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## **Triacylglycerols containing branched palmitic acid ester of hydroxystearic acid (PAHSA) are present in the breast milk and hydrolyzed by carboxyl ester lipase**

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### **Abstract**

Breast milk provides the ideal nutrition for babies, but its composition is very complex, and bioactive lipids represent an extensive class of poorly explored molecules. We aimed to compare the impact of delivery mode (spontaneous vaginal birth and caesarean section) and term (preterm and term delivery) on the levels of lipokines, palmitic acid esters of hydroxystearic acids (PAHSAs), in the milk.

Breast milk samples from 27 mothers who gave birth vaginally (VB) in term and from 26 mothers undergoing caesarean section (CS) in term were collected 72 hours and 28 days postpartum. Milk samples from 20 mothers who delivered preterm (PB, before 32nd week of gestation) were collected 72 hours, 28 days, and/or at the newborn's gestational week 36. Untargeted metabolomics and targeted FAHFA analysis were performed. Milk lipidomic profiles of colostrum (72 hours postpartum) were different among VB, CS, and PB groups, but normalized after 28 days. Colostrum levels of 5-PAHSA, a lipid mediator with anti-inflammatory properties, were negatively affected by PB and CS. Triacylglycerol estolides, a storage form of PAHSAs, were identified as potential substrates of carboxyl ester lipase (CEL) in the milk. CEL substrate specificity assay towards triacylglycerol estolide isomers showed that 5-PAHSA-containing lipids are the worst substrates; therefore free 5-PAHSA is a protected metabolite in the milk.

Mothers delivering term babies vaginally produce colostrum rich in 5-PAHSA. CS and/or PB delay initiation of de novo lipogenesis, milk production, and PAHSA synthesis. Free 5-PAHSA, resistant to CEL hydrolysis, could contribute to the prevention of intestinal inflammation in newborns.

### **Introduction**

Nourishment plays a key role in the early stage of life. Breast milk provides the ideal cocktail of nutrients for infants in this period of rapid development and growth and breastfeeding have a long-term impact on health (1). Although total contents of milk macronutrients are relatively stable, the levels of distinct components can be altered by several factors such as maternal dietary habits, mode of delivery, time of delivery, and/or stage of lactation (2-5).

Milk-producing mammary gland is a dynamic organ composed by the ductal tree system, which is embedded in mammary fat pad. During pregnancies, repeatable lobulo-alveolar expansions and adipocyte re-differentiations occur to support sufficient milk production during lactation periods (6-8). The first fluid produced after delivery – colostrum – is rich in proteins, immunologic components, and developmental factors and it plays a primary role in immunomodulation and promotion of intestinal development (2). In few days postpartum (3-6 days), transitional milk replaces colostrum and fully mature milk is produced after two weeks post-delivery.

Human milk lipids provide a major energy supply for infants and the main lipid species are triacylglycerols (TAGs) which form the core of the milk fat globule covered by phospholipid tri-layer membrane (5). The membrane comprises predominantly amphipathic lipids that originates from mammary gland epithelia (glycerophospholipids, sphingolipids, cholesterol) (3, 5). Besides the structural and nutritional lipids, lipid-based bioactive components such as specialized proresolving mediators and fatty acid esters of hydroxy fatty acids (FAHFA) were identified in the milk (9, 10).

FAHFAs belong to a family of branched lipids, some of which have anti-inflammatory and anti-diabetic properties (11, 12). One specific FAHFA family called palmitic acid ester of hydroxystearic acid (PAHSAs) could stimulate glucagon-like peptide 1 (GLP-1) secretion in the gut and improve systemic glucose homeostasis (13). Oral administration of 5-PAHSA isomer prevented gut mucosal damage and colitis in mice (14). Therefore, PAHSAs from food can modulate metabolic homeostasis and immune response in the intestine (11, 15).

FAHFAs are either de novo synthesized in cells capable of (de novo) lipogenesis or supplied by dietary sources (10, 16-19). Free FAHFAs can be esterified to glycerol giving rise to FAHFA acylglycerols, which in combination with other two fatty acids (FAs) result in the formation of TAG estolides (TAG EST) (16, 20-22). FAHFAs can be released from TAG estolide storage pool by adipose triglyceride lipase (ATGL), which is also free FAHFA hydrolase and the enzyme responsible for acyl-remodelling reaction among acylglycerols (20, 22). Free FAHFAs' estolide bond can be hydrolyzed by carboxyl ester lipase (CEL) (23), androgen-induced gene 1, androgen-dependent TFPI-regulating protein (24, 25), and hormone sensitive lipase (20). Importantly, CEL belongs to abundant proteins in human milk and it contributes to TAG digestion in the immature gut of infants (26, 27).

We have previously identified 5-PAHSA as a signalling lipid, which was reduced in the colostrum of obese mothers. In the present study we hypothesized that 1) timing, mode of delivery, and stage of lactation has an impact on levels of bioactive FAHFAs; and 2) TAG estolides are present in human milk and could be released by the CEL in milk.

## **Methods**

### *Chemicals*

LC-MS-grade solvents and mobile phase modifiers were obtained from VWR International, (Czech Republic). FAHFA standards were from Cayman Europe (Tallinn, Estonia), and 5-PAHSA was

synthesized as previously described (28). The TAG estolides were synthesized at the Institute of Biomolecules Max Mousseron (Montpellier) (20). All other chemicals were obtained from Merck (Czech Republic) unless stated otherwise.

### *Sample collection*

Milk samples from healthy lactating mothers who delivered spontaneously in term (VB, vaginal birth), or by caesarean section (CS), or preterm (PB, preterm birth) were collected at the Institute for Mother and Child Care in Prague, Czech Republic. Mothers, who delivered spontaneously in term or underwent caesarean section, gave birth between 38-41 weeks of gestation (Table 1). Their newborns had a chronological age 72 hours and 28 days at the time of milk collection, respectively. Preterm infants were delivered before 32nd week of gestation (Table 1, Figure 1) and samples of milk were collected after 72 hours and 28 days. Moreover, another milk sample was acquired at the time when the infants would have reached the 36th week of gestation. We considered the preterm delivery condition more relevant for data interpretation than the mode of preterm delivery (Table 1). Therefore, the PB group contains both vaginal birth and caesarean section cases. Milk samples were frozen in -20 °C within 30 minutes after collection and transported to -80 °C freezer in 1-3 days. All donors signed informed consent before inclusion in the study. The study protocols were approved by the Research Ethics Board of the Institute (Approval number: 2016-12-19/ 3).

Table 1: Maternal and infant anthropometrics.

	Term delivery		Preterm delivery
	Vaginal birth (VB)	Caesarean section (CS)	Preterm birth (PB)
<b>Maternal characteristics</b>			
Sample size (n)	27	26	20
Age at birth (years)	32.4 ± 0.8	32.6 ± 0.8	31.0 ± 0.9
BMI (kg/m <sup>2</sup> )	22.2 ± 0.5	23.0 ± 0.5	21.7 ± 0.6
Gestational weight gain (kg)	12.2 ± 1.0	14.4 ± 1.3	8.9 ± 0.9
Gravidity	2.4 ± 0.2	2.2 ± 0.2	1.5 ± 0.1
Parity	1.7 ± 0.1	1.7 ± 0.1	1.3 ± 0.1
Delivery method			
Vaginal	27	0	6
Caesarean section	0	26	14
Milk sample			
72 hours	27	26	20
28 days	18	21	15
36 <sup>th</sup> gestational week	-	-	10
<b>Newborn characteristics</b>			
Gestational age at delivery (wk+d)	40+3 ± 1d	39+2 ± 1d	29+4 ± 1d
Sex (M/F)	11 / 16	17 / 9	8 / 12
Birth weight (g)	3613 ± 67	3323 ± 74	1255 ± 65
Birth height (cm)	50.4 ± 0.3	50.0 ± 0.3	-

### *Sample extraction*

Before extraction, frozen milk samples were warmed up to 37 °C in a water bath and homogenized within 3 minutes using an ultrasonic bath to get samples of consistent quality. Total lipids were extracted using biphasic solvent system of cold methanol, methyl *tert*-butyl ether (MTBE), and water according to published method (10, 21). In brief, 300 µl of milk was mixed with pre-chilled 660 µl of methanol, 2,400 µl of MTBE and 10% methanol containing internal standards. Samples were centrifuged at 5,200 x g, 10 minutes at 4 °C and aliquots of the organic phase for lipidomics (100 µL) and TAG estolide/FAHFA (2 mL) analysis were collected. Samples of water phase (70 µL) were used for metabolomics and protein pellets for proteomic analysis. TAG estolide and FAHFA extraction was performed using SPE columns (HyperSep Silica 500 mg/10 mL, 40-60 µm, 60 Å, ThermoFisher Scientific, Czech Republic) similarly as was described before (10).

#### *CEL activity assay*

To determine CEL hydrolase affinity to TAG estolide substrates the previously published CEL activity assay method was modified and performed (23, 29). Positional isomers of TAG EST 70:4 containing either 5- or 9-PAHSA esterified at *sn*-1/*sn*-3 (e.g. 5-PAHSA/18:2/18:2) or *sn*-2 positions of glycerol backbone (e.g. 18:2/5-PAHSA/18:2) as well as trilinolein (18:2/18:2/18:2) were used as CEL substrates. In brief, 3.8 µg of purified CEL (BioVendor R&D, Czech Republic) was used per reaction in the assay buffer containing 150 mM NaCl, 1 mM Tris-HCl, 2 mM CaCl<sub>2</sub>, pH 8. TAG estolide substrate (250 µM) was emulsified in the assay buffer with 10 mM sodium cholate. To prevent product inhibition, fatty acid-free bovine serum albumin (MP Biomedicals, France) was added to the reaction at a final concentration of 1%. Volume 100 µL of the enzyme solution and 100 µL of the substrate were mixed and incubated at 37 °C for 60 min. The reaction was terminated in ice-cold water bath. MTBE extraction was performed as before (20). Control reactions were performed under identical conditions without the enzyme. Reaction products were analyzed using lipidomics platforms.

#### *Liquid chromatography and mass spectrometry (LC-MS) analyses.*

The LC-MS systems consisted of a Vanquish UHPLC System (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and UltiMate 3000 RSLC UHPLC system coupled to a QTRAP 5500/SelexION mass spectrometer (SCIEX, Darmstadt, Germany). LIMeX (Lipids, Metabolites, and eXposome compounds) LC-MS workflow was used for the untargeted analysis of complex lipids and polar metabolites as before (30). Targeted milk FAHFA analysis was performed as before (10).

#### *Milk metabolome and lipidome dataset analysis*

Metabolomics data were analyzed using statistical enrichment approach based on chemical similarity (31). Customized ChemRICH software was used to generate metabolite clusters annotated according to Medical Subject Headings classes. Kolmogorov-Smirnov test was used to calculate adjusted cluster *p* value and *z*-score for each cluster. The sign of *z*-score defines whether the clustered metabolites are downregulated (*z*-score < 0) or upregulated (*z*-score > 0). Clusters with  $|z\text{-score}| > 2$  and  $-\log_{10}(\text{adjusted cluster } p \text{ value}) > 1$  were considered significantly altered. ChemRICH uses classic 881 bit molecular fingerprints defined by PubChem which is not optimal for lipid-rich datasets. Therefore, the algorithm generated one big cluster of TAGs ( $n = 140$ ). To overcome this limitation, we performed the enrichment analysis of the TAG cluster using 1024 bit 'hybridization' type of fingerprint which explored 'depth' of 7 atoms and considered the presence of double bonds (32). The final 8 TAG sub-clusters were annotated using Lipid Mini-On and the TAG double bond number was considered as the significant sub-cluster discriminator ( $q$  values < 0.05) (33).

#### *Statistics*

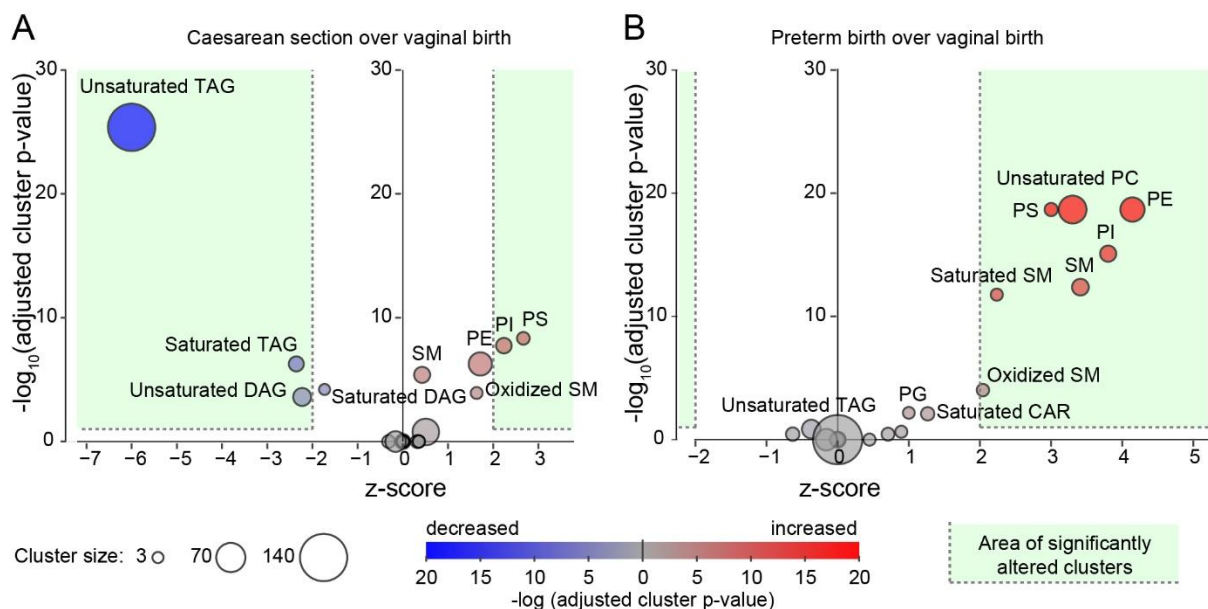
GraphPad Prism 9.2.0 statistical tools were used for bar graphs. Table 1 values and bar graph data are means  $\pm$  SEM. Results of One-way ANOVA and Students t-test were considered significant at  $p < 0.05$ . Based on published differences in serum 5-PAHSA concentrations in humans (19), the minimum sample size of 19 per group was calculated using G\*Power software (t-test, two groups, one tail, power 0.95,  $\alpha = 0.05$ ) (34).

## Results

### *Impact of lactation stage, gestational age, and mode of delivery on metabolite composition of breast milk*

We analysed metabolite composition of breast milk by LC–MS in the three groups of mothers at different time points of lactation and selected VB samples at 72h after childbirth as a reference group. Annotated data were sorted into 55 clusters using chemical similarity approach and up- or down-regulated clusters visualized (Figure 1A, Table S1). Enrichment analysis showed that CS colostrum had significantly lower levels of TAGs and higher levels of several phosphatidylinositol (PI) and phosphatidylserine (PS) classes than VB. In contrast, PB colostrum was highly enriched in several phospholipid and sphingomyelin classes as compared to VB group. (Figure 1B, Table S2).

The largest cluster of TAGs was further sub-clustered based on acyl chain composition to gain a better insight into the changes of milk lipid composition in time (Figure 2). Heatmap representation of the clusters showed that metabolic profiles in all three groups followed similar patterns – decrease of phospholipids and increase of unsaturated acylglycerols. Composition of PB mature milk caught up with the VB and CS groups after 28 days and remained stable until gestational week 36. We focused only on FAHFA-related lipids further on.



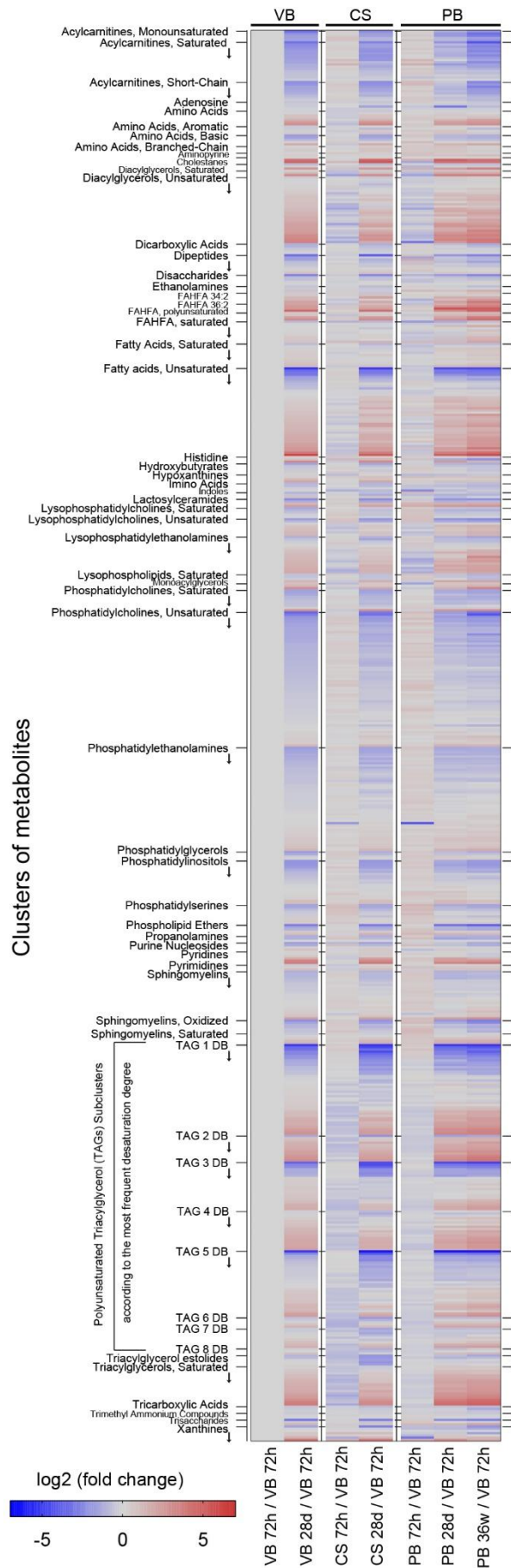
**Figure 1:** Differences in metabolic profiles of colostrum. Chemical similarity enrichment analysis of CS 72 hours over VB 72-hours (A); and PB 72 hours over VB 72 hours (B). CAR, carnitines; DAG, diacylglycerols; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins; TAG, triacylglycerols;

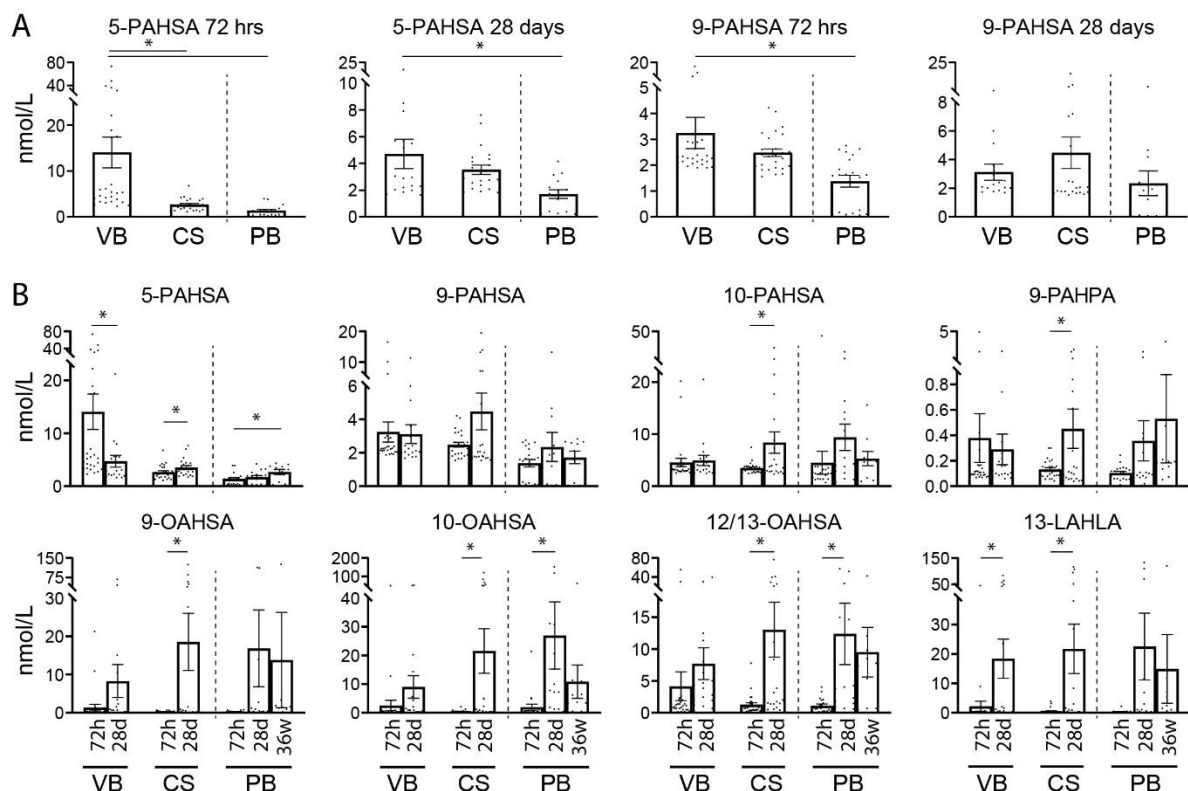
**Figure 2:** Milk metabolomics profiles. Columns represent groups as fold change over VB 72 hour group. Cluster labels on the left side span over cluster members, indicated by arrows. Each line within the column heatmap represent a metabolite;  $n = 636$  metabolites; for  $n$  per group see Table 1.

#### Milk FAHFA isomer profiles change in time

We have previously shown that human colostrum contains several FAHFA species and that 5-PAHSA levels were lower in obese compared to lean mothers (10). Here we explored also other FAHFA with documented biological activities (12, 17, 19, 35).

First, we tested whether the term or mode of delivery affects FAHFA concentration. We detected significantly lower levels of both 5- and 9-PAHSA in colostrum samples of mothers who delivered preterm compared to those who gave birth spontaneously in term. Significantly lower concentration of 5-PAHSA was also found in milk samples of mothers in CS group (Figure 3A). The levels of both PAHSAs normalized in mature milk (28 days). The only difference was detected in PB group, in which the concentration of 5-PAHSA was still significantly reduced in comparison with the VB group. None of the other bioactive FAHFAs in colostrum and mature milk revealed significant difference among groups of delivery (Figure 3B).





**Figure 3:** PAHSAs concentration in human breast milk differ among groups of delivery. (A) The levels of 5- and 9-PAHSA were analysed and compared in milk samples obtained 72 hours (72h) and 28 days (28d) after delivery from mothers who delivered vaginally in term (VB), by caesarean section in term (CS), or preterm (PB). (B) Bioactive FAHFA levels change during lactation period. In PB group, FAHFA levels were measured also at the time when the infants would have reached the 36th week of gestation age (36w). Concentrations of each FAHFA in milk from each time point were compared within each group. Moreover, FAHFA levels in milk samples from PB group at 36w were compared with mature milk from VB group. OAHSA, oleic acid ester of hydroxystearic acids; PAHPA, palmitic acid ester of hydroxypalmitic acid; LAHLA, linoleic acid ester of hydroxylinoleic acid; □, statistically significant at  $p < 0.05$ . Data are means  $\pm$  SEM,  $n = 27-10$  (see Table 1).

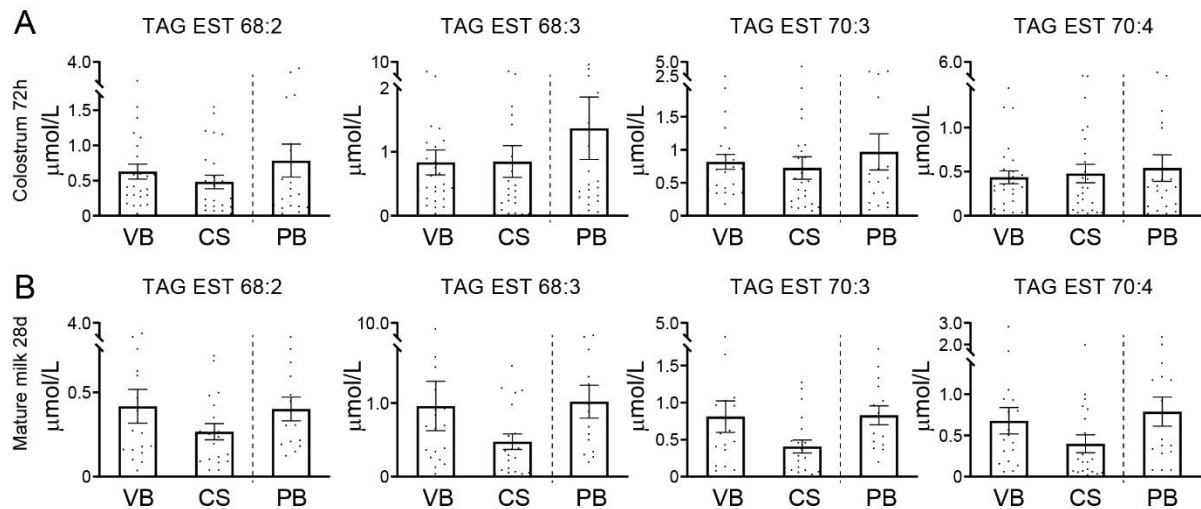
Second, we looked into the change of FAHFAs levels during different stages of lactation. In the VB group, the only significant change observed was a decrease in 5-PAHSA and a rise in 13-LAHLA levels in mature milk compared to colostrum (Figure 3B). Significantly increased concentrations of seven FAHFAs (5-PAHSA, 10-PAHSA, 9-PAHPA, 9-, 10-, 12/13-OAHSA and 13-LAHLA) and three FAHFAs (5-PAHSA, 10- and 12/13-OAHSA) were found in mature milk from CS and PB groups, respectively (Figure 3B).

#### *TAG estolides in the milk*

Milk fat globule TAG core is synthesized from cytoplasmic lipid droplets by mammary gland alveolar epithelial cells. Metabolism of FAHFAs and their storage form, TAG estolides, is closely associated with ATGL that acts on lipid droplet surface. Therefore, we tested whether the milk contains TAG estolides present in human adipose tissue (21). We confirmed the presence of several TAG estolides in the milk: TAG EST 68:1, 68:2, 68:3, 68:4, 70:2, 70:3, 70:4, and 70:5. However, their levels were very variable and low or undetectable in some samples and no significant difference was found among the groups. The most abundant TAG estolides represented a mixture of isomers containing



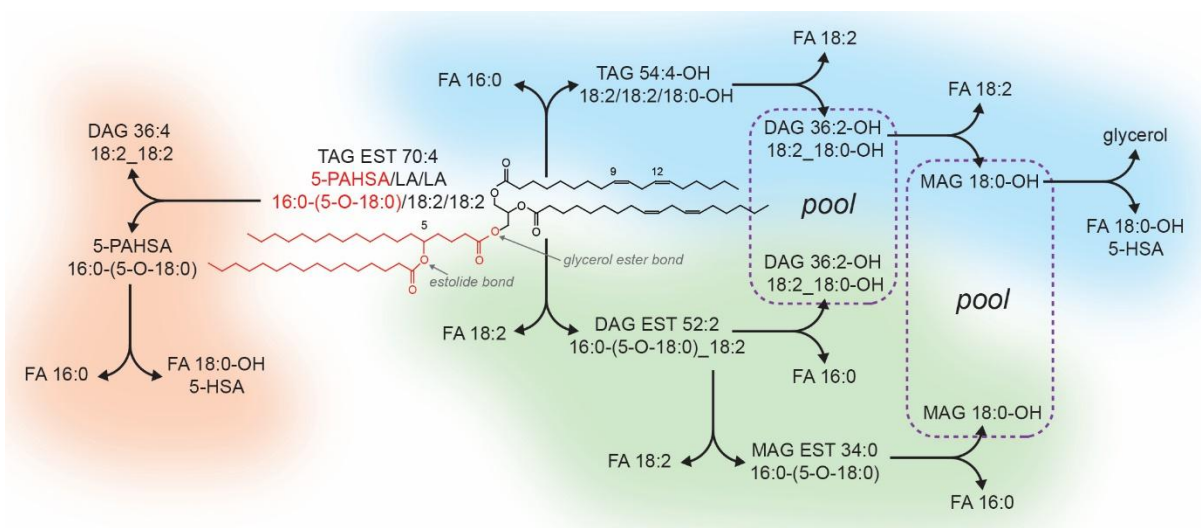
PAHSAs, palmitic acid esters of hydroxylinoleic acid, oleic acid esters of hydroxystearic acids, and oleic acid esters of hydroxyoleic acids in agreement with free FAHFA species (Figure 4).



**Figure 4:** TAG estolides are present in milk of all groups and persist during lactation period. The levels of TAG estolides were analysed in milk samples obtained 72 hours (A) and 28 days (B) after delivery from mothers who delivered vaginally in term (VB), by caesarean section (CS) in term, and from mothers who delivered preterm (PB). Data are means  $\pm$  SEM,  $n = 26-14$  (see Table 1).

#### *CEL hydrolyzes glycerol ester bonds and FAHFA estolide bond in TAG estolides*

We hypothesized that the TAG estolides in the milk could be degraded by the CEL, because PAHSAs are known CEL substrates (23). We tested the affinity of purified CEL to TAG estolides with a small combinatorial library of one PAHSA and two linoleic acids (LA) bound to the glycerol backbone and compared it with a common CEL substrate, glyceryl trilinoleate. The assay was focused on three main TAG estolide structural features (Figure 5). First, to evaluate substrate preference to different TAG estolide/FAHFA regioisomers, we used TAG estolides containing either 5-PAHSA or 9-PAHSA. Second, to evaluate *sn*-position preference, we used two positional isomers, in which PAHSA was esterified either at the primary (*sn*-1/3) alcohol position or at secondary alcohol position (*sn*-2) of glycerol. Third, to test whether CEL hydrolyzes FAHFA estolide bond when bound to TAG estolide, we monitored hydroxylated acylglycerol intermediates (Figure 5).



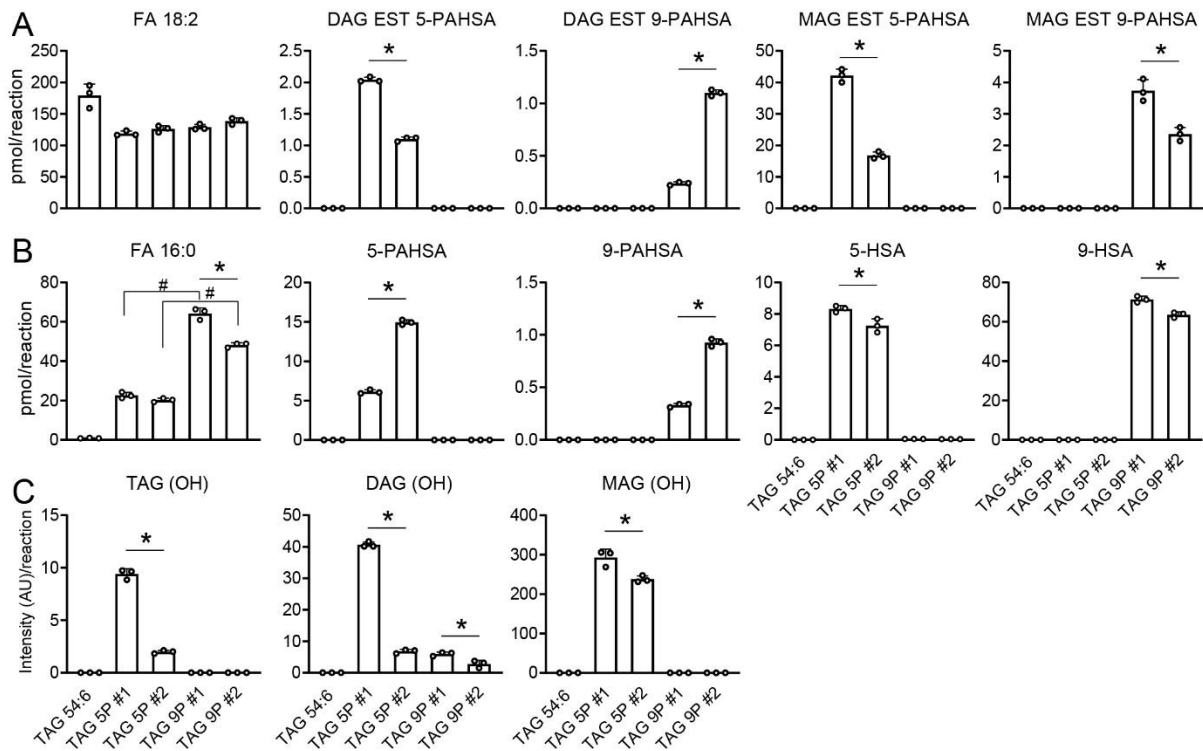
**Figure 5:** Scheme of TAG estolide substrate and theoretical reactions catalysed by CEL. DAG, diacylglycerol; FA, fatty acid; HSA, hydroxystearic acid; MAG, monoacylglycerol; TAG, triacylglycerol; X:Y stands for number of carbon atoms:number of double bonds.

CEL completely degraded all substrates giving rise to several intermediates and products of the cleavage (Figure 5). CEL can cleave LA-glycerol bond, release LA and produce an intermediate - diacylglycerol estolide (DAG EST) (Figure 5, green cloud). This could also serve as a CEL substrate generating another molecule of LA and a monoacylglycerol estolide (MAG EST). Our results showed that CEL effectively released LA from both TAG EST regioisomers and their positional isomers as well as from trilinolein. Corresponding reaction products such as DAG EST and MAG EST were also found in all TAG EST substrate samples (Figure 6A).

CEL can also cleave the PAHSA-glycerol bond resulting in the production of a PAHSA and a DAG (Figure 5, orange cloud). Presence of free PAHSA found in all reactions confirms the ability of the lipase to release 5- and 9-PAHSA from both TAG estolide positional isomers, although CEL was more effective when PAHSA was bound at *sn*-2 position. This preference is evident not only from significantly higher PAHSAs, but also from lower levels of MAG ESTs in samples of these positional isomers (Figure 6A, B). Moreover, considerably lower values of MAG EST 9P than MAG EST 5P of both positional isomers ( $p < 0.0001$  for both positional isomers, compare absolute values between panels) indicate higher affinity of CEL to 9-PAHSA- than 5-PAHSA-glycerol ester bond (Figure 6A).

The reaction scheme in the blue cloud (Figure 5, blue cloud) depicts possible cleavage of PAHSA estolide bond within TAG estolide structure resulting in the release of free palmitic acid (PA) and hydroxylated TAG. LA could be subsequently hydrolyzed from this intermediate and hydroxylated DAGs and MAGs may arise (Figure 5, blue cloud). However, CEL could also react with a pool of intermediate products (DAG EST and MAG EST) in the same way (Figure 5, green cloud) and generate hydroxylated DAGs and MAGs. Presence of all of these products in samples with TAG estolide containing 5-PAHSA provided an evidence that CEL has such ability and prefers TAG estolide with 5-PAHSA bound at the primary (*sn*-1,3) alcohol position in the glycerol (Figure 6C).

Since hydrolysis of 9-PAHSA TAG estolide substrates gave rise to negligible levels of hydroxylated DAG, it is plausible that CEL rather cleaves 9-PAHSA-glycerol ester bond than FAHFA estolide bond (Figure 6C). It has been shown that free 9-PAHSA is more effectively hydrolyzed by CEL than 5-PAHSA (23). This rule also apply to TAG estolide substrates, as significantly higher levels of the final products (9-HSA and PA,  $p < 0.0001$ ), as well as lower levels of free 9-PAHSA ( $p < 0.0001$ ) were found compared to reactions with TAG EST containing 5-PAHSA substrate (Figure 6B).



**Figure 6:** CEL degradation products of TAG EST 70:4 containing either 5-PAHSA (5P) or 9-PAHSA (9P). TAG 5P #1 columns indicate samples, in which TAG EST 70:4 containing 5-PAHSA bound at *sn*-1/3 alcohol position of glycerol was used as a substrate, TAG 5P #2 represents samples with TAG EST 70:4 and 5-PAHSA at *sn*-2 position. TAG 9P #1 and #2 mark positional isomers of TAG EST containing 9-PAHSA. (A) Reaction products of LA-glycerol bond cleavage. (B) Reaction products of PAHSA-glycerol and estolide bond hydrolysis. (C) Products of estolide bond disruption within the TAG EST molecule. TAG 54:6 stands for trilinolein; DAG EST, diacylglycerol estolide; MAG EST, monoacylglycerol estolide; HSA, hydroxystearic acid; TAG (OH), hydroxylated triacylglycerol; DAG (OH), hydroxylated diacylglycerol; and MAG (OH), hydroxylated monoacylglycerol. Data are means  $\pm$  SEM,  $n = 3$ . T-test was used to compare *sn* positions (\* marks) or regioisomers (# marks),  $p < 0.05$ . Regioisomer comparison between panels is described in the text for clarity. No suitable internal standards were available for quantitation of hydroxylated acylglycerols.

## Discussion

Our data suggest that milk from VB group has the optimal balanced combination of free FAHFA and TAG estolides. Premature birth and/or caesarean section represent a situation in which the mammary gland is not ideally stimulated or ready for milk production, but the milk lipid composition normalizes in time.

The caesarean section rate is expected to increase above the medically necessary threshold in future neglecting the potential negative effects for the mother and the child (36). The caesarean section has a negative impact on breastfeeding initiation, duration, and difficulties (4, 37). Prior et al. systematically reviewed association between CS and breastfeeding and concluded that rates of early breastfeeding initiation were lower in CS compared with VB group (38). Delayed initiation of milk production could affect lipid composition of the colostrum acquired at 72 h postpartum (Figure 1A), resulting in higher phospholipid levels and lower TAG levels in CS group. Higher levels of phospholipid and sphingomyelin classes, that form the milk fat globule tri-layer, were obvious also in PB colostrum in agreement with previous observations (39). Differences in TAG and phospholipid

levels or in their ratio could be linked to milk fat globule size and potential digestibility (40, 41). In comparison to colostrum samples, mature milk metabolomic and lipidomic profiles were affected by multiple factors (diet, sample acquisition, etc.), but the differences vanished with advancing lactation.

The logical source of both free FAHFAs and TAG estolides in the milk is the breast white adipose tissue that transdifferentiated into milk-secreting glands during pregnancy (8). FAHFA levels produced into the milk are comparable to serum/plasma FAHFA concentrations (15) and the structure of milk fat globules (5) suggests that the TAG lipid core could store TAG estolides.

FAHFA production is dependent on *de novo* lipogenesis and/or dietary lipids (11, 19). However, the *de novo* lipogenesis in a mammary gland is different from white adipose tissue and the pathway produces mainly saturated fatty acids with 6-14 carbons (42-44). This medium-chain fatty acid synthesis in the human mammary gland starts with parturition, irrespective of length of pregnancy (45). Therefore, fatty acids for FAHFAs containing longer acyl chains (e.g. C16:0 and C18:0 for PAHSAs) are acquired from endogenous sources (liver and adipose tissue DNL) or diet and probably only the estolide bond formation takes place in the mammary gland. Preferential handling of fatty acids for TAG or FAHFA synthesis remains enigmatic. Although colostrum contains comparable levels of e.g. TAG-bound essential LA to mature milk, LA-derived polyunsaturated 13-LAHLA appeared only in mature milk. This might be linked to the unique TAG stereo-specific composition of human milk TAGs which contain conserved proportion of saturated FAs (mainly palmitic acid) at the *sn*-2 position (46).

TAG cores of milk fat globules are synthesized from cytoplasmic lipid droplets budding from the endoplasmic reticulum (5, 47). This pathway could produce also TAG estolides that have been detected only in adipose tissue (presumably in lipid droplets) and not in circulation (16, 21, 22). ATGL is one of several known FAHFA hydrolases, but this enzyme can also remodel acyl chains on TAGs and synthesize FAHFA-TAG combinations (20). ATGL is highly expressed in goat mammary gland, induced during lactation, and regulates TAG formation (48). Therefore, both FAHFAs and TAG estolides can be present within the lipid droplets and their levels can be controlled via ATGL within the epithelial cells or via CEL in the milk. However, it is currently unknown why are FAHFAs and TAG estolides exported to the milk together with their hydrolase CEL.

CEL activity could modulate free FAHFA levels, because TAG estolide concentration is an order of magnitude higher than free FAHFA levels. CEL concentrations are higher in mature milk than in colostrum, but no difference was observed between full term and preterm colostrum (49). We randomly selected 4 and 4 colostrum samples from the VB and PB group and performed proteomic analysis. Relative protein levels of CEL in colostrum were similar in VB and PB groups (Figure S1) in agreement with published data (49). However, the CEL activity was reported higher in preterm colostrum, resulting in a higher fat digesting potential during the early postnatal phase (50). Therefore, preterm colostrum might be optimized to help with the fat digestion at the expense of TAG estolides and FAHFAs.

The CEL *in vitro* assay showed that the enzyme hydrolyzes the glycerol ester and the estolide bonds at all positions of TAG estolide substrates. In agreement with data on CEL substrate preferences (23), we observed lower affinity to 5-PAHSA than 9-PAHSA isomer. This preference explains high levels of 5-PAHSA in VB colostrum samples and also high 5-PAHSA levels in colostrum from an independent study (10). In mouse experiments, 5-PAHSA successfully passed the gastrointestinal tract and reached circulation (10, 16). The privileged presence of 5-PAHSA in the colostrum could be linked to its anti-inflammatory effects and protection against ulcerative colitis (14). A recent screening study

concluded that FAHFAs with lower branching position are more likely to be anti-inflammatory (12), thus could help to prevent necrotizing enterocolitis in a newborn. Future studies will be needed to define whether FAHFA-rich milk from milk banks could be the preferred substitute for preterm newborns at risk for conditions such as necrotizing colitis (14). The degradation products of PAHSA-containing TAG estolides could also serve as precursors for the odour-active molecules in the milk that attract a newborn (51). Milk TAGs containing 5-hydroxy FA could produce stable  $\delta$ -lactones, e.g.  $\delta$ -octadecalactone as semivolatile flavor component in the cream fat (52, 53).

## Conclusion

Here we identified TAG estolides as a new component of human milk and broadened substrate specificity of CEL that can modulate milk FAHFA composition. Mothers and health care professional should be aware of the indication that CS and associated delayed breastfeeding initiation could negatively affect levels of anti-inflammatory lipid mediators in the milk.

## Limitations of the study.

Qualified personnel processed the colostrum samples at the maternity hospital, but mature milk samples were collected by the nursing mothers at home. Therefore, higher variability in milk composition has to be anticipated (mode and time of sample collection, diet, etc.). Our analytical approach is unable to separate TAG estolide isomers and we report the acyl composition of major species based on MS/MS data. The CEL lipolytic activities were measured under optimal in vitro assay conditions. However, the expected physical form of the TAG estolide substrate is a TAG lipid droplet core of a milk fat globule surrounded by a tri-layer phospholipid membrane. Digestion of the substrate in this unique microenvironment is a complex process and cannot be simulated in vitro.

## Clinical perspectives

- Breast milk provides the ideal nutrition for babies, but its composition is very complex and not completely characterized at specific conditions such as preterm birth or caesarean section. Especially bioactive lipids represent an extensive class of poorly explored molecules.
- Colostrum levels of 5-PAHSA, a lipid mediator with anti-inflammatory properties preventing colitis, were negatively affected by PB and CS. Furthermore, new FAHFA species and their TAG estolide storage form were identified in the milk. CEL substrate specificity assay towards TAG estolide isomers confirmed a special role of 5-PAHSA in human milk.
- Mothers delivering term babies vaginally produce colostrum rich in 5-PAHSA. CS and/or PB delay initiation of de novo lipogenesis, milk production, and FAHFA synthesis. Free 5-PAHSA, resistant to CEL hydrolysis, could contribute to prevention of intestinal inflammation in newborns. Our results could indicate a new approach in the use of donor milk in preterm infants.

## Data Availability

All raw LC–MS profiling data have been uploaded to the Zenodo repository and can be accessed under accession number: ZENODO #...

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Supplementary Information

### Supplementary methods

#### Milk protein mass spectrometry analysis

Protein-containing pellets from the MTBE extraction were processed according to Hartmannova et al. (54). Briefly, pellets corresponding to 100  $\mu\text{g}$  of protein were solubilized using sodium deoxycholate and digested sequentially with Lys-C and trypsin. LC-MS data were acquired using Exploris 480 (Thermo Scientific) mass spectrometer. Resulting raw files were processed in MaxQuant. Relative amounts of proteins (label-free quantification) were analyzed in Metaboanalyst 5.0 (55). Samples were log-transformed and auto-scaled.

### Supplementary figures

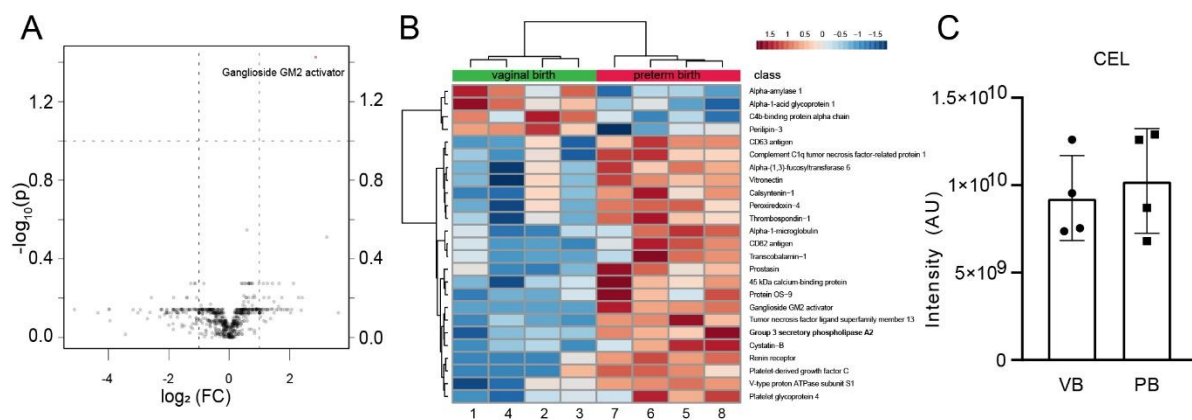


Figure S1: Proteomic profiling of the milk. A) Volcano plot of the identified proteins. False discovery rate set to p-value 0.1 with equal group variance. Volcano plot showed only one significantly altered protein (q-value 0.037) 'Ganglioside GM2 activator' which was higher in PB group. B) Heatmap representation of the 25 top-ranked proteins according to t-test score, distance measure Euclidean, clustering algorithm Ward. Heatmap visualization sorted according to the lowest p-value highlighted a panel of proteins with a higher relative concentration in PB group. Most of the proteins belong to leukocyte mediated immunity, vesicle-mediated transport, and secretion pathways. C) Milk levels of CEL, data are means  $\pm$  S.D.,  $n = 4$ . VB, vaginal birth; PB, preterm birth.

**Table S1**

Cluster name	Cluster size	p values	FDR	Key compound	Altered metabolites	Increased	Decreased	Increased ratio	Altered Ratio
Unsaturated TAG	140	1.5E-27	4.2E-26	TAG 52:6 (3); TAG 14:0_16:0_22:6; [M+NH4] <sup>+</sup>	71	0	71	0	0.5
PS	9	3.2E-10	4.6E-09	PS 38:3; PS 18:0_20:3; [M-H] <sup>-</sup>	8	8	0	1	0.9
PI	20	1.9E-09	0.00000018	PI 38:3 (2); PI 18:0_20:3; [M-H] <sup>-</sup>	10	10	0	1	0.5
PE	49	0.000000075	0.00000054	PE O-38:5 (1); PE O-16:1_22:4; [M-H] <sup>-</sup>	14	13	1	0.9	0.3
Saturated TAG	18	0.000000093	0.00000054	TAG 38:0; TAG 10:0_12:0_16:0; [M+NH4] <sup>+</sup>	10	0	10	0	0.6
SM	22	0.00000085	0.0000041	SM 36:5;2O; [M+H] <sup>+</sup>	4	3	1	0.8	0.2
Saturated DAG	3	0.000015	0.000062	DAG 26:0; DAG 12:0_14:0; [M+NH4] <sup>+</sup>	3	0	3	0	1
Oxidized SM	6	0.000033	0.00012	SM 42:1;3O; [M+H] <sup>+</sup>	4	4	0	1	0.7
Unsaturated DAG	29	0.000078	0.00025	DAG 32:6; DAG 10:0_22:6; [M+NH4] <sup>+</sup>	12	0	12	0	0.4
Unsaturated PC	62	0.059	0.17	PC 32:1; PC 16:0_16:1; [M+H] <sup>+</sup>	4	4	0	1	0.06
Saturated CAR	10	1	1	ACar 4:0; [M] <sup>+</sup>	1	1	0	1	0.1
MAG	4	1	1	MAG 16:0; [M+Na] <sup>+</sup>	0	0	0	0	0
Hex2Cer	4	1	1	Hex2Cer 34:1;2O; Hex2Cer 18:1;2O/16:0; [M+H] <sup>+</sup>	0	0	0	0	0
LysoPE	18	1	1	LPE 18:1 (2); [M+H] <sup>+</sup>	0	0	0	0	0
TAG EST	5	1	1	TAG-EST 68:2; 16:0_18:1_18:1-(O-16:0); [M+NH4] <sup>+</sup>	0	0	0	0	0
Saturated SM	5	1	1	SM 36:0;2O; [M+H] <sup>+</sup>	0	0	0	0	0
Unsaturated CAR	8	1	1	ACar 8:1; [M] <sup>+</sup>	1	1	0	1	0.1
FAHFA	10	1	1	5-PAHSA	1	0	1	0	0.1
O=FA_34_2	4	1	1	9-POHOA	0	0	0	0	0
O=FA_36_2	4	1	1	9-LAHSA	0	0	0	0	0
O=FA_36_4	3	1	1	13-OAHLA	0	0	0	0	0
PG	4	1	1	PG 36:2; PG 18:1_18:1; [M-H] <sup>-</sup>	0	0	0	0	0
Phospholipid ethers	5	1	1	PC O-38:5 (2); [M+H] <sup>+</sup>	0	0	0	0	0
Saturated FA	11	1	1	FA 18:0; [M-H] <sup>-</sup>	0	0	0	0	0
Saturated LPC	5	1	1	LPC 14:0/0:0; [M+H] <sup>+</sup>	0	0	0	0	0
Saturated lysophospholipids	4	1	1	LPE 16:0 (1); [M+H] <sup>+</sup>	0	0	0	0	0
Saturated PC	9	1	1	PC 32:0; PC 16:0_16:0; [M+H] <sup>+</sup>	0	0	0	0	0
Unsaturated FA	39	1	1	FA 16:4 (1); [M-H] <sup>-</sup>	1	0	1	0	0.03
Unsaturated LPC	8	1	1	LPC 18:1/0:0; [M+H] <sup>+</sup>	1	1	0	1	0.1

Table S2

Cluster name	Cluster size	p values	FDR	Key compound	Altered metabolites	Increased	Decreased	Increased ratio	Altered Ratio
PE	49	2.2E-20	2.1E-19	PE 40:6 (2); [M+H] <sup>+</sup>	29	29	0	1	0.6
PS	9	2.2E-20	2.1E-19	PS 38:3; PS 18:0_20:3; [M-H] <sup>-</sup>	9	9	0	1	1
Unsaturated PC	62	2.2E-20	2.1E-19	PC 36:5 (2); [M+H] <sup>+</sup>	26	26	0	1	0.4
PI	20	1.1E-16	8E-16	PI 38:3 (2); PI 18:0_20:3; [M-H] <sup>-</sup>	17	17	0	1	0.8
SM	22	7.2E-14	4.2E-13	SM 32:1 (2);2O; SM 18:1;2O/14:0; [M+H] <sup>+</sup>	16	16	0	1	0.7
Saturated SM	5	3.5E-13	1.7E-12	SM 32:0;2O; [M+H] <sup>+</sup>	5	5	0	1	1
Oxidized SM	6	0.000023	0.000095	SM 34:1 (1);3O; [M+H] <sup>+</sup>	5	5	0	1	0.8
PG	4	0.0019	0.0067	PG 36:1; PG 18:0_18:1; [M-H] <sup>-</sup>	2	2	0	1	0.5
Carnitines	10	0.0025	0.008	ACar 4:0; [M] <sup>+</sup>	4	4	0	1	0.4
Unsaturated DAG	29	0.047	0.14	DAG 34:2; DAG 16:0_18:2; [M+NH4] <sup>+</sup>	2	0	2	0	0.07
Phospholipid ethers	5	0.089	0.23	PC O-38:5 (2); [M+H] <sup>+</sup>	2	2	0	1	0.4
Unsaturated LPC	8	0.16	0.36	LPC 18:1/0:0; [M+H] <sup>+</sup>	2	2	0	1	0.2
O=FA_34_1	10	0.16	0.36	5-PAHSA	2	0	2	0	0.2
MAG	4	1	1	MAG 16:0; [M+Na] <sup>+</sup>	0	0	0	0	0
Hex2Cer	4	1	1	GM3 42:2;2O; [M-H] <sup>-</sup>	0	0	0	0	0
LysoPE	18	1	1	LPE 18:1 (2); [M+H] <sup>+</sup>	0	0	0	0	0
TAG EST	5	1	1	TAG-EST 68:3 (1); 16:0_18:1_18:2-(O-16:0); [M+NH4] <sup>+</sup>	0	0	0	0	0
Unsaturated CAR	8	1	1	ACar 10:1; [M] <sup>+</sup>	0	0	0	0	0
O=FA_34_2	4	1	1	9-PAHOA	0	0	0	0	0
O=FA_36_2	4	1	1	13/12-OAHSA	0	0	0	0	0
O=FA_36_4	3	1	1	13-PAHLA	0	0	0	0	0
Saturated DAG	3	1	1	DAG 28:0; DAG 12:0_16:0; [M+NH4] <sup>+</sup>	0	0	0	0	0
Saturated FA	11	1	1	FA 20:0 (2); [M-H] <sup>-</sup>	0	0	0	0	0
Saturated LPC	5	1	1	LPC 17:0/0:0; [M+H] <sup>+</sup>	1	1	0	1	0.2
Saturated lysophospholipids	4	1	1	LPE 18:0 (2); [M+H] <sup>+</sup>	0	0	0	0	0
Saturated PC	9	1	1	PC 32:0; PC 16:0_16:0; [M+H] <sup>+</sup>	0	0	0	0	0
Saturated TAG	18	1	1	TAG 30:0; TAG 8:0_10:0_12:0; [M+NH4] <sup>+</sup>	0	0	0	0	0
Unsaturated FA	39	1	1	FA 16:4 (1); [M-H] <sup>-</sup>	1	0	1	0	0.03
Unsaturated TAG	140	1	1	TAG 52:6 (3); TAG 14:0_16:0_22:6; [M+NH4] <sup>+</sup>	0	0	0	0	0