FACULTY OF HEALTH AND MEDICAL SCIENCES

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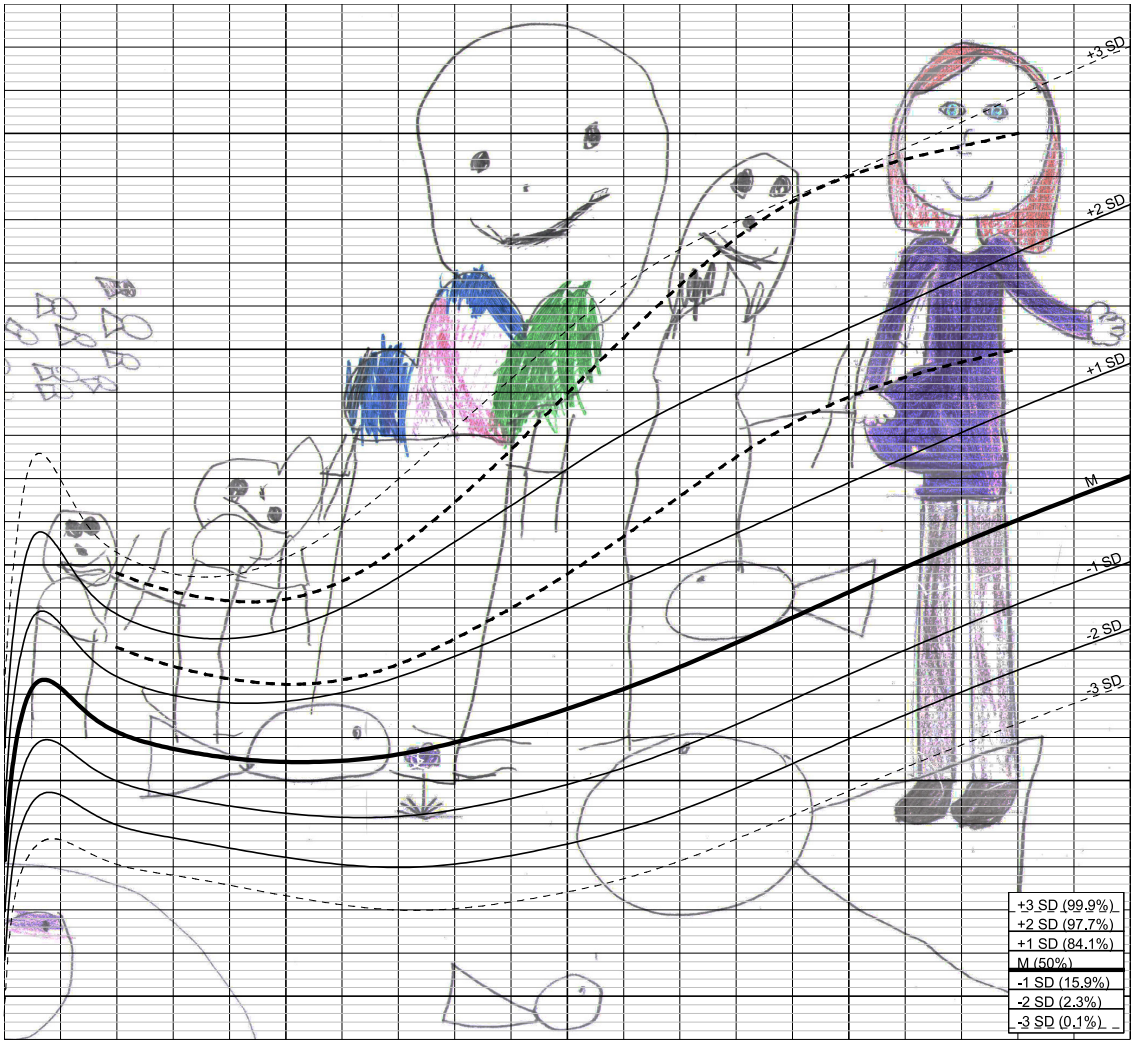
Early life exposures and childhood growth



PhD Thesis by Rebecca Kofod Vinding, MD

COPSAC, (COpenhagen Prospective Studies on Asthma in Childhood),
Danish Pediatric Asthma Center, Copenhagen University Hospital, Gentofte
Department of Pediatrics, Naestved Hospital

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COPENHAGEN PROSPECTIVE STUDIES ON ASTHMA IN CHILDHOOD

Academic advisor:	Hans Bisgaard, Professor, MD, DMSc. Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) Head of the Danish Pediatric Asthma Center Copenhagen University Hospital, Gentofte-Herlev University of Copenhagen
Project advisor:	Jakob Stokholm, MD, PhD. Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) Copenhagen University Hospital, Gentofte-Herlev.
Evaluating committee:	Anders Juul, Professor, MD, DMSc. (Chairman) Department of Clinical Medicine, University of Copenhagen, Rigshospitalet Copenhagen, Denmark
	Ellen Aagaard Nøhr, Professor Odense Universitetshospital, Forskningsenheden for Gynækologi og Obstetrik, Klinisk Institut på Syddansk Universitet,
	Jovanna Dahlgren, Professor, MD, Department of Pediatrics at Institute of Clinical Sciences; Drottning Silvias Barn O Ungdomssjukhus, Sweden
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Summary and Conclusions

The increased prevalence of overweight children cannot be explained by changes in genetic factors, since the great increase has occurred over a short period of time, the causes must be sought in environmental exposures (1,2). Identification of these exposures are important as preventive steps against development of obesity, type 2 diabetes and cardiovascular diseases (3).

We have focused on two exposures in this thesis. Firstly, caesarean section (CS) which has had an increase in prevalence the last decades (4). Two recent meta-analyses have shown associations between delivery by CS and obesity in the off-spring in both child- and adulthood (5,6).

Secondly, we have focused on maternal dietary n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) during pregnancy, since it is an important determinant of adequate child development and health (7). In humans, both observational studies on dietary intake of fish as well as randomized trials of fish oil supplementation in pregnancy have shown longer gestation and higher birth weight in children born to women with a high n-3 LCPUFA intake (8–11). The increased birth weight could solely be caused by the prolonged duration of pregnancy (9,12), but could also be explained by an increased intrauterine growth rate. Animal studies have shown that n-3 LCPUFA supplementation both in pregnancy and the postnatal period affects the proliferation and differentiation of pre-adipocytes, which could theoretically have a positive impact on later adiposity through inhibition of fat tissue development (13,14). However, in humans randomized trials with n-3 LCPUFA supplementation in pregnancy and/or lactation have shown ambiguous results regarding anthropometric outcomes later in childhood (15–17).

The aim of this thesis is to examine pre and perinatal factors influence on childhood growth in the two birth cohorts Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) and COPSAC$_{2010}$.

In **study I** we examined the development of Body Mass Index (BMI) from birth through childhood in children with different delivery mode, to determine if CS were associated with differences in childhood growth and obesity. We collected height/length and weight measurements prospectively from term children until 5 years in COPSAC$_{2010}$ and 13 years in COPSAC$_{2000}$, furthermore dual-energy X-ray absorptiometry (DXA) scans were performed at age 3.5 years and 7 years.

We found that children delivered by CS had a higher mean BMI at 6 months compared to those delivered vaginally. However, there were no differences in BMI trajectory between the two groups ages 5 to 13 years, nor cross-sectional at these ages, nor in fat percentages from DXA scans.

In **study II** we analysed the effect of n-3 LCPUFA supplementation in pregnancy on intrauterine growth, gestational age and birth weight. The study was a double-blinded, RCT. The pregnant women received capsules containing either 2.4 g of n-3 LCPUFA or control daily from pregnancy week 24 until one week after birth.

We found that n-3 LCPUFA compared to control was associated with a longer duration of pregnancy, a higher birth weight and an increased intrauterine growth. We observed no effects on preterm delivery or other pregnancy complications.

In **study III** we examined the effect of n-3 LCPUFA supplementation in pregnancy on childhood anthropometrics outcomes. The study was nested in the above-mentioned intervention study. We used prospectively collected height/length and weight to examine the development of BMI from birth through 6 years of age. At 6 years we evaluated the cross sectional effect on height, weight, BMI, head and waist measurements. We used data from DXA scans at 3.5 years and 6 years.

We found that the n-3 LCPUFA group had a significantly higher mean z-score BMI from 1 week to 6 years of age compared to the controls, leading to a significantly higher z-score BMI at age 6 years and a larger waist circumference.

From DXA scans, we found a higher total lean soft tissue mass and a higher bone mineral content in the n-3 LCPUFA group. There were not a higher number of children in risk of obesity or with a higher fat percentage at 6 years of age in the n-3 LCPUFA group.

In conclusion, our findings have manifested that these exposures during pregnancy and birth are associated with a changed childhood growth.

We saw that children delivered by CS had a higher BMI at 6 months of age, but this did not track into later childhood.

N-3 LCPUFA supplementation from pregnancy week 24 was associated with prolonged gestation and increased intrauterine growth, leading to a higher birth weight. In addition, it led to an increase in BMI from age 1 year to 6 years. It did not lead to a higher number of obese children or a higher fat percentage at 6 years of age.

This thesis adds knowledge to the field regarding early life exposures and metabolic programming. We find associations between two exposures which have been changing during the last decades and childhood growth, both exposures are still modifiable.

Danish Summary (Dansk resumé)

I de sidste tre årtier har forekomsten af overvægt og fedme blandt børn været stigende på verdensplan. Denne stigning af overvægtige børn kan ikke forklares på baggrund af genetiske ændringer, da stigningen har fundet sted i løbet af en kort årrække og derfor skal årsagerne søges i miljøfaktorer (1,2). Identifikation af disse faktorer er vigtig som forebyggende skridt mod udvikling af fedme, type 2 diabetes og hjerte-kar-sygdomme (3).

Vi har fokuseret på to faktorer i denne afhandling. Først har vi set på kejsersnit, som har haft en stigende forekomst på verdensplan de sidste årtier (4). To nylige meta-analyser har vist sammenhænge mellem fødsel ved kejsersnit og risikoen for fedme senere i barndommen og voksenlivet (5,6).

Den anden er kosttilskud med n-3 langkædede flerumættede fedtsyrer (n-3 LCPUFA) under graviditeten, da man ved at tilstrækkelige mængder n-3 LCPUFA i løbet af graviditeten er afgørende for barnets udvikling og vækst (7). I mennesker har både observations studier af kostindtag af fisk samt randomiseret forsøg med fiskeolietilskud i graviditeten vist forlængelse af graviditeten og højere fødselsvægt hos børn født af kvinder med et højt n-3 LCPUFA indtag (8–11). Den øgede fødselsvægt kan udelukkende skyldes forlængelse af graviditeten (9,12), men det kunne også delvist forklares ved en øget intrauterinvækst. Dyreforsøg har vist, at n-3 LCPUFA-tilskud både i graviditeten og i den postnatale periode påvirker dannelsen og differentieringen af pre-adipocytter, dette kunne teoretisk set føre til en nedsat risiko for overvægt senere i livet ved at hæmme udviklingen af fedtvæv (13,14). Randomiseret forsøg hos mennesker, hvor man har givet n-3 LCPUFA-tilskud under graviditet og / eller amme perioden, har imidlertid vist tvetydige resultater i forhold til antropometriske målinger senere i barndommen (15–17).

Formålet med denne afhandling er at undersøge præ- og perinatale faktorers indflydelse på væksten gennem barndommen i de to fødselskohorter; Copenhagen Prospective Studies of Asthma in Childhood 2000 ($COPSAC_{2000}$) og $COPSAC_{2010}$.

I **Studie I** undersøgte vi udviklingen af BMI fra fødslen og gennem barndommen hos børn med forskellige fødselsveje for at afgøre, om kejsersnit var associeret med en ændring i væksten gennem barndommen og den senere risiko for fedme. Vi indsamlede højde/længde og vægtmålinger prospektivt fra fødslen og indtil 5 år i $COPSAC_{2010}$ og 13 år i $COPSAC_{2000}$. Derudover blev der udført (DXA) scanninger på børnene ved 3,5 år og 7 år.

Vi fandt ud af, at de børn, som var født ved kejsersnit, havde et højere gennemsnitligt BMI ved 6 måneders alderen sammenlignet med dem, der blev født vaginalt. Der var imidlertid ingen forskel i BMI over tid mellem de to grupper indtil 5 og 13 års alderen, ej heller ved tværsnits analyser i disse aldre eller i fedtprocents målene fra DXA-scanninger.

I **Studie II** analyserede vi effekten af n-3 LCPUFA-tilskud under graviditet på gestationsalder, fødselsvægt og intrauterin vækst. Undersøgelsen var et dobbeltblindet, randomiseret kontrolleret forsøg. De gravide kvinder modtog kapsler indeholdende enten 2,4 g n-3 LCPUFA eller kontrol dagligt fra graviditets uge 24 og indtil en uge efter fødslen.

Vi fandt at n-3 LCPUFA sammenlignet med kontrol var forbundet med en længere varighed af graviditeten, en højere fødselsvægt og en øget intrauterin vækst. Vi observerede ikke en reduktion i børn født for tidligt eller andre graviditetskomplikationer.

I **studie III** undersøgte vi effekten af n-3 LCPUFA-tilskud under graviditet på vækst i barndommen. Studiet er udført i ovennævnte randomiseringsstudie. Vi brugte prospektivt indsamlet højde/længde og vægtmål for at undersøge udviklingen af BMI fra fødslen til 6 år. Ved 6 år lavede vi tværsnitsanalyser på n-3 LCPUFA-tilskuds effekt på højde, vægt, BMI, hoved- og talje-omfang. Vi undersøgte ligeledes effekten på kropssammensætningen vha. data fra DXA-scanninger ved 3,5 år og 6 år.

Vi fandt at n-3 LCPUFA-gruppen havde en signifikant højere gennemsnitlig z-score BMI fra 1 uge til 6 år sammenlignet med kontrolgruppen, hvilket resulterede i en signifikant højere z-score BMI og større taljeomfang ved 6 år. Der var ikke et højere antal børn i risiko for fedme senere i livet eller med en højere fedtprocent i n-3 LCPUFA-gruppen.

Som konklusion har vi med vores resultater vist, at disse eksponeringer under graviditet og fødsel er associeret med en ændret vækst i barndommen.

Vi fandt at børn født ved kejsersnit havde et højere BMI ved 6 måneders alderen, men dette førte ikke til forskelle i BMI senere barndommen.

N-3 LCPUFA-tilskud fra graviditets uge 24 var forbundet med øget intrauterin vækst og forlængelse af graviditeten, hvilket resulterede i en højere fødselsvægt. Derudover medførte det en stigning i BMI fra 1 års alderen til 6 år. Dette førte ikke til et højere antal fede børn eller en højere fedtprocent ved 6 år. Denne afhandling føjer viden til feltet vedrørende det tidlige miljøs effekt på vækst og metabolisk programmering. Vi finder sammenhæng mellem to miljøfaktorer, kejsersnit og n-3LCPUFA indtag, som har ændret sig i de sidste årtier, og vækst i barndommen, begge faktorer er stadig påvirkelige.

Abbreviations

BMI = Body Mass Index

CI = Confidence Interval

$COPSAC_{2000}$ = COpenhagen Prospective Studies on Asthma in $Childhood_{2000}$;

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

DHA = DocosaHexaenoic Acid;

DXA = Dual-energy X-ray Absorptiometry;

EPA = EicosaPentaenoic Acid;

IOTF = International Obesity Task Force;

LCPUFA = Long chain polyunsaturated fatty acids;

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

RCT = Randomized Controlled Trial;

SE = Standard Error

TBLH = Total Body Less Head

1. Introduction

1.1. Background

The prevalence of overweight and obesity has been increasing worldwide for the last three decades among children (18,19). However, it seems the increase of overweight and obesity in childhood has reached a plateau in western countries in the recent years (20).

Early life is characterized by rapid growth and development and it is known that obesity and extensive weight gain in the first years of life are major risk factors for obesity in adulthood (3). Especially the timing and velocity of infancy BMI peak, which is reached at around age 6-7 months, have been associated with higher BMI later in childhood and cardiovascular disease and type 2 diabetes in early adulthood (21,22). The increased prevalence of overweight children cannot be explained by changes in genetic factors, since the great increase has occurred over a short period, the causes must therefore be sought in environmental exposures in which there have been a shift in the last decades (1,2). There have been major focus on exposures from the intrauterine and early postnatal environment and their potential to initiate permanent changes in the body's structure and function, also named 'metabolic programming', a process "*whereby a stimulus or insult at a critical period of development has lasting or lifelong significance*" (23,24).

In this thesis, we focus on two exposures which have changed in the last decades; delivery by caesarean section (CS) and intake of n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) in pregnancy.

CS has had an increase in prevalence the last decades (4). Two recent meta-analyses have shown associations between delivery by CS and obesity in the off-spring in both child- and adulthood (5,6).

Optimal nutritional intake during pregnancy is of great importance to ensure a healthy pregnancy that benefits both the mother and child. N-3 LCPUFA during pregnancy has shown to be an important determinant of adequate child development and health (7,25). In Denmark and many Western countries, the intake of n-3 long-chain polyunsaturated fatty acids (LCPUFA) is much lower than recommended (26) and there has been a shift in the intake ratio of n-6 and n-3 fatty acids, with a higher intake of n-6 fatty acids (27)

1.2. Infant and childhood growth and later risk of overweight

The growth pattern during infancy and childhood have been linked to multiple growth and metabolic outcome later in life.

Infancy has been found to be a period of high interest. Studies have found that the weight of the infant and rapid growth during infancy, are risk factors of subsequent obesity (28). A large focus has been on infancy BMI peak since it has been found that the timing and velocity of this associates both to childhood and adult obesity and several determinants of cardiovascular disease and type 2 diabetes in early adulthood (1,2).

An explanation to why this period of life is so essential could be the nature of adipogenesis early in life. Adipogenesis begins in mid to late gestation. Adipose tissue growth depends on two actions; an increase in the size of adipocytes and new formation of mature adipocytes from precursor cells, so it is a question about number and size of fat cells. It has been shown that infancy is the time period where the capacity of precursor cells differentiation into mature adipocytes are highest and therefore also the time were the formation of adipose tissue is susceptibility to exposures (29,30).

Another time period in childhood which has been associated to later life overweight and obesity is the timing of adiposity rebound, defined as the second rise in BMI that occurs between 3 and 7 years (31). However, it does not seem like it is the timing of adipositas rebound but in larger extend the growth patterns leading to the early timing and the BMI curve the child ends up at in later childhood. Cole concluded in 2004 "*the age at adiposity rebound reflects the level and rate of change of BMI centile at that age. Upward BMI centile crossing at rebound and other ages in childhood predicts later obesity. So adiposity rebound is not a critical period*" (31).

Finally, it is important to state that many exposures have already been virtually established to the risk of obesity later in childhood and adulthood; increased maternal and paternal BMI, increased gestational weight gain, primi-parity, male sex and short duration of exclusive breastfeeding are independent predictors of greater adiposity in adulthood (32–34).

1.3. Mode of delivery way and childhood growth

In the last three decades an increased prevalence of CS deliveries have been observed, and as observed for obesity this prevalence has reached a plateau in western countries during the last decade (4), however in many countries it is still rising and in a country like Brazil the prevalence have now reach 50 % (35). CS is often needed to reduce or eliminate the potential for medical complications during vaginal births. Yet, various aberrations have been linked to delivery by CS;

short-term effects such as hypoglycaemia, breast feeding problems (36) altered immune responses (37) and long-term effects on immune-related conditions such as asthma (37). Two recent meta-analyses have shown associations between delivery by CS and obesity in the off-spring in both child- and adulthood (5,6). Several hypotheses to what the mechanism behind this possible effect could be have been proposed.

One is the difference in the microbiome, which is transferred to the child during delivery. The hypothesis is that during birth the newborns delivered vaginally are directly exposed to maternal vaginal and intestinal microbiota, whereas the newborns delivered by CS are exposed to non-maternally derived environmental bacteria. Previous studies have indicated that the gut microbiota affects the human metabolism and thereby risk of obesity (38). It has therefore been hypothesized that differences in gut microbiota caused by CS could be an explanation for the differences in BMI (39).

Another explanation to the association could be an altered metabolism just after delivery. It has been reported that children delivered by CS just after birth have lower levels of plasma metabolites such as glucose, glycerol and non-esterified fatty acids (36) compared to children delivered vaginally. This altered early life metabolism could track into later life.

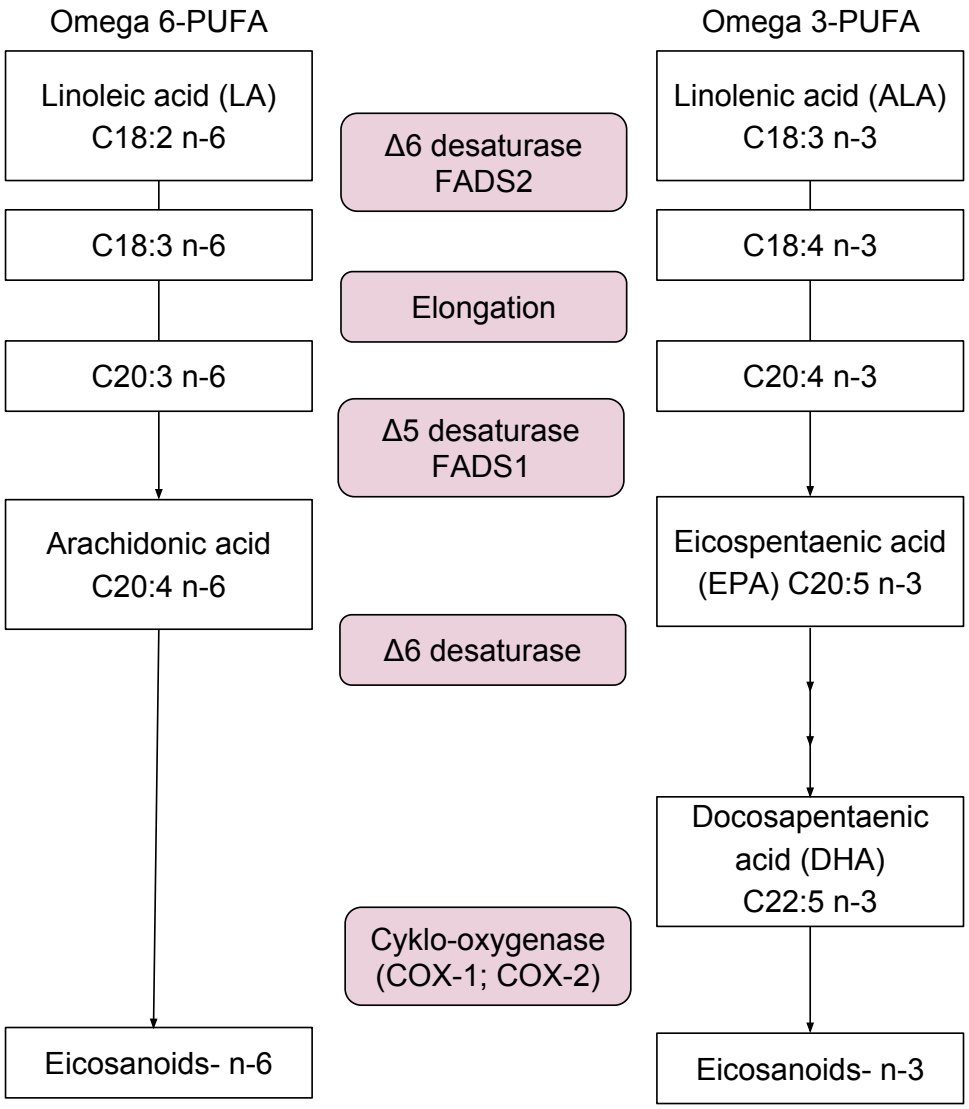
A part of the explanation to the above mentioned could be that vaginal delivery initiates a stress response in the mother and infant measurable by an increase in plasma catecholamine and cortisol levels in the neonate (40). This could alter physiological responses in the infant and thereby alter the metabolism. This change in the hypothalamic–pituitary–adrenal axis could also account for some of the other short and long-term changes that are observed.

1.4. Polyunsaturated fatty acids

As mentioned above N-3 LCPUFA during pregnancy has been shown to be an important determinant of adequate child development and health (7). During the last four decades there has been a shift in the ratio of the dietary intake of the two families of essential fatty acids, n-6- and n3-PUFAs and thereby their elongation and desaturation products, referred to as LCPUFAs (27). There has been a favoring of diet products as nuts, corn oil, eggs and meat which contain n-6 PUFAs, mainly linoleic (LA, 18:2n-6) and the long chained products mainly arachidonic (ARA, 20:4n-6). This in comparison to diet products as marina fish and fish oil with the n-3 long chained derivate eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and in a small extend, diet products as canola oil containing n-3 PUFA mainly linolenic acid (ALA, 18:3n-3). In Denmark

and many Western countries, the intake of N-3 LCPUFA is much lower than recommended (27). The recommend fish or shell fish intake for pregnant women in Denmark are 50 g per day (26) and a study on maternal dietary exposure in pregnancy revealed an average fish intake of 26 g fish and shell fish intake per day. LA and ALA are essential fatty acids and should be obtained from the diet, also their elongation and desaturation products come mainly from the diet but can in some extend be synthesized. The synthesis of ARA, EPA and DHA occur in multiple steps, with the Δ6 desaturase as the rate controlling system, the affinity of Δ6 desaturase for ALA is greater than for LA, but since the concentration of LA typically is higher than for ALA this leads to a greater conversion of LA (**Figure 1**).

Figure 1



Many of the effects from LCPUFA´s are due to their active metabolites, as prostaglandins, prostacyclins and thromboxanes, this group of locally acting hormones are named the eicosanoids. The synthesis of these is dependent upon the cyclooxygenases (COX1 and COX 2) which catalyze the conversion of both the n-6 and n-3 PUFAs to prostanoids and the lipoxygenases which catalyze to leukotrienes.

The importance of the n-6/ n-3 PUFA ratio lies in the competition between the two during their metabolism and thereby the end concentration of their eicosanoids. The different levels in the competition are:

The dietary short chained PUFA competes about the axes to the Δ6 desaturase which metabolizes PUFAs to their respective long-chained derivate. Further down in the metabolism especially ARA and EPA compete for the COXs in relation to the biosynthesis of eicosanoids, and in addition, EPA and DHA have an inhibiting effect on of these enzymes. Finally, eicosanoids from n-3 LCPUFA have antagonistic effect on the eicosanoids derived from n-6 LCPUFA.

The endogenous synthesis of LCPUFA is influenced by genetic variation in the fatty acid desaturase (FADS) gene cluster. FADS SNPs can modulate erythrocyte DHA-status in infancy and some SNPs (rs1535 and rs3834458) have been shown to give rise to increased DHA. These are believed to be important in the effects observed between n-6 and n-3 PUFAs and it has recently been shown that there is a potential modulating effect of FADS SNPs on asthma and allergy risk and cognitive development (41–49).

Polyunsaturated fatty acids effects on gestation and growth.

Maternal nutrition during pregnancy can influence the course of pregnancy and also fetal and childhood growth. This is important as poor intrauterine growth and low gestational age have been associated with increased morbidity and impaired development in children (25). Observational studies in humans have shown prolonged duration of pregnancy and increased birth weight in communities with high fish intake (50,51). The beneficial effects of a high fish intake on pregnancy outcomes have mainly been attributed to the n-3 LCPUFAs, and a number of randomized controlled trials (RCT) have been performed to evaluate the efficacy of n-3 LCPUFA supplementation in pregnancy to increase pregnancy duration and birth weight. A meta-analysis of 21 RCTs of pregnancy supplementation of fish oil showed increased gestational age and reduced risk of preterm delivery (52). However, another recent large meta-analysis of 34 RCTs concluded that there was not enough evidence to support the routine use of omega-3 supplementation during pregnancy to improve mother and child health (53). It has been speculated that the increased birth weight is

caused only by the prolonged duration of pregnancy (12,54), but another explanation could be that the n-3 LCPUFA supplementation also increases the intrauterine growth rate.

The suggested mechanisms behind the effects of n-3 LCPUFA on prolonged gestation involve down-regulation of n-6 LCPUFA-derived prostaglandins (PGE2 and PGF2α), which are involved in initiating the parturition process. Furthermore the increase in production of prostacyclins (PGI2 and PGI3) caused by n-3 LCPUFA could have a relaxing effect on the myometrium (55).

In regards to n-3 LCPUFAs effect on growth, animal studies have shown that n-3 LCPUFA supplementation both in pregnancy and the postnatal period affects the proliferation and differentiation of pre-adipocytes, which could theoretically prevent adiposity development through inhibition of fat tissue development (13,14). A suggested pathway is through "transcriptional factor perioxisome proliferator-activated receptors" (PPARγ´s) in the pre-adipocytes. These receptors are nuclear receptors with gene regulating actions and their ligands include EPA, DHA, ARA and eicosanoids. The PPARγ´s control genes which are involved in regulation of energy metabolism and storage and thereby play a big part in adipogenesis (56,57).

Despite this possible mechanism a recent systematic review of animal studies concluded that the evidence is insufficient to draw any definite conclusions on the role of n-3 LCPUFA supplied during pregnancy and/or lactation on fat mass development in the offspring (54).

A number of RCTs in humans have been performed to evaluate both the efficacy of n-3 LCPUFA supplementation in pregnancy to increase pregnancy duration, birth weight and anthropometric outcomes later in childhood (9,11).

Albeit there have been ambiguous results in regards to gestation age and birth weight, since some studies found no effects of n-3 LCPUFA supplementation (52,53), most studies found a prolonged duration of pregnancy and an increased birth weight (9,10,12).

In regards to growth, randomized trials with n-3 LCPUFA supplementation in pregnancy and/or lactation have shown various results but mostly with no effect. A review from 2014 and a Cochrane review from 2015 concluded that there is no evidence that n-3 LCPUFA supplementation during pregnancy and/or lactation affects BMI or growth development in childhood (15–17).

2. Aim and Objectives

The overall aim of this thesis is to examine pre- and perinatal factors influence on childhood growth. We have focused on two exposures in this thesis; delivery by CS and supplementation with n-3 LCPUFA in pregnancy.
The specific objectives are:

Study I

The study aim is to examine the association between delivery by CS and BMI patterns among children and adolescents in the two birth cohorts Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) and COPSAC$_{2010}$.
We analysed longitudinal BMI data in combination with fat percentage from Dual-energy X-ray absorptiometry (DXA) scans.

Study II and Study III

We aim to investigate whether pregnancy n-3 LCPUFA supplementation compared to control affected:

- Duration of pregnancy, intrauterine growth rate and birth weight of the children.
- Body composition development from birth to age 6 years, by analysing the longitudinal BMI data in combination with data on body composition from Dual-energy X-ray absorptiometry (DXA) scans at 3.5 and 6 years of age. Moreover, anthropometric measurements cross-sectional at 6 years of age.

3. Methodology

3.1. Design, Setting and Participants

Study I in this thesis is based on data from the two birth cohorts Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) and COPSAC$_{2010.}$

Study II and **III** are based on data solely from COPSAC$_{2010.}$

COPSAC$_{2000}$ is a single-centre prospective clinical birth cohort study of 411 children born to asthmatic mothers recruited in 1998–2001 (58). The cohort is designed to assess genetic-environmental interaction in high-risk infants and young children to identify early-life exposures that can be modified to prevent the development of immune diseases.

The children were prospectively seen in the research unit at the age 4 weeks, 6 months and every six months hereafter until the age of 7 years and again at age 13 years (59).

COPSAC$_{2010}$ was designed from the COPSAC$_{2000}$ cohort and is a study of 738 unselected pregnant women and their 700 children followed prospectively until age 6 years (60). The mothers participated in a randomized control trial of fish oil supplementation (N=736) and high dose D-vitamin (N=623) in third trimester of pregnancy (60). We identified pregnant women in the eastern part of Denmark by reviewing the monthly lists of reimbursements to general practitioners for the first pregnancy visits. These women were sent a written invitation to contact the COPSAC clinic by telephone and screened for eligibility. Thereafter, detailed information was sent and the first visit to the clinic was planned within pregnancy weeks 22 through 26 with an additional visit at 36 weeks of pregnancy.

Exclusions criteria in both cohorts were maternal chronic cardiac, endocrinological, nephrological or lung disease other than asthma. In COPSAC$_{2000}$ children born before 36 weeks of gestation were excluded from the study. In COPSAC$_{2010}$ pregnant women with gestational age above week 26 and vitamin D supplementation intake larger than 600IU/day were excluded. In all analysis in this thesis we excluded twins.

In **study I** we excluded children from COPSAC$_{2010}$ with a gestational age <36 weeks. In **study II** we excluded women who did not attended the two pregnancy visits with the exception of women giving birth before pregnancy week 36.

All data were collected according to Good Clinical Practice data management and quality control procedures including external monitoring.

3.2. Study intervention in $COPSAC_{2010}$

The women were randomized 1:1 in a double-blinded design at inclusion to either daily supplementation of 2.4g/day fish oil (55% EPA and 37% DHA), Incromega TG33/22, Croda Health Care, UK) in triacylglycerol form or with identically looking control supplementation capsules of olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid, Pharmatech A/S, Norway). The allocation 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 kept in consecutively numbered closed containers. The fatty acid content and the oxidation levels in both kinds of oil capsules were analysed by the manufacturer at two time-points during the study, showing levels within the expected. The supplementation was continued until one week after birth. The randomization code was unblinded when the youngest child turned three years old.
A subgroup from this pregnancy cohort also participated in a nested, factorial designed, double-blind, RCT of 2,400IU/day vitamin D3 supplementation during late second and third trimester of pregnancy (N=576) and a smaller subgroup participated in a phase IV randomized, participant-blinded comparison of influenza A/California/2009 vaccines (N=142) (61,62).
The intervention with fish oil was used as exposure in **study II** and **III**.

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

3.3. Mode of delivery and intrapartum antibiotics

Information on delivery mode was obtained by personal interview at the child's first visit after birth; furthermore, we asked if the birth was induced by personal interview, the information was validated against the Danish Medical Birth Registry for all of the children. CS was sub-categorized as emergency and elective CS, and vaginal delivery as induced and non-induced.
Information on intrapartum antibiotics was available only in $COPSAC_{2010}$ and obtained by interview 1 week postpartum and birth journal inspection. All women giving birth by CS were treated with prophylactic intrapartum antibiotics.
Mode of delivery and intrapartum antibiotics were used as exposure in **study I**.

3.4. Anthropometrics outcomes

Anthropometrics were assessed at the COPSAC research at age 1 month, 6 months and then every sixth months until age 7 years and then again at 13 years of age for $COPSAC_{2000}$. For $COPSAC_{2010}$ at age 1 week, 1 month, 3 months, 6 months, and every sixth month until age 2 years, and hereafter every 12th month until age 6 years.

Weight was measured without clothes using calibrated digital weight scales.

Length was measured until 2 years using an infantometer (Kiddimeter; Raven Equipment Ltd, Dunmow, Essex, England). Height at later ages and in parents was measured with a stadiometer (Harpenden. Holtain Ltd, Crymych, Dyfed, Wales), which was calibrated yearly.

Waist circumference was measured with a tape measure using the navel as fix point. The mean of two measures during inspiration and expiration was used.

Head circumference was measured with a tape measure, using the largest diameter as measurement.

WHO age- and sex specific BMI z-scores (63) were calculated at all scheduled clinical visits.

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) (64).

In **study I** we analysed 6 months, 1 year, 5 years and 13 years BMI as outcomes. These were defined as the BMI-values closest to 6 months or 1 year ±3 months, 5 years ±6 months and 13 years ±12 months. We calculated IOTF for age 5 and 13 years of age

In **study III** anthropometrics used cross sectional at age 6 years of age were defined as the specific anthropometric measurement closest to 6 ±6 months and we calculated IOTF for age 6 years.

3.5. DXA

DXA is a frequently used technique for body composition measurements and is especially suitable for paediatric patients due to its small radiation dose and short scanning time, and the ability to provide regional body composition analysis (65–67). The underlying principle of DXA is that two X-ray beams with different energy levels are directed through the body in a posterior-to-anterior direction. The attenuation of the photons is directly related to the specific chemical compounds with which they interact. Soft tissue, consisting principally of water and organic compounds restrict the flux to a lesser extent than bone, enabling the system to distinguish between the three components of the body: fat, bone mineral and fat free tissue.

Whole body scans were performed with a 'Lunar iDXA` densitometer (GE Healthcare, Fairfield, CT, USA) The children were DXA scanned at 3.5 years and 6 years in $COPSAC_{2010}$ and at 7 years in $COPSAC_{2000}$ (68).

In **study I** we used the DXA scan to both determine total body fat percentage, calculated as total fat mass divided by body weight at day of scan, extracting the head, since many moved the head during the scan, and moreover body compartment specific fat percentage, using the predefined compartments by the software. We used DXA data at 3.5 years in $COPSAC_{2010}$ and at 7 years in $COPSAC_{2000.}$

In **study III** we used data on body composition from the scans including fat mass, lean soft tissue mass (total mass minus bone mineral content and fat mass), bone mineral content (BMC, g hydroxyapatite) and bone mineral density for the total body less head. In addition, for fat mass and lean soft tissue mass we analysed specific regions of interest predefined by the software. Furthermore, we calculated the percentage of fat mass and lean soft tissue mass for total body less head and regions of interest.

All DXA scan data were scrutinized by an experienced specialist and analysed with enCore™ software (GE-Healthcare, Fairfield, USA).

3.6. Fetal and birth anthropometrics and gestational age

Gestational age: Gestational age was calculated based on expected due date, determined by the scheduled ultrasound scan, which is performed in all pregnant Danish women around pregnancy week 12. In women who had missed the ultrasound scan, due date was calculated from last menstrual period using Naegeles rule. The due date was validated against both the mothers pregnancy record where here doctor had written the date and with data from the Danish foetodatabase. In the few occasions of discrepancy, we used the data from the Danish foetodatabase as first choice, since this data were from the ultrasound clinics. Preterm delivery was defined as birth before week 37+0.

Birth anthropometrics: Birth length and -weight were obtained at the first clinical visit after birth by personal interview and thereafter all of them were validated against the Danish Medical Birth Registry. Furthermore, if there was a difference larger than 10 g or 5 cm, data were validated against the length and weight measures at 1 week from the research clinic to see which value was the most plausible.

Fetal anthropometrics: The fetal weight at mid-pregnancy was estimated from the scheduled ultrasound scans around pregnancy week 20, which is standard prenatal care in Denmark, by the Hadlock equation (69,70) using the fetal biometrics for head, abdomen and femur.
Size for gestational age and intrauterine growth: As an estimate of intrauterine growth we used size for gestational age according to two standards based on Northern European populations: ultrasound-based fetal growth curves according to Marsal (71) and population-based fetal growth curves according to Skjaerven (72). The fetal growth curves were used to calculate which percentage each child birth weight given their gestational age corresponded to on the two standardized curves. Small for gestational age and large for gestational age were defined as below and above two standard deviation on Marsal fetal growth curve. In addition, we calculated the fetal growth rate from week 20 to birth by subtracting the Hadlock weight estimate from the birth weight and dividing this weight gain with the numbers of days from the week 20 ultrasound scan to birth (birth weight (g) – Hadlock estimated weight (g)) / (gestational age at birth (days) - gestational age at scanning (days)).
Pregnancy complications: Information regarding preeclampsia, gestational diabetes mellitus, antibiotics usage in pregnancy, mode of delivery, birth induction and the child's APGAR score were obtained during the scheduled visits and validated against register data. If there were discrepancy, the mother and child's medical record was checked.

3.7. Covariates

Information on race, sex, maternal age at birth, household income, parent's educational level, older siblings, cat or dog in pregnancy, passive smoking and parents asthma status was obtained by personal interviews. Likewise, was parity, smoking during pregnancy, preeclampsia, diabetes in pregnancy, assisted reproduction and days of hospitalization after birth obtained by personal interviews and subsequently validated with register data, in case of inconsistency we checked the medical record from the mother and child.
The social circumstances 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 52% of the variance in $COPSAC_{2000}$ and 55% of the variance in $COPSAC_{2010}$) (73)
Information on breastfeeding was collected by interviewing the mothers on the duration of exclusive and total breastfeeding period and the use of infant formula when the children were 1, 3, 6, 12, 18, 24, 30 and 36 months old at the clinic. As soon as the child's diet was supplemented or replaced by continually use (> 7 days) of infant formula/complementary foods, we considered

exclusive breastfeeding as terminated. If the child had received infant formula for a period of < 7 days as a supplement to breastfeeding, we still considered it exclusive breastfeeding.
Information on pre-pregnancy weight was collected from pregnancy records in $COPSAC_{2010}$, BMI was calculated with the height measured in the clinic.

3.8. Maternal intake and blood levels of EPA, DHA, and fatty acid desaturase (FADS) genotype

The participants completed a validated 360-item food frequency questionnaire assessing dietary intake in the 4 weeks prior to randomization (74–76). Maternal whole blood EPA+DHA levels were assessed at the time of randomization (77,78).
Maternal FADS gene variation was tagged by genotyping of the single nucleotide polymorphism rs1535 (LGC Limited, Hoddesdon, UK) in mothers of European descent. The SNP rs1535 was chosen because it, and its proxies in close linkage disequilibrium, has been associated with n-3 LCPUFA levels in a genome-wide association study (79) and with blood levels of EPA and DHA during pregnancy (80). The risk genotype (GG) has been associated with lower n-3 LCPUFA levels compared to the non-risk genotypes (AA/AG).

3.9. Statistics

All variables were tested for normal distribution. The differences in population characteristics were determined by Student's t-test chi-square tests or Wilcoxon rank-sum test.
Results with a p-value <0.05 were considered significant. Missing data were treated as missing observations. All data analyses were conducted with R v 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria); packages used include lme4; rio; gridExtra; tidyverse; gtools; gtable; plyr; dplyr; cowplot and broom.

Study I:

Covariates with p-values <0.1 were considered potential confounders. We investigated associations between delivery mode and BMI and total fat percentage by Student's t-test and multiple linear regressions. Meta-analysis estimates were calculated with a random effects regression model. Heterogeneity between studies was estimated by I^2-values.
BMI tracking over time were analysed by a mixed model (including repeated measures); using World Health Organization (WHO) sex specific BMI z-scores (81) at every scheduled visit from 1

year to 5 years in $COPSAC_{2010}$ and 13 years in $COPSAC_{2000}$. We used z-scores since BMI does not have a linear development.

Post hoc power analysis

The detectable effect sizes (80% power) were estimated using the T statistics, and they were 0.367, 0.333 and 0.305 for BMI at 6 months, 1 year and 5 years respectively in $COPSAC_{2010}$ and 0.545, 0.516, 0.538 and 1.191 for BMI at 6 months, 1 year, 5 years and 13 years respectively in $COPSAC_{2000}$.

Study II:

Associations between fish oil supplementation and gestational age were analysed using Wilcoxon signed-rank test, since gestational age was not normal distributed.

Power analyses:

The trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the statistical power of the RCT on gestational age, birth weight and birth weight /gestational age was calculated post-hoc based on cohort data.

Our sample size of 699 gave us the opportunity, assuming 80% power, to detect a mean difference of 2 days in relation to gestational age, with a Standard deviation (SD) of 11 days, and a mean increase of 112 g in birth weight, with an SD of 530 g. In regards to difference in birth weight /gestational age, we had power to detect a mean increase of 6.4 %, with an SD of 28 %.

The power/sample size calculation and testing for the study hypotheses were based on a 2-sample, two-tailed t-test at $\alpha=0.05$ level.

Study III:

Associations between supplementation groups and cross-sectional anthropometric outcomes were analysed using Student's t-test for normally distributed continuous variables and chi-square tests for categorical variables.

BMI changes over time were analysed in a random intercept mixed model with BMI z-scores as the outcome. Time trends in the association between intervention and BMI were investigated as an interaction between visit-ages and intervention.

Power analyses

The trial was powered according to the primary outcome: persistent wheeze/asthma. Therefore, the statistical power of the RCT on BMI was calculated post-hoc based on available data. Children who completed the 6 years BMI measurement were used as our sample size. 605 children gave us the opportunity, assuming an 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 at the=0.05 level.

3.10. Ethics

The studies were conducted in accordance with the guiding principles of the Declaration of Helsinki and were approved by the Local Ethics Committee ($COPSAC_{2000}$: KF 01-289/96, $COPSAC_{2010}$: H-B-2008-093), and the Danish Data Protection Agency ($COPSAC_{2000}$ and $COPSAC_{2010}$: 2015-41-3696). Both parents gave written informed consent before enrollment.

4. Results

4.1. Study I

Cesarean Section and Body Mass Index at 6 months and into Childhood

Baseline characteristics

A total of 673 children were included from the $COPSAC_{2010}$ cohort and 21% (N=138) of these were born by CS. In the $COPSAC_{2000}$ cohort a total of 393 children were included and 19% (N=76) of these were born by CS.

Table 1 shows baseline characteristics of the children born by CS compared with children born by vaginal delivery in both cohorts separately.

The mothers, who delivered by CS, were significantly older ($COPSAC_{2000}$ 30.1y vs. 29.5y; p=0.01, $COPSAC_{2010}$ 33.2y vs. 32.1y; p=0.01).

Children born by CS had a lower gestational age ($COPSAC_{2000}$ 278days vs. 281days; p=0.03, $COPSAC_{2010}$ 278days vs. 281days; p<0.001).

In $COPSAC_{2010}$ the children delivered by CS had a higher z-score birth weight/gestational age (0.20 vs. -0.05; p=0.01) and a shorter duration of exclusive breastfeeding (93 days vs. 106 days; p=0.02), in $COPSAC_{2000}$ we did not find these differences.

In $COPSAC_{2010}$ the mothers, who delivered by CS had a higher pre-pregnancy BMI (mean BMI, 25.5kg/m^2 vs. 24.3kg/m^2; p=0.01) and they were more likely to be nulliparous (52.9% vs. 44.7%; p=0.09).

We observed no other differences associated with delivery mode in the cohorts.

All results were therefore adjusted for age at BMI measurement, sex, parity, mother's age, birth weight for gestational age, exclusive breastfeeding duration and in $COPSAC_{2010}$ furthermore for maternal pre-pregnancy BMI.

BMI measurements:

88 % (N=471) of the children in $COPSAC_{2010}$ had BMI measurement at 5 years of age.

Respectively 75 % (N=294) and 82 % (N=324) had BMI measurements at age 5 years and 13 years of age in $COPSAC_{2000}$.

Table 1: Baseline characteristics of children delivered by CS and vaginally in $COPSAC_{2010}$ and $COPSAC_{2000}$.

	$COPSAC_{2010}$					$COPSAC_{2000}$				
	N	**Cesarean section**	**N**	**Vaginal delivery**	**P-value**	**N**	**Cesarean section**	**N**	**Vaginal delivery**	**P-value**
Mode of delivery % (N)		20.4 (138)		79.6 (535)			19.3 (76)		80.7 (317)	
Demographics										
Caucasian % (N)	138	97.8 (135)	535	95.5 (511)	0.22	76	96.1 (73)	317	96.5 (306)	0.84
Female % (N)	138	44.2 (61)	535	50.1 (268)	0.22	76	52.6 (40)	317	50.8 (161)	0.77
Gestational age (days), mean (SD)	138	277.5 (10.1)	535	281.1 (8.6)	**<0.01**	76	277.6 (12.8)	317	280.6 (10.1)	**0.03**
Age at 5 years BMI measurement (years), mean (SD)	118	5.0 (0.1)	471	5.0 (0.1)	**0.04**	61	5.2 (0.2)	233	5.2 (0.2)	0.8
Age at 13 years BMI measurement (years), mean (SD)	-	-	-	-	-	67	12.9 (0.6)	257	12.9 (0.6)	0.96
Social circumstances*	138	0.2 (1.2)	535	0.0 (0.9)	0.43	72	0.1 (1.2)	292	-0.1 (1.1)	0.32
Mother's age at birth, mean (SD)	138	33.2 (4.8)	535	32.1 (4.2)	**0.01**	76	31.0 (4.5)	317	29.5 (4.4)	**0.01**
Nulliparity % (N)	138	52.9 (73)	535	44.9 (240)	0.09	76	44.7 (34)	317	45.4 (144)	0.91
Father height (cm), mean (SD)	121	180.7 (6.8)	505	180.9 (6.7)	0.77	63	181.2 (7.9)	245	180.7 (7.2)	0.63
Mother asthmatic % (N)	137	30.7 (42)	534	24.7 (132)	0.16	76	100 (76)	317	100 (317)	1
Risk factors										
Smoking in pregnancy % (N)	138	8.1 (12)	534	7.5 (40)	0.79	76	19.7 (15)	317	26.5 (84)	0.22
Exclusive breastfeeding (days), mean (SD)	136	93.4 (66.6)	529	106.3 (57.1)	**0.02***	72	110.8 (67.8)	281	114.8 (58.7)	0.61
Mother pre-pregnancy BMI (kg/m2), mean (SD)	121	25.5 (4.8)	488	24.3 (4.2)	**0.01**	-	-	-	-	-
Mother height (cm), mean (SD)	138	166.6 (6.3)	535	167.7 (6.3)	**0.07**	74	167.4 (7.0)	292	167.0 (6.7)	0.73
Gestational diabetes % (N)	138	2.1 (3)	535	2.4 (13)	0.86	-	-	-	-	-
Pre-eclampsia % (N)	138	6.5 (9)	535	3.6 (19)	0.12	76	7.9 (6)	317	4.1 (13)	0.17
Intrapartum antibiotics % (N)	138	100 (138)	532	12.9 (69)	-	-	-	-	-	-

Hospitalization after birth % (N)	138	4.6 (21)	535	4.7 (47)	0.94	-	-	-	-	-
Fish oil supplementation % (N)	137	53.3 (73)	534	48.9 (261)	0.38	-	-	-	-	-
High dose D-vitamin supplementation % (N)	118	51.7 (61)	452	50.0 (226)	0.74					
Anthropometrics										
Birthweight for gestational age z-score (units)**, mean (SD)	138	0.2 (1.2)	534	-0.1 (0.9)	**0.01**	76	0.1 (1.2)	317	-0.1 (1.1)	0.44
Birthweight (Kg), mean (SD)	138	3.6 (0.6)	535	3.6 (0.5)	0.82	76	3.5 (0.6)	317	3.6 (0.5)	0.72
BMI > 85 percentile at 5 years % (N)	118	16.9 (20)	471	14.9 (70)	0.57	61	16.4 (10)	234	15.8 (37)	0.91
BMI > 85 percentile at 13 years % (N)	-	-	-	-	-	67	17.9 (12)	259	13.1 (34)	0.32
BMI > 90 percentile at 5 years % (N)	118	10.9 (14)	471	9.9 (47)	0.72	61	14.8 (9)	234	11.16 (26)	0.44
BMI > 90 percentile at 13 years % (N)		-	-	-	-	67	11.9 (8)	259	9.7 (25)	0.58

* Defined as the first component of a principal component analysis on household income, maternal age and maternal level of education at 2 years with a mean value of zero and standard deviation of one.
**Calculation was based on Marsals intra uterine growth curves.
BMI= Body Mass Index, N=Number.

Delivery mode and BMI development in the 1st year of life

Children born by CS had a higher peak value of mean BMI in infancy in both cohorts compared to children delivered vaginally (**Figure 2**).

In $COPSAC_{2010}$ this difference was most pronounced at age 6 months; 17.6kg/m^2 vs. 17.2kg/m^2 [0.10; 0.61] and subsequently the groups aligned with no difference at age 1 year. In $COPSAC_{2000}$ the BMI values of children born by CS diverged from 6 months compared with children born vaginally the difference increased further reaching its maximum at age 1 year; 17.6kg/m^2 vs. 17.2kg/m^2 [0.01; 0.82]. The differences in BMI at 6 months were significant after adjustment in $COPSAC_{2010}$ (β-coefficient, 0.41kg/m2; [0.12; 0.69]; p=0.01), but not in $COPSAC_{2000}$ (β-coefficient, 0.16kg/ m2; [-0.11; 0.68]; p=0.16). Meta-analysis of BMI at 6 months revealed significant association with CS in the two cohorts (β-coefficient, 0.37kg/ m2; [0.14; 0.60]; p=0.002) (**Table 2**), but no difference in the meta-analysis of BMI at 1 year. Further adjusting the analyses in $COPSAC_{2010}$ for the pregnancy supplementation trials did not change the results (data not shown). We sub-analysed the associations between CS and BMI at months in the 174 children delivered by asthmatic mothers in $COPSAC_{2010}$ β-coefficient, 0.30kg/m2; [-0.20; 0.80]; p=0.22.

Delivery mode and BMI development during childhood

The BMI curves aligned after the gap in the first year (**Figure 1**) and we found no difference in mean BMI at 5 years of age, with regards to mode of delivery ($COPSAC_{2010}$: β-$coefficient_{adjusted}$, -0.03kg/m^2; [-0.27; 0.21]; p=0.81, $COPSAC_{2000}$: β-$coefficient_{adjusted}$, 0.18kg/m^2; [-0.20; 0.56]; p=0.35) (**Table 2**). We found no difference in the meta-analysis at this time point.

Figure 3 illustrates the longitudinal BMI development for the children until 13 years of age in the $COPSAC_{2000}$ cohort according to mode of delivery. We found no difference in mean BMI between the two groups at age 13 years (β-$coefficient_{adjusted}$, -0.03kg/m^2; [-0.87; 0.82]; p=0.95) (**Table 2**). From 1.5 to 13 years of age the curves are almost coherent with the CS curve on top and graphically they reach the time for adipositas rebound (approx. age 4.5 year) simultaneously and continuing with an identical course into puberty.

Using repeated measurements statistics we analysed if there were a difference in mean BMI from infancy through childhood between children delivered by CS and vaginally. We found no difference in mean z-score BMI over time; $COPSAC_{2010}$; 1 year to 5 year of age (β-coefficient -0.05; SE [0.07]; p=0.95) and $COPSAC_{2000}$; 1 year to 13 year of age (β-coefficient 0.12; SE [0.10]; p=0.21). Furthermore, we compared the ratio of children having a BMI-value in the highest 85 percentiles and 90 percentiles at five years and at thirteen years of age and found no differences with regards to delivery mode (**Table 1**).

Figure 2: BMI in first 5 years of life.

Curves showing mean BMI with standard errors according to visit age in the first five years of life for children delivered by cesarean section and vaginally in $COPSAC_{2010}$ and $COPSAC_{2000}$.

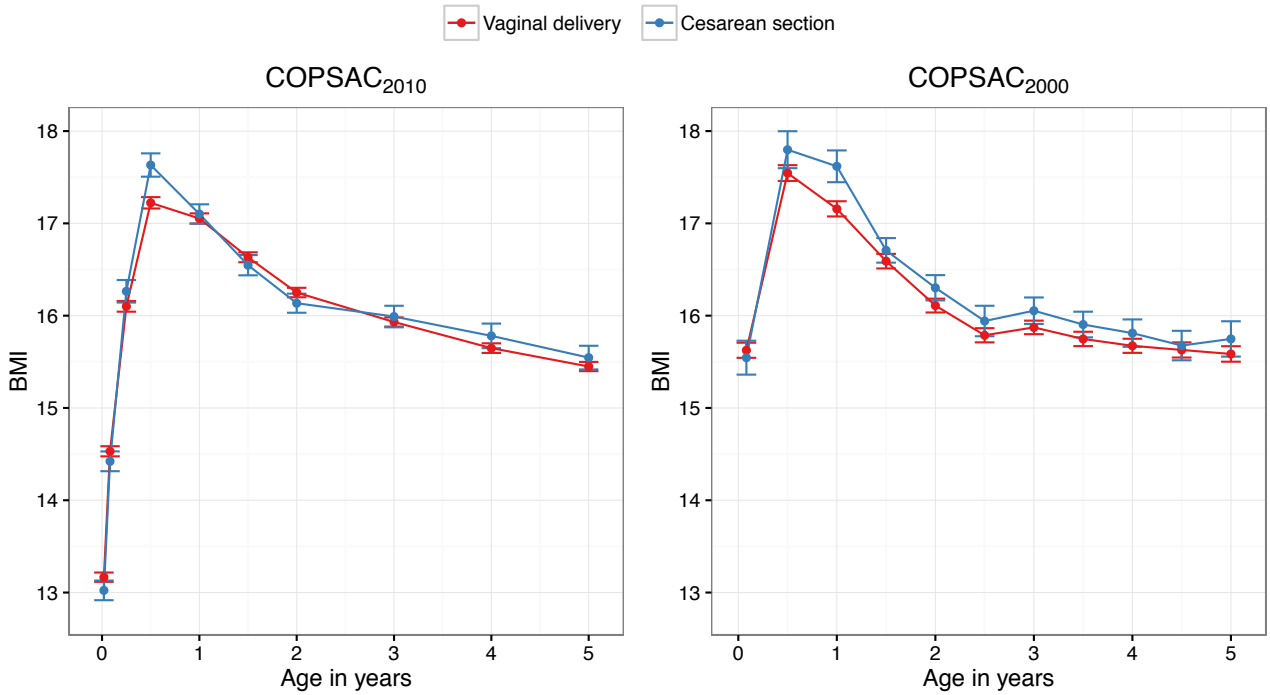


Figure 3: BMI in first 13 years of life.

Curves showing mean BMI with standard errors according to visit age for children delivered by cesarean section and vaginally until 13 years of age in $COPSAC_{2000}$.

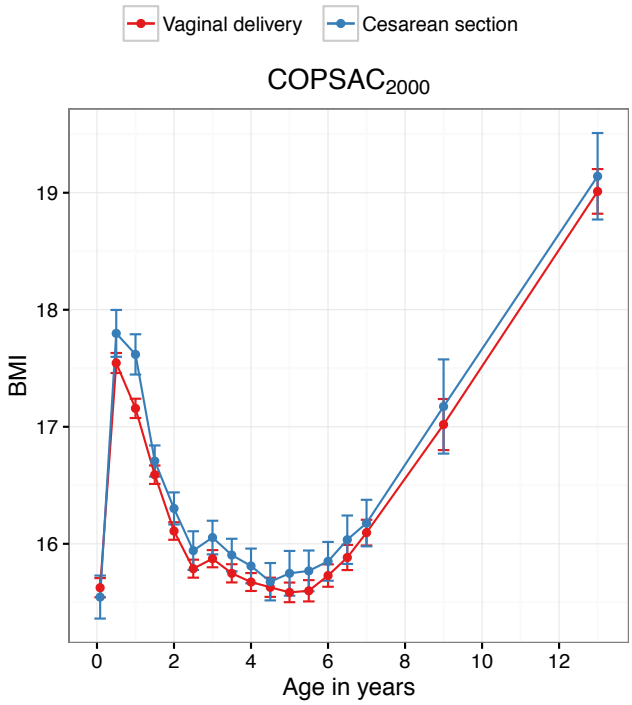


Table 2: Relationship between mode of delivery and 6 months, 1 year, 5 year and 13 year BMI measurement

	$COPSAC_{2010}$				**$COPSAC_{2000}$**				**Meta-analysis**			
	Estimate Crude (95 % CI)	P-value	Estimate adjusted§ (95 % CI)	P-value	Estimate Crude (95 % CI)	P-value	Estimate adjusted# (95 % CI)	P-value	I^2	Hetero-geneity p-value	Estimate* (95 % CI)	P-value
BMI at 6 months	0.36 (0.10;0.61)	**0.007**	0.42 (0.13;0.70)	**0.004**	0.29 (-0.10;0.66)	0.139	0.28 (-0.11;0.68)	0.156	0.0%	0.60	0.37 (0.14;0.60)	**<0.01**
BMI at 1 year	-0.01 (-0.239;0.21)	0.918	-0.05 (-0.30;0.20)	0.695	0.46 (0.01;0.82)	**0.013**	0.50 (0.14;0.87)	**0.007**	73.2 %	0.05	0.18 (-0.35;0.72)	0.51
BMI at 5 years	0.07 (-0.15;0.29)	0.544	-0.03 (-0.27;0.21)	0.811	0.20 (-0.18;0.58)	0.304	0.18 (-0.20;0.56)	0.349	0.0%	0.36	0.03 (-0.17;0.24)	0.76
BMI at 13years	-	-	-	-	0.15 (-0.68;0.98)	0.722	-0.03 (-0.87;0.82)	0.950	-	-	-	-

*Random effects
BMI= Body Mass Index
§ Adjusted for in $COPSAC_{2010}$; Age at BMI measurement, sex, parity, mother's age, mother's pre-pregnancy BMI, birth weight for GA and duration of exclusive breastfeeding.
# Adjusted for in $COPSAC_{2000}$; Age at BMI measurement, sex, parity, mother's age, birth weight for GA and duration of exclusive breastfeeding.

Induction of birth and BMI development during childhood

In $COPSAC_{2010}$ we sub-analysed whether induction of birth in the vaginal delivery group and type of CS could affect the results. We found no differences in BMI at any time point in vaginally delivered children with regards to birth induction. Furthermore, we found no differences in BMI with regards to type of CS (**Table 3**).

Table 3:

A: Relationship between induced vs non-induced birth in vaginal delivery and 6 months, 1 year and 5 year BMI measurement unadjusted in $COPSAC_{2010}$.

B: Relationship between emergency and elective cesarean section and 6 months, 1 year and 5 year BMI measurement unadjusted in $COPSAC_{2010}$.

A: Vaginal delivery			
BMI	Not induced birth Mean (SD)	Induced Birth Mean (SD)	P-value
6 months	17.25 (1.46) N=395	17.13 (1.25) N=129	0.36
1 year	17.04 (1.31) N=394	17.07 (1.15) N=127	0.88
5 years	15.43 (1.11) N=358	15.55 (1.02) N=115	0.26
B: Cesarean section			
BMI	Emergency section Mean (SD)	Elective Section Mean (SD)	P-value
6 months	17.59 (1.45) N=65	17.67 (1.51) N=65	0.73
1 year	17.08 (1.20) N=71	17.12 (1.23) N=64	0.85
5 years	15.64 (1.60) N=58	15.47 (1.16) N=60	0.50

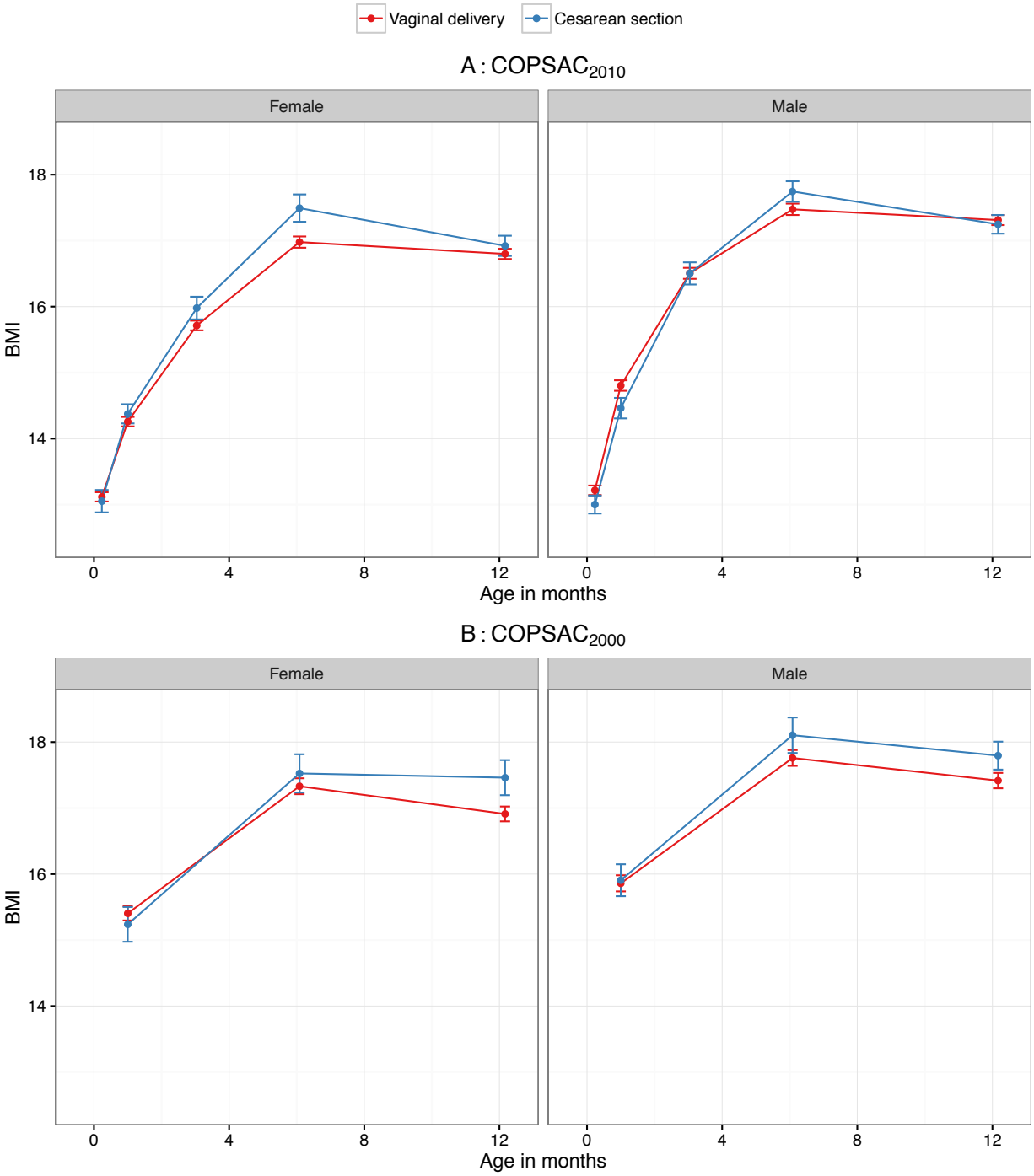
BMI= Body Mass Index, N= Number.

Sex specific growth

Sex specific growth curves for both cohorts are illustrated in **Figure 4** showing the mean BMI-value in the first year of life. We did not find any sex specific growth patterns according to mode of delivery.

Figure 4: BMI in first year of life sex stratified.

Curves showing mean BMI with standard errors according to visit age in the first year of life for respectively boys and girls delivered by cesarean section and vaginally in A:COPSAC$_{2000}$ and B: COPSAC$_{2010}$.



Delivery mode and body fat percentage

CS delivery was not associated with significant differences in the body fat percentage of the children measured by DXA scans at age 3.5 years in $COPSAC_{2010}$ and at age 7 years in $COPSAC_{2000}$ (**Table 4**).

We furthermore sub-analysed the DXA-scans from $COPSAC_{2010}$ and found no significant regional differences in body fat percentage in legs, arms, trunk or the android region between the two delivery groups (**Table 5**).

Table 4 Fat percentage from Dual-energy X-ray absorptiometry scans at 3.5 years in $COPSAC_{2010}$ and at 7 years in $COPSAC_{2000}$

	Cesarean Section Mean (SD)	Vaginal Mean (SD)	Crude[#] Estimate (95 % CI)	P-value	Adjusted[§] Estimate (95 % CI)	P-value
$COPSAC_{2010}$						
Fat-%	28.18 (4.79) N=79	28.77 (4.36) N=272	-0.30 (-1.27;0.67)	0.22	-0.29 (-1.39;0.81)	0.61
$COPSAC_{2000}$						
Fat-%	27.96 (5.16) N=57	28.28 (5.85) N=233	-0.29 (-1.82;1.23)	0.70	-0.53 (-0.21;1.04)	0.51

BMI= Body Mass Index, N= Number.
# adjusted for age at Dual-energy X-ray absorptiometry scans and sex.
§ adjusted for: $COPSAC_{2010}$; Age at BMI measurement, sex, parity, mother's age, mother's pre-pregnancy BMI, birth weight for GA and duration of exclusive breastfeeding.
$COPSAC_{2000}$; Age at BMI measurement, sex, parity, mother's age, birth weight for GA and duration of exclusive breastfeeding.

Table 5

Fat percentage from Dual-energy X-ray absorptiometry scans at 3.5 years in arms, legs, trunk and android region in $COPSAC_{2010}$

	Cesarean Section Mean (SD)	Vaginal Mean (SD)	Crude Estimate (95 % CI)	P-value	Adjusted[§] Estimate (95 % CI)	P-value
Fat-% Legs	36.78 (5.28) N=72	37.18 (4.72) N=266	-0.03 (-1.13;1.08)	0.96	-0.10 (-1.27;1.06)	0.86
Fat-% Trunk	20.14 (4.90) N=72	20.55 (4.58) N=266	-0.02 (-1.10;1.06)	0.46	-0.10 (-1.28;1.08)	0.87
Fat-% Arms	39.13 (5.08) N=72	40.09 (4.68) N=266	-0.61 (-1.74;0.50)	0.28	-0.70 (-1.91;0.49)	0.25
Fat-% Android region	16.92 (4.80) N=72	17.18 (4.72) N=266	0.06 (-1.07;1.19)	0.91	0.06 (-1.18;1.31)	0.92

BMI= Body Mass Index, N= Number.
*adjusted for age at Dual-energy X-ray absorptiometry scans and sex.
§ adjusted for: $COPSAC_{2010}$; Age at BMI measurement, sex, parity, mother's age, mother's pre-pregnancy BMI, birth weight for gestational age and duration of exclusive breastfeeding.

Intrapartum antibiotics and cross-sectional BMI

As all women giving birth by CS were treated with intrapartum antibiotics, we wanted to examine, whether this treatment could be responsible for some of the effects. In $COPSAC_{2010}$, 13% (N=69) of women with vaginal delivery received intrapartum antibiotics. There were no differences in mean BMI at age 6 months or 5 years in children of these women compared to children, whose mothers did not receive antibiotics (**Table 6**).

Table 6: Relationship between usage of intrapartum antibiotics in vaginally delivery children and 6 months BMI and 5 years BMI in $COPSAC_{2010}$

			Crude*		**Adjusted§**	
BMI	AB Mean (SD)	No AB Mean (SD)	Estimate (95 % CI)	P-value	Estimate (95 % CI)	P-value
6 months	17.30 (1.48) N=72	17.19 (1.41) N=462	-0.15 (-0.48;0.20)	0.40	-0.06 (-0.43;0.31)	0.75
5 years	15.38 (1.42) N=47	15.47 (1.08) N=291	0.10 (-0.24;0.43)	0.57	0.04 (-0.26;0.34)	0.78

BMI= Body Mass Index, N= Number.
*adjusted for age at BMI measurement and sex.
§ additional adjusted for: parity, mother's age, mother's pre-pregnancy BMI, birth weight for GA and duration of exclusive Breast feeding.

4.2. Study II and Study III

Study II Fish oil supplementation increases pregnancy duration and intrauterine growth. Randomized controlled trial

Study III: Fish-oil supplementation in pregnancy causes a proportional increase in lean mass, bone mass and fat mass at 6 years: A Randomized, Controlled, Double-Blind, Clinical Trial

Baseline characteristics

We randomized 736 unselected women at pregnancy week 24 to either n-3 LCPUFA or control. Before pregnancy week 36; 27 women were lost to follow-up, we also excluded 10 children born from twin pregnancies and four children with disabling disease. For the analyses in study II we therefore ended up with 699 mother/child pairs allocated with 346 in the n-3 LCPUFA group and 353 in the control group. Additional 11 mother/child pairs were lost to follow up after the visit in pregnancy week 36, leaving 688 children with at least one clinical anthropometrics measurement. These were included in study III allocated with 341 in the n-3 LCPUFA supplementation group and 347 in the control group (**Figure 5**). Enrolment began in November 2008, ended in November 2010, and the last child was born in April 2011.

699 (100 %) of the children had information on gestational age and birth weight. 605 (88%) children had anthropometrics measured and 465 (68%) had DXA scanning data 6 years of age.

The baseline characteristics are presented in **Table 7**, showing a successful randomization ($p>0.05$ in all comparisons, data not shown).

Figure 5. Flow chart of enrollment and allocation of the $COPSAC_{2010}$ pregnancy cohort and follow-up of the $COPSAC_{2010}$ birth cohort.

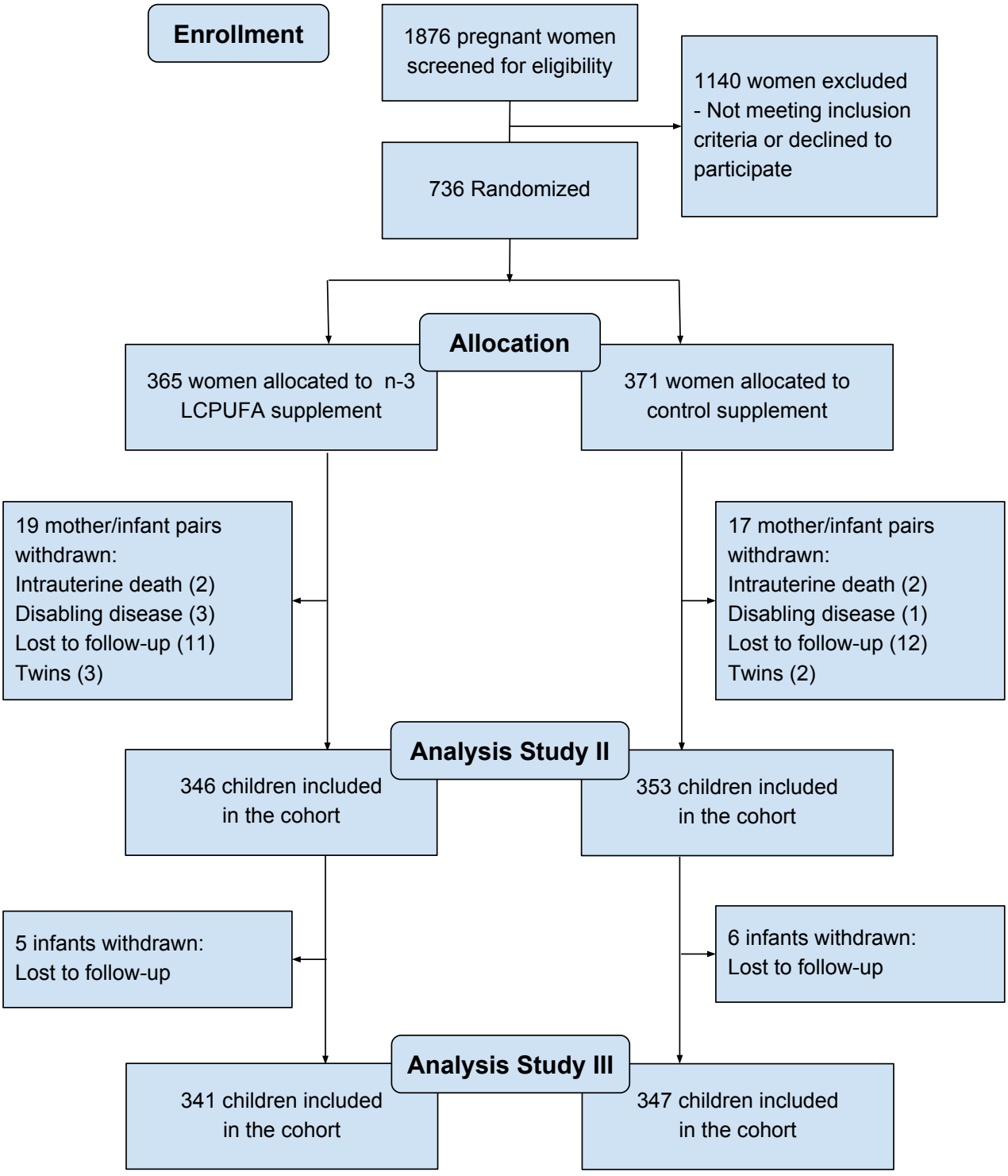


Table 7 Baseline characteristics of the participants in COPSAC$_{2010}$ mother-child cohort.

	All	Randomization	
		n-3 LCPUFA	Control
	699	49% (346)	51% (353)
Child			
Sex, male % (N)	50.5 (353)	48.2 (166)	53.1 (187)
Caucasian % (N)	95.4 (667)	96.8 (335)	95.1 (332)
Season of birth			
Winter % (N)	30.9 (216)	28.3 (98)	33.4 (118)
Spring % (N)	26.8 (187)	27.7 (96)	25.8 (91)
Summer % (N)	21.1 (148)	21.1 (73)	21.2 (75)
Fall % (N)	21.1 (148)	22.8 (79)	19.5 (69)
Exclusive breastfeeding (days), mean (SD)	103 (60)	104 (59)	103 (60)
Age at 6 years BMI measurement (years), mean (SD)	6.0 (0.2)	6.0 (0.2)	6.2 (0.2)
Age at 6 years DXA scanning (years), mean (SD)	6.2 (0.2)	6.2 (0.2)	6.2 (0.2)
Pregnancy			
Primi-parity % (N)	45.4 (317)	43.9 (152)	46.7 (165)
Smoking in pregnancy % (N)	7.9 (55)	5.8 (20)	9.8 (35)
Cat or dog in pregnancy % (N)	34.9 (244)	35.9 (124)	34.2 (120)
Antibiotics in pregnancy % (N)	45.2 (316)	44.7 (155)	46.1 (161)
GA at inclusion, mean (SD), weeks	24.3 (0.7)	24.3 (0.7)	24.3 (0.7)
Hadlock calculated in utero weight[#], mean (SD), g	323.4 (54.0)	321.7 (49.1)	326.0 (58.5)
Assisted reproduction % (N)	9.4 (66)	8.9 (30)	10.6 (36)
Participation in the high dose vitamin D intervention % (N)	84.3 (589)	40.3 (282)	43.69 (307)
Daily fish intake before inclusion mean (SD), g	27.9 (17.5)	27.9 (16.7)	27.8 (18.3)

Maternal pre-treatment blood levels of EPA+DHA*, mean (SD), %	4.9 (1.2)	4.9 (1.2)	4.9 (1.2)
Parental factors			
Maternal age at birth, mean (SD), years	32.2 (4.5)	32.3 (4.4)	32.1 (4.5)
Maternal pre-pregnancy BMI, mean (SD), Kg/m^2	24.6 (4.4)	24.7 (4.3)	24.3 (4.5)
Maternal asthma % (N)£	26.0 (182)	24.4 (84)	27.8 (98)
Paternal height, mean (SD), cm	181.0 (6.7)	181.1 (6.3)	180.8 (7.1)
Social circumstances**	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)

Calculated by the Hadlock equation using the fetal biometrics for head, abdomen and femur.
* Relative percentage of measured blood fatty acids.
£ History of doctor diagnosed asthma
**Defined as the first component of a principal component analysis on household income, maternal age and maternal level of education at 2 years with a mean value of zero and standard deviation of one.
Student's t-test was used for normally distributed continuous variables and chi-square tests for categorical variables to analyse differences between the intervention and the control group. All comparisons were non-significant with p>0.05.
Abbreviations: GA – gestational age; SD – standard deviation; BMI – body mass index;

Adherence

Adherence to the study supplementation defined as an intake of more than 80% of the prescribed dose based upon capsule count was estimated to be 70 % of the included women with no differences between the n-3 LCPUFA and control group.

Outcomes in study II:

Gestational age, fetal growth and birth weight

Gestational age

For the 699 included children, the median gestational age was 281 days.

Supplementation with n-3 LCPUFA showed a significantly higher gestational age of two days; median [IQR] (282 days [274–288] vs. 280 days [273–286]), p=0.02 (**Table 8)**. This prolongation of pregnancy duration did not lead to a reduction in frequency of preterm delivery: 12 children in the n-3 LCPUFA group and 14 children in the control group were born before pregnancy week 37, equal to 4 % in both groups. **Figure 6A** illustrates gestational age in the n-3 LCPUFA and control group, indicating that the effect of n-3 LCPUFA supplementation is evenly distributed across the spectrum of gestational age in the population.

Birth weight

For the 699 children included the mean (SD) birth weight was 3,552 g (533g). Children born to mothers who were supplemented with n-3 LCPUFA in pregnancy had a significantly higher birth

weight compared to the control group; mean (SD) 3,601g (534g) vs. 3,504g (528g), p=0.02; corresponding to a mean increase of 97 g (95% CI: 0.02g; 0.18 g) (**Figure 6B** and **Table 8**). We also found that the children in the intervention group had a non significant higher length at birth cm, mean (SD) 52.1 (2.4) vs 51.8 (2.5), p=0.15 (**Table 8**).

Size for gestational age and intrauterine growth: As a proxy of intrauterine growth, we used size for gestational age. The children were larger for gestational age in the n-3 LCPUFA vs. control group when using standardized growth curves to estimate the size; mean percentage (SD) by Marsal; 51.6%(28.4) vs. 47.6%(28.3); p=0.06 and by Skjaerven; 49.9% (28.3) vs. 44.5% (27.6); p=0.01. (**Figure 6C** and **Table 8**).

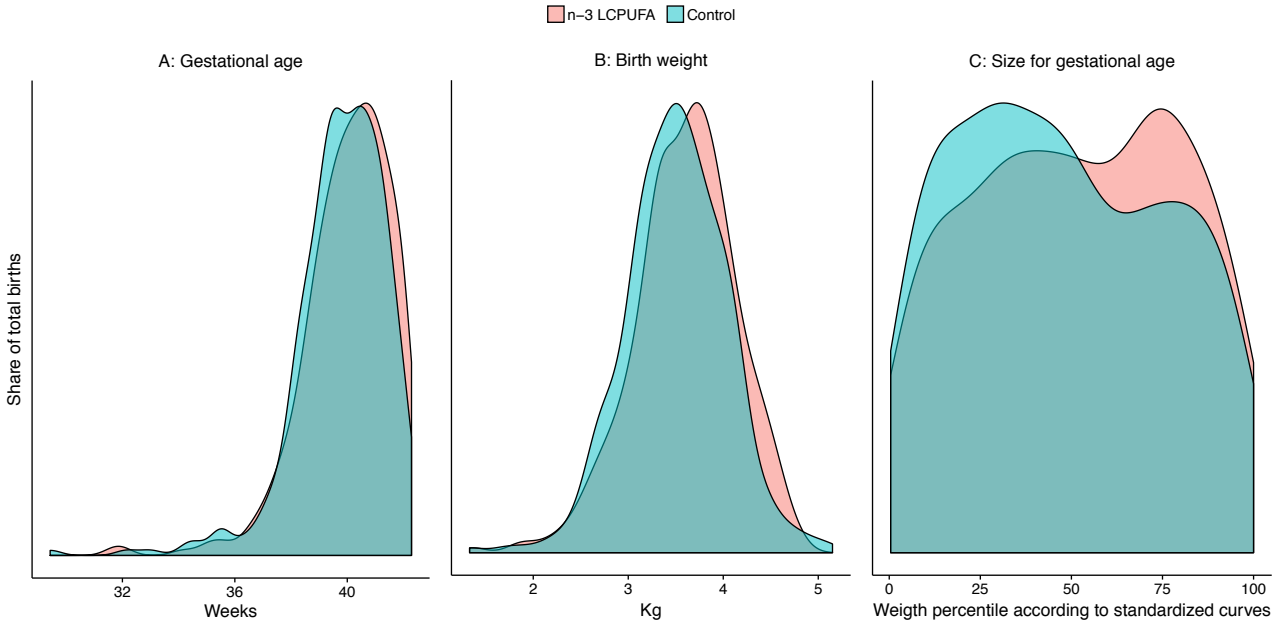
We did not find any significant difference in children born small or large for gestational age according to Marsals definition (71): 8 (2.3 %) children in the n-3 LCPUFA group and 5 (1.4 %) children in the control group were born were born small for gestational age. 13 (3.8 %) children in the n-3 LCPUFA group and 8 (2.3 %) children in the control group were born large for gestational age.

Intrauterine growth based on the difference between Hadlock calculated fetal weight (69) from pregnancy week 20 to weight at birth, was available for 691 (99%) of the children. The average (SD) growth rate from this estimation was 23.19 g/day (3.15) in the n-3 LCPUFA group vs. 22.83 g/day (3.17) in the control group; p=0.13, corresponding to a mean difference of 0.36 g/day (95% CI: -0.11 g/day; 0.83 g/day) (**Table 8**).

Stratifying the analyses on nulliparous and multiparous women yielded the same associations between the n-3 LCPUFA supplementation and gestational age, birth weight and intrauterine growth measures (data not shown).

There was no significant interaction between n-3 LCPUFA and the vitamin D supplementation with regards to the gestational age or growth outcomes (p>0.20).

Figure 6: Density plot illustrating gestational age, birth weight and fetal growth rate stratified by supplementation groups; n-3 LCPUFA and control.



Adverse pregnancy and birth outcomes

The n-3 LCPUFA supplementation had no significant effects on adverse pregnancy outcomes such as preeclampsia or gestational diabetes. Furthermore, there were no differences in delivery complications such as induced birth, emergency or elective cesarean section, and no difference in the APGAR score at 5 minutes (**Table 8)**

Table 8 Effects of fish oil supplementation on primary and secondary end-points

	n-3 LCPUFA N=346	Control N=353	P-value
Primary end-points;			
Gestational age, median [IQR], days	282 [274–288]	280 [273–286]	**0.02**
Birth weight, mean (SD), g	3,601 (535)	3,504 (528)	**0.02**
Birth length, mean (SD), cm	52.1 (2.4)	51.8 (2.5)	0.15
Marsal-percentage*, mean (SD), %	51.6 (28.4)	47.6 (28.3)	0.06
Skjaerven-percentage*, mean (SD), %	49.9 (28.3)	44.5 (27.6)	**0.01**
Secondary end-points;			
Preterm delivery (GA<week 37) % (N)	3.5 (12)	4.0 (14)	0.88
Born small for gestational age# % (N)	2.3 (8)	1.4 (5)	0.55
Born large for gestational age## % (N)	3.8 (13)	2.3 (8)	0.35
Fetal growth from week 20 to birth, mean (SD), g/day**	23.2 (3.2)	22.8 (3.2)	0.13
APGAR score 5 min <10 % (N)	5.0 (17)	4.3 (15)	0.82
Induced birth % (N)	36 (123)	35 (120)	0.81
Emergency cesarean section % (N)	13.9 (48)	10.5 (37)	0.22
Elective cesarean section % (N)	8.9 (31)	9.9 (35)	0.90
Preeclampsia % (N)	4.4 (15)	4.3 (15)	1
Gestational diabetes % (N)	1.8 (6)	2.9 (10)	0.46

* Calculated percentage of expected birth weight at a certain gestational age using the two standardized growth curves.
# Children with a Marsal percentage below – two SD.
Children with a Marsal percentage above two SD.
**Calculated as (birth weight (g) – Hadlock estimated weight (g)) / (gestational age at birth (days) – gestational age at scanning (days))
Student's t-test was used for normally distributed continuous variables and chi-square tests for categorical variables to analyze differences between the intervention and the control group.
Abbreviations, SD – standard deviations; IQR – inter quartile range, GA – gestational age, N- number

Outcomes in study III

n-3 LCPUFA supplementation and BMI development during childhood

Figure 7 illustrates the BMI development from birth to age 6 years according to intervention group. As revealed in Study II the children in the n-3 LCPUFA group had a higher birth weight and here we also see a higher BMI at 1 week compared to the control group, but from 1 month to 6 months, the two groups demonstrated comparable BMI (**Figure 8**). Between 6 months and 1 year the groups separated again with significantly higher BMI values among children in the n-3 LCPUFA supplementation group compared to the control group from age 1 year, a comparable separation continued to age 6 years (**Figure 8**).

Figure 7

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.

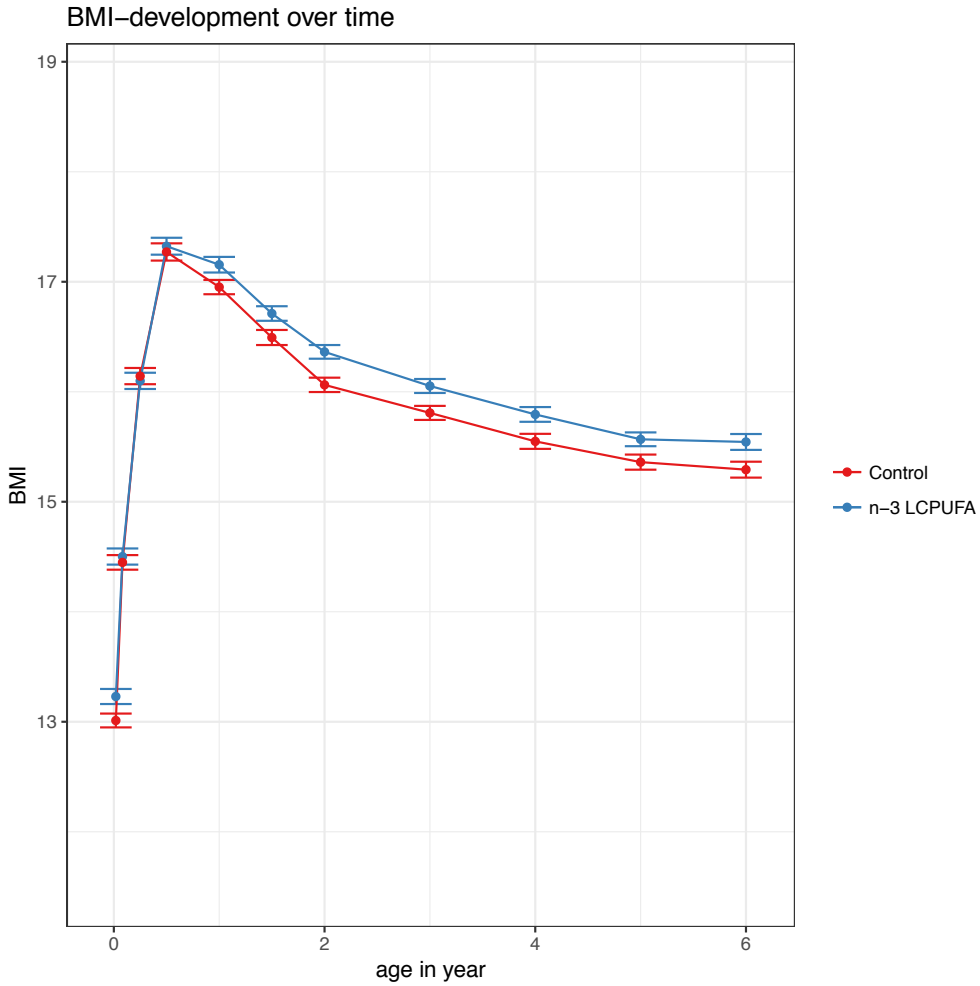
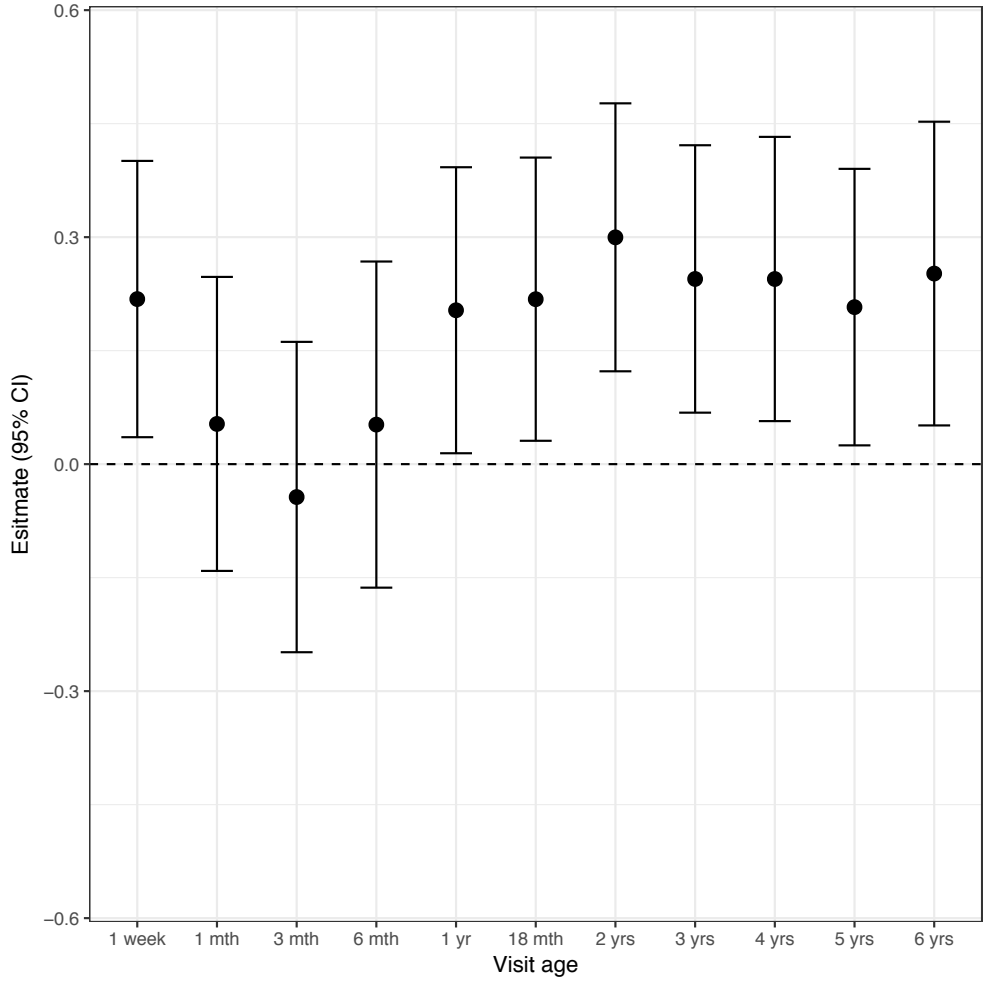


Figure 8

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.



Using the mixed effects model to capture repeated measurements of BMI, we found that the n-3 LCPUFA supplementation group had a significantly higher mean z-score BMI from 1 week to 6 years of age compared to the control group; (β-coefficient 0.14; SE [0.05]; p=0.006). We furthermore observed a significant interaction between age and intervention group (p-value=0.03), which confirmed the growth pattern observed in **Figure 7+8**. In addition, a sub-analysis only including BMI between 1-6 years of age showed no interaction between age and intervention group (p=0.42).

We previously reported differences in asthma risk between the intervention groups [15], but excluding the children with an asthma diagnosis at age 5 years did not affect the supplementation effect on BMI (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 z-score BMI at age 6 years (0.10 vs -0.09, p=0.004) compared to the control group. Furthermore, they had a larger waist circumference (55.46 cm vs. 54.82 cm, p=0.04) and a trend towards a higher weight existed (21.78 kg vs. 21.40 kg, p=0.11), while there were no differences in height or head circumference (**Table 9**).

Figure 9 illustrates the mean z-score BMI distribution between the intervention groups at age 6 years. We found significantly more children from the n-3 LCPUFA supplementation group having a BMI in the highest quartile (29% vs. 21%, p=0.02) and significantly fewer in the lowest quartile (21% vs. 30%, p=0.02) compared to the control group, but 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 9**).

In Study II we found that the supplementation had a positive effect on intrauterine growth, we therefore tried to adjust our analyses for Marsal percentage at birth and this yielded no changes (data not shown).

We found no interaction between the intervention and, the rs1535 genotype and pre-intervention blood levels of EPA+DHA, respectively, on our anthropometric outcomes (data not shown).

Table 9

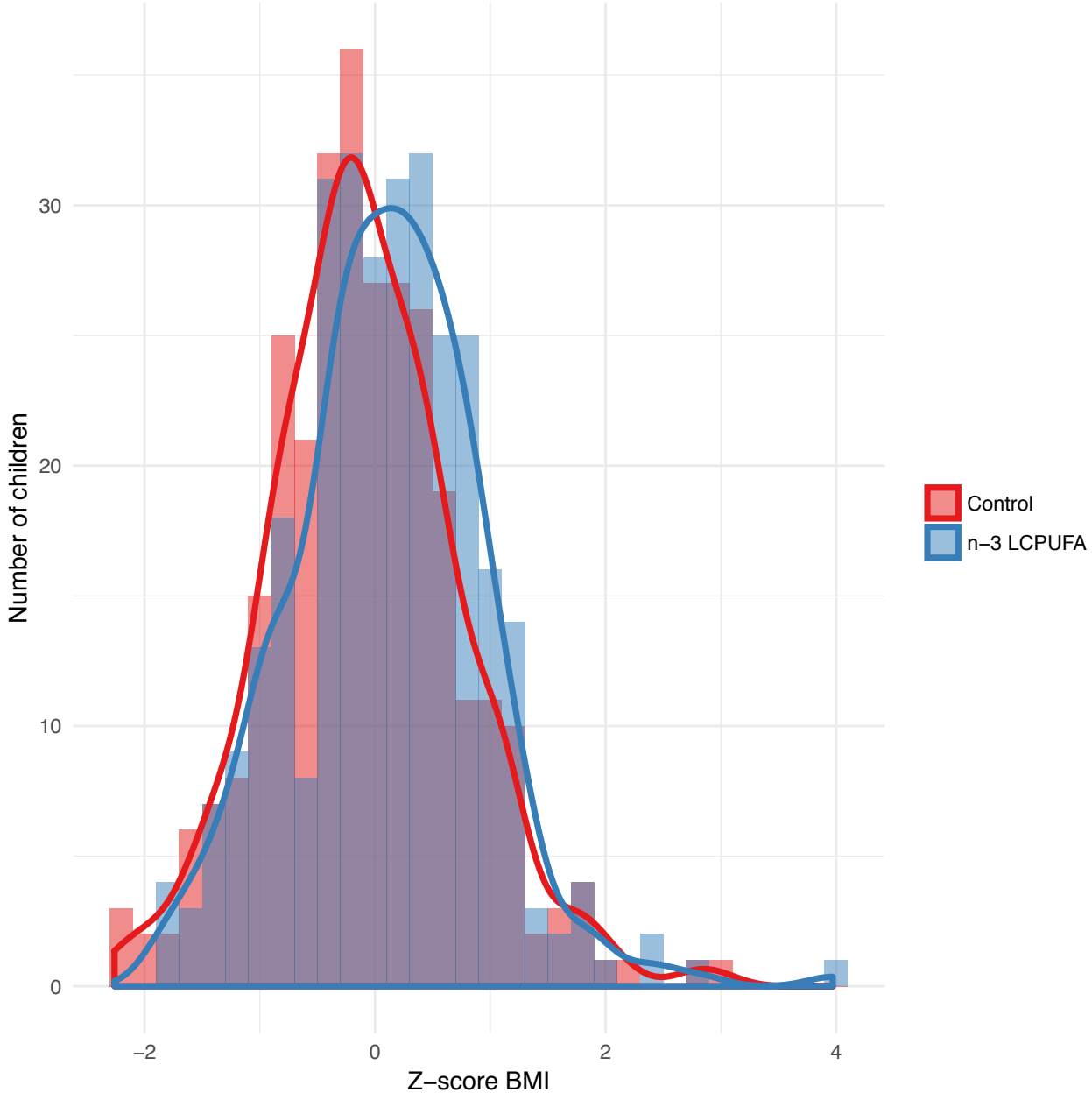
Effects of n-3 LCPUFA on the development anthropometric measurement at 6 years of age.

	N-3 LCPUFA N=304	**Control N=301**	**P-value**
Z-score BMI, Mean (SD)	0.10 (0.82)	-0.09 (0.83)	0.004
Waist, Mean (SD), cm	55.46 (3.77)	54.82 (3.70)	0.04
Weight, Mean (SD), Kg	21.78 (2.94)	21.40 (2.85)	0.11
Height, Mean (SD), cm	118.16 (4.61)	118.18 (5.14)	0.97
Head, Mean (SD), cm	52.11 (1.42)	52.14 (1.44)	0.83
ZBMI <10 / >90 perc. (%)	9 / 11	11 / 10	0.63
ZBMI <25 /> 75 perc. (%)	21 / 29	30 / 21	0.09
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

Figure 9

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.

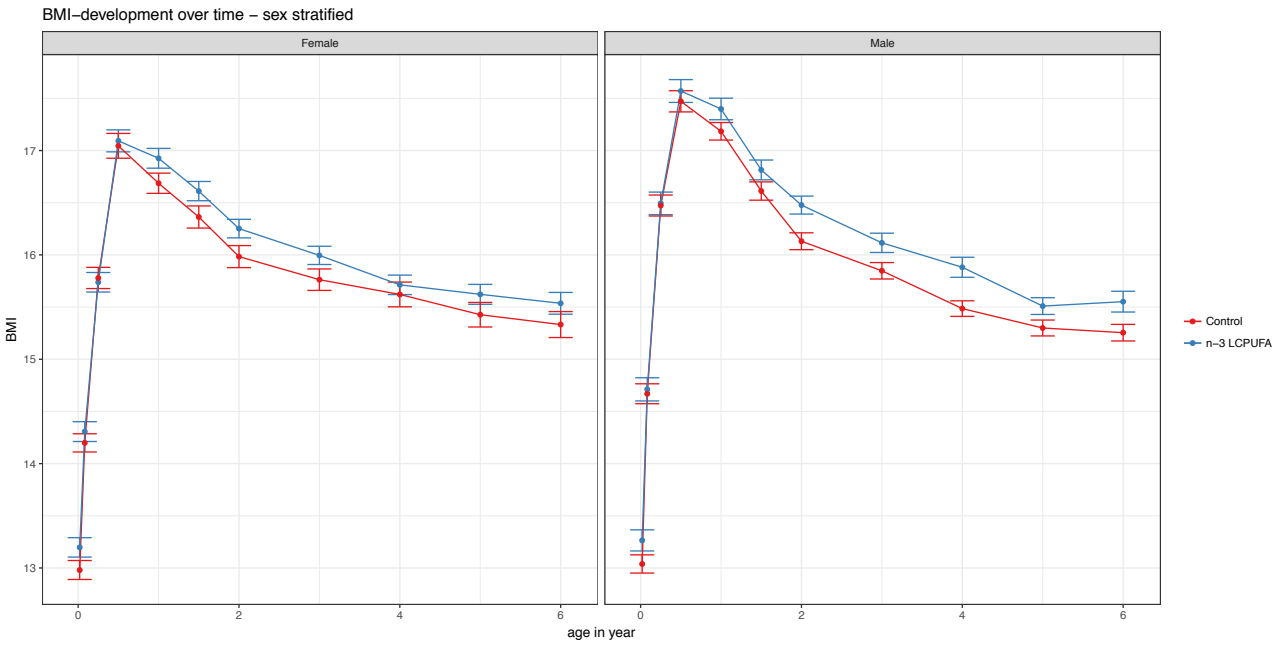


Sex specific growth

We found that the BMI difference between the two intervention groups were similar in boys and girls (**Figure 10**). Furthermore, the repeated measurement analyses demonstrated comparable estimates between the two sexes, however only statistically significant for the boys (girls: β-coefficient 0.11; SE [0.07]; p=0.11 and boys: β-coefficient 0.16; SE [0.07]; p=0.02), but with no significant interaction between sex and intervention group (p=0.79).

Figure 10

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.



Furthermore, stratified analyses on sex showed comparable effects at 6 years of age (**Table 10)**

Table 10

Effects of n-3 LCPUFA on the development anthropometric measurement 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)	0.10 (0.81)	-0.07 (0.90)	0.07	0.11 (0.83)	-0.09 (0.75)	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

n-3 LCPUFA supplementation and body composition

523 children completed DXA scanning at 6 years of age, The n-3 LCPUFA group had a higher total mass on the total body less head (TBLH); 19,36 kg vs. 18.97 kg (height adjusted mean difference of 384g (95 % CI [75 g; 694g]), p=0.03), almost identical of the difference we saw for bodyweight. Sub- analyses on tissue type revealed that the children in the n-3 LCPUFA supplementation group had a significantly higher lean soft tissue mass on the TBLH; height adjusted mean difference of 240 g (95 % CI [38g;422g]), p=0.01. They had a tendency to a higher fat mass on TBLH; height adjusted mean difference of 157 g (95 % CI [-73g; 386g]), p=0.18, but no differences in fat mass on the trunk or in the android region and no difference in total body fat percentage or lean mass percentage, nor when we sub-analysed the composition in sub regions (**Table 12**)

Children in the n-3 LCPUFA supplementation group also had a higher BMC: 11g (95% CI [3g; 18g]), p=0.007 and a trend towards a higher bone mineral density: 0.006 g/cm^{-2} (95 % CI [0.001; 0.012]), p=0.06 in TBLH.

At 3.5 years of age, there were no significant differences in body composition between the intervention groups, but all estimates for lean soft tissue mass, fat mass and BMC where increased in the n-3 LCPUFA supplementation group (**Table 11)**.

We found no interaction between the intervention and the rs1535 genotype and pre-intervention blood levels of EPA+DHA, respectively, on our DXA outcomes (data not shown).

Table 11

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	P-value
Fat (TBLH) Mean (SD); Estimate difference [SE]	4783.90 (1560.02)	4637.59 (1404.35)	156.61 g [116.88]	0.18*
Fat % (TBLH),Mean (SD), Estimate difference [95 CI-interval]	24.41 (5.02)	24.22 (4.86)	0.19 [-0.66;1.04]	0.66
Fat (Trunk) Mean (SD); Estimate difference [SE]	1800.97 (726.51)	1760.25 (679.66)	46.57g [57.33]	0.42*
Fat % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	18.13 (5.22)	18.03 (5.24)	0.10 [-0.80;1.00]	0.83
Fat (Android) Mean (SD); Estimate difference [SE]	209.60 (112.67)	204.40 (105.29)	6.08g [9.04]	0.50*
Fat % (Android), Mean (SD), Estimate difference [95 CI-interval]	15.05 (5.65)	14.92 (5.76)	0.13 [-0.85;1.11]	0.79
Lean soft tissue mass (TBLH) Mean (SD); Estimate difference [SE]	14030.87g (2024.63)	13794.40g (2078.60)	239.65g [97.76]	0.01*
Lean soft tissue mass % (TBLH), Mean (SD), Estimate difference [95 CI-interval]	72.76 (4.93)	72.95 (4.76)	-0.19 [-1.03;0.64]	0.65
Lean soft tissue mass (Trunk) Mean (SD); Estimate difference [SE]	7750.11g (1025.54)	7653.53g (1060.64)	99.97g [54.23]	0.07*
Lean soft tissue mass % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	79.56 (5.19)	79.67 (5.21)	-0.10 [-1.00;0.79]	0.82
Total BMC (TBLH) Mean (SD); Estimate difference [SE]	546.22g (91.25)	535.06g (94.89)	10.93g [4.04]	0.007 **
Total BMD (TBLH) Mean (SD); Estimate difference [SE]	0.56 (0.05)	0.56 (0.05)	0.006 g/cm^2 [0.003]	0.07 **

* Adjusted for height and height^2, ** Adjusted for height

BMC: Bone Mass Content, BMD: Bone Mass Density, CI: Confidence Interval, SE: Standard Error, TBLH: Total Body Less Head

Table 12

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 (SE)	P-value
Fat (TBLH) Mean (SD); Estimate difference [SE]	3767.11 (844.48)	3694.06 (809.35)	53.31 [71.16]	0.45*
Fat % (TBLH),Mean (SD), Estimate difference [95 CI-interval]	28.66 (4.55)	28.60 (4.34)	0.07 [-0.73;0.86]	0.87
Fat (Trunk) Mean (SD); Estimate difference [SE]	1403.95 (412.42)	1386.73 (419.02)	9.73 [36.26]	0.79*
Fat % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	20.34 (4.66)	20.40 (4.63)	0.06 [-0.88;0.76]	0.89
Fat (Android) Mean (SD); Estimate difference [SE]	171.14 (59.67)	170.90 (61.58)	-0.60 [5.36]	0.11*
Fat % (Android), Mean (SD), Estimate difference [95 CI-interval]	17.04 (4.77)	17.11 (4.82)	-0.07 [-0.01;0.01]	0.87
Lean soft tissue mass (TBLH) Mean (SD); Estimate difference [SE]	9043.70g (1271.42)	8888.85g (1284.23)	81.02 [73.11]	0.27*
Lean soft tissue mass % (TBLH), Mean (SD), Estimate difference [95 CI-interval]	69.00 (4.50)	69.07 (4.26)	-0.06 [-0.85;0.72]	0.86
Lean soft tissue mass (Trunk) Mean (SD); Estimate difference [SE]	5329.10g (704.86)	5240.08g (863.98)	49.94 [53.40]	0.35*
Lean soft tissue mass % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	77.60 (4.68)	77.55 (4.62)	0.05 [-0.77;0.87]	0.90
Total BMC (TBLH) Mean (SD); Estimate difference [SE]	309.18g (49.12)	304.28g (49.80)	1.30 [3.20]	0.69**
Total BMD (TBLH) Mean (SD); Estimate difference [SE]	0.45 (0.03)	0.45 (0.03)	0.003 [0.003]	0.36**

* Adjusted for height and height^2 ** Adjusted for height. BMC: Bone Mass Content, BMD: Bone Mass Density, CI: Confidence Interval, SE: Standard Error, TBLH: Total Body Less Head

Maternal FADS genotype and BMI development during childhood

In a sub-analysis, we investigated if the maternal FADS genotype was associated with offspring BMI development and body composition. We stratified the data by supplementation groups and investigated the difference between children born by mothers with the FADS genotypes associated with higher levels of EPA and DHA (AA/AG) to the children born by mothers with the genotype associated with low levels of EPA+DHA (GG). In the control group, we found that the children born to mothers with the AA/AG-genotype tended to have higher BMI values from 1 to 6 years of age compared to children born to mothers with the GG-genotype (β-coefficient 0.21 SE [0.13]; p=0.06) (Figure 11). Similarly, at 6 years of age in the control group, the children born to mothers with the AA/AG-genotype had a significantly higher z-score BMI (mean difference: 0.31; 95 % CI [0.07; 0.54], p=0.01), a higher weight (mean difference: 0.88 kg ; 95 % CI [0.06; 1.70], p=0.04) and trend towards a larger waist circumference (mean difference: 0.9cm 0.; 95 % CI [-0.18; 2.01], p=0.10 compared to children born to mothers with the GG-genotype (Table 13).

There was no difference in BMI development over time or the anthropometrics outcomes at 6 years between the maternal FADS genotypes in the n-3 LCPUFA supplemented group.

Figure 11

Legend: 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.

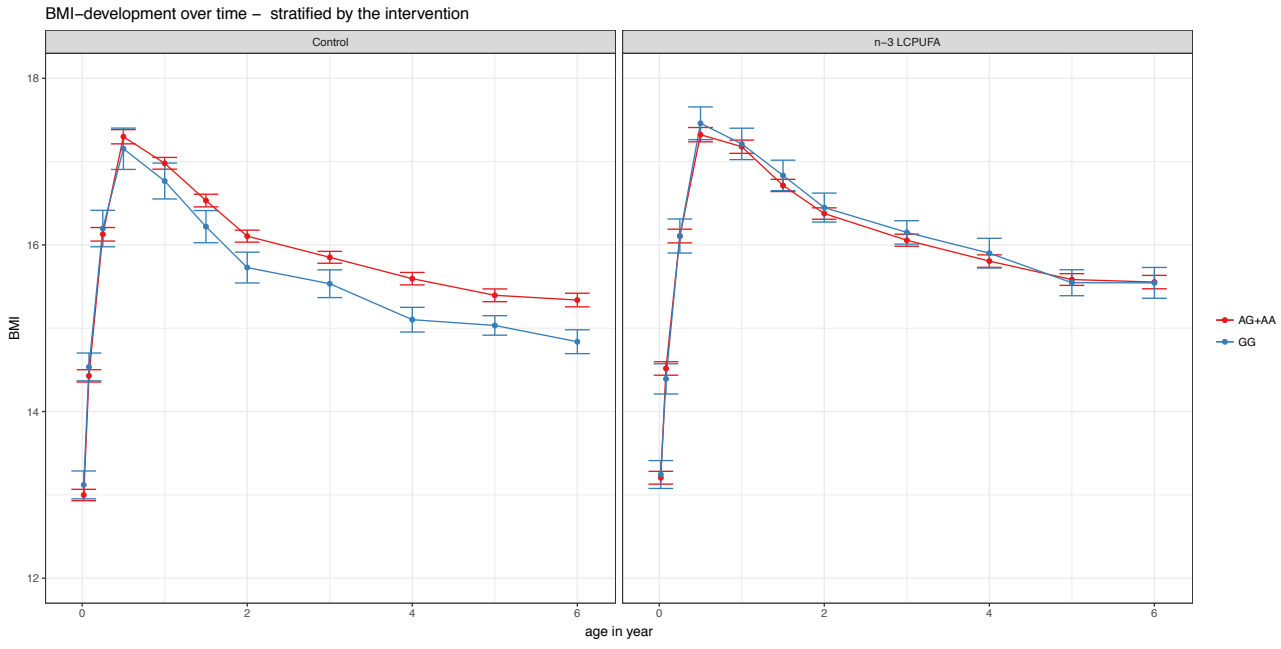


Table 13 Effects of maternal FADS genotype, GG risk allele and non-risk alleles (AA+AG), stratified by the intervention, on the anthropometric measurements at 6 years of age.

	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.

5. Discussion

5.1. Primary findings

We found that children born by CS had an increased BMI peak at 6 months of age compared with children born vaginally. This increased BMI in early childhood did not track into later childhood and adolescence. Furthermore, we found no association between delivery mode and body fat percentage in children at age 3.5 years or 7 years.

We found that daily supplementation with fish oil compared to control from pregnancy week 24 to one week after birth resulted in a two days prolongation of pregnancy and a 97 g higher birth weight in our cohort. The increase in birth weight was not only explained by a longer duration of pregnancy, but also by an increased intrauterine growth. The prolonged pregnancy did not reduce the number of children born preterm or induced any other differences in pregnancy or delivery complications.

Postnatally the supplementation resulted in a higher offspring BMI from age 1 to 6 years and a higher waist circumference at age 6 years compared to children in the control group. Results from DXA scans demonstrated that the increased BMI was not the result of a higher fat percentage but instead reflected a parallel increase in lean soft tissue mass, fat mass and bone mineral content.

5.2. Mode of delivery and the influence on childhood growth in the COPSAC cohorts

We found that children born by CS have higher mean BMI at 6 months of age compared with children born vaginally, leading to a 0.37 kg/m^2 difference in mean BMI. We saw no significant association between mode of delivery and BMI or body composition in later childhood or puberty. These findings suggest that infants delivered by CS have a divergent growth pattern during early infancy, but shows no risk for overweight in later childhood.

Two recent meta-analyses on this subject have results and conclusions that differ from ours. One study found an increased risk of overweight and obesity in offspring delivered by CS in childhood, youth and adulthood (5,6). The other study found that delivery by CS was associated with an increased risk of overweight and obesity and a higher mean BMI in adulthood in unadjusted analyses. However, most of the included studies did not provide information about relevant covariates, such as breastfeeding patterns and mother's BMI, and they used divergent definitions of growth outcomes and age of measurement. In the meta-analyses the main association was found

between CS and obesity, by using either BMI >95th percentile for age and sex or the International Obesity Task Force criteria (34,82–84). In our birth cohorts the variation in BMI was narrow (Table 2) with only 6 of the children having IOTF >1 at 5 years in each cohort and 7 children at 13 years. Our sub-analysis of children with a BMI-value above the 85 percentile and 90 percentile at five years and thirteen years of age showed that the distribution according to mode of delivery was the same as in the rest of the children. The prevalence of childhood obesity was approximately 8.5% in USA (18) and 3.5% in Denmark (19) in 2011. This difference in obesity prevalence could partly account for the ambiguous results.

Other studies have demonstrated findings in line with ours with increased growth in infancy for children born by CS followed by no long-term effects. One study (85) found a greater likelihood of obesity at age 2 years in children delivered by CS, but not later. Another study found an increased risk of obesity for 4 year old boys, but no risk for girls and no increased risk at later ages (86).

Two previous Danish studies show conflicting results. A register study (87) found that 7 years old children delivered by CS had an 15% increased risk of being overweight in unadjusted analyses, but no difference after adjustment. Another study found that men age 18 years delivered by CS had a higher adjusted mean BMI and an increased risk of obesity (88). However, the latter did not adjust for mother's BMI or breastfeeding pattern.

Both short duration of breastfeeding (32) and high maternal pre-pregnancy BMI (33) increases the risk of obesity; we found both of these factors associated with CS. Most children in our cohort were breastfed and the variation of socio-economic circumstances was narrow (Table 1) compared with other countries (83,84,86). We speculate that some of the earlier studies have had a more diverse population and that the lack of sufficient confounder adjustment could partly explain the different associations found between countries (85,87,88).

As mentioned in the introduction it has been hypothesized that differences in gut microbiota caused by CS (38) could be an explanation for the differences in BMI. Our group has shown that there was a difference in the composition of the gut microbiota in regards to delivery way for the children in $COPSAC_{2010}$ (39). Likewise, we only found a diverging BMI in early infancy according to delivery mode, which could be suggested to be explained by the differences in the gut microbiota. In this study, we do not have the microbiome data to answer this question. However, it has been speculated that an explanation to the difference in microbiota could be due to intrapartum antibiotics.

All children in our study, who were delivered by CS, had been exposed to intrapartum antibiotics. Therefore, as an attempt to uncover this we investigated our growth outcomes in relation to intrapartum antibiotics in the children delivered vaginally. Our analysis on BMI in childhood

according to antibiotic administration during vaginal delivery showed no relationship between intrapartum antibiotics and BMI in infancy or later in life (Table 6).
So, our data do not support the hypothesis that CS is merely a proxy for intrapartum antibiotics (82).
Our results may be limited in power when assessing each cohort separately, which might lead to the lack of difference in BMI at 5 and 13 years. However, our meta-analysis clearly shows no difference at age 5 years, and the non-significant higher BMI observed at age 13 years in COPSAC2000, disappears after covariate adjustment, showing only an association between delivery by CS and changes in infant BMI. As mentioned in the introduction infant BMI have been associated to risk of obesity later in life. We therefor hypothesized that CS could be a risk factor if the child grows up in an environment with other risk factors for obesity. Even though we do not see an association between delivery by CS and increased BMI later in our Danish population, could this change in infant growth represent a modifiable link in the prevention of obesity in selected children and possibly also in populations with a higher prevalence of CS.
Our study is observational and we do not have data on microbiome or early life metabolism, so unfortunately we are not able to say anything about the possible mechanism behind the changed infant growth pattern. Our finding suggests that a mechanism should be sought in early infancy.

5.3. Effects of n-3 LCPUFA supplementation in pregnancy on birth outcomes

In our RCT, we confirm earlier findings showing that fish oil supplementation during pregnancy leads to a prolongation of pregnancy and an increase in birth weight (9). Furthermore, we demonstrate a significantly higher birth weight for gestational age in the n-3 LCPUFA supplemented group. This suggests that the increase in birth weight is not solely explained by the prolonged duration of pregnancy, but is also a consequence of increased intrauterine growth. To our knowledge, this has not been shown before.
Our findings of an increased gestational age and birth weight are in line with the results from the majority of other studies demonstrating a prolongation of pregnancy between two and four days and an increase in birth weight between 70 and 170 g from fish oil supplementation (52,53). An explanation for the slightly different effects observed in the previous studies could be differences in time of initiating the supplementation during pregnancy and the amount of fish oil supplied. A recent study with 500 participants, which did not observe similar effects, supplemented from week 18 of pregnancy with a less than one fifth of the fish oil dose used in the present study (89). Another study with 1200 participant using a third of our dose from week 21 of pregnancy found that the

pregnancy was only prolonged by one day and the birth weight only increased with 70 g (12). This could imply that there is a dose-response relationship between the amount of n-3 LCPUFA supplement and the effect on pregnancy length and fetal growth and that a minimum threshold might exist (90).

It has previously been suggested that the fish oil induced increase in birth weight is only caused by the prolongation of pregnancy (9). However, our data shows that n-3 LCPUFA supplementation also has an impact on the intrauterine growth, leading to an increased birth weight for gestational age. A possible mechanism for the increased fetal growth could be that n-3 LCPUFAs increase the ratio of prostacyclins to thromboxanes, thereby reducing blood viscosity and facilitating increased placental blood flow, which benefits fetal growth (91).

A relevant concern of prolongation of pregnancy and higher birth weight is that it could course unwanted complications during pregnancy and birth. However, we found no association between n-3 LCPUFA supplementation and adverse pregnancy outcomes such as induced labour, acute CS, preeclampsia, gestational diabetes or hospitalization after birth, and there was no difference in APGAR score at 5 minutes between the children in the n-3 LCPUFA and control group. A more broad evaluation of the safety profile has been published elsewhere (49), showing no differences between the groups.

Our study was not powered to demonstrate a reduction in preterm delivery or children born small for gestational age after n-3 LCPUFA supplementation in pregnancy, which has been suggested in epidemiological observational studies (90). Future trials should explore whether n-3 LCPUFA supplementation can reduce the amount of preterm deliveries and children born small for gestational age (92).

Our findings that fish oil supplementation leads to a prolonged pregnancy and increased intrauterine growth could have large relevance in countries with low income where an improvement in overall fetal growth and birth weight could have a large impact on children's future health (93). Supplementation with n-3 LCPUFA is inexpensive and safe and could be particularly beneficial in these countries since previous studies have reported an association between low income and low intake of n-3 LCPUFAs(94). The potential benefits from fish oil supplementation for improving fetal growth rate and increasing birth weight in developing countries should be further investigated.

5.4. Effects of n-3 LCPUFA supplementation in pregnancy on postnatal growth

We found that n-3 LCPUFA supplementation in third trimester of pregnancy led to higher offspring BMI and increased waist circumference at 6 years of age. Data from DXA scans revealed that the

children in the n-3 LCPUFA supplementation group had a higher soft tissue mass. Lean and fat compartments seemed equally affected, resulting in no increase in fat or lean mass percentage, and we observed no increase in number of obese children. Furthermore, we observed a positive effect from supplementation on bone mineral content.

This study is the first to show that n-3 LCPUFA supplementation in third trimester pregnancy leads to a higher BMI throughout childhood.

Most randomized trials with n-3 LCPUFA intervention either to the mother during pregnancy or lactation or to the child in infancy have not shown any persistent effects on BMI (95). A review from 2014 and a Cochrane review from 2015 concluded that there is no evidence that n-3 LCPUFA supplementation during pregnancy and/or lactation affects BMI or growth development in childhood (96,97). Only one previous study have reported effects in early infancy in line with ours. Another Danish study supplementing mothers with 1.5 g n-3 LCPUFA (40 % EPA) during the first four months of lactation showed that the children in the n-3 LCPUFA supplementation group had a significantly higher BMI and increased waist circumference at 2.5 years (98), but there were no differences at 7 and 13 years of age (16). There are several potential explanations for the discrepancy between this and previous studies, including differences in timing of the supplementation during pregnancy/lactation/infancy, the dose and type of n-3 LCPUFA supplied, the age at follow-up, and the study size. The dose of n-3 LCPUFA supplied in the current study (2.4 g/d) is higher than in many previous studies (17,54,99,100). Our study is the first large trial with high dose supplement and thereby had a higher statistical power to detect an effect on growth and body composition (95). One other study supplemented from week 30 of pregnancy with the same dose as we did, they completed a follow-up on 243 participant at age 19 years. They found no effect on waist circumference or z-score BMI (101).

Our primary outcome for the randomized trial was asthma in childhood. We found that asthma in the first 5 years of life was reduced with approximately one third in the n-3 LCPUFA supplementation group (49). Children without asthma have a lower burden of infection and are less impacted by infection in childhood (102,103) and it could therefore be speculated that the higher BMI throughout childhood in this same group could be mediated by an effect on asthma; however, we did not find any changes in the effect, when we excluded children with asthma.

The effect we found on intrauterine growth in **study II** could be speculated to be the explanation to this difference in childhood BMI. However, adjusting the main analysis for Marsal percentage did not change the results, and therefore the increased BMI through childhood does not appear to be driven by the increased intrauterine growth.

We did not find any differences on the effect between boys and girls, which is in line with most other studies (96).

We can replicate our finding of a higher BMI in children born to mothers with the non-risk FADS genotypes, which leads to higher n-3 LCPUFA levels. This can be thought of as a proxy for the n-3 LCPUFA supplementation in the control group, which provides a proof of concept for our intervention effects. The reason that this genotype has not previously been found in larger BMI/obesity GWAS studies (104) may be due to the indirect link between the maternal genotype and the offspring BMI, which has not been analysed.

Our BMI curves illustrated a difference in BMI for the two groups at birth and one week followed by no significant differences in BMI till age 6 months, where after the n-3 LCPUFA supplementation group had a sustained higher BMI. This difference in infant growth patterns in the two intervention groups could lead to speculations regarding the long term consequences. As mentioned in the introduction has the timing of the infant BMI peak been associated to later obesity outcomes, where earlier peak is associated to later obesity (3). The lack of BMI difference in the first year of life could reflect that n-3 LCPUFA supplementation delays the infant BMI peak, so despite the higher BMI during childhood this infant growth pattern may associated with a later adiposity rebound and ultimately a lower BMI later in life (38). This is however purely speculative since we did not include enough measurements to establish the infancy peak or rebound.

Our results could raise the concern that n-3 LCPUFA supplementation in pregnancy could lead to obesity in childhood. However, our DXA results do not support this, since we saw no difference in fat or lean soft tissue mass percentages, but rather a larger amount of both lean soft tissue mass and bone mass 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 being in the highest or lowest 10 percentile BMI at 6 years. It appears that it is mainly the children with BMI in the normal range that are affected by the n-3 LCPUFA intervention. These findings suggest that n-3 LCPUFA supplementation is not associated with an increased risk of developing obesity later in life, which is also supported by previous studies suggesting an effect on growth in infancy (98,99) but not later in life (16) The effect of supplementation on bone mineral content and bone mineral density might imply a positive health benefit in terms of decreased risk of later osteoporosis (105), studies have implied that intake of total PUFAs and fish is associated with higher BMD (106). However, the clinical implications of our findings can only be speculative at the current stage. It is important to follow these children through puberty and into adulthood to evaluate the potential long term health effects related to changes in growth and body composition induced by n-3 LCPUFA.

As mentioned was our primary outcome for the RCT asthma where we demonstrated a reduction in childhood asthma risk by one third among children born to mothers, in the fish oil group. This risk was further reduced by more than 50% in children, whose mothers had the lowest blood levels of LCPUFA prior to supplementation (49). Furthermore, the fish oil supplementation also led to affected neurological outcomes with earlier achievement of motor milestones and higher cognitive scores at 2.5 years among boys (Bjarnadóttir et al.; submitted). These findings that fish oil impacts different clinical outcomes such as Asthma, neurological development, and growth, makes future recommendations for fish oil supplementation difficult. Pregnant women in Denmark are recommended to eat fish 1-2 times per week, with the exception of predatory fish and raw fish (107).

Since we saw the highest reduction in asthma for the children of the mothers with low fish intake during pregnancy, could a defensive approach be recommendation of a higher fish intake during pregnancy than the one we have today.

5.5. Strengths and limitations

The main strength of our data and studies relates in a high degree to two facts; the close longitudinal follow up in the two COPSAC cohorts preformed in our clinical research unit and the large RCT on n-3 LCPUFA supplementation in pregnancy preformed in $COPSAC_{2010}$.

This is supported by a very high adherence in our cohort with respectively 88%, whom had anthropometrics measured at age 5 and 6 years and 82 % at 13 years of age. Likewise, adherence to the supplementations in the randomized trial was high among the pregnant women.

All of our growth measurement was performed using the same equipment by trained COPSAC assistants based on standardized procedures and the observed growth curves were similar to previous reports (108). The longitudinal follow up allowed for repeated measurement statistics and in **study I** this advantage gave us the opportunity to compare the longitudinal follow up on growth data in the two cohorts and subsequently recap the results in a meta-analysis.

In addition, we had the opportunity to expand our findings by including DXA scans as an objective measure of body composition. This meticulous approach strengthens especially the findings in **study III**, where the objective measure of fat, lean soft tissue mass and bone mass enable us to disentangle which tissues were affected by the intervention and even though the results from the DXA scans were insignificant at 3.5 years, the estimates showed the same tendency as the findings from the 6 years DXA scans.

Furthermore, we had a broad range of exposures, which were validated by register data and personal interviews with the families.

This availability of covariates allowed for adjustments in **study I** in regards to important potential confounders such as sex, parity, birth weight for gestational age, maternal age, pre-pregnancy BMI and breastfeeding duration (32,33). After covariate adjustment an observed non-significant higher BMI at age 13 years disappears completely, which could otherwise have suggested a possible difference not found due to low power at this time point.

Our large amount of validated data secured that we in **study II** had data on 99.8% of the due dates determined by early ultrasound scans and that the birth weight data we captured by parental interviews in the clinic subsequently were validated against register. The same was the case for the broad range of data on adverse pregnancy and delivery end-points used as secondary outcomes, these were similarly obtained by interviews with the families and validated against register data.

In our randomization studies it gave us the opportunity to assure a successfully randomization including an equal distribution of PUFA lipid levels and diet PUFA intake before randomization between the two groups.

It is a significant strength in **study II** that we find similar results across different measurements for fetal growth, including Marsal percentage, Skjaerven percentage, and estimated growth rate from week 20 of pregnancy based on ultrasound scans. Since a slight weight difference matters much more if the expected birth weight for a preterm infant is very low compared with a normal-weight infant born at term using standardized growth curves increases the confidence in our findings.

A strength in **study III** is the internally validation of the findings done on the intervention that we did with our observational findings of higher BMI values in children born to mothers with the FADS non-risk genotypes that associates with high n-3 LCPUFA levels. This was only observed in the control group and could be seen as an "unconfounded" validation of our results.

Our studies have some limitations.

In **study I** the fact that we compare and subsequently combine our two cohorts in a meta-analysis leads to some potential problems. Firstly, the decade between the enrollments of two cohorts, leading to different time-related environmental impacts. We saw difference in duration of breastfeeding and smoking habits (Table **1**) between the two cohorts, however, the results appear similar, and we believe that we have accounted for the majority of differences by our comprehensive confounder adjustment and by meta-analyses of the individual cohort results.

Secondly, it may be a limitation that the $COPSAC_{2000}$ is an asthma high risk cohort, but in the sub-

analyses of children born by asthmatic mothers we still observed a difference in BMI at 6 months between the two delivery groups with an estimate comparable to the one we found in $COPSAC_{2000}$. Even though our RCT is among the largest on n-3 LCPUFA supplementation in pregnancy, the number of participating mothers (N=699) limited the opportunities to investigate if the increased intrauterine growth lead to a reduction in children born small for gestational age, since this is a rare outcome with only 13 children according to Marsals definition (36). Likewise if the supplementation has the potential to reduce children born premature. The study design precluded investigation of the effect of n-3 LCPUFA supplementation on very preterm delivery (GA <24) since the supplementation was initiated after this point in pregnancy.

Furthermore, is it a limitation that our outcomes were secondary outcomes in our RCT. Our post hoc power calculation indicated that the prolongation of pregnancy and the mean increase in z-score BMI over time that we find for the fish oil group are trustworthy with >80% power, but we do not have the same power to assure that the difference we find for birth weight and size for gestational age are true.

Overall, we have relatively few children in risk of overweight and obesity with only 30 (5%) of 605 children in the IOTF category for risk of overweight at 6 years of age. This in combination with our limited number becomes a limitation in **study III** since we lack the power to assure that the n-3 LCPUFA supplementation did not lead to a higher number of obese children. One could argue that it also is a limitation in **study I,** but since we in that study did not observed a difference in BMI later in childhood it is not essential in the same degree.

6. Conclusions and Perspectives

6.1. Conclusions

Children delivered by CS had an increased mean BMI at 6 months of age, but this did not track into later childhood. At 13 years of age, children born by CS had a non-significant higher BMI but after adjustment this difference disappeared. CS did not associate with childhood fat percentage measured by DXA scans.

Fish oil supplementation during third trimester of pregnancy lead to a prolongation of pregnancy gestation and a higher birth weight. The increase in birth weight was due to an increase in pregnancy duration as well as an increased intrauterine growth.

Supplementation with fish oil in pregnancy had also post-natal consequences and 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 during childhood.

6.2. Perspectives

This thesis adds knowledge to the field regarding early life exposures and metabolic programming and the findings have many perspectives. We find associations between two exposures, CS and n-3 LCPUFA, which have been changing during the last decades and childhood growth.

In regards to CS as exposure we found a divergent growth pattern early in life with no effect in later childhood. We know that the mechanism of getting obese is multifactorial and as mentioned in the introduction; increased growth in infancy and especially a high infant BMI peak have been associated with later risk of overweight and obesity in child and adulthood, potentially through metabolic programming. We hypothesized that CS could be a risk factor if the child grows up in an environment with other risk factors for obesity. This window of growth should therefore be a focus of interest in future studies, since it could represent a modifiable link in the prevention of obesity in selected children. In a worldwide perspective this could have large impact since the prevalence is still rising in many countries and for some countries the majority of children are delivered by CS (109).

The findings regarding the effects of n-3 LCPUFA supplementation have several implications.

In countries with low income, an improvement in overall fetal growth and birth weight could have a large impact on children's future health. Supplementation with n-3 LCPUFA is inexpensive and safe and could be particularly beneficial in these countries since previous studies have reported an association between low income and low intake of n-3 LCPUFAs. The potential benefits from fish oil supplementation for improving fetal growth and increasing birth weight in developing countries should be further investigated.

Our findings regarding n-3 LCPUFA supplementation and increased childhood BMI confirm earlier speculations that fish oil affects metabolic programming leading to changed growth. However, the mechanism seems to be slightly different from expected.

Further studies are needed to both uncover the mechanism behind the effect and establish the long-term implications in regards to growth and metabolism. It is important to do follow-up on the children in $COPSAC_{2010}$ on growth and metabolic measures through adolescence to explore the long term consequences. This knowledge is important in developing new national recommendation regarding fish oil supplementation during pregnancy. A defensive suggestion until further knowledge is established could be a recommendation of a higher fish intake during pregnancy than the one we have today.

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Appendix – Paper I, II & III

Paper I

Cesarean Section and Body Mass Index at 6 months and into Childhood

Cesarean Delivery and Body Mass Index at 6 Months and Into Childhood

Rebecca Kofod Vinding, MD,[a,b] Tobias Steen Sejersen, MD,[a,b] Bo L. Chawes, MD, DMSc,[a] Klaus Bønnelykke, MD, PhD,[a] Thora Buhl, MD, PhD,[c] Hans Bisgaard, MD, DMSc,[a] Jakob Stokholm, MD, PhD[a,b]

abstract

BACKGROUND AND OBJECTIVES: The prevalence of cesarean delivery (CD) is rising worldwide, and so is childhood obesity. Studies have shown associations between these factors. We examined the development of BMI from birth through childhood to determine whether CDs were associated with differences in growth and obesity.

METHODS: Term children from the birth cohorts Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) and COPSAC$_{2010}$ were included. Height, length, and weight measurements were collected prospectively until 5 years in COPSAC$_{2010}$ and until 13 years in COPSAC$_{2000}$. Dual-energy x-ray absorptiometry (DXA) scans were performed at 3.5 and 7 years. Information on relevant covariates were verified during clinical visits. Analyses were adjusted for covariates associating with CD.

RESULTS: In COPSAC$_{2010}$, 20% (N = 138/673) of the children were delivered by CD; 49% were girls. In COPSAC$_{2000}$, 19% (N = 76/393) were delivered by CD; 51% were girls. Children delivered by CD had a higher mean BMI at 6 months compared with those delivered vaginally: COPSAC$_{2010}$ β-coefficient, .41 (95% confidence interval [CI], .12 to .69), P = .01; COPSAC$_{2000}$ β-coefficient, .16 (95% CI, −.11 to .68), P = .16; and meta-analysis β-coefficient, .37 (95% CI, .14 to .60), P = .002. There were no differences in BMI trajectory between the 2 groups by 5 and 13 years, nor cross-sectional BMI at 5 and 13 years, nor in fat percentages from DXA scans.

CONCLUSIONS: Children delivered by CD had a higher BMI at 6 months of age, but this difference did not track into later childhood. Our study does not support the hypothesis that CD leads to later overweight.

[a]*Copenhagen Prospective Studies on Asthma in Childhood, Faculty of Health and Medical Sciences, University of Copenhagen and Danish Pediatric Asthma Center, Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;* [b]*Department of Pediatrics, Naestved Hospital, Naestved, Denmark; and* [c]*Department of Clinical Physiology and Nuclear Medicine, Herlev-Gentofte, Copenhagen University Hospital, Copenhagen, Denmark*

We are aware of and comply with recognized codes of good research practice, including the Medical Research Council's Good Research Practice and the Guidelines for Good Scientific Practice by the Danish Committees on Scientific Dishonesty. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's 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.

Dr Vinding carried out the initial analyses, wrote the first draft of the manuscript, and was responsible for data acquisition, analysis, and interpretation; Drs Sejersen, Chawes, and Bønnelykke contributed substantially to the analysis and interpretation of the data and provided important intellectual input; Dr Buhl examined and validated all data from the DXA scans and critically reviewed the manuscript for important intellectual content; Dr Bisgaard, guarantor of the study from conception and design to conduct of the study, had full access to the data and

WHAT'S KNOWN ON THIS SUBJECT: The rate of cesarean delivery is rising worldwide, and so is obesity. Meta-analyses have found an association between these 2 factors, but the findings are ambiguous, and it is unknown how mode of delivery affects growth throughout childhood.

WHAT THIS STUDY ADDS: Children born by cesarean delivery had a higher mean BMI at 6 months of age, but this difference did not track into later childhood. This window of higher BMI in infancy should be explored.

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The prevalence of overweight and obesity among children has been increasing worldwide for the last 3 decades.[1,2] However, it seems that this increase has reached a plateau in Western countries in recent years.[3] It is known that obesity and extensive weight gain in the first years of life are major risk factors for obesity, type 2 diabetes, and cardiovascular disease in adulthood.[4] Furthermore, the timing and velocity of infancy BMI peak, which is reached at around age 6 to 7 months, have been associated with higher BMI later in childhood and cardiovascular disease and type 2 diabetes in early adulthood.[5,6] The increased prevalence of overweight children cannot be explained by changes in genetic factors, because the great increase has occurred over a short period. The causes must therefore be sought in environmental exposures.[7,8]

Over the same period, an increase in the prevalence of cesarean delivery (CD) has been observed, and as observed for obesity this prevalence has also reached a plateau in Western countries in the last decade.[9] Various aberrations have been linked to CD: short-term effects such as hypoglycemia, breastfeeding problems,[10] altered immune responses,[11] and long-term effects on immune-related conditions such as asthma.[9] Two recent meta-analyses have shown associations between CD and obesity in offspring in both childhood and adulthood.[12,13] However, the included studies were heterogeneous in design, typically including only cross-sectional data measurements.

The study aim was to examine the association between CD and BMI patterns among children and adolescents. We analyzed longitudinal BMI data in combination with data on body composition from dual-energy x-ray absorptiometry (DXA) scans from 2 Danish birth cohorts: the Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) and COPSAC$_{2010}$.

METHODS

Study Population

COPSAC$_{2000}$ is a prospective clinical birth cohort study of 411 children born to asthmatic mothers.[14] The children have been followed prospectively until age 13 years.[15,16]

COPSAC$_{2010}$ was designed from the COPSAC$_{2000}$ cohort and is a study of 738 unselected pregnant women and their 700 children, followed prospectively until age 5 years.[17] The mothers participated in a randomized controlled trial of fish oil supplementation and high-dose vitamin D in the third trimester of pregnancy.[18,19]

Exclusion criteria in both cohorts were maternal chronic cardiac, endocrinologic, nephrologic, or pulmonary disease other than asthma, and for the current study we excluded twins and children with a gestational age <36 weeks. Data validation and quality control followed the guidelines for good clinical practice.

Ethics

The studies were conducted in accordance with the guiding principles of the Declaration of Helsinki and were approved by the Local Ethics Committee (COPSAC$_{2000}$: KF 01-289/96, COPSAC$_{2010}$: H-B-2008-093) and the Danish Data Protection Agency (COPSAC$_{2000}$ and COPSAC$_{2010}$: 2015-41-3696). Both parents gave written informed consent before enrollment.

Primary Outcomes

Anthropometrics were assessed at the COPSAC research facility at age 1 month, 6 months, and every 6 months until age 7 years, and then again at 13 years of age for COPSAC$_{2000}$. For COPSAC$_{2010}$ at age 1 week, 1 month, 3 months, 6 months, and every 6 months until age 2 years, and thereafter every 12 months until age 5 years.

Weight was measured without clothes on calibrated digital scales. Length was measured until 2 years with an infantometer (Kiddimeter; Raven Equipment Ltd, Dunmow, Essex, England). Height at later ages and in parents was measured with a stadiometer (Harpenden; Holtain Ltd, Crymych, Dyfed, Wales), which was calibrated yearly.

We analyzed BMIs at 6 months and 1, 5, and 13 years as outcomes. For each child these BMI values were defined as the BMI measurement closest to 6 months or 1 year ± 3 months, 5 years ± 6 months, and 13 years ± 12 months.

DXA Scans

Whole body scans were performed with a Lunar iDXA densitometer (GE Healthcare, Fairfield, CT) and were used to determine both the total body fat percentage (calculated as total fat mass divided by body weight on the day of scan, except for the head, because many patients moved their heads during the scan), and body compartment–specific fat percentage, based on the compartments predefined by the software.[20–22]

The children were DXA scanned at 3.5 years in COPSAC$_{2010}$ and at 7 years in COPSAC$_{2000}$.[23]

All DXA scan data were scrutinized by an experienced specialist and analyzed with enCore software (GE-Healthcare).

Mode of Delivery and Intrapartum Antibiotics

Information on delivery mode was obtained by personal interview at the child's first visit after birth; furthermore, we asked whether the birth was induced. The information was validated against the Danish Medical Birth Registry for all of the children. CD was subcategorized as emergency or elective CD, and

vaginal delivery was categorized as induced or noninduced.

Information on intrapartum antibiotics was available only in $COPSAC_{2010}$ and was obtained by interview 1 week postpartum and birth journal inspection. All women giving birth by CD were treated with prophylactic intrapartum antibiotics.

Covariates

Information on race, gender, gestational age, maternal age at birth, parity, household income, parents' educational level, older siblings, smoking during pregnancy, preeclampsia, diabetes in pregnancy, passive smoking, and days of hospitalization after birth were obtained by personal interviews and if possible validated with register data.

Birth length and weight were obtained at the first clinical visit after birth by personal interview, and thereafter all data were validated against the Danish Medical Birth Registry. Furthermore, if there was a difference >10 g, data were validated against the length and weight measures at 1 week from the research clinic. Birth weight for gestational age *z* score units were derived from ultrasound-based intrauterine growth curves.[24]

The social circumstances were defined as the first component of a principal component analysis on household income, maternal age, and maternal level of education at 2 years with a mean value of 0 and SD of 1 (which explained 52% of the variance in $COPSAC_{2000}$ and 55% of the variance in $COPSAC_{2010}$) (see Supplemental Tables 8 and 9).

Information on breastfeeding was collected by interviewing the mothers at the clinic on the duration of exclusive and total breastfeeding and the use of infant formula when the children were 1, 3, 6, 12, 18, 24, 30, and 36 months old. As soon as the child's diet was supplemented or replaced by continual use (>7 days) of infant formula or complementary foods, we considered exclusive breastfeeding terminated. If the child had received infant formula for a period of <7 days as a supplement to breastfeeding, we still considered it exclusive breastfeeding.

Information on prepregnancy weight of the mothers was collected from pregnancy records in $COPSAC_{2010}$, and BMI was calculated with the height measured at the clinic.

Statistics

Baseline characteristics were compared between children born by CD and vaginal delivery via a χ^2 test or Student's *t* test. Covariates with $P < .1$ were considered potential confounders. We investigated associations between delivery mode and BMI and total fat percentage by Student's *t* test and multiple linear regressions. Meta-analysis estimates were calculated with a random effects regression model. Heterogeneity between studies was estimated by I^2 values.

BMI tracking over time was analyzed by a mixed model (including repeated measures), based on World Health Organization gender-specific BMI *z* scores[25] at every scheduled visit from 1 to 5 years in $COPSAC_{2010}$ and 13 years in $COPSAC_{2000}$. We used *z* scores because BMI does not have a linear development.

Results with a $P < .05$ were considered significant. Missing data were treated as missing observations. Data processing was conducted in R version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria).

The detectable effect sizes (80% power) were estimated via *t* statistics, and they were 0.367, 0.333, and 0.305 for BMI at 6 months, 1 year, and 5 years, respectively, in $COPSAC_{2010}$ and 0.545, 0.516, 0.538, and 1.191 for BMI at 6 months and 1, 5, and 13 years, respectively, in $COPSAC_{2000}$.

RESULTS

Baseline Characteristics

Table 1 shows baseline characteristics of the children born by CD compared with children born by vaginal delivery in both cohorts separately.

In the $COPSAC_{2010}$ cohort, 21% ($N = 138$) of the children were born by CD, and 19% ($N = 76$) were born by CD in the $COPSAC_{2000}$ cohort.

The mothers who delivered by CD were significantly older (in $COPSAC_{2000}$, 30.1 vs 29.5 years, $P = .01$; in $COPSAC_{2010}$, 33.2 vs 32.1 years, $P = .01$).

Children born by CD had a lower gestational age (in $COPSAC_{2000}$, 278 vs 281 days, $P = .03$; in $COPSAC_{2010}$, 278 vs 281 days, $P < .001$).

In $COPSAC_{2010}$ the children delivered by CD had a higher *z* score birth weight for gestational age (0.20 vs −0.05, $P = .01$) and a shorter duration of exclusive breastfeeding (93 vs 106 days, $P = .02$); in $COPSAC_{2000}$ we did not find these differences.

In $COPSAC_{2010}$ the mothers who delivered by CD had a higher prepregnancy BMI (mean BMI, 25.5 vs 24.3, $P = .01$), and they were more likely to be nulliparous (52.9% vs 44.7%, $P = .09$).

We observed no other differences associated with delivery mode in the cohorts.

All results were therefore adjusted for age at BMI measurement, gender, parity, mother's age, birth weight for gestational age, and exclusive breastfeeding duration, and, in $COPSAC_{2010}$, for maternal prepregnancy BMI.

Delivery Mode and BMI Development in the First Year of Life

Children born by CD had a higher peak value of mean BMI in infancy in

TABLE 1 Baseline Characteristics of Children Delivered by CD and Vaginally in $COPSAC_{2010}$ and $COPSAC_{2000}$

	$COPSAC_{2010}$					$COPSAC_{2000}$				
	N	CD	*N*	Vaginal Delivery	*P*	*N*	CD	*N*	Vaginal Delivery	*P*
Mode of delivery, % (*N*)	—	20.4 (138)	—	79.6 (535)	—	—	19.3 (76)	—	80.7 (317)	—
Demographics										
Caucasian, % (*N*)	138	97.8 (135)	535	95.5 (511)	.22	76	96.1 (73)	317	96.5 (306)	.84
Female, % (*N*)	138	44.2 (61)	535	50.1 (268)	.22	76	52.6 (40)	317	50.8 (161)	.77
Gestational age, d, mean (SD)	138	277.5 (10.1)	535	281.1 (8.6)	<.01	76	277.6 (12.8)	317	280.6 (10.1)	.03
Age at 5-y BMI measurement, y, mean (SD)	118	5.0 (0.1)	471	5.0 (0.1)	.04	61	5.2 (0.2)	233	5.2 (0.2)	.8
Age at 13-y BMI measurement, y, mean (SD)	—	—	—	—	—	67	12.9 (0.6)	257	12.9 (0.6)	.96
Social circumstances	138	0.2 (1.2)	535	0.0 (0.9)	.43	72	0.1 (1.2)	292	−0.1 (1.1)	.32
Mother's age at birth, y, mean (SD)	138	33.2 (4.8)	535	32.1 (4.2)	.01	76	31.0 (4.5)	317	29.5 (4.4)	.01
Nulliparity, % (*N*)	138	52.9 (73)	535	44.9 (240)	.09	76	44.7 (34)	317	45.4 (144)	.91
Father's height, cm, mean (SD)	121	180.7 (6.8)	505	180.9 (6.7)	.77	63	181.2 (7.9)	245	180.7 (7.2)	.63
Mother asthmatic, % (*N*)	137	30.7 (42)	534	24.7 (132)	.16	76	100 (76)	317	100 (317)	—
Risk factors										
Smoking in pregnancy, % (*N*)	138	8.1 (12)	534	7.5 (40)	.79	76	19.7 (15)	317	26.5 (84)	.22
Exclusive breastfeeding, d, mean (SD)	136	93.4 (66.6)	529	106.3 (57.1)	.02[a]	72	110.8 (67.8)	281	114.8 (58.7)	.61
Mother's prepregnancy BMI, mean (SD)	121	25.5 (4.8)	488	24.3 (4.2)	.01	—	—	—	—	—
Mother's height, cm, mean (SD)	138	166.6 (6.3)	535	167.7 (6.3)	.07	74	167.4 (7.0)	292	167.0 (6.7)	.73
Gestational diabetes, % (*N*)	138	2.1 (3)	535	2.4 (13)	.86	—	—	—	—	—
Preeclampsia, % (*N*)	138	6.5 (9)	535	3.6 (19)	.12	76	7.9 (6)	317	4.1 (13)	.17
Intrapartum antibiotics, % (*N*)	138	100 (138)	532	12.9 (69)	—	—	—	—	—	—
Hospitalization after birth, % (*N*)	138	4.6 (21)	535	4.7 (47)	.94	—	—	—	—	—
Fish oil supplementation, % (*N*)	137	53.3 (73)	534	48.9 (261)	.38	—	—	—	—	—
High-dose vitamin D supplementation, % (*N*)	118	51.7 (61)	452	50.0 (226)	.74	—	—	—	—	—
Anthropometrics										
Birth weight for gestational age *z* score, units[a], mean (SD)	138	0.2 (1.2)	534	−0.1 (0.9)	.01	76	0.1 (1.2)	317	−0.1 (1.1)	.44
Birth weight, kg, mean (SD)	138	3.6 (0.6)	535	3.6 (0.5)	.82	76	3.5 (0.6)	317	3.6 (0.5)	.72
BMI >85 percentile at 5 y, % (*N*)	118	16.9 (20)	471	14.9 (70)	.57	61	16.4 (10)	234	15.8 (37)	.91
BMI >85 percentile at 13 y, % (*N*)	—	—	—	—	—	67	17.9 (12)	259	13.1 (34)	.32
BMI >90 percentile at 5 y, % (*N*)	118	10.9 (14)	471	9.9 (47)	.72	61	14.8 (9)	234	11.16 (26)	.44
BMI >90 percentile at 13 y, % (*N*)	—	—	—	—	—	67	11.9 (8)	259	9.7 (25)	.58

[a] Calculation was based on Maršál's intrauterine growth curves.

TABLE 2 Relationship Between Mode of Delivery and 6-mo, 1-y, 5-y, and 13-y BMI Measurement

	$COPSAC_{2010}$			$COPSAC_{2000}$			Meta-analysis		
	Crude Estimate (95% CI)	*P*	Adjusted Estimate[a] (95% CI)	*P*	Crude Estimate (95% CI)	*P*	Adjusted Estimate[b] (95% CI)	*P*	I^2 (%)	Heterogeneity *P*	Estimate[c] (95% CI)	*P*
BMI at 6 mo	0.36 (0.10 to 0.61)	.007	0.42 (0.13 to 0.70)	.004	0.29 (−0.10 to 0.66)	.139	0.28 (−0.11 to 0.68)	.156	0.0	.60	0.37 (0.14 to 0.60)	.002
BMI at 1 y	−0.01 (−0.239 to 0.21)	.918	−0.05 (−0.30 to 0.20)	.695	0.46 (0.01 to 0.82)	.013	0.50 (0.14 to 0.87)	.007	73.2	.05	0.18 (−0.35 to 0.72)	.51
BMI at 5 y	0.07 (−0.15 to 0.29)	.544	−0.03 (−0.27 to 0.21)	.811	0.20 (−0.18 to 0.58)	.304	0.18 (−0.20 to 0.56)	.349	0.0	.36	0.03 (−0.17 to 0.24)	.764
BMI at 13 y	—	—	—	—	0.15 (−0.68 to 0.98)	.722	−0.03 (−0.87 to 0.82)	.950	—	—	—	—

[a] Adjusted for $COPSAC_{2010}$: age at BMI measurement, gender, parity, mother's age, mother's prepregnancy BMI, birth weight for gestational age, and duration of exclusive breastfeeding.
[b] Adjusted for $COPSAC_{2000}$: age at BMI measurement, gender, parity, mother's age, birth weight for gestational age, and duration of exclusive breastfeeding.
[c] Random effects.

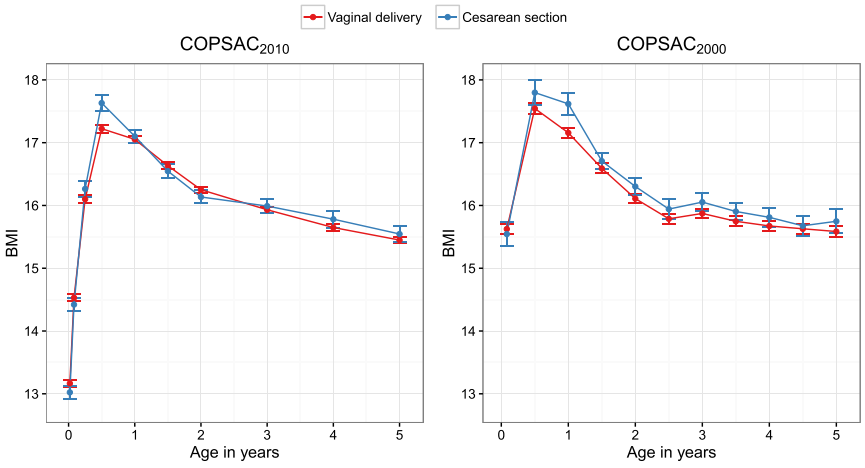

FIGURE 1
BMI in first 5 years of life. Curves showing mean BMI with SEs according to visit age in the first 5 years of life for children delivered by CD and vaginally in $COPSAC_{2010}$ and $COPSAC_{2000}$.

both cohorts compared with children delivered vaginally (Fig 1). In $COPSAC_{2010}$ this difference was most pronounced at age 6 months, 17.6 versus 17.2 (95% confidence interval [CI], 0.10 to 0.61), and subsequently the groups aligned with no difference at age 1 year. In $COPSAC_{2000}$ the BMI values of children born by CD diverged from 6 months compared with children born vaginally, and the difference reached its maximum at age 1 year, 17.6 versus 17.2 (85% CI, 0.01 to 0.82). The differences in BMI at 6 months were significant after adjustment in $COPSAC_{2010}$ (β-coefficient, .41; 95% CI, .12 to .69; $P = .01$) but not in $COPSAC_{2000}$ (β-coefficient, .16; 95% CI, −.11 to .68; $P = .16$). Meta-analysis of BMI at 6 months revealed a significant association with CD in the 2 cohorts (β-coefficient, .37; 95% CI, .14 to .60; $P = .002$) (Table 2) but no difference in the meta-analysis of BMI at 1 year. Adjusting the analyses in $COPSAC_{2010}$ for the pregnancy supplementation trials did not change the results (data not shown).

We subanalyzed the associations between CD and BMI at 6 months of age in the 174 children delivered by asthmatic mothers in $COPSAC_{2010}$ (β-coefficient, .30; 95% CI, −.20 to .80; $P = .22$).

Delivery Mode and BMI Development During Childhood

The BMI curves aligned after the gap in the first year (Fig 1), and we found no difference in mean BMI at 5 years of age with regard to mode of delivery ($COPSAC_{2010}$: $\beta\text{-coefficient}_{adjusted}$, −.03; 95% CI, −.27 to .21; $P = .81$); $COPSAC_{2000}$: $\beta\text{-coefficient}_{adjusted}$, .18; 95% CI, −.20 to .56; $P = .35$) (Table 2). We found no difference in the meta-analysis at this time point.

Figure 2 illustrates the longitudinal BMI development for the children until 13 years of age in the $COPSAC_{2000}$ cohort according to mode of delivery. We found no difference in mean BMI between the 2 groups at age 13 years ($\beta\text{-coefficient}_{adjusted}$, −.03; 95% CI, −.87 to .82; $P = .95$) (Table 2). From 1.5 to 13 years of age the curves are almost coherent, with the CD curve on top, and graphically they reach the time for adiposity rebound (~age 4.5 years) simultaneously and continuing with an identical course into puberty.

Using repeated measurement statistics, we analyzed whether there were a difference in mean BMI from infancy through childhood between children delivered by CD and vaginally. We found no difference in mean *z* score BMI over time:

$COPSAC_{2010}$, 1 to 5 years of age (β-coefficient −.05; SE .07; $P = .95$) and $COPSAC_{2000}$, 1 to 13 years of age (β-coefficient .12; SE .10; $P = .21$).

Furthermore, we compared the ratio of children having a BMI above the 85th and 90th percentiles at 5 and at 13 years of age and found no differences with regard to delivery mode (Table 1).

In $COPSAC_{2010}$ we subanalyzed whether induction of birth in the vaginal delivery group and type of CD could affect the results. We found no differences in BMI at any time in vaginally delivered children with regard to birth induction. Furthermore, we found no differences in BMI with regard to type of CD (Supplemental Tables 5 and 6).

Gender-Specific Growth

Gender-specific growth curves for both cohorts are illustrated in Supplemental Fig 3, showing the mean BMI value in the first year of life. We did not find any gender-specific growth patterns according to mode of delivery.

Delivery Mode and Body Fat Percentage

CD was not associated with significant differences in the body fat percentage of the children measured by DXA scans at age 3.5 years in $COPSAC_{2010}$ and at age 7 years in $COPSAC_{2000}$ (Table 3).

We subanalyzed the DXA scans from $COPSAC_{2010}$ and found no significant regional differences in body fat percentage in legs, arms, trunk, or android region between the 2 delivery groups (Supplemental Table 7).

Intrapartum Antibiotics and Cross-Sectional BMI

Because all women giving birth by CD were treated with intrapartum antibiotics, we wanted to examine whether this treatment could be responsible for some of the effects. In $COPSAC_{2010}$, 13% ($N = 69$) of women with vaginal delivery received intrapartum antibiotics. There were no differences in mean BMI at age 6 months or 5 years in children of these women compared with children whose mothers did not receive antibiotics (Supplemental Table 4).

TABLE 3 Fat Percentage From DXA Scans at 3.5 y in $COPSAC_{2010}$ and at 7 y in $COPSAC_{2000}$

	CD Mean (SD)	Vaginal Mean (SD)	Crude[a] Estimate (95% CI)	*P*	Adjusted[b] Estimate (95% CI)	*P*
$COPSAC_{2010}$						
Fat, %	28.18 (4.79) *N* = 79	28.77 (4.36) *N* = 272	−0.30 (−1.27 to 0.67)	.22	−0.29 (−1.39 to 0.81)	.61
$COPSAC_{2000}$						
Fat, %	27.96 (5.16) *N* = 57	28.28 (5.85) *N* = 233	−0.29 (−1.82 to 1.23)	.70	−0.53 (−0.21 to 1.04)	.51

$COPSAC_{2000}$: age at BMI measurement, gender, parity, mother's age, birth weight for gestational age, and duration of exclusive breastfeeding.

[a] Adjusted for age at DXA scans and gender.

[b] Adjusted for $COPSAC_{2010}$: age at BMI measurement, gender, parity, mother's age, mother's prepregnancy BMI, birth weight for gestational age, and duration of exclusive breastfeeding.

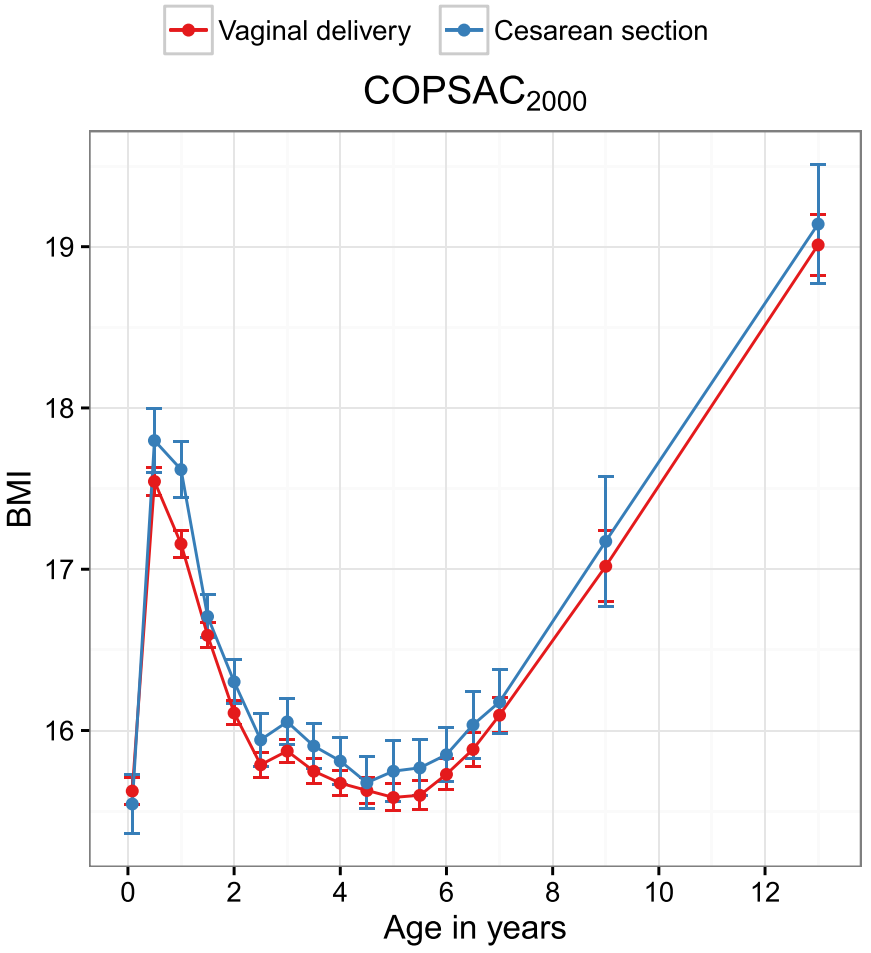

FIGURE 2
BMI in first 13 years of life. Curves showing mean BMI with SEs according to visit age for children delivered by CD and vaginally until 13 years of age in $COPSAC_{2000}$.

DISCUSSION

Primary Findings

We found that children born by CD had a higher BMI peak at 6 months of age compared with children born vaginally. This higher BMI in early childhood did not track into

later childhood and adolescence. Furthermore, we found no association between delivery mode and body fat percentage in children at age 3.5 or 7 years.

Strengths and Limitations

The primary strength of this study was the longitudinal follow-up on growth parameters in 2 comparable cohorts at the same center under a similar design and the subsequent meta-analysis. Each growth measurement was performed with the same equipment by trained COPSAC assistants based on standardized procedures, and the observed growth curves were similar to those of previous reports.[26] The longitudinal follow-up allowed repeated measurement statistics. In addition, we included DXA scans as an objective measure of fat percentage.

Furthermore, we had a broad range of exposures, which were validated by register data and personal interviews with the families. This availability of covariates allowed adjustments for important potential confounders such as gender, parity, birth weight for gestational age, maternal age, prepregnancy BMI, and breastfeeding duration.[27,28] After covariate adjustment, an observed nonsignificant higher BMI at age 13 years disappears completely, which could otherwise have suggested a possible difference not found because of low power at this time point.

It may be a limitation that the $COPSAC_{2000}$ is an asthma high-risk cohort, but in the subanalyzes of children with asthmatic mothers we still observed a difference in BMI at 6 months between the 2 delivery groups, with an estimate comparable to the one we found in $COPSAC_{2000}$.

Another limitation could be the decade between the enrollments of 2 cohorts, leading to different time-related environmental impacts. We saw differences in the duration of breastfeeding and smoking habits (Table 1) between the 2 cohorts; however, the results appear similar, and we believe that we have accounted for the majority of differences through our comprehensive confounder adjustment and meta-analyses of the individual cohort results.

Our post hoc power calculation indicated that the differences we found at 6 months were reliable with >80% power, but we do not have the same power to ensure that the lack of differences we found at 5 and 13 years were true.

Interpretation

We found that children born by CD have higher mean BMI at 6 months of age compared with children born vaginally, leading to a 0.37 difference in mean BMI. We saw no significant association between mode of delivery and BMI or body composition in later childhood or puberty.

These findings suggest that infants delivered by CD have a divergent growth pattern during early infancy but show no risk for overweight in later childhood.

Two recent meta-analyses on this subject have results and conclusions that differ from ours. One study found a greater risk of overweight and obesity in offspring delivered by CD in childhood, youth, and adulthood.[12,13] The other study found that delivery by CD was associated with a greater risk of overweight and obesity and a higher mean BMI in adulthood in unadjusted analyses. However, most of the included studies did not provide information about relevant covariates, such as breastfeeding patterns and mother's BMI, and they used divergent definitions of growth outcomes and age of measurement. In the meta-analyses the main association was found between CD and obesity, according to either BMI >95th percentile for age and gender or the International Obesity Task Force criteria.[29–32] In our birth cohorts the variation in BMI was narrow (Table 2), with only 6 children fulfilling the International Obesity Task Force criteria for risk of obesity at 5 years in each cohort and 7 children at 13 years. Our subanalysis of children with a BMI value above the 85th percentile and 90th percentile at 5 and 13 years of age showed that the distribution according to mode of delivery was the same as in the rest of the children. The prevalence of childhood obesity was ~8.5% in United States[1] and 3.5% in Denmark[2] in 2011. This difference in obesity prevalence could partly account for the ambiguous results.

Other studies have demonstrated findings in line with ours, with increased growth in infancy for children born by CD followed by no long-term effects. One study[33] found a greater likelihood of obesity at age 2 years in children delivered by CD but not later. A study found an increased risk of obesity for 4-year-old boys but no risk for girls and no increased risk at later ages.[34]

Two previous Danish studies showed conflicting results. A register study[35] found that 7-year-old children delivered by CD had a 15% higher risk of being overweight in unadjusted analysis but no difference after adjustment; another study found that men 18 years old delivered by CD had a higher adjusted mean BMI and a higher risk of obesity.[36] However, the latter did not adjust for mother's BMI or breastfeeding pattern.

Both duration of breastfeeding[27] and prepregnancy BMI[28] increase the risk of obesity; we found both of these factors associated with CD. Most children in our cohort were breastfed, and the variation of socioeconomic circumstances was narrow (Table 1) compared with other countries.[30,31,34] We speculate that some of the earlier studies had more diverse populations and that

the lack of sufficient confounder adjustment could partly explain the different associations found between countries.[33,35,36]

Previous studies have indicated that the gut microbiota affects the human metabolism and thereby risk of obesity.[37] It has therefore been hypothesized that differences in gut microbiota caused by CD[38] could explain the differences in BMI. We found a diverging BMI according to delivery mode only in early infancy, which we speculate could be explained partly by differences in the gut microbiota, which in $COPSAC_{2010}$ was apparent only in the first months of life but equalized at 1 year of age.[38]

All children in our study who were delivered by CD had been exposed to intrapartum antibiotics. We analyzed BMI according to antibiotic administration during vaginal delivery but found no relationship between intrapartum antibiotics and BMI in infancy or later in life (Supplemental Table 4). Our data do not support the hypothesis that CD is merely a proxy for intrapartum antibiotics.[29,38]

The mechanism of becoming obese is multifactorial, and increased growth in infancy, especially a high infant BMI peak, has been associated with later risk of overweight and obesity in childhood and adulthood. Our results may have been limited in power when we assessed each cohort separately, which might lead to the lack of difference in BMI at 5 and 13 years. However, our meta-analysis clearly shows no difference at age 5 years, and the nonsignificant higher BMI observed at age 13 years in $COPSAC_{2000}$ disappears after covariate adjustment. It can be hypothesized that CD may be a risk factor only if the child grows up in an environment with other risk factors for obesity. This window of growth should therefore be a focus of interest in future studies, because it could represent a modifiable link in the prevention of obesity in selected children.

CONCLUSIONS

Children delivered by CD had a higher mean BMI at 6 months of age, but this difference did not track into later childhood. At 13 years of age children born by CD had a nonsignificant higher BMI, but after adjustment this difference disappeared. CD did not associate with childhood fat percentage measured by DXA scans. This window of increased BMI in infancy should be explored in populations containing a higher number of obese children.

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ABBREVIATIONS

CD: cesarean delivery
CI: confidence interval
COPSAC: Copenhagen Prospective Studies on Asthma in Childhood
DXA: dual-energy x-ray absorptiometry

had final responsibility for the decision to submit for publication; Dr Stokholm was responsible for data acquisition, analysis, and interpretation and provided important intellectual input; and all authors approved the final manuscript as submitted.

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Address correspondence to Hans Bisgaard, MD, DMSc, Copenhagen Prospective Studies on Asthma in Childhood, Faculty of Health and Medical Sciences, University of Copenhagen and Danish Pediatric Asthma Center, Gentofte Hospital, University of Copenhagen, Ledreborg Alle 34, 2820 Gentofte, København, Denmark. E-mail: bisgaard@copsac.com

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Cesarean Delivery and Body Mass Index at 6 Months and Into Childhood

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Paper II

Fish oil supplementation increases pregnancy duration and intrauterine growth

Randomized controlled trial

Fish oil supplementation increases pregnancy duration and intrauterine growth.
Randomized controlled trial

Authors: Rebecca Kofod Vinding[1+2], MD, Jakob Stokholm[1], MD, PhD, Astrid Sevelsted[1], MSc, Bo L. Chawes[1], MD, DMSc, Klaus Bønnelykke[1], MD, PhD, Malin Barman[3+4], PhD, Bo Jacobsson[3+5], MD, PhD, Hans Bisgaard[1], MD, DMSc;

Affiliation:
1) COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark
2) Department of Pediatrics, Naestved Hospital, Naestved, Denmark.
3) Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Gothenburg, Sweden
4) Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden.
5) Department of Genetics and Bioinformatics, Domain of Health data and Digitalization, Norwegian Institute of Public Health, Oslo, Norway.

Correspondence:
Professor Hans Bisgaard, MD, DMSc
Copenhagen Prospective Studies on Asthma in Childhood, Faculty of Health and Medical Sciences, University of Copenhagen & Danish Pediatric Asthma Center, Gentofte Hospital, University of Copenhagen
Ledreborg Alle 34
2820 Gentofte; Denmark
Tel: (+45) 39777360
Fax: (+45) 39777129
E-mail: bisgaard@copsac.com
Website: www.copsac.com

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Conflict of Interest:
All authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript.

Abbreviations:
$COPSAC_{2010}$ = COpenhagen Prospective Studies on Asthma in $Childhood_{2010}$;
DHA =Docosahexaenoic acid;
EPA= Eicosapentaenoic acid;
LCPUFA= Long chain polyunsaturated fatty acids;
RCT= Randomized Controlled Trial

Table of Contents Summary:
In a randomized-control-trial we found that supplementation with n-3 LCPUFA led to a higher birth weight, by prolongation of pregnancy and increased fetal growth.

What's Known on This Subject.
Randomized controlled trials have shown that supplementation with fish oil in pregnancy may prolong pregnancy and increase birth weight.
The increased birth weight could be due to the prolongation of pregnancy, but another explanation could be an increased intrauterine growth.

What This Study Adds.
In a randomized controlled trial, we found that supplementation with fish oil led to a higher birth weight, by prolongation of pregnancy and increased fetal growth.
Fish oil supplementation's could have a potential for improving fetal and infant health.

Governance:
We are aware of and comply with recognized codes of good research practice, including the Medical Research Council's Good Research Practice and the Guidelines for Good Scientific Practice by the Danish Committees on Scientific Dishonesty (UVVU). 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.

CONTRIBUTORS STATEMENT PAGE

The guarantor of the study is Hans Bisgaard, from conception and design to conduct of the study. Rebecca Vinding were responsible for acquisition of data, data analysis, and interpretation of data.
Rebecca Vinding has carried out the initial analyses and written the first draft of the manuscript. Astrid Sevelsted, Jakob Stokholm, Bo Chawes, Klaus Bønnelykke, Barman and Bo Jacobsson have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input. Hans Bisgaard had full access to the data and had final responsibility for the decision to submit for publication.
All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

ABSTRACT (250 words)

Background
Randomized controlled trials have shown that supplementation with n-3 long-chain polyunsaturated fatty acids (LCPUFAs) in pregnancy may prolong pregnancy and increase birth weight.

Methods
This was a double-blinded, randomized controlled trial conducted among 736 pregnant women and their offspring, from the Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$-cohort. They were recruited in 24 weeks of pregnancy and randomized to either n-3 LCPUFA or control daily until one week after birth. Exclusion criteria were twin pregnancy and intrauterine death.

Main Outcomes: Gestational age, calculated based on expected due date determined by ultrasound in pregnancy week 12. Birth weight, obtained from parental interviews and validated with register records. As a proxy for intrauterine growth, the calculated mean weight percentile for gestational age according to standardized fetal growth curves.

Results
A total of 699 mother-infant pairs were included in the study. n-3 LCPUFA compared to control was associated with a 2 day prolongation of pregnancy (days, median [IQR]; 282[275-288] vs. 280[273-286], p=0.02), a 97 g higher birth weight (g, mean (SD); 3601(534) vs. 3504(528), p=0.02) and an increased intrauterine growth leading to a higher mean weight percentile for gestational age for the population-based growth curves-Skjarven: (%, mean (SD); 49.9(28.3) vs. 44.5(27.6), p=0.01).

We observed no effects on preterm delivery or other pregnancy complications.

Conclusion
n-3 LCPUFA supplementation in pregnancy was associated with prolonged gestation and increased intrauterine growth, leading to a higher birth weight. Future studies should focus on the potential for using n-3 LCPUFA supplementation for improving fetal and infant health.

Trial Registration
ClinicalTrials.gov: NCT00798226.

INTRODUCTION

Pour maternal nutrition during pregnancy can influence the course of pregnancy and fetal growth(1), leading to a poor intrauterine growth and decreased gestational age. This is important since both of these have been associated with increased morbidity and impaired development in children(1). Observational studies have shown prolonged duration of pregnancy and increased birth weight in communities with high fish intake(2,3), which represents a diet especially rich in important nutrients such as selenium, proteins, iodine, vitamin D and n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA). The beneficial effects of a high fish intake on pregnancy outcomes have mainly been attributed to the n-3 LCPUFAs, and a number of randomized controlled trials (RCT) have been performed to evaluate the efficacy of n-3 LCPUFA supplementation in pregnancy to increase pregnancy duration and birth weight. A meta-analysis of 21 RCTs of pregnancy supplementation of fish oil showed increased gestational age and reduced risk of preterm delivery(4). However, another recent large meta-analyis of 34 RCTs concluded that there was not enough evidence to support the routine use of omega-3 supplementation during pregnancy to improve mother and child health(5). It has been speculated that the increased birth weight is caused only by the prolonged duration of pregnancy(6,7), but another explanation could be that the n-3 LCPUFA supplementation also increases the intrauterine growth rate.

In the population-based Danish Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) mother-child cohort, the women participated in a double-blind, RCT of daily supplementation with n-3 LCPUFA (fish oil) or placebo (olive oil) from week 24 of pregnancy to one week postpartum(8). With these novel data we aim to

investigate the effect of fish oil supplementation compared to placebo on the secondary outcomes: duration of pregnancy, intrauterine growth and birth weight of the children.

METHODS

Study Design

In this RCT, we identified pregnant women in Denmark by reviewing the monthly lists of reimbursements to general practitioners for the first pregnancy visits. These women were sent a written invitation to contact the COPSAC clinic by telephone and screened for eligibility. Thereafter, detailed information was sent and the first visit to the clinic was planned within pregnancy weeks 22 through 26 with an additional pregnancy visit at 36 weeks of pregnancy.

Exclusion criteria were gestational age above week 26, any endocrine, cardiovascular, or nephrological disorders and vitamin D supplementation intake larger than 600IU/day. For this analysis, we further excluded women with twin pregnancies and women, who did not attended both of the two pregnancy visits with the exception of women giving birth before pregnancy week 36.

Study intervention

The women were randomized 1:1 in a double-blind design at 24 weeks of pregnancy to either daily supplementation of 2.4 g fish oil (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 identically looking placebo capsules of olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid, Pharmatech A/S, Norway)(see **Table E1** for product specifications). The allocation was performed by simple randomization procedures using a computer-generated list of random numbers prepared by an external investigator. Capsules were kept in closed consecutively numbered containers. At two time-points during the study, the manufacturer analyzed the fatty acid content and the oxidation levels in both kinds of oil capsules showing levels as expected. The

supplementation was continued until one week after delivery, and the code was unblinded when the youngest child turned three years.

A subgroup from this pregnancy cohort also participated in a nested, 2x2 factorial designed, double-blind, RCT of 2400IU/day vitamin D3 supplementation (N=623)(9) and a smaller subgroup participated in a phase IV randomized, participant-blinded comparison of influenza A/California/2009 vaccines (N=130)(9,10).

Adherence

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

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 oral and written informed consent before enrolment. ClinicalTrials.gov number NCT00798226.

End-points

In this analysis we primarily investigated gestational age, birth weight and intrauterine growth. These were all predetermined as secondary endpoints of the n-3 LCPUFA trial with persistent wheeze/asthma as the primary outcome(8,9,11).

Gestational age: Gestational age was calculated based on expected due date, determined by the scheduled ultrasound scan, which is performed in all pregnant Danish women around pregnancy week 12. In women with no ultrasound scan, due date was calculated from last menstrual period using Naegeles rule. The due date was first validated against data from the ultra sound clinics and subsequently against the national Danish

foetodatabase. We used the data from the Danish foetodatabase as first choice. Preterm delivery was defined as birth before week 37+0.

Birth anthropometrics: Birth length and -weight were obtained at the first clinical visit one week after birth by personal interview and thereafter validated against the national Danish Birth Registry. Furthermore, if there was a difference larger than 10g and 5 cm, data were validated against the length and weight measures at 1 week from the research clinic. Weight was measured without clothes using calibrated digital weight scales. Length was measured using an infantometer (Kiddimeter; Raven Equipment Ltd, Dunmow, Essex, England).

Fetal anthropometrics: The fetal weight at mid-pregnancy was estimated from the scheduled ultrasound scans around pregnancy week 20 by the Hadlock equation(12,13) using the fetal biometrics for head, abdomen and femur.

Size for gestational age and intrauterine growth: As an estimate of intrauterine growth we used size for gestational age according to two standards based on Northern European populations: ultrasound-based fetal growth curves according to Marsal(14) and population-based fetal growth curves according to Skjaerven(15). The fetal growth curves were used to calculate which percentage each child birth weight given their gestational age corresponded to on the two standardized curves. Small for gestational age and large for gestational age were defined as below and above two standard deviation on Marsal fetal growth curve.

In addition, we calculated the fetal growth rate from week 20 to birth by subtracting the Hadlock weight estimate from the birth weight and dividing this weight gain with the numbers of days from the week 20 ultrasound scan to birth (birth weight (g) – Hadlock

estimated weight (g)) / (gestational age at birth (days) - gestational age at scanning (days)).

Pregnancy complications: Information regarding preeclampsia, gestational diabetes mellitus, antibiotics usage in pregnancy, mode of delivery, birth induction and the child's APGAR score were obtained during the scheduled visits and validated against register data. If there were discrepancy, the mother and child's medical record was checked.

Covariates

Information on race, sex, maternal age at delivery, parity, household income, parents' educational level, maternal asthma, older siblings, smoking during pregnancy and assisted reproduction were obtained during the scheduled visits. Information on pre-pregnancy weight was collected from the mother's pregnancy records. Mothers body mass index (BMI) was calculated with height measured at the COPSAC clinicic.
The social circumstances 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 (PCA 1 explained 55% of the variance)(16)

Statistics

Power analyses: The trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the statistical power of the RCT on gestational age, birth weight and birth weight /gestational age was calculated post-hoc based on cohort data. Our sample size of 699 gave us the opportunity, assuming a 80% power, to detect a mean difference of 2 days in relation to gestational age, with an SD of 11 days, and a mean increase of 112 g in birth weight, with an SD of 530 g. In regards to difference in

birth weight /gestational age, we had power to detect a mean increase of 6.4 %, with an SD of 28 %.

We used Student's t-test for normally distributed continuous variables and chi-square tests for categorical variables. Associations between fish oil supplementation and gestational age were analyzed using Wilcoxon signed-rank test, since gestational age was not normal distributed. Results with a p-value <0.05 were considered significant. Missing data were treated as missing observations. The data processing was performed using R v 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria) and the package "ggplot2" for graphics. The power/sample size calculation and testing for the study hypotheses were based on a 2-sample, two-tailed t-test at the $\alpha=0.05$ level.

RESULTS

Baseline characteristics

Enrollment began in November 2008, ended in November 2010, and the last child was born in April 2011. We randomized 736 unselected women at pregnancy week 24 to either n-3 LCPUFA or placebo: 27 women were lost to follow-up before pregnancy week 36 and five twin pregnancy and four children with disabling disease were excluded, leaving 699 mother/child pairs for the analyses with 346 in the n-3 LCPUFA group and 353 in the control group (see study group flowchart in **Figure E1**). The baseline characteristics are presented in **Table 1**, showing a successful randomization ($p>0.05$ in all comparisons, data not shown). Adherence to the study supplementation defined as an intake of more than 80% of the prescribed dose based upon capsule count was estimated to be 69 % (N=485) of the included women with no differences between the n-3 LCPUFA and control group.

Gestational age, fetal growth and birth weight

Gestational age

For the 699 included children, the median gestational age was 281 days. Supplementation with n-3 LCPUFA showed a significantly higher gestational age; days, median [IQR] 282[274–288] vs. 280[273–286], $p=0.02$, corresponding to a two days difference between the groups (**Table 2)**. This prolongation of pregnancy duration did not lead to a reduction in frequency of preterm delivery: 12 children in the n-3 LCPUFA group and 14 children in the control group were born before pregnancy week 37, equal to 4 % in both groups. **Figure 1A** illustrates gestational age in the n-3 LCPUFA and control groups, indicating that the effect of n-3 LCPUFA supplementation is evenly distributed across the spectrum of gestational age in the population.

Birth weight

For the 699 children included the mean (SD) birth weight was 3,552 g (533). Children born to mothers who were supplemented with n-3 LCPUFA in pregnancy had a significantly higher birth weight compared to the control group; g, mean(SD) 3,601(534) vs. 3,504(528), p=0.02; corresponding to a mean increase of 97 g (95% CI: 0.02 g; 0.18 g)(**Figure 1B** and **Table 2**). We also found that the children in the intervention group had a nonsignificant higher length at birth cm, mean (SD) 52.1(2.4) vs 51.8(2.5), p=0.15(**Table 2**).

Size for gestational age and intrauterine growth: As a proxy of intrauterine growth, we used size for gestational age. The children were larger for gestational age in the n-3 LCPUFA vs. control group when using standardized growth curves to estimate the size; mean percentage (SD) by Marsal; 51.6%(28.4) vs. 47.6%(28.3); p=0.06 and by Skjaerven; 49.9% (28.3) vs. 44.5% (27.6); p=0.01. (**Figure 1C** and **Table 2**).

We did not find any significant difference in children born small or large for gestational age according to Marsals definition(14): 8 (2.3 %) children in the n-3 LCPUFA group and 5 (1.4 %) children in the control group were born were born small for gestational age. 13 (3.8 %) children in the n-3 LCPUFA group and 8 (2.3 %) children in the control group were born were large for gestational age.

Intrauterine growth based on the difference between Hadlock calculated fetal weight(12) from pregnancy week 20 to weight at birth, was available for 691 (99%) of the children. The average (SD) growth rate from this estimation was 23.19 g/day(3.15) in the n-3 LCPUFA group vs. 22.83 g/day(3.17) in the control group; p=0.13, corresponding to a mean difference of 0.36 g/day (95% CI: -0.11 g/day; 0.83 g/day) (**Table 2**).

Stratifying the analyses on nulliparous and multiparous women yielded the same associations between the n-3 LCPUFA supplementation and gestational age, birth weight and intrauterine growth measures (data not shown).

There was no significant interaction between n-3 LCPUFA and the vitamin D supplementation with regards to the gestational age or growth outcomes ($p>0.20$).

Adverse pregnancy and birth outcomes

The n-3 LCPUFA supplementation had no significant effects on adverse pregnancy outcomes such as preeclampsia or gestational diabetes. Furthermore, there were no differences in delivery complications such as induced birth, emergency or elective cesarean section, and no difference in the APGAR score at 5 minutes (**Table 2)**.

DISCUSSION

Primary findings

Daily supplementation with fish oil compared to placebo from pregnancy week 24 till one week after birth resulted in a two days prolongation of pregnancy and a 97 g higher birth weight among mother-child pairs of the population-based Danish $COPSAC_{2010}$ cohort. The increase in birth weight was not only explained by a longer duration of pregnancy, but also by an increased intrauterine growth. The prolonged pregnancy did not reduce the number of children born preterm or induced any other differences in pregnancy and delivery complications.

Strengths and limitations

Our study is among the largest RCTs on n-3 LCPUFA supplementation in pregnancy, investigating pregnancy length and fetal growth. Still, the number of participating mothers (N=699) limited the opportunities to investigate if the increased intrauterine growth also lead to a reduction in children born small for gestational age, since this is a rare outcome with only 13 children according to Marsals definition(14). A major advantage of our trial is that it is nested in a population-based cohort, which increases the external validity of our findings.

Another advantage of our design is the quality of the end-points with 99.8% of the due dates determined by early ultrasound scans and the birth weight captured by parental interviews and subsequently validated against register data. Additionally, we acquired a broad range of data on adverse pregnancy and delivery end-points, which were similarly obtained by interviews with the families and validated against register data. The study design precluded investigation of the effect of n-3 LCPUFA supplementation on very

preterm delivery (GA <24) since the supplementation was initiated after this point in pregnancy.

It is a significant strength of our study that we find similar results across different measurements for fetal size for gestational age and fetal growth, including Marsal percentage, Skjaerven percentage, and estimated growth rate from week 20 of pregnancy based on ultrasound scans. Since a slight weight difference matters much more if the expected birth weight for a preterm infant is very low compared with a normal-weight infant born at term using standardized growth curves increases the confidence in our findings.

It is a limitation that our outcomes was secondary outcomes in our RCT, our post hoc power calculation indicated that the 2 days prolongation of pregnancy we find for the fish oil group are trustworthy with >80% power, but we do not have the same power to assure that the difference we find for birth weight and size for gestational age are true.

Interpretation

In this RCT, we confirm earlier findings showing that fish oil supplementation during pregnancy leads to a prolongation of pregnancy and an increase in birth weight (4). Furthermore, we demonstrate a significantly higher birth weight for gestational age in the n-3 LCPUFA supplemented group. This suggests that the increase in birth weight is not solely explained by the prolonged duration of pregnancy, but is also a consequence of increased intrauterine growth. To our knowledge this has not been shown before.

Our findings of an increased gestational age and birth weight are in line with the results from the majority of other studies demonstrating a prolongation of pregnancy between two and four days and an increase in birth weight between 70 and 170 g from fish oil supplementation(4,5). An explanation for the slightly different effects observed in the

previous studies could be differences in time of initiating the supplementation during pregnancy and the amount of fish oil supplied. A recent study with 500 participants, which did not observe similar effects, supplemented from week 18 of pregnancy with a less than one fifth of the fish oil dose used in the present study(17) and another study with 1200 participant using a third of our dose from week 21 of pregnancy found that the pregnancy was only prolonged by one day and the birth weight only increased with 70 g (7). This could imply that there is a dose-response relationship between the amount of n-3 LCPUFA supplement and the effect on pregnancy length and fetal growth and that a minimum threshold might exist(18).

The biological mechanism by which n-3 LCPUFAs can prolong pregnancy remains unclear, but many studies suggest that eicosanoids regulate initiation of labor(19) and a proposed explanation has been that fish oil supplementation alters the balance of prostaglandins derived from the n-3 LCPUFAs and the n-6 LCPUFAs involved in parturition(20). It has previously been suggested that the fish oil induced increase in birth weight is only caused by the prolongation of pregnancy(6). However, our data shows that n-3 LCPUFA supplementation also has an impact on the intrauterine growth, leading to an increased size for gestational age. A possible mechanism for the increased fetal growth could be that n-3 LCPUFAs increase the ratio of prostacyclins to thromboxanes, thereby reducing blood viscosity and facilitating increased placental blood flow, which benefits fetal growth(21).

It is a relevant concern that prolongation of pregnancy and higher birth weight could case unwanted complications during pregnancy and birth. However, we found no association between n-3 LCPUFA supplementation and adverse pregnancy outcomes such as induced labor, acute cesarean section, preeclampsia, gestational diabetes or

hospitalization after birth, and there was no difference in APGAR score at 5 minutes between the children in the n-3 LCPUFA and control group. A more broad evaluation of the safety profile has been published elsewhere(11), showing no differences between the groups. Our study was not powered to demonstrate a reduction in preterm delivery or children born small for gestational age after n-3 LCPUFA supplementation in pregnancy, which has been suggested in epidemiological observational studies(11). Future trials should explore whether n-3 LCPUFA supplementation can reduce the amount of preterm deliveries and children born small for gestational age(22).

In countries with low income an improvement in overall fetal growth and birth weight could have a large impact on children's future health(23). Supplementation with n-3 LCPUFA is inexpensive and safe and could be particularly beneficial in these countries since previous studies have reported an association between low income and low intake of n-3 LCPUFAs(24). The potential benefits from fish oil supplementation for improving fetal growth rate and increasing birth weight in developing countries should be further investigated.

Conclusion

Fish oil supplementation during third trimester of pregnancy lead to a prolongation of pregnancy gestation and a higher birth weight. The increase in birth weight was due to an increase in pregnancy duration as well as an increased intrauterine growth. Future studies should focus on fish oil supplementation's potential for improving fetal and infant health.

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TABLES

Table 1 Baseline characteristics of the participants in $COPSAC_{2010}$ mother-child cohort.

	All	Randomization	
		n-3 LCPUFA	Control
	699	49% (346)	51% (353)
Child			
Sex, male % (N)	50.5 (353)	48.2 (166)	53.1 (187)
Caucasian % (N)	95.4(667)	96.8 (335)	95.1(332)
Season of birth			
Winter % (N)	30.9 (216)	28.3 (98)	33.4 (118)
Spring % (N)	26.8 (187)	27.7 (96)	25.8 (91)
Summer % (N)	21.1 (148)	21.1 (73)	21.2 (75)
Fall % (N)	21.1 (148)	22.8 (79)	19.5 (69)
Pregnancy			
Primi-parity % (N)	45.4 (317)	43.9 (152)	46.7 (165)
Smoking in pregnancy % (N)	7.9 (55)	5.8 (20)	9.8 (35)
Cat or dog in pregnancy % (N)	34.9 (244)	35.9 (124)	34.2 (120)
Antibiotics in pregnancy % (N)	45.2 (316)	44.7(155)	46.1 (161)
GA at inclusion, mean (SD), weeks	24.3 (0.7)	24.3 (0.7)	24.3 (0.7)
Hadlock calculated in utero weight[#], mean (SD), g	323.4 (54.0)	321.7 (49.1)	326.0 (58.5)
Assisted reproduction % (N)	9.4 (66)	8.9 (30)	10.6 (36)
Participation in the high dose vitamin D intervention % (N)	84.3 (589)	40.3 (282)	43.69 (307)
Daily fish intake before inclusion mean (SD), g	27.9 (17.5)	27.9 (16.7)	27.8 (18.3)
Maternal pre-treatment blood levels of EPA+DHA[*], mean (SD), %	4.9 (1.2)	4.9 (1.2)	4.9 (1.2)
Parental factors			
Maternal age at birth, mean (SD), years	32.2 (4.5)	32.3 (4.4)	32.1 (4.5)
Maternal pre-pregnancy BMI, mean (SD), Kg/m^2	24.6 (4.4)	24.7 (4.3)	24.3 (4.5)
Maternal asthma % (N)[£]	26.0 (182)	24.4 (84)	27.8 (98)
Paternal height, mean (SD), cm	181.0 (6.7)	181.1 (6.3)	180.8(7.1)
Social circumstances**	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)

[#] *Calculated by the Hadlock equation using the fetal biometrics for head, abdomen and femur.*

***** *Relative percentage of measured blood fatty acids.*

£ *History of doctor diagnosed asthma*

** *Defined as the first component of a principal component analysis on household income, maternal age and maternal level of education at 2 years with a mean value of zero and standard deviation of one. Student's t-test was used for normally distributed continuous variables and chi-square tests for categorical variables to analyze differences between the intervention and the control group. All comparisons were non-significant with p>0.05.*

Abbreviations: GA – gestational age; SD – standard deviation; BMI – body mass index;

Table 2 Effects of fish oil supplementation on primary and secondary end-points

	n-3 LCPUFA N=346	**Control** N=353	**P-value**
Primary end-points;			
Gestational age, median [IQR], days	282 [274–288]	280 [273–286]	**0.02**
Birth weight, mean (SD), g	3,601 (535)	3,504 (528)	**0.02**
Birth length, mean (SD), cm	52.1 (2.4)	51.8 (2.5)	0.15
Marsal-percentage*, mean (SD), %	51.6 (28.4)	47.6 (28.3)	0.06
Skjaerven-percentage*, mean (SD), %	49.9 (28.3)	44.5 (27.6)	**0.01**
Secondary end-points;			
Preterm delivery (GA<week 37) % (N)	3.5 (12)	4.0 (14)	0.88
Born small for gestational age# % (N)	2.3 (8)	1.4 (5)	0.55
Born large for gestational age## % (N)	3.8 (13)	2.3 (8)	0.35
Fetal growth from week 20 to birth, mean (SD), g/day**	23.2 (3.2)	22.8 (3.2)	0.13
APGAR score 5 min <10 % (N)	5.0 (17)	4.3 (15)	0.82
Induced birth % (N)	36 (123)	35 (120)	0.81
Emergency cesarean section % (N)	13.9 (48)	10.5 (37)	0.22
Elective cesarean section % (N)	8.9 (31)	9.9 (35)	0.90
Preeclampsia % (N)	4.4 (15)	4.3 (15)	1
Gestational diabetes % (N)	1.8 (6)	2.9 (10)	0.46

** Calculated percentage of expected birth weight at a certain gestational age using the two standardized growth curves.*

 # Children with a Marsal percentage below – two SD.

Children with a Marsal percentage above two SD.

***Calculated as (birth weight (g) – Hadlock estimated weight (g)) / (gestational age at birth (days) – gestational age at scanning (days))*

Student's t-test was used for normally distributed continuous variables and chi-square tests for categorical variables to analyze differences between the intervention and the control group.

Abbreviations, SD – standard deviations; IQR – inter quartile range, GA – gestational age, N- number

Online tables

Table E1: Product specification for the fish oil supplement (Incromega TG33/22. Croda Health Care. UK).

Parameter	**2008**	**Units**	**Comments**
Unsaponifiable Matter	0.0 – 2.5	%	
Total Omega-3	60.0 – 100.0	%	Levels in the actual product are in the range of approximately 72–76%
Antioxidant. 70% tocopherol	2,500-4,000	ppm	
PCB (7 congener)	0.000 – 0.009	ppm	
Dioxin like PCB / Dioxin like Furans	0 – 4	pg/g WHO -TEQ	
Omega-3 as triacylglycerol	510.0 – 1000.0	mg/g	Levels in the actual product are in range of approximately 620-700 mg/g

Figures

Figure 1: Density plot illustrating gestational age, birth weight and the percentile of weight for gestational age according to Marsal standard growth curves stratified by supplementation groups; n-3 LCPUFA and control.

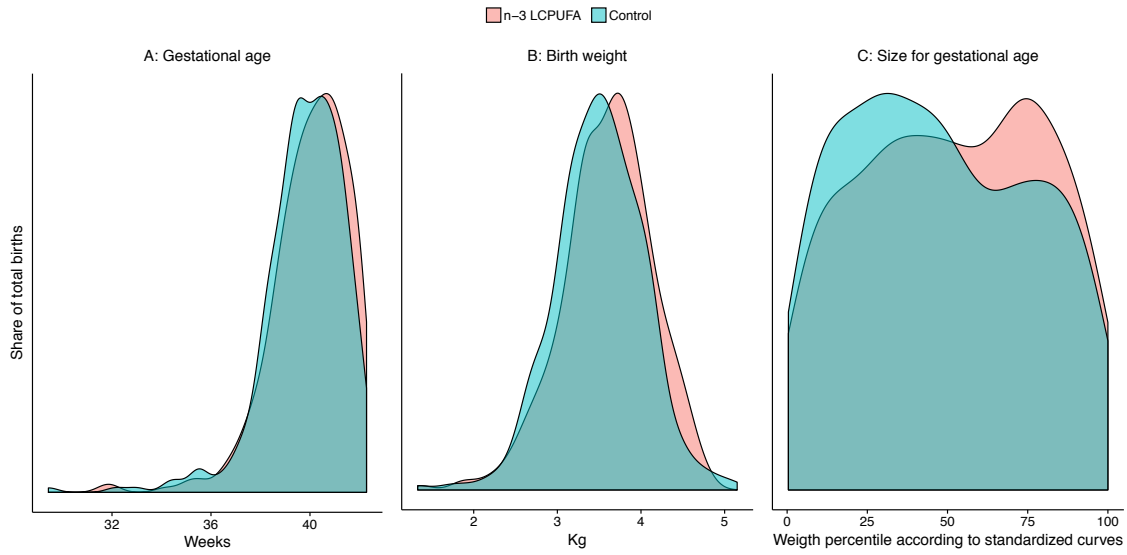
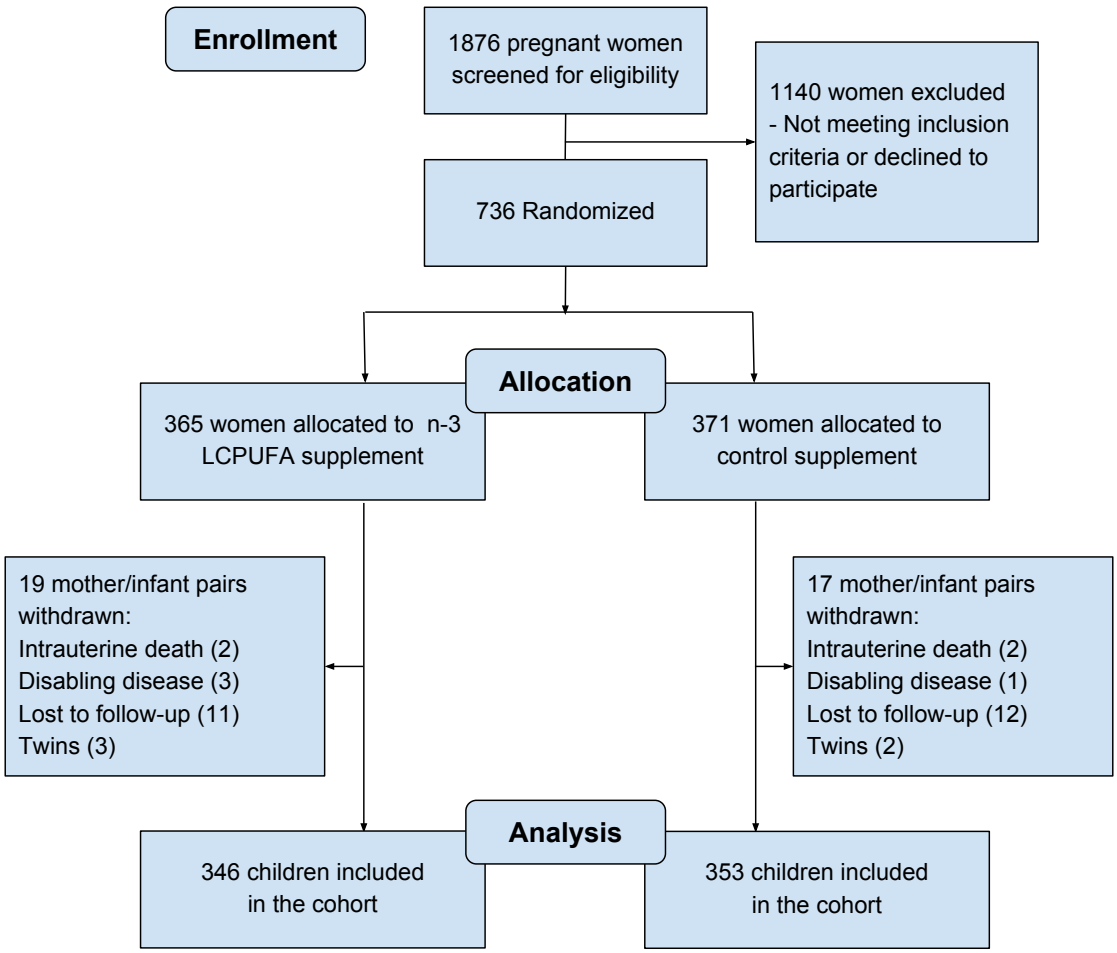


Figure E1. Flow chart of enrollment and allocation of the $COPSAC_{2010}$ pregnancy cohort and follow-up of the $COPSAC_{2010}$ birth cohort.



Paper III

Fish-oil supplementation in pregnancy causes a proportional increase in lean mass, bone mass and fat mass at 6 years: A Randomized, Controlled, Double-Blind, Clinical Trial.

Fish-oil supplementation in pregnancy causes a proportional increase in lean mass, bone mass and fat mass at 6 years: A Randomized, Controlled, Double-Blind, Clinical Trial

Authors: Rebecca Kofod Vinding[1+2], MD, Jakob Stokholm[1], MD, PhD, Astrid Sevelsted[1], MSc, Tobias Sejersen[1+2],MD, Bo Chawes[1], MD, PhD, DMSc, Klaus Bønnelykke[1], MD, PhD, Jonathan Thorsen[1], MD, Laura D Howe[3], PhD, Martin Krakauer, MD, PhD,[4] Thora Buhl, MD, PhD,[4] Hans Bisgaard[1], MD, DMSc;

Affiliation:

1) COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark

2) Department of Pediatrics, Naestved Hospital, Naestved, Denmark.

3) MRC Integrative Epidemiology Unit at the University of Bristol, School of Social and Community Medicine, University of Bristol, Bristol, UK

4) Department of Clinical Physiology and Nuclear Medicine, Herlev and Gentofte Hospital, University Hospital of Copenhagen, Denmark.

Correspondence:

Professor Hans Bisgaard, MD, DMSc

E-mail: bisgaard@copsac.com

Website: www.copsac.com

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Hans Bisgaard: Has consultant arrangements with Chiesi Pharmaceuticals and Boehringer Ingelheim.

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:

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

BMI =Body Mass Index

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

TBLH =Total Body Less Head

ABSTRACT (333 words)

Importance

Some observational studies suggest that maternal fish and fish oil intake during pregnancy affect offspring growth during childhood, but the findings are ambiguous

Objective

To examine the effect of fish oil supplementation in pregnancy on offspring anthropometrics and body composition during childhood in a large randomized trial.

Design, Setting and Participants

A single-center, double blind, randomized controlled trial conducted among 736 pregnant women and their offspring, participating in the Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) mother-child cohort. Exclusion criteria for the present study were twin pregnancy.

Intervention

The pregnant women received capsules containing either 2.4 g of fish oil fish oil or control (olive oil) daily from pregnancy week 24 until one week after birth.

Main Outcomes and Measures

Height/length, weight, head and waist measurements were collected prospectively until 6 years. Dual-energy X-ray absorptiometry (DXA) scans were performed at 3.5 years and 6 years (secondary end-points in the study design).

Results

The fish oil supplementation resulted in significantly higher mean z-score BMI from 0-6 years compared to control: β-coefficient 0.14; 95% confidence interval (CI) [0.13; 0.15]; p=0.006. At 6 years, supplementation was associated with a higher z-score BMI (mean difference fish oil vs. control: 0.19; 95% CI [0.06; 0.32], p=0.004) and a larger waist circumference (0.64cm; 95% CI [0.02; 1.21], p=0.04), but not a higher proportion of children in risk of obesity (5% (N=16) vs 5% (N=14), p=0.89).

The DXA scan at age 6 years showed a higher fat free mass in the fish oil vs. control group (height-adjusted mean difference: 230g (95% CI [37g; 421g]), p=0.02) and a higher bone mineral content (11g (95% CI [3g; 18g]), p=0.007), but no difference in total body fat percentage (0.19%, (95% CI [-0.66; 1.04]), p=0.66.

Conclusion

Fish oil supplementation from 24th week of pregnancy led to an increased BMI from 0-6 years of age, but not an increased risk of obesity, and a body composition at age 6 years characterized by a proportional increase in lean mass, bone mass and fat mass.

Trial Registration

ClinicalTrials.gov: NCT0079822

INTRODUCTION

Diet during pregnancy and infancy is an important determinant of child development and health (1), and in particular, dietary fish intake containing n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) are important for adequate development (2). In humans, both observational studies on dietary intake of fish as well as randomized trials of fish oil (n-3 LCPUFA) supplementation in pregnancy have consistently shown higher birth weight in children born to women with a high n-3 LCPUFA intake (3–6), while the long term effect on growth is more uncertain. Animal studies have shown that n-3 LCPUFA supplementation both in pregnancy and the postnatal period affects the proliferation and differentiation of pre-adipocytes, which could theoretically prevent adiposity development through inhibition of fat tissue development (7,8) . However, there has only been a few randomized trials in humans with n-3 LCPUFA supplementation in pregnancy or during lactation and long term follow-up on growth in childhood and these have shown ambiguous results (9–12).

In the population-based mother-child cohort Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$), we performed a double blind, randomized controlled trial (RCT) of n-3 LCPUFA (fish oil) or control (olive oil) supplementation from week 24 of pregnancy to 1 week postpartum (13). In the present study, we aimed to investigate the effect of n-3 LCPUFA supplementation on growth and body composition in the offspring. BMI development was assessed continuously 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

We identified pregnant women in the eastern part of Denmark by reviewing the monthly lists of reimbursements to general physicians for first pregnancy visits. The screening and information procedure are described earlier(14). Exclusion criteria were gestational age above week 26; any endocrine, cardiovascular, or nephrological disorders, and >600IU/day vitamin D intake.
The primary outcome of the n-3 LCPUFA RCT was persistent wheeze/asthma (13,14). In the current study, we investigated anthropometric measurements through childhood and body composition from DXA scans, which were pre-defined secondary outcomes in the original study protocol (14). For this study, the inclusion criteria was at least one anthropometric measurement and exclusion criteria was twin birth.

Study Intervention

The women were randomized 1:1 in a double-blinded design at inclusion to either daily supplementation of 2.4g/day 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 with identically looking 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 was unblinded when the youngest child reached age 3 years.
A subgroup from this pregnancy cohort also participated in a nested, factorial design, double-blind, RCT of 2,400IU/day vitamin D3 supplementation (N=576) and a smaller subgroup participated in a phase IV randomized, participant-blinded comparison of influenza A/California/2009 vaccines (N=142) (15,16).

Maternal fatty acid desaturase (FADS) genotype

Maternal FADS gene variation was tagged by genotyping of the single nucleotide polymorphism rs1535 (LGC Limited, Hoddesdon, UK) in mothers of European descent. The SNP rs1535 was chosen because it, and it's proxies in close linkage disequilibrium, has been associated with n-3 LCPUFA levels in a genome-wide association study (17) and with blood levels of EPA and DHA during pregnancy (18). The risk genotype (GG) has been associated with lower n-3 LCPUFA levels compared to the non-risk genotypes (AA/AG).

Adherence

See Online methods.

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.

Endpoints

Anthropometrics: Anthropometrics were assessed at the COPSAC research unit at age 1 week, 1 month, 3 months, 6 months, and every sixth month until age 2 years, and hereafter every 1 year until age 6 years.

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

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

Waist circumference was measured with a tape using the navel as fix point. The mean of two measures during inspiration and expiration was used.

Head circumference was measured with a tape, using the largest diameter as measurement. WHO age- and sex specific BMI z-scores(19) were calculated at all scheduled clinical visits from 1 week to 6 years. International Obesity Task Force (IOTF) cut-offs for BMI were used to determine overweight and obesity (above grade zero) and underweight (below grade zero) (20).

Birth length and -weight were obtained at the first clinic visit after birth by personal interview and the values were validated against data from the Danish Medical Birth Registry. If there was a difference larger than 10 g or 5 cm, birth data were further validated against the length and weight measures at 1 week from measured in the COPSAC research clinic.

Birthweight-for-gestational-age percentile rank were derived from Marsal's ultrasound based intrauterine growth curves (21) the scores were calculated as percentage change from the mean size for gestational age of the Marsal population, the mean were set to the value 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 soft tissue mass (total mass minus bone mineral content and fat mass), bone mineral content (BMC, g hydroxyapatite) and bone mineral density for the total body less head (22). In addition, for fat mass and lean soft tissue mass we analyzed specific regions of interest predefined by the software (23–25). Furthermore, we calculated the percentage of fat mass and lean soft tissue mass for total body less head and regions of interest. All DXA scan data were validated by an experienced specialist and analyzed with enCore™ software (GE-Healthcare, Fairfield, USA).

Baseline characteristics

Collection and definition of baseline characteristics of the participants are described in the Online Methods.

Statistical Analysis

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

BMI changes over time were analyzed in a random intercept mixed model with BMI z-scores as the outcome. Time trends in the association between intervention and BMI were investigated in the mixed models by including an interaction-term between visit-ages and intervention group. Missing data were treated as missing observations 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); packages used include lme4; rio; gridExtra; tidyverse; gtools, cowplot. Results with a p-value <0.05 were considered 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 available data from children who completed the 6 years BMI measurement. The 605 children gave us 80% power, to detect a mean difference of 0.19 in z-score BMI, with an 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 began in November 2008 and ended in November 2010. We randomized 736 unselected women at pregnancy week 24 to either n-3 LCPUFA or control supplementation (**Figure E1**); 698 children born to these women were included in the birth cohort[14]. After excluding twins, 688 (XX %) children had at least one available clinical anthropometrics measurement and were included in the study allocated with 341 in the n-3 LCPUFA supplementation group and 347 in the control group. 605 (88%) children had anthropometrics measured at the 6 years visit.

The 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

71% of the women had compliance at minimum 80% with no differences between the n-3 LCPUFA and control group.

n-3 LCPUFA supplementation and BMI development during childhood

Figure 1 illustrates the BMI development from birth to age 6 years according to intervention group, showing that children in the n-3 LCPUFA group had a higher birth weight and BMI at 1 week and a higher BMI from 1 year to 6 years, while no significant difference were seen at ages 1, 3 and 6 months (**Figure 2**).

Using a mixed effects model to capture repeated measurements of BMI, we found that the n-3 LCPUFA supplementation group overall had a significantly higher mean z-score BMI from 1 week to 6 years of age compared to the control group: β-coefficient 0.14; 95% CI [0.04;0.23]; p=0.006. We furthermore observed a significant interaction between age and intervention group (p-value=0.03), which confirmed the growth pattern observed in **Figure 1**. In addition, a sub-analysis only including BMI between 1-6 years of age showed no interaction between age and intervention group (p=0.42).

We found that the difference in BMI development between the intervention groups were similar in boys and girls (**Figure E2**), and there was no significant interaction between sex and intervention group in the repeated measurement analysis (p=0.79).

We previously reported a reduced risk of asthma from n3-LCPUFA supplementation [15], but excluding the children with an asthma diagnosis at age 6 years did not affect the association between n-3 LCPUFA supplementation and BMI (data not shown).

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

523 children completed DXA scanning at 6 years of age, The n-3 LCPUFA group had a higher total mass on the total body less head (TBLH); 19,36 kg vs. 18.97 kg (height adjusted mean difference of 384g (95 % CI [75 g; 694g]), p=0.03), almost identical of the difference we saw for bodyweight. Sub- analyses on tissue type revealed that the children in the n-3 LCPUFA supplementation group had a significantly higher lean soft tissue mass on the TBLH; height adjusted mean difference of 240 g (95 % CI [38g;422g]), p=0.01. They had a tendency to a higher fat mass on TBLH; height adjusted mean difference of 157 g (95 % CI [-73g; 386g]), p=0.18, but no differences in fat mass on the trunk or in the android region and no difference in total body fat percentage or lean mass percentage, nor when we sub-analysed the composition in sub regions (Table 3)

Children in the n-3 LCPUFA supplementation group also had a higher BMC: 11g (95% CI [3g; 18g]), p=0.007 and a trend towards a higher bone mineral density: 0.006 g/cm-2 (95 % CI [0.001; 0.012]), p=0.06 in TBLH.

At 3.5 years of age, there were no significant differences in body composition between the intervention groups, but all estimates for lean soft tissue mass, fat mass and BMC where increased in the n-3 LCPUFA supplementation group (Table E2).

We found no interaction between the intervention and the rs1535 genotype and pre-intervention blood levels of EPA+DHA, respectively, on our DXA outcomes (data not shown).

n-3 LCPUFA supplementation and body composition

523 children completed DXA scanning at 6 years of age, The n-3 LCPUFA group had a higher total mass on the total body less head (TBLH); 19,36 kg vs. 18.97 kg(height adjusted mean difference of 384g (SE [158]), p=0.02), almost identical of the difference we saw for bodyweight. Sub- analyses on tissue type revealed that the children in the n-3 LCPUFA supplementation group had a significantly higher lean soft tissue mass on the TBLH; height adjusted mean difference of 230 g (95 % CI [38g;422g]), p=0.02. They had a tendency to a higher fat mass on TBLH; height adjusted mean difference of 144 g (SE: 116), p=0.22, but no differences in fat mass on the trunk or in the android region and no difference in total body fat percentage or lean mass percentage, nor when we sub-analyzed the composition in sub regions (**Table 3**)

Children in the n-3 LCPUFA supplementation group also had a higher BMC: 11g (95% CI [3g; 18g]), p=0.007 and a trend towards a higher bone mineral density: 0.006 g/cm^{-2} (95 % CI [0.001; 0.012]), p=0.06 in TBLH.

At 3.5 years of age, there were no significant differences in body composition between the intervention groups, but all estimates for lean soft tissue mass, fat mass and BMC where increased in the n-3 LCPUFA supplementation group (**Table E2)**.

We found no interaction between the intervention and rs1535 genotype and pre-intervention blood levels of EPA+DHA, respectively, on our DXA outcomes (data not shown).

Maternal FADS genotype and BMI development during childhood

In a sub-analysis, we investigated if the maternal FADS genotype was associated with offspring BMI development and body composition. We stratified the data by supplementation groups and investigated the difference between children born by mothers with the FADS genotypes associated with higher levels of EPA and DHA (AA/AG) to the children born by mothers with the genotype associated with low levels of EPA+DHA (GG). In the control group, we found that the children born to mothers with the AA/AG-genotype tended to have higher BMI values from 1 to 6 years of age compared to children born to mothers with the GG-genotype (β-coefficient 0.21 SE [0.13]; p=0.06) (**Figure E3**). Similarly, at 6 years of age in the control group, the children born to mothers with the AA/AG-genotype had a significantly higher z-score BMI (mean difference: 0.31; 95 % CI [0.07; 0.54], p=0.01), a higher weight (mean difference: 0.88 kg ; 95 % CI [0.06; 1.70], p=0.04) and trend towards a larger waist circumference (mean difference: 0.9cm 0.; 95 % CI [-0.18; 2.01], p=0.10 compared to children born to mothers with the GG-genotype **(Table E3)**. There was no difference in BMI development over time or the anthropometrics outcomes at 6 years between the maternal FADS genotypes in the n-3 LCPUFA supplemented group.

DISCUSSION

Primary Findings

In this RCT, supplementation with n-3 LCPUFA in third trimester of pregnancy resulted in a higher offspring BMI from age 1 to 6 years and a higher waist circumference at age 6 years compared to children in the control group. Results from DXA scans demonstrated that the increased BMI was not the result of a higher fat percentage but instead reflected a parallel increase in lean soft tissue mass, fat mass and bone mineral content.

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, which increases the external validity of our findings. Adherence to the supplementations was high among the women and the study adherence for the children was also high with 88% having anthropometrics measured at age 6 years.

The longitudinal clinical follow-up including a broad range of anthropometrics to assess growth development and body composition is a significant strength of the study. Each growth measurement was performed using the same equipment by trained COPSAC assistants based on standardized procedures, and the observed growth curves were similar to previous reports (32). In addition, we included DXA scans as an objective measure of fat, lean soft tissue mass and bone mass enabling us to disentangle which tissues were affected by the intervention, to our knowledge we are the first to do so. Even though the results from the DXA scans were insignificant at 3.5 years, the estimates showed the same tendency as the findings from the 6 years DXA scans. The observation of a higher BMI and weight in children born to mothers with FADS genotypes leading to higher n-3 LCPUFA levels provides "genetic support" of our findings from the n3-LCPUFA intervention. The reason that this genotype has not previously been found in larger BMI/obesity GWAS studies (33) may be due to the indirect link between the maternal genotype and the offspring BMI, which has not been analysed.

We had relatively few children in risk of overweight and obesity with only 30 (5 %) of 605 children in the IOTF category for risk of overweight. This is a limitation of our study reducing the statistical power to assure that the n-3 LCPUFA supplementation did not lead to a higher risk of obesity.

Interpretation

We found that n-3 LCPUFA supplementation in third trimester of pregnancy led to higher offspring BMI and increased waist circumference at 6 years of age. Data from DXA scans revealed that the children in the n-3 LCPUFA supplementation group had a higher soft tissue mass. Lean and fat compartments seemed equally affected, resulting in no increase in fat or lean mass percentage, and we observed no increase in number of obese children. Furthermore, we observed a positive effect from supplementation on bone mineral content.

This study is the first to show that n-3 LCPUFA supplementation in third trimester pregnancy leads to a higher BMI throughout childhood. Most randomized trials with n-3 LCPUFA intervention either to the mother during pregnancy or lactation or to the child in infancy have not shown any persistent effects on BMI (34). A review from 2014 and a Cochrane review from 2015 concluded that there is no evidence that n-3 LCPUFA supplementation during pregnancy and/or lactation affects BMI or growth development in childhood (35,36). Only one previous study have reported effects in early infancy in line with ours. Another Danish study supplementing mothers with 1.5 g n-3 LCPUFA (40 % EPA) during the first four months of lactation showed that the children in the n-3 LCPUFA supplementation group had a significantly higher BMI and increased waist circumference at 2.5 years (37), but there were no differences at 7 and 13 years of age (10). There are several potential explanations for the discrepancy between this and previous studies, including differences in the dose and type of n-3 LCPUFA supplied. The dose of n-3 LCPUFA supplied in the current study (2.4 g/d) is higher than in many previous studies (9,11,38,39). Our study is the first large trial with high dose supplement and thereby had a higher statistical power to detect an effect on growth and body composition(34). One other study supplemented from week 30 of pregnancy with the same dose as we did and completed a follow-up on 243 participant at age 19 years. They found no effect on waist circumference or z-score BMI (40).

We previously reported that persistent wheeze/asthma in the first 5 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 throughout childhood in this same group of children could be mediated by an effect on asthma, as it is well known that asthma and asthma treatment may affect childhood growth. However, we did not find any changes in the effect, when we excluded children with asthma.

We have also previously reported that the n-3 LCPUFA supplementation resulted in a prolonged pregnancy duration of 2 days and enhanced intrauterine growth, and as a result of this a higher birth

weight (manuscript submitted) and a difference in birth weight related to gestational age **(**Marsal percentage). However, adjusting the main analysis for Marsal percentage did not change the results, and therefore the increased BMI through childhood does not appear to be driven by the increased intrauterine growth. We did not find any differences on the effect between boys and girls, which is in line with most other studies (35).

Our BMI curves illustrated a difference in BMI for the two groups at birth and one week followed by no significant differences in BMI till age 6 months, where after the n-3 LCPUFA supplementation group had a sustained higher BMI. This difference in infant growth patterns in the two intervention groups could lead to speculations regarding the long term development in BMI. In infancy the BMI peaks at a certain age, and the timing of this peak has been associated to later obesity outcomes, where earlier peak is associated to later obesity The lack of BMI difference in the first year of life could reflect that n-3 LCPUFA supplementation delays the infant BMI peak, so despite the higher BMI during rest of childhood this infant growth pattern may associates with a later adiposity rebound and ultimately end led to a lower BMI later in life (38). This is however purely speculative since we did not include enough measurements to establish the infancy peak or neither have a long enough follow-up to establish adipositas rebound.

Our results could raise the concern that n-3 LCPUFA supplementation in pregnancy could lead to obesity in childhood. However, our DXA results do not support this, since we saw no difference in fat or lean soft tissue mass percentages, but rather a larger amount of both lean soft tissue mass and bone mass 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 being in the highest or lowest 10 percentile BMI at 6 years. It appears that it is mainly the children with BMI in the normal range that are affected by the n-3 LCPUFA intervention. These findings suggest that n-3 LCPUFA supplementation is not associated with an increased risk of developing obesity later in life, which is also supported by previous studies suggesting an effect on growth in infancy (37,38) but not later in life (10) The effect of supplementation on bone mineral content and bone mineral density might imply a positive health benefit in terms of decreased risk of later osteoporosis (42),. Previous studies have implied that intake of total PUFAs and fish is associated with higher BMD (43). However, the clinical implications of our findings can only be speculated on at the current stage. It is important to follow these children through puberty and into adulthood to evaluate the potential long term health effects related to changes in growth and body composition induced by n-3 LCPUFA.

CONCLUSION

Supplementation with n-3 LCPUFA 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, fat mass and bone mass at 6 years. Our findings confirm that n-3 LCPUFA affects fetal programing leading to changed growth during childhood. Further studies are needed to establish the long-term health implications of our findings.

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TABLES

Table 1

	All	Randomization	
		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)	-0.26 (28.4)	1.52 (28.4)	-2.20 (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)	6.2 (0.2)
Age at 6 years DXA scanning (years), mean (SD)	6.2 (0.2)	6.2 (0.2)	6.2 (0.2)
Parent			
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 (Kg/m^2), mean (SD)	24.6 (4.4)	24.7 (4.2)	24.4 (4.6)
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)	45 (310)	49 (151)	51 (159)
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 Marsals intra uterine growth curves.*

£ History of doctor diagnosed asthma

[#]Relative percentage of measured blood fatty acids.

BMI= Body Mass Index, DXA= Dual-energy X-ray Absorptiometry, 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)	0.10 (0.82)	-0.09 (0.83)	0.004
Waist, Mean (SD), cm	55.46 (3.77)	54.82 (3.70)	0.04
Weight, Mean (SD), Kg	21.78 (2.94)	21.40 (2.85)	0.11
Height, Mean (SD), cm	118.16 (4.61)	118.18 (5.14)	0.97
Head, Mean (SD), cm	52.11 (1.42)	52.14 (1.44)	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= Intenational 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 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	P-value
Fat (TBLH) Mean (SD); Estimate difference [SE]	4783.90 (1560.02)	4637.59 (1404.35)	156.61 g [116.88]	0.18*
Fat % (TBLH),Mean (SD), Estimate difference [95 CI-interval]	24.41 (5.02)	24.22 (4.86)	0.19 [-0.66;1.04]	0.66
Fat (Trunk) Mean (SD); Estimate difference [SE]	1800.97 (726.51)	1760.25 (679.66)	46.57g [57.33]	0.42*
Fat % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	18.13 (5.22)	18.03 (5.24)	0.10 [-0.80;1.00]	0.83
Fat (Android) Mean (SD); Estimate difference [SE]	209.60 (112.67)	204.40 (105.29)	6.08g [9.04]	0.50*
Fat % (Android), Mean (SD), Estimate difference [95 CI-interval]	15.05 (5.65)	14.92 (5.76)	0.13 [-0.85;1.11]	0.79
Lean soft tissue mass (TBLH) Mean (SD); Estimate difference [SE]	14030.87g (2024.63)	13794.40g (2078.60)	239.65g [97.76]	0.01*
Lean soft tissue mass % (TBLH), Mean (SD), Estimate difference [95 CI-interval]	72.76 (4.93)	72.95 (4.76)	-0.19 [-1.03;0.64]	0.65
Lean soft tissue mass (Trunk) Mean (SD); Estimate difference [SE]	7750.11g (1025.54)	7653.53g (1060.64)	99.97g [54.23]	0.07*
Lean soft tissue mass % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	79.56 (5.19)	79.67 (5.21)	-0.10 [-1.00;0.79]	0.82
Total BMC (TBLH) Mean (SD); Estimate difference [SE]	546.22g (91.25)	535.06g (94.89)	10.93g [4.04]	0.007**
Total BMD (TBLH) Mean (SD); Estimate difference [SE]	0.56 (0.05)	0.56 (0.05)	0.006 g /cm^2 [0.003]	0.07 **

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

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)	0.10 (0.81)	-0.07 (0.90)	0.07	0.11 (0.83)	-0.09 (0.75)	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 (SE)	P-value
Fat (TBLH) Mean (SD); Estimate difference [SE]	3767.11 (844.48)	3694.06 (809.35)	53.31 [71.16]	0.45*
Fat % (TBLH),Mean (SD), Estimate difference [95 CI-interval]	28.66 (4.55)	28.60 (4.34)	0.07 [-0.73;0.86]	0.87
Fat (Trunk) Mean (SD); Estimate difference [SE]	1403.95 (412.42)	1386.73 (419.02)	9.73 [36.26]	0.79*
Fat % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	20.34 (4.66)	20.40 (4.63)	0.06 [-0.88;0.76]	0.89
Fat (Android) Mean (SD); Estimate difference [SE]	171.14 (59.67)	170.90 (61.58)	-0.60 [5.36]	0.11*
Fat % (Android), Mean (SD), Estimate difference [95 CI-interval]	17.04 (4.77)	17.11 (4.82)	-0.07 [-0.01;0.01]	0.87
Lean soft tissue mass (TBLH) Mean (SD); Estimate difference [SE]	9043.70g (1271.42)	8888.85g (1284.23)	81.02 [73.11]	0.27*
Lean soft tissue mass % (TBLH), Mean (SD), Estimate difference [95 CI-interval]	69.00 (4.50)	69.07 (4.26)	-0.06 [-0.85;0.72]	0.86
Lean soft tissue mass (Trunk) Mean (SD); Estimate difference [SE]	5329.10g (704.86)	5240.08g (863.98)	49.94 [53.40]	0.35*
Lean soft tissue mass % (Trunk), Mean (SD), Estimate difference [95 CI-interval]	77.60 (4.68)	77.55 (4.62)	0.05 [-0.77;0.87]	0.90
Total BMC (TBLH) Mean (SD); Estimate difference [SE]	309.18g (49.12)	304.28g (49.80)	1.30 [3.20]	0.69**
Total BMD (TBLH) Mean (SD); Estimate difference [SE]	0.45 (0.03)	0.45 (0.03)	0.003 [0.003]	0.36**

** Adjusted for height and height^2,** Adjusted for height*
BMC: Bone Mass Content, BMD: Bone Mass 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

Figures

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.

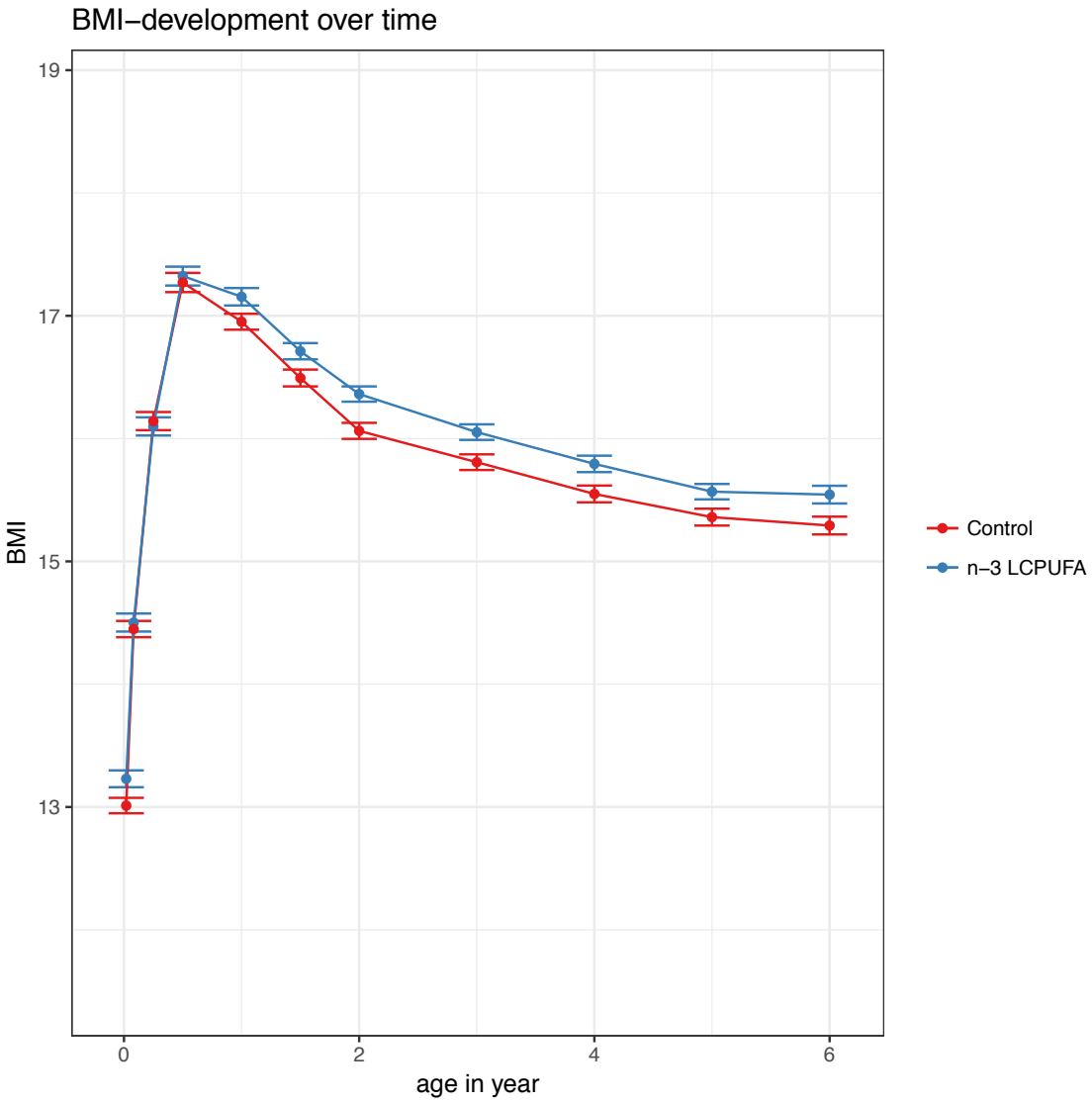


Figure 2

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.

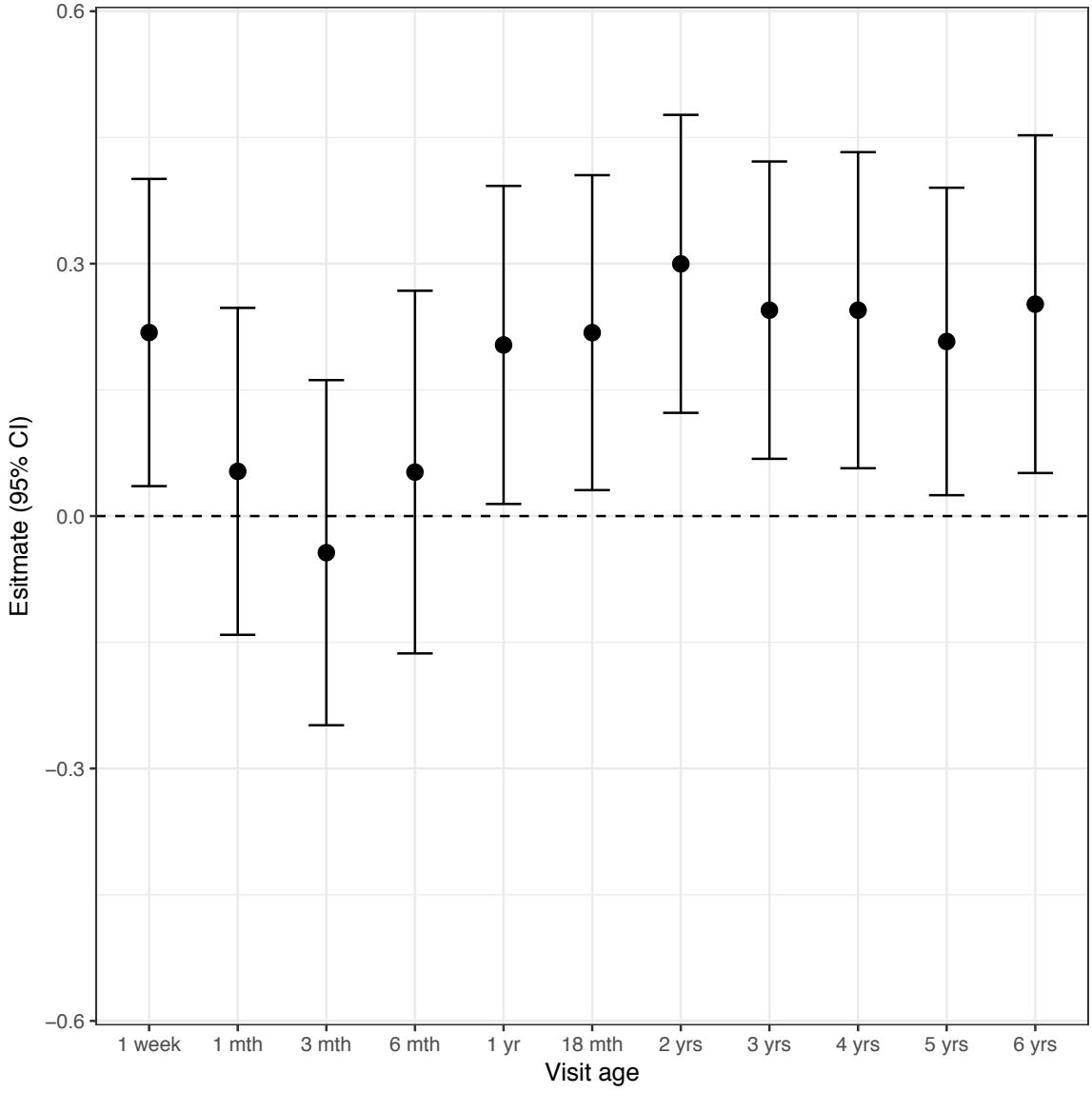


Figure 3

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.

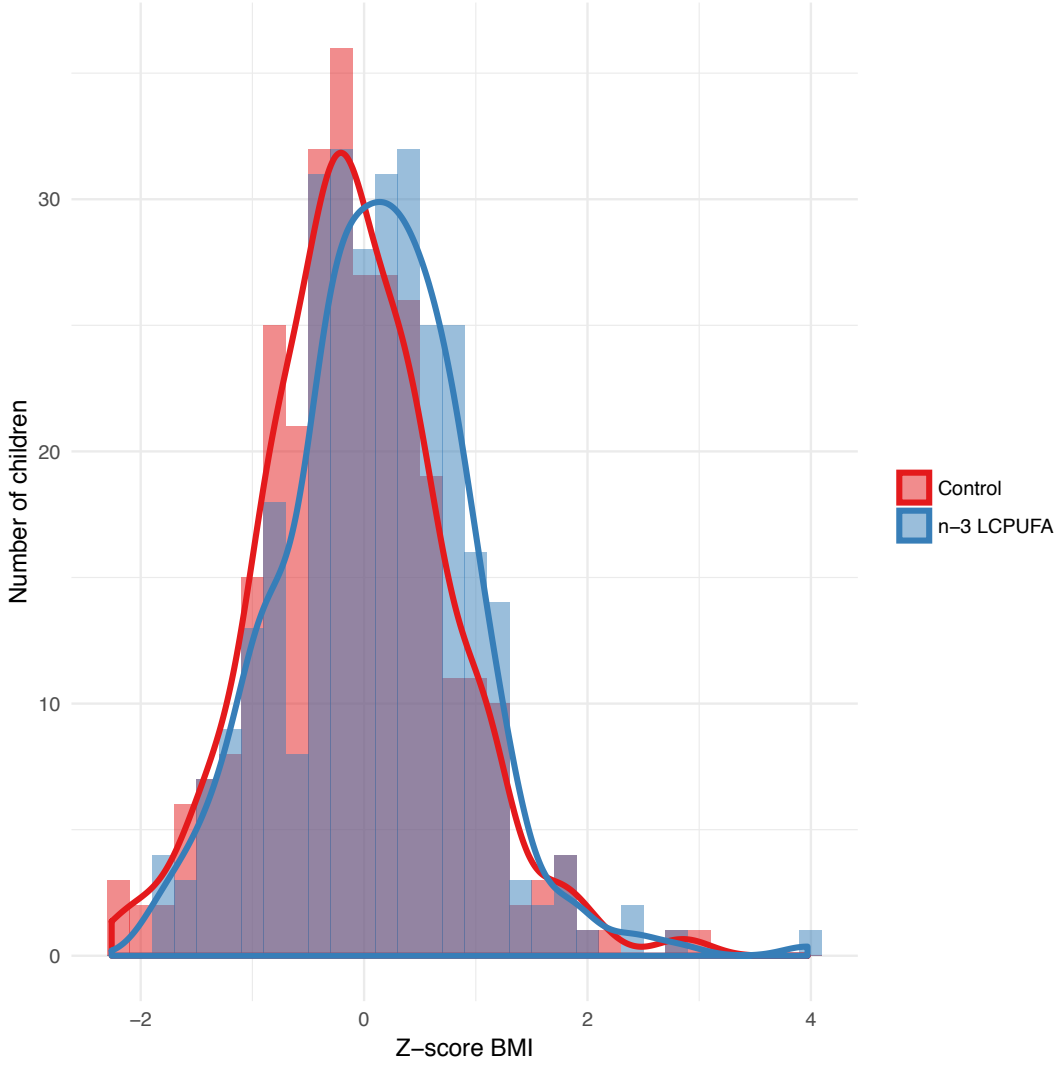


Figure E1

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

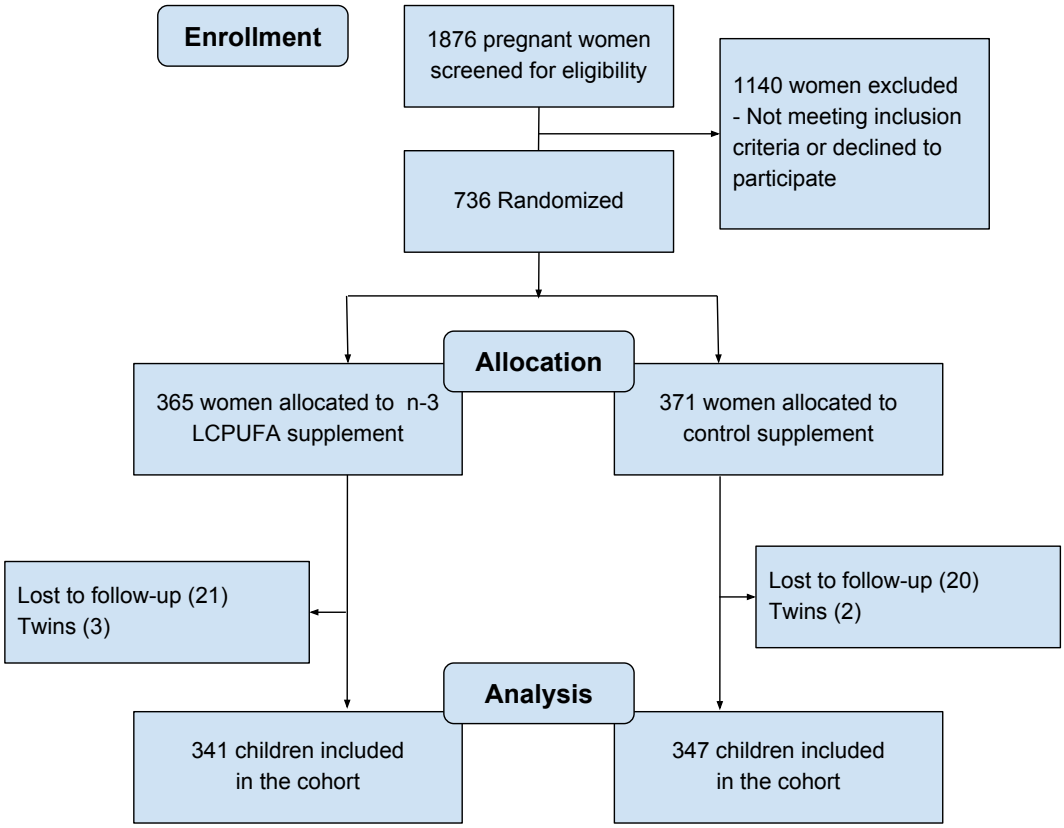


Figure E2

Legend: 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.

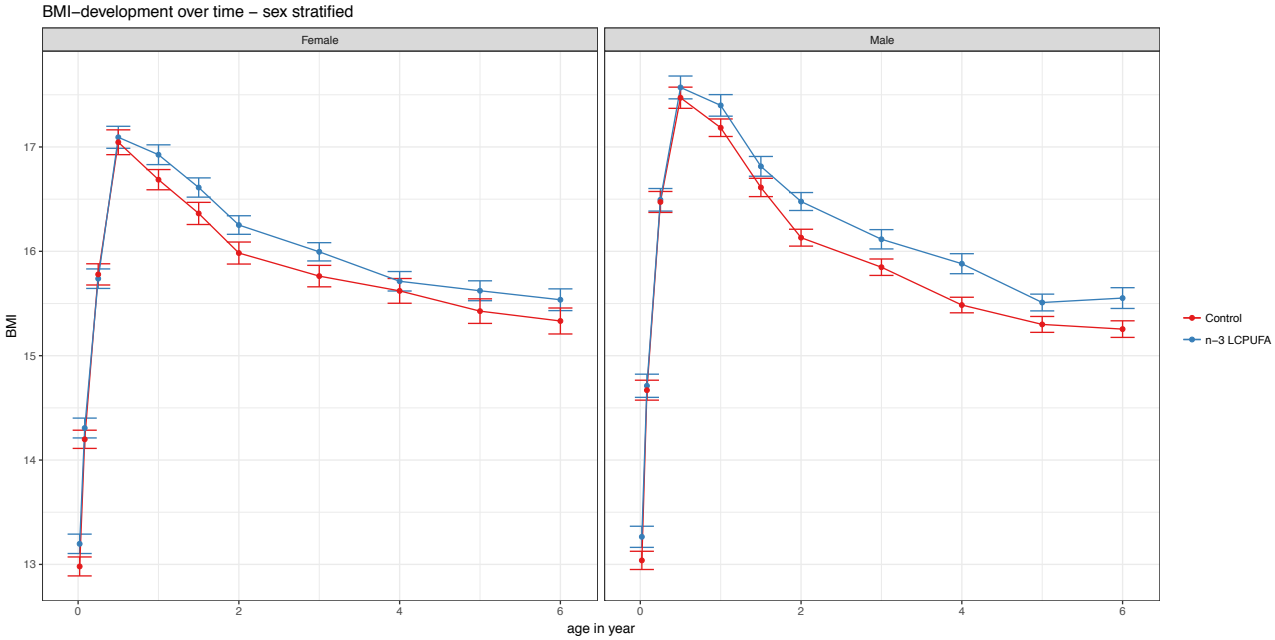
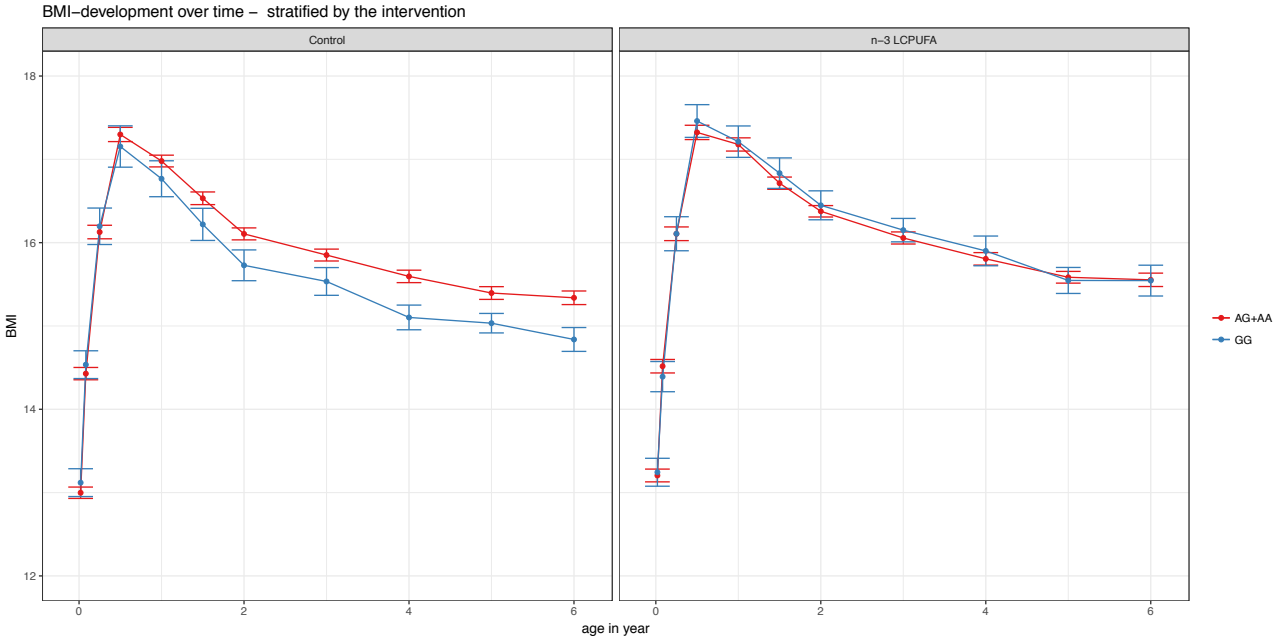


Figure E3

Legend: 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.



GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
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DECLARATION OF CO-AUTHORSHIP

Information on PhD student:	
Name of PhD student	Rebecca Kofod Vinding
E-mail	Rebecca.vinding@dbac.dk
Date of birth	28-09-1983
Work place	Næstved børneafdeling/ Dansk Børne Astma Center
Principal supervisor	Hans Bisgaard

Title of PhD thesis:
Early life exposures and childhood growth

This declaration concerns the following article:
Cesarean Section and Body Mass Index at 6 months and into Childhood

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

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Signature of the co-authors:			
Date:	Name:	Title:	Signature:
	Tobias steen Sejersen	MD	
4/9-17	Bo L Chawes	MD,DMSc	
	Klaus Bønnelykke	MD, PhD	
31/08-17	Thora Buhl	MD, PhD	Thora Buhl

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	Jakob Stokholm	MD, PhD	

Signature of the PhD student and the principal supervisor:	
Date: 6/9-2017 **PhD student:** Rebecca Vinding	**Date:** **Principal supervisor:**

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4/9-17	Jakob Stokholm	MD, PhD	Jakob Stokholm
	Astrid Sevelsted	MSc	
4/9-17	Bo L. Chawes	MD, DMSc	
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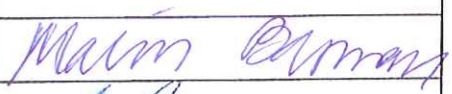
	Malin Barman	PhD	
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4/9-2017	Klaus Bønnelykke	MD, PhD	

1/9-2017	Malin Barman	PhD	
1/9-2017	Bo Jakobsson	MD, PhD	
4/9-2017	Hans Bisgaard	MD, DMSc	

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Date: 4/9-2017 PhD student:	Date: Principal supervisor:

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DECLARATION OF CO-AUTHORSHIP

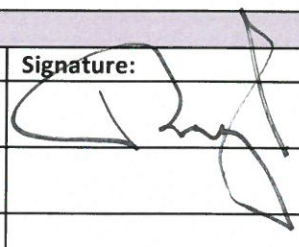
Information on PhD student:	
Name of PhD student	Rebecca Kofod Vinding
E-mail	Rebecca.vinding@dbac.dk
Date of birth	28.09.1983
Work place	Næstved børneafdeling/ Dansk Børne Astma Center
Principal supervisor	Hans Bisgaard

Title of PhD thesis:
Early life exposures and childhood growth

This declaration concerns the following article:
Fish-oil supplementation in pregnancy causes a proportional increase in lean mass, bone mass and fat mass at 6 years: A Randomized, Controlled, Double-Blind, Clinical Trial

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	C
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

Signature of the co-authors:			
Date:	Name:	Title:	Signature:
4/9-2017	Hans Bisgaard	MD, DMSc	

Signature of the PhD student and the principal supervisor:	
Date: 5/7-2017 PhD student:	Date: 4/9-2017 Principal supervisor: