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ORIGINAL CONTRIBUTION

Increased linoleic acid/α-linolenic acid ratio in Swedish cord blood samples collected between 1985 and 2005

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Abstract
Background Cord serum (CS) phospholipid fatty acid composition is associated with maternal diet during foetal life, and maternal intake of linoleic acid (LA, C18:2ω-6) and α-linolenic acid (LNA, C18:3 ω-3) has been shown to influence the LA and LNA levels in CS. A possible connection between the increased incidence of atopic diseases and increased intake of LA and decreased intake of LNA in the Western world has been proposed.
Aim The aim of this study was to explore phospholipid fatty acid proportions and total IgE levels in CS from Swedish children, collected from 1985 to 2005, a period with increasing frequency of allergic diseases in Sweden, and reveal possible changes over time.
Method Phospholipid fatty acids and total IgE antibodies were analysed with gas chromatography and UniCAP® technology, respectively, in 300 CS samples.
Results The proportions of LA and LNA decreased significantly from 1985 to 2005 ($p < 0.001$ for both). However, the LA/LNA ratio did increase ($p < 0.001$), revealing a relatively larger decrease in LNA than in LA. No correlations were found between ω-6 and ω-3 fatty acids and total IgE antibodies in CS from newborn children.
Conclusions The LA/LNA ratio increased ($p < 0.001$) in cord serum samples collected between 1985 and 2005, and no correlations between fatty acids and total IgE were found.

Keywords Cord blood · Fatty acids · Immunoglobulin E

Introduction

The essential fatty acids, that is, the omega(ω)-6 long-chain polyunsaturated fatty acid (PUFA) linoleic acid (18:2 ω-6, LA) and the ω-3 PUFA α-linoleic acid (18:3 ω-3, LNA), and their metabolites are important for foetal growth and development [1]. During foetal life, both ω-6 and ω-3 LCPUFA, particularly docosahexaenoic acid (22:6 ω-3, DHA) and arachidonic acid (20:4 ω-6, AA), are deposited in the central nervous system [2]. Membrane structure and stability, that is, fluidity within different cellular compartments, are essential for neuronal maturation and metabolic function as well as for advanced CNS activities [3]. The most abundant LCPUFA in the brain is DHA from the ω-3 series, which is concentrated at nerve cell synapses and is important for neural cell signalling and neurotransmitter processes [4]. The fatty acid demand of the foetus is met by maternal diet, maternal fat stores and metabolism [5]. Furthermore, fatty acid translocase (FAT/CD36), plasma membrane fatty acid–binding protein (FABPpm), a family of placental fatty acids transfer proteins (FATPs 1–6), and intracellular FATP support the preferential demand DHA and AA leading to higher relative concentrations of DHA and AA in the foetal than in the maternal circulation [6]. Thus, the fatty acid composition in cord serum (CS) gives

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an indication of fatty acid exposure during foetal life. The ω-6 and the ω-3 PUFA and their derivates have a broad range of other influences in humans: as facilitators of signal transmission in CNS [7], as regulators of gene transcription [8] and as the precursors of proinflammatory and anti-inflammatory molecular families [9].

The prevalence of allergic diseases, for example, asthma, allergic rhinoconjunctivitis and eczema, among children has increased during recent decades and is still increasing in many parts of the world [10, 11]. The rise in allergic diseases in some parts of the world during recent decades has been proposed to be associated with a coincident increase in the dietary intake of ω-6 PUFA [12, 13]. This association is based on the hypothesis that LA is metabolised to AA, which is the precursor of 2-series prostaglandins and 4-series leukotrienes involved in the pathogenesis of allergic disease [14]. This hypothesis has been supported by several other studies [15–17]. We have, however, previously reported a relationship between low levels of ω-3 PUFA and higher LA/LNA and AA/EPA (eicosapentaenoic acid, 20:5 ω-3) ratios in breast milk [18], maternal and infant serum phospholipids [19] and the development of allergic disease in infancy, suggesting that it is particularly the ω-6/ω-3 PUFA ratio that is important for early development of allergic disease.

Similar to the worldwide reported increase of allergic disease [10, 11], a recently published Swedish study have reported an increase in the prevalence of positive SPT (skin prick test) from 21 % in 1996 to 30 % in 2006, although the prevalence of allergic symptoms did not change [20]. Cord serum samples collected from all newborn babies in Linköping, Sweden, from 1985 to 2005 made it possible to assess changes in the levels of ω-6 and the ω-3 PUFA serum phospholipids over a period of time partly coinciding with the increase in allergic diseases.

The primary aim of this study is to investigate changes in ω-6 and the ω-3 PUFA phospholipid fatty acid profile in unselected CS samples collected from 1985 to 2005 and secondarily to asses a possible relation between PUFA phospholipids and the level of CS total IgE. We hypothesise that the CS proportions of ω-6 and the ω-3 PUFA phospholipids have changed with increasing proportions of LA and AA and decreasing proportions of LNA, resulting in increasing ratio of ω-6/ω-3 PUFA (LA/LNA) and that the increased ratio is related to CS increasing total IgE levels.

Materials and methods

Study population

This is a descriptive, prospective and observational pilot study. Umbilical cord serum samples were collected as part of a daily routine at the maternity ward, in cooperation with the department of paediatrics, at the University Hospital in Linköping, Sweden, from 1985 to 2006. The staff at the maternity ward was instructed to collect samples after normal deliveries of healthy full-term neonates. After delivery of the placenta, the umbilical cord at the placental site was carefully cleaned in order to avoid contamination with maternal blood. The umbilical cord was then squeezed and the cord blood was collected in tubes. The cord blood sample was collected immediately after delivery, stored in refrigerator until they were moved to the research laboratory where serum was separated by centrifugation and CS was stored at −20 °C. A consecutive number, date of birth and sex were recorded on the tubes. No additional data on the child are available. The CS sample collection comprises a total of approximately 39,000 samples. Samples ($n = 300$) from the first ten children (five boys and five girls) born during uneven months (i.e. January, March, May, July, September and November) every fifth year (i.e. 1985, 1990, 1995, 2000 and 2005) were selected for analyses. One sample, from a girl born in March 1990, was excluded from statistical calculations because of an exceptionally high total IgE value (44.3 kU/l) in combination with an unexpected CS phospholipid fatty acid chromatogram possibly owing to either contamination with maternal blood or most probably a maternal blood sample misplaced among the cord blood samples by mistake.

Analysis of fatty acids in phospholipids

Fatty acids with a chain length of 12–22 carbon atoms were identified in serum in phospholipids. The fatty acids LA (18:2 ω-6), LNA (18:2 ω-3), DHGLA (20:3 ω-6), AA (20:4 ω-6), EPA (20:5 ω-3) and DHA (22:4 ω-6) are reported here.

Total lipids from 200 µl freeze-dried serum and 25 µl of internal standard (C17:0 as phospholipid, 570 mg/l) were extracted three times according to Folch [21] using 0.01 % butylated hydroxytoluene (BHT, VWR International, Stockholm, Sweden) as an antioxidant. The lipids were evaporated with N_2 and reconstituted in 0.4 ml chloroform. Lipid fractions were separated on a SEP-PAK aminopropyl cartridge (Waters Sverige AB, Sollentuna, Sweden) according to a method originally described by Kaluzny et al. [22] and later adapted by Pietsch and Lorenz [23]. Total lipids were applied to cartridges preconditioned twice with 1 ml n-hexane. Neutral lipids, triglycerides and cholesterol were eluted with chloroform : isopropanol 2:1 (v/v) and discarded. Subsequently, the free fatty acids were eluted with diethyl ether : acetic acid 98:2 (v/v) and discarded. Finally, the phospholipids were eluted with methanol and subsequently dried in N_2 and transmethylated in methanolic HCl 3N (VWR) at 80 °C for 4 h. The fatty acid

methyl esters were extracted twice with *n*-hexane (VWR), washed twice with water and dried with water-free $MgSO_4$ (VWR). Finally, the extractions were dried with N_2 and reconstituted in 1.4 ml n-hexane Uvasol (VWR). The fatty acids methyl esters were separated by Agilent Technologies 6890N Network GC System gas chromatograph (Agilent Technologies, Stockholm, Sweden), equipped with a fused silica capillary column (45 m × 0.25 mm × 0.2 μm film thickness) (Supelco SP™-2380, Sigma-Aldrich). Helium was used as carrier gas and the injection was splitless. The injector and detector temperature was 250 °C. Column oven temperature was programmed from 60 °C with a heating rate of 8 °C/min up to 155 °C, 1.5 °C/min up to 180 °C and finally 6 °C/min up to 230 °C. The data were recorded and analysed with the Agilent Technologies Chemstation Software (Agilent Technologies). The fatty acids methyl esters were identified by comparing the retention times of the peaks with those of a known standard (GLC-461, Nu-Chek Prep.Inc, Elysian, MN, USA). The samples were analysed randomly to minimise methodological bias. The proportions were expressed as wt%. As a control regarding the quality of the method, a serum sample from a blood donor was analysed in every run. The interassay coefficient of variance was 1.2 % for LA (C18:2 ω-6), 1.7 % for AA (C20:4 ω-6) and 2.7 % for EPA (C20:5 ω-3) ($n = 24$).

Analysis of IgE antibodies

The presence of cord serum total IgE antibodies was assessed with the ImmunoCAP™ Total IgE Low Range (Phadia, Uppsala, Sweden), according to the manufacturer's instructions. The detection limit of the method was 0.05 kU/l.

Ethical aspects

The study was approved by the regional ethics board at the Faculty of Health Sciences, Linköping University, Linköping, Sweden.

Statistics

In an interventional study [24], we report the mean level of LA to be 6.3 ±1.0 mol% in CS phospholipids. To reveal a difference of 1 % unit on LA concentrations over a 20-year period with a p value <0.05 and 80 % power, 60 samples were needed. However, 300 samples were analysed in order to overcome possible quality problems. The phospholipid fatty acids were normally distributed, and changes over time were analysed with one-way analysis of variance (ANOVA) with Bonferroni as post hoc test. Total IgE levels were not normally distributed, and comparisons over time were therefore performed with the Kruskal–Wallis test. The total IgE levels were dichotomised, and the Spearman correlation was applied to assess an association between PUFA proportions and total IgE levels above the point of dichotomisation. The Mann–Whitney U test was used to compare PUFA levels of children displaying values above and below the points of total IgE dichotomisation. Statistical analyses were performed using Stat View for Windows Version 5.9 (SAS Institute Inc., Cary, North Carolina, USA).

Results

The amount of total fatty acids and mean relative proportions of LA, LNA, DHGLA, AA, EPA and DHA in CS phospholipids as well as total levels of IgE antibodies in CS is presented in Table 1. The absolute amount of total fatty acids in CS phospholipids was similar during the study period. Although the proportion of both LA (Fig. 1a; Table 1) and LNA (Table 1) decreased between the years 1985 and 2005 ($p < 0.01$ for both), the LA/LNA ratio increased ($p < 0.01$, Fig. 1b) during the same time period.

The ω-6 PUFA DHGLA (Table 1) and AA (Fig 1c) as well as ω-3 PUFA EPA and DHA (Table 1) proportions in CS phospholipids remained unchanged between 1985 and 1995 and increased later until 2005. The AA/DHA and AA/EPA (Table 1) ratio on the contrary seemed to increase between 1985 and 1995 and decreased later until 2005. This was more obvious for AA/EPA (Fig. 1d).

Total IgE levels ≥0.1 kU/L were detectable in 231/299 (77 %) of the samples, and levels ≥1.0 kU/L were detectable in 36/299 (12 %). CS total IgE levels did not change over time from 1985 to 2005 (Table 1). Correlations between CS IgE were analysed with cut-off levels set to both ≥0.1 kU/L and ≥0.5 kU/L and the relative concentrations of LA, LNA, DHGLA, AA, EPA or DHA at all time points (data not shown), and no correlation was found (data not shown). The relative levels of LA, DHGLA, AA, LNA, EPA and DHA and the ratios LA/LNA and AA/EPA were similar in CS phospholipids from children below as compared with children above the cut-off points for total IgE antibodies (data not shown).

Discussion

In this study, we report decreasing LA and LNA proportions in CS phospholipids of Swedish children during 2 decades. The LA/LNA ratio in CS during the same period of time, however, increased. The proportions of the longer ω-6 and ω-3 PUFA remained unchanged between 1985 and 1995 and increased later. No relationship was found

Table 1 Cord serum total IgE levels and proportions of phospholipid (PL) fatty acids in samples collected from 1985 to 2005

	1985 ($n = 60$) Median (25th–75th percentiles)	1990 ($n = 59$) Median (25th–75th percentiles)	1995 ($n = 60$) Median (25th–75th percentiles)	2000 ($n = 60$) Median (25th–75th percentiles)	2005 ($n = 60$) Median (25th–75th percentiles)	p value
Total IgE (kU/L)	0.21 (0.08–0.52)	0.23 (0.10–0.48)	0.22 (0.11–0.60)	0.19 (0.08–0.53)	0.22 (0.11–0.46)	ns[†]
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	p value
Total PL fatty acids (mg/l)	653 (141)	700 (154)	658 (138)	681 (139)	656 (137)	ns[‡]
w-6 PUFA (wt%)						
LA (C18:2 ω-6)	7.3 (0.94)[cde]	7.2 (1.2)[cde]	6.5 (0.9)[abe]	6.6 (1.2)[abe]	5.5 (1.1)[abcd]	***[‡]
DHGLA (C20:3 ω6)	4.43 (0.66)[e]	4.45 (0.73)[de]	4.30 (0.61)[d]	4.84 (0.78)[abc]	5.04 (0.80)[abc]	***[‡]
AA (C20:4 ω-6)	11.6 (1.58)[de]	11.9 (1.43)[de]	12.1 (1.64)[de]	13.5 (1.46)[abc]	14.0 (1.95)[abc]	***[‡]
w-3 PUFA (wt%)						
LNA (C18:3 ω-3)	0.09 (0.03)[bcde]	0.08 (0.02)[acde]	0.07 (0.02)[abde]	0.06 (0.01)[abc]	0.05 (0.01)[abc]	***[‡]
EPA (C20:5 ω-3)	0.31 (0.16)[de]	0.26 (0.12)[de]	0.27 (0.17)[de]	0.38 (0.15)[abc]	0.39 (0.15)[abc]	***[‡]
DHA (C22:6 ω-3)	4.49 (1.08)[de]	4.50 (1.08)[de]	4.34 (1.17)[de]	5.34 (1.40)[abce]	5.93 (1.35)[abcd]	***[‡]
Ratio ω-6/ω-3 PUFA						
LA/LNA	86 (31)[bcde]	100 (28)[ade]	102 (33)[ade]	127 (32)[abc]	115 (38)[abc]	***[‡]
AA/DHA	2.71 (0.69)	2.77 (0.63)	2.95 (0.82)[e]	2.69 (0.71)	2.47 (0.58)[c]	***[‡]
AA/EPA	43 (13)[bc]	53 (20)[ade]	53 (25)[ade]	41 (15)[bc]	41 (15)[bc]	***[‡]

ns Non significant

[†] Kruskal–Wallis

[‡] One-way ANOVA with Bonferroni

*** $p < 0.001$

[a] Significantly different from 1985

[b] Significantly different from 1990

[c] Significantly different from 1995

[d] Significantly different from 2000

[e] Significantly different from 2005

between PUFA phospholipid proportions and total IgE antibodies in CS from the children.

Contrary to the first part of the current study′s hypothesis, we found a significant decrease over time in both LA and LNA proportions in CS phospholipids in samples collected from Swedish newborns from 1985 to 2005. However, the second part of the hypothesis was supported: LNA decreased more than LA, resulting in an increased LA/LNA ratio over this period of time. In addition, the proportion of DHGLA and AA increased as well as EPA and DHA proportions between the years 1995 and 2005. The decrease of LA proportions in the current study was surprising as the common assumption claims that the intake of LA has increased markedly during recent decades [25] and that the increased dietary intake of LA ought to be reflected in the CS phospholipid PUFA profile, as shown by others [5].

The FAO Statistical Database (FAOSTAT, http://faostat.fao.org) provides statistics from over 200 countries on agriculture, nutrition, fish industry, forestry, food aid, land use and population. They present food balance sheets that represent a comprehensive picture of the pattern of a country's food supply during a specified reference period. The main source of LA is from vegetable oils, such as soybean oil, sunflower oil and safflower oil. Fat supply (g/capita/day) from soybean oil and to some extent also vegetable oils has decreased substantially in Sweden between 1985 and 2005 (Fig. 2), and this may explain the unexpected results in this study. We found a significant correlation between mean LNA proportions in CS as measured in this study and the average fat supply quantity (g/capita/day) from soybean oil at the same time points as presented by FAOSTAT ($r = 0.99$, $p = 0.023$, unpublished data) and a corresponding trend for LA proportions and soy bean oil supply ($r = 0.87$, $p = 0.056$, unpublished data). In addition, there was a trend to correlation between mean EPA and DHA proportions and the average fat supply quantity (g/capita/day) from fish and seafood, $r = 0.76$, $p = 0.14$ and $r = 0.76$, $p = 0.13$, respectively (unpublished data). We are well aware that the data from FAO are crude and only represent an estimation for the whole population and not for pregnant women, but as diet recommendations for pregnant women do not include

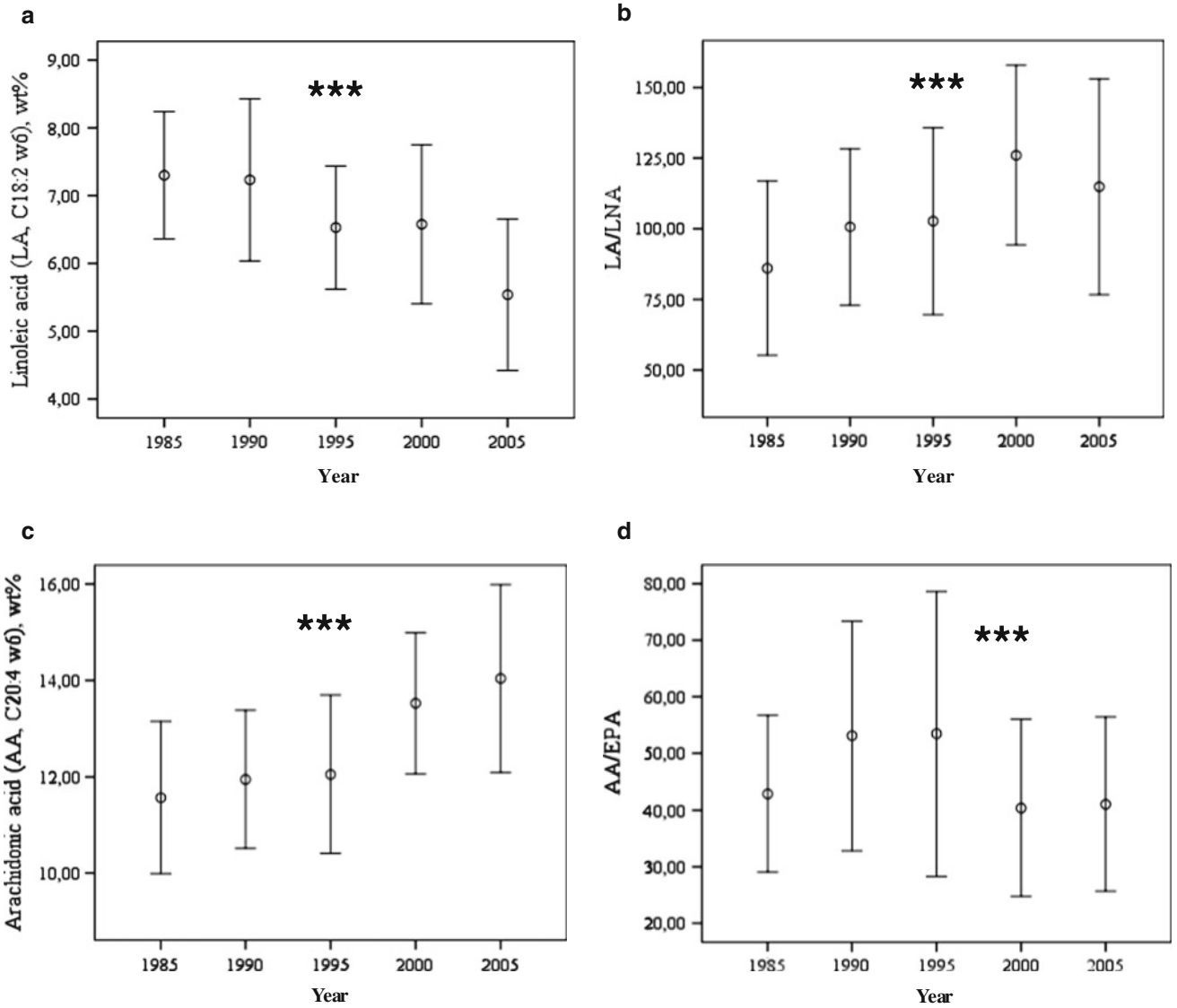

Fig. 1 Relative levels (wt%) of **a** linoleic acid (C18:2 ω-6) and **c** arachidonic acid (C20:4ω-6) in cord serum phospholipids of newborn babies analysed every fifth year from 1985 to 2005. **b** LA/LNA and **d** AA/EPA ratio in cord serum phospholipids of newborn babies analysed every fifth year from 1985 to 2005. Mean $\pm$ 1 SD is given. Significance level for ANOVA is given. For significance levels for differences between time points, see Table 1

vegetable oil, one might suspect that the intake is an average also for pregnant women. Nevertheless, the decrease in fat supply from vegetable oils corresponds well with decreasing LA levels in CS PUFA phospholipids reported in this study. Although Sweden seems to have a declining intake of vegetable oil, the levels of intake are still relatively high.

The 'Hodge and Black hypothesis' [26] postulates that the increased incidence and prevalence of allergic disease could be caused by a parallel increased consumption of LA. Data from the FAO food balance sheet have been related to ISAAC (International Study of Asthma and Allergies in Childhood) data on the prevalence of asthma, rhinitis and eczema [12]. There are multiple epidemiological and case control studies supporting the relationship between high LA intake and the prevalence of allergic disease [15, 17, 27]. The proposed causal link is based on increased conversion of LA to AA and thereby increased AA content in cells of tissues followed by increased formation of the 2-series prostaglandins, promoting the development of atopic disease. However, studies on AA exposure in early life measured as cord blood and breast milk AA content are inconclusive [28]. Based on our previous studies, we believe that it is in particular a high ω-6/ω-3 PUFA ratio (i.e. LA/LNA and AA/EPA) that seems to be of most interest concerning the relation between PUFA and the development of allergy in infancy [18, 19, 24, 29]. In this context, it is interesting that the increased LA/LNA ratio in

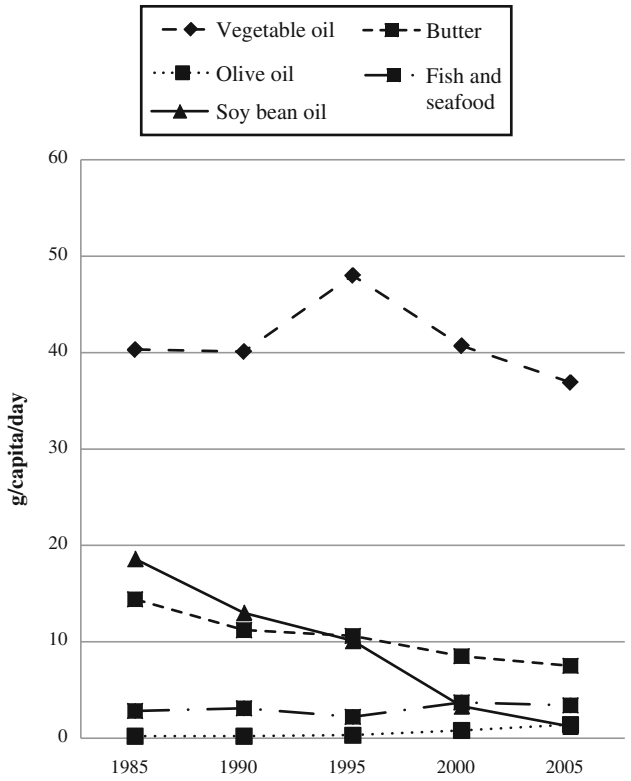

Fig. 2 Changes in fat supply quantity in g/capita/day in Sweden from different foods between 1985 and 2005 according to statistics from the FAO Statistical Database (FAOSTAT, http://faostat.fao.org/)

CS phospholipids of Swedish children in the current study coincided with a period of time with increasing allergic sensitisation in childhood in Sweden [20].

In this study, we could not find any correlation between CS phospholipid PUFA and CS total IgE antibodies. One could speculate that the study material was too small to observe a correlation. If there is such a relationship, it should not be influenced by time. No correlations were found even after pooling the data, suggesting that the lack of correlation is true. The similar levels of PUFA in CS phospholipids from children above and below the cut-off levels for total IgE (both 0.1 and 0.5 kU/L) verify the findings. Cord serum total IgE was chosen as a predictor for allergic disease. This parameter has proven to be a good predictor for IgE-mediated disease in previous studies, but the value has also been questioned [30–33]. An alternative method could have been to analyse chemokines in CS samples, as they can readily be measured in serum. A recent study showed that development of allergic disease during the first 2 years of life was associated with a more Th2-like immunity, that is, with increased levels of Th2-associated CCL22 and higher ratios of CCL22/CXCL10 and CCL22/CXCL11 in cord serum [32].

This observational pilot study is to the best of our knowledge the first study exploring changes in phospholipids fatty acid composition in CS of newborn children over two decades. The sample collection is unique with continuously collected samples from inhabitants in the same city exposed to a similar environment and lifestyle. The catchment area for the maternal ward at the University Hospital in Linköping comprises both rural and urban areas with an approximate population size of 200,000. However, there are some limitations. First, the samples have been frozen in −20 °C for a varying time ranging from 3 to 23 years. There is a risk of oxidisation of fatty acids and dehydration of the samples with subsequent risk of degeneration of proteins such as immunoglobulins. This is a major flaw, but cannot be avoided in these kinds of trials surveying such long time. Analysing the samples continuously over the last 20 years would probably have been connected with other disadvantages such as discontinuous staff, technical equipments and chemicals. A previous study assessing the relationship between serum phospholipid PUFA and breast cancer analysed samples stored for more than 10 years and reported some quantitative and qualitative fatty acid degradation over this time period [34]. Prior to our study, we analysed samples collected in 1985 and compared them with five recently collected CS samples. Comparisons of the chromatograms showed no differences, that is, no new peaks appeared or were missing, and the peaks were similar in height and area. Furthermore, cord serum total fatty acids phospholipid concentration and also the concentrations of individual fatty acids were in agreement with previous data from other groups [35] as well our group [24]. Taken together, this suggests that the phospholipids are intact although they have been stored for many years. Secondly, the lack of demographic data, clinical data regarding allergic heredity, maternal nutrition and allergic status of the children may be other major flaws, but this was beyond the aims of this study.

In conclusion, the proportions of LA in CS phospholipids decreased between 1985 and 2005 possibly as a result of a decreased maternal intake of vegetable oils. However, the LA/LNA ratio and AA, EPA and DHA proportions increased during the same period of time. No relationship between PUFA phospholipids and total IgE antibodies in CS from newborn children could be established.

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Conflict of interest The authors declare no conflict of interest.

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