Title: Extremely preterm infants receiving standard care receive very low levels of arachidonic and docosahexaenoic acids

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Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid

Abstract

Background & aims: Adequate supply of arachidonic (ARA) and docosahexaenoic (DHA) acids is essential for brain development, and extremely preterm infants may be at risk of deficiency. Current levels of ARA and DHA given to extremely preterm infants and the amounts available for accretion have not been established, although recent evidence suggests DHA intake is at a level likely to lead to severe deficits. This study quantified the omega-6 and omega-3 polyunsaturated fatty acid (PUFA) intakes from all sources in the first six weeks of life of preterm infants in standard care. In addition, the relationship between blood levels of circulating cytokines and PUFAs was explored.

Methods: Single centre longitudinal study with omega-6 and omega-3 PUFA intake data analysed from all sources for 17 infants born < 28 weeks gestation. At six weeks of age the infants' whole-blood fatty acid levels were measured along with a range of cytokines and chemokines analysed by Luminex® multiplex array.

Results: ARA intake was significantly below international recommendations in weeks 1-5 (all $p < 0.05$), and DHA intake was significantly below recommendations in week 1 ($p < 0.0001$). The amounts of ARA and DHA available for accretion were significantly below estimated accretion rates in all weeks (all $p < 0.001$). Mean ARA and DHA intakes were correlated with their respective blood levels ($r = 0.568$, $p = 0.017$ and $r = 0.704$, $p = 0.002$). There were significant relationships between MIP-1β and blood DHA levels ($rs = 0.559$, $p = 0.02$) and between RANTES and omega-6:omega-3 PUFA ratio ($rs = -0.498$, $p = 0.042$).

Conclusions: This study establishes that extremely preterm infants receive insufficient intakes of ARA and DHA. Moreover, blood fatty acid levels may provide a useful measure of intake, where establishing sufficient consumption could have clinical importance. There may also be important interactions between long-chain PUFA status and markers of inflammation, which requires further study.

Keywords: arachidonic acid, docosahexaenoic acid, breast milk, preterm infants, inflammation

1. Introduction

The brain is enriched in arachidonic (ARA) and docosahexaenoic (DHA) acids, long-chain polyunsaturated fatty acids (LC-PUFAs) of omega-6 and -3 series, respectively, with both essential for optimum brain development [1]. Fetal demand for ARA and DHA is high, especially in the last trimester, the period of maximal brain growth [2]. Prior to birth ARA and DHA are provided by placental transfer, and thereafter from breast milk and/or infant formula [3]. Although preterm infants are capable of synthesising ARA from linoleic acid (LA) and DHA from α-linolenic acid (ALA) the conversion is extremely limited [4]. Moreover, analyses of human infant autopsy tissue suggests that preterm infants are especially at risk of the developing fatty acid imbalances in the brain and retina in response to low ARA and DHA intake [5].

The Committee on Nutrition of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) have set recommendations for enteral nutrition in preterm infants for LA, ALA, ARA, DHA and eicosapentaenoic acid (EPA) [6]. However, these guidelines do not consider the greater requirements needed to compensate for early nutritional deficits and malabsorption or indeed reflect *in utero* accretion rates [7]. There are a range of estimated values for *in utero* ARA and DHA accretion rates depending on the background assumptions made [2, 8, 9]; however, the most recent estimates for accretion rates of ARA and DHA in the last trimester are 212 and 45 mg/kg/day, respectively [2], which are far higher than the ESPGHAN recommended intakes for ARA and DHA of 18 to 42 and 12 to 30 mg/kg/day, respectively [6].

To help to identify the optimum feeding regimes needed for extremely preterm infants to meet these recommendations and establish the potential deficits in ARA and DHA compared to *in utero* accretion rates it is necessary to quantify actual omega-6 and omega-3 PUFA intake from all sources. A recent study suggests DHA intake is at a level likely to lead to severe deficits [7]; however, the breast milk fatty acid composition was not directly measured in this study, nor importantly was ARA intake evaluated. This present study therefore extends these important initial observations by directly quantifying the omega-6 and omega-3 PUFA intake from all sources in extremely preterm infants born at < 28 weeks gestational age. Since the LC-PUFA composition of maternal milk varies widely [10], and ARA and DHA levels decline over time in the transition from colostrum to mature milk [11], the fatty acid composition of maternal breast milk was longitudinally measured at six time-points over the study. The levels of intake are presented as absolute intake levels of LA, ARA, ALA, EPA and DHA and levels of ARA and DHA available for accretion, which are calculated from the metabolizable levels and the amount endogenously synthesized, as recommended [7]. In addition, whole-blood fatty acid levels measured in week six were examined as potential useful biomarkers for fatty acid intake.

In the second part of the study, the relationship between blood LC-PUFA levels and markers of inflammation in the preterm infants was explored. Infection and inflammatory conditions are a major source of morbidity and mortality in premature infants and the vulnerability of the preterm infant to infection is well-described. Therefore, a robust immune response is essential for survival. In the neonate the blood brain barrier is more permeable than in the adult, and cytokines may gain direct access to the brain from the circulation [12] and pro-inflammatory cytokines have been shown to exert a toxic effect on the developing brain: [13].

DHA and EPA have well characterised anti-inflammatory properties [14] and the omega-6 to omega-3 PUFA balance has been shown to be a predictor of neonatal morbidities in preterm infants [15]. The aim of this analysis was to identify if there were any relationships between potential biomarkers of inflammation and blood omega-3 and -6 PUFA levels.

2. Materials and methods

2.1 Participants

This was a monocentric longitudinal study conducted in a tertiary, surgical neonatal unit in London, U.K. Participation was offered to all infants either inborn or transferred into the unit within 3 days of birth at < 28 weeks gestation. Infants with major congenital abnormalities, life-limiting conditions, from families who were not able to access the study information in English, or with mothers who were < 18 years of age at the start of the study were excluded. The West of Scotland Research Ethics Committee gave ethical approval, host site approval was confirmed by the Hospital's Joint Research & Enterprise Office and the University of Roehampton Ethics Committee. All mothers gave informed consent and the study was conducted according to the Declaration of Helsinki guidelines.

2.2 Study design

Intensive care and high dependency days were recorded as defined in SEND (standardised electronic neonatal database) for each infant. Daily weights, volumes of maternal, banked and formula milk consumed and parenteral lipid administered were recorded prospectively.

2.3 Determination of fatty acids in breast milk

Breast milk samples (0.5 – 5 mL) were collected at six time points in order to allow colostrum, transitional and mature milk to be sampled. For colostrum and transitional milk,

hand expression was used and for mature milk machine expression (Axifeed Fisio R, Orthofix Ltd, UK) was used. The time points were: sample 1, day 0 - 4; sample 2, day 5 - 9; sample 3, day 10 - 15; sample 4, day 16 - 23; sample 5, day 24 - 33 and sample 6, day 34 - 42. Where possible, expressions from more than one time-point on the day were pooled to allow for differences in milk expression during the course of 24 hours. All samples were frozen at -70^{0}C on the day of collection and analysed within two months.

The initial lipid extraction was from 0.5 mL of milk using the Bligh and Dyer method [16]. Tricosanoic acid was added at 0.5 mg/mL as an internal standard. Fatty acid methyl esters were prepared and analyzed by gas chromatography coupled with flame ionisation detector (Agilent Technologies, 7820A) using an Omegawax^{TM} column (30 m x 0.2 µm x 0.2 mm i.d., Sigma-Aldrich, UK), as described previously [17].

2.4 Whole blood fatty acid analysis

A drop of whole-blood for fatty acid analysis was collected from the infants by heel prick (about 20 µL) during the last study week and analysed as described previously [17]. Briefly, the samples were collected onto Whatman filter paper impregnated with 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene, BHT), at 50 mg / 100 mL in ethanol. The paper was air-dried for one hour and then wrapped in foil and sealed in polythene bag and kept at -80°C until analysis, which was typically within two weeks.

2.5 Blood cytokine and chemokine analysis

The blood samples for cytokine and chemokine analysis were collected in parallel with the samples for fatty acid analysis. Between 0.4 - 0.6 mL was collected into a SST serum separator gel microtainer. The sample stood at room temperature for 30 min, centrifuged at

3000 rpm for 10 min, and the serum frozen at -70°C. A bead-based multiplex assay (Luminex®, R&D systems) was used according to the manufacturer's instructions. The pro-inflammatory markers analysed were: tumour necrosis factor α (TNFα), Interleukin 1β (IL-1β), Interleukin 2 (IL-2), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 8 (IL-8), other inflammatory proteins: Interferon γ (IFN-γ), Granulocyte-macrophage colony-stimulating factor (GMCSF), Granulocyte colony stimulating factor (GCSF), Monocyte chemoattractant protein 1 (MCP-1), Macrophage inflammatory protein-1β (MIP-1β) and Regulated on Activation, Normal T Expressed and Secreted (RANTES). The anti-inflammatory markers were: Interleukin 10 (IL-10), Interleukin 1 receptor antagonist IL- 1ra), Interleukin 4 (IL-4), Interleukin 17 (IL-17). The Luminex® Performance Human Cytokine Panel A was used, and samples were read using the Luminex® Flexmap 3D analyser. All samples were analysed on the same plate.

2.6 Quantification of LA, ALA, ARA, EPA and DHA intake

The total LA, ALA, ARA, EPA and DHA intake from all sources was measured. Milk and parenteral lipid intake was recorded. Fatty acid intakes from formula milk were calculated based on manufacturer's published values, and milk samples of the infants' majority intake were analysed at six time points within the first six weeks of life. These values were compared with ESPGHAN guidelines [6].

2.7 Estimation of ARA and DHA available for metabolism and accretion

Estimations of ARA and DHA available for metabolism and accretion were based on previously published assumptions [7]. The amount of ARA and DHA available for metabolism was calculated based on intestinal absorption of rates of 80% for both PUFAs [18]. Absolute ARA and DHA synthesis in preterm infants at 1 month old fed LC- PUFAs

has been reported to be 27 ± 4 and 13 ± 4 mg/kg/day, respectively [19]. In our calculations values of 27 and 13 mg/kg/day for ARA and DHA synthesis were set when total energy intake was ≥ 100 kcal/kg/day and 9 and 4 mg/kg/day when intake was < 100 kcal/kg/day, respectively. The amounts of ARA and DHA available for accretion were calculated from the sum of metabolizable and endogenously synthesized values. These values were compared with published estimated accretion rates [2].

2.8 Statistical analysis

The results are reported according to STROBE guidelines [20]. Descriptive statistics were calculated for infant and maternal characteristics. Unless otherwise stated the results are presented as means (SD). Groups were compared by one-way ANOVA followed by the Tukey post hoc test. Fatty acid values were compared with published guidelines by one sample t-test. The cytokine and chemokine data were not normally distributed and are presented as median values (IQR) and correlations were evaluated using the two-sided Spearman test. This is an exploratory study and in all analyses $p < 0.05$ was considered statistically significant and there was no adjustment of p values for multiple comparisons to avoid Type-II errors, as recommended [21]. Statistical analysis was performed using either SPSS (IBM SPSS Inc., v.20.0) or GraphPad Prism (Version 6.07, GraphPad Software Inc.) Figures were prepared using GraphPad Prism.

3. Results

3.1. Participant characteristics

Participant flow is shown in Figure 1, where it can be seen that 24 preterm infants were recruited. 17 completed the study, all of whom were included in the final analyses. Maternal and infant clinical characteristics and infant clinical outcomes are shown in Table 1. The

infants' mean number of days to reach full feeds (150 mL/kg/day) was 19 (9.2) days, and the infants had a mean of 18 (9.1) days of parenteral nutrition support.

3.2 Wide variability of maternal breast milk fatty acid content over the first six weeks

The mean omega-6 and omega-3 PUFA concentrations of maternal breast milk are shown in Table 2. Over the six weeks of the study there were significant differences in the mean ARA and DHA content as determined by repeated measures one-way ANOVA ($F(2.046, 32.74) = 119.3$, $p < 0.0001$ and $F(2.120, 33.92) = 42.13$, $p < 0.001$), respectively. A Tukey post-hoc test revealed that the mean ARA content significantly decreased over the period of the study ($p < 0.05$), with the mean values of sample 6 comprising only 65% of sample 1. Similarly, there were also significant decreases in the DHA content over the study ($p < 0.05$), with sample 6 content only 64% of the value of sample 1. There was wide variability in ARA and DHA content between the individual mother's milks, as shown in Figure 2. There were no significant differences in the content of any of the other omega-6 PUFAs over the study. Although there were significant differences in the ALA between the groups ($F(3.093, 49.49) = 3.521$, $p < 0.02$), there were no observable trends across the study. There were also significant differences in the EPA concentrations ($F(2.659, 42.54) = 3.298$, $p < 0.03$); however, many of the values were at the limit of quantification and these results should be interpreted with caution.

3.3 LA, ARA, ALA, EPA and DHA intake levels in the preterm infants

The week-by-week total mean intakes of LA, ARA, ALA, EPA and DHA from both parenteral and enteral sources are given in Table 3, along with the metabolizable amounts (parenteral and enteral intake available for absorption) and amount of ARA and DHA available for accretion (metabolizable plus endogenously synthesized amounts). There were

significant differences in LA intake across the study ($F(5, 96) = 8.204$, $p < 0.0001$), with intakes significantly lower in weeks four, five and six, than weeks one and two (all at $p < 0.05$). LA intake was significantly below the minimum ESPGHAN recommended intake levels of 385 mg/kg/day in week 6 ($t = 2.9782$, $p = 0.009$) [6]. ALA intake significantly differed across the study ($F(5, 96) = 9.392$, $p < 0.0001$), with intakes significantly lower in weeks four, five and six, than weeks one and two (all at $p < 0.05$). ALA was significantly below the ESPGHAN guidelines of > 55 mg/kg/day in week six ($t = 6.2188$, $p < 0.0001$). The mean intake of ARA differed across the study ($F(3.318, 53.09) = 14.52$, $p < 0.0001$) with significant increases from the first to the second week ($p < 0.05$) and then remained at similar levels for the remainder of the study. The ARA intake was significantly below the ESPGHAN minimum intake levels of 18 mg/kg/day in weeks one to five (all $p < 0.05$). DHA intake differed across the study ($F(3.863, 61.81) = 7.933$, $p < 0.0001$) increasing significantly from the first to the second week ($p < 0.05$) and then remaining at similar levels. DHA was significantly below the minimum ESPGHAN intake guidelines of 12 mg/kg/day in week one only ($t = 16.0801$, $p < 0.001$). EPA intake levels were not significantly different across the study, and were within EPSGHAN recommended levels.

3.4 The ARA and DHA content available for accretion leads to deficits in the preterm infants

The mean ARA and DHA available for accretion significantly increased between the first and second weeks, ($F(5, 96) = 9.415$, $p < 0.001$ and $F(5, 96) = 6.760$, $p < 0.001$, respectively), and then both remained at about these levels for the remaining period of the study (Figure 3). These values were all significantly below the values estimated for ARA and DHA provided *in utero* (all values $p < 0.0001$). The values for the cumulative mean ARA and DHA deficits produced over the six weeks of the study were calculated by successively adding the weekly deficits derived from the individual daily deficits, and are shown in Figure 4. The ARA

values at the end of the 6 weeks represented 13.5% of the levels that should have been provided *in utero*, whereas for DHA the value was 36.6%. To compensate for these deficits an additional 183.4 mg/kg/day of ARA and 28.5 mg/kg/day of DHA available for accretion would be required to match levels provided *in utero*.

3.5 Relationships between intake and whole-blood fatty acid levels

The mean (SD) whole-blood levels of the omega-6 and omega-3 PUFAs of the preterm infants at six weeks and the mean intakes of LA, ARA, ALA, EPA and DHA are shown in Table 4. The mean haemoglobin level in week six was 110.9 g/L (15.1 g/L), and none of the infants were considered clinically anaemic. Blood samples were not taken until week six to limit complications due to the number of transfusions given. The mean number of transfusions over the study was 6.4 (3.4), with the mean number in week one 1.8 (1.3), week two, 1.3 (1.2), weeks three and four, 2.2 (1.5) and weeks five and six, 1.1 (0.9). The strength of relationship between mean intake levels and blood fatty acid levels was estimated by Pearson product-moment correlation coefficient. There were significant positive correlations between mean DHA intake and blood DHA levels ($r = 0.704$, $p = 0.002$), mean ARA intake and blood ARA levels ($r = 0.568$, $p = 0.017$) and mean EPA intake and blood EPA levels ($r = 0.572$, $p = 0.016$). There were no significant correlations with LA or ALA levels and their respective blood levels.

3.6 Relationships between whole-blood DHA and cytokine and chemokine levels

In the final part of the study the relationship between the whole-blood levels DHA levels and a range of pro- and anti-inflammatory cytokine and chemokines was explored at six weeks. As the cytokine and chemokine values were not normally distributed relationships were estimated using Spearman’s rank order tests. There were significant correlations between

MIP-1β and DHA (r_s = 0.559, p = 0.02) and MIP-1β and AA/DHA ratio (r_s = -0.690, p = 0.002), as well as between MIP-1β and the omega-6:omega-3 PUFA ratio (r_s= -0.716, p = 0.001). The omega-6:omega-3 PUFA ratio was also negatively correlated with RANTES (r_s = -0.498, p = 0.042). There were no significant correlations with any of the other cytokines or chemokines.

4. Discussion

This is the first study to quantify the omega-6 and omega-3 PUFA intake from all sources of extremely preterm infants born at less than 28 weeks over the first six weeks of care. From these results it can be seen that the infants were receiving intakes below ESPGHAN guidelines for LA, ALA, ARA and DHA and furthermore, the intakes of ARA and DHA were well below estimated *in utero* accretion rates. Importantly, these deficits occurred in spite of the infants receiving maternal breast milk from an early stage, and may be due to the wide variability in ARA and DHA content of the breast milk between the mothers, as has been shown by others [10]. These results are of clinical importance as low ARA and DHA levels in preterm infants may adversely affect neural development and health outcomes [22].

It has been suggested that the smallest infants at threshold viability and birth weight have the greatest relative deficit in LC-PUFAs, due to the low level of provision of preformed ARA and DHA and limited efficiency in the conversion of LA and ALA to the LC-PUFAs [4]. Much research has been focused on the effects of supplementing preterm infants with DHA and ARA, with heterogeneous result. These mixed results may be due to a range of methodological issues, but also importantly due to a lack of recruitment of sufficient numbers of very small, very immature infants [4]. Furthermore, most supplementation trials have attempted to supplement human or formula milk to reach levels typical of the ‘average’ DHA

in human milk for term infants. During pregnancy there is a preferential transfer of ARA and DHA across the placenta [23], which is very different to the supply available in term breast milk.

Recommendations for enteral nutrient intake aim to provide levels needed to achieve growth similar to fetal growth and satisfactory functional development [6]. However, these recommendations do not consider the potential additional needs required to compensate for early nutritional deficits and it may therefore be more appropriate to consider intake levels compared to *in utero* accretion rates. In the present study, therefore in addition to quantifying the absolute intake of omega-6 and omega-3 PUFAs and comparing these to ESPGHAN recommendations, the amounts of ARA and DHA available for metabolism and accretion were also calculated, based on previously published assumptions [7]. In both of the analyses the results confirm the previous observation that current feeding practices provide levels of DHA intake that are likely to lead to severe deficits; however, the present results also provides the first evidence that deficits in ARA are likely to be of an even greater magnitude. The effects of these intake levels were investigated on whole blood fatty acid levels, as a surrogate marker for whole body stores.

Lower blood levels of ARA and DHA are associated with increased risk of neonatal morbidities in preterm infants [15]. It is therefore important to establish whether the low level of intake found in the present study is reflected in the blood levels. However, analysis of blood levels in this patient group is complicated due to the number of transfusions they typically receive. For this reason whole-blood fatty acid analysis was not undertaken until week six of the study and it must be acknowledged that even by this time only two infants did not receive transfusions in the two weeks preceding the sampling. However, only three

infants received more than one transfusion and significant correlations were found for ARA, DHA and EPA intake and blood levels, suggesting whole-blood samples may provide useful clinical information, even in extremely preterm infants.

As blood levels were not taken until week six it was not possible to assess whether the level of intake led to decreases in LC-PUFA levels over the study and comparisons must be made to published values. Martin and co-workers report significant rapid declines in DHA and ARA composition in preterm infants after birth [15]. The DHA values reported in their study at week four are similar to those observed in the present study at week six, consistent with a need for DHA supplementation. Whereas the values we report for ARA are similar to those shown at week one, and are therefore not consistent with deficiency. Similarly, compared to the ARA and DHA values reported in a recent study by Baack and co-workers, the results in the present study do not suggest a deficiency [24].

However, comparisons with published values are complicated due the results being expressed as percent composition values based on area normalisation. With this approach the composition values do not provide absolute concentrations and are strongly interdependent and vary depending on the number of fatty acids analysed. Omissions or additions of fatty acids in the analyses will affect the values of the other fatty acids reported. This and other methodological differences may explain the apparent anomaly with blood values not apparently indicative of deficiency. These types of difficulties comparing whole-blood fatty acid results between studies have been reported by others [25]. Therefore, overall with data based on the single whole-blood samples taken at six weeks it is not possible to confirm that the DHA and ARA intake was insufficient to compensate for *in utero* provision; however, with the low levels of intake and equivocal nature of the comparisons to other studies

supplementation of extremely preterm infants with additional preformed DHA and ARA may be prudent.

The results show that assuming an intestinal absorption rate of 80% additional intakes of 230 mg/kg/day of ARA and 36 mg/kg/day of DHA would be needed to provide levels similar to those found *in utero*. Whilst this level of DHA intake has been achieved in intervention studies, ARA supplementation is typically either given at doses similar to those of DHA, or not at all [26]. However, a recent study reported beneficial effects on blood fatty acid status and psychomotor development of extremely preterm infants provided with ARA at twice the levels of DHA [27]. Future trials should seek to identify the effects of higher levels of ARA intake which have been identified from enteral intake requirements.

Most supplementation trials to date have used the enteral route once feeds are established. In our cohort of extremely small infants, the mean duration to reach full feeds was 19 days, which is representative of reported time to full feed intervals for infants of this gestation [28]. This leaves an important 2-3 week gap in which reduced amounts of preformed ARA or DHA are available. Newer parenteral lipid formulations which include fish oils as their source of LC-PUFAs are potentially available for use in neonates. There is encouraging evidence for their safety and tolerance to date [28]. However, long-term parenteral nutrition is not the norm for the extremely preterm infant, and adequate supplementation once enteral feeds are established remains a concern.

Finally, the relationship between circulating cytokine and chemokines and blood PUFA levels was investigated. This exploratory part of the study identified some potential relationships between blood omega-6 and omega-3 PUFA status and markers of

inflammation. The most consistent observations were seen with MIP-1β. MIP-1β is a chemotactic cytokine produced by macrophages, dendritic cells and lymphocytes and has chemotactic and pro-inflammatory effects, but can also promote homeostasis [29]. Significant positive correlations were shown between MIP-1β and DHA, with negative correlations between MIP-1 β and the ARA/DHA ratio, as well as between MIP-1 β and omega-6/omega-3 PUFA ratio. These data suggest that as the omega-3 PUFA status increases, the infant's ability to produce MIP-1β increases to that seen in a well term infant [30]. The omega-6:omega-3 PUFA ratio was also negatively correlated with RANTES. RANTES is a chemoattractant for monocytes, memory T-helper cells and eosinophils [31]. Plasma RANTES levels are significantly lower in preterm infants with sepsis, disseminated intravascular coagulation and necrotising enterocolitis (NEC) [31]. These data suggest that a normal (as opposed to a low) level of RANTES is seen with higher omega-3 PUFA status. Overall, these data are suggestive of interactions between LC-PUFA status and markers of inflammation; however, further research with larger numbers studied longitudinally is required to confirm these observations.

Important strengths of this study are that it was conducted at a neonatal centre which is also a surgical centre, and consequently infants with a wide range of conditions were included, and the milk fatty acid composition was measured at six time-points, so the subtle changes in the fatty acid profile over time were identified. Moreover, the omega-6 and omega-3 PUFA intake was quantified from all sources, and importantly the amount of ARA available for accretion was quantified for the first time. Blood fatty acid status was measured by a validated dried blood spot method, which is preferable for the assessment of circulating fatty acid fractions in preterm infants [24]; however, as noted above, blood fatty acid levels were only assessed at one time-point, six weeks. Furthermore, the large number of transfusions

400 received by some of the infants in this study may constitute a source of LC-PUFAs, which
401 was not considered in the analysis. This study was conducted on extremely preterm infants,
402 and therefore the results may not be applicable to infants born at a later gestational age;
403 however, as information on this group is underrepresented in the literature this focus should
404 be considered a strength of the study. It must be acknowledged that the study was only
405 conducted at one site on a small number of infants; however, the nature and extent of the low
406 DHA intake is consistent with results reported by others [7], suggesting the observations of
407 low levels of DHA and ARA intake are applicable to other sites in the UK and across Europe.
408 The results do however need to be confirmed by further research on a larger number of
409 infants at other sites. Finally, a number of assumptions were used in calculating levels of
410 ARA and DHA available for accretion; however, these assumptions were based on previous
411 literature [7].
412
413 The results indicate that omega-6 and omega-3 PUFA intake in extremely preterm infants
414 receiving standard care is likely to lead to deficits in ARA and DHA. These results confirm
415 and extend previous observations that current parenteral and enteral nutritional practices for
416 the extremely preterm infant are likely to fall below the levels of LC-PUFAs provided *in*
417 *utero*. There is growing evidence of the importance of optimising ARA and DHA provision
418 postnatally not only for brain function, but also to potentially reduce the morbidity and
419 mortality from conditions such as NEC, bronchopulmonary dysplasia (BPD) and retinopathy
420 of prematurity (ROP) [4] and the present results highlight the need for supplementing preterm
421 infants with preformed ARA and DHA. Furthermore, the positive correlations between intake
422 of ARA, DHA and EPA and blood levels suggests preterm infants are responsive to different
423 levels of intake, and that measuring blood levels may provide a useful clinical marker of
424 sufficiency.

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Statement of authorship

SD and LDR designed the research; SD, LDR and HH undertook the research and analysed the data; the paper was written by SD and LDR,SD was responsible for the final content of the paper and the final manuscript was read and approved by all of the authors.

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Figures Legends and Tables

Figure 1 Flow of participants through the study

Figure 2 (A) ARA and (B) DHA breast milk content from individual mothers. Sample 1, days 0 - 4; sample 2, days 5 - 9; sample 3, days 10 - 15 sample 4, days 16 - 23; sample 5, days 24 - 33 and sample 6, days 34 - 42

Figure 3 (A) ARA and (B) DHA available for metabolism (i.e. parenteral and absorbed) and available for accretion (i.e. metabolizable and endogenously synthesized) for the 17 infants. Values expressed as mean (SD). Estimated ARA and DHA *in utero* accretions rates of 212 and 45 mg/kg/day, respectively, are shown for reference.

Figure 4. Cumulative (A) ARA and (B) DHA deficits of the 17 infants over the 6 weeks of the study compared to estimated *in utero* accretion rates. The cumulative deficits were calculated based on estimated *in utero* accretion rates of 212 and 45 mg/kg/day for ARA and DHA, respectively using previously published assumptions [7].

547 Table 1. Clinical characteristics and clinical outcomes of the 17 preterm infants born at < 28

548 weeks gestational age

Clinical characteristics	Mean (SD) / number (%)
Maternal age (yr)	31.8 (6)
Antenatal steroid treatment	14 (82)
Pregnancy complications	
Pre eclampsia	1 (6)
Chorioamnionitis	6 (35)
Antepartum haemorrhage	6 (35)
Pre-labour rupture of membranes	3 (18)
Multiple pregnancy	2 (12)
Gestational age (weeks)	25.3 (1.1)
Gender, boys	9 (53)
Birth weight (g)	770 (135)
Birth weight z score	0.12 (0.45)
Clinical outcomes	
PDA	13 (76)
NEC, Bell's stage 2 and above	2 (12)
IVH grade 3 or 4	2 (12)
PVL	1 (6)
Sepsis requiring 5 days antibiotic treatment	16 (94)
ROP requiring laser therapy	5 (29)
CLD	
Oxygen therapy at 28 days of life	17 (100)
Non-invasive respiratory support at 6 weeks of age	9 (53)

Abbreviations: PDA, patent ductus arteriosus; NEC, necrotising enterocolitis; IVH, intraventricular haemorrhage; PVL, peri-ventricular leukomalacia, ROP, retinopathy of prematurity, and CLD, chronic lung disease.

573 Table 2. Mean (SD) omega-3 and omega-6 PUFA content of maternal breast milk of 17

574 mothers of extremely preterm infants[1]

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
18:2n-6	1.601	1.493	1.483	1.665	1.583	1.611
(LA)	(0.302)	(0.269)	(0.370)	(0.266)	(0.330)	(0.346)
18:3n-6	0.018	0.019	0.020	0.018	0.018	0.020
	(0.006)	(0.006)	(0.007)	(0.007)	(0.006)	(0.009)
20:2n-6	0.043	0.046	0.049	0.046	0.052	0.047
	(0.012)	(0.010)	(0.013)	(0.011)	(0.009)	(0.011)
20:3n-6	0.059	0.057	0.059	0.059	0.061	0.066
	(0.014)	(0.012)	(0.013)	(0.017)	(0.016)	(0.022)
20:4n-6	0.151	0.137	0.127	0.116	0.107	0.098
(ARA)	(0.034)	(0.031)	(0.031)	(0.033)	(0.033)	(0.033)
22:4n-6	0.024	0.030	0.027	0.028	0.026	0.029
	(0.008)	(0.011)	(0.007)	(0.013)	(0.009)	(0.012)
18:3n-3	0.107	0.101	0.097	0.094	0.104	0.118
(ALA)	(0.026)	(0.026)	(0.022)	(0.018)	(0.026)	(0.027)
20:3n-3	0.014	0.018	0.016	0.018	0.014	0.016
	(0.009)	(0.009)	(0.006)	(0.010)	(0.008)	(0.010)
20:5n-3	0.006	0.006	0.010	0.009	0.013	0.013
(EPA)	(0.007)	(0.008)	(0.009)	(0.007)	(0.005)	(0.005)
22:6n-3	0.123	0.110	0.103	0.096	0.088	0.079
(DHA)	(0.051)	(0.051)	(0.048)	(0.045)	(0.041)	(0.041)

575 [1] Results are expressed in mg/mL

576

577 Table 3. Mean (SD) omega-6 and omega-3 PUFA intakes of the 17 preterm infants for each
578 week of the study (mg/kg/day)

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
			Parenteral and enteral intakes			
LA	670.1	798.2	447.7	366.4	349.7	306.7
	(249.5)	(365.9)	(377.5)	(294.5)	(233.8)	(108.4)
ALA	101.2	112.4	50.1	40.8	40.7	27.7
	(37.9)	(61.5)	(63.0)	(48.3)	(42.8)	(18.1)
ARA	4.5 (2.1)	13.0 (5.7)	14.1 (7.1)	15.0 (5.3)	14.3 (6.5)	14.5 (7.1)
EPA	0.6 (1.6)	1.3 (2.3)	1.1 (1.1)	2.1 (1.5)	1.8 (1.1)	1.4 (1.1)
DHA	4.2 (2)	11.6 (6.9)	11.4 (7.7)	11.6 (5.9)	10.7 (6.6)	10.6 (6.8)
			Metabolizable intakes (i.e. parenteral intake and absorbed enteral intake)			
LA	665.0	772.2	409.0	324.6	317.7	269.2
	(248.1)	(377.7)	(385.9)	(297.2)	(236.8)	(101.2)
ALA	100.8	110.5	47.9	35.3	33.6	25.5
	(39.3)	(62.4)	(63.6)	(48.0)	(42.2)	(16.6)
ARA	3.9 (1.7)	10.8 (4.3)	11.3 (5.7)	11.1 (4.7)	11.6 (5.1)	12.4 (6.5)
EPA	0.2 (0.4)	1.0 (1.8)	0.9 (0.9)	1.6 (1.2)	1.4 (0.9)	1.1 (0.9)
DHA	3.7 (1.7)	9.7 (5.4)	9.3 (6.1)	9.4 (4.7)	8.7 (5)	8.8 (5.2)
			Available for accretion (metabolizable intake and endogenously synthesized values)			
ARA	14.5 (3.4)	27.4 (10.3)	29.6 (12.7)	32.0 (10.1)	33.4 (9.8)	34.8 (10.8)
Deficit[1]	-198.6	-185.3	-183.1	-180.1	-178.7	-177.5
	(2.6)	(10.5)	(12.6)	(10.0)	(10.1)	(10.9)
DHA	8.1 (2.2)	17.1 (7.2)	17.6 (8.0)	18.5 (6.5)	18.6 (6.9)	19.0 (7.2)

Deficit[1]	-36.9 (2.3)	-27.9 (7.2)	.27.3 (8.0)	-26.5 (6.8)	-26.4 (6.9)	-26.0 (6.8)

[1] Mean deficit compared to estimated *in utero* accretion rates of 212 mg/kg/day for ARA and 45 mg/kg/day for DHA [2]

599 Table 4. Mean (SD) whole-blood omega-6 and omega-3 PUFAs at 6 weeks and mean (SD)

600 intakes of LA, ARA, ALA, EPA and DHA over the study of the 17 infants

Fatty Acid	% Total fatty acids	Mean intake (mg/kg/day)
LA	8.79 (1.46)	474.5 (122.0)
18:3n-6	0.30 (0.06)	
20:2n-6	0.36 (0.06)	
20:3n-6	2.22 (0.29)	
ARA	16.74 (1.36)	12.6 (4.4)*
22:4n-6	3.21 (0.22)	
22:5n-6	1.36 (0.14)	
ΣN-6	32.97 (2.72)	
ALA	0.13 (0.05)	80.8 (79.0)
EPA	0.46 (0.11)	1.4 (0.6)*
22:5n-3	1.23 (0.24)	
DHA	3.88 (0.82)	10.0 (6.0)*
ΣN-3	5.70 (0.92)	
Omega-6:omega-3	5.8 (1.2)	

601 * indicates significant correlation. ARA: r = 0.568, p = 0.002; EPA: r = 0.572, p = 0.016;

602 DHA: r = 0.704, p = 0.002.

603