of associations and reduces false positive discoveries. Although many metabolic associations were detected using our combined analysis, most of them were present either in the tertiles analysis or in the linear regres-

sion analysis suggesting a complex, nonlinear association probably modulated by many external factors. In fact, only at birth, the three tertiles follow a progressive trend from tertile 1 to tertile 2 and later tertile 3 whereas, at 6 and 12 months of age, there seems to be a discrimination of the high or the low tertile with respect to the others. The interpretation of these results is far from simple, but the scores plots suggest some metabolic shifts that happen during the first 12 months of life that can be modulated by maternal GWG.

The infant metabolome is influenced by a range of factors, including genetics, diet, and environmental exposures, and undergoes significant changes during the first year of life as the infant grows and develops [19]. Maternal GWG has been associated with alterations in the infant amino acid metabolome [20] and the lipid metabolome [21]. The metabolites studied in this study cover a wider and rather comprehensive spectrum of compounds. By studying 43 metabolites, which include amino acids, sugars, ketone bodies, lipid moieties, short-chain fatty acids, and general cellular and circulating compounds, at birth and two more early ages, we aimed to provide a broader view of the impact of maternal weight gain during pregnancy on the infant metabolome. Our study revealed that the influence of maternal weight gain during pregnancy on the early metabolic profile of the spring is not linear and most likely is multifactorial. Although some differences between tertiles of maternal weight gain appear during birth, its influences peaks at 6 months of age and most of them are resolved at 12 months of age. The impact in the long term and in other biological processes of these changes is unknown and deserves further investigation.

Among the metabolites affected by maternal weight gain in early life, butyrate derivatives and, specifically, 3-hydroxybutyrate, appears as predominant. During the first few days of life, new-borns experience a physiological state known as neonatal ketosis, characterized by high levels of circulating ketone bodies, including 3-hydroxybutyrate. This is a normal response to the sudden cessation of glucose supply from the mother, which after a few days stabilizes to low levels. However, infants who are born prematurely may have abnormal levels of 3-hydroxybutyrate during the first year of life [19]. 3-hydroxybutyrate has been shown to inhibit the activity of histone deacetylases, which are enzymes that remove acetyl groups from histone proteins and therefore regulate epigenetic modifications [22]. 3-hydroxybutyrate also has important cell signalling and regulatory functions, including pathways involved in reducing oxidative stress and inflammation, and are potentially linked to the differences in glycoproteins levels observed in our study [23]. Glycoproteins, which appear affected by maternal weight gain at birth, 6 and 12 months, reflect leukocyte activation and are associated with inflammation. A previous study found that higher maternal weight gain during pregnancy was associated with an increase in leucocyte activation in the umbilical cord blood of newborns [24], which may be related to our findings on glycoproteins and 3-hydroxybutyrate. Most recently, Jacopo et al., found lower levels of beta-hydroxybutyrate in both SGA and low birth weight placenta's metabolome, which may reflect poorer tolerance to hypoxia [25,26]. Finally, butyrate's derivatives, like aminobutyrates and oxobutyrates, are bacterial co-metabolites that are initially produced by the gut microbiota and further processed by human cells [27]. These facts combined with our results suggest a relevant role of this metabolite in the potential impact of maternal weight gain on infant health.

Our analysis revealed other, less studied, metabolites in serum associated to maternal weight gain. Lactate and acetate at birth appear as linearly correlated with maternal weight gain. In a previous study, higher maternal weight gain was associated with higher lactate levels in the cord blood of new-borns [28]. Phenylalanine, which shows statistically significant differences between tertiles at birth in our study, is an essential amino acid that is necessary for proper growth and development in infants. There is some evidence to suggest that maternal phenylalanine intake during pregnancy may be associated with increased birth weight and adiposity in offspring [29], but the underlying causes are unclear. Leucine is a branched-chain amino acids (BCAAs) typically associated with metabolic diseases e.g., type 2 diabetes or obesity in adults [30]. However, its role in

early life is controversial. In our study, babies from mothers with lower maternal weight gain exhibit lower levels of leucine. Despite high levels of BCAAs in breast milk, the concentration of leucine and isoleucine in the blood of newborns is relatively low. Leucine levels increase rapidly during the first few months of life, reaching adult levels by around six months of age but its association with maternal weight gain or with the infant health is poorly understood.

The reported levels of choline in newborns are relatively high compared to adults because choline is essential for brain development, which is rapid during the first few months of life [31]. By the time a baby is one year old their choline requirements are like those of an adult. Our results show that these findings may extend to other choline-containing related compounds, like phosphocholine and phosphatidylcholine. Phosphocholine is also a metabolite that partially comes from the processing in the gut of dietary carnitine into trimethylamines and choline-containing compounds [32]. Our results suggest that maternal weight gain during pregnancy affects the bacterial ecosystem of the baby. Microbial co-metabolism can affect nutrient availability and absorption, as well as immune system development, and be pivotal for many of the metabolic effects we observed in this study. While the initial colonization of gut microbiota is strongly influenced by the mother's microbiota during delivery and breastfeeding, the microbiota evolves and becomes more diverse based on a variety of factors throughout life.

On the one hand, the main strength of our study lies in the novelty of analysing the offspring metabolomic profiles according to maternal GWG and adjusting them by other common confounders of both the mother (obesity, diabetes during gestation and adherence to the Mediterranean diet) and child (sex, weight at birth and type of feeding). Moreover, this analysis is not limited to the moment of birth but continues up to the age of one year, identifying what could be markers of cardiovascular risk later in life.

On the other hand, our study has several limitations. First, the high cost of metabolomic studies and the difficulty of obtaining subjects has caused the initial sample size to be modest which could have played a role in not finding clinical differences and in missing some metabolomic differences among the three groups. Second, the progressive loss of subjects during follow-up, particularly affected by the COVID pandemic, led the sample set at different ages (6 months and especially at 12 months of age) to have a smaller and different number of subjects. Finally, we also lack information about other potential co-variables such as data on the infant microbiome, the exact duration of exclusive breastfeeding, the total formula intake, and the order of introduction of other complementary foods and its quantity.

Overall, the mechanisms underlying the relationship between maternal weight gain and metabolic profiles in infants are not well understood, but it has been hypothesized that higher maternal weight gain may lead to alterations in placental function and metabolism, which could in turn affect fetal and infant metabolism. We identified several metabolites associated with maternal weight gain either linearly or non-linearly by tertiles which could help in identifying metabolic pathways and clusters involved in long-term implications. Our metabolite set enrichment analysis (Figure 5) suggests that studying the hypoxic-like metabolism, ketone bodies production, and ammonia-related metabolic pathways may help in understanding this connection. The presence of high amino derivatives of short-chain fatty acids, methylamines, and choline-containing compounds in all our metabolic significant sets supports a relevant role of nitrogen metabolism in this network of interactions. Further research will be needed to elucidate the mechanisms by which maternal GWG modulates these changes in children's metabolome as well as the long-term effects in offspring life. It is very difficult to translate these metabolomic findings into clinical routine with our current knowledge, but further investigation may help to identify at-risk infants on whom to target more personalized dietary and growth monitoring interventions.

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Informed Consent Statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical reasons.

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