Maternal fish oil supplementation in pregnancy modifies neonatal leukotriene production by cord-blood-derived neutrophils

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ABSTRACT

Fish oil supplementation has been shown to reduce neutrophil production of inflammatory LTB4 (leukotriene B4) in adults. The present study is the first to examine the effects on neonatal neutrophil function following supplementation in pregnancy. Pregnant women with allergic disease (n = 98) were randomized to receive either fish oil [3.7 g of n−3 long-chain PUFAs (polyunsaturated fatty acids)/day] or a placebo supplement for the final 20 weeks of pregnancy. Leukotriene production by neonatal neutrophils was measured after stimulation with the calcium ionophore A23187. This was examined in relation to supplementation, cell membrane fatty acid levels and mononuclear cytokine production. Neutrophil LTB4 production was significantly reduced in neonates whose mothers had received fish oil in pregnancy. This was most evident for isomer 2 of LTB4 (P = 0.031), although this was also observed for total LTB4 (P = 0.051) and isomer 1 (P = 0.088). There was also a trend for lower production of other PUFA metabolites, namely 5-HETE (5-hydroxyeicosatetraenoic acid; P = 0.054) in the fish oil group. Accordingly, LTB4 levels were inversely related to membrane n−3 PUFA levels. Less inflammatory products (LTB5) were only produced at very low levels, although there was a trend for higher levels of this metabolite in the fish oil group. Consistent with this, LTB5 levels were positively correlated with n−3 PUFA membrane levels, particularly EPA (eicosapentanoic acid) and negatively correlated with n−6 PUFA levels. Neonates with lower neutrophil LTB4 production also had lower production of pro-inflammatory IL (interleukin)-6 responses (r = 0.35, P = 0.005) and regulatory IL-10 responses (r = 0.37, P = 0.003) by LPS (lipopolysaccharide)-stimulated neonatal mononuclear cells. In conclusion, maternal dietary changes can modify neonatal neutrophil function. This has implications for the early immune programming, which can be influenced by the inflammatory milieu of local tissues during initial antigen encounter. It also provides evidence of another pathway through which long-chain PUFAs status can influence early immune development.

INTRODUCTION

Diet-derived long-chain PUFAs (polyunsaturated fatty acids) are fundamental elements of all cell membranes and form the metabolic substrates of key inflammatory mediators in host defence. Variations in the dietary long-chain PUFAs have significant implications for membrane fluidity, cell signalling and the patterns of mediator

Key words: allergic disease, cytokine, fish oil, n−3 long-chain polyunsaturated fatty acid, leukotriene, neonatal immune response, T-cell.

Abbreviations: AA, arachidonic acid; APC, antigen-presenting cell; CBMC, cord blood mononuclear cell; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; IFN-γ, interferon-γ; IL, interleukin; LA, linoleic acid; LPS, lipopolysaccharide; LTB, leukotriene B; MFI, mean fluorescence intensity; PG, prostaglandin; PHA, phytohaemagglutinin; PUFA, polyunsaturated fatty acid; rIFN-γ, recombinant IFN-γ; TLR4, Toll-like receptor 4.

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production. Dietary strategies to increase the $\alpha -3$ (omega-3) long-chain PUFA membrane composition have been used to reduce inflammatory responses associated with a number of disease states, including autoimmune diseases and asthma. Most of these anti-inflammatory effects are mediated by competitive inhibition of the activities of more inflammatory $\alpha -6$ (omega-6) long-chain PUFAs such as AA (arachidonic acid) (reviewed by [1]). AA is the progenitor of highly inflammatory leukotrienes [LTB$_4$ (leukotriene B$_4$) and prostaglandins [PGE$_2$ (prostaglandin E$_2$)], which are produced through the 5-lipoxygenase and cyclo-oxygenase enzymatic pathways respectively, and contribute to pain, erythema and swelling. The $\alpha -3$ homologue of AA, EPA (eicosapentaenoic acid), acts competitively through these enzymatic pathways to produce LTB$_4$ and PGE$_2$, which have very little inflammatory activity compared with the LTB$_4$ and PGE$_2$ $\alpha -6$ products. In human studies, $\alpha -3$-rich fish oil supplementation has been associated with a reduction in LTB$_4$ production by neutrophils from asthmatic subjects with corresponding increases in LTB$_4$ production [2]. This was also associated with a clinically relevant improvement in exercise-induced bronchoconstriction [2].

Although there is some evidence that these effects have clinical benefits in the treatment of established disease, there is also growing interest in the role earlier in development for disease prevention. Diet and nutrition in pregnancy have a fundamental influence on fetal development, and there is growing focus on the role of key dietary nutrients in subsequent health or disease. Exposures during pregnancy, when the immune system is less mature, may have greater potential to influence subsequent immune programming, levels of tissue inflammation and the risk of tissue damage. We have shown previously [3] that supplementation with high doses of fish oil in pregnancy can modify T-cell immune function in the offspring. We now speculate that alterations in neonatal long-chain PUFAs may influence local inflammatory responses through effects on the production of lipid-derived inflammatory mediators by local tissue neutrophils. The aim of the present study was to examine the effects of maternal $\alpha -3$ long-chain PUFA supplementation on the patterns of neutrophil leukotriene production by newborns from the same cohort.

**MATERIALS AND METHODS**

**Study design**
In this trial, pregnant women with allergic disease ($n = 98$) were randomized to receive either fish oil (3.7 g of $\alpha -3$ PUFA/day) or a placebo supplement for the final 20 weeks of pregnancy, as described in detail elsewhere [3–5].

**Participants**
Maternal atopy was defined as a doctor-diagnosed clinical history of asthma, allergic rhinitis or eczema plus a positive SPT (skin prick test) to one or more common allergens (house dust mite, grass pollens, cat, dog, feathers, moulds and cockroach extracts; Hollister-Stier Laboratories). Women were ineligible for the study if they smoked, had other medical problems or pregnancy complications, delivered prior to 37 weeks of gestation, or were already taking fish oil supplements or ingesting more than two servings of oily fish/week.

Ethical approval for the study was granted by Princess Margaret Hospital for Children, King Edward Memorial Hospital, St John of God Hospital and Mercy Hospital Ethics Committees, and all women gave written informed consent.

**Assignment**
The groups were block-randomized according to parity (no previous term childbirth compared with one or more), pre-pregnancy BMI (body mass index), age and maternal allergy (allergic rhinitis or asthma). Randomization and allocation of capsules occurred at a different centre, separate from the recruitment of participants.

**Intervention**
Women in the fish oil group received four (1 g) fish oil capsules/day (Ocean Nutrition), comprising a total of 3.7 g of $\alpha -3$ PUFAs with 56.0 % as DHA (docosahexaenoic acid) and 27.7 % as EPA (confirmed by GC). The control group received four (1 g) capsules of olive oil/day (containing 66.6 % $\alpha -9$ oleic acid and <1 % $\alpha -3$ PUFAs; Pan Laboratories). The staff dispensing the capsules were blinded to the allocation. The capsules in the two groups were image-matched and the participants, research scientists and paediatrician remained blinded to group allocations for the duration of the study. Compliance was monitored by measuring the incorporation of DHA and EPA into the cell membranes of erythrocytes.

**Fatty acid measurements**
Phospholipid fatty acid analyses were carried out as described previously [6]. Briefly, total lipids were extracted from both neonatal and maternal (at 37 weeks of gestation) erythrocyte membranes with methanol and chloroform. The phospholipid fraction was obtained from total lipid extracts by TLC, and the fatty acid methyl esters were analysed by GLC. The fatty acids were expressed as a percentage of the weight of the total fatty acids measured (C14 to C22). The total sum of $\alpha -3$ PUFAs (C20:5$\omega -3$, C22:5$\omega -3$ and C22:6$\omega -3$) and $\alpha -6$ PUFAs (C18:2$\omega -6$, C20:4$\omega -6$, C22:4$\omega -6$, C22:5$\omega -6$ and C22:6$\omega -6$), as well as $\alpha -3/\alpha -6$ fatty acid ratio, were also expressed.
Neutrophil purification

Neutrophils were isolated by density gradient separation using Lymphoprep (Nycomed Pharma), followed by dextran (6%) sedimentation. Contaminating erythrocytes were removed by hypotonic lysis. The purity of the neutrophils was >90%. Purified neutrophils (1 × 10^7 cells) were incubated in RPMI/0.1% HSA (human serum albumin) with or without the calcium ionophore A23187 (2.5 µg/ml) at 37 °C for 10 min. The supernatant was stored at −80 °C.

Leukotriene production by neutrophils

The cell supernatant was analysed for leukotriene metabolites using HPLC [7]. An internal standard PGB 

\(\text{µl}) \) was added to each neutrophil supernatant, which was then acidified with formic acid. LTB 

4 and its metabolites were extracted into ethyl acetate and evaporated under nitrogen. The extract was reconstituted in methanol/acetonitrile/water (1:1:2, by vol.), and LTB4 was evaporated under nitrogen. The extract was reconstituted and was stored at −80 °C.

Detection of cytokine responses to specific and polyclonal T-cell stimulation

Fetal mononuclear cells (CBMCs) (cord blood mononuclear cells) were isolated from cord blood using Lymphoprep (Nycomed Pharma) gradient centrifugation and were cryopreserved for subsequent batch analysis [3,8]. Upon thawing, 1 × 10^6 CBMCs/l were cultured in serum-free AIM V medium (Life Technologies) [9] for 48 h with or without 1 µg/ml PHA (phytohaemagglutinin) mitogen (HA16; Murex). Supernatants were analysed for IL (interleukin)-5 using an in-house ELISA, as described previously [9]. IL-10, IL-13 and IFN-γ (interferon-γ) were quantified by an in-house ELISA using a TRF (time-resolved fluorimetry) detection system (DELPHIA; PerkinElmer) [3]. The limit of detection was 3 pg/ml. Cytokine data are expressed (in pg/ml) as the difference between the stimulated culture and the control.

Assessment of innate immune function

CBMCs (1 × 10^6 cells/l) were cultured in the presence of bacterial stimuli as a measure of innate immune function. CBMCs (1 × 10^6 cells/l) were cultured in RPMI (Life Technologies)/10% (v/v) FCS (fetal calf serum) for 24 h with or without 10 ng/ml rIFN-γ (recombinant IFN-γ; Pharmingen) and/or 10 ng/ml LPS (lipopolysaccharide; Escherichia coli 055:B5; Sigma). IL-6 and IL-10 were analysed as described above. To determine HLA-DR expression on APCs (antigen-presenting cells), CBMCs were cultured as above with or without 10 ng/ml rIFN-γ (Pharmingen), and then monocytes were labelled with FITC-conjugated anti-CD14 (Becton Dickinson) and PE (phycoerythrin)-conjugated anti-(HLA-DR) (Becton Dickinson), as described previously [14]. MFI (mean fluorescence intensity) of expression was examined using a FACS Calibur and Cell Quest software (Becton Dickinson).

Statistical analysis

Leukotriene results were normally distributed, and differences between the groups were assessed by independent Student’s t tests. Fatty acid levels were also normally distributed. Continuous cytokine results were log (natural)-transformed to obtain a normal distribution. Values are expressed as geometric means [95% CIs (confidence intervals)] or means (S.E.M.). Pearson’s correlation coefficients were used to identify associations between variables when the groups were combined. All statistical analyses were performed using SPSS software (version 11.0 for Mac OS X). A P value of <0.05 was considered statistically significant for all analyses. Comments on the use of correction factors for multiple comparisons are made in the Discussion section.

RESULTS

Of the 98 women enrolled in the study, 83 completed the supplementation and were assessed at birth. The reasons for dropout included nausea (n = 8), preterm delivery (n = 4), unrelated neonatal disease (n = 2), and one woman was lost to follow-up. Of those who completed the study, 64 had samples available for the present analyses. The characteristics of these women and their infants (with available samples) are shown in Table 1. The groups were well-matched for age, parity and socio-economic status. The groups had similar birth weight and head circumference, Apgar scores and rate of postnatal infections [3,4]. Fish oil supplementation achieved significantly higher proportions of n-3 PUFA s in neonatal erythrocyte membranes compared with the control group (P < 0.001), as shown in Table 1 and described in detail elsewhere [3,4].

Effects of fish oil on leukotriene production by neonatal neutrophils

Neutrophil LTB4 production was significantly lower in neonates whose mothers had received fish oil in pregnancy (Figure 1). This was most evident for isomer 2 of LTB4 (P < 0.031), although this was also observed for total LTB4 (P = 0.051) and isomer 1 (P = 0.088). The formation of the other major lipoxygenase product 5-HETE (5-hydroxyeicosatetraenoic acid) also tended to be lower in neutrophils from the fish oil group (P = 0.054). There was no detectable LTB4 or other eicosanoid metabolites released in unstimulated neutrophils.

LTB4 was only produced at low levels and was not detectable in many of the samples; however, it was the
Table 1  Population characteristics

<table>
<thead>
<tr>
<th></th>
<th>Fish oil group</th>
<th>Placebo group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>31.0 (0.7)</td>
<td>32.7 (0.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Maternal weight at 36 weeks (kg)</td>
<td>75.7 (2.1)</td>
<td>76.7 (1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Gestation (days)</td>
<td>274 (1.5)</td>
<td>274 (1.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Parity (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primigravid</td>
<td>53</td>
<td>47</td>
<td>NS</td>
</tr>
<tr>
<td>Multigravid</td>
<td>47</td>
<td>53</td>
<td>NS</td>
</tr>
<tr>
<td>Delivery method (%)</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Vaginal</td>
<td>73</td>
<td>80</td>
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<tr>
<td>Caesarean</td>
<td>27</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>349 (63)</td>
<td>3423 (66)</td>
<td>NS</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>50.4 (0.4)</td>
<td>50.0 (0.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Infant gender (%)</td>
<td></td>
<td></td>
<td>NS</td>
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<tr>
<td>Male</td>
<td>43</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>57</td>
<td>47</td>
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</table>

Maternal fatty acids (% of total)

<table>
<thead>
<tr>
<th></th>
<th>Fish oil group</th>
<th>Placebo group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (C18:2n-6)</td>
<td>7.4 (0.24)</td>
<td>8.50 (0.16)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>AA (C20:4n-6)</td>
<td>10.3 (0.33)</td>
<td>13.63 (0.15)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total n-6</td>
<td>21.07 (0.63)</td>
<td>28.21 (0.32)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>EPA (C20:5n-3)</td>
<td>2.56 (0.12)</td>
<td>0.7 (0.03)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>DHA (C22:6n-3)</td>
<td>10.7 (0.40)</td>
<td>6.52 (0.19)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total n-3</td>
<td>22.3 (0.70)</td>
<td>16.2 (0.30)</td>
<td>P &lt; 0.001</td>
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</table>

Neonatal fatty acids (% of total)

<table>
<thead>
<tr>
<th></th>
<th>Fish oil group</th>
<th>Placebo group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA (C18:2n-6)</td>
<td>3.85 (0.10)</td>
<td>3.58 (0.06)</td>
<td>P = 0.032</td>
</tr>
<tr>
<td>AA (C20:4n-6)</td>
<td>14.78 (0.27)</td>
<td>17.26 (0.21)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total n-6</td>
<td>24.93 (0.36)</td>
<td>29.35 (0.25)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>EPA (C20:5n-3)</td>
<td>1.39 (0.10)</td>
<td>0.38 (0.05)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>DHA (C22:6n-3)</td>
<td>10.22 (0.22)</td>
<td>7.30 (0.16)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Total n-3</td>
<td>18.1 (0.30)</td>
<td>13.72 (0.21)</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Relationship between membrane fatty acids and leukotriene production

The purpose of the correlation analysis was to allow for further variations in long-chain PUFAs due to potential variations in background diets and compliance. It is therefore valid to combine the groups in order to assess the effects of a range of PUFA status against the outcomes of interest.

There were consistent relationships between long-chain PUFA composition and the patterns of leukotriene production. LTB4 production was consistently associated with higher levels of the n-3 PUFAs (significant for EPA and similar trends for DHA and total n-3 PUFAs) and lower n-6 PUFAs (as shown in Table 2 for maternal fatty acids and Table 3 for neonatal fatty acids). Conversely, LTB4 isomers (both 1 and 2) and 5-HETE were consistently associated with lower n-3 PUFAs (as shown on Figure 2 for the LTB4 isomer 2 in relation to total n-3 PUFAs). These relationships were highly consistent (for virtually all correlations), and most were statistically significant (more particularly in relation to maternal fatty acid status). LTB4 isomers were associated with higher C22:6n-3 PUFA, with consistent trends for the other n-6 PUFAs [with the exception of LA (linoleic acid; C18:2n-6)]. These correlation results serve to reinforce that the ‘group’ effect was due to the variations in fatty acids.

Relationship between leukotriene production and aspects of neonatal immune function

In the whole population, we assessed the propensity for leukotriene production in relation to other aspects of neonatal immune function, including innate APC responses and responses to polyclonal T-cell activation.

APC responses

First, we assessed leukotriene production in relation to neonatal APC responses to a ubiquitous microbial product (LPS endotoxin), which activates innate immune defence mechanisms through TLR4 (Toll-like receptor 4). When primed with IFN-γ and LPS, CBMCs produced an abundant cytokine response dominated by high levels of the pro-inflammatory cytokine IL-6 [25 560 (95 % CI, 22 775–28 687) pg/ml]. We also detected IL-12 [589 (95 % CI, 492–706) pg/ml], which inhibits allergic differentiation, as well as the production of IL-10 [185 (95 % CI, 153–224) pg/ml], a key regulatory cytokine.

Notably, the inflammatory IL-6 cytokine response was significantly correlated with the propensity for neutrophil LTB4. This was observed for both LTB4 isomers (1 and 2) as shown in Table 4. A parallel relationship was seen between the IL-6 response and other long-chain PUFA metabolites, namely 5-HETE (Table 4), which was also down-regulated in response to fish oil supplementation (as shown in Figure 1).

Interestingly, there were also positive relationships between the production of both LTB4 isomers 1 and 2 and the level of IL-10 induction during the innate responses (Table 4). This is consistent with the strong positive associations between IL-6 and IL-10 production in response to this microbial activation (r = 0.439, P < 0.001). IL-12 responses were not significantly related to leukotriene production.

There were no significant relationships between LTB4 and cytokine responses.

We also assessed the ability of LPS to up-regulate antigen presentation (MHC class II expression on the
Maternal fish oil inhibits neonatal leukotriene B₄ production

Figure 1 PUFA metabolites produced by neonatal neutrophils stimulated by the calcium ionophore A23187

Values are means ± S.D. Production of long-chain PUFA metabolites following calcium ionophore stimulation of neonate neutrophils in the fish oil group (n = 30; closed bars) was compared with control (olive oil) group (n = 34; open bars). P < 0.05 was considered statistically significant, as determined using a Student’s t test.

Table 2 Pearson’s correlation between leukotriene release from stimulated neutrophils and fatty acid content in maternal erythrocyte membranes in 63 neonates

Table 3 Pearson’s correlation between leukotriene release from stimulated neutrophils and fatty acid content in neonatal erythrocyte membranes in 63 neonates

surface of APCs). As anticipated, expression of MHC class II HLA-DR on CD14⁺ monocytes significantly increased (MFI ± S.D. from 303.5 ± 106.9 to 923.3 ± 291.8; P < 0.001) following stimulation with IFN-γ/LPS. We also observed that the magnitude of this up-regulation was inversely related to LTB₄ isomers 1
Relationship between total $n-3$ fatty acid levels in maternal red cell membranes and LTB$_4$ (isomer 2)

Individual data points are shown from both groups. RBC, red blood cell.

Table 4  Pearson's correlation between leukotriene release from stimulated neutrophils and innate (TLR4-induced) immune response in 64 neonates

Neutrophils were stimulated in vitro with a calcium ionophore for 10 min, and CBMCs were stimulated with IFN-$\gamma$ + LPS for 24 h at 37 $^\circ$C. $^*$ $P < 0.05$, $^{**} P < 0.01$ and $^{***} P < 0.005$.

<table>
<thead>
<tr>
<th>Leukotriene metabolites from neutrophils</th>
<th>CBMC response</th>
<th>S-HETE</th>
<th>LTB$_4$</th>
<th>LTB$_4$ isomer 1</th>
<th>LTB$_4$ isomer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.259$^*$</td>
<td>0.052</td>
<td>0.327$^{**}$</td>
<td>0.350$^{**}$</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.183</td>
<td>0.008</td>
<td>0.340$^{**}$</td>
<td>0.368$^{***}$</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>0.014</td>
<td>0.178</td>
<td>0.078</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>Mean HLA-DR</td>
<td>$-0.202$</td>
<td>0.03</td>
<td>$-0.287^*$</td>
<td>$-0.276^*$</td>
<td></td>
</tr>
</tbody>
</table>

Responses to polyclonal T-cell simulation

Secondly, we examined relationships between leukotriene production and T-cell responses. A polyclonal T-cell stimulant (PHA) was used to assess the production of Th (T-helper) cell cytokines, including Th1 cytokine IFN-$\gamma$ and Th2 cytokine IL-13, and the regulatory cytokine IL-10 from CBMCs in culture. There was a trend for lower IFN-$\gamma$ and IL-13 responses in the fish oil group compared with the control group, although this did not reach statistical significance. The multiple comparisons used in the present study raises the issues of statistical correction factors that are likely to abolish most relationships, even if biologically significant. However, it has been validly argued [12] that, where there are consistent, repeated, coherent and plausible patterns, the results reinforce, rather than

$LTB_4$ ratio ($r = 0.362$, $P = 0.035$), total $LTB_4$ ($r = -0.309$, $P = 0.013$) and 5-HETE ($r = -0.421$, $P = 0.001$). There were no relationships between leukotriene production and polyclonal Th1 or Th2 responses.

DISCUSSION

The present study population was the very first pregnancy intervention study to explore the effects of altering maternal long-chain PUFA status to modify neonatal immune function. The key purpose of the cohort was to provide exploratory 'proof of concept' results with the intention of informing the larger more definitive studies which have now been initiated. The key novel finding of the present analysis is that fish oil supplementation in pregnancy significantly altered the pattern of neonatal long-chain PUFA metabolites, notably with inhibition of pro-inflammatory leukotrienes. Although the most notable effect was a significant reduction in neutrophil $LTB_4$ (isomer 2) production and the biological activity of this isomer is not known, there were multiple and consistent influences on other $LTB_4$ metabolites, which support a significant biological effect (Figure 1). This is also plausible and highly consistent with similar observations in adults ([2,10,11] and others). This now confirms that the maternal diet can have effects on the propensity of neonatal neutrophils to generate inflammatory products. Fish oil supplementation has also been associated with increased production of less inflammatory leukotrienes, namely $LTB_5$. However, although $LTB_5$ was the only metabolite to show a trend for increased production in the present study (Figure 1), it was only produced at very low levels and was not detectable in stimulated neutrophils from many neonates.

Consistent with these observations and our hypothesis, there were consistent coherent relationships between the patterns of neutrophil leukotriene production and variations in membrane fatty acid composition that occurred as the probable result of fish oil supplementation. Specifically, $n-3$ PUFA status was consistently associated with the lower production of inflammatory metabolites ($LTB_4$ isomers and 5-HETE) and higher production of the less inflammatory product ($LTB_5$). Many of these relationships were statistically significant (particularly in relation to maternal fatty acid status) and, even those that were not, had a high level of consistency, reinforcing a biological effect that is also in keeping with the known downstream biochemical effects of modifying long-chain PUFA substrate composition. The multiple comparisons used in the present study raises the issues of statistical correction factors that are likely to abolish most relationships, even if biologically significant. However, it has been validly argued [12] that, where there are consistent, repeated, coherent and plausible patterns, the results reinforce, rather than
detract, from each other. We argue that this may be the case with the consistent relationships between \(n-3\) PUFA status and the multiple independent measures of leukotriene production observed in the present study. These relationships are also consistent with the known properties of fatty acids and leukotrienes described in the literature. This is an exploratory study, and we propose that these observations collectively support a biologically plausible relationship that needs to be explored further.

The underlying basis for the present study was our interest in how the propensity for local tissue inflammation might influence immune programming. Although we acknowledge that it is difficult to extrapolate relationships observed in cord blood to local tissues, it is not ethically possible to do more invasive studies in humans. Immune programming typically begins in local tissues where surveying APCs digest encountered antigens and present them as peptides (via MHC class II receptors) to effector T-cells, after migration to the regional lymph nodes. The local tissue conditions during antigen encounter play an important part in determining the maturation and activity of APCs with downstream effects on T-cell programming. It has already been reported that LTB\(_4\) has an influence on APC (dendritic cell) migration [13]. We have shown previously [14] that \(n-3\) PUFA supplementation in pregnancy is associated with reduced neonatal oxidative stress in vivo (with significantly lower \(F_2\)-isoprostane levels; \(P < 0.0001\)). In the present study, we also demonstrate (in vitro) that this intervention was also associated with reduced production of LTB\(_4\) from cells (neutrophils) that are known to be present in local tissues during inflammatory events. Both of these potentially interrelated effects of fish oil (in addition to others) could logically influence the local tissue inflammatory milieu during antigen presentation and early immune programming.

Although there were relationships between the pattern of leukotriene production and cellular immune function, it is not possible to draw any conclusions about the causality of these associations, as changes in membrane long-chain PUFA composition are known to influence immune function through multiple pathways. The most notable and consistent relationships were seen with APC responses, which are typically initiated in proximity to tissue inflammation (as opposed to T-cell activation in the regional nodes). The propensity to produce more inflammatory long-chain PUFA metabolites (5-HETE, and LTB\(_4\) isomer 1 and isomer 2) was associated with increased production in the potent inflammatory cytokine IL-6 by APCs following microbial (TLR4) stimulation. It is of note that this potent innate stimulation also induced inhibitory IL-10 responses in parallel, arguably to counterbalance or regulate an excessive response. The induction of IL-10 is in keeping with the known properties of microbial products in promoting regulatory function [15]. Thus, although increased LTB\(_4\) isomer (1 and 2) production was associated with increased inflammatory IL-6 production, it was also associated with reduced antigen-presenting capacity (lower HLA-DR expression) and IL-10 production, both of which could limit responses. Again, although these observations are of interest, we acknowledge the difficulties of extrapolating this to in vivo events.

It is even more difficult to extrapolate the significance of the T-cell responses which occur in regional nodes downstream from tissue events. LTB\(_4\) receptors (BLT1) are highly expressed on Th2 lymphocytes, and animal models have demonstrated that LTB\(_4\) is required for the development of allergic Th2 immune responses [16]. In children with established allergic disease (asthma), local LTB\(_4\) production in airways (detected in breath condensates) is a measure of disease activity and airway inflammation [17]. Animal models also show that genetic ablation of the LTB\(_4\) signalling pathway ameliorates airway hyper-responsiveness and Th2 immune responses [16], suggesting that other interventions to modify LTB\(_4\) production (such as fish oil) could have therapeutic relevance. The significance of this in earlier life is even less clear. There are reports that neutrophil release of LTB\(_4\) is higher in neonates compared with adults [18], but it is not clear whether this has any direct relevance to the Th2 skewed responses that are normal in the neonatal period [19]. In the present study, we did not observe any relationships between leukotriene production and the propensity for either Th2 or Th1 T-cell responses assessed by this polyclonal method. It is not clear why there is a very different relationship between neutrophil leukotriene production and IL-10 release following direct polyclonal T-cell stimulation; with significant inverse relationships with LTB\(_4\) and 5-HETE (unlike the positive relationships seen following innate activation by LPS). This method of polyclonal stimulation measures T-cell ‘capacity’ and does not necessarily reflect specific responses in vivo, which develop in the regional nodes, rather than within the local tissues where the antigens are initially encountered. Furthermore, it is more likely that changes in membrane long-chain PUFAs had a direct effect on neonatal T-cell function, as we have demonstrated previously [3,20].

In summary, our present findings demonstrate that changes in maternal dietary long-chain PUFAs in pregnancy can directly influence the pattern of leukotriene production by neonatal neutrophils following an inflammatory trigger. We speculate that variations in the activity of these cells during early inflammatory events (such as respiratory tract infections) could influence the activity of other cells involved in immune programming. Although our observations support relationships between leukotriene production and other aspects of immune function, this is more difficult to interpret because of the multifaceted influences of long-chain PUFAs in vivo. However, our findings support more detailed in vivo studies (in neonatal animal models) to
examine (i) how the inflammatory propensity of local tissue neutrophils influence perinatal immune development, and (ii) the pathways through which changes in long-chain PUFA composition influences these processes. This is highly relevant to curtailing the mounting burden of inflammatory immune-mediated diseases in early life.

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