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Original article

# Distinct infant feeding type-specific plasma metabolites at age 3 months associate with body composition at 2 years

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SUMMARY

*Background & objectives:* Early life is a critical window for adiposity programming and metabolic profile may affect this programming. We investigated if plasma metabolites at age 3 months were associated with fat mass, fat free mass and abdominal subcutaneous and visceral fat outcomes at age 2 years in a cohort of healthy infants and if these associations were different between infants receiving exclusive breastfeeding (EBF) and those with exclusive formula feeding (EFF).

*Methods:* In 318 healthy term-born infants, we determined body composition by Dual Energy X-ray absorptiometry (DXA) and visceral fat by abdominal ultrasound at 2 age years. High-throughput metabolic profiling was performed on blood samples collected at age 3 months. Tertiles were generated for each body composition outcome and differences in plasma metabolite levels at age 3 months between infants with high and low body composition outcomes at age 2 years were evaluated in general, as well as separately in EBF- and EFF-infants.

*Results:* Distinct plasma metabolite variables identified at age 3 months were associated with body composition at 2 years. These metabolites included several classes of lyso-phospholipids. Associations between the metabolites at age 3 months and fat mass index, fat mass percentage, fat free mass index and visceral fat at 2 years were predominantly found in EBF-infants.

*Conclusion:* Associations between plasma metabolite levels at age 3 months and high body fat mass at 2 years depend on infant feeding type. These findings contribute to our insight into the importance of infant feeding on adiposity programming in early life.



## 1. Introduction

Childhood obesity is a global public health threat [1]. Excess adiposity at young age has not only short-term, but particularly long-term morbidity, such as insulin resistance, type 2 diabetes mellitus and cardiovascular disease [1,2]. The first months of life are a critical window for adiposity programming, affecting adult metabolic health and body composition [3]. Multifactorial risk factors influence this course, but the exact mechanisms have not yet been elucidated. Our research group found that especially rapid weight gain in the first 6 months of life is an important risk factor for a higher fat mass and an unfavorable metabolic profile in young adults [3]. In addition, infants with a rapid rise in fat mass in the first 6 months of life have higher fat mass percentage trajectories up until at least age 2 years [4]. Recently, we have added that fat mass and fat free mass track already from age 3 months to at least age 4 years [5]. Infant feeding is also reported to influence body composition during the first 6 months of life. Infants with exclusive

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breastfeeding have different growth trajectories with more subcutaneous fat accumulation, different serum concentrations of appetite regulating hormones and less tracking of fat mass percentage compared with formula fed infants [5–7]. It remains unclear how dietary exposure in the first months of life affects later life body composition and we seem to be lacking the tools to study this. Unravelling the risk factors involved in adiposity programming will increase the ability to identify infants at risk of excess adiposity at an early age and may thus provide the opportunity to develop more targeted preventative strategies.

Excessive body and visceral fat in children and adults are associated with adverse plasma lipid profiles [8,9]. Especially branched chain amino acids (BCAAs) concentrations were reported to be higher in children with obesity [10]. In infants, the metabolic profile is also associated with growth patterns [11,12]. At age 3 months, plasma levels of phosphatidylcholine (PC) (20:4/18:0), PC plasmalogen (36:4) and sphingomyelin (d18:1/16:0) have been associated with poor weight gain, while PC (18:1/16:0) and PC plasmalogen (34:1) were associated with accelerated weight gain [11,12]. Recently, we have reported that LysoPC(22:2), dimethylarginine and several other plasma metabolites at age 3 months modestly predict the truncal-to-peripheral skinfold ratio (T:P-ratio) at age 2 years [13]. The metabolic profile in the first months of life could, therefore, potentially be involved in adiposity programming. However, the association between early life plasma metabolites and measured body composition including visceral fat in later in life has never been described.

The metabolic profile in early life is strongly influenced by infant feeding, since differences in metabolic profiles have been described between children with exclusive breastfeeding and exclusive formula feeding [11–14]. In breastfed infants, total PC levels in plasma are higher and linoleic acid is less incorporated than palmitate into the phospholipid fraction as compared to that of formula fed infants [12,15]. However, after the cessation of breastfeeding and the introduction of complementary food, these differences in metabolic profile dissolve [12].

The metabolic profile in early life, whether or not influenced by infant feeding type, could have consequences for adiposity programming. The primary objective of the current study was to investigate if plasma metabolites at age 3 months are associated with fat mass, fat free mass, abdominal subcutaneous fat (ASF) and visceral fat outcomes at age 2 years in a cohort of healthy infants. Secondly, we investigated if these associations were different between infants with exclusive breastfeeding and those with exclusive formula feeding at age 3 months. We hypothesized that specific metabolites in early life are associated with body composition at age 2 years and that these associations depend on infant feeding type.

## 2. Methods

### 2.1. Subjects

The study population consisted of 318 healthy infants participating in the Sophia Pluto study, a birth cohort aiming to provide detailed data on early growth- and body composition trajectories in infancy and childhood. Infants were recruited from several maternity wards in Rotterdam, The Netherlands. All participants met the following inclusion criteria: born term (≥37 weeks of gestation) and singleton. Exclusion criteria were severe asphyxia (defined as an Apgar-score below 3 after 5 minutes), sepsis or the need for respiratory ventilation or any maternal disease or medication use that could interfere with fetal growth, including maternal corticosteroids, diabetes mellitus or intrauterine infection, and known congenital or postnatal disease that could interfere with infant growth. The Medical Ethics Committee of Erasmus Medical Centre approved the study. We obtained written informed consent of all parents or caregivers with parental authority.

### 2.2. Data collection and measurements

Outpatient clinic visits were scheduled at age 3 months and 2 years. The same trained staff carried out the measurements according to local standard procedures. Birth data were taken from hospital and midwife records. Parental characteristics and feeding type were obtained by interviews at the clinic visits and by questionnaires. Exclusive breast feeding (EBF) was defined as receiving only mother's milk at the 3 months study visit. Exclusive formula feeding (EFF) was defined as receiving only infant formula at the 3 months study visit. Mixed feeding (mix) was defined as receiving both mother's milk and infant formula at the 3 months study visit.

### 2.3. Anthropometrics

Weight was measured to the nearest 5 g by an electronic infant scale (Seca 717, Hamburg, Germany). Length was measured in supine position to the nearest 0.1 cm by an infantometer (Seca 416). SD-scores of weight, length and weight-for-length were calculated using Dutch references [16] by Growth Analyser software (www.growthanalyser.org).

### 2.4. Body composition

Body composition was measured with air-displacement plethysmography (ADP) by Peapod (Infant Body Composition System (COSMED)) during the visits at age 3 months, as described in detail elsewhere [17]. ADP was conducted using the same machine. It was used and calibrated daily according to the user's manual [18]. ADP was validated earlier against a reference 4-compartment model and reliability was determined with a CV of 7.9% for fat mass percentage (FM%) [19].

At age 2 years, body composition was measured by DXA scan (GE Prodigy Advance R000279 and encore v14.1 software). Children wore only underwear and were swaddled in a cotton blanket. A vacuum cushion (465 75,100, Schmidt, Germany) was used to avoid movement artifacts, which has similar results at age 6 months compared to ADP [20]. During the study, the same DXA machine was used and calibrated daily, according to the protocol recommended by the supplier. CV for FM% was 3.2% [21]. Fat mass index (FMI) was calculated as fat mass divided by length in meters squared (kg/m$^2$) and fat free mass index (FFMI) as fat free mass divided by length in meters squared (kg/m$^2$).

### 2.5. Abdominal fat

Visceral and subcutaneous fat depth were determined at age 3 months and 2 years, using ultrasound (Prosound 2 ultrasound with a UST-9137 convex transducer (both Hitachi Aloka Medical, Zug, Switzerland). Assessments were made in supine position, with the transducer on the intercept of the xiphoid line and the waist circumference measurement plane. Visceral fat was measured in the longitudinal plane from the peritoneal boundary to the corpus of the lumbar vertebra with a probe depth of 9 cm and subcutaneous fat in the transverse plane from the cutaneous boundary to the linea alba with a probe depth of 4 cm. Minimal pressure was applied. Validity and reproducibility of measurements were confirmed in the Cambridge Baby Growth Study (CBGS), the relative interobserver technical error of measurement was 3.2% for visceral depth and 3.6% for subcutaneous depth [22]. If the

vertebrae were not visualized, measurement was considered unsuccessful and were excluded from analyses.

### 2.6. Sample collection

Blood samples were collected at age 3 months and 2 years by capillary toe or finger prick sampling after the infants had fasted for ≥2 hours. Blood was collected in heparin microtubes (BD Microtainer®) and centrifuged to prepare plasma. The samples were stored at −80 °C until transportation on dry ice to the University of Cambridge (United Kingdom (UK)) for metabolomics analysis.

### 2.7. Metabolic profiling

Metabolic profiling was performed with the high throughput platform at the Metabolic Research Laboratories of the Institute of Metabolic Science, University of Cambridge, UK. The samples were analysed using a liquid chromatography mass spectrometry method [23] ultimately yielding results of the absolute and relative concentration of 349 individual metabolites and lipids. The protein precipitation liquid extraction protocol was performed as described previously [23]. Briefly, 50 μL of plasma was transferred into a 2 mL screw cap Eppendorf plastic tube (Eppendorf, Stevenage, UK). Immediately, 650 μL of chloroform was added to each sample, followed by thorough mixing. Then, 100 μL of the internal standards (5 μM in methanol), 100 μL of the carnitine internal standards (5 μM in methanol) and 150 μL of methanol was added to each sample, followed by thorough mixing, after which 400 μL of acetone was added to each sample. The samples were vortexed and centrifuged for 10 minutes at ~20,000*g* to pellet any insoluble material. The supernatant was pipetted into separate 2 mL screw cap amber-glass auto-sampler vials (Agilent Technologies, Cheadle, UK). The organic extracts were evaporated to dryness using a Concentrator Plus system (Eppendorf, Stevenage, UK) run for 60 minutes at 60 °C. The samples were reconstituted in 100 μL of a 2: 1: 1 mixture of propan-2-ol, acetonitrile and water, and then thoroughly vortexed. The reconstituted sample was transferred into a 250 μL low-volume vial insert inside a 2 mL amber glass auto-sample vial ready for liquid chromatography with mass spectrometry detection (LC-MS) analysis.

Chromatographic separation was achieved using Shimadzu HPLC System (Shimadzu UK Limited, Milton Keynes, UK) with the injection of 10 μL onto a Waters Acquity UPLC® CSH C18 column (Waters, Hertfordshire, UK); 1.7 μm, I.D. 2.1 mm × 50 mm, maintained at 55 °C. Mobile phase A was 6:4 acetonitrile and a 10 mM ammonium formate solution in water. Mobile phase B was 9:1 propan-2-ol and a 10 mM ammonium formate solution in acetonitrile. The flow was maintained at 500 μL per minute through the following gradient: 0.00 minutes_40% mobile phase B; 0.40 minutes_43% mobile phase B; 0.45 minutes_50% mobile phase B; 2.40 minutes_54% mobile phase B; 2.45 minutes_70% mobile phase B; 7.00 minutes_99% mobile phase B; 8.00 minutes_99% mobile phase B; 8.3 minutes_40% mobile phase B; 10 minutes_40% mobile phase B. The sample injection needle was washed using 9:1, propan-2-ol and acetonitrile. The mass spectrometer used was the Thermo Scientific Exactive Orbitrap with a heated electrospray ionisation source (Thermo Fisher Scientific, Hemel Hempstead, UK). The mass spectrometer was calibrated immediately before sample analysis using positive and negative ionisation calibration solution (recommended by instrument manufacturer). Additionally, the mass spectrometer scan rate was set at 4 Hz, giving a resolution of 25,000 (at 200 $m/z$) with a full-scan range of $m/z$ 100 to 1800 with continuous switching between positive and negative mode.

### 2.8. Data processing

All.RAW files were converted to.mzXML files using msConvert (ProteoWizard) [24]. Converted files were subsequently processed in R (v3.3.1) using the CAMERA package [25] with peak picking performed using the centWave method as this enables for the deconvolution of closely eluting and slightly overlapping peaks. Metabolite variables included within the final dataset were defined as peaks that had an intensity at least 3 times higher in analytical samples relative to the extraction blanks and that was present in at least 90% of the analyzed samples. For each sample all signals are expressed relative to the total signal strength. If possible, metabolite variables were putatively annotated by matching measured accurate masses to entities in the Human Metabolome database (www.hmdb.ca) and the metabolomics workbench (www.metabolomicsworkbench.org). Resulting identities were cross-checked manually based on retention time and isotopic pattern and additional ion adducts (see Supplementary Information Table 1).

### 2.9. Statistical analysis

Because body composition outcomes have sex-specific differences from an early age onwards, tertiles for FM%, FMI, FFMI, ASF and visceral fat were generated for boys and girls separately. These were subsequently merged into group tertiles for 'high', 'moderate' and 'low' (Supplementary Table 2) [5]. Comparison was done using Mann–Whitney U-test for non-parametric parameters, as most metabolite signals were not normally distributed based on manual checks of histograms. Differences between metabolic biomarkers, in 'high' versus 'low' tertiles, were calculated using non-parametric Wilcoxon rank-sum tests, for the total group (EBF, EFF and mix fed infants combined) and for EBF- and EFF-infants separately. To correct the false discovery rate, we used a lenient p-value cut-off $p < 0.005$, based on half the square root of the signals and the high degree of co-dependency between the signals as described previously [11].

## 3. Results

Clinical characteristics are presented in Table 1. Of the total study population, 55.0% was male, 71.8% was Caucasian. One hundred twenty one infants were exclusively breastfed (EBF) and 130 exclusively formula fed (EFF) at age 3 months. FMI and abdominal subcutaneous fat (ASF) were highest in EBF-infants at age 3 months. At age 2 years, there were no differences in clinical characteristics between EBF and EFF.

### 3.1. Plasma metabolites at age 3 months and body composition at 2 years

At age 3 months, two plasma metabolites were associated with FMI and FM% at age 2 years (Table 2). At age 3 months, dimethylarginine plasma level was higher in EBF-infants with high FMI at age 2 years compared with those with low FMI. Oxidised triglyceride (TG 55:10; O) levels at age 3 months were higher in the total group and associated with high FMI at age 2 years. The levels were also elevated at age 3 months in EBF infants and associated with high FM% at 2 years. For EFF-infants, these metabolites at 3 months did not show a relationship with FMI or FM% at 2 years.

Three other metabolites at age 3 months were associated with FFMI at age 2 years. LPE 18:2 levels at age 3 months were higher in EBF-infants with high FFMI at age 2 years, compared to EBF-infants with low FFMI. Two, not further identified, phospholipids species showed higher levels at age 3 months in the total group with high

**Table 1**
Clinical characteristics.

| | Exclusive breast feeding at age 3 months | Exclusive formula feeding at age 3 months | p-value |
|---|---|---|---|
| N | 121 | 130 | |
| Boys (%) | 62 (51.2%) | 76 (58.5%) | 0.250 |
| Gestational age (weeks) | 39.91 (1.15) | 39.45 (1.22) | **0.002** |
| Ethnicity | | | 0.054 |
| Caucasian | 76 (62.8%) | 95 (73.1%) | |
| Black | 8 (6.6%) | 9 (6.9%) | |
| Asian | 1 (0.8%) | 1 (0.8%) | |
| Latin-American | 1 (0.8%) | 0 | |
| Mix | 32 (26.4%) | 15 (11.5%) | |
| Missing | 3 (2.5%) | 10 (7.7%) | |
| Birth weight SDS | 0.29 (1.12) | 0.36 (1.16) | 0.630 |
| Birth length SDS[a] | 0.89 (1.03) | 0.40 (1.34) | **0.020** |
| Age 3 months | | | |
| Weight SDS | 0.58 (1.14) | 0.46 (1.06) | 0.371 |
| Length SDS | 0.43 (0.80) | 0.34 (0.90) | 0.390 |
| FM% | 23.46 (4.57) | 22.05 (4.84) | **0.021** |
| FMI (kg/m$^2$) | 3.79 (0.99) | 3.54 (10.96) | 0.051 |
| FFMI (kg/m$^2$) | 12.20 (0.87) | 12.37 (0.94) | 0.153 |
| ASF (cm) | 0.43 (0.12) | 0.39 (0.11) | **0.005** |
| Visceral fat (cm) | 2.29 (0.56) | 2.41 (0.60) | 0.121 |
| Age 2 years | | | |
| Weight SDS | −0.26 (1.06) | −0.22 (1.03) | 0.746 |
| Length SDS | 0.20 (0.97) | 0.30 (0.96) | 0.393 |
| FM% | 17.56 (3.75) | 18.02 (3.72) | 0.391 |
| FMI (kg/m$^2$) | 2.82 (0.77) | 2.89 (0.75) | 0.550 |
| FFMI (kg/m$^2$) | 13.09 (0.88) | 13.00 (0.85) | 0.439 |
| ASF (cm) | 0.35 (0.09) | 0.33 (0.10) | 0.292 |
| Visceral fat (cm) | 2.15 (0.58) | 2.22 (0.60) | 0.361 |

Data expressed as mean (SD) or N(%). Significant p-values are boldfaced.
Abbreviations: ASF = abdominal subcutaneous fat, FM% = fat mass percentage, FMI =Fat Mass Index, FFMI = Fat Free Mass Index, SDS = Standard Deviation Score.
[a] Birth length available in 75 infants with exclusive breastfeeding, 64 infants with exclusive formula feeding.

FFMI at age 2 years compared to those with low FFMI. Again, none of these 3 metabolites showed a relationship with FFMI at 2 years in EFF-infants.

### 3.2. Plasma metabolites at age 3 months and abdominal fat at 2 years

Three metabolites at age 3 months associated with abdominal subcutaneous fat (ASF) at age 2 years (Table 3). Oxycholesterol levels at age 3 months were lower in infants with high ASF at age 2 years in all infants. PC 42:8 levels were higher at age 3 months in all and in EBF-infants with high ASF at age 2 years, whereas PC 38:3 levels at age 3 months were only higher in EFF-infants with high ASF at age 2 years.

Twelve other metabolites at age 3 months were associated with visceral fat at 2 years (Table 3). These were mainly lysophosopholipids (LysoPC 14:0, LysoPC 16:3, LysoPC 16:1, LysoPC 16:0, LysoPS 21:1, LysoPS 25:6 and LysoPA 23:1), as well as dimethylarginine, diacylglycerol (DG 40:10) and sphingomyelin (SM 35:2; O2). All plasma levels of LysoPCs and the DG were higher at age 3 months in infants with high visceral fat at age 2 years, while all three LysoPSs, LysoPA, SM and dimethylarginine levels were higher at age 3 months in infants with low visceral fat at 2 years. These results were mostly driven by EBF-infants. Only for LysoPA 23:1, the difference between the tertiles was more prominent in EFF-infants.

## 4. Discussion

Our data show that several plasma metabolites at age 3 months are associated with progression towards a high FMI, FM%, FFMI and visceral fat at age 2 years. The associated metabolites are mainly different classes of lyso-phospholipids. Most of the associations were strongly dependent of infant feeding type. We found most

**Table 2**
Relative metabolite levels at age 3 months associated with body composition at age 2 years.

| Metabolite | Feeding type | Relative plasma metabolite levels at age 3 months (mol ratio; mean ± SD) | | p-value |
|---|---|---|---|---|
| **FMI (kg/m$^2$) at age 2 years** | | **Low tertile** | **High tertile** | |
| **Dimethylarginine** | All | 2.92E-05 (±3.27E-05) | 3.83E-05 (±4.22E-05) | 0.1331 |
| | **EBF** | 1.89E-05 (±2.04E-05) | 5.43E-05 (±4.91E-05) | **0.0007** |
| | EFF | 4.5E-05 (±4.04E-05) | 2.85E-05 (±3.26E-05) | 0.0950 |
| **TG 55:10; O (M + Na$^+$)** | **All** | 4.30E-05 (±1.11E-05) | 4.78E-05 (±0.87E-05) | **0.0030** |
| | EBF | 4.24E-05 (±1.09E-05) | 4.82E-05 (±0.85E-05) | 0.0207 |
| | EFF | 4.26E-05 (±1.09E-05) | 4.76E-05 (±0.84E-05) | 0.0571 |
| **FM% at age 2 years** | | **Low tertile** | **High tertile** | |
| **TG 55:10; O (M + Na$^+$)** | **All** | 4.24E-05 (±1.13E-05) | 4.78E-05 (±0.80E-05) | **0.0007** |
| | **EBF** | 4.17E-05 (±1.10E-05) | 4.88E-05 (±0.68E-05) | **0.0030** |
| | EFF | 4.22E-05 (±1.09E-05) | 4.73E-05 (±0.82E-05) | 0.0476 |
| **FFMI (kg/m$^2$) at age 2 years** | | **Low tertile** | **High tertile** | |
| **LysoPE 18:2 (M+1)** | All | 0.000230 (±0.00012) | 0.000280 (±0.00016) | 0.0275 |
| | **EBF** | 0.000199 (±0.00006) | 0.000282 (±0.00013) | **0.0010** |
| | EFF | 0.000260 (±0.00016) | 0.000260 (±0.00017) | 0.9918 |
| **$C_{48}H_{94}N_2O_8P$** | **All** | 0.001587 (±0.00051) | 0.001842 (±0.00036) | **0.0004** |
| | **EBF** | 0.001606 (±0.00062) | 0.002005 (±0.00030) | **0.0025** |
| | EFF | 0.001556 (±0.00044) | 0.001712 (±0.00035) | 0.1218 |
| **$C_{48}H_{96}N_2O_8P$** | **All** | 0.000776 (±0.00029) | 0.000912 (±0.00025) | **0.0020** |
| | EBF | 0.000834 (±0.00023) | 0.000964 (±0.00025) | 0.0329 |
| | EFF | 0.000779 (±0.00029) | 0.000859 (±0.00024) | 0.2343 |

Metabolite variables (protonated ions (M + H$^+$) or as given, further details in suppl info table 1) associated with FMI, FM% and FFMI. Metabolite levels are provided as molar ratio. Normalised to total signal strength data for each metabolite signal. Tertiles were merged from sex-specific tertiles (further details in suppl info table 2). p-value is difference in plasma level between high and low tertile. Significant p-value <0.005 (corrected for FDR) are boldfaced. Abbreviations: EBF: exclusive breastfed infants, EFF: exclusive formula fed infants, FFMI = fat free mass index, FMI = fat mass index, FM% = Fat mass percentage, LysoPE = lysophosphatidylethanolamine, TG.O = oxidised triglyceride.

**Table 3**
Relative metabolite levels at age 3 months associated with abdominal subcutaneous and visceral fat at 2 years.

| Metabolite | Feeding type (n) | Relative plasma metabolite levels at age 3 months (mol ratio; mean ± SD) | | p-value |
|---|---|---|---|---|
| **Subcutaneous fat at age 2 years** | | **Low tertile** | **High tertile** | |
| **Oxid Chol (M-2$H_2O$)** | **All** | 3.17E-06 (±1.59E-06) | 2.55E-06 (±1.51E-06) | **0.0032** |
| | EBF | 2.63E-06 (±1.31E-06) | 2.57E-06 (±1.64E-06) | 0.8617 |
| | EFF | 3.19E-06 (±1.63E-06) | 2.45E-06 (±1.42E-06) | 0.0344 |
| **PC 42:8** | **All** | 0.000734 (±0.00030) | 0.000892 (±0.00028) | **0.0002** |
| | **EBF** | 0.000687 (±0.00037) | 0.000951 (±0.00023) | **0.0013** |
| | EFF | 0.000762 (±0.00023) | 0.000823 (±0.00031) | 0.3177 |
| **PC 38:3** | **All** | 0.012896 (±0.00355) | 0.014161 (±0.00318) | 0.0104 |
| | EBF | 0.014895 (±0.00423) | 0.015504 (±0.00356) | 0.5220 |
| | **EFF** | 0.011468 (±0.00218) | 0.012929 (±0.00150) | **0.0009** |
| **Visceral fat at age 2 years** | | **Low tertile** | **High tertile** | |
| **Dimethylarginine** | **All** | 4.79E-05 (±4.84E-05) | 2.93E-05 (±3.45E-05) | **0.0025** |
| | EBF | 5.16E-05 (±4.58E-05) | 2.66E-05 (±3.28E-05) | 0.0122 |
| | EFF | 5.06E-05 (±5.20E-05) | 2.98E-05 (±3.53E-05) | 0.0498 |
| **LysoPC 14:0 (M+1)** | **All** | 2.06E-05 (±0.94E-05) | 2.62E-05 (±1.25E-05) | **0.0005** |
| | EBF | 2.0E-05 (±0.99E-05) | 2.49E-05 (±1.19E-05) | 0.0760 |
| | EFF | 2.1E-05 (±0.91E-05) | 2.57E-05 (±1.32E-05) | 0.0691 |
| **LysoPC 16:3** | **All** | 5.02E-05 (±2.04E-05) | 6.15E-05 (±2.24E-05) | **0.0003** |
| | EBF | 4.67E-05 (±1.62E-05) | 5.86E-05 (±2.16E-05) | 0.0150 |
| | EFF | 5.43E-05 (±2.28E-05) | 5.94E-05 (±2.17E-05) | 0.3270 |
| **LysoPC 16:1** | **All** | 0.000106 (±5.01E-05) | 0.000125 (±4.14E-05) | **0.0046** |
| | **EBF** | 0.000109 (±4.08E-05) | 0.000139 (±3.72E-05) | **0.0020** |
| | EFF | 8.99E-05 (±3.87E-05) | 0.000104 (±3.35E-05) | 0.0971 |
| **LysoPC 16:0** | **All** | 0.006089 (±0.0016) | 0.006783 (±0.0014) | **0.0020** |
| | EBF | 0.005690 (±0.0014) | 0.006414 (±0.0015) | 0.0459 |
| | EFF | 0.006257 (±0.0017) | 0.007016 (±0.0014) | 0.0430 |
| **LysoPC 16:0 (M+1)** | **All** | 0.001566 (±0.00064) | 0.001831 (±0.00049) | **0.0016** |
| | EBF | 0.001434 (±0.00055) | 0.001608 (±0.00062) | 0.2352 |
| | EFF | 0.001688 (±0.00064) | 0.001986 (±0.00037) | 0.0181 |
| **LysoPA 23:1** | **All** | 0.000155 (±0.00016) | 9.10E-05 (±0.00012) | **0.0069** |
| | EBF | 0.000179 (±0.00016) | 8.25E-05 (±0.00012) | 0.0665 |
| | **EFF** | 0.000159 (±0.00016) | 9.6E-05 (±0.00012) | **0.0016** |
| **LysoPS 21:1 (M+1)** | **All** | 0.000375 (±0.00018) | 0.000283 (±0.00015) | **0.0002** |
| | **EBF** | 0.000408 (±0.00016) | 0.000278 (±0.00013) | **0.0006** |
| | EFF | 0.000371 (±0.00019) | 0.000301 (±0.00017) | 0.0900 |
| **LysoPS 22:2** | **All** | 1.78E-05 (±1.17E-05) | 1.35E-05 (±0.88E-05) | **0.0044** |
| | EBF | 1.94E-05 (±1.20E-05) | 1.26E-05 (±0.85E-05) | 0.0096 |
| | EFF | 1.75E-05 (±1.15E-05) | 1.36E-05 (±0.85E-05) | 0.1034 |
| **LysoPS 25:6** | **All** | 0.000164 (±8.41E-05) | 0.000124 (±7.04E-05) | **0.0005** |
| | **EBF** | 0.000169 (±8.48E-05) | 0.000112 (±6.24E-05) | **0.0023** |
| | EFF | 0.000169 (±8.86E-05) | 0.000138 (±7.52E-05) | 0.1088 |
| **DG 40:10 (M + $NH_4^+$)** | **All** | 0.000286 (±0.00017) | 0.000359 (±0.00018) | **0.0033** |
| | EBF | 0.000244 (±0.00011) | 0.000286 (±0.00012) | 0.1490 |
| | EFF | 0.000374 (±0.00023) | 0.000401 (±0.00022) | 0.6005 |
| **SM 35:2; $O_2$** | **All** | 0.000956 (±0.00018) | 0.000881 (±0.00018) | **0.0040** |
| | EBF | 0.001029 (±0.00013) | 0.000955 (±0.00016) | 0.0432 |
| | EFF | 0.000909 (±0.00019) | 0.000815 (±0.00016) | 0.0232 |

Metabolite variables (protonated ions (M + $H^+$) or as given, and further details in suppl info table 1) associated with FMI, FM% and FFMI. Metabolite levels are provided as molar ratio. Normalised to total signal strength data for each metabolite signals. Tertiles were merged from sex-specific tertiles (further details in suppl info table 2). p-value is difference in plasma level between high and low tertile. Significant p-value <0.005 (corrected for FDR) are boldfaced. Abbreviations: DG = diacylglycerol, EBF: exclusive breastfed infants, EFF: exclusive formula fed infants, LysoPA = lysophosphosphatidic acid, LysoPC = lysophosphatidylcholine, LysoPS = lysophosphatidylserine, Oxid Chol = Oxycholesterol, PC = phosphatidylcholine, SM = sphingomyelin.

differences in plasma metabolite levels at age 3 months, between low and high tertile body fat mass infants at age 2 years to be apparent in EBF-infants, while only a few associations were seen for EFF-infants.

Lyso-phospholipids are phospholipids with only one fatty acid attached to the glycerol backbone and are mostly the result of the hydrolysis of phospholipids by lipases [26] and through the autotaxin-LysoPA-lipid phosphate phosphatase 3 axis [27]. Several of the identified metabolites (dimethylarginine, LysoPA, LysoPS, LysoPE and LysoPG) are known to be associated with pro-inflammatory pathways in preadipocytes [28,29] and with inflammatory processes [30–32], mainly via interaction with Toll-like receptor dimers [33] and peroxisome proliferator-activated receptor gamma (PPAR-γ) [34], and/or the development of cardiovascular disease via G protein-coupled receptors (GPCRs) [35]. Our findings could, therefore, suggest that the identified metabolites at age 3 months are involved in adiposity development and systemic low-grade inflammatory processes, seemingly starting from early age onwards.

Our findings also show that the association between metabolite profile at age 3 months and body composition at age 2 years is dependent of infant feeding type. From our previous work, we know that feeding type and milk composition has a major impact on metabolic profiles of infants [11–14]. We now add that these differences in metabolic profile in early life, can potentially be involved in differences in adiposity programming between EBF- and EFF-infants.

Differences between plasma metabolite levels at age 3 months and 'high' or 'low' FM%, FMI, FFMI and visceral fat at 2 years were predominantly seen in EBF-infants. In EBF-infants, early life plasma

metabolites which associated with later body composition were predominately LysoPC, LysoPE and sphingomyelin (SM). These polyunsaturated phospholipids were previously reported to be related to weight-to-age and weight-to-length trajectories until age 2 years [36]. Also, LysoPC and SM plasma levels are reported to be different between infants with EBF and EFF [37,38]. This could potentially be linked to the milk fat globule membrane (MFGM), which coats fat droplets in human milk, but is absent in formula feeding in The Netherlands [37,38]. This phospholipid layer consists mostly of PC and PE in combination with sphingolipids, predominantly in SM form [38]. The MFGM is associated with neurodevelopmental outcomes and microbiome development [38]. Our findings suggest that the MFGM could potentially also be involved in adiposity programming.

In EFF-infants, LysoPA was associated with visceral fat outcomes. Like LysoPC and LysoPE, this metabolite is a lyso-phospholipid, but from a different class. Lyso-phospholipids levels are a marker for phospholipid turnover and the product of lipases [39]. While LysoPA in EFF-infants is generally not a major phospholipid in the circulation, it is involved in biosynthesis of the more abundant classes of phospholipids like the PC lipids [40]. Together, these findings suggest that phospholipids are important in body composition development and fat distribution and that the supply and incorporation of phospholipids is different between EBF- and EFF-infants.

With the rapid increase in childhood obesity, it is imperative that we understand what factors predispose infants to unfavourable body composition, so that we can develop approaches to identify infants at risk for excess adiposity and adequate preventive tools. Our work shows that already at age 3 months, metabolic processes occur that could be involved in differences in future body composition. Moreover, it shows that caution is needed to combine the results for EBF-infants with those from EFF-infants. Our findings could contribute to more targeted and individualized preventive strategies, as well as an opportunity for early treatment options by improving infants diet, either directly for formula, or indirectly through the mother for breastmilk. Further work in this area is, therefore, crucial.

Our work has several strengths and weaknesses. It is important to note the exceptional quality of the body composition data that was crucial to show how both breast- and formula feeding have very different long-term effects on body composition, through the infant's metabolism. Another strength was the use of tertiles which were balanced for sex, which helped to assess the relationship between plasma metabolite variables and body composition outcomes. However, from these observational data it is difficult to infer causality. The differences between the plasma metabolites precede the body composition differences, but can be either the cause or the result of mechanisms and processes in metabolism that influence later body composition. The impact of feeding type, although expected, was much clearer than anticipated. As none of the metabolite associations with body composition outcomes were independent from feeding type, analysis of the whole cohort diluted any signal, whereas testing of the EBF and EFF sub-groups reduced the statistical power. Another limitation was its scoping nature in relation to the specific metabolites involved. However, there was no clear evidence in the literature to enable us to focus the metabolite profiling. Our findings show that especially lyso-phospholipid analysis is important, hence future projects can use more targeted and quantitative methods to externally validate these findings in other, more diverse study populations. In addition, more research is needed to further understand these relationships and study how these relationships hold in infants that receive both breast- and formula (mixed) feeding.

In conclusion, distinct plasma metabolites at age 3 months are associated with body composition at 2 years, measured as FMI, FM %, FFMI and abdominal fat distribution. Associations between plasma levels at age 3 months and high or low body composition outcomes were infant feeding type specific and were predominantly found in EBF-infants. These findings contribute key insights into the importance of the type of infant feeding on adiposity programming in early life.

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## Author contributions

AK, AHK, SB and KO were in charge of designing the study. IvB and AHK were in charge of the cohort, design, and collecting of the data and samples. AK performed the metabolomics and bioinformatic analysis. Drafting the manuscript was primarily done by IvB under supervision of AHK and AK. All authors were involved in writing the manuscript and had final approval of the submitted version.

## Conflict of interest

None of the authors declare conflict of interest.

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*Sophia Pluto.*

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.04.015.

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