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Breast milk lipidome is associated with early growth trajectory in preterm infants.

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Abstract

Human milk is recommended for feeding preterm infant. Yet the potential impact of specific breast-milk lipid components on the initial growth rate of very-preterm infants has received scant attention. The current pilot study aims to determine whether breast-milk lipidome had any impact on the early growth pattern of preterm infants fed their own mother's milk. A prospective monocentric observational birth cohort was established, enrolling 147 preterm infants, who received their own mother's breast-milk throughout hospital stay. Among that cohort, infants who experienced slow (n=15) or fast (n=11) growth were selected, based on the change in their weight Z-score between birth and hospital discharge (-1.54± 0.42 and -0.48± 0.19 Z-score, respectively). Liquid chromatography-high resolution-mass spectrometry was used to obtain lipidomic signatures in breast-milk. Multivariate analyses made it possible to identify breast-milk lipid species that allowed clear-cut discrimination between the 2 infants' groups. Validation of the selected biomarkers was performed by means of various multidimensional statistical techniques, false-discovery rate and ROC curve computation. Breast-milk associated with fast growth contained more medium chain-saturated fatty acid and -sphingomyelin, dihomo-γ-linolenic acid (DGLA)-containing phosphethanolamine, and less oleic acid-containing triglyceride and DGLA-oxylipin. Their predictive ability of preterm early-growth rate was validated in presence of confounding clinical factors.

Key words: breast milk lipidome, preterm infant, growth trajectory.

Abbreviations used:

AA: arachidonic acid; ALNA: alpha-Linolenic acid; AoV-PLS, analysis of variance combined to partial least squares regression; AUC, area under the curve; CL : cardiolipine;

DG: diacylglycerol ; DGLA: dihomo-gamma-linolenic acid; DHA: docosahexanoic acid; GA, gestational age; GLNA : gamma-linolenic acid; EPA: eicosapentaenoic acid; ESI, electrospray ionization; FDR, false discovery rate; LA: Linoleic acid; LC-HR-MS, Liquid-Chromatography-High-Resolution-Mass-Spectrometry; LC-PUFA: Long-Chain PUFA ; MCSAT, medium chain-saturated fatty acid; MG PLS-DA, multi-group partial least squares discriminant analysis; MLR, multiple linear regression; MCSAT: medium chain saturated fatty acids; MUFA , mono-unsaturated fatty acids; PC: phosphocholine; PE: phsosphethanolamine; PG: phosphatidylglycerol; PI: phosphoinositol; PS : phosphoserine ; PUFA: Polyunsaturated fatty acid; SAT: saturated fatty acids; ROC, receiver operating-characteristic; SD : standard deviation; SM: sphingomyéline; TG: triacylglycerol ; W4M , Workflow4Metabolomics®.

Introduction

A large body of epidemiologic evidence supports the short- and long-term benefit of breastfeeding. In full-term infants, breastfeeding indeed is associated with a lower incidence of gastroenteritis in the first year of life, better cognitive development, and a lower incidence of obesity during childhood and adolescence [1-3]. The World Health Organization therefore recommends exclusive breastfeeding for the first 6 months of life [4]. Human milk contains a host of bioactive components [5], which may mediate the health benefits of breastfeeding through an impact on immune system, microbiota, or metabolism [6, 7]. In preterm infants, maternal milk feeding is strongly recommended as well [8] since it was shown: (i) to reduce the incidence and mortality of necrotizing enterocolitis [9] and (ii) to improve neurodevelopment [9, 10]. Breast milk alone, however, due to its relatively low protein and energy content, cannot cover the tremendous needs of preterm infants, and exclusive

breastfeeding is often associated with extra-uterine growth restriction (EUGR) [10, 11]. As impaired initial growth could have dramatic consequences since preterm birth *per se* is a risk factor for developmental delay [12, 13], the European Society for Paediatric Gastroenterology Hepatology and Nutrition (EPSGHAN) committee on nutrition recommends human milk fortification for preterm infants [14]. Notwithstanding, a large range of variation is still observed regarding growth during hospital stay [15] even among preterm infants receiving protein-fortified human milk. Literature reports large inter-subject variations in the composition of expressed own mother's milk [16]. Indeed, the fat composition can be directly impacted by numerous factors, including maternal diet [17, 18], body mass index (BMI) [19], parity [20], or stage of lactation [21, 22]. Regarding gestational age, it has been reported to impact total milk fat content only in the first stage of lactation [23, 24]. Milk lipid supplies not only 45% - 55% of the total energy requirement of a healthy infant [25], but also building blocks for tissue growth. The effects of milk fatty acids and phospholipids on neurodevelopment have been widely studied [25, 27]. For example, the supplementation of infant formulae with sphingomyelin and phosphatidylcholine was reported to protect from gastrointestinal infections in early childhood [28]. There is, however, scarce data on the potential health benefits of specific lipid components of human milk [25], with only a few trials either involving small sample sizes [29] on term infants [30, 32], or focusing on the comparison between breastmilk and formula composition [33, 34]. Metabolomics and lipidomics seem to have promising applications in neonatology [35, 36] to explore the broad range of concentrations of various components [37], although it has been used only in a limited number of clinical applications [38, 39, 40]. Furthermore, to the best of our knowledge, no previous study has explored in depth the relationships between lipid components in human milk and the early growth of preterm infants during their hospital stay. To fill this gap, the current study aims at shedding light on the relationships between breast

milk lipidome and growth pattern of preterm infants nourished by their own mother's milk. Comprehensive lipidomic signatures of human milk collected from mothers delivering a preterm infant were obtained by means of liquid chromatography-high resolution-mass spectrometry (LC-HR-MS). To emphasize the difference in growth patterns, two groups of infants, presenting either fast *vs* slow growth rate during hospital stay, were selected within the ongoing LACTACOL birth cohort. Multivariate analysis, involving techniques from partial least squares (PLS) family, allowed analysis of multiple variables (all lipid species) together, to avoid loss of information. It resulted in the identification of those lipid species which contributed to clear separation between infants' groups. Furthermore, their statistical significance and predictive ability related to infant growth was assessed by means of several techniques encompassing cross-validation and bootstrapping procedures, together with ROC curves. In a final step, the reliability of the selected lipid species was confronted to confounding clinical factors.

Subjects and Methods

Study design and population

The mono-centric prospective population-based LACTACOL birth cohort (registered at www: clinicaltrails.gov as NCT01493063) of preterm infant - mother dyads was recruited from October 2011 to March 2016. A written consent was obtained from all participants at enrolment. One hundred and thirty eight infants born between 26-36 weeks of gestational age, with no major congenital disease except prematurity, were included, for a total of 118 mothers finally enrolled in the LACTACOL cohort (Figure 1).

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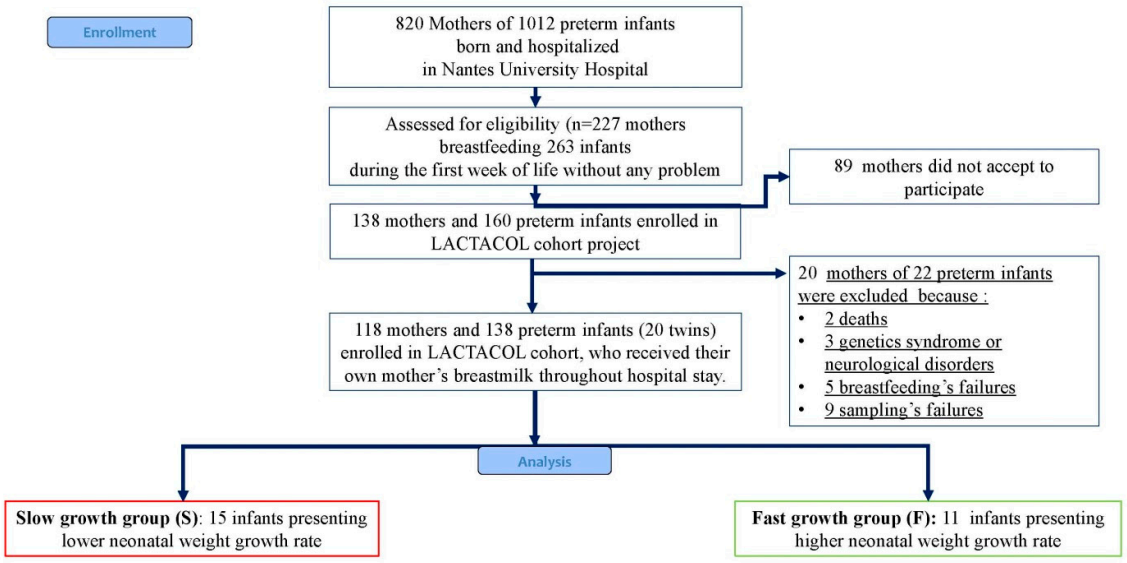

Figure 1: Flowchart of infants enrolled in the ancillary study of the mono-centric prospective population-based LACTACOL.

Infants, admitted to the Neonatal Intensive Care Unit at Nantes University Hospital, were eligible if they received human milk as their sole feeding for more than 28 days. Clinical characteristics both on mothers and infants were reported: maternal age, educational level, pre-gravid BMI, adverse events during pregnancy and delivery, infants' gestational age, birth weight and head circumference, growth trajectory through hospital discharge, and events during hospital stay in neonatology. Parenteral nutritional supply was recorded daily, as well as enteral intake: volume of milk delivered per feeding session, fortifiers used and fortification level (according to the EPSGHAN recommendations [14], mean daily energy and macronutrients intakes (expressed as kcal/kg body weight/d and g/kg body weight/d, respectively). Preterm infants received parenteral and minimal enteral feeding, predominantly with expressed breast milk, for a minimum of two weeks. After completion of the cohort, a pilot study was conducted: two groups of infants were selected among those who experienced very low growth rate (S group: 15 infants with 'slow growth') or very high growth rate (F group: 11 infants with 'fast growth') throughout hospital stay (Figure 1).Two sets of twins

belonged to the slow growth group and two others sets of twins followed opposite trajectories regarding their weight Z-score difference between birth and hospital discharge, i.e. one of twin belonged to the fast growth group whereas the other twin belonged to the slow growth group. Clinical characteristics of mother-infant dyads are summarized in Table 1. Although gestational age (30-31 weeks) and length of hospital stay (49-51 days) did not differ between the groups, the group of infants with fast growth had a 25% lower birth weight combined with a 69% greater gain in weight Z-score between birth and discharge. This negative correlation between birth weight and weight Z-score at time of discharge has long been known, and was previously reported in the large LIFT cohort of 2277 preterm infants by our team [41] and in another cohort [42]. This would lead to an inconsistent stratification of our preterm infants in the LACTACOL cohort if birth anthropometry homogeneity was considered jointly with an opposite growth velocity.

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Table 1: Maternal and preterm infants' characteristics

	Slow growth rate	Fast growth rate	p-value
Maternal characteristics	**11**	**11**	
Age	30.00 ± 4.12 [26.00; 33.00]	29.00 ± 4.52 [25.00; 35.00]	0.908
BMI before gestation	24.00 ± 5.11 [20.83; 30.80]	22.32± 5.26 [19.14; 28.91]	0.789
Infants characteristics at birth	**15 (10 males and 5 females)**	**11 (7 males and 4 females)**	
Gestational age (w)	30.00 ± 1.68 [29.00; 32.00]	31.00 ± 1.37 [30.0; 32.00]	0.288
Hospital stay (d)	49.50 ± 4.21 [36.75; 54.75]	51.50 ± 3.16 [37.25; 56.25]	0.849
Birth weight (kg)	1.605 ± 0.211 [1.465; 1.705]	1.200 ± 0.293 [1.020; 1.445]	**0.005**
Birth length (cm)	41.20 ± 1.38 [39.00; 41.60]	38.00 ± 1.84 [37.00; 40.00]	**0.004**
Birth head circumference (cm)	28.00 ± 1.53 [27.00; 29.00]	26.20 ± 2.07 [25.505; 28.20]	0.202
Birth weight Z-score (SD)	0.564 ± 0.718 [-0.290; 0.842]	-1.592 ± 0.958 [-2.079; -0.571]	**0.000**
Birth length Z-score (SD)	0.210 ± 0.725 [-0.522; 0.746]	-0.793 ± 0.939 [-2.245; -0.129]	**0.003**
Birth head circumference Z-score (SD)	-0.036 ± 0.795 [-0.686; 0.386]	-1.528 ± 1.212 [-1.651; -0.128]	**0.015**
BMI at birth (kg/m^2)	9.455 ± 0.857 [8.843; 9.900]	7.694 ± 1.573 [7.139; 9.884]	0.161
Discharge weight (kg)	2.565 ± 0.270 [2.355; 2.720]	2.340 ± 0.320 [2.029; 2.520]	**0.041**
Discharge length (cm)	45.00 ± 1.529 [44.10; 46.00]	44.00 ± 2.543 [41.00; 45.50]	0.219
Discharge head circumference (cm)	33.00 ± 1.412 [32.50; 34.00]	33.50 ± 1.511 [32.00; 34.00]	0.787
Discharge weight Z-score (SD)	-1.142 ± 0.682 [-1.552; -0.953]	-1.878 ± 0.857 [-2.264; -1.127]	0.146
Discharge length Z-score (SD)	-1.800 ± 0.713 [-2.242; -1.251]	-2.349 ± 1.054 [-2.803; -1.096]	0.466
Discharge head circumference Z-score (SD)	-0.681 ± 0.775 [-1.184; 0.301]	- 0.216 ± 0.754 [-1.010; 0.1074]	0.655
BMI at Discharge (kg/m^2)	12.67 ± 0.955 [11.78; 13.36]	11.98 ± 0.485 [11.66; 12.28]	**0.047**
Fat mass at discharge (%)	11.00 ± 1.34 [9.15; 16.70]	12.70 ± 0.91 [10.30; 15.85]	0.845
Difference between discharge and birth weight Z-score (SD)	-1.538 ± 0.417 [-1.953; -1.230]	-0.479 ± 0.189 [-0.668; -0.294]	**<0.001**
Difference between discharge and birth length Z-score (SD)	-2.010 ± 0.752 [-2.474; -1.278]	-0.940 ± 0.723 [-1.822; -0.343]	**0.015**
Difference between discharge and			

Values are medians and 25% and 75% percentiles. P values for comparison between fast and slow growth groups were derived by using Mann-Whitney *U* test.

Ethics

This research study, referenced BRD/11/02-Y at Nantes University Hospital, was approved, on 28 February 2011, by the National Data Protection Authority (Commission Nationale de l'Informatique et des Libertés, N° 8911009) and, on 19 July 2011, by the appropriate ethics Committee for the Protection of People Participating in Biomedical Research (CPP- Ouest I (Tours, France), reference CPP RCB-2011-AOO292-39). The LACTACOL cohort was registered at www: clinicaltrials.gov_under # NCT01493063 and the current data were

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obtained in the corresponding ancillary study N°3. The milk biobank was approved by the Committee for the Protection of Persons in medical research (approval was granted 24 June 2010, reference CPP CB-2010-03). Parents received oral and written information in the maternity ward or neonatal unit, lactation support and training on proper sample collection from the study lactation consultant. A written consent was obtained from all parents at enrolment.

Assessment of infant growth and body composition

Body weight was measured weekly from birth to discharge (accuracy of 0·1 g). Weight Z-score was calculated using the Lambda Mu Sigma (LMS) method [43] and Olsen's preterm infant growth chart [44] was applied with respect to birth and discharge measurements. Weight gain during hospitalization was computed as the weight Z-score difference (expressed in units of standard deviation (SD) using the SD of the term category as the benchmark) between birth and discharge. Infants with 'slow growth' were defined as those who experienced a large decline in weight Z-score between birth and discharge; infants with 'fast growth' were defined as those who experienced a moderate decline, or even an increase, in Z-score between birth and discharge. In the present study, the medians of weight Z-score difference of the preterm infants identified in 'slow growth' group and 'fast growth' group were -1.54 SD and -0.48 SD, respectively (Table 1). Body composition was assessed by air displacement plethysmography (PEA POD®, COSMED) [45] at discharge. See Supplementary Material under "Supplemental data" online for detailed information about clinical measurements.

Human milk collection and targeted fatty acids analysis:

Weekly breast milk collection was performed manually by mothers at home, using a Medela Manual Breast pump (Medela Inc., Etampes, France). Representative 24-h mature milk

samples were obtained by pooling breast milk sampled from 5 to 6 bottles brought daily to the Milk Bank of the Nantes University Hospital. The whole milk pool was homogenized with a disruptor (Polytron, Lucerne, Switzerland) and kept frozen at -80°C until analysis. Total milk fat was measured using the MIRIS® human milk analyzer (Miris AB®, Uppsala, Sweden), based on mid-infrared methodology [46]. The modified liquid–liquid extraction method of Bligh-Dyer [47] was used to extract lipophilic metabolites, and total fatty acids were analyzed by gas chromatography using an Agilent Technologies 7890A® instrument (Perkin Elmer, France), following trans-esterification [48]. See Supplementary Material under "Supplemental data" online for detailed information about biochemical analysis and materials used.

Breast milk Liquid Chromatography-High-Resolution-Mass Spectrometry (LC-HRMS)–based lipidomic profiling

Following Bligh-Dyer extraction, the organic layers were dried and subsequently reconstituted in acetonitrile-isopropanol-water (ACN: IPA: H_2O 65:30:5, v/v/v). A 1200 infinity series® high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, California, USA) coupled to an Exactive Orbitrap® mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray (H-ESI II) source (operating in polarity switch mode) was used for lipid profiling. The full instrument calibration was performed using a MSCAL6 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg® (Sigma–Aldrich). Xcalibur 2.2® (Thermo Fisher Scientific, San Jose, CA, USA) was used for data acquisition and analysis. Lipid species separation was performed on a reverse phase CSH® C_{18} (100 x 2.1 mm^2 i.d., 1.7 µm particle size) column (Waters Corporation, Milford, MA) using ACN:H_2O (60:40) and IPA:ACN:H_2O (88:10:2) as solvent A and B, respectively; both containing 10 mM ammonium acetate and 0.1 % acetic acid [49]. The precision associated with sample preparation and LC-HRMS measurement was

determined on the basis of a quality control (QC) consisting of a pool of 10 mothers' milk provided by the milk bank of Nantes Hospital Center. Summary assay procedures have been detailed in Supplementary Material.

Data analysis and lipid species characterization

LC–ESI (positive/negative) HRMS raw data files were initially preprocessed with Xcalibur 2.2® (Thermo Fisher Scientific, San Jose, CA, USA), converted to the (*.mzXML) cross-platform open file format, using MSConvert® [50], and processed using Workflow4Metabolomics® (W4M) (http://workflow4metabolomics.org) [51]. Lipidomic data were extracted using i) pre-processing with the open-source XCMS® [52] within W4M [51], for nonlinear retention time alignment and for automatic integration and extraction of the peak intensities for each detected features (ions of given mass-to charge ratio and retention time [m/z; RT]), combined to CAMERA® [53], for annotation of isotopes and adducts and ii) normalization of intra- and inter-batch effects by fitting linear or local polynomial regression models to QC samples [54]. The resulting XCMS [m/z; RT] features for each sample was subsequently manually sorted out according to their quality of integration and filtered by a 30% relative SD cutoff within the repeated pooled QC injections (see Dunn et al.'s recommendations [55]). Thereafter, accurate mass measurement of each putative metabolite was submitted to LIPID Metabolites And Pathways Strategy (LipidMaps®, www.lipidmaps.org) annotation. Moreover, the use of all ion fragmentation, when reverse phase chromatography was applied, helped us identify the proposed lipids by examination of the (pseudo) tandem mass spectrometry spectrum generated [49], combined with the use of an in-house reference databank [56].

Statistical analyses.

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Statistical analyses were carried out using GraphPad Prism® software version 6.00 (La Joya, California, USA), SIMCA P® version 13 (Umetrics AB, Sweden) and R version 3.2.5 (R Development Core Team, 2013; http://www.R-project.org). For all data analyses, the significance level (α) was set to 5%, and 10% for Multiple Linear Regression. Multivariate statistical models were applied on pre-processed QC-filtered lipidomic mother x time (rows) by [m/z; RT] features (columns) data matrix considering the *a priori* structure into 'fast' *vs* 'slow' infants' growth groups. Lipidomic features were column-wise centered and scaled with a Log Pareto scaling [57]. A statistical workflow (Figure 2) was set up to: (i) select the most reliable lactation points, (ii) identify the lipid biomarkers providing a clear separation between the two infant postnatal growth subgroups, (iii) check the selected biomarkers predictive ability and (iv) confront them to the various confound clinical variables.

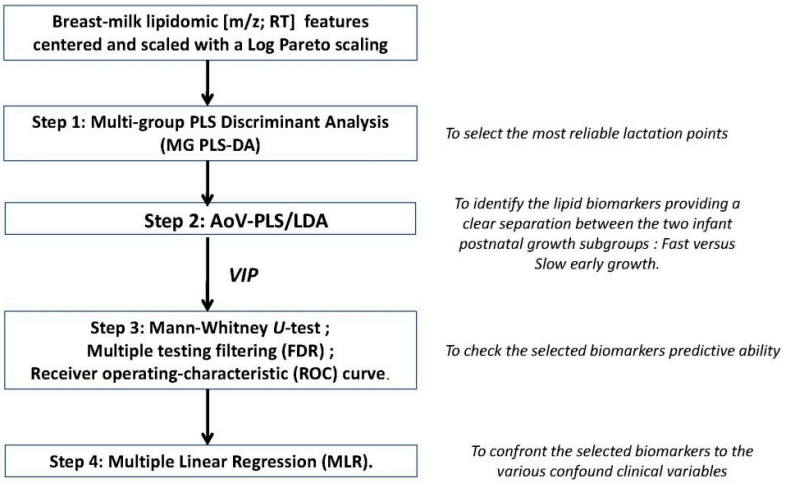

Figure 2: Statistical workflow applied on breast-milk LC-ESI^{+}/ESI^{-}-HRMS profiles.

Step 1: **Selection of the most reliable lactation points**. **Multi-group PLS Discriminant Analysis (MG PLS-DA)** [58] was performed to explain the *a priori* infants growth trajectory taking into account the longitudinal character of the data (weekly measurements) as groups

among the rows (multigroup package in R) [59]. As a matter of fact, MG PLS-DA model revealed the time lactation points presenting the best differentiation between the two groups (fast *vs* slow infant growth). Thereafter, only the most discriminant time lactation points were considered in the subsequent analyses and the significance of the growth factor was assessed. To cope with multi-colinearity inherent in metabolomics data [60, 61], the AoV-PLS procedure proposed by El Ghaziri et al. [62] was applied.

Step 2: **Identification of the lipid species biomarkers. AoV-PLS** aimed at: i) exploring how fast *vs* slow preterm infants' growth structure was explained by milk lipidomic signatures and ii) pinpointing the most discriminant features related to the infants' growth pattern during hospital stay. This selection was performed on the basis of the variables of importance (for the milk clustering) indices (VIP>1.0) [62] reflecting the contribution of each variable ([m/z; RT] feature) to the separation of the two infant groups. The appropriate number of components retained for the AoV-PLS model was chosen on the basis of the RMSEP (root mean square error of prediction) value obtained with a leave-one-out cross-validation procedure. In a subsequent stage, the selected AoV-PLS components were subjected to a Fisher's linear discriminant analysis (LDA) to assess the significance of this model with respect with the two *a priori* groups (fast *vs* slow infant growth) (function linDA of the DiscriMiner package in R [63]).

Step 3: Validation of the predictive ability of the selected biomarkers. To determine the more robust putative biomarkers of infant growth during hospital stay, lipid species that had been selected on the basis of AoV-PLS/LDA VIP indices in a multivariate scope were submitted to: (i) a subsequent univariate Mann-Whitney *U*-test, (ii) multiple testing filtering (FDR) and (iii) receiver operating-characteristic (ROC) curve (GraphPad Prism®). The parameter associated to the area under the curve (AUC) was set at 0.5 while the α-threshold was set at 0.05.

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Step 4: –Introduction of confounding clinical variables. Finally, confounding variables encompassing maternal and infant clinical data were introduced together with every selected putative biomarker to validate its reliability. The association between selected breast milk lipid species and child's growth in terms of weight and head circumference gain during hospital stay (delta Z-score) or fat mass at discharge were investigated by means of Multiple Linear Regression (MLR), taking into account these confounding variables.

RESULTS

A distinct breast milk lipidomic signature is associated with infant growth rate during hospital stay

Selected features of lipidomic LC-HRMS (ESI^+/ESI^-) profiles performed on breast milk samples from week 2 to week 7 of lactation were processed with MG PLS-DA (**Step 1**).

On the corresponding score plots obtained in positive (Figure 3) and negative (Supplemental Figure S1) ionization mode (**3451** and **903 features** respectively, 118 observations), the milk samples associated with the two groups (fast *vs* slow growth) are plotted separately for each weekly sampling time point (*i.e.* weeks 2 to 7). The first three sampling points at weeks 2, 3 and 4 (*i.e.* from the 12^{th} to the 24^{th} day (median values)) allowed the best separation between the two groups of milks. This result led us to further restrict the multivariate analysis to data from samples obtained between week 2 and week 4.

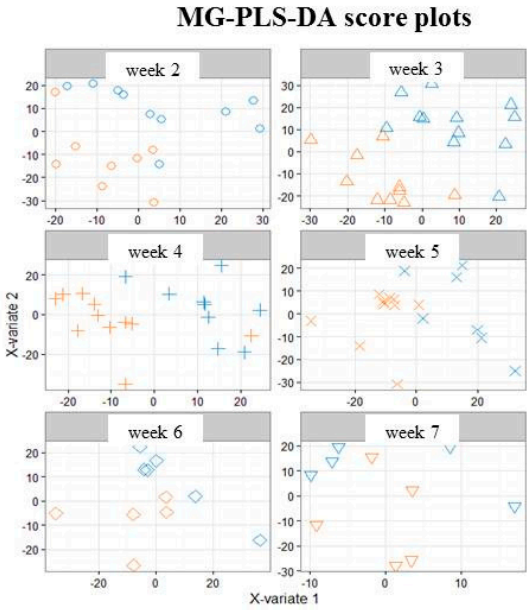

Figure 3: Multi-group PLS-DA score plots based on the LC-ESI$^+$-HRMS profiles (3451 features, 118 milks) obtained on human preterm milk. Representation of the individuals : milk provided to preterm infants who experienced fast (orange) or slow (blue) growth from week 2 to week 7 of lactation. MB-PLS-DA score plots: ○, week 2; Δ, week 3; +, week 4; x, week 5; ◊, week 6;▽, week 7.

AoV-PLS (**Step 2**) was applied separately on the LC-HRMS (ESI$^+$/ESI$^-$) to assess the association between the metabolites and the *a priori* grouping structure (fast *vs* slow growth). The score plots clearly highlight the separation between breast milk lipidotypes associated with fast or slow infant growth in both positive (Figure 4a) and negative (Supplemental Figure S2a) ionization modes. Interestingly, the breast milk lipidomic profiles, corresponding to two sets of twins with a concordant low growth rate, were plotted in the "slow growth" milk cluster. The lipidomic profiles of the breastmilks provided to two sets of twins with

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discordant weight Z-score difference, one corresponding to fast (-0,668 and -0,479 SD) and the other to slow (-1,23 and -1,53 SD) growth, were found to be in an intermediate location between the two lipidotypes (depicted with blue symbols in Figure 4a and Supplemental Figure 2). Then, the selected appropriate components of AoV-PLSs (for both ionization modes) were subjected to a Fisher's linear discriminant analysis (LDA) to test the significance of growth factor.

Figure 4a

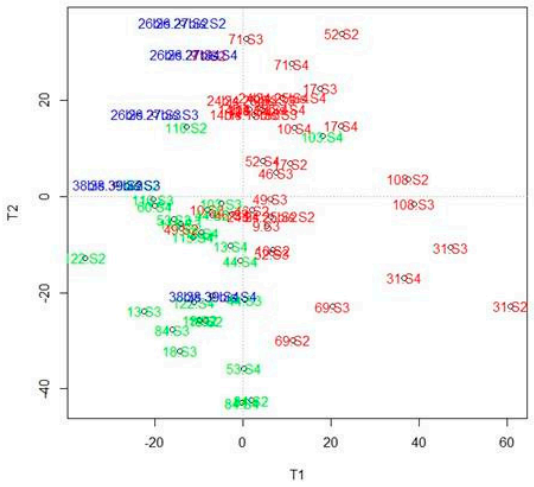

Figure 4b

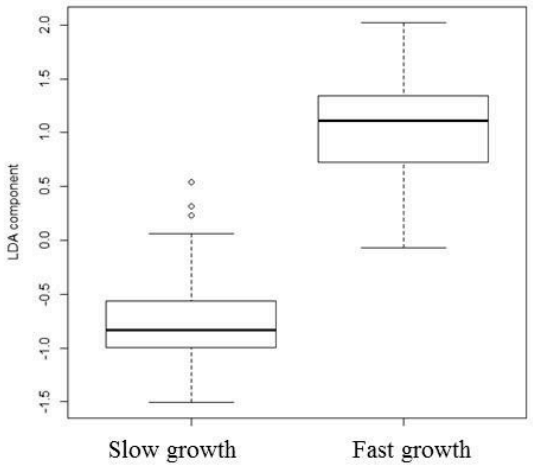

Figure 4: AoV-PLS and LDA models, based on the LC-ESI$^+$-HRMS profiles of human preterm milk, on the factor weight Z-score (discharge - birth) : AoV-PLS score plot with 45% of variance (R2Y=38%) on components 1-2 (**Figure 4a**) and LDA (built on 10 components of AoV-PLS) with a p-value=0) (**Figure 4b**). Breast milk provided to preterm infants who experienced fast (green) or slow (red) growth and to twin infants with discordant growth rate, one with high growth rate and one with low growth rate, (blue).

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The correlation ratio associated with the LDA canonical variable was equal to 71%, with respect to the positive mode (Figure 4b), and 61%, with respect to the negative mode (Supplemental Figure S2b), while their cross validation error rates were both equal to 7.14%. The most discriminant features associated with infant growth during hospital stay corresponded to a cluster of **1006 (resp. 256) VIP-based lipid species**, in the positive (resp. negative) ionization mode.

Characterization of preterm breast milk lipidotypes in the first month of lactation

Milk provided to the 'fast' growth group contained more total fat (4.75 g/100ml) than the milk provided to infants with a slower growth rate (3.55 g/100ml) from week 2 to week 4 (Table 2) (Supplemental Table S1, for each sampling time, W2, W3 and W4 of lactation). This is, mainly due to a higher abundance of total saturated fatty acids (SAT) (free and triglycerides- and phospholipids-bound fatty acids) and particularly, medium chain-SAT (MCSAT). In contrast, total mono-unsaturated fatty acids (MUFA), the second most abundant class of milk fatty acids, were lower in the 'fast growth' group than in the 'slow' group, essentially due to lower oleic acid content. Milk provided to infants who experienced 'fast growth' contained more overall n-3 long-chain PUFA, such as docosahexanoic acid and its precursors (eicosapentaenoic and docosapentaenoic acids). Finally, the essential FA content (i.e. linoleic and α-linolenic acids), was similar in both groups during this W2-W4 period.

Table 2. Concentration levels of total fatty acids (free and triglycerides- and phospholipids-bound) in breast milk provided to preterm infants with fast or slow growth during the W2-W4 lactation period.

Fatty acids (%)	W2 to W4		Mann-Whitney p-value From W2 to W4	FDR corrected q-value from W2 to W4
	Slow growth (n=38)	Fast growth (n=29)		
8:0	0.176 [0.151-0.211]	0.198 [0.157-0.236]	0.201	0.183
10:0	1.654 [1.512-1.911]	1.791 [1.591-2.082]	0.101	0.132
12:0	6.203 [5.817-6.866]	7.100 [6.069-8.127][a]	**0.022**	***0.055***
14:0	7.049 [6.053-8.045]	8.039 [6.909-9.629] [a]	**0.013**	**0.046**
16:0	23.19 [19.48-24.84]	23.15 [21.21-24.84]	0.677	0.353
18:0	6.872 [6.313-7.556]	6.934 [5.883-7.435]	0.406	0.279
20:0	0.195 [0.170-0.211]	0.196 [0.178-0.211]	0.872	0.379

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SAT	45.74 [41.97-48.51]	48.08[45.81-49.54] [a]	**0.027**	***0.051***
MCSAT	8.050 [7.506-8.871]	8.976 [7.772-10.210] [a]	**0.019**	***0.051***
16:1$_{n-9}$	0.479 [0.419-0.530]	0.4573 [0.393-0.514]	0.192	0.183
16:1$_{n-7}$	2.331 [2.030-2.737]	2.239 [2.098-2.698]	0.852	0.379
17: 1$_{n-7}$	0.222 [0.175-0.247]	0.228 [0.190-0.261]	0.310	0.249
18:1$_{n-9}$	34.79 [32.25-37.88]	32.82 [31.02-34.78] [a]	**0.027**	***0.055***
18:1$_{n-7}$	1.720 [1.495-1.993]	1.818 [1.556-2.007]	0.468	0.287
20:1$_{n-9}$	0.532 [0.483-0.567]	0.520 [0.462-0.597]	0.801	0.379
MUFA	41.10 [37.77-44.35]	39.58 [36.87-40.82] [a]	**0.047**	***0.059***
MUFA/SAT	0.91 [0.79-1.04]	0.82 [0.74-0.91] [a]	**0.037**	***0.059***
18:1$_{n-9 \text{ and } n-7}$	36.67 [34.09-39.79]	34.78 [32.76-36.61] [a]	**0.028**	***0.051***
cis 18:2$_{n-6}$(LA)	9.651 [8.754-12.41]	8.881 [7.859-11.64]	***0.078***	0.117
cis 18:3$_{n-6}$ (GLNA)	0.107 [0.086-0.133]	0.099 [0.078-0.125]	0.370	0.276
cis 20:2$_{n-6}$	0.295 [0.252-0.332]	0.295 [0.235-0.343]	0.615	0.337
cis 20:3$_{n-6}$ (DGLA)	0.349 [0.317-0.443]	0.404 [0.317-0.456]	0.429	0.279
cis 20:4$_{n-6}$(AA)	0.502 [0.425-0.580]	0.467 [0.379-0.591]	0.544	0.315
cis 22:2$_{n-6}$	0.048 [0.040-0.061]	0.048 [0.039-0.060]	0.791	0.379
cis 22:4$_{n-6}$	0.113 [0.091-0.135]	0.108 [0.086-0.134]	0.945	0.394
Total n-6 PUFA	11.44 [10.20-14.14]	10.38 [9.434-13.32]	***0.091***	***0.088***
cis 18:3$_{n-3}$ (ALNA)	0.831 [0.609-1.140]	0.947 [0.720-1.285]	0.210	0.183
***cis* 20:5$_{n-3}$ (EPA)**	0.058 [0.042-0.077]	0.076 [0.061-0.092] [b]	**0.006**	**0.046**
cis 22:5$_{n-3}$ (DPA)	0.141 [0.114-0.172]	0.164 [0.140-0.180] [a]	**0.057**	***0.098***
***cis* 22:6$_{n-3}$ (DHA)**	0.320 [0.220-0.390]	0.383 [0.316-0.477] [a]	**0.013**	**0.046**
Total n-3 PUFA	1.598 [1.411-1.976]	1.881 [1.466-2.213] [a]	***0.058***	***0.067***
Total PUFA	12.96 [11.74-16.40]	12.29 [11.15-15.65]	0.201	**0.159**
Unsaturated/saturated fatty acid	1.18 [1.06-1.38]	1.08 [1.02-1.18] [a]	**0.023**	***0.052***
PUFA/SFA	0.29 [0.24-0.38]	0.26 [0.22-0.32]	0.101	***0.088***
n-6/n-3 PUFA	7.03 [5.83-8.23]	5.71 [5.04-6.87][2]	**0.005**	***0.052***
LC-PUFA	2.088 [1.768-2.321]	2.129 [1.978-2.383]	0.268	0.200
Essential FA (LA+ALNA)	10.73 [9.46-13.81]	9.83 [8.79-13.18]	0.101	***0.088***
LA/ALA	10.86 [9.30-15.66]	9.99 [8.35-11.69] [a]	**0.047**	***0.059***
AA/DHA	1.679 [1.251-2.191]	1.307 [0.983-1.690] [b]	**0.010**	***0.052***
BCFA	29.82 [28.65-30.99]	31.25 [29.99-32.51]	0.104	***0.088***
Total lipids (Miris)(g/100ml)	3.55 [3.12-4.57]	4.75 [3.97-5.65] [a]	**0.027**	***0.052***

PUFA: Polyunsaturated fatty acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexanoic acid; DGLA : dihomo-gamma-linolenic acid ; GLNA : gamma-linolenic acid ; LA: Linoleic acid; ALNA: alpha-Linolenic acid. SAT: saturated fatty acids; MCSAT: medium chain saturated fatty acids (C8:0 to C12:0); MUFA: monounsaturated fatty acid; LC-PUFA: Long-Chain PUFA (polyunsaturated fatty acid that contains at least 20 carbons); BCFA: branched-chain fatty acids (C14:0, C15:0; C17:0 and C16:0).
Values (expressed as % of total identified FA) are median and [25% and 75% percentile] and are given for fatty acids present at > 0.05% of total fatty acids in milk. Values of p-values (assessed by Mann Whitney *U* test) between fast and slow growth groups were reported with [a] or [b] significantly different, $p<0.05$ or $p<0.01$, respectively. n: milk samplings between week 2 and week 4 of lactation. Fatty acids in bold font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups over the entire W2- W4 lactation period.

Considering the untargeted lipidomic LC-HRMS signatures, among the 1262 features, selected as described above, **162 discriminant lipid species** were annotated and are listed comprehensively in Table 3**,** for the overall W2-W4 lactation period, and in Supplemental Table S2, for each sampling time, W2, W3 and W4 of lactation. Most of the annotated lipids were more abundant in the breastmilk provided to infants with fast postnatal growth.

Table 3. Abundance (10^6) of annotated lipids that discriminated lipidotypes of breast milk provided to preterm infants with fast or slow growth during the W2-W4 lactation period.

Lipids	mz	median [25% and 75% percentile] from W2 to W4		Mann-Whitney *U* p-value From W2 to W4	FDR corrected q-value from W2 to W4
		Slow growth (n=38)	**Fast growth (n=29)**		
Fatty acid		9.47 [7.40-12.84]	8.39 [5.53-13.00]	0.1910	0.192
Anandamide (C18:3. n-6)	339.2889 $[M+NH_4]^+$	8.80 [7.02-11.77]	7.43 [5.11-11.47]	0.119	0.133
3-Hydroxyadipic acid	161.0455$[M-H]^-$	17.17 [14.02-20.25]	11.85 [5.68-15.09]	**0.000**	**0.000**
N-formylmaleamic acid	142.0203$[M-H]^-$	1.26 [0.84-1.64]	0.73 [0.59-0.97][c]	**0.000**	**0.000**
Dodecatetraenedioic acid	221.0667 $[M-H]^-$	0.77 [0.53-0.88]	0.55 [0.34-0.73][b]	**0.000**	**0.001**
Linderic acid	187.1340 $[M-H]^-$	0.52 [0.38-1.43]	0.76 [0.42-1.80]	0.282	***0.066***
alpha-hydroxy lauric acid	215.1653 $[M-H]^-$	4.06 [2.39-8.23]	5.08 [2.51-13.03]	0.433	***0.087***
2-hydroxy palmitic acid	271.2281 $[M-H]^-$	38.63 [22.48-48.63]	37.44 [29.98-50.32]	0.769	0.126
3-oxo-4-pentenoic acid	113.0243 $[M-H]^-$	1.43 [1.18-1.71]	0.94 [0.69-1.28] [c]	**0.000**	**0.000**
Dehydrocholic acid	401.2312 $[M-H]^-$	0.43 [0.21-0.73]	0.52 [0.33-0.97]	0.211	**0.054**
7R.9.14R-trimethyl-2E.4E.8E.10E-hexadecatetraenoic acid	289.2169 $[M-H]^-$	0.19 [0.10-0.48]	0.27 [0.14-0.46]	0.326	***0.072***
Ceramide		15.56 [12.98-18.63]	20.27 [17.34-23.79] [c]	**0.000**	**0.000**
Cer (18:1/22:0)	622.6123 $[M+H]^+$ 604.6017 $[M+H-H2O]^+$ 644.5491 $[M+Na]^+$	1.03 [0.89-1.38]	1.36 [1.14-1.69] [b]	**0.005**	**0.032**
Cer (d18:1/24:0)	632.6326 [M-H2O]+ 650.643$[M+H]^+$	14.59 [12.11-17.50]	18.78 [16.21-22.15] [c]	**0.000**	**0.011**
GlucosylCeramide		511.4 [487.8-574.5]	519.5 [483.9-558.1]	0.764	0.385
Glucosylceramide (d18:2/14:0)	685.5361$[M+NH_4]^+$	32.05 [13.43-46.52]	26.70 [13.73-41.90]	0.519	0.343
Galactosylceramide (d18:1/18:1)	743.614 $[M+NH_4]^+$	322.7 [309.6-355.8]	340.8 [316.5-348.9]	0.543	0.355
Galactosylceramide (d18:1/20:0)	773.652 $[M+NH_4]^+$	31.36 [29.58-33.78]	31.52 [30.08-33.87]	0.734	0.424

Glucosylceramide (d18:1/16:0)	717.5892[M+NH_4]$^+$	42.06 [34.50-48.39]	42.43 [35.90-48.07]	0.9440	0.492
Glucosylceramide (d18:1/18:0)	728.5481 [M+H]$^+$	2.28 [1.54-2.87]	1.85 [1.18-2.24] [b]	**0.004**	**0.032**
Glucosylceramide (d18:1/20:0)	773.6614 [M+NH_4]$^+$	20.89 [19.20-22.66]	19.24 [15.94-22.67]	***0.092***	0.116
Glucosylceramide (d18:1/24:0)	854.7266 [M+Na]$^+$	57.94 [54.82-62.99]	62.46 [56.55-66.47] *t*	***0.068***	***0.099***
Phosphocholine		357.0 [315.6-456.2]	453.3 [370.1-568.0] [b]	**0.006**	**0.009**
PC (18:0/18:1)	788.5863 [M+H]$^+$	2.59 [1.55-4.34]	4.16 [3.06-5.25] [a]	**0.025**	***0.064***
PC (14:0/16:0)	706.5391 [M+H]$^+$	1.54 [1.09-2.63]	1.830 [1.47-3.3] [f]	0.134	0.142
PC (14:0/16:1)	704.5237 [M+H]$^+$	0.20 [0.09-0.40]	0.25 [0.13-0.50]	0.277	0.224
PC (20:0/20:2)	842.636 [M+H]$^+$	1.49 [1.11-2.33]	2.33 [178-3.33] [b]	**0.017**	**0.053**
PC (16:1/18:1)	1620.1146 [M+HPO_3+2H]$^+$	1.06 [0.89-1.21]	1.03 [0.89-1.14]	0.551	0.358
PC (16:1/18:0)	1542.1426 [2M+Na]$^+$	11.76 [8.45-13.37]	10.58 [9.23-11.88]	0.387	0.277
PC (16:1/18:2)	1512.1349 [2M+H]$^+$	2.33 [1.70-2.96]	3.23 [1.762-3.79] [f]	**0.030**	***0.069***
PC (18:0/18:1)	1576.2229 [2M+H]$^+$	12.09 [7.30-23.45]	16.68 [8.09-28.81]	0.150	0.153
PC (18:1/18:1)	1572.1908[2M+H]$^+$	103.2 [84.86-131.5]	127.6 [99.45-164.00] [a]	**0.036**	***0.074***
PC (18:0/18:2)	786.5899 [M+H]$^+$	7.02 [5.92-9.61]	10.60 [8.13-13.21] [c]	**0.000**	**0.016**
PC (16:0/20:3)	784.5833 [M+H]$^+$	51.58 [41.81-65.60]	65.96 [52.12-85.89] [b]	**0.004**	**0.030**
PC (18:0/20:1)	816.6456 [M+H]$^+$	11.92 [7.23-18.88]	12.22 [8.59-20.67]	0.386	0.277
PC(18 :0/20 :3)	812.6143 [M+H]$^+$	30.80 [25.22-44.50]	46.59 [37.16-55.24] [3]	**0.000**	**0.016**
PC (18 :0/20 :5)	808.5806 [M+H]$^+$	93.34 [74.64-112.8]	94.55 [78.87-119.9]	0.343	0.256
PC (20:1/20:4)	836.6145 [M+H]$^+$	3.72 [2.96-4.54]	4.88 [3.77-6.26] [a]	**0.007**	**0.037**
PC (18:0/22:6)	834.596 [M+H]$^+$	11.95 [8.65-15.21]	12.61 [10.11-17.45]	0.153	0.154
PC (20:3/22:6)	856.5807 [M+H]$^+$	5.42 [3.95-6.97]	6.46 [5.06-7.62] [a]	**0.013**	**0.047**
PC (16:0/22:6)	806.5678 [M+H]$^+$	12.08 [7.25-16.08]	16.07 [12.04-24.40] [a]	**0.007**	**0.037**
PC-plasmalogen		8.16 [6.53-10.52]	8.88 [6.97-12.23]	0.273	0.236
PC (*P*-18:0/18:0)	796.6199 [M+Na]$^+$	1.72 [1.19-2.49]	2.32 [1.85-3.09] [b]	**0.008**	**0.037**
PC (*P*-16:0/18:2)	742.5723 [M+H]$^+$	2.18 [1.61-2.98]	1.59 [1.09-2.52]*t*	0.104	0.124
PC (O-16:0/18:1)	768.5514 [M+H]$^+$	4.11 [3.36-5.58]	4.82 [3.42-6.81]	0.114	0.131
Phosphoethanolamine		279.4 [253.2-317.5]	316.5 [283.1-356.3] [b]	**0.002**	**0.004**

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PE (16:0/16:1)	690.5054 $[M+H]^+$	1.79 [1.59-2.22]	2.15 [1.93-2.45] [b]	**0.005**	**0.032**
PE (16:1/20:0)	1492.1294 $[2M+H]^+$	6.72 [5.45-8.88]	8.38 [6.85-10.43] [a]	**0.037**	***0.074***
PE (16:0/20:2)	1488.0975 $[2M+H]^+$	21.16 [18.3-27.46]	27.62 [18.61-34.14] [a]	**0.039**	***0.075***
PE (16:0/20:2)	744.5519 $[M+H]^+$	142.2 [132.7-168.4]	166.6 [151.1-179.3] [a]	**0.016**	**0.050**
PE (16:0/20:4)	779.5379 $[M+Na]^+$	0.67 [0.57-0.87]	0.81 [0.61-1.19] [a]	**0.044**	***0.079***
PE (18:0/20:0)	776.60 $[M+H]^+$	1.68 [1.29-2.50]	1.52 [1.25-1.73]*t*	0.131	0.141
PE (20:1/20:4)	794.5676 $[M+H]^+$	7.45 [6.07-10.05]	9.41 [8.22-10.29] [a]	**0.030**	***0.069***
PE (18:0/20:4)	1536.0968 $[2M+H]^+$	4.93 [3.62-7.23]	7.71 [5.28-9.57] [b]	**0.004**	**0.030**
PE (22:0/20:3)	826.6047 $[M+H]^+$	2.09 [1.53-2.61]	2.51 [2.18-3.48] [b]	**0.006**	**0.033**
PE (18:1/18:2)	1484.067 $[2M+H]^+$	2.79 [1.97-3.99]	3.14 [2.07-4.36]	0.665	0.398
PE (16:0/20:4)	740.5208 $[M+H]^+$	7.75 [5.31-9.68]	7.18 [5.44-9.98]	0.468	0.321
PE (18:2/18:2)	740.5213 $[M+H]^+$	4.76 [3.58-5.70]	6.21 [4.92-7.41] [b]	**0.006**	**0.035**
PE (20:0/18:1)	774.599 $[M+H]^+$	6.92 [5.61-9.12]	9.24 [7.24-11.69] [b]	**0.004**	**0.030**
PE (18:0/20:4)	768.5495 $[M+H]^+$	13.52 [12.42-14.25]	14.14 [13.13-15.75]*t*	***0.094***	0.118
PE (20:4/20:0)	796.5836 $[M+H]^+$	6.13 [4.98-8.72]	8.63 [6.81-10.52] [b]	**0.007**	**0.035**
PE (22:6/18:0)	792.5517 $[M+H]^+$	17.55 [12.31-22.26]	20.02 [15.51-25.01]	***0.085***	0.112
PE (20:3/22:6)	814.5336 $[M+H]^+$	2.63 [1.96-3.06]	3.14 [2.58-3.59] [c]	**0.002**	**0.023**
PE (22:0/22:6)	848.6566 $[M+H]^+$	4.69 [3.55-6.02]	4.25 [2.64-4.98]	0.131	0.141
PE (18:0/20:3)	770.5672 $[M+H]^+$	12.72 [10.13-16.13]	17.21 [14.84-20.82] [c]	**0.000**	**0.001**
PE-plasmalogen		39.91 [31.47-49.92]	38.83 [34.83-52.40]	0.592	0.385
PE (*P*-16:0/20:5)	722.5102 $[M+H]^+$	4.73 [3.96-7.45]	5.92 [4.46-7.69]	0.119	0.133
PE (P-16:0/18:0)	726.5323 $[M+Na]^+$	10.82 [7.93-14.26]	9.44 [5.92-13.33]	0.186	0.173
PE (P-16:0/20:0)	754.5636 $[M+Na]^+$	4.11 [2.58-5.23]	3.94 [2.25-4.96]	0.343	0.256
PE (P-16:0/20:3)	726.5417 $[M+H]^+$	10.49 [8.45-12.70]	11.39 [9.41-14.01]	0.117	0.132
PE (O-18:0/20:5)	752.5551 $[M+H]^+$	7.84 [6.04-9.98]	9.95 [7.30-12.09] [a]	**0.019**	**0.056**
Phosphatidylglycerol		16.41 [15.59-18.36]	18.85 [16.90-22.25] [b]	**0.002**	**0.004**
PG (16:0/16:0)	723.5101 $[M+H]^+$	3.31 [2.77-3.90]	3.57 [2.85-3.79]	0.475	0.324
PG (18:0/20:4)	799.5425 $[M+H]^+$	2.11 [1.72-2.36]	1.90 [1.48-2.25]	0.202	0.181
PG (P-16 : 0/22 : 4)	783.5586 $[M+H]^+$	1.58 [1.26-1.76]	1.93 [1.47-2.26] [a]	**0.021**	***0.058***

PG (18 : 2/20 : 5)	810.523 $[M+NH_4]^+$	6.52 [5.64-7.40]	7.67 [6.61-9.94] [b]	**0.001**	**0.016**
PG (22 : 2/22 : 6)	892.5991 $[M+NH_4]^+$	3.20 [2.97-3.61]	3.56 [3.12-3.91] [a]	**0.035**	***0.074***
Phosphinositol					
PI (36:0) PI(18:0/18:0)	889.5716 $[M+Na]^+$	0.92 [0.78-1.39]	1.26 [0.89-2.20] [a]	**0.055**	***0.087***
Phosphoserine					
PS (40:5)	838.552 $[M+H]^+$	2.72 [2.52-3.06]	2.95 [2.64-3.11]	0.186	0.173
PS (18:0/20:4)	812.5419 $[M+H]^+$	2.13 [1.59-2.77]	2.79 [2.29-3.26] [b]	**0.002**	**0.023**
Retinol	287.2362 $[M+H]^+$	2.02 [1.33-2.97]	1.57 [1.10-3.11]	0.752	0.429
Diacylglyceride		104.0 [66.98-163.6]	92.39 [52.86-126.5]	0.183	0.192
DG (14:0/18:3)	580.5373 $[M+Na]^+$	8.52 [3.30-15.47]	4.92 [2.30-9.16] [a]	**0.025**	***0.064***
DG (16:0/16:1)	567.4974$[M+H]^+$	6.61 [3.88-7.99]	5.26 [3.05-11.07]	0.656	0.395
DG 18:0/18:1)	645.544 $[M+Na]^+$	7.56 [5.03-7.58]	6.90 [5.51-11.53]	0.797	0.444
DG (18:0/18:2)	638.5708 $[M+NH_4]^+$	55.02 [36.04-87.52]	40.60 [22.83-66.09] *t*	**0.045**	***0.079***
DG (18:0/18:1)	634.5395 $[M+NH_4]^+$	10.80 [5.53-17.81]	10.46 [7.75-17.81]	0.582	0.370
DG (20:4/19:0)	681.5402 $[M+Na]^+$	0.92 0.47-1.60]	1.07 [0.60-2.03]	0.292	0.233
DG (20:3:/20:0)	***697.5727*** *$[M+Na]^+$*	4.98 [2.91-9.25]	2.74 [1.64-4.81] [a]	**0.027**	***0.065***
DG (18:3/20:0)	647.5574 $[M+H]^+$	6.81 [2.59-13.46]	4.06 [1.51-9.17] *t*	***0.079***	0.107
DG (20:4/22:5)	713.5103 $[M+Na]^+$	0.56 [0.24-0.79]	0.26 [0.15-0.56] [a]	**0.040**	***0.076***
Triglyceride		7758 [7304-8118]	7572[7236-8496]	0.717	0.385
TG (14:0/16:1/17:2)	804.7056 $[M+NH_4]^+$	55.94 [47.00-60.12]	59.35 [52.54-69.66] [a]	**0.011**	**0.043**
TG (14:0/14:1/14:1)	741.5983 $[M+Na]^+$	415.0 [358.3-520.8]	415.2 [371.5-448.9]	0.972	0.499
TG (14:0/16:0/16:0)	796.7277 $[M+NH_4]^+$	337.3 [308.2-373.3]	381.5 [340.2-439.9] [b]	**0.003**	**0.030**
TG (14:1/14:1/18:1)	790.6895 $[M+NH_4]^+$	1074 [923.6-1296]	1074 [911.2-1330]	0.788	0.442
TG (14:0/14:1/19:1)	806.712 $[M+NH_4]^+$	9.17 [7.68-10.01]	9.81 [8.58-10.81] [a]	**0.012**	**0.046**
TG (13:0/14:1/20:5)	798.6639 $[M+NH_4]^+$	117.1 [114.0-124.0]	118.9 [111.6-124.5]	0.743	0.427
TG (16:0/16:1/16:1)	820.7365 $[M+NH_4]^+$	2033 [1831-2139]	2142 [1937-2447] [a]	**0.028**	***0.067***

TG (14:0/16:0/16:0)	801.6948 $[M+Na]^+$	25.93 [21.63-28.64]	29.18 [24.40-36.14] [b]	**0.019**	***0.057***
TG (16:1/16:1/16:1)	823.676 $[M+Na]^+$	257.9 [246.2-272.9]	258.8 [244.0-270.8]	0.639	0.388
TG (16:1/16:1/17:1)	832.7368 $[M+NH_4]^+$	74.98 [64.31-82.97]	76.59 [69.19-90.90]	0.178	0.169
TG (14:0/15:0/20:5)	828.7134 $[M+NH_4]^+$	10.48 [8.74-11.70]	11.27 [9.79-12.47]	0.178	0.169
TG (16:0/16:1/18:0)	850.7742 $[M+NH_4]^+$	320.2 [306.7-334.3]	325.7 [308.6-364.5]	***0.099***	0.121
TG (16:0/17:1/18:1)	862.8205 $[M+NH_4]^+$	3.37 [2.70-5.18]	2.93 [2.00-3.59]*t*	**0.051**	***0.083***
TG (16:0/16:0/18:1)	850.7655 $[M+NH_4]^+$	12.01 [10.70-13.14]	11.06 [9.80-12.04] [1]	**0.021**	***0.058***
TG (16:1/16:1/17:2)	830.7291 $[M+NH_4]^+$	10.40 [9.81-10.85]	10.65 [9.44-11.54]	0.606	0.378
TG (18:1/20:1/22:1)	986.9093 $[M+NH_4]^+$	3.29 [2.50-4.13]	2.51 [1.83-4.16]	0.111	0.128
TG (16:1/16:1/18:2)	844.7364 $[M+NH_4]^+$	147.3 [130.4-182.8]	182.8 [156.9-240.3] [b]	**0.002**	**0.022**
TG (16:1/16:1/17:2)	835.6764 $[M+Na]^+$	3.64 [3.20-4.61]	3.53 [3.09-3.89]	0.178	0.169
TG (16:0/16:0/16:1)	827.7101 $[M+Na]^+$	19.01 [15.59-21.21]	21.55 [17.11-25.06] *t*	**0.036**	***0.074***
TG (16:1/18:4/18:4)	862.6902 $[M+NH_4]^+$	4.35 [2.97-6.41]	6.37 [3848-9.41] [a]	**0.014**	**0.047**
TG (16:0/17:2/18:1)	860.7679 $[M+NH_4]^+$	184.9 [156.0-222.3]	190.3 [171.0-220.9]	0.320	0.247
TG (16:0/16:0/17:2)	839.7451 $[M+Na]^+$	1.57 [1.37-1.71]	1.47 [1.32-1.64]	0.114	0.131
TG (18:1/18:3/20:0)	935.7928 $[M+Na]^+$	3.76 [3.35-4.49]	4.00 [3.24-4.74]	0.639	0.388
TG (18:0/18:1/20:3)	933.7851$[M+Na]^+$	19.46 [17.47-22.81]	18.90 [17.36-23.78]	0.963	0.497
TG (18:2/18:2/20:0)	930.8363 $[M+NH_4]^+$	21.21 [18.33- 23.40]	17.47 [15.95-23.86] *t*	**0.055**	***0.088***
TG (18:0/18:1/18:1)	904.83 $[M+NH_4]^+$	387.1 [333.1- 451.4]	341.6 [267.7- 385.0] [a]	**0.015**	**0.049**
TG (18:0/18:1/18:1)	906.8364 $[M+NH_4]^+$	81.24 [74.07-95.84]	75.22 [56.50-84.79] [a]	**0.025**	***0.064***
TG (18:1/18:1/18:1)	902.8144 $[M+NH_4]^+$	956.4 [818.7-1069]	878.3 [810.2-1074]	0.242	0.203
TG (16:0/18:0/18:1)	883.761 $[M+Na]^+$	41.08 [33.90-44.66]	42.83 [40.77-45.25]	0.233	0.198
TG (18:1/18:1/18:2)	900.7896 $[M+NH_4]^+$	246.9 [224.0-277.0]	225.1 [190.1-236.1] [a]	**0.005**	**0.032**
TG (18:1/18:2/18:2)	898.7739 $[M+NH_4]^+$	120.3 [75.92-128.3]	96.31 [70.34-119.4] *t*	**0.034**	***0.073***
TG (18:0/18:1/18:2)	902.8052 $[M+NH_4]^+$	260.5 [233.8-281.8]	243.6 [211.4-295.7]	0.246	0.205
TG (16:0/17:1/20:5)	882.7478 $[M+NH_4]^+$	8.63 [8.18-9.64]	8.31 [7.60-8.83] [a]	**0.001**	**0.016**
TG (18:1/20:1/22:3)	982.8773 $[M+NH_4]^+$	2.52 [1.96-3.30]	1.94 [1.43-3.51]	0.119	0.133
TG(18:1/18:2/20:0)	930.8455 $[M+NH_4]^+$	78.44 [71.25-95.93]	71.54 [58.23-93.06] *t*	***0.059***	***0.091***
TG(18:2/20:4/20:4)	944.7669 $[M+NH_4]^+$	39.93 [29.65-52.66]	29.27 [22.13-49.46]	***0.094***	0.118
TG(18:1/20:4/20:4)	946.7742 $[M+NH_4]^+$	8.65 [6.97-10.87]	6.77 [5.28-11.25]	0.101	0.124
TG (20:0/20:0/20:4)	989.8484 $[M+Na]^+$	2.27 [1.65-2.85]	1.68 [1.41-2.63]	0.1064	0.1255
TG (18:2/20:1/20:1)	956.8607 $[M+NH_4]^+$	18.75 [14.96-25.95]	13.52 [11.70-23.93]	0.0716	0.1021

TG (18:1/20:2/20:4)	950.814 $[M+NH_4]^+$	88.26 [75.04-95.26]	74.00 [62.15-90.20] [a]	**0.0225**	***0.0597***
TG (18:0/20:3/20:5)	953.7908 $[M+Na]^+$	1.89 [1.72-2.32]	1.87 [1.77-2.48]	0.8976	0.4773
TG (18:2/20:1/20:4)	955.8051 $[M+Na]^+$	2.89 [2.46-4.61]	3.50 [2.50-4.72]	0.3315	0.2502
TG (16:0/18:0/18:0)	880.8213 $[M+NH_4]^+$	119.5 [108.1-154..4]	110.0 [82.96-128.8]*t*	***0.0773***	0.1070
TG (18:1/20:0/20:0)	967.8653 $[M+Na]^+$	1.69 [1.34-2.62]	1.98 [1.67-3.13]	0.3315	0.2502
TG (20:1/20:1/20:4)	980.8615 $[M+NH_4]^+$	3.64 [2.69-4.41]	2.63 [2.16-4.60]	0.1197	0.1331
TG (18:0/20:1/20:4)	959.801 $[M+NH_4]^+$	9.16 [8.57-10.72]	8.57 [7.13-11.61]	0.2978	0.2355
TG (18:0/20:3/22:0)	991.8645 $[M+NH_4]^+$	2.61 [1.95-3.24]	1.96 [1.69-3.57]	0.1535	0.1542
TG (20:2/20:4/20:4)	977.7533 $[M+NH_4]^+$	3.58 [2.55-4.31]	2.76 [2.17-4.20]	0.137	0.1442
Sphingomyéline		178.6 [122.6.-220.0]	188.4 [157.9-245.9]	0.480	0.363
SM (d18:0/12:0)	651.5340 $[M+H]^+$ (isotopic peak)	0.88 [0.52-1.02]	1.04 [0.68-1.54] [a]	**0.011**	**0.044**
SM (d18:1/12:0)	647.5119 $[M+H]^+$	3.80 [2.89-5.56]	5.24 [3.99-7.00] [b]	**0.003**	**0.029**
SM (18:1/14:0)	675.5425 $[M+H]^+$	72.48 [45.46-98.60]	86.28 [68.91-111.24] [a]	**0.036**	***0.074***
SM (d18:1/16:0)	725.5552 $[M+Na]^+$	41.85 [31.37-52.33]	39.63 [32.05-50.87]	0.907	0.481
SM (d18:1/16:1)	701.5583 $[M+H]^+$	13.98 [10.63-20.40]	15.97 [12.92-17.79]	0.574	0.367
SM (d16:1/18:1)	723.5399 $[M+Na]^+$	2.76 [2.15-4.12]	2.96 [2.47-3.65]	0.682	0.405
SM (18:1/20:1)	779.6015 $[M+Na]^+$	3.82 [3.20-4.93]	3.29 [2.50-4.30] [b]	**0.026**	***0.065***
SM (d18:1/20:2)	755.5768 $[M+H]^+$	1.49 [1.19-2.17]	1.15 [1.01-1.44] [a]	**0.010**	**0.042**
SM (18:1/20:1)	757.6205 $[M+H]^+$	13.82 [11.32-18.54]	14.51 [12.06-19.27]	1.000	0.506
SM (d18:1/23:0)	801.6827 $[M+H]^+$	5.97 [3.64-7.23]	7.21 [5.42-9.30] [a]	**0.048**	***0.081***
SM (d18:1/24:0)	815.6983 $[M+H]^+$	7.59 [5.81-10.37]	7.50 [5.59-9.53]	0.433	0.302
Eicosanoid					
10.11-dihydro-20-trihydroxy-leukotriene B4	385.2364 $[M-H]^-$	9.88 [6.00-12.19]	5.66 [4.12-7.09] [c]	**0.000**	**0.000**
20-Trihydroxy-leukotriene-B4	383.2208 $[M-H]^-$	11.22 [4.99-24.61]	9.62 [7.27-15.02]	0.566	0.104
HETE	319.2278 $[M-H]^-$	2.32[1.04-0.90]	2.31 [1.31-4.47]	0.607	0.109
Leukotriene B4	335.2227 $[M-H]^-$	0.51 [0.28-0.94]	0.32 [0.22-0.60]	0.277	***0.065***
7.8-epoxy-17S-HDHA	357.2051 $[M-H]^-$	1.78 [0.80-3.71]	1.60 [0.90-2.47]	0.691	0.119
15S-HpEDE	339.2537 $[M-H]^-$	1.68 [1.29-2.45]	1.50 [1.27-2.00]	0.292	***0.068***
11-deoxy-16.16-dimethyl-PGE2	363.252 $[M-H]^-$	52.64 [32.17-70.29]	40.19 [25.54-50.40] [a]	**0.014**	**0.007**
9-deoxy-9-methylene-16.16-dimethyl -PGE2	377.2676 $[M-H]^-$	1.38 [0.92-3.30]	0.99 [0.72-1.56]	***0.099***	**0.031**
PGF2alpha	353.2314 $[M-H]^-$	4.89 [3.26-7.50]	4.12 [2.76-6.73]	0.150	**0.042**

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11-dehydro-2.3-dinor-TXB2	339.2001 $[M-H]^-$	2.98 [1.54-4.17]	3.29 [2.23-4.75]	0.147	**0.041**
Lyso-PC/PE		31.55 [25.78-38.13]	29.07 [20.34-40.91]	0.658	0.385
LysoPC (16 :0)	454.2921 $[M+H]^+$	3.76 [2.85-4.60]	3.21 [2.42-4.48]	***0.0990***	0.121
LysoPC (14:0)	468.3079 $[M+H]^+$	4.40 [3.16-6.06]	5.39 [3.52-9.12]	0.131	0.141
LysoPE (16:1)	452.3133 $[M+H]^+$	1.37 [1.03-1.66]	1.65 [1.25-2.02] [a]	***0.056***	***0.088***
LysoPE (18:1)	480.3079 $[M+H]^+$	11.25 [7.84-16.01]	8.82 [6.71-12.77]	***0.079***	0.107
LysoPE (20:5)	500.274 $[M+H]^+$	3.34 [2.75-4.56]	2.94 [2.13-5.07]	0.198	0.179
LysoPE (20:3)	504.3058 $[M+H]^+$	2.96 [2.23-3.56]	3.09 [2.42-4.50]	0.122	***0.058***
LysoPE (20:4)	502.2902 $[M+H]^+$	3.58 [2.49-4.38]	2.43 [1.74-3.27] [a]	**0.020**	***0.043***
LysoPS (22:0)	580.3535 $[M-H]^-$	0.51[0.32-1.07]	0.43 [0.18-0.86]	0.157	***0.001***
LysoPG (22:4)	559.2853 $[M-H]^-$	0.70 [0.49-0.89]	0.30 [0.20-0.68] [c]	**0.000**	***0.019***
LysoPA (20:0)	465.3048 $[M-H]^-$	4.19 [3.39-4.93]	5.75 [3.75-8.59] [a]	**0.052**	
Cardiolipine					
CL (18:1/18:1/20:4/18:0)	739.5129 $[M-2H]^-$	2.40 [1.32-3.57]	2.74 [2.04-3.22]	0.419	***0.086***
CL (18:2/20:0/20:0/20:4)	767.5439 $[M-2H]^-$	3.21 [1.45-4.41]	2.87 [1.68-4.54]	0.842	0.134

PC: phosphocholine; PE: phsosphethanolamine; PG: phosphatidylglycerol; PI: phosphoinositol; PS : phosphoserine ; SM: sphingomyéline; DG: diacylglycerol ; TG: triacylglycerol ; CL : cardiolipine.

Values are median and [25% and 75% percentile]. Values of p-values (assessed by Mann Whitney *U* test) between fast and slow growth groups were reported with [a], [b], [c] significantly different, $p<0.05$, $p<0.01$ or $p<0.001$, respectively and *t*, a trend, $0.050<p<0.10$. Lipids in bold font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups over the entire W2- W4 lactation period.

Indeed, these milks contained higher levels of medium- or long-chain sphingomyelins and ceramides [such as Cer (d18:1/24:0), SM(d18:0/12:0)], in several phosphatidylcholines, phosphatidylethanolamines or plasmalogen-derivatives containing palmitic (C16:0) or palmitoleic (C16:1) acid [such as PE (16:0/16:1), PE (16:1/20:0)], stearic acid (C18:0) [such as PC (18:0/18:1), PC (18:0/18:2) and PE (*O*-18:0/20:5)], dihomo-γ-linolenic acid (DGLA, C20:3) [PE (20:3/22:6), PE (22:0/20:3)], or docosahexaenoic acid (DHA, C 22:6) [PE (20:3/22:6)]. In addition, breastmilk associated with fast growth contained more medium-chain triglycerides (TG) [TG (14:0/14:1/19:1)] but less long-chain TG containing oleic acid (C18:1) [such as TG (18:1/18:1/18:2) and TG (18:0/18:1/18:1)]. Finally several eicosanoids, including many DGLA-derived oxylipins [15S-HpEDE, 11-deoxy-16, 16-dimethyl- and 9-deoxy-9-methylene-16,16-dimethyl-PGE2], were lower in the breastmilk provided to preterm infants with fast growth.

Reliability of maternal milk lipids biomarkers regarding postnatal infant's growth

To assess the reliability of putative biomarkers as predictors of preterm infants' growth during hospital stay, we considered the multiple testing filtering (*i.e.* adjusted FDR p-value < 0.05) (**Step 3**), combined, only for lipidomic LC-HRMS signatures, to the multivariate selection operated by the previously described AoV-PLS/LDA model. This approach led to the selection of **4 selected fatty acids** [MCSAT and oleic acid (with a p-value of 0.051), EPA and DHA] and **46 robust biomarkers** of infant postnatal growth, that were annotated and are reported in bold font in Tables 2 and 3, respectively. In addition, the reliability of these selected biomarkers to predict postnatal growth rate was evaluated using a multiple linear regression (MLR) analysis (**step 4**) to explain the change in weight Z-score between birth and hospital discharge. Several confounding clinical factors (birth weight, gestational age, complementary parenteral and enteral nutrition, mother's body mass index) were introduced

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in MLR models. The resulting MLR p-values of all these putative biomarkers are listed in Table 4.

Table 4: Predictive ability of tentative lipid and fatty acid biomarkers on infant' growth (defined based on the difference between discharge and birth weight, head circumference Z-score, and infant' body fat mass (%) at discharge).

	Infant' weight growth between birth and discharge (SD) (p-value corrected – MLR)	**Infant' head circumference growth between birth and discharge (SD) (p-value corrected – MLR)**	**Infant' body fat mass (%) at discharge (p-value corrected – MLR)**
Fatty acids (targeted analysis)			
12:0	**0.0548**	0.2990	0.9761
14:0	**0.0910**	0.2411	0.6165
SAT	0.9940	0.8838	0.9929
MCSAT	**0.0544**	0.3515	0.8425
18:1_{n-9}	0.3695	0.7646	0.5717
MUFA	0.7250	0.4404	0.7999
MUFA/SFA	0.9627	0.7043	0.8068
18:$1_{n-9\ et\ n-7}$	0.5096	0.7195	0.6274
cis 20:5_{n-3} (EPA)	0.1990	0.9540	0.5121
cis 22:5_{n-3} (DPA)	0.2900	0.9234	0.9727
cis 22:6_{n-3} (DHA)	0.2576	0.6421	0.5709
Total n-3 PUFA	0.1104	0.7182	0.8281
Unsaturated/saturated fatty acid	0.9077	0.9379	0.9116
n-6/n-3 PUFA	0.3955	0.1852	0.5731
LA/ALA	0.5149	0.9840	0.7330
AA/DHA	0.4421	0.4522	0.9609
Fatty acids (lipidomics analysis)			
3-Hydroxyadipic acid	0.4888	0.0215	0.1228
N-formylmaleamic acid	0.1539	0.8550	**0.0831**
Dodecatetraenedioic acid	0.1069	0.0211	0.1465
Linderic acid	**0.0631**	0.2197	0.9116
alpha-hydroxy lauric acid	**0.0553**	**0.0928**	**0.0792**
3-oxo-4-pentenoic acid	0.2843	**0.0874**	0.1668
Dehydrocholic acid	0.2779	0.3958	0.3805
7R,9,14R-trimethyl-2E,4E,8E,10E-hexadecatetraenoic acid	0.2239	0.2322	**0.0805**
Ceramide			
Cer(d18:1/24:0)	**0.0735**	0.9820	0.8891
Sphingomyeline			
SM (d18 :0/12 :0)	**0.0192**	0.9988	0.4412
SM (d18 :1/12 :0)	0.9640	0.5514	0.4909
SM (18 :1/14 :0)	0.5331	0.1678	0.9231
SM (18 :1/20 :1)	0.5874	0.6646	0.6519
SM (d18 :1/20 :2)	0.4859	0.9478	0.6689

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SM (d18 :1/23 :0)	0.4519	0.2199	0.5212
SM (d18 :1/24 :0)	0.3047	0.9461	0.4036
Glucosy/Galactosyl-Ceramide			
GlucosylCeramide (d18 :1/18 :0)	0.8384	0.6769	0.1141
Phosphatidylcholine			
PC (18 :0/18 :1)	**0.0846**	0.9925	0.5848
PC (20 :0/20 :2)	0.8796	0.9506	0.3652
PC (16 :1/18 :2)	0.7326	0.1714	0.9567
PC (18 :1/18 :1)	0.6045	0.8982	0.5574
PC (18 :0/18 :2)	0.5854	**0.0825**	0.8515
PC (16 :0/20 :3)	0.6372	0.1802	0.2627
PC (18 :0/20 :3)	0.3791	0.1735	0.7289
PC (20 :1/20 :4)	0.6725	0.7472	0.9858
PC (20 :3/22 :6)	0.2389	0.2229	0.6475
PC (16 :0/22 :6)	0.5579	0.8700	0.7159
PC-plasmalogen			
PC (P-18 :0/18 :0)	0.3871	0.3175	0.3281
Phosphatidylethanolamine			
PE (16 :0/16 :1)	0.8436	0.3558	**0.0285**
PE (16 :1/20 :0)	0.9493	0.8600	**0.0197**
PE (16 :0/20 :2)	0.6331	0.1396	0.1289
PE (18 :0/20 :4)	0.8224	0.6572	0.9686
PE (22 :0/20 :3)	0.5384	**0.0635**	0.5931
PE (18 :2/18 :2)	0.5179	0.3161	0.4904
PE (20 :0/18 :1)	0.4043	0.2576	0.7201
PE (20 :4/20 :0)	0.3322	0.3989	0.8096
PE (20 :3/22 :6)	**0.0311**	0.6385	0.6067
PE (18 :0/20 :3)	0.1498	0.9979	0.7610
PE-plasmalogen			
PE (O-18 :0/20 :5)	**0.0916**	**0.0340**	0.8537
Phosphatidylglycerol			
PG (P-16:0/22:4)	0.8864	0.2704	0.3261
PG (18:2/20:5)	0.4292	0.5861	0.8835
PG (22:2/22:6)	0.1065	0.9632	0.8748
Diacylglyceride	0.1569	0.7014	0.9421
DG (20:3:/20:0)			
Triglyceride			
TG (14:0/16:0/16:0)	0.9384	0.1592	0.3541
TG (14:0/14:1/19:1)	0.5518	0.7615	**0.0128**
TG (16:0/16:1/16:1)	0.9021	0.6373	0.1674
TG (14:0/16:0/16:0)	0.7508	0.5671	0.3760
TG (16:0/17:1/18:1)	**0.0096**	0.2851	0.1884
TG (16:1/16:1/18:2)	0.7736	0.7006	0.3158
TG (16:0/16:0/16:1)	0.7244	0.4945	0.2347
TG (16:1/18:4/18:4)	**0.0913**	0.7318	0.8405
TG (18:2/18:2/20:0)	0.2827	0.7397	0.8076
TG (18:0/18:1/18:1)	**0.0136**	0.3839	**0.0374**
TG (18:1/18:1/18:2)	**0.0380**	0.2897	0.8946

TG (18:1/18:2/18:2)	0.5058	0.4183	0.9026
TG (16:0/17:1/20:5)	0.7399	0.1535	0.1707
TG (18:1/18:2/20:0)	0.1927	0.5581	0.6932
TG (18:1/20:2/20:4)	0.5488	0.2420	0.6740
Eicosanoïd			
10,11-dihydro-20-trihydroxy-leukotriene B4	0.7581	0.5496	0.1347
Leukotriene B4	0.7026	0.7121	**0.0920**
15S-HpEDE	**0.0017**	0.1461	0.2579
11-deoxy-16,16-dimethyl-PGE2	**0.0060**	0.2715	0.7307
9-deoxy-9-methylene-16,16-dimethyl -PGE2	**0.0047**	0.2870	0.4182
PGF2alpha	0.1125	0.7673	0.9819
11-dehydro-2,3-dinor-TXB2	0.5377	0.3015	**0.0936**
Lyso PS/PG			
LysoPS (22:0)	**0.0641**	0.3482	0.3598
LysoPG (22:4)	**0.0163**	0.9320	0.5419
LysoPA (20:0)	0.4313	0.4373	0.7483
Cardiolipine			
CL (18:1/18:1/20:4/18:0)	0.6416	0.9790	**0.0316**

Values of p-values were calculated using multiple linear regression analysis, by taking into account several confounding factors (infant's birth weight, mother's BMI, gestational age, milk enrichment with lipid, protein and calories). Lipids in bold and red font presented a statistical significance set to a confidence level of P<0.10.

Among the 50 AoV-PLS/LDA- and/or FDR-selected biomarkers, **7 lipid species** appeared of paramount interest, due to their significant (10%) MLR p-value for delta weight Z-score [two MCSAT, lauric (C12:0) and myristic (C14:0) acids; one MUFA, linderic (C12:1) acid; a long-chain ceramide, Cer (d18:1/24:0) and a medium-chain SM, SM (d18:1/12:0); a PC- and a PE-plasmalogen containing stearic acid [PC (18 :0/18 :1) and PE (O-18 :0/20 :5)]; a PE-containing DGLA and DHA, PE (20:3/22:6); DGLA-derived oxylipins [15S-HpEDE and two deoxy-dimethyl-PGE2] and two TG-containing oleic acid [TG (18:1/18:1/18:2) and TG (18:0/18:1/18:1)]. The AUC of ROC curve of these 7 lipid species ranged between 0.6 and 0.7, indicating a reasonably good performance of these selected biomarkers to predict preterm infant weight growth during their first four postnatal weeks (as illustrated in Figure 5).

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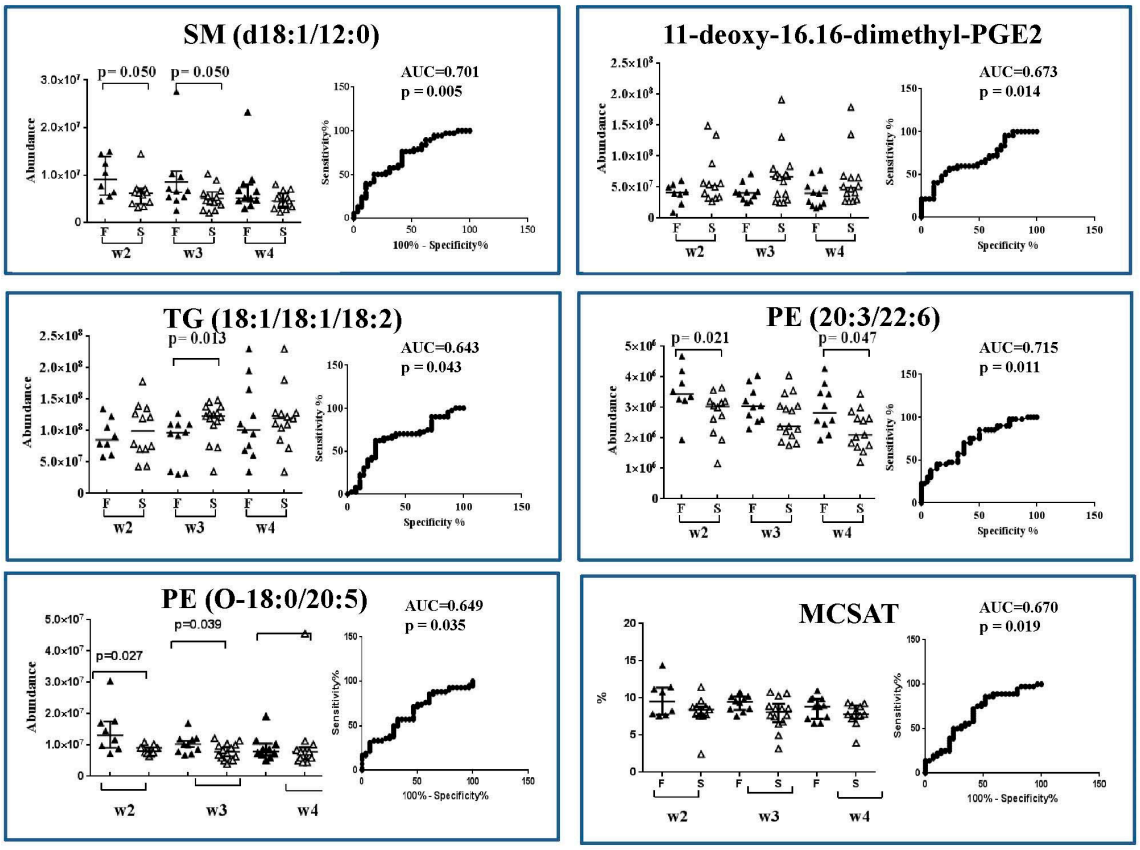

Figure 5 : Scatter plot and ROC plot analysis using lipids biomarkers abundance [SM (d18:1/12:0), TG (18:1/18:1/18:2), PE (O-18:0/20:5), , PE (20:3/22:6), MCSAT and one deoxy-dimethyl-PGE2] in breast milk provided to preterm infants who experienced fast (black triangle, F group) *versus* slow (white triangle, S group) growth during hospital stay. Scatter plot (median): from w2 to w4 of lactation period; ROC plot: over the entire W2-W4 lactation period.

Interestingly, among these 50 selected biomarkers, 6 lipid species presented a significant MLR p-value either for (i) delta head circumference Z-score [alpha-hydroxy lauric acid; a PE containing plasmalogen, PE (*O*-18:0/20:5); a PC-containing stearic acid, PC (18 :0/18 :2); a PE-containing DGLA, PE (22 :0/20 :3)] or (ii) fat mass at discharge [again alpha-hydroxy lauric acid; hexadecatetraenoic acid; two PE-containing palmitic acid, PE (16:0/16:1) and PE (16:1/20:0); two eicosanoids, leukotriene B4 and 11-dehydro-2,3-dinor-TXB2; one

cardiolipin-containing stearic and oleic acid, CL (18:1/18:1/20:4/18:0); and again TG (18:0/18:1/18:1)] (Table 4).

Discussion

To the best of our knowledge, the current pilot study is first to demonstrate that clear-cut differences in breast milk lipidomic signature are associated with the early growth pattern of preterm infants receiving their own mother's breast milk as their sole source of enteral feeding for the first month of life. Our strategy was to compare two groups of preterm infants who presented opposite growth velocity during their hospital stay. Infants in our fast growth group (mean birth weight Z-score -1.59 SD) lost less than 0.67 SD weight Z-scores between birth and discharge, indicating clinically nearly optimal growth during their hospitalization. In contrast, infants in our slow growth group (mean birth weight Z-score 0.56 SD) lost more than -1.10 SD (range -1.953; -1.230) weight Z-scores between birth and discharge, indicating sub-optimal growth. These mean weight Z-score at discharge are nearly identical to the first and third tertile identified in a larger preterm cohort described earlier by our team [41]. A negative correlation between birth weight Z-score early growth (between birth and hospital discharge) has long been known in cohorts of preterm infants [41, 42]. We therefore introduced birth weight as one of the clinical parameters in the adjustment variables in Multiple Linear Regression (MLR) analysis. This latter model made it possible to check the reliability of potential biomarkers. In the following discussion, we focus on the biomarkers that remained significant after adjustment in MLR.

Fast growth during hospital stay is associated with a specific maternal milk lipidomic signature

We are aware of the fact that breast milk lipidomic signatures require *ad hoc* methods to assess the relevance of putative lipid biomarkers of preterm infant growth. For this purpose, we combined different multivariate statistical supervised models (MG PLS-DA, AoV-PLS followed to LDA), with various validation procedures [64]. These models allowed us to retrieve a set of 162 annotated lipids that contributed to breast-milk lipidotypes' clustering associated to infants presenting fast or slow growth. The intermediate clustering of lipidotypes in AoV-PLS score plot for breast milk provided to the two sets of twins with discordant growth rates, suggests that postnatal growth rate is obviously multifactorial, and postnatal feeding is undoubtly one of these factors. Stringency in our analysis was ensured by taking into account confounding clinical factors for the selection of biomarkers. Then, among the 50 FDR-selected lipids biomarkers, 7 displayed good performance measures of infant's weight gain during hospital stay, computed through a ROC curve [65], and a significant corrected MLR p-value for weight delta Z-score. The performance of ROC curve remained unchanged when discarding the two sets of twins who had discordant growth trajectories. This result suggests the reliability of the selected lipid species in the prediction of postnatal infant growth. These lipids present in breastmilk therefore could be considered as robust candidate biomarkers of infant growth during hospital stay, in addition to classical determinants of postnatal growth of preterm infants during hospitalization such as birth weight, gestational age, and protein/energy ratio in nutrition support [41]. It should be noted that the volume of milk intake, known to have a strong impact on early growth, was introduced in the Multiple Linear Regression model through the estimation of the total caloric intake. In the following, we therefore address the relevance of such putative MLR-significant biomarkers for preterm infant growth.

The abundance of MCSAT in breast milk is associated with fast growth during hospital stay

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Milk provided to preterm infants with fast growth contained more total MCSAT than milk provided to infants with low growth MCSAT may be important for the normal maturation of the gastrointestinal tract [66] and its protection from infection [67, 68]. Moreover, MCSAT, and particularly lauric and myristic acids, are extensively oxidized [7]. Such an extensive oxidization leads to produce a dose-dependent rise in plasma ketones [69]. Ketones are a major source of both energy and acetyl-CoA not only for the brain development [69] but also for overall body growth. Such role of MCSAT could explain the association of MCSAT-rich milk with fast growth in our cohort.

Medium- and long- chain- ceramides/sphingomyelins and choline-containing phospholipids in breastmilk reliably predict early growth in preterm infant

Preterm infants with a fast weight growth received breast milk with a higher content in a long-chain ceramide **Cer (d18:1/24:0**), a medium-chain sphingomyelin **SM (d18:0/12:0)** and long-chain phosphatidylcholine **(PC (18:0/18:1)**) and phosphatidylethanolamine **(PE (20:3/22:6)**). In adults, medium- and long-chain ceramides were found to enhance insulin sensitivity and improve glucose homeostasis [70, 71]. Such an impact has not been yet explored in infants. Phosphatidylcholine and sphingomyelin were reported to protect against gastrointestinal infection, during early childhood, and to play a key role in gut barrier function [68]. With regard with phospholipids containing PUFAs, they may act as antioxidants in gut mucosa [72]. Finally, the abundance of phosphatidylcholine and sphingomyelin in breast milk implies that human milk supplies large amounts of choline, which is essential for neurodevelopment [27].

Enhanced breast milk levels of palmitoleic acid-, DHA-, dihomo-γ-linolenic acid- and plasmalogen-containing PE are associated with fast growth in preterm neonates

Breast milk provided to preterm infants with fast growth contained more DGLA-, DHA- and palmitoleic acid- containing PE: **PE (20:3/22:6)** displayed an ability to predict growth during

hospital stay, and **PE (16:0/16:1)** and **PE (16:1/20:0)** predicted fat mass at discharge. Such finding is consistent with the beneficial effects of DGLA reported in the treatment of inflammatory disorders and in cholesterol homeostasis [73]. Palmitoleic acid, a 'new' lipokine, was found to affect metabolism [74], which could, in turn, enhance infant growth. Interestingly, our lipidomics study confirms the presence of plasmalogens from the PE and PC (Pls-PEs and PCs) family. This has been previously reported in human milk [67, 75] with a potential impact on infant health [67]. With respect with our data, the **plasmalogen PE (O-18:0/20:5)** containing EPA, a precursor of DHA, presented a significant MLR p-value corrected for both weight gain and head circumference growth in preterm infants. In contrast, breast milk total EPA and DHA content did not predict head growth in our infants, even though higher levels of EPA and DHA were found in the fast growth group. Taken together, these findings suggest dietary DHA, provided as a phospholipid may be more efficient than DHA supplied as part of a triglyceride, for brain DHA accretion, as reported in piglets [76]. Human milk plasmalogens may be involved in infant brain development since brain Pls-PEs were recently shown to accumulate postnatally and to be enriched in long-chain PUFA, particularly DHA [77]. Brain plasmalogen content was reported to increase between the 32nd week of gestation and the 4th and 6th postnatal month [67]. Moreover, plasmalogens exert an antioxidant effect [67]. Indeed, the lower erythrocyte levels of plasmalogens reported in neonates, compared to older children could be particularly detrimental in preterm infants who have decreased anti-oxidant defense [78, 79]. However, the biological determinants of Pls-PE FAs and physiological relevance to the breastfed infant remain to be elucidated [77].

Decreased breast milk levels of eicosanoids and oleic acid-containing triglycerides are associated with early weight gain in preterm neonates

Whereas higher amounts of medium chain TG, containing myristic acid, correlated with body fat mass at discharge, decreased levels in long chain TG containing oleic or linoleic acids [**TG**

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(18:0/18:1/18:1), TG (18:1/18:1/18:2) and TG (16:0/17:1/18:1)] predicted infant growth, and, concerning the former TG, fat mass at discharge as well. Dietary substitution of medium chain- for long chain- triglycerides was shown to affect energy balance, promoting weight reduction in obese, adult humans [7]. In preterm infants, the use of lipid emulsions containing 50% medium chain-TG in parenteral nutrition was found to be associated with a lesser rate of protein accretion, compared to emulsions containing 100% long chain-TG [80].The potential impact of oleic acid has been evidenced for its anti-microbial activity and its protection of digestive tract against infections [75]. However, it still remains largely unexplored in infants [81]. Finally, among several eicosanoids, that were less abundant in the breastmilk provided to preterm infants' with fast growth, three **dihomo-γ-linolenic acid (DGLA)-derived oxylipins (15S-HpEDE and two deoxy-dimethyl-PGE2)** displayed predictive ability on infant growth during hospital stay. These oxylipins are signaling lipids very recently found in human milk [73, 82], and may play a role inpreterm infant as pro- and/or anti-inflammatory mediators which could, in turn, exert key roles in maternal–infant biochemical imprinting [82].

Taken in aggregate, the current findings suggest a striking inter-subject variation in the lipidome of breast milk among mothers who deliver preterm infants. Moreover, they suggest that such heterogeneity may impact early infant's growth. Limitations of the study stem from the relatively small sample size of the sample, the exploratory character of the study, and slight differences in birth weight between the groups. Our finding in twins, who followed opposite trajectories during their hospital stay, illustrates the fact that breast milk lipid composition is only one among the many factors that determine early growth in preterm infants. The main strength of the study lies in the rigorous and comprehensive approach used to discriminate breast milk lipidomic patterns between the infants groups that experienced fast growth *versus* slow growth in their first 4 postnatal weeks. Robust lipid biomarkers, i.e.

medium-chain sphingomyelin [SM (d18:1/12:0)], phospholipid containing DGLA and DHA [PE (20:3/22:6)], a DGLA-derived oxylipin, TG-containing oleic acid [TG (18:0/18:1/18:1) and TG (18:1/18:1/18:2)] and MCSAT, displayed a good ability to predict weight gain during hospital stay, likely through their role in energy homeostasis, or the defense against oxidative stress, gastrointestinal tract infection, or inflammation. In particular, we confirm the presence of some new bioactive lipids (oxylipin) recently evidenced in human milk with suggested biological relevance to preterm infant health [83]. Further research on milk bioactive-lipid components is warranted to improve our understanding of the biological role of breast milk fat and its impact on infant development and health. Such understanding could, in turn, open the way to the manipulation of maternal diet to produce desired changes in breast milk, and/or to the use of specific lipid supplements in the personalized nutritional care of preterm infants in the neonatal intensive care unit.

Supplementary Materials: Supplemental Material and Methods ; Figure S1: Multi-group PLS-DA score plots based on the LC-ESI$^-$-HRMS profiles obtained on human preterm milk ; Figure S2 : AoV-PLS and LDA models, based on the LC-ESI$^-$-HRMS profiles of human preterm milk, on the factor weight Z-score (discharge - birth) ; Table S1: Concentration levels of total fatty acids expressed as % of total identified FA, measured in human preterm milk at W2, W3, W4 of lactation and provided to newborns with fast or slow growth ; Table S2: Abundance of annotated discriminant lipids measured in breast milk at week 2 (W2), W3, W4 of lactation provided to preterm infants with fast or slow growth are available from the "Online Supporting Material".

Clinical Trial Register Number: LACTACOL cohort registered at www: clinicaltrials.gov under # NCT01493063.

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Authors' contributions: M-CA-G, C-YB, J-CR: had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis designed research; C-YB, J-CR, CB, DD, AL: conceived the LACTACOL study and design; CB, HB and C-YB: managed the LACTACOL cohort; M-CA-G: conceived and supervised the present lipidomics study conducted on a subset of infants of LACTACOL cohort; TM, VC, EMQ, MM: conceived the appropriate statistical tools and/or performed the statistical analysis; J-PA, AD, YG, MC: conducted lipidomic analysis; M-CA-G, TM, VC, J-CR, C-YB and DD: drafted the manuscript; DD: critically revised the manuscript for important intellectual content; and all authors: acquired, analyzed, or interpreted the data, and read and approved the final manuscript.

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Legends

Figure 1: Flowchart of infants enrolled in the ancillary study of the mono-centric prospective population-based LACTACOL.

Figure 2: Statistical workflow applied on breast-milk LC-ESI$^{+/}$ESI^{-}-HRMS profiles.

Figure 3: Multi-group PLS-DA score plots based on the LC-ESI^{+}-HRMS profiles (3451 features, 118 milks) obtained on human preterm milk. Representation of the individuals : milk provided to preterm infants who experienced fast (orange) or slow (blue) growth from week 2 to week 7 of lactation. MB-PLS-DA score plots: ○, week 2; Δ, week 3; +, week 4; x, week 5; ◊, week 6;▽, week 7.

Supplemental Figure S1: Multi-group PLS-DA score plots based on the LC-ESI^{-}-HRMS profiles (903 features, 118 milks) obtained on human preterm milk. Representation of the individuals : milk provided to preterm infants who experienced fast (orange) or slow (blue) growth from week 2 to week 7 of lactation. MB-PLS-DA score plots: ○, week 2; Δ, week 3; +, week 4; x, week 5; ◊, week 6; ▽ , week 7.

Figure 4: AoV-PLS and LDA models, based on the LC-ESI^{+}-HRMS profiles of human preterm milk, on the factor weight Z-score (discharge - birth) : AoV-PLS score plot with 45% of variance (R2Y=38%) on components 1-2 (**Figure 4a**) and LDA (built on 10 components of AoV-PLS) with a p-value=0) (**Figure 4b**). Breast milk provided to preterm infants who experienced fast (green) or slow (red) growth and to twin infants with discordant growth rate, one with high growth rate and one with low growth rate, (blue).

Supplemental Figure S2: AoV-PLS and LDA models, based on the LC-ESI^{-}-HRMS profiles of human preterm milk, on the factor weight Z-score (discharge - birth): AoV-PLS score plot with 45% of variance (R2Y=50%) on components 1-2 **(Supplemental Figure S2a)** and LDA

(built on 4 components of AoV-PLS) with a p-value=0) **(Supplemental Figure S2b).** Breast milk provided to preterm infants who experienced fast (green) or slow (red) growth and to twin infants with discordant growth rate, one with high growth rate and one with low growth rate, (blue).

Figure 5 : Scatter plot and ROC plot analysis using lipids biomarkers abundance [SM (d18:1/12:0), TG (18:1/18:1/18:2), PE (O-18:0/20:5), , PE (20:3/22:6), MCSAT and one deoxy-dimethyl-PGE2] in breast milk provided to preterm infants who experienced fast (black triangle, F group) *versus* slow (white triangle, S group) growth during hospital stay. Scatter plot (median): from w2 to w4 of lactation period; ROC plot: over the entire W2-W4 lactation period.

Table 1: Maternal and preterm infants' characteristics

Values are medians and 25% and 75% percentiles. P values for comparison between fast and slow growth groups were derived by using Mann-Whitney *U* test.

Table 2. Concentration levels of total fatty acids (free and triglycerides- and phospholipids-bound) in breast milk provided to preterm infants with fast or slow growth during the W2-W4 lactation period.

PUFA: Polyunsaturated fatty acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexanoic acid; DGLA: dihomo-gamma-linolenic acid; GLNA: gamma-linolenic acid ; LA: Linoleic acid; ALNA: alpha-Linolenic acid. SAT: saturated fatty acids; MCSAT: medium chain saturated fatty acids (C8:0 to C12:0); MUFA: monounsaturated fatty acid; LC-PUFA: Long-Chain PUFA (polyunsaturated fatty acid that contains at least 20 carbons); BCFA: branched-chain fatty acids (C14:0, C15:0; C17:0 and C16:0).

Values (expressed as % of total identified FA) are median and [25% and 75% percentile] and are given for fatty acids present at > 0.05% of total fatty acids in milk. Values of p-values

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(assessed by Mann Whitney *U* test) between fast and slow growth groups were reported with [a] or [b] significantly different, $p<0.05$ or $p<0.01$, respectively. n: milk samplings between week 2 and week 4 of lactation. Fatty acids in bold font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups over the entire W2-W4 lactation period.

Table 3. Abundance (10^6) of annotated lipids that discriminated lipidotypes of breast milk provided to preterm infants with fast or slow growth during the W2-W4 lactation period.

PC: phosphocholine; PE: phsosphethanolamine; PG: phosphatidylglycerol; PI: phosphoinositol; PS: phosphoserine; SM: sphingomyéline; DG: diacylglycerol; TG: triacylglycerol; CL: cardiolipine.

Values are median and [25% and 75% percentile]. Values of p-values (assessed by Mann Whitney *U* test) between fast and slow growth groups were reported with [a], [b], [c] significantly different, $p<0.05$, $p<0.01$ or $p<0.001$, respectively and [t], a trend, $0.050<p<0.10$. Lipids in bold font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups over the entire W2- W4 lactation period.

Table 4: Predictive ability of tentative lipid and fatty acid biomarkers on infant' growth (defined based on the difference between discharge and birth weight, head circumference Z-score, and infant' body fat mass (%) at discharge).

Values of p-values were calculated using multiple linear regression analysis, by taking into account several confounding factors (infant's birth weight, mother's BMI, gestational age, milk enrichment with lipid, protein and calories). Lipids in bold font presented a statistical significance set to a confidence level of $P<0.10$.

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Supplemental Table 1. Concentration levels of total fatty acids (free and triglycerides- and phospholipids-bound) expressed as % of total identified FA, measured in human preterm milk at W2, W3, W4 of lactation and provided to newborns with fast or slow growth.

PUFA: Polyunsaturated fatty acid; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexanoic acid; DGLA: dihomo-gamma-linolenic acid; GLNA: gamma-linolenic acid; LA: Linoleic acid; ALNA: alpha-Linolenic acid. SAT: saturated fatty acids; MCSAT: medium chain saturated fatty acids (C8:0 to C12:0); MUFA: monounsaturated fatty acid; LC-PUFA: Long-Chain PUFA (polyunsaturated fatty acid that contains at least 20 carbons); BCFA: branched-chain fatty acids (C14:0, C15:0; C17:0 and C16:0).

Values (expressed as % of total identified FA) are median and [25% and 75% percentile] and are given for fatty acids present at > 0.05% of total fatty acids in milk. Values of p-values (assessed by Mann Whitney *U* test) between fast and slow growth groups were reported with [a] or [b] significantly different, $p<0.05$ or $p<0.01$, respectively and [t], a trend, $0.050<p<0.10$. n: milk samplings at week 2, week 3 and week 4 of lactation. Fatty acids in bold and red font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups on the total W2 to W4 lactation period.

Supplemental Table 2. Abundance (10^6) of annotated discriminant lipids measured in breast milk at week 2 (W2), W3, W4 of lactation provided to preterm infants with fast or slow growth.

Values are median and [25% and 75% percentile]. Values of p-values (assessed by Mann Whitney *U* test) between fast and slow growth groups are reported with [a], [b], [c] significantly different at same stage of lactation, $p<0.05$, $p<0.01$ or $p<0.001$, respectively and [t], a trend,

0.050<p<0.10. Lipids in bold and red font presented a corrected p-value <0.05, using the *post hoc* control of the type I error rate (False discovery Rate procedure), between the two infant groups on the total W2 to W4 lactation period.