Lipidomic Analyses, Breast- and Formula-Feeding, and Growth in Infants

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Objective To evaluate lipidomic differences between breast- and formula-fed infants.

Study design We utilized high-resolution mass-spectrometry methods to analyze 3.2 mm dried blood spot samples collected at ages 3 months (n = 241) and 12 months (n = 144) from a representative birth cohort study. Lipidomic profiles were compared between infants exclusively breast-fed, formula-fed, or mixed-fed, and related to 12-month infancy weight. Data analysis included supervised multivariate statistics (partial least squares discriminant analysis), and univariate analysis with correction for multiple testing.

Results Distinct differences in 3-month lipidomic profiles were observed between exclusively breast-fed and formula-fed infants; mixed-fed infants showed intermediate profiles. Principle lipidomic characteristics of breast-fed infants were lower total phosphatidylcholines (PCs), with specifically lower short chain unsaturated PC but higher long chain polyunsaturated PC; higher cholesterol esters; and variable differences in sphingomyelins. At 12 months, lipidomic profiles were markedly different to those at 3 months, and differences between the earlier breast/formula/mixed-feeding groups were no longer evident. However, several specific lipid species, associated with breast-feeding at 3 months, also correlated with differences in 3- to 12-month weight.

Conclusions State-of-the-art dried blood spot sample lipidomic profiling demonstrated striking differences between breast-fed and formula-fed infants. Although these changes diminished with age, breast-fed lipidomic profiles at 3 months were associated with infancy weight and could potentially represent biomarkers of infant nutrition. *(J Pediatr 2015;166:276-81)*.

Links between early life exposures and later health outcomes may in part be due to nutritional programming in infancy. This hypothesis is supported by observed long-term benefits associated with breast-feeding, such as better cognitive development in childhood, and lower risks of obesity and high blood pressure in later life.[1] Effects of early nutritional interventions in infancy, using nutrient-enriched milk formulas, include increased later risk of metabolic disease.[2] However, the underlying mechanisms are unknown and are difficult to study.

Previous work has shown that the biochemical phenotype of infants differs according to feeding practice (breast- vs formula-feeding). Higher total cholesterol levels,[3-7] higher low density lipoproteins,[5] and lower high density lipoproteins[7] have been reported in breast-fed infants, and may lead to lower cholesterol levels in adulthood.[5,8,9] However, more detailed lipidomic profiling in breast- vs formula-fed infants has not yet been performed. We, therefore, utilized technological developments in lipidomics for metabolic phenotyping[10] to obtain a detailed lipidomic profile from dried blood spot (DBS) samples in infants recruited into the Cambridge Baby Growth Study (CBGS). Our aim was to investigate the relationships between infancy feeding and age on specific lipids across multiple lipid classes.

Methods

The CBGS is a prospective observational birth cohort, focusing on the antenatal and early postnatal determinants of infancy growth. Mothers were recruited during early pregnancy from a single antenatal center in Cambridge between 2001 and 2009.[11] Their infants were seen at birth by trained research nurses and then followed-up at 3 and 12 months of age, with weight measurements.

CBGS	Cambridge Baby Growth Study
CE	Cholesterol ester
DBS	Dried blood spot
LC-PUFA	Long chain polyunsaturated fatty acid
PC	Phosphatidylcholine
PC-O	1-alkyl,2-acylglycerophosphocholine
PC-P	1-(alkenyl),2-acylglycerophosphocholin
PLS-DA	Partial least squares-discriminant analysis
SM	Sphingomyelin
TG	Triglyceride

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DBS samples were collected when parents consented, using capillary heel prick sampling, dropping blood spots onto untreated filter paper cards (Ahlstrom 226; ID Biological Systems, Greenville, South Carolina). Infancy feeding (exclusive breast-, mixed-, or exclusive formula-feeding) was assessed by questionnaire at age 3 months. All children were on a full mixed diet by 12 months of age. The study was approved by the Cambridge local research ethics committee, and all mothers gave informed written consent.

DBS samples on filter paper cards were air dried at ambient temperature overnight, before being stored in zip-loc storage bags at −20°C until analysis, when a single 3.2 mm spot was punched from the larger DBS samples. Individuals with sufficient DBS samples for multiple analyses were selected for this nested study.

We recently reported that large-scale lipidomic profiling platforms established for use on plasma samples[10] can be successfully adapted to DBS samples.[12] In brief, a 3.2 mm DBS was extracted in methanol in a well of a glass-coated 2.4 mL deep well plate (Plate+TM; Esslab, Hadleigh, United Kingdom), and lipids were partitioned into methyl tertiary butyl ether. After centrifugation, the organic layer was concentrated and used for lipid analysis. Samples were infused into a Thermo Exactive benchtop orbitrap (Hemel; Hampstead, United Kingdom), using an Advion Triversa Nanomate (Ithaca, New York) and data acquired in both positive (1.2 kV voltage) and negative modes (−1.5 kV). All experiments were run with blank controls and 2 different quality control samples. In total, 94 lipid species could be detected using this method.

Statistical Analyses

Lipidomic profiles were compared between infants exclusively breast-fed, mixed-fed, and formula-fed, between age 3 vs 12 months, and also related to infancy weight at age 12 months. The obtained lipid signals were semiquantitative, with the signal intensity of each lipid expressed relative to the total lipid signal intensity, for each individual, per thousand (‰). Raw high-resolution mass-spectrometry data were processed using XCMS (www.bioconductor.org) and Peakpicker v 2.0 (an in-house R script). All lipid species where >30% of cases had a value of zero were excluded from further analyses; consequently, 78 lipids were analyzed at 3 months, and 90 lipids were compared between 3- and 12-month samples. Multivariate analysis allowed analysis of multiple variables (all lipid species) together, to avoid loss of information and to identify underlying trends. These data were analyzed in SIMCA-P (v. 13; Umetrics AB, Umeå, Sweden). Data were normalized using log transformation and unit variance scaled. Principal component analysis was used first to observe overall patterns and detect outliers. Partial least squares-discriminant analysis (PLS-DA) plots were then constructed, using Q^2 values for cross validation. The PLS-DA algorithm was chosen as this maximizes separation between lipid variables for each categorical outcome (infancy feeding/age), enabling clear determination of variables contributing to any separation. Receiver operating characteristic curves were plotted to assess the predictive ability of obtained models (http://www.roccet.ca/).[13]

Univariate analysis, using Mann-Whitney U for exclusively breast-fed vs formula-fed infants, and Wilcoxon signed ranks tests for matched age 3 vs 12 month samples, was performed in SPSS v 19 (SPSS Inc, Chicago, Illinois). A stringent Bonferroni corrected P value (<.0006) was used to identify significant associations with individual lipids. This was calculated by dividing the significance threshold of .05 by the number of variables, in this case the lipids analyzed (78 at 3 months; 90 comparing 3 and 12 months). Spearman correlations were used for correlations between DBS lipids and infancy weight.

Results

Two hundred forty-one infants (110 male) had DBS lipidomic profiles measured at age 3 months (mean ± SD: 97 ± 9 days of age); 141 infants (77 male) at 12 months (373 ± 12 days), with 45 paired samples from the same infants at both time points. The cohort characteristics are summarized in Table I, and the sample was representative of the total CBGS (N = 1340 at 3 months), with mean ± SD birth weight 3.49 ± 0.55 kg, at 39.8 ± 1.6 weeks gestation, 3-month weight 6.15 ± 0.83 kg, and 12-month weight 9.97 ± 1.19 kg.

Infants transported the majority of long chain polyunsaturated fatty acids (LC-PUFAs) as phospholipids, rather than as cholesterol esters (CEs) or triglycerides (TGs). Overall in these infant samples, phosphatidylcholines (PCs) contributed 35% of the total lipid signal; TGs in contrast only contributed to approximately 10% of the total lipid signal. DBS samples had been stored for 2.1-9.5 years at −20°C. Duration of storage was significantly positively correlated with only 3 lipid species: lyso PC(18:0), PC(32:0), and PC(34:3); none were inversely correlated. There was no significant difference between the lipidomic profiles of male and female infants, at either 3 or 12 months of age.

Of the 241 infants at 3 months of age, 81 were exclusively breast-fed, 66 mixed-fed, and 84 formula-fed (4 infants

Table I. Demographics of infancy subgroups at 3 and 12 months of age

	All 3 months (N = 241), mean ± SD	3-month breast-fed (N = 81), mean ± SD	3-month formula-fed (N = 84), mean ± SD
Sex (% male)	46%	42%	46%
Gestational age (wk)	39.9 ± 1.5	40.2 ± 1.3	39.8 ± 1.5
Birth weight (kg)	3.50 ± 0.52	3.54 ± 0.43	3.50 ± 0.57
3 m weight (kg)	6.13 ± 0.80	5.96 ± 0.66	6.38 ± 0.76*
3 m weight SDS	−0.07 ± 1.05	−0.19 ± 0.88	0.25 ± 0.96*
	All 12 mo (N = 141), mean ± SD	**12 mo initially breast-fed (N = 38), mean ± SD**	**12 mo initially formula-fed (N = 50), mean ± SD**
Sex (% male)	55%	47%	56%
Gestational age (wk)	40.0 ± 1.4	40.1 ± 1.2	39.9 ± 1.4
Birth weight (kg)	3.47 ± 0.52	3.58 ± 0.43	3.43 ± 0.62
12 m weight (kg)	10.03 ± 1.31	9.72 ± 1.26	10.32 ± 1.41*
12 m weight SDS	0.07 ± 1.21	−0.19 ± 1.20	0.34 ± 1.24*

*P < .05 between breast-fed and formula-fed infants.