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Abbreviations:

AA: Arachidonic acid

ALA : Alfa-linolenic acid

CD: Cluster of differentiationdGLA: Dihomo-γ-linolenic acid

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FA: Fatty acid

FADS: Fatty acids desaturase gene cluster

GLA: Gamma-linolenic acid

IFN: Interferon

IL: Interleukin

LA: Linoleic acid

LCPUFA: Long-chain PUFA

PBMC: Peripheral blood mononuclear cells

PC(A): Principal component (Analyses)

PUFA: Polyunsaturated Fatty Acid

SNP: Single nucleotide polymorphism

Th: T-helper cell

TNF: Tumor necrosis factor

Abstract

Breast-milk long-chain polyunsaturated fatty acids (LCPUFA) have been associated with changes in early life immune responses and may modulate T-cell function in infancy. We studied the effect of maternal fatty acid desaturase (*FADS*) genotype and breast-milk LCPUFA levels on infant's blood T cell profiles and *ex vivo* produced cytokines after anti-CD3/CD28-stimulation of PBMCs in 6 months old infants from the $COPSAC_{2000}$ birth cohort. LCPUFA concentrations of breast-milk were assessed at 4 weeks of age and *FADS* single nucleotide polymorphisms (SNPs) were determined in both mothers and infants (n=109). In general, breast milk arachidonic acid (AA) levels were inversely correlated with the production of IL-10 (r=-0.25; p=0.004), IL-17 (r=-0.24; p=0.005), IL-5 (r=-0.21; p=0.014), and IL-13 (r=-0.17; p=0.047), while eicosapentaenoic acid (EPA) was positively correlated with the counts of blood Treg and cytotoxic T-cells, and decreased T helper cell counts. The minor *FADS* allele was associated with lower breast-milk AA and EPA, and infants of mothers carrying the minor allele of *FADS* SNP rs174556 had higher production of IL-10 (r=-0.23; p=0.018), IL-17 (r=-0.25; p=0.009) and IL-5 (r=-0.21; p=0.038) from *ex vivo* activated immune cells. We observed no association between T-cell distribution and maternal or infant *FADS* gene variants. We conclude that increased maternal LCPUFA synthesis and breast-milk AA is associated with decreased levels of IL-5, IL-13 (Type 2 related), IL-17 (Type 17-related) and IL-10 (regulatory immune responses), but not to IFN-γ and TNF-α, which could be due to an unconfounded effect of the maternal *FADS* variants on the offspring immune response being transferred via breast milk LCPUFA.

Introduction

Long-chain polyunsaturated fatty acids (LCPUFA) in breast-milk, specifically arachidonic acid (AA, 20:4 *n*-6) and docosahexaenoic acid (DHA, 22:6 *n*-3), may have beneficial effects on the development of the immune system in breastfed infants [(1)]. This is speculated because LCPUFA's are precursors of a range of immune-modulating signaling molecules [(2,3)]. The diet is the most important determinant of breast-milk LCPUFA content [(4)], but the content is also affected by the maternal capacity to convert the precursor molecules to LCPUFA products [(5,6)] catalyzed by desaturases and elongases.

Studies have reported that especially marine *n*-3 LCPUFA can modify immunological responses and change the Th1/Th2 balance [7,8] also in infants [9,8,10] It has been suggested that such alterations might have an epigenetic origin [11] thus imposing more sustained changes on the immunological phenotype. *In vitro* studies have further confirmed the effect of LCPUFAs on cytokine production and proliferation of T-cells [12].

Maternal intake of PUFA during lactation has been shown to modulate breast-milk DHA content and the immune responses in infants in a number of observational as well as a few randomized controlled trials [(11–14)]. However, assessing diet is connected to a high risk of confounding interactions and the randomized trials have not been conclusive. Moreover, to our knowledge, no one has reported on the influence of LCPUFA in breast-milk in relation to other than Th1 and Th2 based responses, such as the IL-17-producing Th17, and IL-10 from regulatory T-cells (Treg). Since most cytokines are produced by more than one cell type within peripheral blood mononuclear cells, we will use the terms Type 1 immune responses to refer to cytokines produced by Th1, cytotoxic T-cells, Natural Killer (NK) cells and innate lymphoid cells (ILC) type 1 (ILC1) with cardinal cytokines being interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), while Type 2 responses are based on Th2 cells and ILC2 and the cytokines IL-4, IL-5 and IL-13. Type 17 responses are based on Th17 and ILC3 producing IL-17A, and regulatory type responses on regulatory T cells (Treg) and regulatory dendritic cells and monocytes, all producing IL-10.

Recent studies have shown that single nucleotide polymorphisms (SNPs) in the fatty acid desaturase gene cluster (FADS) can affect maternal plasma and breast-milk PUFA composition (15,16). As the genetic variants are "randomized" during meiosis, the FADS SNPs can serve as a randomizing instrument independent of possible confounders (17).FADS have been shown to modify the effect of dietary fatty acid intake on health, that including the allergic diseases in children [20]. Moreover, it has been shown that the polymorphism of FADS genes can influence markers of the metabolic syndrome, and was demonstrated to influence the plasma total cholesterol, High and Density Lipids

and triglycerides levels in children [21,22] and therefore modulate the risk of cardiovascular diseases later in life. Further, these genes have been linked to neurodevelopmental outcomes in breastfed, although the results are still not unequivocal [5,6,23,24]. Also in relation to the breast milk composition, FADS genes seem to alter the effect of breastfeeding on risk of asthma, and for the homozygotes of the major allele no significant effect of breastfeeding on the development of asthma have been shown [25]

The aim of this study was to analyze the influence of maternal FADS SNPs on the profile and function of T-cells isolated from infants at 6 months of age enrolled in the $COPSAC_{2000}$ birth cohort. We also analyzed the associations between maternal *FADS* SNPs and breast-milk LCPUFA content as well as the association between breast-milk LCPUFA levels and infant immune responses.

Methods and materials

Cohort

The Copenhagen Prospective Study of Asthma in Childhood (COPSAC2000) is a single-center, birth cohort study, with the objective to assess the interaction between genes and environment in infants and young children with high-risk of asthma to identify early-life exposures with a putative role in the development and course of atopic diseases. The cohort consists of 411 children of mothers with asthma recruited between 1998 and 2001. Premature children (gestational age <36 weeks) as well as children suspected of chronic diseases or lung symptoms prior to inclusion were excluded. The COPSAC study is conducted in accordance with the Declaration of Helsinki and is approved by the Copenhagen Ethics Committee (KF 01-289/96 and KF 11-107/02) and the Danish Data Protection Agency (2008-41-1754). Oral and written informed consent was obtained from both parents at enrollment (18).

Breast-milk samples

Breast-milk samples (2-5ml) were collected at approximately four weeks post-partum. After addition of 0.01% 2.6-di-tert-butyl-4-methylphenol (Sigma Chemical Co, St Louis, MO), the breast-milk aliquots were frozen at -80°C. The analyses of the samples were completed within one year after the collection. The FA composition of the breast-milk was determined by extraction, KOH-catalyzed trans-methylation, gas-liquid chromatography (Hewlett-Packard Inc., Waldbronn, Germany) and use of commercial standards (Nu-Chek-Prep Inc, Elysian, MN) as previously described (19). Total values of the *n*-3 and *n*-6 PUFA families as well as the total of PUFAs were calculated using the full set of 11 PUFAs (19), but in this study we focused on LA, γ-linolenic acid

(GLA, 18:3 *n*-6), dihomo-γ-linolenic acid (dGLA, 20:3 *n*-6), AA, ALA, eicosapentaenoic acid (EPA, 20:5 *n*-3) and DHA.

T cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from 4ml peripheral blood drawn at the 6-month routine visit (mean age at the visit 6.35, SD=0.64) to the clinical research unit. The PBMCs were stored at -140°C for up to 12 years until the cells were analyzed during 2011. The overall composition of the T-cell compartment in PBMCs was assessed using flow cytometry with a predefined gating strategy: Cytotoxic T-cells (Tc; CD3+CD8+), T-helper cells (Th; CD3+CD4+), and regulatory T-cells (Treg; CD3+CD4+CD127^{lo}CD25^{hi}). The fraction of Tc- and Th-cells were calculated relative to total CD3+ cells, and Treg cells relative to CD3+CD4+ cells. Briefly, the methodology was as follows: Unstimulated PBMCs (5 x 10^5 cells) were washed once in FACS buffer (PBS containing 0.1% Sodium Azide and 1% heat-inactivated fetal bovine serum). Cells were stained with a pre-titrated fluorochrome-conjugated antibody mixture designed to characterize the T-cell compartment (CD3/eFlour450 (clone UCHT1), CD8/FITC (clone OKT8), CD127/APC-eFlour780 (clone A7R34) (all from eBioscience; San Diego, CA), and CD4/V500 (clone RPA-T4, BD Biosciences, San Jose, CA), CD25/PC7 (clone B1.49.9, Beckman Coulter, Brea, CA). 400,000 cells were analyzed on a BD FACSCanto™ II flow cytometer (BD Biosciences, Franklin Lakes, CA).

Polyclonal T-cell activation and cytokine analysis

PBMC (>95 % viable) were cultured in a concentration of 2x10^5 cells/200ul culture medium (RPMI 1640 containing 2mM L-glutamine (Cambrex, East Rutherford, NJ); 0.1M HEPES (Lonza); 100U/ml penicillin/streptomycin (Lonza) and 10% heat-inactivated fetal bovine serum (Cambrex)) in U-bottomed 96-well plates. T-cells were activated polyclonally by anti-CD3/28 expander beads (Dynabeads Human T-Activator CD3/CD28, Invitrogen) added at a beads-to-cell ratio of 1:2. Unstimulated cells were added culture medium alone. The PBMCs were stimulated for 40 hours in a humidified 37°C, 5% CO2 incubator and the response was assessed as cytokine production in cell-free culture supernatants. Supernatant cytokines interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-17A, IL-5, IL-13, IL-10 and IL-2 were analyzed using a custom multiplex assay from Meso Scale Discovery, and read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA). The cytokine levels detected in unstimulated PBMCs were subtracted from that of activated cells. All concentrations were above zero, also upon adjustment for background cytokine production.

FADS gene variants

Using a QIAamp DNA Blood Maxi Kit (QiagenInc, Valencia, CA), DNA was purified from blood samples of children and parents and stored at -80°C. Genotyping was performed in both the child and mothers, by high throughput genome-wide SNP genotyping, using the IlluminaInfinium II HumanHap550 BeadChip technology (Illumina, San Diego) as described previously [20–22] and hereafter imputed by use of the 1000 genomes reference CEU panel as previously described [31]. Five candidate SNPs were chosen based on previous studies [23] shown to be associated with PUFA metabolism. To reduce redundancy and because these SNPs are highly correlated (all in linkage with $r^2>0.5$), we took forward rs174546 and rs174556, the two SNPs that were originally genotyped while the remaing three were purely imputed. The two SNPs furthermore had a high degree of linkage disequilibrium with each other ($r^2=0.866$).

Data handling and statistics

From the $COPSAC_{2000}$ cohort of 411 children, 90 infants were excluded as they were not breastfed more than one month (n=38) or their mothers failed to provide a breast-milk sample (n=52). A further 190 infants did not have data on T-cell immunology, leaving 131 infants with complete milk and T-cell immunology data. Of these, genotyping data was unavailable in 22 mothers, resulting in 109 with complete data (Supplemental Figure 1).

Samples were handled in a random manner by the researchers performing the laboratory analyses. Results are presented as mean±SD or median (25-75 percentile) for normally and non-normally distributed data, respectively. Due to non-normal distribution of the cytokines, all correlations between breast-milk PUFAs, *FADS* SNPs, cytokines and T-cell distribution were tested using the non-parametric Spearman method. SNPs were analyzed in additive genetic models (effect per minor allele). For tables genotypes were converted to best guess genotypes. The statistical data analysis was performed with SPSS (version 20.0; SPSS Inc, Chicago, IL). To examine the patterns in the cytokine profile we used Principal Component Analysis (PCA) performed using Matlab (version 2013a; Mathworks) and subsequently used the two first principal components as continuous variables. Spearman correlations were used also to study correlations of the PCA scores and the FAs, T-cells count and SNPs. The content of individual FAs are presented as a percentage by weight of total FA content (FA%).Microsoft Office Excel 2007 software was used to create the heat-maps illustrating the correlation patterns between breast-milk PUFA, *FADS* SNPs and cytokines. The results were considered significant at the level $p\leq0.05$.

Results

The baseline description of the study population compared to the infants with incomplete data is shown in **Table 2.**

Infant immune profiles at 6 months of age

The T-cell phenotype distribution and mean *ex vivo* cytokine production after polyclonal activation in infants at 6 months of age are represented in **Table 1.** The overall infant cytokine response was analyzed by use of a multivariate principal component analysis (PCA) and the two principal components (PC1 and PC2) were found to explain 74% and 8.5% of the variation, respectively. PC1 separated the infants based on the overall level of cytokines produced, whereas PC2 segregated infants with a profile dominated by IFN-γ (Type 1) and IL-2 from infants with a profile associated mainly with IL-5 and IL-13 (Type 2), IL-17 (Type 17) and IL-10 (Regulatory cells) (**Figure 1a**). We analyzed the overall cytokine profile in the infants in relation to the maternal *FADS* genotype by color-coding according to the rs172556 gene variants. Some clustering of the infant cytokine profiles appeared based on maternal *FADS* genotype, and PC1 (overall cytokine level) correlated significantly with maternal gene variants of rs174556 (**Figure 1b and Figure 2**). The separation was however far from complete and the associations was studied further by univariate correlation analysis.

Maternal FADS SNP effects on immunological responses in infants

All individual cytokines were negatively correlated with the maternal *FADS* SNPs (Figure 2 and **Supplemental Figure 2**); and the correlations were significant for IL-17 vs. both *FADS*, and for IL-10, IL-5 and PC1 (which is a score for all cytokines production) with rs174556. No significant correlation was found between *FADS* SNPs in the infants and the *ex vivo* induced cytokine production (Figure 2).

Breast-milk concentrations of PUFAs

The overall FA composition of the breast-milk was 42.0±4.3 FA% saturated fatty acids, 40.5±3.4 FA% monounsaturated fatty acids and the mean total content of PUFAs was 13.7±2.6 FA% with a *n*-6/*n*-3 PUFA-ratio of 6.0±1.8. The breast-milk PUFA distribution was associated with the maternal *FADS* SNPs (**Table 3**), as minor allele carrying mothers of both SNPs had lower AA levels in their breast-milk. The breast-milk level of EPA was also decreased in minor allele carriers of rs174546 and tended to be so for rs174556, whereas no significant effects were seen on other PUFAs. Notably, none of the PUFAs were found to be highest in breast-milk from minor allele carriers.

Breast-milk PUFA and immunological response in infants

Univariate correlation analyses of breast-milk PUFAs and *ex vivo* produced cytokines revealed that generally breast-milk PUFAs were inversely associated with the cytokine production, except for breast-milk EPA that was significantly associated with an increased number of Tc and less Th cells and associated (although not significantly) with increased cytokine secretion from activated T-cells (Figure 2). AA was inversely correlated with IL-10, IL-17, IL-5, and IL-13, and GLA was inversely correlated with IL-17, IL-5, and IL-13.

When analyzing correlations between the relative numbers of Tc, Th and Treg in blood from the infants and maternal breast-milk PUFAs, we found an overall negative correlation between all breast-milk PUFAs and Treg counts; which was significant for dGLA and total *n*-6 PUFAs (Figure 2 and Supplemental Figure 2). As expected, PUFAs showed opposing correlations with %Tc and %Th, but this was only significant for EPA, which was negatively correlated with %Th and positively correlated with %Tc (Figure 2 and Supplemental Figure 2). The combined cytokine profile from PC1 (Figure 1a and 1b) revealed a significant inverse correlation with breast-milk GLA and AA, whereas PC2 was positively correlated with dGLA, but not with any other of the studied breast-milk PUFAs (Figure 2 and Supplemental Figure 2).

Discussion

Our results showed that infants of mothers carrying minor alleles of *FADS* rs174556 had higher *ex vivo* stimulated production of IL-10, IL-17 and IL-5 from PBMCs. We also found that higher breast-milk LCPUFA content was generally associated with lower infant cytokine production. The association was significant for AA and GLA levels, and IL-5 and IL-13, IL-17 and IL-10 (), but not for IFN-γ. AA levels in breast-milk were associated with maternal *FADS* gene variants.

The main strength of our study is that unlike most studies on the role of PUFA in the regulation of infant immunological responses, our study did not include oil supplementation, but rather focused on maternal *FADS* genotype and natural LCPUFA concentrations in the breast-milk. Thus, our study simulated a randomized study using the genetic variants as a randomization variable independent of possible confounding factors (Mendelian Randomization Analysis). The accuracy of our findings would probably be improved if we had performed repeated breast-milk sampling, to rule out short-term fluctuations in LCPUFA content. Fluctuations are largest in the *n*-3 LCPUFAs that to a greater extent depend on the dietary intake [(4)] and thus also are more prone to confounding. One limitation of our study is the relatively small sample size. Moreover, the long-term storage of PBMCs have been shown to affect the *ex vivo* cytokine responses [(24)], but as all samples were stored in the same way, and we found viability to be >95%, this factor is not likely to change the associations with maternal *FADS* and breast-milk PUFAs.

We examined a large amount of associations, but did not correct for multiple testing due to the strong correlations between the cytokines and between the SNPs and LCPUFAs, which allows for treating them as non-independent variables; instead we focused on the consistency in the results to exclude type-1 errors. A high consistency observed in described correlations is unlikely to be the effect of chance. The same cytokines (IL-10, IL-5, IL-13 and IL-17) that showed to be significantly associated with the GLA and/or AA were also significantly associated (borderline tendency for IL-13) with maternal SNP rs174556. Furthermore, we found more association than would be expected by chance.

The breast-milk fatty acid composition was determined prospectively 5 month before the assessment of infant cytokine profile and a shorter period or simultaneous measurements might perhaps result in stronger associations. However, the time gap indicates that the effect of infant LCPUFA supply remains significant over time and that *in utero* or early PUFA intake may have a programming effect on the immune response. Randomized trials with maternal *n*-3 LCPUFA supplementation indicate that the former could be the most likely [(25)], while it cannot be neglected

that more long-lasting effects can be imposed on the immune system via epigenetic changes, as was previously demonstrated for *n*-3 LCPUFA in the study by Lee et al. (2013).

FADS variants affect lipid metabolism and levels of specific PUFAs and served here as a randomization instrument, which indicated that the observed effect of maternal polymorphism on child's immunity could be due to changes in the breast milk LCPUFA. Experimental studies are necessary to explore the putative causal effect of this association. Although both the associations between maternal *FADS* SNPs and breast-milk AA and breast-milk AA and infant *ex vivo* cytokine production were more pronounced than associations with any of the other LCPUFA, this does not necessarily indicate that the effect is due to AA. Other studies of maternal *FADS* polymorphism and the PUFA composition in breast-milk also indicate that the content of AA is influenced by *FADS* variants to a larger extent than that of *n*-3 LCPUFA [26,27]. A longitudinal study of *FADS* variants and breast-milk PUFA found significant associations with AA and the AA/dGLA-ratio, but no associations for *n*-3 LCPUFA [28]. However, Xie and Innis reported that *FADS* minor allele homozygotes had lower breast-milk levels of both AA and EPA, but one of the examined SNPs also decreased DHA [29] and another study reported lower proportions of breast-milk DHA in *FADS* minor allele homozygous mothers [30]. *FADS* SNPs have been found to influence changes in the breast-milk fatty acid composition during the course of lactation, as the concentrations of saturated and *trans*-fatty acids increase markedly over time in the milk of minor allele carrying mothers, while no effect was seen on the time course in AA [28]. Colostrum AA levels have also been found to be decreased in minor allele carriers of a number of *FADS* SNPs, but a few of the SNPs was also reported to result in a decrease in colostrum DHA [30]. Furthermore, our recent study of *FADS* SNPs and LCPUFA status in infants showed that the effect of various SNPs may differ and vary over time [31]. It is therefore, not possible to determine neither the timing of the effect nor the responsible LCPUFA from the current study.

Studies on the role of breast-milk PUFA composition on offspring health have shown conflicting results [32–34]. Most of the studies investigating the effect of dietary PUFAs on infant cytokine responses have focused on the effect of fish oil-supplementation. D'Vaz et al. [9] found that direct supplementation of the infants with fish oil from birth resulted in decreased Th2-responses (IL-13 and IL-5) to allergens, and an increased polyclonal Th1-response as seen by increased IFN-γ and TNF-α, but no effect was seen on the IL-17 in response to mitogenic stimulation of PBMCs at 6 months of age. Another study on a fish oil-supplementation of healthy infants from 9 to 12 month of age demonstrated higher IFN-γ and a tendency for lower IL-10 production in stimulated whole-blood cultures [35]. An increased production of IFN-γ was also observed in lipopolysaccharide-stimulated whole blood cultures from 2½-year-old children of mothers supplemented with fish oil

during the first 4 months of lactation, but there was no difference in the IL-10 production [36]. In the present study, we observed significant changes in the production of IL-17, IL-5 and IL-13, which all correlated negatively with breast-milk AA and GLA.EPA was associated with an increase in the serum Tc% and concomitant decrease in Th as well as with an overall, although insignificant increase in the infant cytokine production. As Tc% also correlated positively with IFN-γ levels, it could be speculated that EPA may have an effect on T-cell immunology in the same direction as observed in the fish-oil supplementation studies. However, with the indicated opposing effect of EPA and AA and the enhancement of both EPA and AA by maternal FADS genotypes it will presumably be difficult to observe a significant effect of EPA under non-supplemented conditions. Regarding the potential effect of n-3 LCPUFA, the fact that the association with cytokine production was mainly seen for EPA, rather than DHA, does not mean that only EPA is promoting the effect, as EPA could merely be a better indicator of the variation in n-3 LCPUFA metabolism.

Studies have indicated an association between infant *FADS* variants and the development of eczema and asthma [37–39]. These variants have similarly been shown to alter the association between breastfeeding and risk of childhood asthma as well as the impact of dietary fatty acids on the risk of asthma, which has been shown to be strongest among homozygous major allele carriers [23,37]. We speculate that the altered capacity of cytokine secretion might influence predisposition for development of immune-mediated diseases such as asthma. We have observed the association of breast-milk PUFAs to be strongest in regard to reduction of the cytokines mostly attributed to the activity of Type 2, Type 17 and regulatory responses as opposed to those from Type 1 responses . Aberrant Type 2 cell responses is commonly associated with asthmatic inflammation and additionally the Type 17 cytokines has been shown to enhance Type 2 cell mediated allergic inflammation, contributing to severe asthma [40]. Our results therefore, suggest that the decrease of asthma and allergy risk by LCPUFA supply could be due to reduced Type 2 and and Type 17 activity in early life.

In conclusion, our results indicate that maternal *FADS* SNPs through a higher infant supply of LCPUFA are associated with a decrease in the production of cytokines related to Type 2, Type 17 and regulatory immune activity in the infant. Furthermore, our results indicate that breast-milk *n*-6 and *n*-3 LCPUFA may have opposite effect and that breast-milk *n*-3 LCPUFA stimulate overall infant cytokine production. By changing the type of the immune cell response, infant LCPUFA supply could modulate the susceptibility of the child to immune disorders, such as asthma and allergies, but further studies are necessary to elucidate the mechanisms.

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Conflict of interest statement

No authors declare a conflict of interest.

Authors Contributions

HB was responsible for design, initiation and conduct of the $COPSAC_{2000}$ cohort. LL, HB, SBi, SBr and EKM designed the present sub-study. JML conducted the PBMC activation analyses. MM and SBi was responsible for the statistical analyses. MM and LL drafted the manuscript. All authors contributed to writing of the manuscript, the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication.

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571 **Table 1.** Infant blood T-cell counts (as percentage of CD3+ cells) and *ex vivo* stimulated cytokine
572 production (pg/mL) at 6 month.

573 Abbreviations: Tc- Cytotoxic T cell, Th- T helper cell, Treg- regulatory T cell, IFN- γ- Interferon
574 gamma, TNF- α- Tumor Necrosis Factor alpha, IL- Interleukin.

575

	Median	**25;75 percentile**	**Min**	**Max**
%Tc	25.8	20.8;31.5	11.3	43.1
%Th	66.8	61.8;72.4	46.5	81.5
%Treg of Th	3.3	2.7;4.0	0.8	6.7
IFN-γ	743	409;1090	18.3	3270
IL-2	16158	6914;24358	5.3	809539
TNF-α	899	507;1360	19.0	2303
IL-10	37.0	17.2;68.4	1.0	247.3
IL-17	386	141;685	2.5	5136
IL-5	8.0	2.7;16.8	0.1	135.2
IL-13	207	87.8;387	0.3	1069

576 **Table 2.** Demographic characteristics of the studied infants and the rest of the COPSAC2000 birth
577 cohort.

		Included (n=131)	**Not included** (n=280)	**p**
Sex (% male)		51.1	48.6	0.627[1]
Gestational age (weeks)		39.95±1.61	39.88±1.55	0.634[2]
Birth weight (kg)		3.62±0.57	3.52±0.60	0.109[2]
Birth length (cm)		52.48±2.22	52.21±2.36	0.277[2]
Delivery (% natural, vacuum & cesarean)		62.6, 18.3 & 19.1	68.2, 10.4 & 21.4	0.080[1]
Apgar Score at 5 min (% ≥9)		95.3	97.1	0.357[1]
Breastfed period (weeks)		36.5±20.9	40.7±33.6	0.122[2]
Solely breastfed period (weeks)		21.7±25.6	39.3±50.2	**<0.01[2]**
Breastfed at blood sampling (% yes)		84 (64.1)	151 (53.9)	0.055[1]
Solely breastfed at blood sampling (% yes)		13 (9.9)	12 (4.3)	**0.044 [1]**
Ethnicity (% Caucasian)		96.9	96.4	0.787[1]
Mothers Education[3]	Low	81 (63.3)	144 (57.8)	0.593[1]
	Medium	31 (24.2)	69 (27.7)	
	University	16 (12.5)	36 (14.5)	
Mothers Occupation	Unemployed	12 (9.4)	26 (10.2)	0.097[1]
	Student	17 (13.4)	25 (9.8)	
	Non-professional	37 (29.1)	91 (35.7)	
	Professional	61 (48.0)	113 (44.3)	
Mothers age (years)		29.73±4.25	30.17±4.68	0.364[2]
Fathers age (years)		31.71±5.00	31.52±5.25	0.730[2]

578

579 Data are shown as mean±SD or n (%)

580 [1] Analyzed by Pearson's chi-squared test.

581 [2] Analyzed by independent samples T-test,

582 [3] Low (elementary school or college graduate), Medium (tradesman or medium
583 length), High (university candidate).

584

585 **Table 3.** Breast-milk PUFA composition at 4 weeks post-partum in *FADS* SNP major allele
586 homozygotes (MM), heterozygotes (Mm) and minor allele homozygotes (mm).

	mm	**Mm**	**MM**	**p**
rs174546	GG (n=15)	CG (n=38)	CC (n=56)	
LA[1]	9.29±2.03	9.57±2.08	10.04±2.05	0.342
GLA[2]	0.12 (0.08;0.22)	0.12 (0.06; 0.19)	0.16 (0.09;0.26)	0.215
dGLA[2]	0.41 (0.37;0.49)	0.41 (0.31;0.50)	0.41 (0.35;0.45)	0.927
AA[1]	0.49±0.12	0.55±0.14	0.63±0.14	**0.001**
Total n6 [1]	10.79±2.26	11.17±2.23	11.83±2.27	0.186
ALA[2]	0.19 (0.79;1.33)	1.33 (1.02;1.61)	1.26 (0.98;1.48)	0.502
EPA[2]	0.07 (0.00;0.10)	0.10 (0.07;0.16)	0.10 (0.08;0.15)	**0.028**
DHA[2]	0.41 (0.28;0.47)	0.46 (0.31;0.60)	0.49 (0.36;0.66)	0.124
Total n3[2]	1.93 (1.49;2.10)	2.03 (1.70;2.55)	2.08 (1.71;2.60)	0.237
rs174556	TT (n=14)	TC (n=37)	CC (n=58)	
LA[1]	9.17±2.05	9.72±2.04	9.95±2.08	0.443
GLA[2]	0.13 (0.09;0.22)	0.12 (0.06;0.20)	0.16 (0.09;0.25)	0.310
dGLA[2]	0.40 (0.36;0.50)	0.41 (0.31;0.50)	0.41 (0.34;0.45)	0.942
AA[1]	0.50±0.12	0.54±0.14	0.62±0.13	**0.001**
Total n6 [1]	10.70±2.32	11.32±2.19	11.73±2.30	0.283
ALA[2]	1.22 (0.99; 1.39)	1.35 (0.98;1.62)	1.26 (0.97;1.47)	0.649
EPA[2]	0.08 (0.04;0.10)	0.09 (0.07;0.17)	0.10 (0.08;0.15)	0.071
DHA[2]	0.41 (0.31;0.48)	0.44 (0.30;0.60)	0.48 (0.36;0.65)	0.209
Total n3[2]	1.94 (1.51;2.10)	1.99 (1.70;2.55)	2.08 (1.71;2.55)	0.448

587 Data are given as % by weight of all breast-milk fatty acids; means±SD or if not normally
588 distributed as median (25;75 percentile).
589 [1] Statistical comparisons were performed by One-way ANOVA test.
590 2 Statistical comparisons were performed by Kruskal-Wallis test.
591 Abbreviations: LA- Linoleic Acid, GLA-gamma Linolenic Acid, dGLA- dihomo Gamma Linolenic
592 Acid, AA- Arachidonic Acid, ALA- alpha Linolenic Acid, EPA- Eicosapentaenoic Acid, DHA-
593 Decosahexaenoic Acid, PUFA- Polyunsaturated Fatty Acid.
594

595 **Figures' legends**

596 **Figure 1.** Multivariate principal component analysis of cytokines produced by *ex vivo* stimulation
597 of infant PBMC at 6 month of age. The data are shown as the score plot displaying the dispersion of
598 each individual (as each dot represents one individual) in regard to the combined production of the
599 seven cytokines color-coded according to the maternal *FADS* rs172556 genotype (black,
600 diamond=MM, light gray, square=Mm and dark gray, circle=mm). The cytokines separated as
601 shown in the inserted loading plot. Variance explained by each component is placed on the axes.

602

603 **Figure 2.** Heat-map illustrating the patterns in correlations between breast-milk PUFA, maternal
604 and infants' *FADS* variants (rows) and *ex vivo* induced cytokines, T-cell counts, as well as
605 combined cytokine profiles from PC1 and PC2, derived from Figure 2 (columns). White boxes
606 represent negative correlations and black boxes positive correlations. The saturation of the color is a
607 representation of the size of the Spearman correlation coefficients. Bold underlined values indicate
608 that the correlation is statistically significant. Results are presented as correlation coefficient with
609 the p value placed in the brackets below.

610