

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

3

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20 Abbreviations: ALA,  $\alpha$ -linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid;

21 EPA, eicosapentaenoic acid; LA, linoleic acid

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25 Abstract

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

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

38 Results: ARA intake was significantly below international recommendations in weeks 1-5  
39 (all  $p < 0.05$ ), and DHA intake was significantly below recommendations in week 1 ( $p <$   
40  $0.0001$ ). The amounts of ARA and DHA available for accretion were significantly below  
41 estimated accretion rates in all weeks (all  $p < 0.001$ ). Mean ARA and DHA intakes were  
42 correlated with their respective blood levels ( $r = 0.568$ ,  $p = 0.017$  and  $r = 0.704$ ,  $p = 0.002$ ).  
43 There were significant relationships between MIP-1 $\beta$  and blood DHA levels ( $r_s = 0.559$ ,  $p =$   
44  $0.02$ ) and between RANTES and omega-6:omega-3 PUFA ratio ( $r_s = -0.498$ ,  $p = 0.042$ ).

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

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

52

### 53 1. Introduction

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

64

65 The Committee on Nutrition of the European Society for Paediatric Gastroenterology,  
66 Hepatology, and Nutrition (ESPGHAN) have set recommendations for enteral nutrition in  
67 preterm infants for LA, ALA, ARA, DHA and eicosapentaenoic acid (EPA) [6]. However,  
68 these guidelines do not consider the greater requirements needed to compensate for early  
69 nutritional deficits and malabsorption or indeed reflect *in utero* accretion rates [7]. There are  
70 a range of estimated values for *in utero* ARA and DHA accretion rates depending on the  
71 background assumptions made [2, 8, 9]; however, the most recent estimates for accretion  
72 rates of ARA and DHA in the last trimester are 212 and 45 mg/kg/day, respectively [2],  
73 which are far higher than the ESPGHAN recommended intakes for ARA and DHA of 18 to  
74 42 and 12 to 30 mg/kg/day, respectively [6].

75

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

92

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

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

104

## 105 2. Materials and methods

### 106 2.1 Participants

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

116

### 117 2.2 Study design

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

121

### 122 2.3 Determination of fatty acids in breast milk

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

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

131

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

137

#### 138 2.4 Whole blood fatty acid analysis

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

145

#### 146 2.5 Blood cytokine and chemokine analysis

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

150 3000 rpm for 10 min, and the serum frozen at -70°C. A bead-based multiplex assay  
151 (Luminex®, R&D systems) was used according to the manufacturer's instructions. The pro-  
152 inflammatory markers analysed were: tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), Interleukin 1 $\beta$  (IL-  
153 1 $\beta$ ), Interleukin 2 (IL-2), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 8 (IL-8), other  
154 inflammatory proteins: Interferon  $\gamma$  (IFN- $\gamma$ ), Granulocyte-macrophage colony-stimulating  
155 factor (GMCSF), Granulocyte colony stimulating factor (GCSF), Monocyte chemoattractant  
156 protein 1 (MCP-1), Macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ) and Regulated on  
157 Activation, Normal T Expressed and Secreted (RANTES). The anti-inflammatory markers  
158 were: Interleukin 10 (IL-10), Interleukin 1 receptor antagonist IL-1ra), Interleukin 4 (IL-4),  
159 Interleukin 17 (IL-17). The Luminex® Performance Human Cytokine Panel A was used, and  
160 samples were read using the Luminex® Flexmap 3D analyser. All samples were analysed on  
161 the same plate.

162

## 163 2.6 Quantification of LA, ALA, ARA, EPA and DHA intake

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

169

## 170 2.7 Estimation of ARA and DHA available for metabolism and accretion

171 Estimations of ARA and DHA available for metabolism and accretion were based on  
172 previously published assumptions [7]. The amount of ARA and DHA available for  
173 metabolism was calculated based on intestinal absorption of rates of 80% for both PUFAs  
174 [18]. Absolute ARA and DHA synthesis in preterm infants at 1 month old fed LC- PUFAs

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

181

## 182 2.8 Statistical analysis

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

194

## 195 3. Results

### 196 3.1. Participant characteristics

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



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

202

### 203 3.2 Wide variability of maternal breast milk fatty acid content over the first six weeks

204 The mean omega-6 and omega-3 PUFA concentrations of maternal breast milk are shown in  
205 Table 2. Over the six weeks of the study there were significant differences in the mean ARA  
206 and DHA content as determined by repeated measures one-way ANOVA ( $F(2.046, 32.74) =$   
207  $119.3, p < 0.0001$  and  $F(2.120, 33.92) = 42.13, p < 0.001$ ), respectively. A Tukey post-hoc  
208 test revealed that the mean ARA content significantly decreased over the period of the study  
209 ( $p < 0.05$ ), with the mean values of sample 6 comprising only 65% of sample 1. Similarly,  
210 there were also significant decreases in the DHA content over the study ( $p < 0.05$ ), with  
211 sample 6 content only 64% of the value of sample 1. There was wide variability in ARA and  
212 DHA content between the individual mother's milks, as shown in Figure 2. There were no  
213 significant differences in the content of any of the other omega-6 PUFAs over the study.  
214 Although there were significant differences in the ALA between the groups ( $F(3.093, 49.49)$   
215  $= 3.521, p < 0.02$ ), there were no observable trends across the study. There were also  
216 significant differences in the EPA concentrations ( $F(2.659, 42.54) = 3.298, p < 0.03$ );  
217 however, many of the values were at the limit of quantification and these results should be  
218 interpreted with caution.

219

### 220 3.3 LA, ARA, ALA, EPA and DHA intake levels in the preterm infants

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

225 significant differences in LA intake across the study ( $F(5, 96) = 8.204, p < 0.0001$ ), with  
226 intakes significantly lower in weeks four, five and six, than weeks one and two (all at  $p <$   
227  $0.05$ ). LA intake was significantly below the minimum ESPGHAN recommended intake  
228 levels of 385 mg/kg/day in week 6 ( $t = 2.9782, p = 0.009$ ) [6]. ALA intake significantly  
229 differed across the study ( $F(5, 96) = 9.392, p < 0.0001$ ), with intakes significantly lower in  
230 weeks four, five and six, than weeks one and two (all at  $p < 0.05$ ). ALA was significantly  
231 below the ESPGHAN guidelines of  $> 55$  mg/kg/day in week six ( $t = 6.2188, p < 0.0001$ ). The  
232 mean intake of ARA differed across the study ( $F(3.318, 53.09) = 14.52, p < 0.0001$ ) with  
233 significant increases from the first to the second week ( $p < 0.05$ ) and then remained at similar  
234 levels for the remainder of the study. The ARA intake was significantly below the  
235 ESPGHAN minimum intake levels of 18 mg/kg/day in weeks one to five (all  $p < 0.05$ ). DHA  
236 intake differed across the study ( $F(3.863, 61.81) = 7.933, p < 0.0001$ ) increasing  
237 significantly from the first to the second week ( $p < 0.05$ ) and then remaining at similar levels.  
238 DHA was significantly below the minimum ESPGHAN intake guidelines of 12 mg/kg/day in  
239 week one only ( $t = 16.0801, p < 0.001$ ). EPA intake levels were not significantly different  
240 across the study, and were within EPSGHAN recommended levels.

241

242 3.4 The ARA and DHA content available for accretion leads to deficits in the preterm infants  
243 The mean ARA and DHA available for accretion significantly increased between the first and  
244 second weeks, ( $F(5, 96) = 9.415, p < 0.001$  and  $F(5, 96) = 6.760, p < 0.001$ , respectively),  
245 and then both remained at about these levels for the remaining period of the study (Figure 3).  
246 These values were all significantly below the values estimated for ARA and DHA provided  
247 *in utero* (all values  $p < 0.0001$ ). The values for the cumulative mean ARA and DHA deficits  
248 produced over the six weeks of the study were calculated by successively adding the weekly  
249 deficits derived from the individual daily deficits, and are shown in Figure 4. The ARA

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

254

### 255 3.5 Relationships between intake and whole-blood fatty acid levels

256 The mean (SD) whole-blood levels of the omega-6 and omega-3 PUFAs of the preterm  
257 infants at six weeks and the mean intakes of LA, ARA, ALA, EPA and DHA are shown in  
258 Table 4. The mean haemoglobin level in week six was 110.9 g/L (15.1 g/L), and none of the  
259 infants were considered clinically anaemic. Blood samples were not taken until week six to  
260 limit complications due to the number of transfusions given. The mean number of  
261 transfusions over the study was 6.4 (3.4), with the mean number in week one 1.8 (1.3), week  
262 two, 1.3 (1.2), weeks three and four, 2.2 (1.5) and weeks five and six, 1.1 (0.9). The strength  
263 of relationship between mean intake levels and blood fatty acid levels was estimated by  
264 Pearson product-moment correlation coefficient. There were significant positive correlations  
265 between mean DHA intake and blood DHA levels ( $r = 0.704$ ,  $p = 0.002$ ), mean ARA intake  
266 and blood ARA levels ( $r = 0.568$ ,  $p = 0.017$ ) and mean EPA intake and blood EPA levels ( $r =$   
267  $0.572$ ,  $p = 0.016$ ). There were no significant correlations with LA or ALA levels and their  
268 respective blood levels.

269

### 270 3.6 Relationships between whole-blood DHA and cytokine and chemokine levels

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

275 MIP-1 $\beta$  and DHA ( $r_s = 0.559$ ,  $p = 0.02$ ) and MIP-1 $\beta$  and AA/DHA ratio ( $r_s = -0.690$ ,  $p =$   
276  $0.002$ ), as well as between MIP-1 $\beta$  and the omega-6:omega-3 PUFA ratio ( $r_s = -0.716$ ,  $p =$   
277  $0.001$ ). The omega-6:omega-3 PUFA ratio was also negatively correlated with RANTES ( $r_s =$   
278  $-0.498$ ,  $p = 0.042$ ). There were no significant correlations with any of the other cytokines or  
279 chemokines.

280

#### 281 4. Discussion

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

291

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

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

303

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

317

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

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

328

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

338

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

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

352

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

361

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

371

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

375 inflammation. The most consistent observations were seen with MIP-1 $\beta$ . MIP-1 $\beta$  is a  
376 chemotactic cytokine produced by macrophages, dendritic cells and lymphocytes and has  
377 chemotactic and pro-inflammatory effects, but can also promote homeostasis [29]. Significant  
378 positive correlations were shown between MIP-1 $\beta$  and DHA, with negative correlations  
379 between MIP-1  $\beta$  and the ARA/DHA ratio, as well as between MIP-1  $\beta$  and omega-6/omega-  
380 3 PUFA ratio. These data suggest that as the omega-3 PUFA status increases, the infant's  
381 ability to produce MIP-1 $\beta$  increases to that seen in a well term infant [30]. The omega-  
382 6:omega-3 PUFA ratio was also negatively correlated with RANTES. RANTES is a  
383 chemoattractant for monocytes, memory T-helper cells and eosinophils [31]. Plasma  
384 RANTES levels are significantly lower in preterm infants with sepsis, disseminated  
385 intravascular coagulation and necrotising enterocolitis (NEC) [31]. These data suggest that a  
386 normal (as opposed to a low) level of RANTES is seen with higher omega-3 PUFA status.  
387 Overall, these data are suggestive of interactions between LC-PUFA status and markers of  
388 inflammation; however, further research with larger numbers studied longitudinally is  
389 required to confirm these observations.

390

391 Important strengths of this study are that it was conducted at a neonatal centre which is also a  
392 surgical centre, and consequently infants with a wide range of conditions were included, and  
393 the milk fatty acid composition was measured at six time-points, so the subtle changes in the  
394 fatty acid profile over time were identified. Moreover, the omega-6 and omega-3 PUFA  
395 intake was quantified from all sources, and importantly the amount of ARA available for  
396 accretion was quantified for the first time. Blood fatty acid status was measured by a  
397 validated dried blood spot method, which is preferable for the assessment of circulating fatty  
398 acid fractions in preterm infants [24]; however, as noted above, blood fatty acid levels were  
399 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.

425

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429 supporting the study.

430

431 Statement of authorship

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

435

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522 Figures Legends and Tables

523 Figure 1 Flow of participants through the study

524

525 Figure 2 (A) ARA and (B) DHA breast milk content from individual mothers. Sample 1, days  
526 0 - 4; sample 2, days 5 - 9; sample 3, days 10 - 15 sample 4, days 16 - 23; sample 5, days 24 -  
527 33 and sample 6, days 34 - 42

528

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

533

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

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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)

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

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573 Table 2. Mean (SD) omega-3 and omega-6 PUFA content of maternal breast milk of 17  
 574 mothers of extremely preterm infants<sup>1</sup>

	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 <sup>1</sup> Results are expressed in mg/mL

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 (249.5)	798.2 (365.9)	447.7 (377.5)	366.4 (294.5)	349.7 (233.8)	306.7 (108.4)
ALA	101.2 (37.9)	112.4 (61.5)	50.1 (63.0)	40.8 (48.3)	40.7 (42.8)	27.7 (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 (248.1)	772.2 (377.7)	409.0 (385.9)	324.6 (297.2)	317.7 (236.8)	269.2 (101.2)
ALA	100.8 (39.3)	110.5 (62.4)	47.9 (63.6)	35.3 (48.0)	33.6 (42.2)	25.5 (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 <sup>1</sup>	-198.6 (2.6)	-185.3 (10.5)	-183.1 (12.6)	-180.1 (10.0)	-178.7 (10.1)	-177.5 (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)

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Deficit <sup>1</sup>	-36.9 (2.3)	-27.9 (7.2)	.27.3 (8.0)	-26.5 (6.8)	-26.4 (6.9)	-26.0 (6.8)
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579 <sup>1</sup> Mean deficit compared to estimated *in utero* accretion rates of 212 mg/kg/day for ARA and

580 45 mg/kg/day for DHA [2]

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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