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# Fish Oil Supplementation in Pregnancy Lowers $F_2$-isoprostanes in Neonates at High Risk of Atopy

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The anti-inflammatory properties of n-3 polyunsaturated fatty acids (n-3 PUFA) have suggested a potential role of these nutrients in dietary modification for prevention of allergic disease in early life. As oxidative stress is known to modify antigen presenting cell (APC) signalling and resulting immune responses, we examined the effects of maternal n-3 PUFA supplementation in pregnancy on markers of oxidative stress and APC function in neonates at high risk of allergy.

Eighty-three pregnant atopic women were randomised to receive 4 g daily of either fish oil ($n = 40$) or olive oil ($n = 43$) capsules in a controlled trial from 20 weeks gestation until delivery. Plasma (cord blood) and urinary $F_2$-isoprostanes were measured as markers of lipid peroxidation. Cord erythrocyte fatty acids and markers of APC function (HLA-DR expression and cytokine responses) were measured and related to levels of plasma $F_2$-isoprostanes.

Maternal fish oil supplementation lowered plasma ($p < 0.0001$) and urinary ($p = 0.06$) $F_2$-isoprostanes. HLA-DR expression on APC was not different between the groups. In multiple regression analysis, 28.8% of the variance in plasma $F_2$-isoprostanes was explained by positive relationships with erythrocyte arachidonic acid (AA) and monocyte HLA-DR expression and a negative relationship with erythrocyte eicosapentaenoic acid (EPA).

This study shows that maternal supplementation with fish oil can attenuate neonatal lipid peroxidation. Clinical follow-up of these infants will help to determine if there are sustained effects on postnatal oxidative stress and expression of allergic disease.

*Keywords*: Fish oil; Pregnancy; Atopy; Neonate; Oxidative stress; F2-isoprostanes

## BACKGROUND

There has been recent concern that dietary changes, particularly declining intakes of n-3 PUFA and antioxidants may contribute to rising rates of allergic disease in "Westernised" populations through adverse effects on oxidative status. While inflammation and associated oxidative stress are clearly implicated in the established allergic disease,[1,2] adverse oxidative status in early life may also influence patterns of immune development and play a role in the pathogenesis of allergic disease. As such, this is becoming an area of increasing interest in targeting prevention of these increasingly common diseases. Allergic diseases are strongly associated with altered immune responses to environmental proteins (allergens) encountered at epithelial surfaces, with a characteristic increased propensity for allergic Type 2 helper cell (Th2) responses.[3] Although the processes responsible for this pattern of cell differentiation are not clear, antigen presenting cells (APC) provide essential signals which determine the pattern of T cell maturation and differentiation during immune development. Recent studies demonstrate that changes in oxidative status can significantly alter the pattern of APC signalling (IL-12, IL-10, IL-6 and TNFα production) and alter resulting patterns of T cell development.[4,5] Specifically, APC in an "oxidative" microenvironment appear to favour the development of Th2 responses compared with

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"reductive" APC. Thus, factors which modify "oxidative" status during early life are of key interest.

Measurement of $F_2$-isoprostanes, free radical oxidation products of arachidonic acid (AA), is a useful tool for assessment of *in vivo* lipid peroxidative damage in humans.[6] In addition, some $F_2$-isoprostanes also have biological activity. In the kidney 8-iso-prostaglandin $F_{2\alpha}$ is a potent vasoconstrictor and has bronchoconstrictor actions.[6] Levels of 8-iso-prostaglandin $F_{2\alpha}$, have been shown to be elevated in plasma[7] and breath exudate[8] of asthmatic subjects.

The n-3 PUFA inhibit allergic immune responses by inhibiting lymphoproliferation, pro-inflammatory cytokine responses, $PGE_2$ and APC function (expression of MHC class II antigens).[9] Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two principle n-3 fatty acids, have also been shown to decrease urinary $F_2$-isoprostanes in controlled trials of subjects with Type 2 diabetes mellitus[10] and in hyperlipidemic subjects.[11] Moreover, recent evidence suggests an association between the anti-inflammatory effects of n-3 fatty acids and $F_2$-isoprostanes in Type 2 diabetes mellitus.[12]

There is some evidence that lower levels of n-3 PUFA in breast milk are associated with infant atopy,[13] and we have shown that maternal fish oil supplementation during pregnancy results in decreased concentrations of IL-13 in cord blood.[14] This report examines the effect of maternal fish oil supplementation during the latter half of pregnancy on lipid peroxidation in neonates by measuring levels of $F_2$-isoprostanes in cord blood and urine.



## MATERIALS AND METHODS

### Subject Recruitment and Study Design

Eighty-three pregnant women with allergic disease were recruited before 20 weeks of pregnancy from St. John of God Hospital, Subiaco, Western Australia between January 2000 and September 2001. The study was approved by the ethics committees of St John of God Hospital and Princess Margaret Hospital. All women gave their written consent to participate. Allergic disease was defined as a history of doctor-diagnosed allergic rhinitis and/or asthma and one or more positive skin prick tests to common allergens (house dust mite, grasses, moulds, cat, dog, feathers and cockroach); (Hollister-Stier Laboratories, Spokane, WA, USA.) A weal size of greater than 3 mm above the negative control was considered positive. Women were excluded from the study if they were smokers, had other pre-existing medical conditions, had complicated pregnancies, had seafood allergies or were consuming more than two fish meals per week. The women were stratified on the basis of allergy (allergic rhinitis or asthma), parity (nulliparous versus primiparous and multiparous), age and pre-pregnancy body mass index (BMI). They were then randomly assigned to receive either a daily fish oil supplement 4 (1 g) capsules (Ocean Nutrition, Halifax, Nova Scotia, Canada) or olive oil 4 (1 g) capsules (Pan Laboratories, Moorebank, NSW, Australia) in a double blind placebo controlled parallel study from 20 weeks of pregnancy until delivery. The fish oil group received a total of 4 g of oil with 56% as DHA and 27.7% as EPA; the olive oil group received 4 g of olive oil containing 67% oleic acid and less than 1% n-3 PUFA.

### Samples and Measurements

Cord blood samples were collected from the placental vessels by venipuncture immediately after delivery. Cord blood for plasma $F_2$-isoprostanes was collected into EDTA and reduced glutathione, centrifuged at 4°C and the plasma stored at −80°C after the addition of butylated hydroxytoluene (BHT) 200 μg/ml to prevent *ex vivo* oxidation. A spot urine sample was collected from 68 babies during the first week post partum using a paediatric collection bag. Urinary creatinine was measured in the Department of Clinical Biochemistry at Royal Perth Hospital. Plasma and urinary $F_2$-isoprostanes were extracted purified and assayed using electron capture negative ionisation gas chromatography mass spectrometry (ECNI-MS) as previously described.[15] Erythrocyte fatty acids were extracted from washed red cell membranes and assayed by gas chromatography as previously described.[16] Cord blood mononuclear cells (CBMC) were isolated and cryopreserved as previously described.[17]

#### *Flow Cytometry of HLA-DR Expression on Neonatal APC*

At the time of assay CBMC were thawed and resuspended in RPMI culture medium. HLA-DR expression in $1 \times 10^6$ CBMC/ml was determined immediately (Time "0") and after incubation for 24 h in RPMI/10% heat inactivated FCS with or without rhIFNγ (10 ng/ml, Pharmagen). After incubation, monocytes were labelled with anti-CD14 conjugated fluorescein isothiocyanate (FITC) (Pharmingen, Becton Dickinson [BD], CA, USA) expression of HLA-DR identified with anti-HLA-DR coupled to phycoerythrin (PE) (BD, CA, USA). B-lymphocytes were identified by their expression of CD19 (anti-CD19 FITC, Pharmingen, BD, CA, USA) and were also counterstained with anti-HLA-DR (PE). The percentage of monocytes (CD14+) or B lymphocytes (CD19+) expressing HLA-DR and

the mean fluorescence intensity of expression (MFI) was examined using a FACSCalibur (Becton-Dickinson) and CellQuest software.

### *Cytokine Responses by Neonatal APC*

For analyses of APC cytokine responses to lipopolysaccharide (LPS, *Escherichia coli* 055:B5; 10 ng/ml, Sigma Australia). $1 \times 10^6$ CBMC/ml cultured in RPMI (Gibco, Life Technology, UK) plus 10% fetal calf serum (not heat inactivated) (Australian Biosearch, Australia) for 24 h with or without LPS and/or rhIFNγ. Cytokines (IL-10 and IL-12p70) were analysed in culture supernatants by time resolved fluorimetry (TRF).[18] Cytokine data was expressed as the difference between the stimulated culture and unstimulated culture.

### Statistics

Values are expressed as mean and standard error of the mean. Where data was not normally distributed values are expressed as geometric means and 95% confidence intervals (GM, 95% CI). Between group differences in cord plasma and urinary $F_2$-isoprostanes were assessed using a general linear model with adjustment for erythrocyte AA. Multiple regression analysis was used to find the model that best explained the variance in cord plasma $F_2$-isoprostanes.

## RESULTS

Eighty-three women completed the study and cord blood was collected from 35 women who had taken fish oil during pregnancy and 41 women who had taken olive oil. Age, pre-pregnancy BMI, and the percentage of nulliparous women were similar between the two groups of women (Table I). The numbers of women in each group with asthma was similar (Table I). Birth weight of the babies was not different between the groups (Table I). The newborns of mothers who took fish oil during pregnancy had raised levels of both erythrocyte EPA ($p < 0.001$) and DHA ($p < 0.001$) compared with those whose mothers took olive oil (Table I). Neonatal erythrocyte AA levels in the fish oil supplemented group were lower than in neonates whose mothers received olive oil but this did not achieve statistical significance ($p = 0.054$) (Table I). Neonatal erythrocyte oleic acid (18:1) levels were not different between the groups (Data not shown). Plasma $F_2$-isoprostanes were significantly lower in the offspring of women who had taken fish oil during pregnancy compared with those who took olive oil (Fig. 1). These differences were independent of erythrocyte AA levels. For the groups combined, cord plasma $F_2$-isoprostanes were significantly negatively correlated with erythrocyte EPA ($r = -0.351$, $p = 0.001$) but not DHA ($r = -0.177$, $p = 0.11$).

TABLE I Characteristics of women supplemented during pregnancy with olive oil or fish oil, and measurements in their neonates

| | Olive oil | Fish Oil |
|---|---|---|
| *Maternal characteristics* | | |
| Age (yrs) | 32.4 ± 0.5 | 31.0 ± 0.6 |
| Pre-pregnancy BMI (kg/m$^2$) | 24.1 ± 0.6 | 23.7 ± 0.6 |
| % nulliparous women | 47% | 45% |
| % women with asthma | 39.5% | 42.5% |
| *Neonatal measurements* | | |
| Birth weight (g) | 3430 ± 57 | 3503 ± 53 |
| *Erythrocyte fatty acids* ($\mu g/10^6 cells$) | | |
| AA** | 14.29 ± 0.62 | 12.47 ± 0.57 |
| EPA* | 0.32 ± 0.04 | 1.09 ± 0.08 |
| DHA* | 6.02 ± 0.31 | 8.53 ± 0.43 |
| Urinary $F_2$-isoprostanes***,† (pmol/mmol creatinine) | 23,878 (20,183–28,248) | 20,749 (17,218–24,888) |

Urinary $F_2$-isoprostanes are expressed as geometric means and 95% confidence intervals. All other values are expressed as mean ± SEM. *$p < 0.001$, **$p = 0.054$, ***$p = 0.064$ for between group comparison using a general linear model. †($n = 37$) for olive oil, ($n = 31$) for fish oil.

Urinary $F_2$-isoprostanes corrected for creatinine excretion tended to be lower in infants whose mother took fish oil during pregnancy compared with levels of infants whose mother took olive oil but this did not achieve statistical significance, either before ($p = 0.064$, Table I) or after adjusting for erythrocyte AA ($p = 0.107$). In the groups combined, urinary $F_2$-isoprostanes corrected for creatinine excretion were negatively correlated with both erythrocyte EPA ($r = -0.290$, $p = 0.017$) and DHA

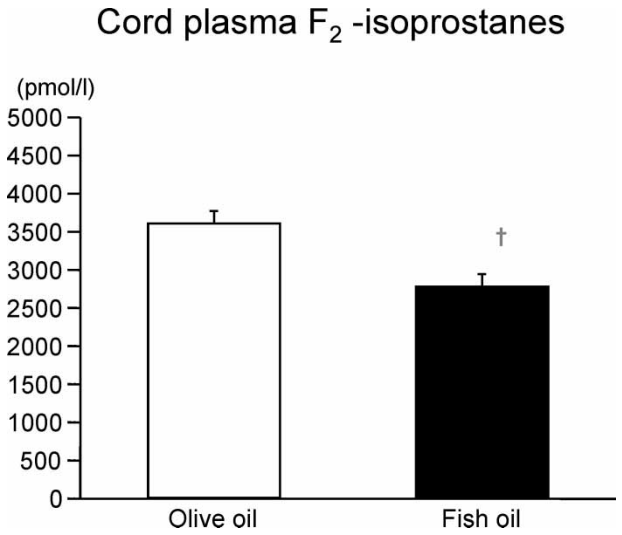


FIGURE 1 Cord plasma $F_2$-isoprostanes in neonates whose mothers were fed fish oil or olive oil during pregnancy. Between groups differences assessed using a general linear model ($p < 0.001$), after adjustment for erythrocyte AA.

TABLE II Basal and stimulated HLA-DR expression on antigen presenting cells in neonates of women who were supplemented with either olive oil or fish oil during pregnancy

| | Olive oil | Fish Oil |
|---|---|---|
| *Neonatal monocyte HLA-DR* | | |
| *Basal expression (time = 0)* | | |
| % monocytes | 92.7 ± 1.0 | 93.0 ± 0.9 |
| MFI | 68.2 ± 4.9 | 67.4 ± 5.2 |
| *After 24 h in cell culture* | | |
| % monocytes | 85.9 ± 1.8 | 84.1 ± 2.2 |
| MFI | 310 ± 14 | 295 ± 20 |
| *After stimulation (IFNγ 10 ng/ml, 24 h)* | | |
| % monocytes | 95.8 ± 0.7 | 95.4 ± 0.5 |
| MFI | 902 ± 44 | 947 ± 49 |
| *Neonatal B-lymphocyte HLA-DR* | | |
| *Basal expression (time = 0)* | | |
| % B lymphocytes | 97.6 ± 0.3 | 97.9 ± 0.2 |
| MFI | 335 ± 23 | 343 ± 21 |
| *After 24 h in cell culture* | | |
| % B lymphocytes | 98.4 ± 0.1 | 98.4 ± 0.1 |
| MFI | 706 ± 30 | 661 ± 29 |
| *After stimulation (IFNγ 10 ng/ml, 24 h)* | | |
| % B lymphocytes | 98.1 ± 0.2 | 98.3 ± 0.2 |
| MFI | 718 ± 33 | 680 ± 24 |

($r = -0.241$, $p = 0.05$) but were not significantly correlated with erythrocyte AA.

Fish oil supplementation during pregnancy did not alter either the mean intensity fluorescence or the percentage of cord blood monocytes or B-lymphocytes expressing HLA-DR, either with or without stimulation with rhIFNγ (Table II). However, for both groups combined, cord plasma $F_2$-isoprostanes were significantly positively correlated with basal ($t = 0$) monocyte HLA-DR expression, either expressed as the percentage of cells ($r = 0.342$, $p = 0.003$) or MFI ($r = 0.230$, $p = 0.049$). Cord plasma $F_2$-isoprostanes were not significantly correlated with HLA-DR expression on B-lymphocytes. After stimulation with IFNγ there was no significant correlation between cord plasma $F_2$-isoprostanes and HLA-DR expression on either monocytes or B-lymphocytes. Intensity of HLA-DR expression on (rhIFNγ treated) monocytes was positively correlated with the IL-12 response to LPS in both the olive oil group ($r = 0.503$, $P = 0.001$) and the fish oil group ($r = 0.345$, $P = 0.036$). However, there was no association between IL-12 (or IL-10) response to LPS and cord plasma $F_2$-isoprostane levels.

Multiple regression analysis was used to determine the model that best explained the variance in neonatal plasma $F_2$-isoprostane levels. Plasma $F_2$-isoprostanes were significantly and independently positively related to erythrocyte AA, negatively related to erythrocyte EPA and positively related to percentage of unstimulated monocytes expressing HLA-DR. This model accounted for 28.8% of the variance in plasma $F_2$-isoprostanes (Table III).

TABLE III Regression model examining relations between log cord plasma $F_2$ isoprostanes, erythrocyte AA, erythrocyte EPA and basal CB monocyte HLA-DR expression in the combined groups

| Independent variable | B | (95% CI for B) | Significance |
|---|---|---|---|
| Cord erythrocyte EPA (μg/$10^6$ Cells) | −0.205 | (−0.307−−0.104) | 0.0001 |
| Cord erythrocyte AA (μg/$10^6$ Cells) | 0.018 | (0.005–0.031) | 0.009 |
| Basal CB Monocyte HLA-DR expression (% cells) | 0.014 | (0.003–0.024) | 0.01 |
| Constant | 6.651 | (5.671–7.631) | 0.0001 |
| Adjusted $R^2 = 0.288$ | | | |

## DISCUSSION

This study examined lipid peroxidation measured as $F_2$-isoprostanes in neonates whose mothers took either fish oil or olive oil during the latter half of pregnancy. Maternal fish oil supplementation was associated with significantly lower neonatal plasma $F_2$-isoprostanes. Although the levels of erythrocyte AA were lower in offspring of women who took fish oil, the difference in $F_2$-isoprostanes between the groups was independent of the level of AA, suggesting that fish oil may act to decrease oxidative stress by mechanisms other than by reducing AA substrate. Fish oil supplementation in humans is known to decrease inflammatory cell responses, such as neutrophil chemotaxis and superoxide production, monocyte chemotaxis, and MHC class II expression on monocytes as well as decreasing the levels of pro-inflammatory cytokines.[9] Dampening of any of these inflammatory responses are likely to lead to decreased lipid peroxidation.

To our knowledge, this is the first report of urinary $F_2$-isoprostanes in newborn infants after maternal fish oil supplementation during pregnancy. Urinary $F_2$-isoprostanes measured in a spot urine sample in the first week of life tended to be lower in infants whose mothers took fish oil, mirroring the effects seen in cord plasma and suggesting that maternal fish oil supplementation during pregnancy might protect against oxidative stress in the infant soon after birth. This supports our previous studies where urinary $F_2$-isoprostanes were reduced by 20% in Type 2 diabetes patients given a daily fish meal providing 3.6 g/day of n-3 fatty acids,[10] and both EPA and DHA reduced 24 h urinary $F_2$-isoprostanes by 27 and 26% in hyperlipidemia men[11] and in patients with Type 2 diabetes.[10] In a study of infants fed a preterm formula with and without long chain polyunsaturated fatty acids, Stier *et al.* did not find differences in urinary $F_2$-isoprostanes after 3 weeks feeding.[19] In our study, urinary $F_2$-isoprostanes in both groups of infants are many times higher than those reported

by us in healthy men (365 ± 5 pmol/mmol creatinine) and smokers (981 ± 138 pmol/mmol creatinine)[15] using the same assay methodology.

Neonatal plasma $F_2$-isoprostanes in both groups were also elevated relative to plasma $F_2$-isoprostanes in healthy subjects (952 ± 38 pmol/l)[15] but were similar to those of healthy pregnant women (2042 ± 319 pmol/l).[20] This confirms observations by Berger *et al.*, who showed that regardless of whether or not infants had bleomycin detectable iron present, plasma $F_2$-isoprostanes were higher in infants than adults.[21] Our study suggests that regardless of maternal intake of fish oil during pregnancy the newborn is under relative oxidative stress, which results in raised cord plasma levels of $F_2$-isoprostanes. The only other report measuring umbilical cord 8-iso-prostaglandin $F_{2\alpha}$ showed that concentrations of organic hydroperoxides and umbilical cord arterial 8-iso-prostaglandin $F_{2\alpha}$ were raised in cases of fetal distress.[22] The very high levels of urinary $F_2$-isoprostanes in the neonates during the first week of life most likely reflect the oxidative challenge presented at birth, when there is transition from a relatively low intrauterine oxygen environment to a significantly higher oxygen extrauterine environment. The oxidative challenge is likely to be exacerbated by the low efficiency of natural anti-oxidant systems in the newborn.[23] Another possible source of oxidant stress in the newborn infant may come from the release of free iron from erythrocytes, which occurs even in healthy newborn infants[24,25] more readily, than release of free iron from erythrocytes in healthy adults.[25] The very high levels of urinary $F_2$-isoprostanes in the newborn infant are most likely a homeostatic response to rapidly remove products of free radical damage produced at birth.

The current finding of reduced cord plasma and urinary $F_2$-isoprostanes support our previous results[10–12] and those of others[26,27] in demonstrating that lipid peroxidation is reduced following n-3 fatty acid intake. A study in pigs showed that compared to beef tallow high dose n-3 fatty acids decreased plasma $F_2$-isoprostanes after coronary occlusion.[26] A fall in plasma $F_2$-isoprostanes was also shown in post-menopausal women given n-3 fatty acids compared with oleate or linoleate enriched diets.[27] However, these differences were eliminated when $F_2$-isoprostanes were corrected for plasma AA concentrations.[27]

The origin cord plasma $F_2$-isoprostanes and the mechanism by which they are lowered after fish oil supplementation remains unknown. It is possible that cord plasma $F_2$-isoprostanes are in part derived from the maternal circulation. Although we did not measure maternal $F_2$-isoprostanes in this study, our previous studies supplementing with either fish,[10] or purified EPA or DHA[11,12] consistently show that levels of urinary $F_2$-isoprostanes are lower than in subjects supplemented with olive oil. It is, therefore, likely that maternal supplementation with fish oil would also result in lower maternal plasma $F_2$-isoprostanes. There have been no studies examining transport of $F_2$-isoprostanes across the placental barrier. The fatty acid substrate for $F_2$-isoprostanes synthesis can be synthesised in fetal tissue, however, about 50% of fetal fatty acid requirements are maternally derived and readily cross the placenta as free fatty acids.[28] Therefore, it is possible that cord blood $F_2$-isoprostanes could be derived as a result of either oxidative damage in the maternal circulation or oxidation in the fetus or a combination of both.

Another possible explanation for reduced cord plasma $F_2$-isoprostane levels after fish oil supplementation is a redirection of lipid oxidation to EPA[29] or DHA[30,31] resulting in the formation of $F_3$ and $F_4$-isoprostanes, respectively. Whilst this remains a possibility we have not been able to quantify levels of $F_3$-isoprostanes after dietary fish meals[10] or purified EPA or DHA supplementation for 6 weeks.[11,12]

Expression of MHC class II molecules such as HLA-DR is a pre-requisite for the antigen-presenting function of monocytes and B-lymphocytes. In our study fish oil feeding did not alter unstimulated or IFNγ stimulated expression of either monocyte HLA-DR or B-lymphocyte HLA-DR. Previous *in-vitro* studies in the mouse[32] and a small study in humans reported lower HLA-DR expression after fish oil,[33] but a larger study of fish oil supplementation in patients with Type 2 diabetes also showed no effect of fish oil on monocyte HLA-DR expression.[34] We found no significant correlations between HLA-DR expression on monocytes or B lymphocytes and erythrocyte EPA or DHA. However, in regression analysis the significant independent positive predictors of neonatal plasma $F_2$-isoprostanes were the percentage of unstimulated monocytes expressing HLA-DR and erythrocyte AA, whilst the level of erythrocyte EPA was independently and negatively related to plasma $F_2$-isoprostanes. This model accounted for 28.8% of the variance in plasma $F_2$-isoprostanes. This model suggests that neonatal plasma $F_2$-isoprostanes levels are in part explained by the available substrate level reflected by AA, markers of immune function such as monocyte HLA-DR expression and the level of EPA. The relationship of plasma $F_2$-isoprostanes with monocyte HLA-DR expression might be explained by their common association with oxidative stress.[35] We showed that urinary $F_2$-isoprostanes were reduced by 20% in Type 2 diabetes patients given a daily fish meal providing 3.6 g/day of n-3 fatty acids.[10] In addition, both EPA and DHA reduced 24 h urinary $F_2$-isoprostanes by

27% and 26% in hyperlipidemia men[11] and in patients with Type 2 diabetes[10] and may reflect a normal immune response.

Although previous studies in animals have documented that reducing oxidation status can enhance IL-12 production by APC and inhibit the development of Th2 responses,[4,5] fish oil supplementation and $F_2$-isoprostane levels were not associated with altered IL-12 and IL-10 responses in this study.

Overall, these findings suggest fish oil supplementation during pregnancy reduces oxidant stress. In addition, there may be additional physiological benefits in lowering levels of cord plasma $F_2$-isoprostanes in infants susceptible to atopy, as one of the $F_2$-isoprostanes, 8-isoprostane (15-$F_{2t}$-isoP), is known to have direct bronchoconstrictor actions,[6] and levels of plasma 8-isoprostane have been related to disease severity.[7] Further assessment and clinical follow-up of these infants will help determine if there are any effects on postnatal oxidative stress and expression of allergic disease.



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