Prenatal omega-3 fatty acid supplementation does not affect offspring telomere length and F₂-isoprostanes at 12 years: a double blind, randomized controlled trial

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Abbreviations: telomere length (TL) ; deoxyribonucleic acid (DNA); omega-3 (n-3); long chain polyunsaturated fatty acids (LCPUFA); olive oil (OO); docosahexaenoic acid (DHA); eicosapentaenoic acid (EPA); arachidonic acid (AA); absolute telomere length (aTL); maximum likelihood estimation (MLE)

ABSTRACT

Background: Oxidative stress and nutritional deficiency may influence the excessive shortening of the telomeric ends of chromosomes. It is known that stress exposure in intrauterine life can produce variations in telomere length (TL), thereby potentially setting up a long-term trajectory for disease susceptibility.

Objective: To assess the effect of n-3 LCPUFA supplementation during pregnancy on telomere length and oxidative stress in offspring at birth and 12 years of age (12y).

Design: In a double-blind, placebo-controlled, parallel-group study, 98 pregnant atopic women were randomised to 4g/day of n-3 LCPUFA or control (olive oil [OO]), from 20 weeks gestation until delivery. Telomere length as a marker of cell senescence and plasma and urinary F₂-isoprostanes as a marker of oxidative stress were measured in the offspring at birth and 12y.

Results: Maternal n-3 LCPUFA supplementation did not influence offspring telomere length at birth or at 12y with no changes over time. Telomere length was not associated with F₂-isoprostanes or erythrocyte total n-3 fatty acids. Supplementation significantly reduced cord plasma F₂-isoprostanes ($P<0.001$), with a difference in the change over time between groups ($P=0.05$). However, the differences were no longer apparent at 12y. Between-group differences for urinary F₂-isoprostanes at birth and at 12y were non-significant with no changes over time.

Conclusions: This study does not support the hypothesis that n-3 LCPUFA during pregnancy provides sustained effects on postnatal oxidative stress and telomere length as observed in the offspring.

Keywords: n-3 fatty acid supplementation, pregnancy, oxidative stress, telomere length, infants

48 **Summary**

49 This study aimed to assess the effect of n-3 fatty acid supplementation during pregnancy on
50 telomere length(TL) and oxidative stress in the offspring. In a double-blinded study, 98 pregnant
51 atopic women were randomised to 4g/day of n-3 fatty acid or olive oil, from 20 weeks' gestation
52 until delivery. TL as a marker of cell senescence and plasma and urinary F₂-isoprostanes as a
53 marker of oxidative stress were measured in the offspring at birth and 12y. Maternal n-3 fatty
54 acid supplementation did not influence offspring TL or urinary F₂-isoprostanes from birth to 12y.
55 TL was not associated with F₂-isoprostanes or erythrocyte total n-3 fatty acids. Supplementation
56 significantly reduced cord plasma F₂-isoprostanes ($P<0.001$), with a between-group difference in
57 the change over time ($P=0.05$). However, the differences were no longer apparent at 12y. Thus,
58 n-3 fatty acid during pregnancy does not provide sustained effects on postnatal TL and oxidative
59 stress in the offspring.

(1) Introduction

Telomeres are tandem repeats of the sequence TTAGGG that stabilise and protect the ends of chromosomes and thus maintain chromosome and genome stability. There are two major determinants of telomere length – the newborn telomere length which represents the origin of the individual's telomere biology system and telomere length attrition over time[1]. A reduction in the newborn telomere length could therefore confer greater susceptibility in later life for pathophysiological outcomes. A growing body of empirical evidence suggests the origins of susceptibility for many of the pathophysiological outcomes leading to non-communicable diseases may be traced back to the intrauterine period of life [2, 3]. The “developmental origins of health and disease” (DOHaD) hypothesis asserts that factors that modify physiological processes during the sensitive period in early life may result in structural and/or functional changes in multiple organ systems that have important long-term consequences for subsequent health and disease susceptibility[4, 5]. In this context, we proposed that impaired telomere maintenance leading to cell senescence and apoptosis may represent a pathway linking suboptimal conditions during embryonic and fetal life with subsequent child and adult health outcomes.

In many cell types, senescence and subsequent apoptosis often occur when telomere length reaches a critical value[6]. Sampson *et al.*[7] postulated that increased oxidative stress would lead to telomere damage, shortening, and cellular senescence. Reactive oxygen species are a source of oxidative stress and can produce modified bases (mainly 8-oxoguanine) and single strand breaks anywhere in the genome. It has been estimated that 100-500 8-oxoguanine lesions are formed per day in the ‘normal’ state[8]. The high incidence (25%) of guanine residues in telomeric deoxyribonucleic acid (DNA) sequences means telomeres are potentially subjected to oxidative damage[8]. Thus, reactive oxygen species are capable to cause excessive telomere

attrition [8], and the anti-oxidant action of omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) has the potential to reduce reactive oxygen species, thereby reducing the attack of reactive oxygen species on telomeric DNA[9].

Comprehensive lifestyle changes incorporating daily consumption of n-3 LCPUFA have shown increased activity of telomerase, an enzyme responsible for preserving telomere length to maintain healthy cells and long-term immune function[10]. In a cohort of adults with coronary heart disease, omega-3 fatty acid concentrations were inversely associated with the rate of telomere shortening over 5 years[11]. Thus, it is possible that n-3 LCPUFA could influence telomere length through their antioxidant effects. As evidenced from animal and human studies, the association between adverse conditions during fetal development with newborn telomere length provides support for the notion that telomere length may be 'programmed' *in utero* [12].

This study tested the hypothesis that n-3 LCPUFA in pregnancy maintains telomere length in the offspring, that these effects would be sustained into early childhood at 12 years of age (12y), and that telomere length would be inversely associated with oxidative stress measured as plasma and urine F2-isoprostanes. This study aims to ascertain if effects of maternal n-3 LCPUFA in this critical time of plasticity are sustained in the offspring beyond the intervention period.

(2) Participants and Methods

2.1 Study Design and Participants

The study design, methodology, inclusion and exclusion criteria have been published [13-19]. Briefly, 83 atopic pregnant women were recruited before 20 weeks of pregnancy from St. John of God Hospital, Subiaco, Western Australia between January 2000 and September 2001.

Maternal atopy was defined by a positive skin prick test and a history of physician-diagnosed allergic rhinitis and/or asthma. 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.

2.2 Randomization and Intervention

The study was single-centred and stratified by parity (no previous term childbirth vs one or more), prepregnancy BMI, age and maternal allergy (allergic rhinitis or asthma) with block randomization [1:1]. The study was double-blind, with mothers randomly assigned to receive either 4 (1g) n-3 LCPUFA capsules (Ocean Nutrition Ltd, Mulgrave, Nova Scotia, Canada) or olive oil (OO) capsules (Pan Laboratories, Moorebank, NSW, Australia) as the control. Independent persons from those performing the allocation dispensed image and scent-matched n-3 LCPUFA or OO capsules to the participants. Supplementation commenced at 20 weeks of gestation and ceased at delivery. The n-3 LCPUFA capsules provided a total of 3.7 g of n-3 LCPUFA with 56.0% as docosahexaenoic acid (DHA; 22:6n-3) and 27.7% as eicosapentaenoic acid (EPA; 20:5n3) (confirmed by gas chromatography). Placebo capsules provided 4g of OO with 66.6% as n-9 oleic acid and <1% n-3 PUFA. This dose was based on previous trials studying the effect of n-3 LCPUFA supplementation in pregnancy[20], on high-risk pregnancies[21], on BP[22] and our previous studies in adults[23]. Participants completed a validated, semi-quantitative food frequency questionnaire at 20- and 30-week gestation to monitor fish consumption. Compliance with the treatment regime was determined by measuring erythrocyte DHA and EPA composition of the mothers at 30 and 36-week gestation and 6 weeks postnatally[16].

2.3 Samples and Measurements

Cord blood was collected from the placental vessels by venipuncture immediately after delivery. Peripheral blood from the offspring was obtained by venipuncture after an overnight fast at 12y. Blood for telomere length was collected into heparinized tubes and processed immediately after the clinic visit[13]. Cord blood mononuclear cells (CBMC) at birth and peripheral blood mononuclear cells (PBMC) were isolated and cryopreserved as previously described[13]. DNA was isolated using a QIAGEN DNeasy Kit with minor modifications to prevent DNA oxidation, as previously described [24]. Absolute TL (aTL) was measured in triplicate samples by determining the number of TTAGGG hexamer repeats using quantitative real-time polymerase chain reaction as previously described[24]. aTL values in kb/diploid genome were calculated using a synthesised TTAGGG 84 mer oligonucleotide to generate a standard curve; the number of diploid genome copies per reaction was determined using the single copy gene 36B4. Using our internal control (1301 cell line DNA), we estimated that the interexperimental variability was $\leq 7\%$ (n = 35) and the intraexperimental variability 1.1% (n = 16). Blood for plasma F₂-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 (200mg/ml) to prevent *ex vivo* oxidation. A spot urine sample was collected from the babies during the first week post partum using a paediatric collection bag and a urine sample was collected from the children at the 12y follow-up visit. Urinary creatinine was measured in the PathWest Laboratory at Royal Perth Hospital. F₂-isoprostanes were extracted purified and assayed using electron capture negative ionisation gas chromatography mass spectrometry (ECNI-MS) as previously described for cord and urine[17]. In a modification of the previously described method 8,12-iso-iPF₂ α -VI-d₁₁ was used as the internal standard for urinary F₂-isoprostanes [25]. Erythrocyte fatty acids were extracted from washed red cell membranes assayed by gas chromatography as previously

described[26]. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committees of St John of God Hospital and Princess Margaret Hospital, Perth, Western Australia. Written informed consent was obtained from all the mothers.

2.4 Statistics

Data were summarised using counts, percentages, means and standard deviations as appropriate. Log transformations were applied to outcomes when the data were not normally distributed. For these variables, geometric means and standard deviations were calculated. Differences between group characteristics at 12y for dichotomous data were assessed using chi-square tests, and *t*-tests for continuous data. Outcomes measured at multiple time points were analysed using linear mixed models with maximum likelihood estimation (MLE) which produces unbiased estimates. MLE handles missing data by using complete and incomplete data as well as intra class correlation to estimate the most likely mean for the whole sample at each time point. A *P* value of <0.05 (2-tailed) was considered statistically significant for all analyses. Adjustments for gender, maternal age and cigarette smoke exposure during pregnancy were applied for telomere length while adjustments for erythrocyte concentrations of arachidonic acid (AA) was applied for plasma F₂-isoprostanes by including these variables in a multivariable model. Associations between telomere length and F₂-isoprostanes/erythrocyte total n-3 fatty acids [sum of EPA + DHA + docosapentaenoic acid (DPA; 22:5n3)] were investigated with three-way and two way interactions of group, year and the variable of interest. Non significant interactions were not reported. All statistical analyses were performed using the Stata 13.1 statistical package (Stata Corp, College Station, TX).

(3) Results

3.1 Study demographics

Eighty three women and their healthy full-term babies completed the study at birth (40 in the n-3 LCPUFA group and 43 in the OO group) as reported previously[13]. Figure 1 shows the flow of the participants through the trial. The characteristics of the offspring who had bloods analysed for telomere length and F₂-isoprostanes and their mothers are shown in Table 1 (39 in the n-3 LCPUFA group and 43 in the OO group). There were no significant differences in maternal age, pre-pregnancy BMI, atopic status or parity between the women in the n-3 LCPUFA and control groups. Maternal education was borderline significantly different ($P=0.05$) between groups where the mothers in the OO group were more highly educated compared to the n-3 LCPUFA group. There were no significant differences in gestational age, gender, birth weight, birth length and head circumference between the neonates in the two groups (Table 1).

3.2 Analysis of offspring measures repeated over time

3.2.1 Telomere Length

Telomere length was not significantly different between the groups at birth ($P=0.76$) and 12y ($P=0.21$). There was no difference in the change over time between groups ($P=0.38$) (Table 2). Adjustments for gender, maternal age and cigarette smoke exposure during pregnancy did not alter the results (Table 2).

3.2.2 F₂-isoprostanes

Cord plasma F₂-isoprostanes were significantly lower in the offspring of women supplemented with n-3 LCPUFA during pregnancy compared with those who took OO ($P<0.001$) (Table 2). Plasma F₂-isoprostanes were significantly raised at birth compared with 12y and declined in both groups from birth to 12y ($P<0.001$). The difference in the change over time between groups was borderline significant ($P=0.05$). The differences in plasma F₂-isoprostanes at birth were independent of erythrocyte AA levels ($P=0.008$) but the difference in the change over time was no longer significant following adjustment for erythrocyte AA levels ($P=0.20$) (Table 2). At 12y, plasma F₂-isoprostanes were lower, albeit not significantly different, in infants whose mothers were supplemented with n-3 LCPUFA compared with OO during pregnancy (696.9 pmol/L and 773.6 pmol/L, respectively) ($P=0.08$) (Table 2). The difference remained non-significant after adjusting for erythrocyte AA ($P=0.15$).

Urinary F₂-isoprostanes corrected for creatinine excretion were not significantly different between the groups at birth and 12y (Table 2). Urinary F₂-isoprostanes were significantly raised at birth compared with 12y and decreased over time ($P<0.001$) in both groups. The fall in urinary F₂-isoprostanes over time differed significantly between groups ($P=0.03$) (Table 2).

3.3 Association between telomere length and F₂-isoprostanes from birth to 12y

There were no significant interactions between group, year and plasma or urinary F₂-isoprostanes on log telomere length. Log-transformed telomere length was not significantly associated with plasma F₂-isoprostanes ($P=0.89$) or urinary F₂-isoprostanes ($P=0.62$) after adjustments for group and difference over time.

3.4 Association between telomere length and erythrocyte n-3 fatty acids from birth to 12y

There were no significant interactions between group, year and erythrocyte n-3 fatty acids on log telomere length. Log-transformed telomere length was not significantly associated with erythrocyte n-3 fatty acids after adjustments for group and difference over time ($P=0.40$).

(4) DISCUSSION

To our knowledge, this is the first study that has examined whether maternal n-3 LCPUFA supplementation during pregnancy affects offspring telomere length at birth and into early childhood, and whether there is any relationship between telomere length and oxidative stress. We have shown that n-3 LCPUFA supplementation from 20 weeks of gestation till delivery significantly reduced plasma F₂-isoprostanes in the newborn offspring at birth but these differences were no longer apparent at 12y. However, leukocyte telomere length was not different between the two groups at birth or at 12y. These findings are in accordance with a randomized controlled trial in which 1.25 or 2.5g/day n-3 LCPUFA given to healthy sedentary overweight middle-aged subjects had no effect on telomere length but oxidative stress as measured by plasma F₂-isoprostanes was significantly reduced (29).

To date there are limited data on the effects of omega-3 fatty acids on telomere length. Omega-3 fatty acid concentrations has been shown to be inversely associated with the rate of telomere shortening over 5 years in a cohort of adults with coronary heart disease [11]. In our study, telomere length was maintained in both groups from birth to 12y. While age-related reductions in telomeres are expected, it may be too early to observe telomere attrition at such an early age. Recent studies show that telomeres can both shorten and elongate *in vivo*, and

leukocyte telomere length can change within a period of months[27, 28]. There is a possibility that OO could also slow the process of telomere shortening as part of a complex healthy dietary pattern. The Nurses' Health Study recently reported an association between adherence to a Mediterranean diet and longer telomeres, but no associations between the individual dietary components and telomere length[29]. The Mediterranean diet in the Nurses' Health Study was characterized by a high intake of OO and a moderately high intake of fish, vegetables, fruits, nuts, legumes, and grains, a low intake of dairy products, meat, and poultry, and a regular but moderate intake of alcohol[29].

Decreased cord plasma F₂-isoprostane concentrations following maternal n-3 LCPUFA supplementation are in accordance with other randomised controlled trials. Two interventions in adults, one providing daily fish meals and the other EPA or DHA, demonstrated that urinary F₂-isoprostanes were significantly reduced by 20-27%[30]. These results demonstrated that n-3 LCPUFA can reduce *in vivo* oxidant stress in humans[30]. Our finding that cord plasma and urinary F₂-isoprostanes are significantly raised at birth compared with 12y suggests that the newborn is subjected to relatively high oxidative stress. The fatty acid substrate for F₂-isoprostanes synthesis, arachidonic acid, can be synthesised in fetal tissue. However, about 50% of fetal fatty acid requirements are maternally derived and readily cross the placenta[31]. Therefore, it is possible that cord plasma F₂-isoprostanes could derive as a result of either oxidative damage in the maternal circulation or oxidation in the fetus or a combination of both. The very high concentrations of urinary F₂-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 newborn infant most likely homeostatically responds to rapidly remove products of free radical damage produced at birth.

The long term follow-up of this study is a strength, however we acknowledge that the results of the study are limited by the high dropout rate by 12y. However, we accounted in statistical analyses for the missing outcomes at each of the time points to address this limitation using mixed linear models. We note the study was unable to assess telomerase activity, a measure that could provide insight into the mechanisms by which n-3 LCPUFA could influence telomere structure and function. Paternal age and maternal folate concentrations were not obtained in this study. Studies have shown that paternal age at birth correlates positively with offspring telomere length[32, 33] and that maternal total folate concentration in early pregnancy is significantly and positively associated with newborn cord blood telomere length[34].

In conclusion, this study has shown that maternal n-3 LCPUFA supplementation in late pregnancy is significantly and negatively associated with newborn cord plasma F₂-isoprostanes but these differences were no longer apparent at 12y. We found no evidence for effects of maternal n-3 LCPUFA affecting offspring telomere length at birth and 12y. Future larger studies are warranted that incorporate the features that limited our study outcomes along with further follow-up during childhood and beyond.

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289 interpretation of results. None of the authors had conflicts of interest.

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TABLE 1. Table 1 Characteristics of the study population^a

Characteristics	n-3 Fatty acids (n=39)	OO (n=43)
<i>Antenatal characteristics</i>		
Maternal age, years	31.0 (4.0)	32.4 (3.5)
Prepregnancy BMI, kg/m ²	23.7 (3.8)	24.1 (4.0)
Maternal allergic status		
• Asthma	16 (41.0)	17 (39.5)
• Rhinitis	23 (59.0)	26 (60.5)
Parity		
• Nulliparous	18 (46.2)	21 (48.8)
Delivery method		
• Normal labour	29 (74.4)	35 (81.4)
Maternal smoked before		
• Yes	13 (33.3)	21 (48.8)
Maternal passive smoking		
• Yes	21 (53.8)	24 (55.8)
Partner smoking status		
• Yes	5 (12.8)	4 (9.3)
Maternal education (years)		
• 10-12	16 (41.0)	9 (20.9)
• >12	23 (59.0)	34 (79.1)

Gestational age, days	274.7 (8.3)	274.2 (7.8)
<i>Birth characteristics</i>		
Gender		
• Male	23 (59.0)	21 (48.8)
Birth weight, g	3502.7 (342.1)	3430.1 (371.8)
Birth length, cm	50.5 (2.0)	49.7 (1.9)
Head circumference, cm	^b 35.2 (1.3)	34.7 (1.2)
Apgar score	^c 8 (1)	^d 8 (1)

^a Data are means(SD) for continuous variables or numbers(%) for dichotomous variables.

^bData available for n = 38 participants

^c Data available for n = 29 participants

^d Data available for n = 34 participants

Table 2 Telomere length, plasma and urinary F₂-isoprostane concentrations of participants over time^{1,2}

	Birth			12y			
	n-3			n-3			
	LCPUFA	OO	<i>P</i> -value	LCPUFA	OO	<i>P</i> -value	<i>P_{interaction}</i>
TL,	150.6	156.7	0.76	153.1	174.8	0.21	0.38
Kb/genome	(1.4)	(1.4)	(0.85) ³	(1.6)	(1.4)	(0.24) ³	(0.39) ⁴
Plasma F₂-isoprostane,	2680.4	3499.3	<0.001	696.9	773.6	0.08	0.05
pmol/L	(1.3)	(1.3)	(0.008) ⁵	(1.2)	(1.2)	(0.15) ⁵	(0.20) ⁶
Urinary F₂-isoprostane,	20746.0	24188.9	0.26 ⁷	404.8	344.6	0.08 ⁷	0.03 ⁸
pmol/mmol creatinine	(1.7)	(1.6)		(1.5)	(1.4)		

¹ Results are given as geometric means (geometric SD)

² *P*-value and *P_{interaction}* are obtained from mixed linear models

³ *P* values test the hypothesis of no difference between the treatment groups (adjusted for gender, maternal age and cigarette smoke exposure)

⁴ *P* values test the hypothesis of no difference between the changes in treatment groups from birth to 12y (adjusted for gender, maternal age and cigarette smoke exposure)

⁵ *P* values test the hypothesis of no difference between the treatment groups (adjusted for erythrocyte AA levels)

⁶ P values test the hypothesis of no difference between the changes in treatment groups from birth to 12y (adjusted for erythrocyte AA levels)

⁷ P values test the hypothesis of no difference between the treatment groups (adjusted for erythrocyte AA levels)

⁸ P values test the hypothesis of no difference between the changes in treatment groups from birth to 12y (adjusted for erythrocyte AA levels)

Figure Legends

Figure 1.

The consort diagram showing volunteer recruitment and randomization

Figure 1