Serum, plasma and erythrocyte membrane lipidomes in infants fed formula supplemented with bovine milk fat globule membranes.

Running title Infant blood lipidomes

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Conflict of interest Olle Hernell and Bo Lönnérdal are members of Hero and Semper scientific advisory boards. Tove Grip, Thomas S Dyrlund, Linda Ahonen, Magnus Domellöf, Tuulia Hyötyläinen, Mikael Knip, Matej Orešič and Niklas Timby declare no conflicts of interest.

Category of study Randomized clinical trial
Abstract

**Background** Supplementation of formula with bovine milk fat globule membranes has been shown to narrow the gap in immunological and cognitive development between breastfed and formula-fed infants.

**Method** In a double-blinded randomized controlled trial 160 formula-fed infants received an experimental formula (EF), supplemented with bovine milk fat globule membranes, or standard formula until 6 months of age. A breast-fed reference group was recruited. Lipidomic analyses were performed on plasma and erythrocyte membranes at 6 months and on serum at 4 and 12 months of age.

**Results** At 6 months of age, we observed a significant separation in the plasma lipidome between the two formula groups, mostly due to differences in concentrations of sphingomyelins (SM), phosphatidylcholines (PC) and ceramides, and in the erythrocyte membrane lipidome, mostly due to SMs, phosphatidylethanolamines and PCs. Already at 4 months, a separation in the serum lipidome was evident where SMs and PCs contributed. The separation was not detected at 12 months.

**Conclusion** The effect of MFGM supplementation on the lipidome is likely part of the mechanisms behind the positive cognitive and immunological effects of feeding the EF previously reported in the same study population.
Introduction

Human milk is the optimal nutrition during early infancy, and WHO, UNICEF and the ESPGHAN Committee on Nutrition all recommend exclusive breastfeeding until 6 month of age (1). If human milk is not available, in developed countries infant formula is the safe alternative even if observational studies have revealed small health disadvantages in formula-fed compared to breastfed infants. Even after adjustment for socio-economic factors, breast-fed infants have better cognitive development (2, 3), and a lower incidence of infections (4) compared with formula-fed suggesting that components of human milk, lacking or present at lower concentrations in infant formula, are needed for optimal development of the brain and the immune system. Some of the likely components are present in the milk fat globule membrane (MFGM). Sphingomyelin, gangliosides, sialic acid, cholesterol and choline are all present in higher/altered concentrations in human milk compared to infant formulas, are components of the MFGM, and have been associated with improved brain function in humans and in animal models (5-15). Further, several of the close to 200 proteins of the MFGM (16), e.g. lactoferrin (17), mucins and butyrophilin (18), and components of the lipid fraction (19) have immunological and/or antimicrobial functions. The MFGM fraction has historically been discarded during formula manufacturing when milk fat has been replaced by blends of vegetable oils. Recently different MFGM concentrates from bovine milk have become available and possible to add to infant formulas.

We recently performed a double-blinded randomized controlled trial exploring the effects of feeding infants an experimental formula (EF) supplemented with a bovine MFGM concentrate from <2 to 6 months of age. Infants fed the EF performed better on cognitive testing at 12 months
of age (20) and had lower incidence of otitis media during the intervention (21) compared to infants fed a standard formula (SF).

The aim of the present study was to investigate the lipidome in serum/plasma and erythrocyte membranes of infants fed EF compared to infants fed SF and a breast-fed reference (BFR) group. Our hypothesis was that MFGM supplementation would affect the lipidome in serum/plasma and cell membranes, and thus be a likely contribution to the mechanisms mediating the positive effects of feeding EF on cognitive development and defense against infections.

**Methods**

*Sample collection and formulas*

As described previously (20), 160 formula-fed infants, 80 boys and 80 girls were randomized to receive either the EF or SF from inclusion until 6 months of age. Eighty breast-fed infants, 40 boys and 40 girls, were recruited as a reference group. Inclusion criteria were age <2 months, gestational age 37-42 weeks, birth weight 2500-4500 g, and no chronic illness. Infants were either exclusively formula-fed or exclusively breast-fed at inclusion. BabySemp (Semper AB, Sundbyberg, Sweden) was used as SF and the EF was modified from this by lowering the energy density from 66 to 60 kcal/100 mL, the protein concentration from 1.27 to 1.20 g/100 mL and by supplementation with a bovine MFGM-fraction (Lacprodan MFGM-10; Arla Foods ingredients, Viby, Denmark), contributing 4% (wt/wt) MFGM protein of the total protein content. In both formulas long-chain polyunsaturated fatty acids were added to the same concentration (arachidonic acid (20:4n-6, ARA) 15 mg/100 ml and docosahexaenoic acid (22:6n-3, DHA) 9 mg/100 ml). Infants in the EF group totally compensated for the lower energy and protein
contents of the EF formula by larger ingested volumes resulting in identical total energy and protein intakes for the EF and SF groups and only marginally higher total fat intake for the EF group (20). The intervention was blinded for the study staff and parents until all infants had passed the study visit at 12 months of age. Blood samples were collected at 4, 6 and 12 months of age and were obtained >2 h after the latest meal. The study was approved by the Regional Ethical Review Board in Umeå. Complete oral and written information was given to the parents/caregivers, and written consent was obtained from parents/caregivers of all infants before inclusion. This study was registered with number NCT00624689 at clinicaltrials.gov. A study flow chart is presented in Figure 1. A CONSORT checklist is available as supplemental material (Supplemental Table S1).

Sample preparation

Venous blood was collected in EDTA and SST tubes. Serum at 4 and 12 months were obtained by centrifugation at 1,300 x g for 10 minutes and frozen. At 6 months plasma and erythrocytes were separated by centrifugation at 2,000 x g for 10 min at 4° C. After removal of plasma, the erythrocyte pellet was washed three times with cold 0.15 M NaCl-1 mM EDTA, pH 7.4. The erythrocytes were resuspended in the EDTA-NaCl-solution and 10 µl DL-α-tocopherol (1.6 mg/ml ethanol) was added. Samples were stored at −80 ° C. The plasma, erythrocyte and serum samples lipid extraction was according to Folch et al (22).

To 20 µl of plasma 110 µl of CHCl₃:MeOH (2:1 v/v) including internal standard solution was added. Samples were shaken for 2 min, and allowed to stand for 30 min at room temperature
before centrifugation (14000 rpm, 3 min, 4 °C). Fifty µl of the lower phase was collected and mixed with 70 µl of CHCl3:MeOH (2:1 v/v). Extracts were stored at −80 °C.

To 10 µL of serum 10 µL of 0.9% NaCl, 40 µL of CHCl3:MeOH (2:1, v/v) and 80 µL of an 3.5 µg mL⁻¹ internal standard solution was added. Samples were vortexed and allowed to stand on ice for 30 min after which they were centrifuged (9,400 × g, 3 min, 4 °C). Sixty µL from the lower layer of each sample was transferred to a glass vial with an insert and 60 µL of CHCl3:MeOH (2:1, v/v) was added. Samples were randomized before analysis.

To 20µl of erythrocytes 30 µl of 0.15 M NaCl and 250 µl of CHCl3:MeOH (2:1 v/v) including internal standards was added. Samples were shaken for 2 min, and allowed to stand for 30 min at room temperature before centrifugation (14000 rpm, 3 min, 4 °C). After centrifugation 120µl of the lower phase was collected and this ready-to-use extract was stored at −80 °C.

**Lipidomic analyses and data processing**

**Plasma at 6 months of age**

The following compounds were used as internal standards: Phosphatidic acid (PA) (17:0/17:0), Phosphatidylethanolamine (PE) (17:0/17:0), Phosphatidylglycerol (PG) (17:0/17:0), Monoacylglycerol (MG) (17:0/0:0/0:0), Diacylglycerol (DG) (17:0/0:0/17:0), Triacylglycerol (TG) (17:0/17:0/17:0), Phosphatidylcholine (PC) (19:0/19:0), Lyso-PC(17:0/0:0), TG(16:0/16:0/16:0)-13C3. QC-samples (Quality control sample, mix of extract from the samples), dilution series of QC and LIMA-samples (pooled plasma samples from the lab) were used as a quality check. The samples were analyzed using ultra-high performance liquid
chromatography-quadrupole time-of-flight/mass spectrometry (UHPLC-QTOF/MS) system (Agilent Technologies, Santa Clara, CA). Lipids were separated on a ACQUITY UPLC® CSH C18 column (2.1×50mm, 1.7µm) (Waters, Milford, MA) using a method modified from (23), and detected by an 6540 Q-TOF mass spectrometer in positive ion mode. Targeted MS data processing was performed using Agilent MassHunter ProFinder B.06 software (Agilent Technologies). In-house libraries with masses and internal retention times were used for lipid annotation according to lipid class and the total number of carbons and double bond. For some lipids each specific fatty acid tail was detected and is in those cases presented. An analytic effect in intensity of detection was observed and data were therefore normalized using the internal standards.

*Serum at 4 and 12 months of age*

The following compounds were used as internal standards: PA(17:0/17:0), PG(17:0/17:0), PE(17:0/17:0), TG(19:0/19:0/19:0), PC(14:0)-d13, PC(17:0/17:0), TG(16:0/16:0/16:0)-13C3 , Cer(d18:1/17:0), Sphingomyelin (SM) (d18:1/17:0), Lyso-PC(17:0/0:0), TG(8:0/8:0/8:0)-13C3, PC(16:0-d31/18:1), Cholesteryl ester (CE) (17:0), Phosphatidylserine (PS) (17:0/17:0) and TG(15:0/15:0/15:0). The lipidomics analyses were performed on a UHPLC-QTOF/MS system (Agilent Technologies) in positive ion mode using a previously described method (24). Data processing was performed using MZmine 2.17 including detection and alignment of peaks, peak integration, normalization and peak identification (25). Data imputation of missing values was performed with half of the rows minimum. Lipids were annotated according to lipid class, total number of carbon and double bonds. For some lipids each specific fatty acid tail was detected and
is in those cases presented. As a batch effect was observed, i.e. lipid concentrations varied among batches, the data were normalized using internal standards.

_Erythrocyte membranes at 6 months of age_

Two internal standards were used, TG(16:0/16:0/16:0)-13C3 (in positive mode) and Cer(d18:1/16:0-d31) (in positive and negative mode). QC-samples and dilution series of QC was used as a quality check. Samples were analyzed by UHPLC-QTOF/MS (Agilent 6550) in positive and negative ion mode. For analysis details and data processing (in ProFinder B.08) see above for plasma at 6 months. There was a concentration effect, i.e. a variation in total lipid concentration among samples, and the data were normalized using the total sum of lipids.

_Statistics_

The multivariate analyses at 6 months of age were based on identified lipids and at 4 and 12 months on both identified and unidentified lipids. To detect any outliers, principal component analysis (PCA) was performed. Strong outliers were identified by Hotelling’s T2. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed to detect any class variation in the lipid profile between the groups. To validate the model cross-validation, permutation test and CV-ANOVA were used. Significance level was set to CV-ANOVA p< 0.001 (26). Multivariate analyses were performed using SIMCA (SIMCA-P+ 14.1, Umetrics, Umeå, Sweden). Differences in relative concentrations of specific lipids were analysed using the two-sided t-test (SPSS statistics 25) of log transformed relative concentrations and reported as unadjusted p values. To highlight specific lipids of interest, a combination of OPLS-DA loadings,
variable importance in the projection (VIP) and the two-sided t-test were used. Significant levels were set to VIP >1 and unadjusted p < 0.001.

Results

Plasma lipidome at 6 months of age

Of the 220 infants still in the study at 6 months of age, lipidomic analyses of plasma were performed in 213 infants (73 EF, 70 SF, 70 BFR) (Figure 1). No outliers were detected. There was a clear separation between the EF and SF groups in the OPLS-DA (Figure 2). The separation was mostly due to SM, PC, Cer and TG. Most of the phospholipids (PL) and TG species important for the separation were higher in relative concentration in the EF group compared to the SF group. Individual lipids with the highest VIP values were SM, PC and Cer species (Table 1).

Serum lipidome at 4 and 12 months of age

Analyses were performed on serum samples from a randomly selected subgroup (n=90; 30 SF, 30 EF and 30 BFR) at 4 and 12 months of age (Figure 1). At 4 and 12 months of age, one sample was excluded due to analytical problems. No outliers were detected. At 4 months, there was a clear separation between the EF and SF groups in the OPLS-DA. The separation was transient and not detected at 12 months, 6 months after the intervention ended (Figure 3). The group separation at 4 months of age was mostly due to SM and PC, whereas PE and TG contributed less. Most of the PLs important for the separation were higher in the EF group compared to the SF group. Individual lipids with the highest VIP values were SM and PC species (Table 1).
Lipidomic analyses on erythrocyte membranes were performed in 213 infants (70 SF, 73 EF, 70 BFR) at 6 months of age (Figure 1). No outliers were detected. There was a clear separation between the two formula groups in the OPLS-DA (Figure 4). Lipids important for the separation were mostly SM, PE and PC species. Relative concentrations of most of the PLs important for the separation were higher in the EF-group (Table 1).

**Discussion**

We found significant differences in the serum/plasma lipidome at 4 and 6 months of age in infants fed the EF compared to infants fed SF. This separation was also detected in erythrocyte membranes at 6 months, but did not remain in sera collected at 12 months of age, 6 months after the end of the intervention. SM and PC species contributed most to the separation in serum/plasma and erythrocyte membranes at 4 and 6 months of age. At 6 months Cer also contributed to the separation in plasma. Most PCs important for the separation were higher in the EF-group as opposed to Cer and SM species having a more heterogeneous pattern. In the erythrocyte membrane, PEs also contributed to the separation and were higher in the EF group. These separations are reasonably caused by differences in lipid intake between the two formula groups. We have previously presented, in the same study population, that the EF group had moderately higher total serum cholesterol concentration than the SF group at 6 months of age (27) due to higher cholesterol intake during the intervention. The conspicuous difference in the lipidomic profile between the EF and SF groups at 6 months of age in the present study is
unlikely caused by the higher total fat intake in the EF group, which was mainly caused by a higher TG intake, but is rather explained by a clear difference in fat quality due to the MFGM supplementation of the EF as the lipids important for the separation between the EF and SF groups are polar lipids present at high concentrations in the MFGM.

Type of feeding, i.e. breastfeeding or formula-feeding, and type of fat consumed affect blood lipid profiles in infants. In a prospective observational cohort study, breast-fed, compared to formula-fed infants, had a different lipid profile and three lipid molecular species, PC (35:2), SM (36:2) and SM (39:1), were suggested to be used collectively as biomarkers indicating whether infants were formula- or breast-fed (28). In the present study these lipids had high VIP values and showed the same pattern when comparing the SF and BFR groups at 4 months, supporting the role of these lipids as biomarkers of standard formula feeding vs breastfeeding. However, the pattern of these lipids differ between the EF and SF groups indicating that these biomarkers might not be valid when comparing infants fed MFGM supplemented formula with breastfed infants.

A previous study showed that feeding preterm infants sphingomyelin-fortified formula resulted in higher SM concentrations and more favorable neurodevelopment (15), and feeding piglets a formula supplemented with phospholipids and gangliosides resulted in improved spatial learning and growth of brain tissue (29). Several differences in the lipidome between the EF and SF groups in our study are interesting in relation to our previous finding that the EF group had improved cognitive development compared to the SF group at 12 months of age (20). Higher relative concentrations of PCs and some SMs contributed to the separation between the groups, both in plasma, serum and erythrocyte membrane. The concentrations of several Cer, a metabolite of SMs and other sphingolipids (30) were affected indicating that the metabolism of
sphingolipids was altered. Cognitive function reflects brain development from fetal life until the
time of measurement. A positive effect on brain development at any time before 12 months of
age may theoretically explain the positive effect on cognitive function seen in the EF group at
this time point. Thus, the clear lipidomic differences between the EF and SF groups during the
intervention period could be relevant for the effect on cognitive function even if the effect on the
lipidome was transient and not present at 12 months of age.

The erythrocyte membrane lipidome, which is less affected by the recent dietary intake and rather
reflects long-term lipid intake (31), was clearly different between the EF and SF groups. The
separation was mostly due to SM, PC and PE species. A dietary effect on the erythrocyte
membrane lipidome is likely a proxy for effects on cell membranes in other tissues. Extrapolating
lipidomic observations to the central nervous system must however be made with caution, since
the transport of lipids and fatty acids over the blood-brain barrier (BBB) is not fully understood.
Two different transport mechanisms have been implicated, i.e. passive diffusion or transporter
proteins (32). Animal and in vitro studies have addressed the uptake and metabolism of DHA
over the BBB. In a recent report, fatty acid transport protein 1 (FATP 1), localized in the basal
membrane of brain microvessels, was suggested to contribute to the transport of DHA, taurine
and biotin into the brain (33). DHA attached to Lyso-PC seems to be the preferred form of DHA
taken up by the brain (34) and might be mediated by the transporter Mfsd2a (35). These studies
indicate that not only the concentration of a fatty acid is of importance, but also in which form,
esterified or non-esterified, and if esterified, to which lipid affect its transport across the BBB.
The association between the lipidome in blood and central nervous system is not fully
understood. However, in a Finnish study, erythrocytes, retinal cells and cells from the optic nerve
from nine humans were collected. Several lipids of the erythrocytes correlated with lipids in ocular tissue (36).

With respect to immunological effects, it is known that PLs play an important role in modulating gut inflammation (37), and also affect the inflammatory response in the lungs during pulmonary infection (38). Sphingolipid metabolites, Cer and sphingosine-1-phosphate, regulate immune cell trafficking and epithelial integrity and therefore are important in inflammation and inflammatory diseases (30). Changes in SM and its metabolite Cer affect T-cell response, both in early activation steps and effector functions (39). Our intervention did affect the lipidome of erythrocyte membranes, where alteration of SMs contributed, thus suggesting that the lipid composition of cell membranes of immune cells are affected similarly. We also found an effect on SM and Cer in plasma at 6 months of age. An improved cell function due to the altered lipid composition of the cell membrane is a likely part of the mechanism behind the positive effect on the immune system previously reported in the same study population (21).

A strength of the current study is the double-blinded randomized design and the longitudinal aspect. Besides the analyses of plasma and erythrocyte membranes at 6 months of age, we analyzed the serum lipidome both at 4 months of age, when the intervention was at its maximum, and at 12 months of age, 6 months after the intervention. This made it possible to study both direct and possible long-term effects on the lipidomes. Another strength was the low drop-out rate.

All analyses were performed on an intention-to-treat basis. As previously described (20), a majority of the parents introduced taste portions of solid food between 4 and 6 months of age in accord with the Swedish National Food Agency recommendations. Some parents stopped the study formula due to various symptoms, and breastfeeding was partly or totally stopped for some
of the infants in the BFR group. This dilution of the intervention is a limitation of the study but gives an estimate of the effect on the population level.

The blood samples were stored at −80°C for 3-9 years until analysis. As previously described, the lipid components are stable during long-term storage at -80°C (40), although some effect has been shown on the concentration of LPC (41). Since the formula-fed infants were recruited randomly the in between group separation should not be affected by the storage time.

Since we are using a multivariate analysis method and our method is limited to identify class of lipid and number of carbons and bonds, the results regarding specific lipids should be interpreted with caution. The main purpose of the analysis was to detect potential differences in the lipidomic profile, not to identify specific lipids. To confirm and validate specific lipids of interest, and to match specific lipids between the MFGM fraction and blood samples, a targeted method should be used.

The intervention of the study is complex including both supplementation with a bovine MFGM concentrate and adjustments of macronutrients as previously described (20). Any effect of the intervention may be due to a number of different mechanisms and it is not possible to separate one part of the intervention from another. The complex intervention is a limitation for the interpretation of mechanisms behind the functional outcomes, which can only be speculative.

In conclusion, we found that feeding infants a formula supplemented with a bovine MFGM fraction clearly affects their serum/plasma and erythrocyte membrane lipidomes. Infants fed the EF had higher concentrations of many PCs and altered concentrations of SMs. It is reasonable that these changes reflect or are part of the mechanisms mediating the positive effects on cognitive development and immune defense previously shown in the study population. However,
to verify this, further studies are needed to confirm the observed effects of MFGM supplemented infant formula on infant lipid metabolism and to study the mechanism behind the functional outcomes in dept.

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Grip T and Timby N had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author contribution

Substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; Grip T, Dyrlund TS, Ahonen L, Domellöf M, Hernell O, Hyötyläinen T, Knip M, Lönnerdal B, Orešić M, Timby N
Drafting the article or revising it critically for important intellectual content; Grip T, Dyrlund TS, Ahonen L, Domellöf M, Hernell O, Hyötyläinen T, Knip M, Lönnerdal B, Orešič M, Timby N

Final approval of the version to be published; Grip T, Dyrlund TS, Ahonen L, Domellöf M, Hernell O, Hyötyläinen T, Knip M, Lönnerdal B, Orešič M, Timby N

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**Disclosure**

Hernell O and Lönnerdal B are members of Hero and Semper scientific advisory boards. Grip T, Dyrlund TS, Ahonen L, Domellöf M, Hyötyläinen T, Knip M, Orešič M, Timby N, declare no conflicts of interest.
References


Table 1. Identified lipids with the highest VIP values in the OPLS-DA.

<table>
<thead>
<tr>
<th>Time</th>
<th>Lipid</th>
<th>Lipid class</th>
<th>VIP value</th>
<th>P-value EF vs SF</th>
<th>EF/SF&lt;sup&gt;1&lt;/sup&gt;</th>
<th>BFR/SF&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 months, plasma</strong> (182 metabolites, all identified)</td>
<td>SM(d18:21:0)</td>
<td>SM</td>
<td>2,79</td>
<td>5,41E-20</td>
<td>1,84</td>
<td>0,61</td>
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<td></td>
<td>SM(d18:1/14:0)</td>
<td>SM</td>
<td>2,43</td>
<td>6,30E-13</td>
<td>1,50</td>
<td>0,88</td>
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<tr>
<td></td>
<td>PC(40:2)</td>
<td>PC</td>
<td>2,33</td>
<td>1,10E-12</td>
<td>1,29</td>
<td>0,84</td>
</tr>
<tr>
<td></td>
<td>SM(d18:1/15:0)</td>
<td>SM</td>
<td>2,15</td>
<td>1,49E-9</td>
<td>1,35</td>
<td>0,98</td>
</tr>
<tr>
<td></td>
<td>HexoseCer(d18:1/24:1)</td>
<td>Cer</td>
<td>2,05</td>
<td>2,74E-8</td>
<td>0,75</td>
<td>1,48</td>
</tr>
<tr>
<td></td>
<td>SM(d18:1/20:0)</td>
<td>SM</td>
<td>2,00</td>
<td>5,39E-8</td>
<td>1,35</td>
<td>0,79</td>
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<tr>
<td></td>
<td>SM(d18:0/14:0)</td>
<td>SM</td>
<td>1,92</td>
<td>2,20E-8</td>
<td>1,25</td>
<td>1,09</td>
</tr>
<tr>
<td></td>
<td>Cer(d18:1/20:0)</td>
<td>Cer</td>
<td>1,89</td>
<td>3,36E-7</td>
<td>1,35</td>
<td>0,91</td>
</tr>
<tr>
<td></td>
<td>Cer(d18:1/23:0)</td>
<td>Cer</td>
<td>1,84</td>
<td>1,43E-8</td>
<td>1,30</td>
<td>0,81</td>
</tr>
<tr>
<td></td>
<td>Cer(d18:1/22:0)</td>
<td>Cer</td>
<td>1,60</td>
<td>6,39E-5</td>
<td>1,21</td>
<td>0,91</td>
</tr>
<tr>
<td><strong>4 months, serum</strong> (484 metabolites in the model, 187 identified)</td>
<td>SM(d39:1) 102</td>
<td>SM</td>
<td>2,39</td>
<td>2,88E-13</td>
<td>1,70</td>
<td>0,57</td>
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<td></td>
<td>PC(42:6) 2084</td>
<td>PC</td>
<td>2,29</td>
<td>4,18E-9</td>
<td>2,08</td>
<td>0,85</td>
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<td></td>
<td>SM(d18:0/14:0) 747</td>
<td>SM</td>
<td>2,16</td>
<td>9,08E-11</td>
<td>1,49</td>
<td>1,26</td>
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<tr>
<td></td>
<td>SM(d32:1) 91</td>
<td>SM</td>
<td>2,11</td>
<td>2,16E-9</td>
<td>1,40</td>
<td>0,90</td>
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<td></td>
<td>SM(d33:1) 149</td>
<td>SM</td>
<td>2,08</td>
<td>5,62E-8</td>
<td>1,32</td>
<td>0,92</td>
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<td></td>
<td>PC(36:2) 288</td>
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<td>1,54</td>
<td>0,94</td>
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<td>PC</td>
<td>1,96</td>
<td>2,56E-7</td>
<td>1,58</td>
<td>1,28</td>
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<tr>
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<td>PC(35:2) 156</td>
<td>PC</td>
<td>1,95</td>
<td>4,34E-7</td>
<td>1,55</td>
<td>1,26</td>
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<tr>
<td></td>
<td>SM(d18:2/24:1) 62</td>
<td>SM</td>
<td>1,95</td>
<td>9,36E-7</td>
<td>0,77</td>
<td>1,49</td>
</tr>
<tr>
<td></td>
<td>PC(35:1) 254</td>
<td>PC</td>
<td>1,93</td>
<td>5,33E-6</td>
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<td><strong>6 months, Erythrocyte membrane</strong> (112 metabolites, all identified)</td>
<td>SM(d18:1/14:0)</td>
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<sup>1</sup> Mean relative concentration in EF group divided by mean relative concentration in SF group.

<sup>2</sup> Mean relative concentration in BFR group divided by mean relative concentration in SF group.
Legends to figures

Figure 1: Study flow chart

Figure 2: OPLS-DA plot of plasma lipidomics at 6 months for the SF (green circles) and EF (blue circles) groups. OPLS-DA (1+4, R2X (cum) 0.651, R2Y (cum) 1, Q2 (cum) 0.515) CV-ANOVA <0.001 (uv-scaled), 182 lipids were included in the model. There was a clear separation between the formula groups.

Figure 3: OPLS-DA plot of serum lipidomics at 4 (circles) and 12 (triangles) months for the SF (green) and EF (blue) groups. OPLS-DA (2+4, R2X (cum) 0.588, R2Y (cum) 0.667, Q2 (cum) 0.414) CV-ANOVA <0.001 (uv-scaled), 484 lipids, both identified and unidentified, were included in the model. There was a clear separation between the SF and EF group at 4 months but not at 12 months of age.

Figure 4: OPLS-DA plot of erythrocyte membrane lipidomics at 6 months for the SF (green circles) and EF (blue circles) groups. OPLS-DA (1+1, R2X (cum) 0.238, R2Y (cum) 1, Q2 (cum) 0.585) CV-ANOVA <0.001 (uv-scaled), 112 lipids are included in the model. There was a clear separation between the formula groups.
Figure 3
Figure 4