Fish-Oil Supplementation in Pregnancy Causes a Proportional Increase in Bone-, Lean- and Fat Mass at 6 Years:
A Randomized Clinical Trial

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Fish-Oil Supplementation in Pregnancy Causes a Proportional Increase in Bone-, Lean- and Fat Mass at 6 Years: A Randomized Clinical Trial

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Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical

Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. We follow national and international rules on the processing of personal data, including the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Abbreviations:

BMC = Bone Mineral Content

BMD = Bone Mineral Density

BMI = Body Mass Index

$COPSAC_{2010}$ = COpenhagen Prospective Studies on Asthma in $Childhood_{2010}$

CI = Confidence Interval

DHA = DocosaHexaenoic acid

DXA = Dual-energy X-ray Absorptiometry

EPA = EicosaPentaenoic acid

IOTF = International Obesity Task Force

n-3 LCPUFA = n-3 long chain polyunsaturated fatty acids

RCT = Randomized Controlled Trial

TBLH = Total Body Less Head

What is already known on this subject

Animal studies have shown that n-3 LCPUFA supplementation both in pregnancy and the postnatal period affects adipogenesis. However, in humans randomized trials with n-3 LCPUFA supplementation in pregnancy have shown ambiguous results regarding anthropometric outcomes later in childhood.

What this study adds

Our study suggest that N-3 LCPUFA supplementation in pregnancy led to increased BMI in childhood with sustained elevated BMI from age 1 year till 6 years. We saw no difference in fat percentage but a proportional increase in lean mass, bone mass and fat mass at 6 years. Our findings confirm that n-3 LCPUFA affects fetal programming leading to changed growth.

ABSTRACT (281 words)

Importance

Observational studies suggest that maternal n−3 long-chain polyunsaturated fatty acids (LCPUFAs) intake during pregnancy may increase offspring growth during childhood.

Objective

To examine the effect of n-3 LCPUFA supplementation in pregnancy on offspring anthropometrics and body composition.

Design, Setting and Participants

This was a double-blinded, randomized controlled trial conducted in 736 pregnant women and their offspring, from the Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$-cohort

Intervention

The pregnant women were randomized to n-3 LCPUFA (fish oil) or control (olive oil) daily from pregnancy week 24 until one week after birth.

Main Outcomes and Measures

Height/length, weight, head, waist measurements and bodycomposition from dual-energy X-ray absorptiometry (DXA) (all pre-specified secondary end-points of the n-3 LCPUFA trial, the primary outcome for the trial was persistent wheeze/asthma.)

Results

The mean BMI z-score was increased from 0-6 years compared to control: 0.14[0.13; 0.15];p=0.006. At 6 years, supplementation was associated with a higher z-score BMI (0.19 [0.06; 0.32], p=0.004) and a larger waist circumference (0.6cm [0.0; 1.2];p=0.04), but not a higher proportion of obese children. The DXA scan at age 6 years showed a higher lean mass in the supplementation vs. control group (280.7g[98.9;462.4];p=0.002), a higher bone mineral content (10.3g [2.3;18.1];p=0.01), and a non-significant higher fat mass (116.3g [-92.9; 325.5];p=0.28), but we observed no differences in total body fat- or lean mass-percentage.

Conclusion

Fish-oil supplementation from 24th week of pregnancy led to an increased BMI in the offspring from 0-6 years of age, but not an increased risk of obesity at age 6. The body composition at age 6 years in fish-oil supplemented children was characterized by a proportional increase in lean-, bone- and fat mass suggesting a general growth stimulating effect of n-3 LCPUFAs.

Trial Registration

ClinicalTrials.gov: NCT00798226

INTRODUCTION

Diet during pregnancy and infancy is an important determinant for child development and health[1], and in particular, fish intake containing n-3 long-chain polyunsaturated fatty acids (LCPUFAs) is important for adequate development[2]. In humans, both observational studies on dietary intake of fish as well as randomized controlled trials (RCTs) of n-3 LCPUFA (fish oil) supplementation in pregnancy and during lactation have consistently shown higher birth weight in children born to women with higher n-3 LCPUFA intake[3–6], while the long-term effect on growth during childhood is uncertain[7–10]. Mechanistic studies in rats have shown that n-3 LCPUFA supplementation both in pregnancy and the postnatal period affects the proliferation and differentiation of pre-adipocytes, which theoretically could prevent adiposity through inhibition of fat tissue[11,12]. However, it is unknown how n-3 LCPUFA supplementation during pregnancy affects offspring body composition during childhood.

In the population-based mother-child cohort Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$), we performed a double blind RCT of n-3 LCPUFA (fish oil) vs. control (olive oil) supplementation from week 24 of pregnancy to 1 week postpartum[13]. The primary end-point asthma or persistent wheeze demonstrated a 31% reduced risk in the group receiving fishoil [14]. As a secondary end-point we aimed to investigate the effect of n-3 LCPUFA supplementation on growth and body composition in the offspring. Body Mass Index (BMI) development was assessed at 11 clinical visits from birth to age 6 years and body composition was assessed from dual-energy X-ray absorptiometry (DXA) scans at 3.5 and 6 years of age.

METHODS

Study Design

This was a single-centre, double-blind, placebo-controlled, parallel-group study[14]. The recruitment procedure is detailed in the supplementary materials.

The primary outcome of the n-3 LCPUFA RCT was persistent wheeze/asthma[13,14]. As a pre-defined secondary end-point we investigated anthropometric measurements through childhood and body composition by DXA scans[14].

Study Intervention

The women were randomized 1:1 in a double-blind design at pregnancy week 24 to either daily supplementation of 2.4g n-3 LCPUFA (55% eicosapentaenoic acid (EPA, 20:5 n-3) and 37% docosahexaenoic acid (DHA, 22:6 n-3), Incromega TG33/22, Croda Health Care, UK) in triacylglycerol form or look-alike control supplementation capsules of olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid, Pharmatech A/S, Norway). The supplementation was continued until one week after birth and the study was un-blinded when the youngest child reached age 3.

A subgroup from this pregnancy cohort also participated in a nested; factorial designed, double-blind, RCT of 2,400IU/day of vitamin D3 supplementation (N=576)

Maternal fatty acid desaturase (FADS) genotype

Maternal FADS gene variation was tagged by genotyping of the single nucleotide polymorphism (SNP) rs1535 (LGC Limited, Hoddesdon, UK) in mothers of European descent (supplementary materials).

Adherence

See supplementary materials.

Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment.

Anthropometrics

Anthropometrics were assessed at the COPSAC research unit at age 1 week, 1 month, 3 months, 6 months, and every sixth months until age 2 years, and hereafter every year until age 6 years; in total 11 visits to our clinic.

Weight was measured without clothes using calibrated digital weight scales. Length was measured until age 2 years using an infantometer (Kiddimeter; Raven Equipment Ltd, Dunmow, Essex, England). Height from age 2 years and parental height was measured with a stadiometer (Harpenden, Holtain Ltd, Crymych, Dyfed, Wales), which was calibrated yearly.

Head circumference was measured with a tape, using the largest diameter as end-point. Waist circumference was measured with a tape using the navel as fix point. The mean of two measures during inspiration and expiration was used. WHO age- and sex specific BMI z-scores[23] were calculated for all measurements from 1 week to 6 years of age. International Obesity Task Force (IOTF) cut-offs for BMI were used to determine risk of overweight and obesity (above grade zero) and underweight (below grade zero)[24].

Birth length and weight were obtained at the first clinical visit after birth by personal interview and the values were validated against data from the Danish National Birth Registry.

Birth weight-for-gestational-age percentile rank was derived from Marsal's ultrasound-based intrauterine growth curves[25] and used as size for gestational age. The scores were calculated as percentage change from the mean size for gestational age of Marsal's population, the mean was set to zero.

DXA scans

Whole body scans were performed with a 'Lunar iDXA' densitometer (GE Healthcare, Fairfield, CT, USA) at 3.5 years and 6 years of age. We analysed data on fat mass, lean mass (total mass minus bone mineral content (BMC) and fat mass), BMC and bone mineral density (BMD) for the total body less head (TBLH)[26]. In addition, for fat mass and lean mass we analyzed specific regions of interest[27–29]. Furthermore, we calculated the percentage of fat mass and lean mass for TBLH and regions of interest. All analysis on DXA scan data were adjusted for sex and age at measurement. All DXA scan data were validated by an experienced specialist and analysed with enCore™ software.

Baseline characteristics

Collection and definition of baseline characteristics of the participants are described in the supplementary materials.

Statistical Analysis

We included children with at least one anthropometric measurement at age 0-6 years and excluded twins.

The effect of n-3 LCPUFA supplementation on cross-sectional anthropometric outcomes was analysed using Student's t-test for normally distributed continuous variables and chi-square tests for categorical variables.

Anthropometrics used for cross-sectional analyses at age 6 years were defined as the specific anthropometric measurement closest to 6 years ± 6 months.

BMI changes over time were analysed in a random intercept mixed model with BMI z-scores as the outcome. Age-related trends in the association between intervention and BMI were investigated in the mixed models by including an interaction-term between age and intervention group. Missing observation were treated as missing data and excluded from analyses. The analyses were performed for all children and stratified by sex. All data analyses were conducted with R v 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Results with a p-value <0.05 were considered statistically significant.

The trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the power of the RCT on BMI was calculated post-hoc based on the 605 children who had available 6-year BMI data. This resulted in 80% power, to detect a mean difference of 0.19 in z-score BMI, with a SD of 0.82. The power/sample size calculation and testing were based on a 2-sample, two-tailed t-test with an alpha of 0.05.

RESULTS

Baseline Characteristics

Enrolment went from November 2008 to November 2010. We randomized 736 women at pregnancy week 24 to either n-3 LCPUFA or control supplementation (**Figure E1**); after excluding twins, 688 children with at least one available anthropometric measurement were included in the study, 341 (49%) in the n-3 LCPUFA supplementation group and 347 (51%) to the control group. 605 (88%) of these children had anthropometrics measured at the 6 years visit.

Baseline characteristics of the pregnant women and their children are presented in **Table 1** showing a successful randomization (p>0.05 in all comparisons).

Adherence

From pill counts 71% (N=487) of the women took at least 80% of the prescribed supplement with no differences between the n-3 LCPUFA (N=242) and control group (N=245).

n-3 LCPUFA supplementation and BMI development during childhood

The n-3 LCPUFA supplementation group had a significantly higher BMI from 1 week to 6 years of age compared to the control group using a mixed effects model of the repeated measurements of BMI: mean z-score difference 0.14, 95% confidence interval (CI) [0.04; 0.23], p=0.006. There was a significant interaction between age and intervention group (interaction p-value=0.03). **Figure 1** illustrates BMI development from birth to age 6 years according to intervention group, showing that children in the n-3 LCPUFA group had a higher BMI at age 1 week and a higher BMI from 1 year to 6 years, whereas there was no clear separation in BMI in the age range 1 week to 6 months (**Figure 2**).

The effects of the intervention on BMI development was similar in boys and girls (**Figure E2**), and there was no significant interaction between sex and intervention group (p=0.79).

Because n-3 LCPUFA supplementation also reduced the risk of asthma and lower respiratory tract infections[14], we performed a sub-analysis excluding children with asthma at age 6 years and/or with lower respiratory tract infections before age 3 years. This did not affect the association between n-3 LCPUFA supplementation and BMI development (data not shown).

n-3 LCPUFA supplementation and anthropometric measurements at 6 years of age

Children in the n-3 LCPUFA supplementation group had a significantly higher BMI z-score at age 6 years compared to the control group: mean difference, 0.19, 95% CI [0.06; 0.32], p=0.004 and a larger waist circumference, 0.6 cm, 95% CI [0.0; 1.2], p=0.04) and a trend towards a higher weight, 0.4 kg, 95% CI [-0.1;0.8], p=0.11, while there were no differences in height or head circumference (**Table 2**).

Figure 3 illustrates the mean z-score BMI distribution between the intervention groups at age 6 years. A higher proportion of children from the n-3 LCPUFA supplementation group had a BMI in the highest quartile (29% (N=91) vs. 21% (N=66), p=0.02) and fewer had a BMI in the lowest quartile (21% (N=62) vs. 30% (N=90), p=0.02) compared to the control group. However, there were no significant differences between the intervention groups among children with the highest or lowest 10 percent BMI and no differences in prevalence of over- or underweight children according to IOTF grades (**Table 2**).

Adjusting the analyses for size for gestational age yielded comparable results (data not shown). There was no interaction between the intervention and sex, size for gestational age or maternal pre-intervention blood levels of EPA and DHA in relation to the anthropometric outcomes (data not shown).

n-3 LCPUFA supplementation and body composition

At 6 years of age, 523 (76%) of 688 children completed a DXA scan. The n-3 LCPUFA group had a higher total body mass in the compartment TBLH compared to controls; 19361.0g vs. 18967.0g (height adjusted mean difference of 395.4g; 95 % CI [86.6; 704.3], p=0.01). This was similar to the measured weight difference at age 6 years.

Sub-analyses on tissue type revealed that the children in the n-3 LCPUFA supplementation group had a significantly higher lean mass in the TBLH compartment (mean difference 280.7g; 95% CI [98.9; 462.4]; p=0.002) and on the trunk (127.2g [29.4; 242.9]; p=0.01). The n-3 LCPUFA supplementation group had a non-significant higher fat mass in TBLH: 116.3g [-92.9; 325.5]; p=0.28, but there were no difference in total body fat percentage or lean mass percentage (**Table 3**). Children in the n-3 LCPUFA supplementation group had a higher TBLH BMC (10.2g [1.8; 23.2]; p=0.01) and a trend towards a higher BMD (0.005g/cm^2 [-0.001; 0.012], p=0.08).

At 3.5 years of age, 356 (52%) of 688 children had available DXA scan data. There were no significant differences in body composition between the intervention groups, but all estimates for

lean mass, fat mass and BMC where higher in the n-3 LCPUFA supplementation group compared to the control group (**Table E2**).

There was no interaction between the intervention and sex, size for gestational age or maternal pre-intervention blood levels of EPA and DHA in relation to the body composition outcomes at 6 years (data not shown).

Maternal FADS genotype and BMI development during childhood

In a sub-analysis, we investigated if maternal FADS genotype was associated with offspring BMI development and body composition. We stratified the data by intervention groups and investigated the difference in BMI between children born by mothers with the FADS genotypes associated with higher levels of EPA and DHA (AA/AG) and those with the genotype associated with lower levels (GG). In the control group, we found that the children born to mothers with AA/AG-genotype tended to have higher BMI values from 1 to 6 years of age (mean difference 0.2, 95% CI [-0.0;0.5], p=0.08) and higher z-score BMI at 6 years of age (mean difference 0.3, 95% CI [0.1; 0.5], p=0.01) compared to children born to mothers with GG-genotype (**Figure E3** and **Table E3**). In contrast, there was no association between FADS genotype and BMI or other anthropometrics outcomes in the n-3 LCPUFA supplemented group. No significant interactions were found between FADS genotype and effect from the intervention in relation to BMI or other anthropometric outcomes (data not shown).

DISCUSSION

Primary Findings

Supplementation with n-3 LCPUFA in third trimester of pregnancy resulted in a higher BMI in the children from age 1 to 6 years, but not an increase in number of obese children. Body composition assessed by DXA scans confirmed that the higher BMI was not the result of a higher fat percentage, but reflected a proportional increase in lean mass, bone mass, and fat mass, suggesting a general growth stimulating effect of the n-3 LCPUFA supplementation.

Strengths and Limitations

Our study is among the largest RCTs on n-3 LCPUFA supplementation in pregnancy. It is nested in a population-based cohort, increasing the external validity of our findings. Both adherence to the supplementations (71%) and to the study was high, with 88% of the children having anthropometrics measured at age 6 years.

The longitudinal clinical follow-up with 11 visits during the first 6 years of life, including a broad range of anthropometrics measures, is a significant strength of the study. Each growth measurement was performed using the same equipment by trained COPSAC assistants based on standard operating procedures, and the observed growth curves were similar to previous reports[30].

Another strength is the DXA scans providing objective measures of fat mass, lean mass and bone mass enabling us to disentangle which tissues were affected by the intervention. The DXA estimates showed the same tendency at 3.5 years as the findings from the 6 years DXA scans.

The observation of a higher BMI and higher weight in children born to mothers with the FADS genotypes causing higher EPA and DHA levels in the control group provides indirect support of our findings from the n-3 LCPUFA intervention as this genetic variation is a marker not confounded by maternal EPA and DHA intake.

Interpretation

This study is the first to show that n-3 LCPUFA supplementation in third trimester pregnancy leads to a higher offspring BMI through childhood, whereas previous trials[7–9] and systematic reviews[31,32] showed no effect of n-3 LCPUFA supplementation during pregnancy and/or lactation on BMI or growth development in childhood[31,32]. Potential explanations for the discrepancy between our findings and previous studies, includes differences in the dose and type of n-3 LCPUFA supplied, timing of the supplementation, study design and accuracy of measurements. The dose of n-3

LCPUFA in our study was 2.4g per day, which is higher than most previous studies, where 900mg or 1.5g[7,9,33,34] were administered and the high number of participants increased the statistical power to detect effects on growth and body composition compared to previous studies[35]. One other study supplemented with a dose similar to ours from week 30 of pregnancy and completed follow-up for 243 participants at age 19 years, finding no effect on waist circumference or z-score BMI, which could be caused by low numbers[36]. In line with our findings, one previous study supplementing mothers with 1.5g n-3 LCPUFA (40% EPA) during the first four months of lactation demonstrated a significantly higher BMI and increased waist circumference in the n-3 LCPUFA supplemented children at 2.5 years[37], but with no differences at 7 or 13 years of age[8].

We previously reported that persistent wheeze/asthma in the first years of life was reduced with approximately one third in the n-3 LCPUFA supplemented group[14]. It could therefore be speculated that the higher BMI through childhood could be mediated by an effect on asthma/airway diseases. However, we did not find any changes in the effect when excluding children with asthma and/or lower respiratory tract infections.

The BMI development curves and the significant interaction with age suggest that the n-3 LCPUFA supplementation effect on BMI was most prominent after age 1 year. Risk of later obesity has been associated with early onset of infant peak BMI[38], which usually occurs at age 6 months. The lacking effect of n-3 LCPUFA in the first year of life in our study could therefore reflect that n-3 LCPUFA supplementation has a general growth stimulating effect, which does not increase the risk of overweight or obesity.

We have previously reported that the n-3 LCPUFA supplementation resulted in prolonged pregnancy duration of 2 days, a higher birth weight and increased size for gestational age (manuscript in review). However, adjusting the main analysis for size for gestational age did not change the results, and therefore the increased BMI through childhood does not appear to be driven by the increased intrauterine growth. Furthermore, we did not find any differences between boys and girls, which is in line with most other studies[31].

The n-3 LCPUFA supplementation in pregnancy lead to an increased weight of 0.4 kg at age 6 years, but our DXA data obtained at 3.5 and 6 years showed no difference in bone-; fat- or lean mass percentages. Instead, we observed a proportional increased in all 3 compartments in the children from the n-3 LCPUFA supplemented group. Furthermore, we did not find any differences between the intervention groups with regard to IOTF grades or children in the highest or lowest 10

BMI percentile at 6 years. This suggests that mainly children with a BMI in the normal range were affected by the n-3 LCPUFA intervention.

Finally, the effect of n-3 LCPUFA supplementation on BMC and BMD might imply a positive health benefit in terms of decreased risk of later fragile bones[39].

The cohort will be followed into adulthood to evaluate the potential long-term effects on growth and body composition induced by n-3 LCPUFA supplementation in pregnancy.

CONCLUSION

Supplementation with n-3 LCPUFA in pregnancy led to increased weight by 0.4 kg in the first 6 years of life, but not an increased risk of overweight or obesity. The n-3 LCPUFA supplementation resulted in a proportional increase in lean mass, bone mass and fat mass, suggesting that n-3 LCPUFA affects fetal programing leading to general growth stimulation in the three compartments during early childhood.

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TABLES

Table 1

Baseline characteristics of the $COPSAC_{2010}$ mother-child pairs, who participated in the n-3 LCPUFA RCT.

	All	n-3 LCPUFA	Control
	688	49% (341)	51% (347)
Child			
Sex, male % (N)	51 (351)	49 (166)	53 (185)
Caucasian % (N)	96 (660)	97 (330)	95 (330)
Season of birth			
Winter % (N)	31 (210)	28 (96)	33 (114)
Spring % (N)	27 (184)	28 (94)	26 (90)
Summer % (N)	21 (147)	21 (73)	21 (74)
Fall % (N)	21 (147)	23 (78)	20 (69)
Exclusive breastfeeding (days), mean (SD)	103 (60)	104 (59)	103 (60)
Marsal percentage*, mean differences (SD)	-0.3 (28.4)	1.5 (28.4)	-2.2 (28.3)
Born before week 37 % (N)	4 (26)	4 (12)	4 (14)
Age at 6 years BMI measurement (years), mean (SD)	6.0 (0.2)	6.0 (0.2)	6.0 (0.2)
Age at 6 years DXA scanning (years), mean (SD)	6.2 (0.2)	6.2 (0.2)	6.2 (0.2)
Parents			
Maternal age at Birth (years), mean (SD)	32.2 (4.5)	32.3 (4.4)	32.1 (4.5)
Social circumstances, mean (SD)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)
Maternal pre-pregnancy BMI	24.6 (4.4)	24.7 (4.2)	24.4 (4.6)

(kg/m^2), mean (SD)			
Maternal Asthma % (N)[£]	26 (181)	25 (84)	28 (97)
Father height (cm), mean (SD)	181 (6.7)	181 (6.3)	181 (7.1)
Daily fish intake before inclusion (g), mean (SD)	28 (18)	28 (17)	28 (18)
Maternal pre-treatment blood levels of EPA+DHA[#] (%), mean (SD)	4.9 (1.2)	4.9 (1.3)	4.9 (1.2)
Pregnancy			
Primiparity % (N)	46 (314)	44 (151)	47 (163)
Preeclampsia % (N)	4 (15)	4 (15)	4 (15)
Smoking in pregnancy % (N)	8 (52)	6 (20)	9 (32)
Antibiotics in pregnancy % (N)	37 (239)	37 (119)	36 (120)
Hadlock calculated in utero weight (g), mean (SD)	322.9 (53.4)	320.7 (49.2)	325.2 (57.2)
High dose D-vitamin intervention % (N)	42 (291)	41 (141)	43 (150)

**Calculation was based on Marsal's intrauterine growth curves.*

£ History of doctor diagnosed asthma

[#]Relative percentage of measured blood fatty acids.

BMI= Body Mass Index, DXA= Dual-energy X-ray Absorptiometry, DHA= DocosaHexaenoic acid, EPA= EicosaPentaenoic acid, N=Number, SD – Standard Deviation

Table 2

Effects of n-3 LCPUFA on the anthropometric measurements at 6 years of age.

	n-3 LCPUFA N=304	Control N=301	P-value
Z-score BMI, Mean (SD)	0.1 (0.8)	-0.1 (0.8)	0.004
Waist circumference, Mean (SD), cm	55.5 (3.8)	54.8 (3.7)	0.04
Weight, Mean (SD), kg	21.8 (2.9)	21.4 (2.9)	0.11
Height, Mean (SD), cm	118.2 (4.6)	118.2 (5.1)	0.97
Head circumference, Mean (SD), cm	52.1 (1.4)	52.1 (1.4)	0.83
ZBMI <10 / >90 perc. (%)	9 / 11	11 / 10	0.63
ZBMI <25 perc. % (N)	21 (66)	30 (90)	0.02
ZBMI> 75 perc. % (N)	29 (91)	21 (62)	0.02
IOTF-grade* >0, % (N)	5 (16)	5 (14)	0.89
IOTF-grade** <0, % (N)	8 (26)	10 (30)	0.62

ZBMI= Z-score Body Mass Index, IOTF= International Obesity Task Force, N=Number, SD – Standard Deviation

*IOTF above grade zero means that the child is at risk of adulthood overweight and obesity.

**IOTF below grade zero means that the child is at risk of adulthood underweight.

Table 3
Effects of n-3 LCPUFA on the dual-energy X-ray absorptiometry measurements at 6 years of age.

	Crude		Adjusted	
	n-3 LCPUFA N=263	Control N=260	Estimate [95 CI-interval]	P-value
Fat (TBLH), Mean (SD), g	4783.9 (1560.0)	4637.6 (1404.4)	116.3* [-92.9;325.5]	0.28
Fat % (TBLH),Mean (SD)	24.4 (5.0)	24.2 (4.9)	0.1 [-0.7;0.8]	0.83
Fat (Trunk), Mean (SD), g*	1801.0 (726.5)	1760.3 (679.7)	21.6 [-83.1;126.4]	0.69
Fat % (Trunk), Mean (SD)	18.1 (5.2)	18.0 (5.2)	0.2 [-0.6;1.0]	0.83
Fat (Android), Mean (SD), g*	209.6 (112.7)	204.4 (105.3)	3.2 [-13.8;20.3]	0.71
Fat % (Android), Mean (SD)	15.1 (5.7)	14.9 (5.8)	0.1 [-0.8;1.0]	0.79
Lean mass (TBLH), Mean (SD), g *	14030.9 (2024.6)	13794.4 (2078.6)	280.7 [98.9;462.4]	0.002
Lean mass % (TBLH), Mean (SD)	72.8 (4.9)	73.0 (4.8)	0.0 [-0.7;0.7]	0.94
Lean mass (Trunk), Mean (SD), g*	7750.1 (1025.5)	7653.5 (1060.6)	152.2 [26.4;223.9]	0.01
Lean mass % (Trunk), Mean (SD)	79.6 (5.2)	79.7 (5.2)	-0.1 [-0.9;0.7]	0.76
Total BMC (TBLH), Mean (SD), g **	546.2 (91.3)	535.1 (94.9)	10.2 [2.3;18.1]	0.01
Total BMD (TBLH), Mean (SD), g/cm^2 **	0.56 (0.05)	0.56 (0.05)	0.005 [-0.006;0.01]	0.08

Adjusted for age and sex
**Additional adjusted for height and height^2, ** Additional adjusted for height,*
BMC: Bone Mineral Content, BMD: Bone Mineral Density, CI: Confidence Interval, SE: Standard Error, TBLH: Total Body Less Head

Online Tables

Table E1

Effects of n-3 LCPUFA on the anthropometric measurements at 6 years of age; sex stratified

	Female			Male		
	n-3 LCPUFA N=159	Control N=144	P-value	n-3 LCPUFA N=147	Control N=157	P-value
Z-score BMI, Mean (SD)	0.1 (0.8)	-0.1 (0.9)	0.07	0.1 (0.8)	-0.1 (0.8)	0.03
Waist, Mean (SD), cm	55.5 (3.9)	54.9 (4.1)	0.19	55.4 (3.6)	54.7 (3.3)	0.10
Weight, Mean (SD), Kg	21.7 (3.1)	21.3 (3.0)	0.19	21.8 (2.8)	21.5 (2.8)	0.32
Height, Mean (SD), cm	118.0 (4.8)	117.8 (5.0)	0.63	118.3 (4.39)	118.6 (5.27)	0.64
Head, Mean (SD), cm	51.8 (1.4)	51.8 (1.3)	0.84	52.5 (1.4)	52.4 (1.5)	0.83
ZBMI <10 perc. % (N)	9 (15)	11 (16)	0.77	10 (15)	12 (18)	0.80
ZBMI <25 perc. % (N)	19 (30)	32 (47)	0.009	23 (34)	28 (43)	0.39
ZBMI> 75 perc. % (N)	28 (45)	23 (33)	0.35	31 (46)	21 (32)	0.06
ZBMI> 90 perc. % (N)	9 (15)	11 (16)	0.77	11 (17)	9 (14)	0.62
IOTF* >0, % (N)	6 (10)	7 (10)	0.98	4 (6)	2 (4)	0.66
IOTF** <0, % (N)	10 (16)	12 (18)	0.60	7 (10)	7 (12)	0.96

ZBMI= Z-score Body Mass Index, IOTF= International Obesity Task Force, N=Number, SD – Standard Deviation

*IOTF above grade zero means the child are in risk of adulthood overweight and obesity.

**IOTF below grade zero means the child are in risk of adulthood underweight.

Table E2

Effects of n-3 LCPUFA on the dual-energy X-ray absorptiometry measurements at 3.5 years of age.

	Crude		Adjusted difference	
	n-3 LCPUFA N=176	Control N=180	Estimate [95 CI-interval]	P-value
Fat (TBLH), Mean (SD), g	3767.1 (844.5)	3694.1 (809.4)	45.1 [83.2;173.4]	0.49
Fat % (TBLH),Mean (SD)	28.7 (4.6)	28.6 (4.3)	0.1 [-0.6;0.7]	0.87
Fat (Trunk), Mean (SD), g*	1404.0 (412.4)	1386.7 (419.0)	3.3 [-63.4;70.0]	0.92
Fat % (Trunk), Mean (SD)	20.3 (4.7)	20.4 (4.6)	-0.1 [-0.8;0.7]	0.81
Fat (Android), Mean (SD), g*	171.1 (59.7)	170.9 (61.6)	-0.4 [10.4;9.5]	0.93
Fat % (Android), Mean (SD)	17.0 (4.8)	17.1 (4.8)	-0.1 [-0.8;0.7]	0.86
Lean mass (TBLH), Mean (SD), g *	9043.7 (1271.4)	8888.9 (1284.2)	93.3 [-45.5;232.0]	0.19
Lean mass % (TBLH), Mean (SD)	69.0 (4.5)	69.1 (4.3)	0.0 [-0.7;0.6]	0.90
Lean mass (Trunk), Mean (SD), g*	5329.1 (704.9)	5240.1 (864.0)	53.0 [-41.2:147.2]	0.27
Lean mass % (Trunk), Mean (SD)	77.6 (4.7)	77.6 (4.6)	0.1 [-0.7;0.8]	0.81
Total BMC (TBLH), Mean (SD), g **	309.2 (49.1)	304.3 (49.8)	3.0 [-2.8;8.8]	0.31
Total BMD (TBLH), Mean (SD), g/cm^2 **	0.45 (0.03)	0.45 (0.03)	0.005 [-0.001;0.01]	0.09

Adjusted for age and sex
**Additional adjusted for height and height^2, ** Additional adjusted for height,*
BMC: Bone Mineral Content, BMD: Bone Mineral Density, CI: Confidence Interval, SE: Standard Error, TBLH: Total Body Less Head

Table E3

Effects of maternal FADS genotype, GG risk allele and non-risk alleles (AA+AG), stratified by the intervention, on the anthropometric measurements at 6 years of age.

	n-3 LCPUFA			Control		
	GG N=40	AA+AG N=257	P-value	GG N=36	AA+AG N=252	P-value
Z-score BMI, Mean (SD)	0.11 (0.82)	0.11 (0.82)	0.98	-0.36 (0.62)	-0.05 (0.84)	**0.01**
Waist, Mean (SD), cm	55.6 (3.9)	55.4 (4.1)	0.81	54.0 (2.9)	54.9 (3.8)	0.10
Weight, Mean (SD), Kg	21.5 (2.8)	21.8 (2.9)	0.49	20.61 (2.2)	21.49 (3.0)	**0.04**
Height, Mean (SD), cm	117.5 (4.9)	118.2 (4.6)	0.36	117.8 (5.6)	118.2 (5.1)	0.66

ZBMI= Z-score Body Mass Index, N=Number, SD – Standard Deviation

Figure legends

Figure 1

Curves showing mean BMI with standard errors according to visit age for children in the n-3 LCPUFA supplementation group and control group until 6 years of age.

Figure 2

Effects of n-3 LCPUFA on BMI through infancy and childhood illustrated by mean difference in BMI z-score at each visit and 95% confidence intervals.

Figure 3

Histogram with overlaying density graph both illustrating the BMI value for the children and the proportion of children with a specific BMI stratified by supplementation groups; n-3 LCPUFA and control.

Online Figure legends:

Figure E1

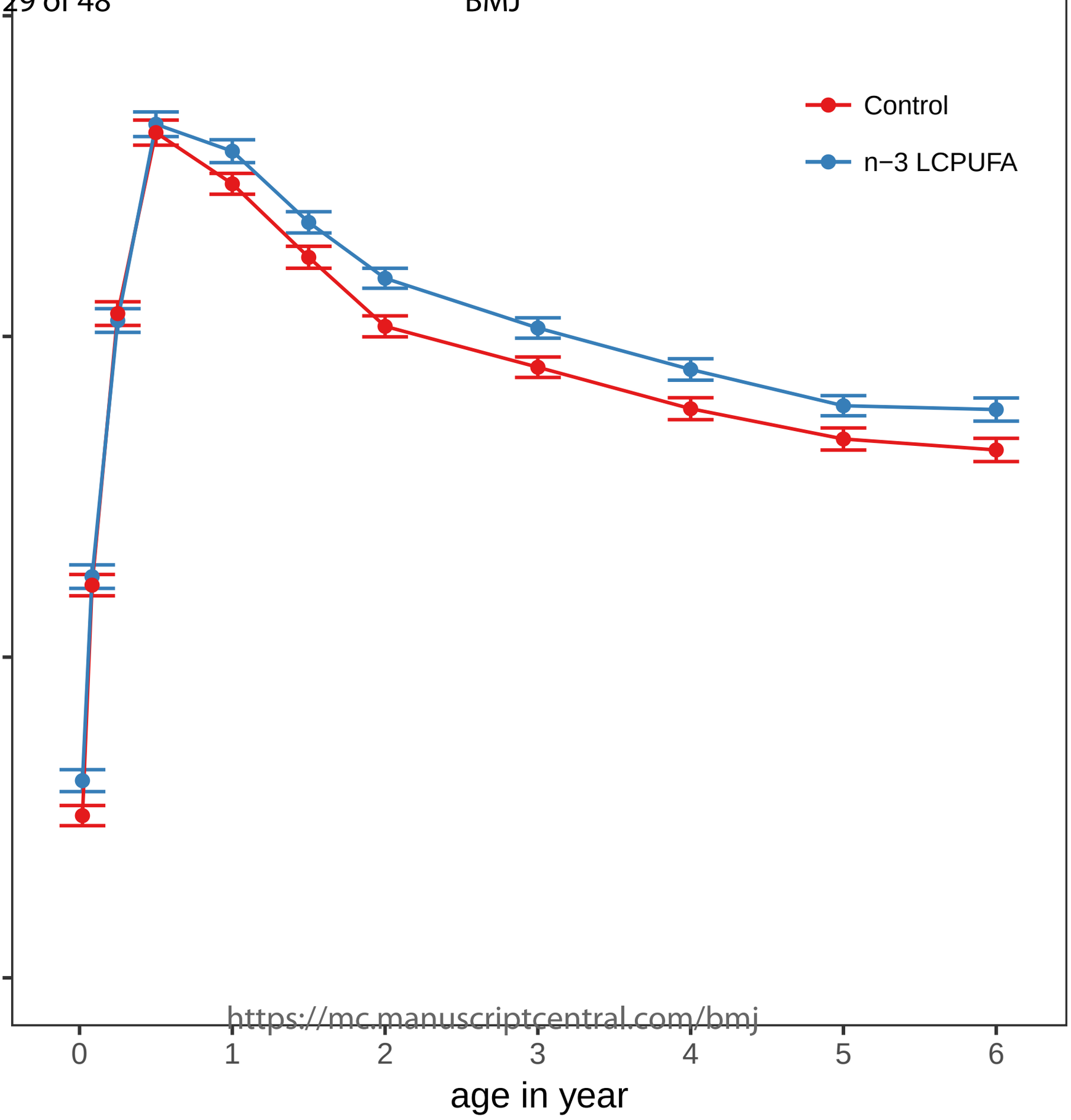
Flow chart of enrollment and allocation of the $COPSAC_{2010}$ pregnancy cohort and follow-up of the $COPSAC_{2010}$ birth cohort.

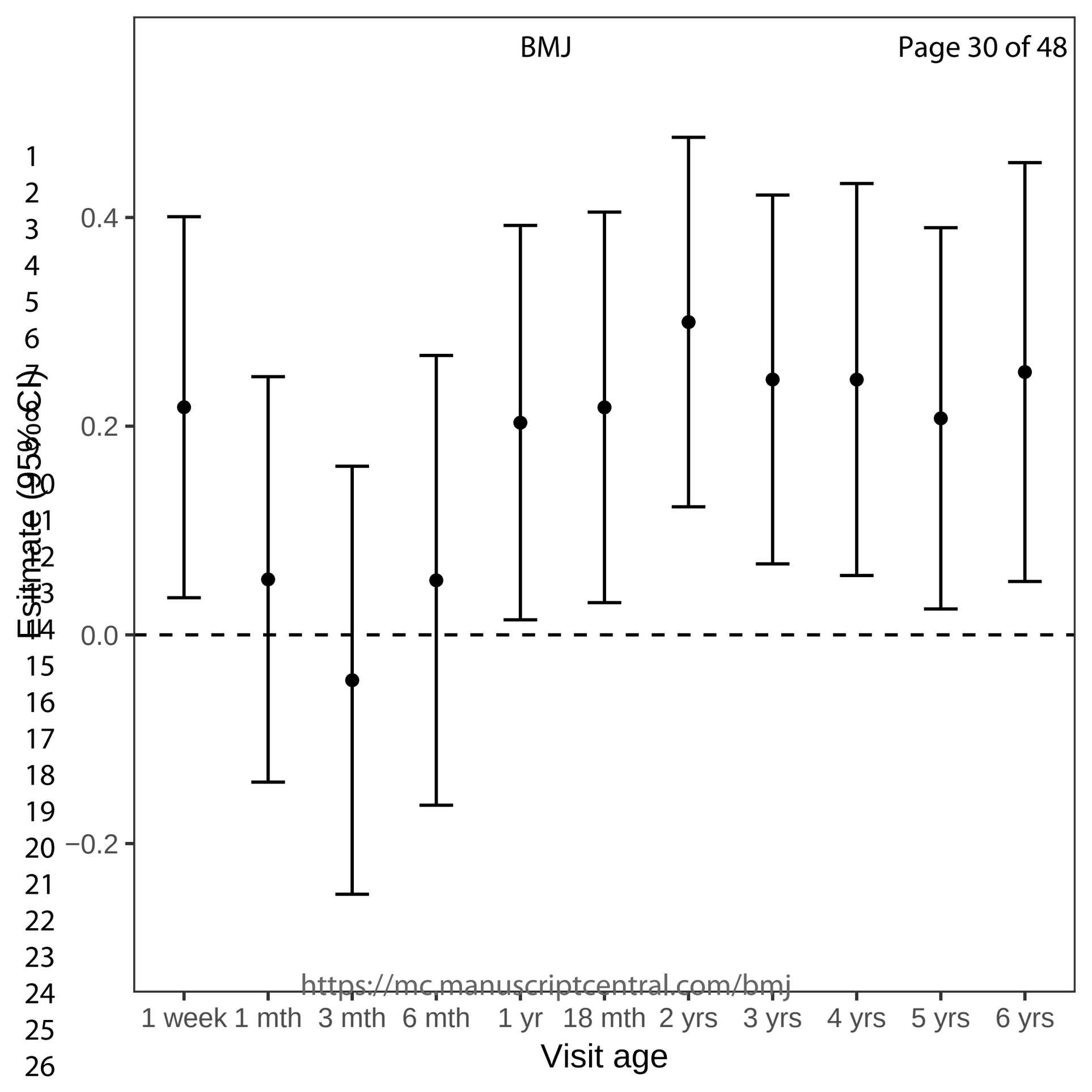
Figure E2

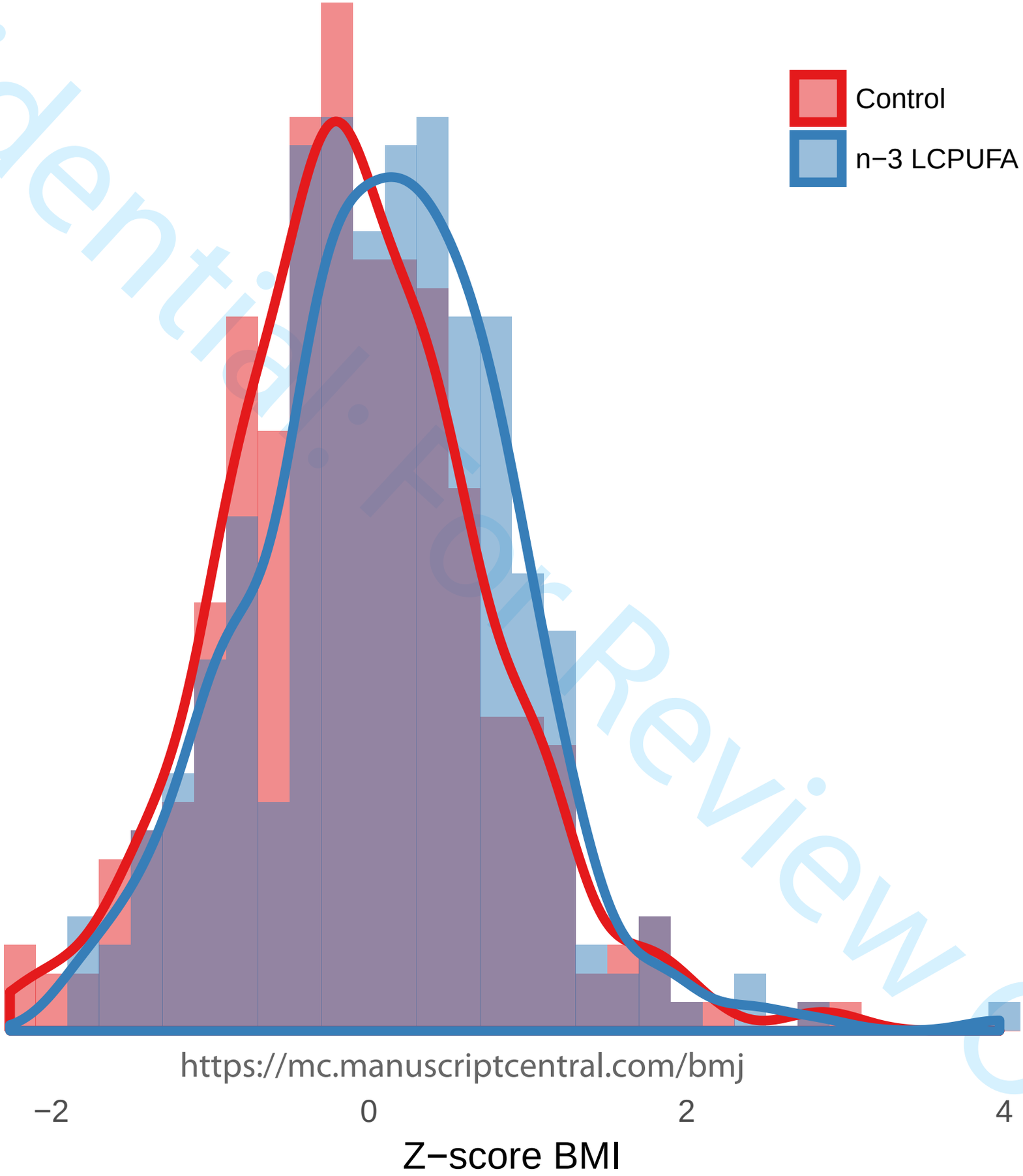
Curves showing mean BMI with standard errors according to visit age for children in the n-3 LCPUFA supplementation group and control group until 6 years of age, stratified by sex.

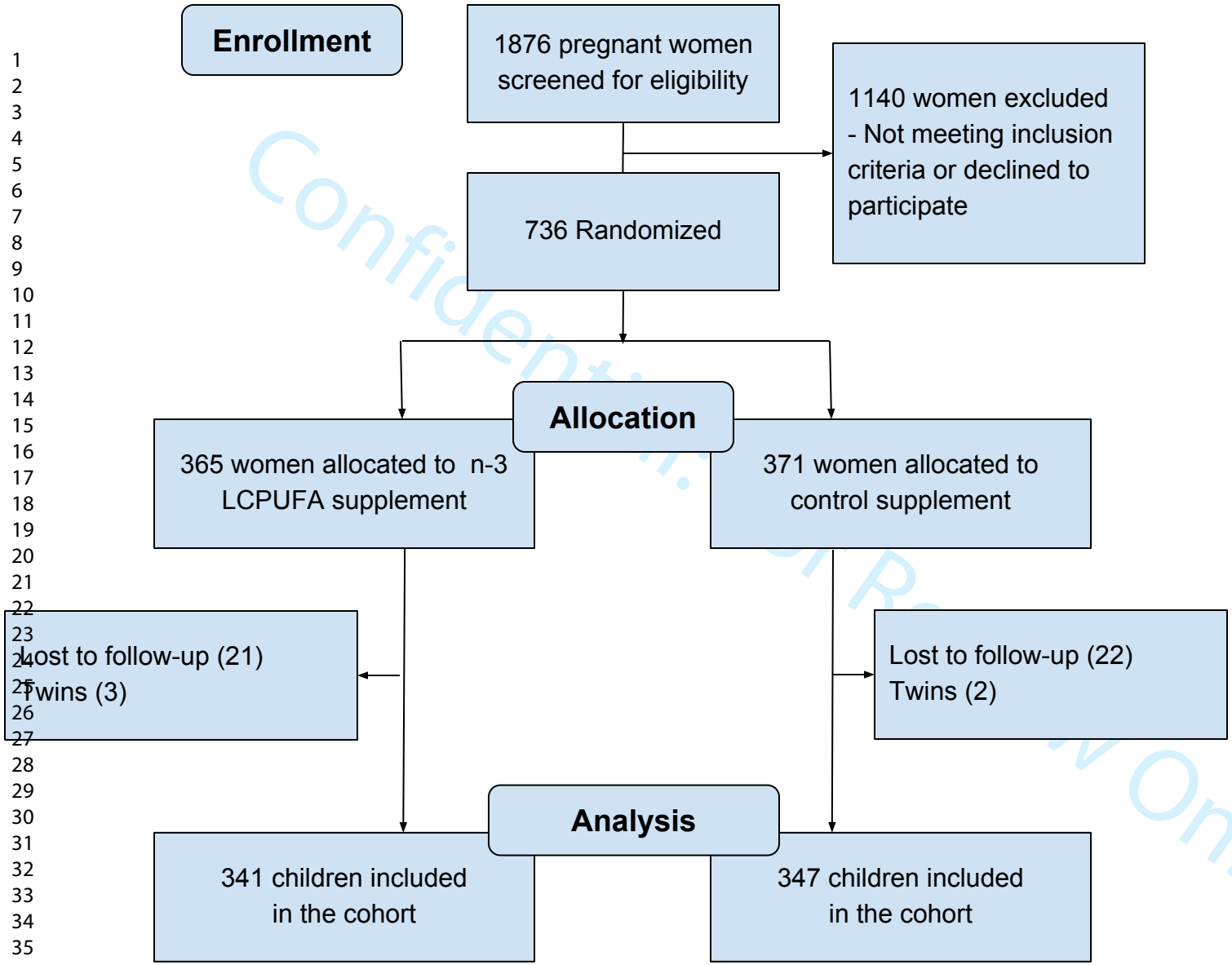
Figure E3

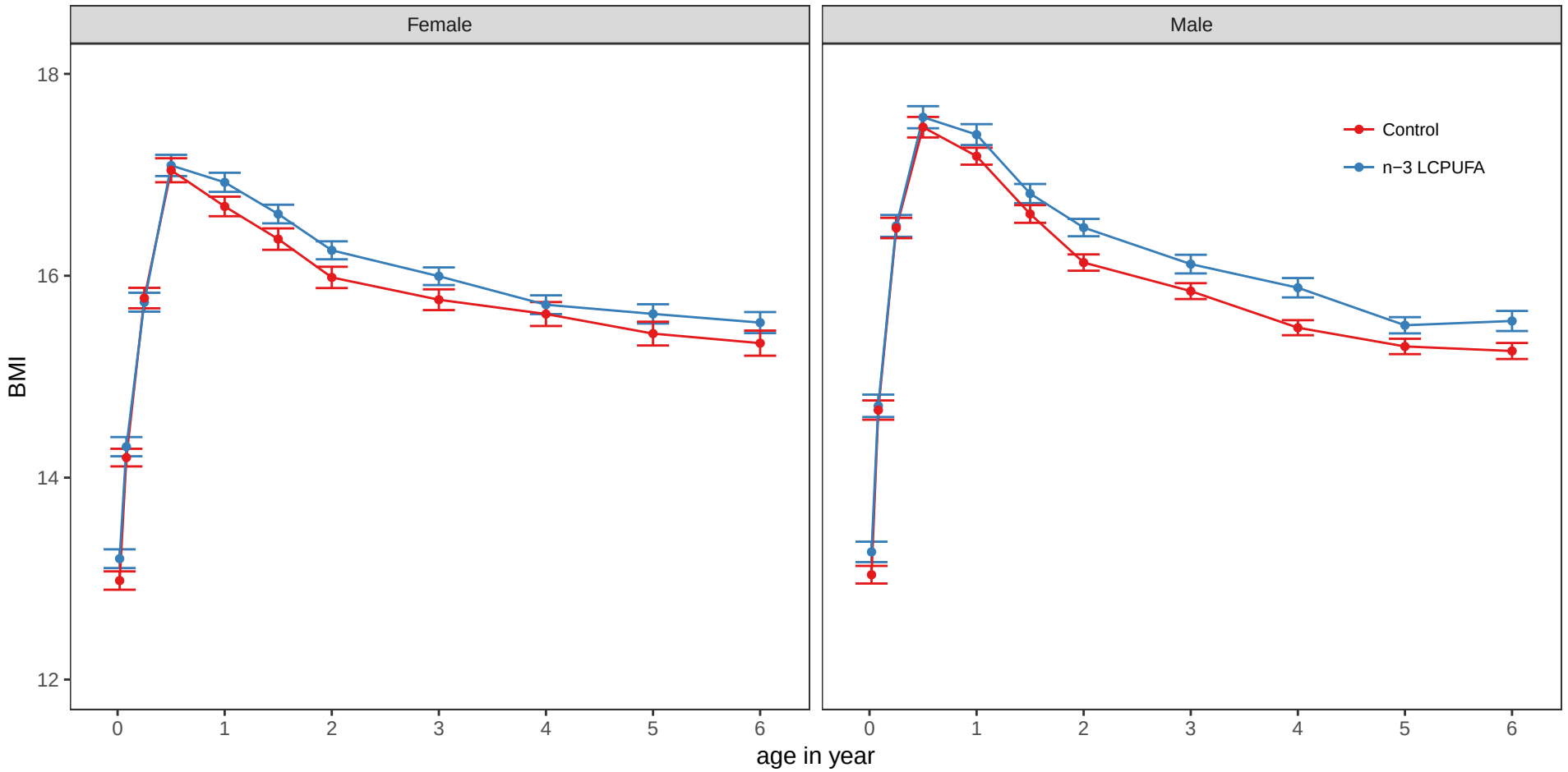
Curves showing mean BMI with standard errors according to visit age for children born by mothers with GG risk allele and non-risk alleles (AA+AG) until 6 years of age stratified by the intervention.







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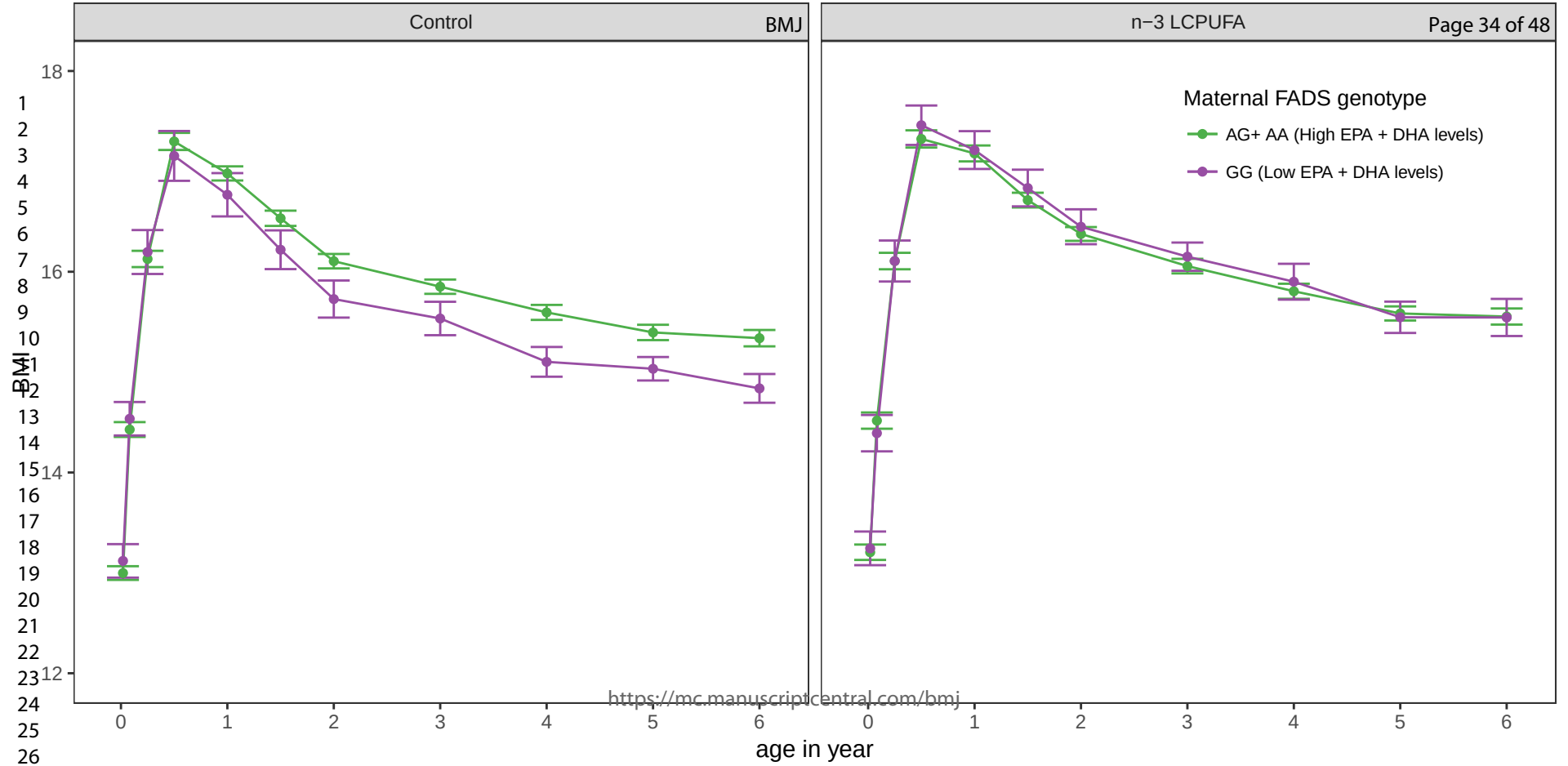
n−3 LCPUFA



Online methods

Study design:

We identified pregnant women in the eastern part of Denmark by reviewing the monthly lists of reimbursements to general practitioners for first pregnancy visits.The screening and information procedure has previously been described [14]. Exclusion criteria were gestational age above week 26; any endocrine, cardiovascular, or nephrological disorders; and >600IU/day vitamin D intake.

Study allocation

The allocation procedure was performed by simple randomization procedures using a computer-generated list of random numbers prepared by an external investigator with no other involvement in the trial. Capsules were consecutively numbered and kept in closed containers. The fatty acid content and the oxidation levels in both kinds of oil capsules were analyzed by the manufacturer at two time-points during the study, showing levels within the expected.

Adherence

Adherence to the intervention was assessed by comparing the number of returned capsules against the expected.

Maternal fatty acid desaturase (FADS) genotype

rs1535 was chosen because this SNP, and its proxies in close linkage disequilibrium, has been associated with n-3 LCPUFA levels in a genome-wide association study [15] and with blood levels of EPA and DHA during pregnancy [16]. The risk genotype (GG) has been associated with lower n-3 LCPUFA levels compared to the non-risk genotypes (AA/AG).

Baseline characteristics

Information on the baseline characteristics race, sex, gestational age, maternal age at birth, parity, older siblings, maternal asthma, smoking during pregnancy, mode of delivery, antibiotics during pregnancy, preeclampsia and diabetes in pregnancy was obtained by personal interviews and when possible validated with registry data.

The social circumstances in the household were defined as the first component of a principal component analysis (PCA) on household income, maternal age and maternal level of education at 2 years with a mean value of zero and standard deviation of one (explained 55% of the variance) [17].

Information on breastfeeding was collected prospectively including duration of exclusive and total breastfeeding period and the use of infant formula. As soon as the child's diet was supplemented or replaced by continual use (>7 days) of infant formula/complementary foods, we considered the exclusive breastfeeding period terminated.Information on pre-pregnancy weight of the mother was collected from pregnancy records and BMI was calculated from the height measured in the clinic. The participants completed a validated 360-item food frequency questionnaire assessing dietary intake in the 4 weeks prior to randomization [18–20]. Maternal whole blood EPA+DHA levels were assessed at the time of randomization [21,22].

This supplement contains the following items:

1. Original protocol, final protocol, summary of changes

2. Original statistical analysis plan, final statistical analysis plan, summary of changes

1. Original protocol

Omega 3's effect on the development of asthma in children and adolescents.
(Supplementary protocol to the ABC (COPSAC2010) birth cohort study.)

The following is an English translation of the original protocol in Danish.

Aim

To investigate whether intake of fish oil capsules rich in Omega 3 fatty acids (n-3 fatty acids) in the last three months of pregnancy has a favorable effect on the development of atopy in the offspring.
Secondary objectives are to investigate whether consumption of fish oil reduces the risk of preterm birth.

Inclusion criteria

In order to participate in the ABC (COPSAC2010) study it is a criterion for the pregnant women to agree to participate in the fish oil-intervention. Studies in pregnant women have previously shown that ingestion of fish have a beneficial effect on their children's development of asthma. These studies have, however, been primarily based on filled questionnaire, where women retrospectively have replied to dietary questions regarding their consumption of fish and fish products. Since this study approach comprises some uncertainty, we wish to include fish oil intervention in this prospective study.

Background

n-3 fatty acids are essential fatty acids, i.e. fatty acids necessary for the human organism, but which we cannot produce and therefore have to get through diet. There are 2 groups of essential fatty acids - n-3 and n-6. The fatty acids have two functions - first, they are included as structural components of the cell walls, secondly they are converted to the important active molecules called prostaglandins. There are 3 types of prostaglandins - PG1, PG2 and PG3.
PG1 has several important properties and inhibits e.g. agglomeration of red blood cells and reduces the degree of inflammation.
PG2 has the opposite effect of PG1. PG2 increases inflammation, contracting blood vessels and promotes agglomeration of red blood cells. These properties are important in, for example, wound healing but can be harmful to the body when in abundance.
PG3 has mixed functions; one of the most important is to reduce the rate at which PG2 is formed.
PG3 is therefore, like PG1, described as having anti-inflammatory properties.
The conversion of essential fatty acids into prostaglandins is carried out by enzymes. The same set of enzymes converts both n-3 and n-6 fatty acids, but the enzymes favor n-3, i.e. n-3 is converted first, if both the n-3 and n-6 are present.
n-6 fatty acids can either be converted to the anti-inflammatory PG1, or to arachidonic acid which is then further

converted to PG2 (inflammatory). The conversion of PG1 is carried out without enzymes, whereas conversion to arachidonic acid is enzyme dependent.
In a diet rich in n-3 fatty acids the majority of the enzymes will be used to convert the n-3 and only a small fraction is available for the conversion of n-6 to arachidonic acid and thence to PG2. n-6 will therefore mainly being converted to the PG1, and a n-3 rich diet would hence reduce the degree of inflammation. Conversely, a diet deficient in n-3 leads to increased conversion of n-6 to arachidonic acid and thus PG2, i.e. increasing the degree of inflammation. n-6 is not in itself harmful, but the ratio between the intake of n-3 and n-6 fatty acids is crucial. Unfortunately, the typical Western diet is rich in n-6 and low in n-3 fatty acids, and thus favors the formation of the inflammatory prostaglandins (1-3).
Prostaglandins also affect our immune system. As stated in the main protocol, the process whereby the immune system is altered from the innate antibody-producing Th2 response to the more mature and less allergy-generating cell-mediated Th1 response is essential to reduce the risk of developing asthma and allergies. PG2 shifts the ratio between Th1 and Th2 in Th2's favor, and thus affects the immune system towards a more allergy prone response. The Th1: Th2 ratio is particularly important in early life, probably already in the intrauterine stage (4, 5).

A diet deficient in n-3 fatty acids, relative to n-6 will thus via PG2 on one hand increase the overall level of inflammation and on the other displace the immune system towards Th2 and to a trajectory towards asthma and allergies.

Studies have shown that maternal dietary fish intake- rich in n-3 fatty acids - in the last part of pregnancy affects the child's later risk of developing asthma. The higher the intake of fish the lower the risk of asthma (6-11). These studies, however, have all been questionnaire-based, some in a retrospective way several years later, where the mother has been interviewed on her diet and associated it with child's later risk of developing atopy. In one study (Olsen et al (10)), randomized supplements of fish oil or olive oil placebo were given to pregnant women, and later found that fewer of the children of the women who received fish oil subsequently developed asthma and allergies. However, this statement was made retrospectively with the complications that are associated therewith.

To our knowledge, no randomized prospective intervention studies with the prospect of reducing the risk of developing asthma and allergies by supplementation with fish oil (rich in n-3 fatty acids) to mothers in the 3rd trimester have previously been presented.
Therefore, we want to complement the main protocol with this study of the possibility of reducing the child's later risk of developing atopy, by supplementing the mother with n-3 fatty acids, in the form of fish oil, and thereby affecting the interaction between prostaglandins, Th1 and Th2 early in life in the intrauterine milieu.
Olsen et al (12, 13) also examined whether n-3 fatty acids via their effect on prostaglandins have a positive effect on preterm birth. The conclusion in terms of preterm birth is ambiguous, but it suggests that n-3 fatty acids in general may prolong pregnancy with 3-4 days. However, it is still not known whether n-3 fatty acids can also prevent preterm birth. This we will likewise investigate.

Method and trial procedure

The 800 pregnant women are randomized in a 1:1 ratio to intake either fish oil or control olive oil. The women are recruited so that they can start the intervention at the beginning of the third trimester (pregnancy week 24) and intervention is continued until 1st visit to the COPSAC clinic after birth (weeks 1-2, see main protocol). Olive oil is used as placebo control. The method is copied from the aforementioned retrospective intervention study by Olsen et al (10), which both increases the likelihood of a positive out-come and increases the potential for the best possible subsequent comparison the two studies.

The fish oil supplements are planned to be issued as four capsules of Incromega[1] (Omega 3: 60% min; EPA: 33% min; DHA: 22% min). Placebo control will be given as four capsules of 1 g olive oil, containing 72% oleic acid (n-9) and 12% linoleic acid (n-6).

The amount of n-6 fatty acid in olive oil is less than 3% of an average woman's daily intake, and therefore does not affect the ratio of n-3 and n-6. On the other hand, the content of n-3 fish oil-in the capsules corresponds to approx. 10 times the average daily intake of n-3 fatty acids.

The pregnant women will be provided with all the capsules for their own use during the visit at week 24 and will be instructed to return packaging and unused capsules. The capsules will be marked with randomization number and it will not be visible whether the capsules contain fish oil or olive oil. The women are informed of this. The women will be assigned to either fish oil or placebo capsules via a random randomizer generator.

In connection with the inclusion interview in week 24 at Gentofte or Næstved Hospital, the women will have a blood test, wherein the content of omega-3 in the erythrocyte membrane is measured. Similarly, a blood test at the completion of the intervention, i.e. at the first visit after birth, will be performed. The blood samples are taken in order to later be able to statistically account for the women who already have a high intake of fish / fish oil and therefore have naturally high blood levels of omega-3 and also to be able to monitor the women's compliance to the project.

Risks and disadvantages

The study will be based on that pregnant women should take fish oil or placebo / olive oil capsules, respectively, every day for the last half of pregnancy. This intervention is safe and previously tested in several studies. There are no known risks from consuming fish oil or olive oil for either mother or baby.

Ethical aspects

As fish oil has already been given to pregnant women and it is shown no risks for either the child or the mother, we do not find that there are any ethical issues in conjunction with this project. Those women who are randomized to fish oil will benefit from a potential preventive effect in their children. However, this is not yet scientifically verified (which the reason for this study) and we do not consider it ethically problematic to half of the women receiving olive oil placebo treatment, as they simply carry out a normal pregnancy.

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1. Final protocol

The effect of Omega 3 on the development of asthma in children and adolescents. (Supplementary protocol to the ABC (COPSAC2010) birth cohort study.)

The following is an English translation of the original protocol in Danish including subsequent changes.

Aim

The aim is to investigate whether intake of fish oil capsules rich in Omega 3 fatty acids (n-3 fatty acids) in the last three months of pregnancy has a protective effect on the development of atopy in the offspring. A secondary objective is to investigate whether consumption of fish oil reduces the risk of preterm birth.

Inclusion criteria

To participate in the ABC (COPSAC2010) study it is an inclusion criterion for the pregnant women to agree to participate in the fish oil-intervention. Studies in pregnant women have previously shown that intake of fish has a protective effect on their children's development of asthma. These studies have, however, primarily been based on filled questionnaire, where women retrospectively have answered dietary questions regarding their consumption of fish and fish products. As this type of study approach encompasses a certain amount of biases, we wish to include a controlled fish oil intervention in this prospective study.

Background

n-3 fatty acids are essential fatty acids, i.e. fatty acids necessary for the human organism, but which it cannot produce and therefore needs to get through diet. There are 2 groups of essential fatty acids: n-3 and n-6. The fatty acids have two functions: First, they constitute structural components of the cell wall, secondly they are converted into important biologically active molecules called prostaglandins, of which there are 3 types: PG1, PG2 and PG3. PG1 has several important properties as inhibits the clotting of red blood cells and reduces the level of inflammation. PG2 has the opposite effect of PG1. PG2 increases inflammation, promotes contracting of blood vessels and clotting of red blood cells. These properties are important in, for example, wound healing but can be harmful to the body when predominant. PG3 has mixed functions; one of the most important being to reduce the rate at which PG2 is formed. PG3 is therefore, like PG1, considered to have anti-inflammatory properties.

The conversion of essential fatty acids into prostaglandins is carried out by enzymes. The same set of enzymes converts both n-3 and n-6 fatty acids, but the enzymes favor n-3, i.e. n-3 is converted first, if both n-3 and n-6 fatty acids are present. n-6 fatty acids can either be converted to the anti-inflammatory PG1, or to arachidonic acid, which is then further converted to PG2 (inflammatory). The conversion of PG1 is carried in an enzyme-free process, whereas conversion to arachidonic acid is enzyme-dependent. In a diet rich in n-3 fatty acids, the majority of the enzymes will be used to convert n-3 and only a small fraction is available for the conversion of n-6 into arachidonic acid and subsequently to PG2. n-6 will therefore mainly be converted to the PG1, and a n-3 rich diet would hence reduce the level of inflammation. Conversely, a diet deficient in n-3 leads to increased conversion of n-6 to arachidonic acid and thus PG2, i.e. increasing the degree of inflammation. n-6 is not in itself harmful, but the ratio

between the intake of n-3 and n-6 fatty acids is crucial. Unfortunately, the typical Western diet is rich in n-6 and low in n-3 fatty acids, and thus favors the formation of the inflammatory prostaglandins (1-3).
Prostaglandins also affect our immune system. As stated in the main protocol, the process whereby the immune system is altered from the innate antibody-producing Th2 response to the more mature and less allergy-generating cell-mediated Th1 response is essential to reduce the risk of developing asthma and allergies. PG2 shifts the ratio between Th1 and Th2 in Th2's favor, and thus affects the immune system towards a more allergy-prone response. The Th1:Th2 ratio is particularly important in early life, probably already in utero (4, 5).
A diet deficient in n-3 fatty acids relative to n-6, will thus via PG2 on one hand increase the overall level of inflammation and on the other shift the immune system towards Th2 and to a trajectory towards asthma and allergies.
Studies have shown that maternal dietary fish intake- rich in n-3 fatty acids - in the last part of pregnancy affects the child's later risk of developing asthma with an inverse effect of lower risk of asthma with higher intake of fish (6-11). These studies, however, have all been questionnaire-based, some in a retrospective manner several years after birth, as the mother were interviewed on her diet during pregnancy, which was then associated with the child's later risk of developing atopy. In one study (Olsen et al (10)), randomized supplements of fish oil or olive oil placebo were given to pregnant women and later found that fewer of the children of the women who received fish oil subsequently developed asthma and allergies. However, this statement was made retrospectively with the complications that are associated therewith.

To our knowledge, no randomized, prospective intervention studies with the prospect of reducing the risk of developing asthma and allergies by supplementation with fish oil (rich in n-3 fatty acids) to women in the 3rd trimester of pregnancy have previously been presented. Therefore, we wish to complement the main study protocol with this study of the prospect of reducing the child's later risk of atopy, by supplementing the mother with n-3 fatty acids as fish oil and thereby affecting the interaction between prostaglandins, Th1 and Th2 early in life in the intrauterine milieu. Olsen et al (12, 13) also examined whether n-3 fatty acids via their effect on prostaglandins have a positive effect on preterm birth. The conclusions from other reports on preterm birth are ambiguous, but suggest that n-3 fatty acids in general may prolong pregnancy with 3-4 days. However, it is still not known whether n-3 fatty acids can prevent preterm birth. We will likewise investigate this matter.

Method and trial procedure

The 800 pregnant women are randomized in a 1:1 ratio to intake either fish oil or control olive oil. The women are recruited in order to start the intervention at the beginning of the third trimester (pregnancy week 24) and intervention is continued until 1st visit to the COPSAC clinic after birth (weeks 1-2, see main protocol). Olive oil is used as placebo control. The method is copied from the aforementioned retrospective intervention study by Olsen et al (10), which both increases the likelihood of a positive out-come and the potential for the best possible subsequent comparison of the two studies. The fish oil supplements are planned to be issued as four capsules of Incromega1 (Omega 3: 60% min; EPA: 33% min; DHA: 22% min). Placebo control will be given as four capsules of 1 g olive oil, containing 72% oleic acid (n-9) and 12% linoleic acid (n-6). The amount of n-6 fatty acid in olive oil is less than 3% of an average woman's daily intake, and therefore does not affect the ratio of n-3 and n-6. On the other hand, the content of n-3 fish oil-in the capsules corresponds to approx. 10 times the average daily intake of n-3 fatty acids.

The pregnant women will be provided with all the capsules for their personal use during the visit at week 24 of pregnancy and will be instructed to return packaging and unconsumed capsules. The capsules will be marked with a randomization number and whether the capsules contain fish oil or olive oil will not be visible. The participating women are informed of this. The women will be assigned to either fish oil or placebo capsules via a randomization generator.

In connection with the inclusion interview at pregnancy week 24 at Gentofte or Næstved Hospital, the women will be subject to a blood test, wherein the content of omega-3 in the erythrocyte membrane is measured. Similarly, a blood test at the completion of the intervention, i.e. at the first visit after birth, will be performed. The blood samples are taken in order to later be able to statistically account for the women who already have a high intake of fish / fish oil and therefore have naturally high blood levels of omega-3 and secondly to be able to monitor the women's compliance to the project.

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The study will be based on the pregnant women taking fish oil or placebo / olive oil capsules, respectively, daily for the last half of pregnancy. This intervention is safe and previously tested in several studies. There are no known risks from consuming fish oil or olive oil for either mother or baby.

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As fish oil is already being given to pregnant women and has shown no risks for either the child or the mother, we do not find that there are any ethical issues in conjunction with this project. Those women who are randomized to fish oil will benefit from a potential preventive effect in their children. However, this is not yet scientifically verified (which is the reason for this study) and we do not consider it ethically problematic that half of the women receive olive oil placebo treatment, as they simply carry out a normal pregnancy.

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Outcome definitions

Primary outcome

Persistent wheeze
Description: Age at onset of persistent wheeze diagnosed according to predefined algorithm of recurrent troublesome lung symptoms, response to treatment and relapse after withdrawal of treatment

Secondary outcomes

Asthma exacerbations
Description: Age at onset of severe asthma exacerbations diagnosed by predefined criteria of acute severe asthma requiring oral/high dose inhaled steroids or acute hospital contact

Eczema
Description: Age at onset of eczema diagnosed prospectively by research doctors according to predefined algorithm based upon Hanifin and Rajka criteria

Allergic sensitization
Description: Allergic sensitization at 6 and/or 18 months of age assessed by skin prick test and specific IgE in blood

Infections
Description:
Main analysis: Number of lower respiratory tract infections registered in daily diaries
Secondary analyses: Acute otitis media, number of upper respiratory tract infections, number of other infections, total number of infections

Additional secondary outcomes

Neurological development 0-3 years
Description:
Main analysis:

- Cognitive development assessed at 2½ years using the cognitive part of Bayley Scales of Infant and Toddler Development, third edition

Secondary analyses:

- Milestone development monitored prospectively by the parents using a registration form based on The Denver Development Index and WHO milestones registration (combined assessment by principal component analysis)
- Language development assessed at 1 and 2 years of age with the Danish version of The MacArthur Bates Communicative Developmental Inventory (CDI)
- The child´s general development (language, fine and gross motor, social and problem solving) at 3 years of age assessed with Ages and Stages Questionnaire, third edition (ASQ-3)

Growth

Description:

- Body composition (fat mass and bone mineral density) assessed by DEXA scan at 3 years of age
- Development of BMI from birth to 3 years assessed longitudinally in the research clinic

Systemic immune status at 18 months

Description:

Main analysis:

- Immune status at 18 months measured in stimulated whole blood as cytokine release (combined assessments by principal component analyses)

Secondary analyses:

- Composition of immune cell subsets in whole blood at birth and at 18 months of age

Airway mucosal immune status at 1 month and 2 years

Description:

Immune status measured in airway mucosal lining fluid at 1 month and 2 years of age (combined assessments by principal component analyses for each age point)

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1. Summary of changes

Changes to the original protocol are indicated in www.clinicaltrials.gov/ct2/archive/NCT00798226
Briefly, these encompass introduction of novel assessments, including neurological development, growth, systemic immune status and airway mucosal immune status**.**

2. Original statistical analysis plan

Power calculation

We assume that the true odds ratio based on exposure is 0.5 and the prevalence of the disease is 15% in an unselected cohort.

In a 1:1 unpaired case-control study 337 cases (fish oil) and 337 controls (placebo) will be needed to detect an effect of exposure to 80% chance of a 5% significance level (normal approximation test). After taking into account drop-outs and the like a cohort of 800 people is thus still appropriate.

Statistical analysis

The main analysis of effect on the primary outcome (persistent wheeze) is the intention-to-treat analysis including all children whose mothers are recruited to the interventional trial. The effect of n-3 LCPUFA intervention is assessed by Kaplan-Meier curves and quantified by Cox proportional hazards regression (P values correspond to Wald tests). The children are retained in the analysis from birth until age of diagnosis, drop-out, or age at their last clinic visit at completion of the RCT (when the youngest included child is 3 years of age), whichever came first.

Planned sub-analyses are: stratified analyses based upon pre-intervention EPA+DHA levels, confounder-adjusted analyses (adjusted for gender, Vitamin D RCT group and pre-intervention EPA+DHA level), and a cross sectional analysis of current persistent wheeze/asthma by age 3 years or older.

Interaction with the concurrent vitamin D RCT is analyzed and a P-value of interaction < 0.05 is considered significant and will result in a stratified main analysis by vitamin D RCT group.

Secondary outcomes with age-at-onset information (LRI and eczema) are analyzed similarly to the main end-point by cox regression analysis. Associations between n-3 LCPUFA intervention and binary outcomes are assessed using logistic regression.

A significance level of 0.05 is used in all types of analyses.

2. Final statistical analysis plan (for the current study)

Power calculation

Power analyses: The trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the power of the RCT on BMI was calculated post-hoc based on the 605 children who had available 6-year BMI data. This resulted in 80% power, to detect a mean difference of 0.19 in z-score BMI, with a SD of 0.82. The power/sample

size calculation and testing were based on a 2-sample, two-tailed t-test with an alpha of 0.05.

Statistics

We included children with at least one anthropometric measurement at age 0-6 years and excluded twins.
The effect of n-3 LCPUFA supplementation on cross-sectional anthropometric outcomes was analysed using Student's t-test for normally distributed continuous variables and chi-square tests for categorical variables. Anthropometrics used for cross-sectional analyses at age 6 years were defined as the specific anthropometric measurement closest to 6 years ± 6 months.
BMI changes over time were analysed in a random intercept mixed model with BMI z-scores as the outcome. Age-related trends in the association between intervention and BMI were investigated in the mixed models by including an interaction-term between age and intervention group. Missing observation were treated as missing data and excluded from analyses. The analyses were performed for all children and stratified by sex. All data analyses were conducted with R v 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Results with a p-value <0.05 were considered statistically significant.

2. Summary of changes

Addition of Body Mass Index to the original statistical plan.
Power calculation was performed based upon the available number of children available for the current study.