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Neurological Development in the COPSAC$_{2010}$ Birth Cohort and the Effect of Fish Oil Supplementation During Pregnancy

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COPSAC

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I. Elín Bjarnadóttir, Jakob Stokholm, Bo Chawes, Anna-Rosa Cecilie Mora-Jensen, Jonathan Thorsen, Maja Deleuran, Klaus Bønnelykke, Lotte Lauritzen, Hans Bisgaard. Determinants of Neurological Development in Early Childhood. *Submitted to* ***Child Development*** *November 2016.*

II. Elín Bjarnadóttir, Jakob Stokholm, Bo Chawes, Anna-Rosa Cecilie Mora-Jensen, Jonathan Thorsen, Klaus Bønnelykke, Lotte Lauritzen, Hans Bisgaard. n-3 Polyunsaturated Fatty Acid Supplementation during Pregnancy and Neurodevelopment during Childhood. A Randomized Controlled Trial. *Submitted to* ***JAMA*** *November 2016.*

Contents

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Summary

Early life exposure may play a role shaping neurological development and be an important indicator of the child's subsequent life achievements[1–4]. During the last trimester of fetal life and the first two years of childhood, the brain undergoes a period of rapid growth where it reaches 80% of its adult weight. Throughout this period, the brain development is particularly vulnerable to the pre- and perinatal environment including not just biological factors like lack of oxygen or extreme prematurity, but also other factors like socio-demographic determinants, stress and nutrition.

The central nervous system is highly enriched with n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), specifically docosahexaenoic acid (DHA). n-3 LCPUFA is accumulated in the central nervous system during the brain growth spurt from the second half of pregnancy throughout the first two years of life and is dependent on the dietary intake of n-3 LCPUFA. Maternal intake of DHA rich seafood during pregnancy has been associated with improved neurodevelopmental outcome but intervention studies have been ambiguous and inconclusive. Despite this, these findings have caused many women to use fish oil supplement during pregnancy.

The aim of this thesis was to investigate which pre and perinatal factors influence the child's neurological development in the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother-child cohort with focus on the effect of fish oil supplementation during pregnancy.

In **study I** we described the overall neurological development of the cohort children. We explore whether early neurological development was affected by maternal age, maternal education, smoking during pregnancy, gestational age, being a first-born, gender, duration of breastfeeding, paternity leave and age at start daycare and. Furthermore, we examined potential effects of persistent wheeze, eczema and number of sick days in the first years of life.
We found that earlier age of milestone achievement was related to male gender, maternal age, gestational age and paternity leave. A higher 1-year language score was associated with female gender and maternal smoking. The 2-year language score was associated by female gender and being first born. Cognitive score at 2 ½ years was found only to be associated with gender, with the

girls achieving a higher score than the boys, wheras neurodevelopmental scores were unrelated to breastfeeding, persistent wheeze, eczema, and number of sick days.

In **study II** we analyzed the effect of n-3 LCPUFA supplementation during 3rd trimester of pregnancy on the child's neurodevelopmental outcome. The study was double-blinded, randomized placebo controlled trial. The pregnant women received capsules containing 2.4g/day n-3 LCPUFA or matched olive oil capsules from pregnancy week 24 until one week after birth. We found a trend of higher cognitive score in the n-3 LCPUFA group compared to the control group with a significant gender interaction. Gender stratified analysis showed that the boys of the n-3 LCPUFA supplemented mothers scored higher on the cognitive test compared to boys in the control group and achieved motor milestones at a younger age, whereas no differences were seen among the girls.

Conclusion: We have found that gender seems to be the strongest predictor of neurological development during the first years in life. Supplementation with n-3 LCPUFA in 3rd trimester of pregnancy improved cognitive scores and motor development of boys, but did not have any effect on girls. Prescription of dietary fish-oil supplements to pregnant women may therefore optimize neurodevelopment of male offspring.

Sammendrag (Danish Summary)

Eksponering tidligt i livet kan spille en vigtig rolle for den neurologisk udvikling og kan være en vigtig indikator for barnets senere præstation i livet. I sidste graviditets trimester og de første to år af livet, gennemgår hjernen en periode med hurtig vækst, hvor den opnår 80% af sin voksne vægt. I denne periode er hjernen særligt sårbare over for det præ- og perinatale miljø, ikke kun vel kendte biologiske faktorer som iltmanglen eller ekstrem præmaturitet, men også andre faktorer som socio-demografiske determinanter, stress og ernæring.

Det centrale nervesystemet er beriget med n-3 langkædede polyumættede fedtsyrer (n-3 LCPUFA), især docosahexaensyre (DHA). n-3 LCPUFA akkumuleres i det centrale nervesystemet under hjernens vækst fra anden halvdel af graviditeten igennem de første to leveår og er afhængig af indtagelsen af n-3 LCPUFA. Moderens indtag af fisk og skaldyr med højt DHA indehold under graviditeten har været associeret med forbedret neurologisk udvikling hos deres børn, men interventionsstudier har været tvetydige og inkonklusive. Alligevel har disse fund forårsaget at mange kvinder vælger at indtage fiskeolie tilskud under graviditeten.

Formålet med denne afhandling var at undersøge hvilke præ- og perinatale faktorer har indflydelse på barnets neurologiske udvikling i et dansk fødselskohorte (Copenhagen Prospective Studies on Asthma in Childhood 2010, $COPSAC_{2010}$), med fokus på effekten af fiskeolie kosttilskud under graviditeten.

I **studie I** beskriver vi den overordnede neurologiske udvikling af $COPSAC_{2010}$ børnene. Vi har undersøgt, om den tidlige neurologiske udvikling er påvirket af varigheden af moderens alder og uddannelse, rygning under graviditeten, gestationsalder, at være første-fødte, køn, amning, fædreorlov samt alder ved start dagpleje. Derudover har vi undersøgt potentiel effekt af vedvarende hvæsen, eksem og antallet af sygedage i de første år af livet.
Vi fandt, at tidligere opnåede milepæle var relateret til det mandlige køn, moderens alder, gestationsalder og fædreorlov. En højere 1-års sprog score var forbundet med kvindelige køn og mødrenes rygning. Den 2-årige sprog score var forbundet med kvindelige køn og at være første-født. Kognitive score ved 2 ½ års alder blev kun fundet at være forbundet med køn, hvor vi fandt at

pigerne opnåede højere score end drengene. Vi fandt ingen relation mellem de neurologiske test og amning, vedvarende hvæsen, eksem eller antallet af sygedage.

I **studie II** analyserede vi effekten af n-3 LCPUFA tilskud under 3. graviditets trimester på barnets neurologisk udvikling. Denne del af studiet var et dobbelt-blindet, randomiseret placebo kontrolleret forsøg. De gravide kvinder fik kapsler indeholdende 2,4 g / dag n-3 LCPUFA eller tilsvarende olivenolie kapsler fra 24. graviditets uge indtil en uge efter fødslen.
Vi fandt en tendens til højere kognitive score i n-3 LCPUFA gruppen sammenlignet med kontrolgruppen med en signifikant køn interaktion. Køns stratificerede analyser viste at drengene i interventionsgruppen scorede højere på det kognitive test i forhold til kontrolgruppen og opnåede motoriske milepæle i en yngre alder. Til gengæld, var der ingen forskel blandt pigerne.

Konklusion: Vi har fundet, at køn synes at være den stærkeste prædiktor for neurologisk udvikling i de første leveår. n-3 LCPUFA tilskud i 3. trimester af graviditeten forbedrede kognitive scores og motorisk udvikling af drenge, men havde ikke nogen effekt på piger. Fiskeolietilskud til gravide kvinder kan derfor optimere neurologisk udvikling af deres drengebørn.

Abbreviations

AA	Arachidonic acid
ALA	Alpha linoleic acid
Bayley-III	Bayley Scales of Infant and Toddler Development, Third Edition
CI	Confidence interval
$COPSAC_{2010}$	COpenhagen Prospective Study on Asthma in $Childhood_{2010}$
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
LCPUFA	Long chain polyunsaturated fatty acids
PCA	Principal Component Analysis
PC	Principal Component
PPCA	Probabilistic principal component analysis
PUFA	Polyunsaturated fatty acids
WHO	World Health Organization

1. Introduction

1.1. Background

In recent years there has been increasing understanding of health and disease across lifespan and more focus on the early environmental influences that can leave a lasting signature on the genetic predisposition that affect emerging brain architecture and long-term health [5]. Healthy early childhood (including the pre-and postnatal period) has been shown to play a significant role in shaping neurological development and to be an important indicator of the child's subsequent academic and life achievement [1–4].

Neurological development, including cognitive function such as intelligence and language; fine and gross motor skills all depend on the prenatal development of the brain, which is effected by both environmental and genetic factors. During the last trimester of fetal life and the first two years of childhood, the brain undergoes a period of rapid growth termed the "brain growth spurt" [6] and by the age of two the brain has reached 80% of its adult weight. Throughout this period the brain development is particularly vulnerable to the pre- and perinatal environment, not just factors like lack of oxygen or extreme prematurity, but also other factors like socio-demographic determinants [7], nutrition and stress [1,4].

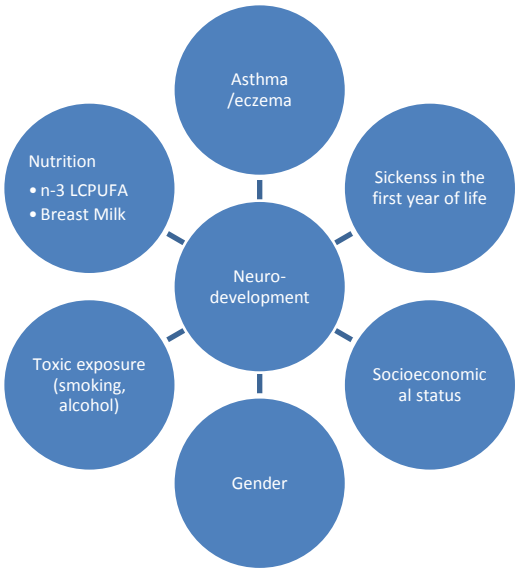
It is unlikely that a single socioeconomic or biological factor leads to developmental delay, but identification of key factors that influence neurodevelopment may allow early identification of developmental problems, prevent complications and possibly enhance the child's neurodevelopment [7] and thereby improve their life prospects.

1.2. Factors influencing neurodevelopment

1.2.1. Gender Differences in Neurodevelopment

There are many factors that can shape the child's neurodevelopment in the first years of life (**Figure 1**) and one of them is gender. Imaging studies have shown that there is gender differences in cerebral anatomy and localization of cerebral functions in children[8] and it is well known that there is difference in several cognitive domains (like verbal and certain spatial skills[9]) between girls and boys from early life.

Figure 1. Schematic presentation of factors that may influence neurodevelopment



Studies on perinatal and neonatal events and exposure have had focus on the premature children. They have shown gender-specific responses on the disadvantage for the boys, but explanation for the differences is unknown[8,10]. It is important to examine if early life exposure effects the genders differently in a healthy cohort-study. Particularly if you want to investigate the effect of an intervention that could affect the genders (in the cultural understanding) and sex (in the biological understanding) differently and, emphasizing the importance that if there is difference in the

outcomes, it is of high importance to be able to analyse boys and girls separately in subsequent studies.

1.2.2. Breastfeeding and Neurodevelopment

The beneficial effect of breastfeeding on infant health is well described, and WHO recommends at least 6 months of exclusive breastfeeding for children globally[11].

The duration of breastfeeding has in previous studies been suggested to influence subsequent neurodevelopment and later academic skills. There are observational studies dating back from 1929 reporting positive effect of breastfeeding on children's intelligence and from 1950 on motor milestone development [12]. These early studies have been followed by large observational studies, two randomized trials and a meta-analysis published on behalf of WHO and all suggest a positive effect in intelligence tests in childhood and adolescence [12].

Many potential confounding variables have been identified to mediate the positive effect of breastfeeding. Social status is one of them. In most countries, social status is related to duration of breastfeeding. But even in countries where breastfeeding is not associated to socioeconomic status, duration of breastfeeding has been found to be directly related to cognition [13]. Stimulation at home has been shown to be related to performance in intelligence tests[14,15] and some studies have suggested that breastfeeding mothers are more likely to stimulate their infants[16].

In studies where it has been possible to adjust for maternal IQ, various socioeconomic variables and the home environment of the child, the beneficial effect of duration of breastfeeding had little or no effect on the children's intelligence[16,17]. It is therefore obvious, that these confounding factors contribute substantially to the positive effect of breastfeeding.

It has however been suggested that the beneficial effect of breastfeeding may be related to is components in breast milk. Breastmilk contains long-chain polyunsaturated fatty acids (LCPUFA), including docosahexanoic acid (DHA) and arachindonic acid (AA). The positive effect of fatty acids on brain development is discussed later. In most countries, mothers of higher social status have higher consumption of fish and fish products that are the main source of n-3 LCPUFA, and this increases the DHA content of breastmilk.

When considering the effect of breastfeeding it is also important to take into consideration what the alternative feed for the children has been. The composition of infant formula has changed over the decades and it was not until the early 2000s that commercially available infant formula in the USA included LCPUFA [18], which has been suggested to be one of the most important beneficial factor of breastfeeding.

In a high income welfare country like Denmark, social status is relatively homogeneous, breastfeeding initiation and continuation is high[19], fish and fish product consumption is moderate[20] and access to high quality infant formula as an alternative feed is easy, it is of importance to explore if there is an association between breastfeeding duration and neurological development.

1.2.3. Asthma, Eczema and Sickness in the First Years of Life

Delayed neurodevelopment in childhood has been associated with chronic inflammatory disease such as asthma, eczema and allergic sensitization in infancy [21]. Most studies have focused on the relationship between neuro-behaviour (such as attention deficit and hyperactivity disorder) and atopic disease[22] and conversely delayed neurodevelopmental scores have also been shown to predate later development of such disorders[23], though in general the studies have shown ambiguous results[22,24].

The interaction between neurological and immunological development may take place in early life, though the mechanism is still not understood.

Several hypotheses are attempting to explain the mechanisms between atopic disease and neurodevelopment and behaviour, either through a common physiologic pathway, e.g. dysregulation of the stress response of the hypothalamus-pituitary-adrenal system (HPA) [21,23,25,26], or from a possible effect of the clinical manifestation and treatment for atopic disease [27].

In **Paper I** we studied the association between different pre- and early life risk factors like gender, breastfeeding and persistent wheeze, eczema and number of sick days in the first years of life have on early neurodevelopment.

1.3. Fish Intake Recommendations and Consumption

In 2007 British Journal of Nutrition published a consensus statement from European and international expert panel groups regarding dietary fat intake recommendation by pregnant and lactating women[28]. They recommend that dietary fat intake during pregnancy and lactation, as a proportion of energy intake, should be the same as that recommended for the general population. They should aim to achieve a dietary intake of n-3 LC-PUFA that supplies a DHA intake of at least 200 mg/d, by consuming one or two portions of sea fish per week (230-340 g), including oily fish. This intake of oily fish rarely exceeds the tolerable intake of environmental contaminants like methylmercury and dioxins[29] that many pregnant women are worried about. However, levels of contaminants in fish, including methylmercury and dioxins, can have large regional differences. It is therefore critical that authorities have information on level of contaminants in fish consumed in their region[29]. In Denmark, pregnant women are advised not to eat more than one can of regular tuna a week and not to eat canned white-tuna or albacore-tuna. Because of high concentration of dioxin in salmon from the Baltic Sea, pregnant women are advised not to consume more than one portion (125 gr) once a month. Lastly they are advised not to consume cuts from large predatory fish because risk of high concentration of methylmercury.

The typical Western diet is notably deficient with respects to n-3 LC-PUFA. It has been estimated that only 19% of American adults consume the recommended 2 servings of fish each week[30] and there has been a decline in fish consumption of pregnant women after a federal advisory regarding methylmercury contamination in certain fish species[31]. A large Danish birth cohort study collected data on maternal dietary exposure[32]. The mothers answered a food frequency questionnaire in gestational week 25. The mothers consumed on average 26 g fish or shellfish per day. That is about half of the recommended amount in Denmark, which is 50 g fish or shellfish per day[33]. A recent national survey shows a general increase in fish consumption, about quarter of the Danish population eat more than the recommended 50 g/d and women (18-75 years of age) consume on average 34 g/dag[34].

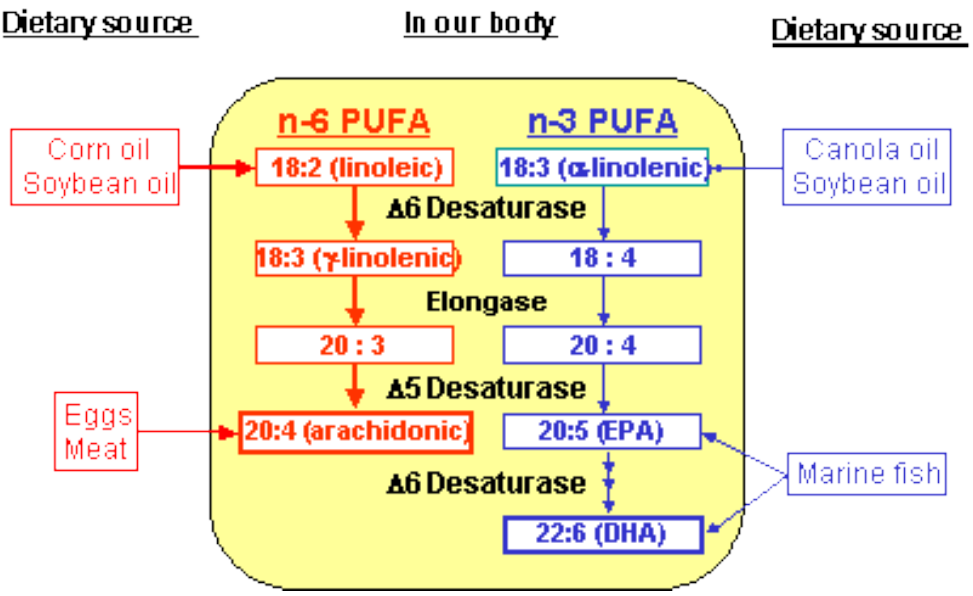
1.4. Fish Oil and the Central Nervous System

The central nervous system is highly enriched in n-3 LCPUFA, specifically DHA, which are accumulated during the brain growth spurt in the second half of pregnancy and the first two years of life [35–37]. DHA play an important role for brain structure, function and metabolism, including neuronal growth and survival, synaptogenesis, membrane fluidity, myelination and expression of genes regulating cell differentiation and growth [38–40].

Brain accretion of n-3 LCPUFA has been shown to be dependent on the dietary intake of n-3 LCPUFA [41,42].

DHA (and eicosapentaenoic acid (EPA)) can be synthesized from the precursor alfa-linoleic acid (ALA) through several steps of desaturation and elongation (**Figure 2**).

Figure 2 - The synthesis of DHA and EPA from alfa-linoleic acid (18:3)[43]



But the synthesis of n-3 LCPUFA, especially that of DHA, appears to be inefficient and has been estimated to <1% of ALA [44,45] though it appears to be higher in infants (in the order of 1%) compared to adults [46] and greater in females compared to males [47]. The endogenous synthesis of DHA is estimated to be too low to cover our needs, specifically in infants and during pregnancy, and is therefore regarded as an essential nutrient[48], that is has to come from the diet. During pregnancy, the fetus relies on maternal supply of DHA which is transported actively in a

preferential maternofoetal manner across the placenta from the mother to the fetus[49,50] in amount depending on the supply of n-3 LCPUFA from the mothers diet. The main source of n-3 LCPUFA is fatty fish, followed by meat, poultry and eggs[51]. Hence, a low maternal fish intake implies a potential risk of inadequate n-3 LCPUFA status in the fetus and neonate.

Epidemiological studies have shown that maternal intake of seafood during pregnancy is associated with improved developmental outcomes of the young child [52,53].

A number of randomized controlled trials (RCT's) [54–62] and a recent meta-analysis [63] have been conducted in order to prove a causal relationship between n-3 LCPUFA intake during pregnancy and neurodevelopment of the young child, but the results have been mixed and inconclusive:

- Helland et al investigated the effect of supplementation with 10 mL of cod liver oil vs corn oil on Norwegian mothers from week 18 in pregnancy until 3 months after delivery[56,57] on the cognitive development of their offspring's at age 6 and 9 months and 4 and 7 years of age. 541 women were randomized, 262 children were evaluated at 6 and 9 months, 84 at the age of 4 years and 143 at the age of 7 years. There was no difference between the two groups at 6 and 9 months of age, but at 4 years the children from the supplemented mothers scored higher on an intelligence test (Kaufman Assesment Battery for Children) in the order of 4.1 points (mean=100). At age 7, the children were tested again with the same test, but they did not find any significant differences in the scores between the two groups.
- Dunstan et al investigated the effect of supplementation with 2.2 g DHA and 1.1 g EPA vs olive oil on Australian women from pregnancy week 20 until birth on neurodevelopment of their offsprings[58]. 98 women were randomized and 72 children were evaluated at 2 ½ years of age. They did not find differences in the childrens language development (Peabody Picture Vocabulary Test) or behavior (Child behavior Checklist), but in Griffihts mental developmental test, the children from the supplemtet mothers scored significantly higher in one out of 7 subtest measuring eye and hand coordination.
- Makrides et al investigated the effect of supplementation with 0.8 g DHA and 0.1 g EPA vs vegetable oil on Australian women from pregnancy week 19 until birth on neurodevelopment of the children. 2399 mothers were randomized and 726 children were evaluated at 18 months of age[54]. They did not find any difference in outcomes form the Bayley Scales of Infant and Toddler Developmental test, third edition between the two groups. However, when analyzing

the genders separately they found that the girls of the supplemented mothers had significantly lower language scores than the girls in the control group and where more likely to have delayed language development. Furthermore, they found that significantly fever boys from the supplemented mothers had a delayed cognitive development. In a follow up study of these children at four years of age (No=703) the differences seen at 18 months could no longer be detected[55].

- Campoy et al investigated the effect of supplementation with 0.5 g DHA and 0.15 g EPA from pregnancy week 20 until 6 months of age on neurodevelopment of the children[60]. The study took place in Spain, Hungary and Germany. 270 mothers were randomized and 154 children were evaluated at 6.5 years of age using Kaufman Assesment Battery for Children. They did not find any differences between the two groups.
- Tofail et al investigated the effect of supplementation of fish-oil or soy oil (4 g/day) during the last trimester of pregnancy on neurodevelopment outcome of infants at 10 months of age[59]. The study took place in Bangladesh and 249 children were assessed using the Bayley Scales of Toddler and Infant Development. They did not find any significant differences between the two groups in any of the outcomes.
- Van Goor et al investigated the effect of supplementation with 220 mg/d DHA, 220 mg/d DHA+AA or placebo, from pregnancy week 16 until 3 months post-partum, on neurodevelopment at 18 months of age[61]. The study took place in The Netherlands. 183 women were randomized and 114 infants were assessed using the Bayley Scales of Infant and Toddler Developmental test, second edition. They found no difference between the groups.
- Ramakrishnan et al investigated the effect of supplementation with 0.4 g DHA vs placebo on pregnant Mexican women, from pregnancy week 18-22 until birth, on neurodevelopment of their children[62]. 1094 mothers were randomized and 730 children were evaluated at 18 months of age using the Bayley Scales of Infant and Toddler Developmental test, second edition. They did not find any differences between the two groups.

Despite the fact that results from the above-mentioned intervention studies have been far from conclusive, fish oil supplementation during pregnancy has become widespread in the westernized world. Probably supported be the fact that many women are concerned about the possible environmental contaminants in fish, but feel pressured to meet the dietary recommendation because

of the supposedly positive effect their unborn child and the fact that it has been shown to reduce the risk of premature birth[64]. It is of high interest to investigate if there are benefits of fish oil supplementation for a child in a country with moderate fish intake, and with the possibility to explore if the effect is different for boys or girls, as done in **Paper II**.

2. Aim and Objectives

The overall aim of this PhD thesis was to investigate which pre and perinatal factors influence the child's subsequent neurological development in their first three years of life.

The specific objectives were:

- In **paper I** we studied pre-natal and early life risk factors of neurodevelopment. We describe the neurological development of the $COPSAC_{2010}$ birth cohort and explored whether early neurological development was affected by duration of breastfeeding, gender, being a first-born, paternity leave, smoking during pregnancy, gestational age, maternal age, age at start day-care, maternal education and furthermore examined potential effects of persistent wheeze, eczema and number of sick days in the first years of life.
- In **paper II** the objective was to determine whether n-3 LCPUFA supplementation during 3^{rd} trimester of pregnancy affects neurodevelopmental outcomes during early childhood. We furthermore wanted to explore if the effect was different for girls and boys.

3. Methodology

3.1. Design, Setting and Participants

COPSAC$_{2010}$

Both studies in this thesis are based on data from the novel Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC$_{2010}$)

COPSAC$_{2010}$ is an ongoing Danish cohort study of 700 unselected children followed prospectively from pregnancy week 24 in a protocol designed from the first COPSAC birth cohort (COPSAC$_{2000}$) [65–67]. Participants were recruited during 2008-2010. Written invitations were sent to pregnant women in Zealand. Women who were interested in participating were interviewed by telephone and subsequently attended the COPSAC clinical research units at Gentofte and Naestved University Hospitals for enrolment at gestational week 24 in a protocol previously described in details [68]. The key exclusion criterions were chronic cardiac, endocrinological, nephrological or lung disease other than asthma; participating in other clinical trial; or not being able to speak fluent Danish. The women were randomized 1:1 to fish oil supplements and a concomitant factorial designed double blind RCT of high dose vitamin D during their pregnancy. Children with a neurological diagnosis, born <37 weeks of gestation and birth weights <2500g where excluded from the studies in this thesis.

The main research focus of this ongoing cohort study is the effect of perinatal exposure and maternal nutrition during pregnancy on immune modulation towards a trajectory of chronic inflammatory disease such as asthma, eczema and allergy. The focus of this thesis, the children's neurodevelopment is a secondary outcome. The families attended the research unit for 2 planned visits in pregnancy and 9 planned visits after birth until the age of 3 years of age, following the principles of longitudinal accumulation of data.

Data validation and quality control followed the guidelines for "Good Clinical Practice". Data was collected during visits to the clinical research unit and stored into a dedicated online database. This

database was double-checked against source data and subsequently locked. An audit trail was run routinely.

In the following sections, procedures, methods and variables of relevance for this PhD thesis will be explained in further detail.

3.2. The Randomized Controlled Trial

From pregnancy week 24 to 1 week after delivery, the pregnant women where randomized 1:1 in a a double-blind, placebo-controlled, parallel-group study to receive a daily dose of either 4 grams of fish oil supplement, providing 2.4 g/day n-3 LCPUFA (55% eicosapentaenoic acid (EPA) and 37% DHA), or identically looking control supplementation of olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid (LA)). The dose of the n-3 LCPUFA was estimated to increase the daily intake of n-3 LCPUFA 6-fold relative to the normal daily intake of pregnant Danish women [32]. The olive oil supplement was estimated to contribute 3% of normal daily intake of linoleic acid. The women were instructed to take the capsules from the day of randomization until one week after delivery and to return any unused study capsules. Allocation was done by a simple randomization procedure using a computer-generated list of random numbers prepared by an external investigator with no other involvement in the trial. The study intervention was maintained double blinded until the youngest child in the cohort turned 3 years. Recruitment for the trial began November 10, 2008, and ended November 17, 2010, and the youngest child was born March 17, 2011.

The high dose D vitamin intervention, which is not a subject of this thesis, was conducted parallel to the fish oil intervention. The mothers were randomized 1:1 to a daily dose of 2400 IU vitamin D_3 supplementation or matching placebo tablets.

3.3. Baseline Characteristics

Information regarding baseline characteristics was collected prospectively during the scheduled visits to the COPSAC clinics. This included information regarding pregnancy and birth (i.e. gender, ethnicity, anthropometric data, congenital disease, gestational age, maternal smoking during pregnancy and maternal age at delivery), home environment (i.e. older siblings, language spoken), socioeconomic and educational status, paternal leave and age at start in daycare. Information on

breastfeeding was obtained longitudinally by interviews and investigated as duration of exclusive breastfeeding and total breastfeeding period. Breastfeeding was evaluated as both continuous variables and grouped: Exclusive breastfeeding (0-1 month, 1-4 months, 4-6 months, >6 months) and total breastfeeding (0-1 month, 1-4 months, 4-6 months, 6-12 months, >12months). In study II we used the definition ”social circumstances” which is a PCA component that consists of household income, maternal age and maternal educational level at the age of 2 in the baseline table.

3.4. Assessment of Neurodevelopment

Milestones

At the child's 1 week visit the parents received a registration form, with thorough instructions, based on The Denver Development Index [69] and WHO (World Health Organization) milestones registration [70]. Dates of achievement of 13 predefined milestones were registered by the parents and reviewed at each visit to the research clinic. The registration form contained a description and an illustration of the milestones. Any difficulties in remembering the specific date were registered as “missing”. The clinical staff carefully reviewed the forms with the parents in order to standardize the registration, and thus minimize differences in interpersonal interpretations. Implementation of milestone registration started after the first 500 children were born, thus some of the milestones were registered retrospectively.

Language development

Language development was assessed with the Danish version of The MacArthur Bates Communicative Developmental Inventory (CDI), which is a well-recognized and validated tool to assess monolingual children´s lexical growth by a standardized parent reporting system [71]. The test was performed as a web-based questionnaire filled out by parents around the child’s 1 year (CDI-WG: Words and gesticulation) and 2 years birthdays (CDI-WS: Words and sentences). The 1-year test evaluates language comprehension, early word production and gestural communication. Language comprehension was assessed by counting the number of words that the parents think the child understands from a list of 409 words which are commonly found in the vocabulary of Danish children around 1 year of age. The assessment of word production was based on the same list and counts the number of words the child actually says or does a lingual imitation of. Gestural communication was assessed by questions regarding the use of gestures typical of early and later communicative development (numbers used out of 18 and 45, respectively). The 2-year test

assesses vocabulary, grammatical skills, syntax and morphology. Vocabulary was assessed by counting the number of words the child pronounces from a list of 725 common words. Grammatical skills were determined from the use of past, future, abstract, plural, possessive and past tense and the number of irregular and over-regularized words. Syntax and morphology was assessed according to whether the child combines words and the mean length of the three longest utterances (M3L: calculated from number of morphemes per utterance in the 3 longest sentences the child has said in the previous two weeks). The CDI was not performed in the first 209 participants as it was implemented in the cohort after they had completed their 1 year visit. Children who were considered bilingual (regularly in contact with another language than Danish at home) where excluded from the language development analysis (40 from the 1 year test and 51 from the 2 years test). Language tests completed when the child was more than 3.5 months older than intended were excluded (9 children).

Cognitive development

Cognitive development was assessed at 2½ years of age, using the cognitive part of the 3rd edition of the Bayley Scales of Infant and Toddler Development (BSID-III) [72]. The test was given on an individual basis lasting 20–60 minutes. The scale includes items that assess abilities such as sensorimotor development, exploration and manipulation, object relatedness, concept formation, memory, and other aspects of cognitive processing. The examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on its performance, the child was given a composite score, which was standardized by use of a normalization material of age corrected means of 100 and standard deviation of 15 (range 50-150). Ten examiners were involved in performing the test and they were all trained by a single expert in the Bayley test procedure (first author). The sessions were video-recorded and the Bayley expert continuously reviewed the videos and supervised the test-persons in order to achieve consistency in all aspects of the testing procedure.

General development

At three years of age the parents filled out the Danish version [73] of the ASQ-III questionnaires [74] which is a brief measure of the child's current skills and development in the areas of communication, gross motor skills, fine motor skills, problem solving, personal-social skills. The questionnaire consists of 30 items (six in each category) and each item is scored depending upon whether the child performs the item consistently (10 points), sometimes (5 points), or not yet (0

points). Scores for each area where then summed. The ASQ-III was not performed in the first 124 participants as they were too old when the testing was implemented (>3 years and 3 months).

3.5. Clinical Predictors of Neurodevelopment

Respiratory and skin symptoms were recorded by the parents in daily diaries from birth to 3 years.

Persistent wheeze was diagnosed according to a previously validated quantitative algorithm[66,75] requiring all of the following:

1) Recurrent troublesome lung symptoms (verified diary recordings of ≥5 episodes of troublesome lung symptoms lasting ≥3 days within 6 months or continuous troublesome lung symptoms >4 weeks)
2) Typical symptoms of asthma, e.g. exercise induced symptoms, prolonged nocturnal cough, persistent cough outside common cold
3) Need for intermittent bronchodilator
4) Response to a 3-month trial of inhaled corticosteroids and relapse upon cessation[66].

Eczema diagnosis was based on the criteria of Hanifin and Rajka, which requires the presence of 3 of 4 major criteria and ≥3 of 23 minor signs[76].

Diagnoses of persistent wheeze and eczema at any time point before age 3 were used as dichotomized end-points.

Sick days: Infections, categorized as common cold, pneumonia, pharyngitis, otitis, fever, gastro-intestinal infection were monitored in the daily diaries. The number of days with either troublesome lung symptoms and/or infection in the child's first year was used as a continuous variable to define "number of sick days in first year of life".

3.6. Maternal whole-blood LCPUFA levels

Adherence: Adherence to the n-3 LCPUFA intervention was assessed by comparing the number of capsules provided for the intervention period with the number returned. In addition, maternal whole-blood % EPA+DHA levels (relative percent of total fatty acids) were assessed at the time of randomization and at completion of the RCT [77,78].

Whole blood samples were collected by veni-puncture in the presence EDTA at time of randomization and at completion. Samples (500 µL) were aliquoted to cryovials and mixed with 50µL 0.1% 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene; Sigma-Aldrich, St. Louis, MO, USA) in ethanol. Samples were then purged with nitrogen, and frozen and stored at -80 °C for a maximum of one year. Fatty acid composition was determined as described previously [79]. Briefly, fatty acid methyl esters were prepared by direct transesterification using 14% boron trifluoride in methanol (Pierce Chemicals, Rockford, IL, USA) with hexane containing butylated hydroxytoluene (50 µg/mL) and an internal standard (22:3n-3 ethyl ester; Nu-Check Prep, Elysian, MN). Fatty acid methyl esters were recovered and analysed using fast gas chromatography. The data was quantified and then expressed as the percentage of each fatty acid with the total fatty acid sum. Maternal EPA+DHA relative percentage of blood fatty acids pre- and post-intervention was calibrated for the blood sample storage time at –80° C prior to analysis using the regression coefficient of EPA+DHA vs. storage time standardizing for the mean storage time.

Maternal whole-blood levels of LCPUFA including EPA+DHA were maintained double-blinded until the youngest child in the cohort turned 3 years.

3.7. Statistical Analysis

All variables were tested for normal distribution. The differences in the population characteristics were determined by Chi-square test, Student's t-test, or Wilcoxon rank-sum test.

In **paper 1** when analysing the predictors of neurodevelopmental test scores a linear or logistic regression analysis was used where appropriate. All the *a priori* factors identified as possible predictors of neurodevelopment where included in the model (gender, parity, smoking during pregnancy, gestational age, maternal age at delivery, maternal educational level, breastfeeding duration, paternal leave, and age at start in daycare). Analyses of clinical predictors (persistent wheeze, eczema and sick days) were adjusted for gender.

In both papers we used principal component analysis (PCA) to extract underlying latent components (principal components, PCs), which describe the systematic part of the variation across the original milestones (used in **paper I and II**) and language variables (used in **paper I**) in fewer uncorrelated variables. For the milestone data we used probabilistic PCA (R) to generate the PCs, as this analysis can analyze full data set with missing values, assuming that the missing values are at random. No transformation of data was needed for language data as there were no missing values.

To examine correlations between neurological endpoints in **paper I**, a heatmap was drawn based on spearman correlations between all variables, using pairwise complete observations. For presentation, the variables were clustered using hierarchical clustering of euclidean distances, and drawn using the R package "pheatmap"[80]

In **paper II** the effect of the n-3 LCPUFA intervention on the composite BSID-III cognitive score was assessed using linear regression models. Probabilistic principal component analysis (pPCA) for the milestone were used to assess if there was an overall effect on milestone development with subsequent analysis by linear regression analysis of individual milestones for interpretation. Intervention effects on language development were assessed using Poisson regression and the effect on ASQ-3 general development was analyzed using Wilcoxon signed-rank test.

Effect modification of gender was done *a priori* by inclusion of a treatment-gender interaction term, because previous studies have suggested a gender specific effect of n-3 LCPUFA supplementation [54,81] . The results were presented for all children and stratified by gender with p-values for both main effects and treatment-gender interaction.

Analyses were also performed with adjustment for maternal pre-intervention whole blood EPA+DHA levels as a continuous variable and the vitamin D RCT allocation. Where appropriate results are reported with 95% confidence intervals [CI]. All P values are 2-sided, and the level of significance was set at 0.05. Missing data was treated as missing observations, except in the milestone pPCA as previously described.

The data processing was conducted using SAS version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA) and R version 3.3.0 (R core team 2016), with the packages "ggplot2", "ggbiplot2" and "ggrepel"[82].

Study Power (**Paper II**)

The intervention trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the statistical power of the RCT on neurodevelopment was calculated *post-hoc* based on the eligible children for such sub-analysis. A total of 649 children were included in the neurodevelopmental outcome assessment and 600 of these completed the BSID-III cognitive test.

The power calculation based on 600 children demonstrated over 80% power (α=0.05, two-tailed) to detect a 5 points difference[54] between the two intervention groups for boys and girls, separately. Additional methodological details are outlined in the $COPSAC_{2010}$ design paper [68].

3.8. Ethics statement

This study was performed according to the principles of the Declaration of Helsinki and the main pregnancy- and birth cohort study was approved by the Local Ethics Committee (H-B-2008-093) and the Danish Data Protection Agency (2008-41-2599). The n-3 LCPUFA supplementation RCT in pregnant women and assessment of the children's neurodevelopment was approved separately under the same protocol numbers and registered at ClinicalTrials.gov (NCT00798226). Written informed consent was obtained from all families.

4. Results

4.1. Paper I – Determinants of Neurological Development in Early Childhood

Baseline

A total of 700 children were included in the $COPSAC_{2010}$ birth cohort with a clinical follow-up rate of 98% at age 1 year; 95% at age 2 years; and 94% at age 3 years. 34 children were excluded from the neurodevelopmental analysis: 5 because of a neurological diagnosis, 24 because they were born prematurely and 5 because of low birth weight (<2500 gr). Furthermore, 16 children did not complete any of the neurological tests, leaving 650 eligible children with neurodevelopmental assessment. **Figure 1** shows the flow of participants throughout the study.

Table 1 shows the baseline characteristics of the participants. The children were solely breastfeed for 104 days (SD 60) and the total length of breastfeeding was 245 days (SD 155). During the first year of life, the children had a median of 48 (IQR, 28-79) days with either an infection and/or troublesome lung symptoms. By the age of 3 years, persistent wheeze had been diagnosed in 19% (123 children), and eczema in 25% (165 children). There were no significant differences in the baseline characteristics between the children, who completed the neurological endpoints and the ones, who did not.

Figure 1. Flow chart of the study participants through the trial and main outcome measures

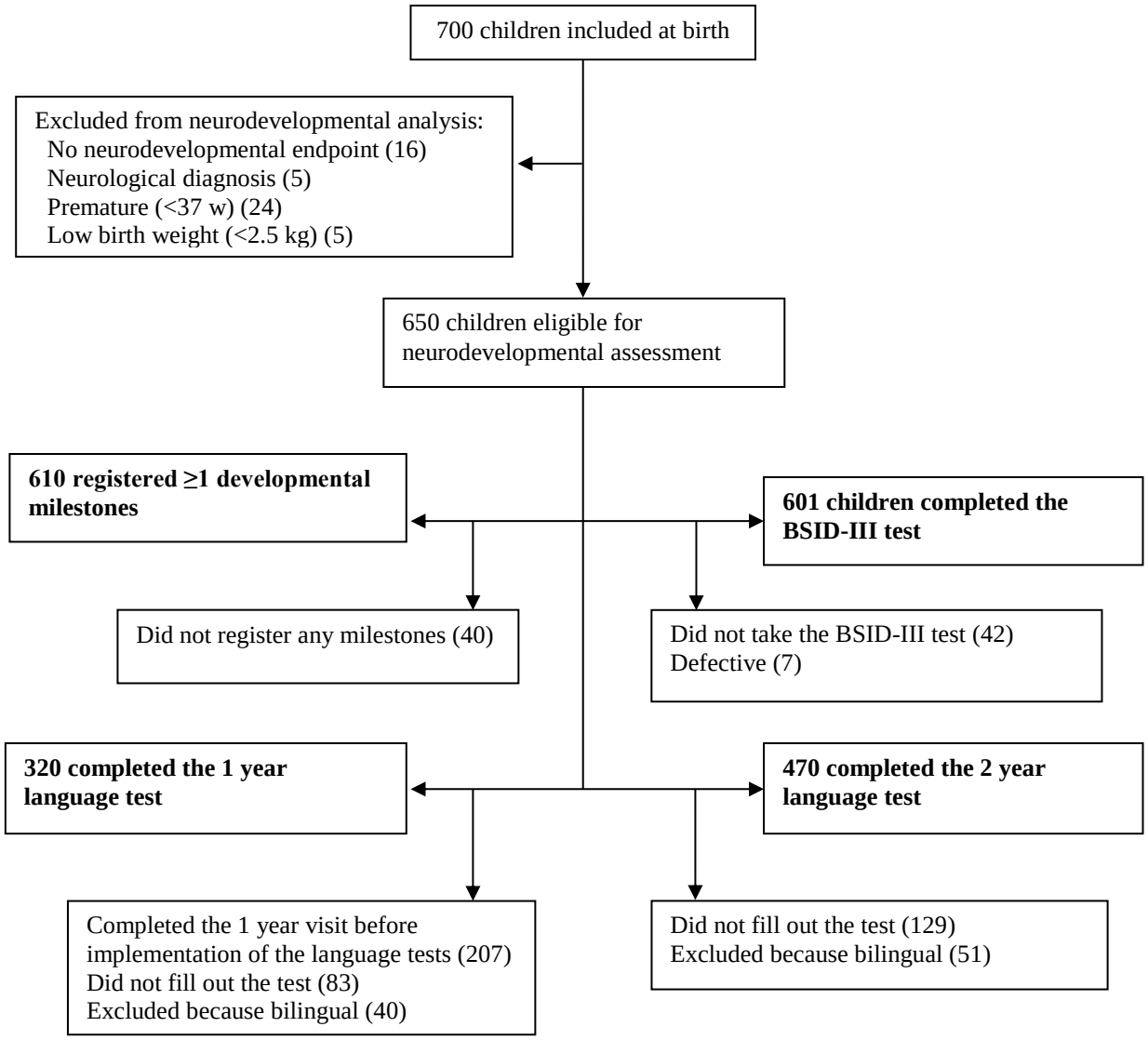


Table 1. Baseline characteristics of the $COPSAC_{2010}$ cohort and the children that completed the different neurological test outcomes.

	All n=700	Milestones n=610	1 y language n=320	2y language n=470	Bayley n=601
Birth cohort					
Boys, % (n)	51% (360)	51% (297)	50% (160)	51% (236)	51% (293)
Mother's age at birth, mean ±SD, years	32.3 ±4.4	32.3 ± 4.3	32.3 ± 4.2	32.3 ± 4.2	32.3 ± 4.3
Caucasian % (n)	96% (669)	96% (588)	97% (323)	98% (463)	96% (580)
Pregnancy and birth					
Gestational age, mean ±SD, weeks	39.9 ± 1.7	40.1 ± 1.3	40.1 ± 1.3	40.1 ± 1.2	40.1 ± 1.2
Weight 1 week, mean ± SD, kg	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5
Length 1 week, mean ± SD, cm	52.1 ±2.2	52.1 ± 2.1	52.2 ± 2.1	52.1 ± 2.2	52.1 ± 2.1
Head circumference at 1 week, mean ± SD, cm	35.7 ±1.4	35.7 ± 1.3	35.7 ± 1.3	35.7 ± 1.3	35.7 ± 1.4
Apgar score at 5 min., mean ±SD	9.9 ±0.34	9.9 ± 0.33	9.9 ± 0.23	9.9 ± 0.33	9.9 ± 0.33
Exposure					
Birth order, % first born, (n)	46% (323)	45% (279)	44% (145)	43% (205)	46% (277)
Smoking during pregnancy, % (n)	7.7%(54)	7.2% (44)	6.0% (20)	6.1% (29)	7.4% (43)
Duration of solely breastfeeding, mean ± SD, days	104 ± 60	104 ± 59	105 ± 59	107 ± 58	105 ± 59
Duration of total breastfeeding, mean ± SD, days	245 ± 155	246 ± 147	248 ± 140	251 ± 142	248 ± 152
Age at start in daycare, mean ± SD, months	10.8 ± 3.1	10.8 ± 3.1	10.9 ± 3.2	10.7 ± 3.1	10.8 ± 3.0
Paternity leave >4 weeks, % (n)	53% (354)	54% (323)	55% (179)	54% (249)	53% (313)
Socioeconomic variables					
Maternal educational level (% Low: Medium: High) [1]	8:64:28	7:65:28	7:64:30	7:64:29	8:65:28
Annual household income (% Low: Medium: High) [2]	10:53:37	10:52:38	9:51:40	10:51:40	10:52:38
Clinical predictors[3]					
Persistent wheeze, % (n)	19% (123)	18% (108)	19% (61)	18% (83)	18% (108)
Eczema, % (n)	25% (165)	26% (153)	26%(85)	26% (121)	26%(154)
Days sick in first year of life, median (25^{th}-75^{th} percentile)	48(28-79)	49 (28-80)	47 (28-74)	50 (28-82)	48 (28-80)

[1]*: Low (elementary school or college graduate), Medium (tradesman or medium length), and High (university candidate)*
[2]*: Low (<*55.000 *Euros/year), High (>110.000 Euros/year)*
[3] *Prevalence of persistent wheeze and eczema in the 660 children who have full follow up to 3 years. Days sick in first year of life is given for the 522 children with a full diary registration (365 days)*

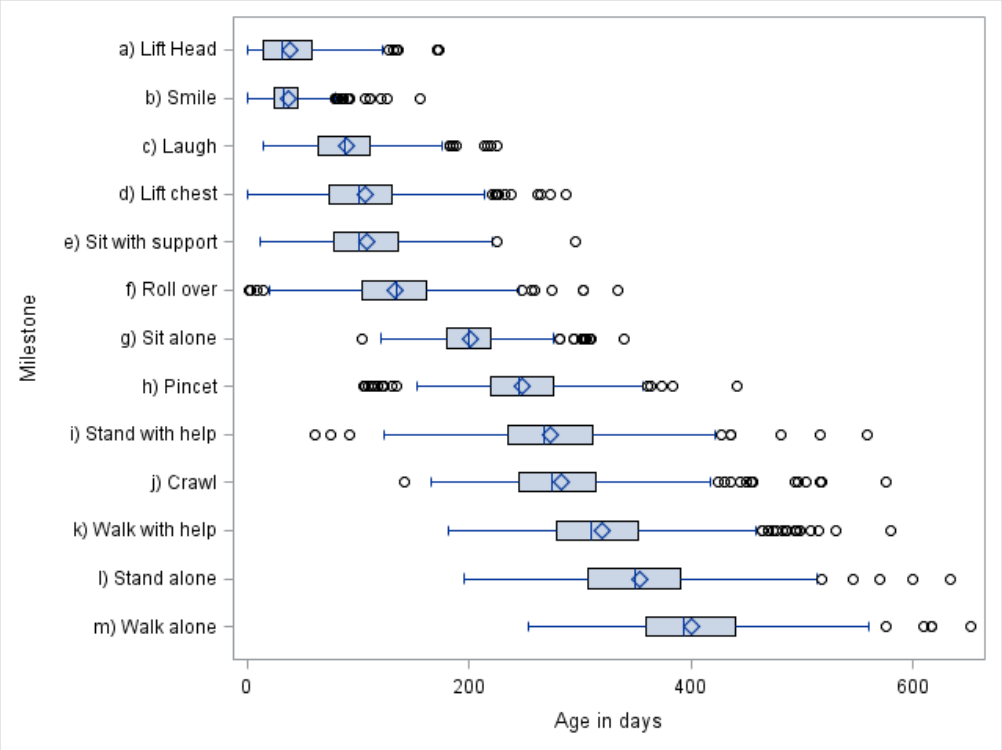
Neurodevelopment

For all the multivariate predictor analysis of neurodevelopment, the estimate for each predictor is adjusted for all the other predictors using a multiple linear regression model. The effects are expressed as either differences in means (categorical variables) or β (continuous variables) with (95% CI) using the neurodevelopmental score as the outcome.

Milestones

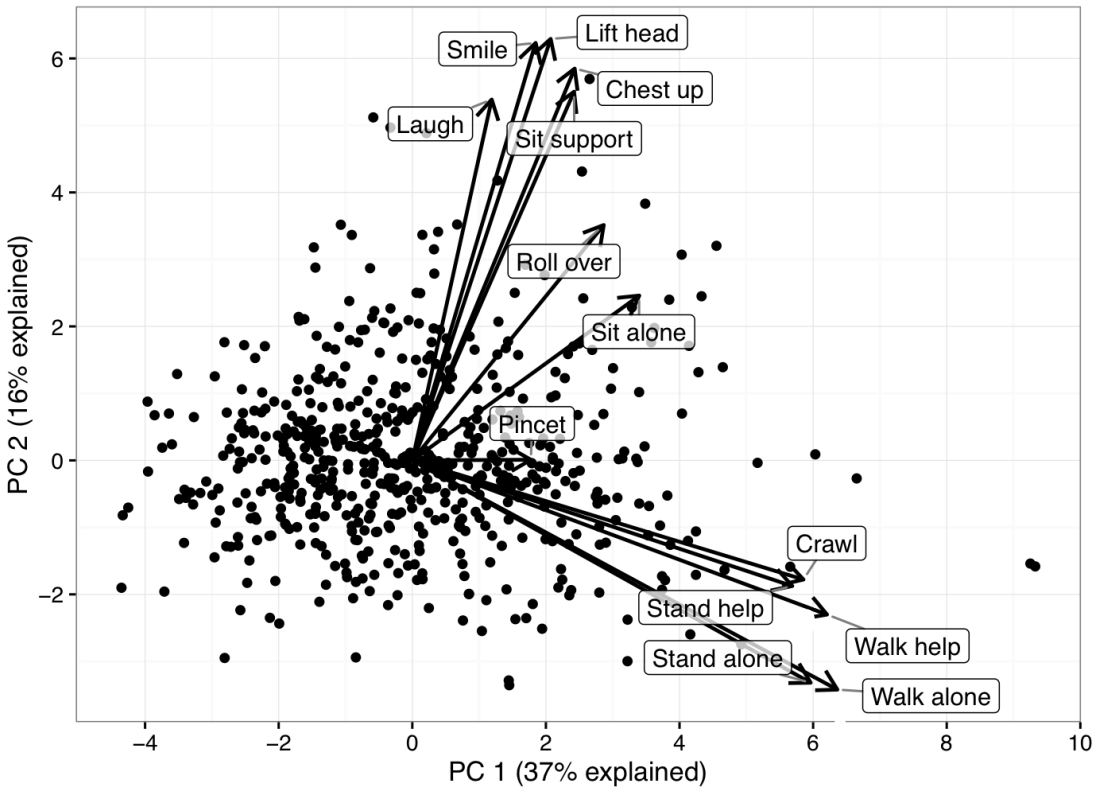
610 families registered at least one of the 13 milestones. **Figure 2** shows the age of milestone achievement in these children.

Figure 2. Box-plot of the window of achievement of developmental milestones



In the PCA all the milestones were positively correlated in the first principal component (PC1), which explained 37% of the variation and was driven primarily by the late gross-motor milestones (crawling, walking and standing). The second principal component (PC2) explained 16% of the variation and was primarily driven by the early milestones (smiling, lifting the head, and sitting with support) and the later milestones in opposite directions (**Figure 3**). PC1 and PC2 were used as the main milestone variables in our predictor analysis.

Figure 3. Biplot from principal component analysis of all the 13 milestones.



As can be seen in **Table 2**, early age at milestone achievement was associated with male gender (PC1, p=0.05), lower maternal age at birth (PC1, p=0.02), as well as higher gestational age (PC1, p<0.001; PC2, p=0.01) and paternity leave (PC2, p=0.01).

Table 2. Multivariate analysis of factors influencing overall age of milestone development

Predictor	**PC 1** Effect (95% CI)	**PC 2** Effect (95% CI)
Gender (Female/Male)	0.32 (0.00, 0.63) **P=0.05**	0.02 (-0.20, 0.22) P=0.96
First born (Y/N)	-0.04 (-0.38, 0.30) P=0.83	-0.11 (-0.34, 0.11) P=0.33
Paternity leave (Y/N)	-0.01 (-0.33, 0.31) P=0.96	-0.28 (-0.50, -0.06) **P=0.01**
Smoking during pregnancy (Y/N)	0.15 (-0.48, 0.78) P=0.64	0.30 (-0.13, 0.72) P=0.17
Gestational age (weeks)	-0.23 (-0.36, -0.11) **P<0.001**	-0.11 (-0.20, -0.03) **P=0.01**
Maternal age at birth (years)	0.05 (0.01, 0.09) **P=0.02**	0.02 (-0.01, 0.05) P=0.22
Age start daycare (months)	-0.02 (-0.07, 0.03) P=0.37	-0.02 (-0.05, 0.01) P=0.26
Mother's education	P=0.60	P=0.56
-High	0.18 (-0.20, 0.55)	-0.06 (-0.31, 0.19)
-Medium (ref)	0	0
-Low	0.16 (-0.46, 0.79)	-0.22 (-0.64, 0.20)

Abbreviation: CI= confidence intervals, PC= Principal Component from PCA in Figure 3

Analysis of the original milestone variables by conventional statistics confirmed these findings as 9 out of the 13 milestones showed early attainment correlated with high gestational age (p-values: <0.001 to 0.017), and boys achieved 9 out of 13 milestones at a younger age than the girls (in the amount of 1-8 days earlier, but all the p-values where > 0.05) (additional results **figure E1**).

Language development

Complete language data at 1 and 2 years of age was obtained from 323 and 470 participants, respectively. **Table 3** shows the overall results from the language tests. In the table the genders are presented separately if there is a statistical difference in the outcomes (p<0.05). The girls had significantly higher scores on several of the outcomes, both on the 1 and 2 year tests.

Table 3. Language development at 1 and 2 years of age assessed by MacArthur Bates Communicative Developmental Inventory

1 year language test n=320	
Age at test (months), mean ± SD	12.2 ± 0.3
Starting to talk (labeling and/or imitation) (%)	47.4
Early gestures (n out of 18), mean ± SD	11.2 ± 2.6
Girls - early gestures	11.6 ± 2.6
Boys – early gestures	10.8 ± 2.6
Late gestures (n out of 45), mean ± SD	8.9 ± 5.2
Phrases understood (n out of 26), mean ± SD	9.8 ± 5.2
Vocabulary comprehension (n of words from a list of 409), median (25^{th} -75^{th} percentile)	39 (20-66)
Vocabulary production (n of words from a list of 409), median (25^{th} -75^{th} percentile)	3 (1-7)
2 year language test n=470	
Age at test (months), mean ± SD	24.2 ± 0.3
Vocabulary production (n of words from a list of 725), median, (25^{th} – 75^{th} percentile)	250 (121-364)
Girls – vocabulary production	287 (162-401)
Boys – vocabulary production	197 (79-327)
Use of abstract words (n from a list of 5), median (25^{th}- 75^{th} percentile)	5 (4-5)
Uses grammar (%)	67.2
Girls – uses grammar	73.7
Boys – uses grammar	61.0
Irregular words (n from a list of 29), median (25^{th} – 75^{th} percentile)	2.0 (0-6)
Girls – irregular words	3 (1-7)
Boys – irregular words	2 (0-5)
Overregularized words (n from a list of 61), median (25^{th} – 75^{th} percentile)	0 (0-2)
Length of longest sentences (n of morphemes), mean ± SD	3.6 ± 1.6
Girls – longest sentence	4.0 ± 1.5
Boys – longest sentence	3.2 ± 1.5
Sentence complexity (n of complex from a list of 33 pairs), median (25^{th} – 75^{th} percentile)	3 (0-7)
Girls – sentence complexity	4 (1-9)
Boys – sentence complexity	1 (0-5)

The 1-year and 2-year language scores were all positively correlated in the respective PCA models. For the 1-year scores, PC1, which is a measure of general early language development, explained 53% of the overall variation in the dataset (**figure 4**) and was therefore used as a combined measure for 1-year language development. For the 2-years scores, PC1 explained 58 % of the variation in the dataset (**Figure 5)** and was used as a combined measure for 2-year language development.

A higher 1-year language score was associated with female gender (p=0.02) and maternal smoking during pregnancy (p=0.01) (**Table 4**). A higher 2-year language score was associated with female gender (p<0.001) and being first born (p=0.01).

Analysis of word production at 1-year as the endpoint (data not shown) confirmed that maternal smoking during pregnancy was the strongest predictor for the 1-year language development (mean difference=5.3 words (95% CI= 2.4, 8.2), p<0.001).

Analysis of word production at 2-years as the endpoint (data not shown) confirmed that female gender was the strongest predictor for 2-year language development (mean difference=63 words (34-91), p<0.001).

Figure 4. Biplot from principal component analyses of all the 1-year language development scores.

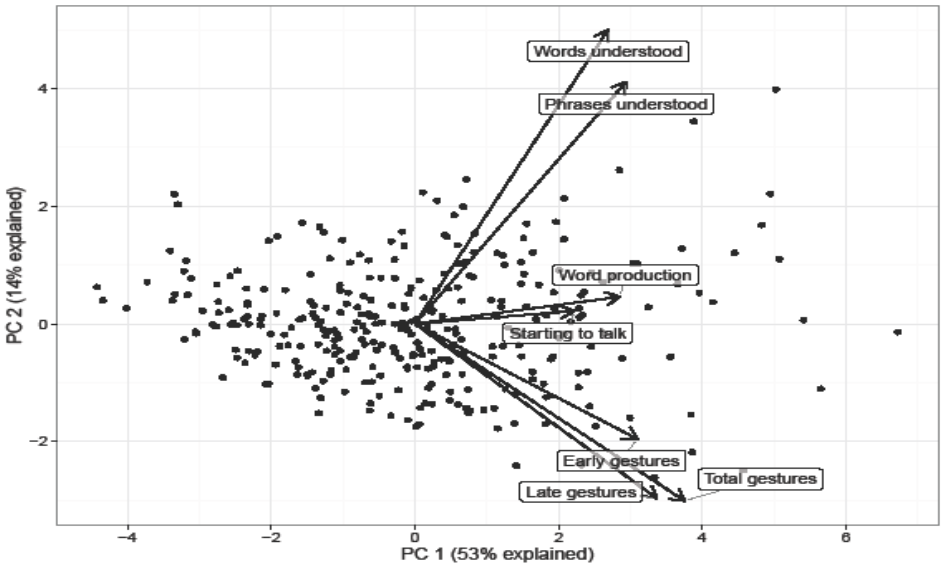


Figure 5. Biplot from principal component analyses of all the 2-year language development scores.

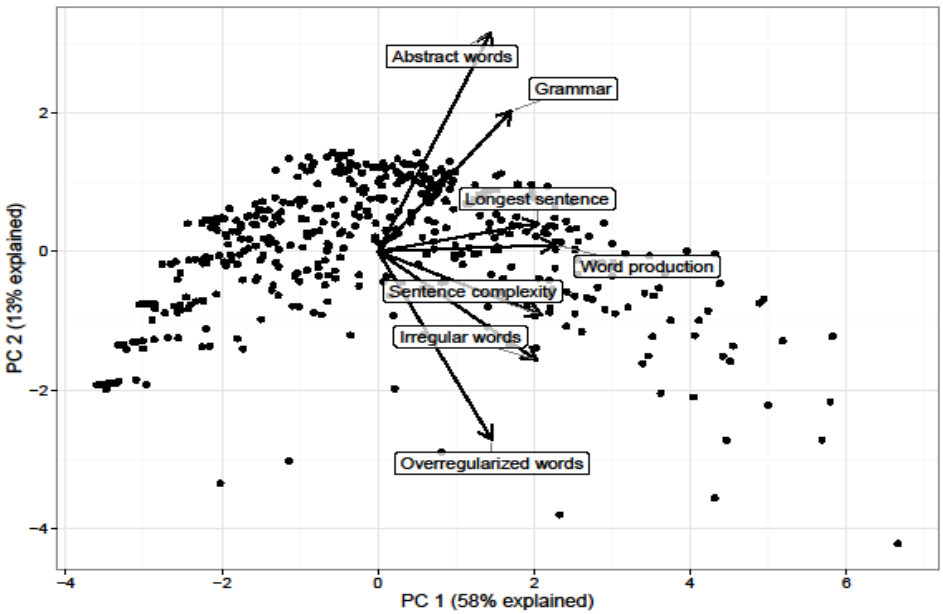


Table 4. Factors influencing 1 and 2 years language

	Language 1 year	**Language 2 years**
Predictor	PC1 Effect (95% CI)	PC1 Effect (95% CI)
Gender (F/M)	0.50 (0.08, 0.92) P=**0.02**	0.87 (0.50, 1.23) **P<0.001**
First born (Y/N)	0.21 (-0.23, 0.66) P=0.37	0.48 (0.09, 0.86) **P=0.02**
Paternity leave (Y/N)	0.01 (-0.42, 0.44) P=0.97	0.09 (-0.29, 0.46) P=0.65
Smoking during pregnancy (Y/N)	1.19 (0.28, 2.09) **P=0.01**	0.49 (-0.29, 1.28) P=0.22
Gestational age (weeks)	0.05 (-0.12, 0.23) P=0.56	0.01 (-0.14, 0.16) P=0.91
Maternal age at birth (years)	-0.05 (-0.10, 0.01) P=0.10	-0.04 (-0.09, 0.01) P=0.10
Age start daycare (months)	0.04 (-0.03, 0.10) P=0.29	0.03 (-0.03, 0.09) P=0.37
Mother's education	P=0.23	P=0.37
-High	-0.44 (-0.93, 0.06)	-0.01 (-0.44, 0.42)
-Medium	0	0
-Low	-0.04 (-0.94, 0.85)	-0.52 (-1.24, 0.21)

Abbreviation: CI= confidence intervals; PC= Principal Component from PCA in figure 4

Cognitive score

601 children completed the BSID-III test. As seen in **Table 5** the mean composite score was 104.9 (SD 9.8). There were no differences in scores among the 10 persons performing the tests (**Figure E3** – additional results). Nevertheless, we included test person as a possible confounding factor in our analysis. A higher cognitive score was only significantly associated with female gender (p=0.02); girls scored 1.88 (95% CI: 0.30-3.47) points higher than boys (**Table 6**).

Table 5. Cognitive scores at 2½ years of age assessed by Bayley III.

Bayley test at 2 ½ years n=601	
Age at test (months), mean ± SD	30.5 ± 0.92
Cognitive composite score (standardized), mean ± SD	104.9 (9.80)
Girls – composite score	106.0 (10.4)
Boys – composite score	103.8 (9.9)

Table 6. Factors influencing Bayley scores at 2 ½ years of age

	Bayley composite score
Predictor	Composite score Effect (95% CI)
Gender (F/M)	1.88 (0.30, 3.47) **P=0.02**
First born (Y/N)	1.08 (-0.65, 2.82) P=0.22
Paternity leave (Y/N)	0.66 (-0.97, 2.29) P=0.43
Smoking during pregnancy (Y/N)	-0.89 (-4.05, 2.26) P=0.58
Gestational age (weeks)	0.44 (-0.21, 1.08) P=0.18
Maternal age at birth (years)	-0.08 (-0.29, 0.13) P=0.47
Age start daycare (months)	-0.03 (-0.29, 0.24) P=0.85
Mother's education	P=0.15
-High	1.93 (-0.01, 3.87)
-Medium	0
-Low	0.36 (-2.83, 3.56)

Abbreviation: CI= confidence intervals

Neurological development and breastfeeding period

As seen in **Table 7**, none of the neurological endpoints was significantly associated with the continuous variables of exclusive or total breastfeeding duration. The same was found when categorizing the exclusive and total breastfeeding periods as <1 month, 1-4 months, 4-6 months, >6 months.

Table 7. Correlations between neurological development and exclusive and total duration of breastfeeding[1].

	Age at milestone (PC1)	Age at milestone (PC2)	Language at 1 year (PC1)	Language at 2 years (PC1)	Bayley composite score
Exclusively breastfeeding (months)	-0.01 (-0.09, 0.08) P=0.86	-0.05 (-0.11, 0.01) P=0.08	-0.05 (-0.17, 0.06) P=0.39	0.00 (-0.10, 0.09) P=0.93	0.05 (-0.37, 0.48) P=0.80
<1 month (N=137)	REF	REF	REF	REF	REF
1-4 months (N=166)	0.21 (-0.26, 0.67) P=0.38	0.15 (-0.16, 0.47) P=0.33	-0.06 (-0.69, 0.58) P=0.86	-0.48 (-1.03, 0.08) P=0.09	0.39 (-2.00, 2.78) P=0.75
4-6 months (N=322)	-0.18 (-0.60, 0.23) P=0.39	-0.12 (-0.41, 0.16) P=0.40	-0.30 (-0.88, 0.27) P=0.30	-0.11 (-0.60, 0.39) P=0.67	0.49 (-1.65, 2.64) P=0.65
6+ months (N=32)	0.71 (-0.11, 1.53) P=0.09	-0.43 (-0.99, 0.13) P=0.13	-0.22 (-1.26, 0.81) P=0.67	0.01 (-0.86, 0.88) P=0.98	0.14 (-3.93, 4.21) P=0.95
Total breastfeeding (months)	-0.03 (-0.07, 0.00) P=0.07	-0.03 (-0.04, 0.00) P=0.09	0.03 (-0.02, 0.08) P=0.22	0.04 (-0.00, 0.08) P=0.05	0.06 (-0.12, 0.24) P=0.54
<1 month (N=46)	REF	REF	REF	REF	REF
1-4 months (N=77)	0.25 (-0.54, 1.03) P=0.54	-0.10 (-0.63, 0.42) P=0.70	-0.22 (-1.29, 0.85) P=0.68	-0.09 (-1.07, 0.89) P=0.86	-1.63 (-5.66, 2.38) P=0.42
4-6 months (N=91)	-0.12 (-0.88, 0.64) P=0.76	0.12 (-0.39, 0.62) P=0.64	-0.34 (-1.36, 0.67) P=0.50	0.20 (-0.74, 1.14) P=0.68	-0.68 (-4.56, 3.20) P=0.73
6-12 months (N=340)	0.27 (-0.40, 0.94) P=0.43	-0.16 (-0.61, 0.29) P=0.49	-0.54 (-1.47, 0.37) P=0.24	0.37 (-0.44, 1.23) P=0.40	-1.32 (-4.81, 2.17) P=0.46
12+ months (N=102)	-0.29 (-1.06, 0.49) P=0.47	-0.10 (-0.62, 0.42) P=0.70	0.35 (-0.69, 1.39) P=0.51	0.79 (-0.17, 1.75) P=0.11	0.77 (-3.21, 4.75) P=0.70

Abbreviation: PC= Principal Component from PCAs in Figures 3 and 4

Neurological development and clinical predictors in the first years of life

None of the neurological endpoints was significantly associated with any of the clinical predictors: persistent wheeze 0-3yrs, eczema 0-3yrs or number of sick days in the first year of life (**Table 8**).

Table 8. Correlations between neurological development and persistent wheeze, eczema and sickness in the first years of life

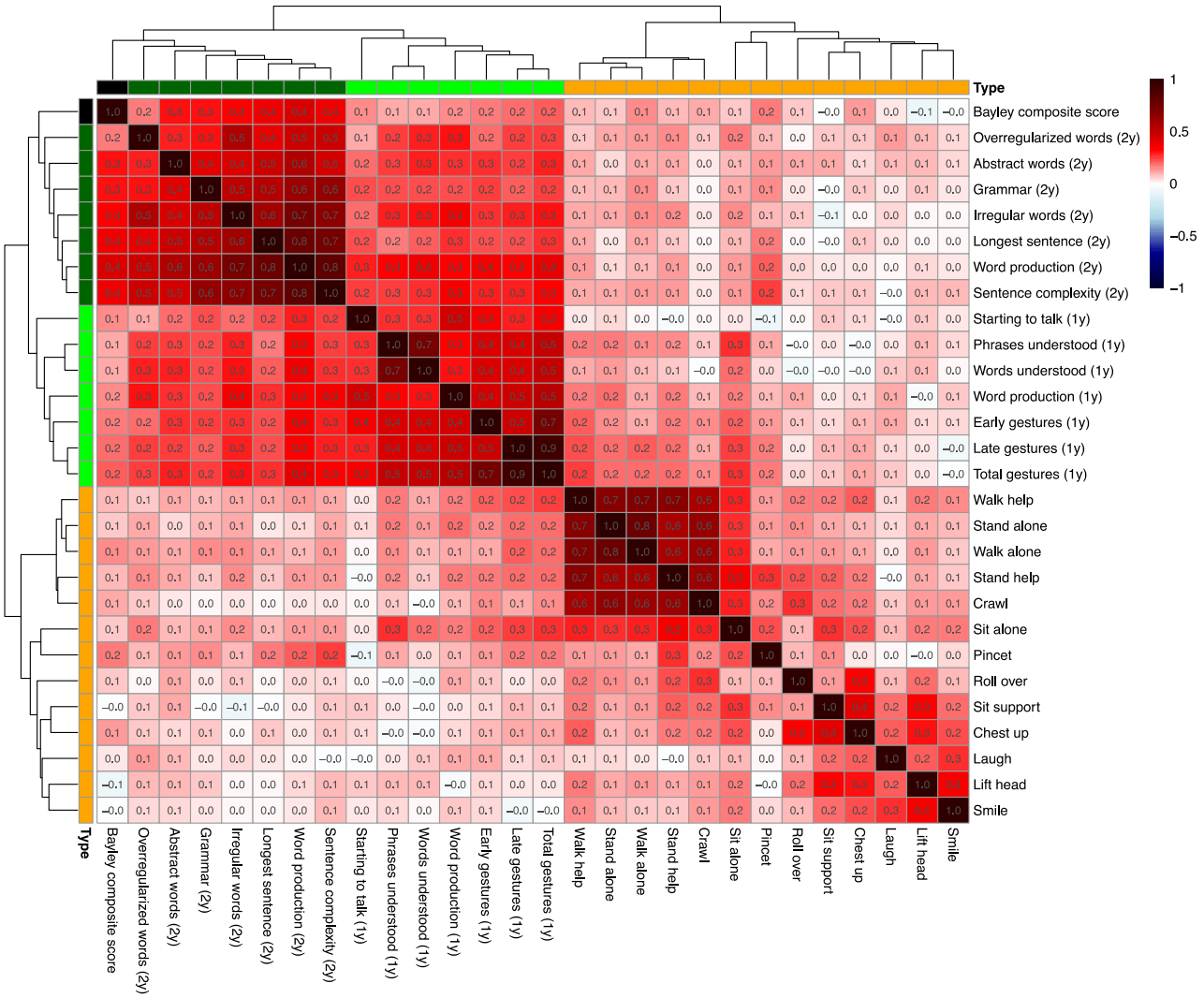
	Age at milestone (PC1)	Age at milestone (PC2)	Language at 1 year (PC1)	Language at 2 years (PC1)	Bayley composite score
Persistent wheeze (yes/no)	0.17 (-0.25, 0.59) p=0.54	0.01 (-0.29, 0.27) p=0.95	0.34 (-0.21, 0.90), p=0.23	0.001 (-0.47, 0.48) p=0.98	-0.55 (-2.61, 1.51) p=0.60
Eczema (yes/no)	0.02 (-0.34, 0.39) p=0.90	0.09 (-0.15, 0.34) p=0.46	-0.28 (-0.77. 0.21) p=0.26	-0.15 (-0.56, 0.26) p=0.48	-0.58 (-2.38, 1.22) p=0.53
Sick days in first year of life (weeks)	-0.03 (-0.06, 0.002) p=0.07	0.002 (-0.02, 0.02) p=0.81	0.04 (-0.01, 0.08) p=0.09	0.02 (-0.01, 0.05) p=0.52	-0.05 (-0.20, 0.09) p=0.47

Abbreviation: PC= Principal Component from PCAs in Figures 4 and 5

Correlation between neurodevelopmental endpoints

A heat map with all the original milestones, 1- and 2-year language scores and the BSID-III composite score (**Figure 6**), showed that all the language scores and all the milestone were inter-correlated and that the composite score of the BSID-III was highly correlated with the 2-year language scores.

Figure 6. Heatmap based on spearman correlations showing the correlation between the neurological endpoints



4.2. Paper II - n-3 Polyunsaturated Fatty Acid Supplementation During Pregnancy and Neurodevelopment During Childhood. A Randomized Controlled Trial

Baseline

A total of 736 population-based women were randomized at pregnancy week 24 to either n-3 LCPUFA or control supplementation. 43 women (6%) were withdrawn from the study before the child was born. 698 infants where included in the cohort (5 pairs of twins). 49 children (7%) were excluded from the present study (15 did not have any neurodevelopmental outcome; 5 had a neurological diagnosis; 24 were born preterm; and 5 had low birth weight), leaving 649 children in the final study group (**Figure 7**).

Table 9 depicts the baseline characteristics of the pregnant women and their children showing as well as compliance and rates of follow-up assessments in the two treatment groups. Adherence is illustrated by 461 (71%) of the women taking >80% of the prescribed capsules with no difference between the 2 groups, demonstrating a similar prevalence in both groups and higher levels of post-randomization whole blood levels of EPA+DHA in women receiving n-3 LCPUFA vs. control: 6.64% of all fatty acids (SD 1.91) vs. 4.21% (SD 1.08), $p<0.0001$.

Figure 7: Flow chart of enrolment and allocation of the $COPSAC_{2010}$ pregnancy cohort and follow-up of the $COPSAC_{2010}$ birth cohort.

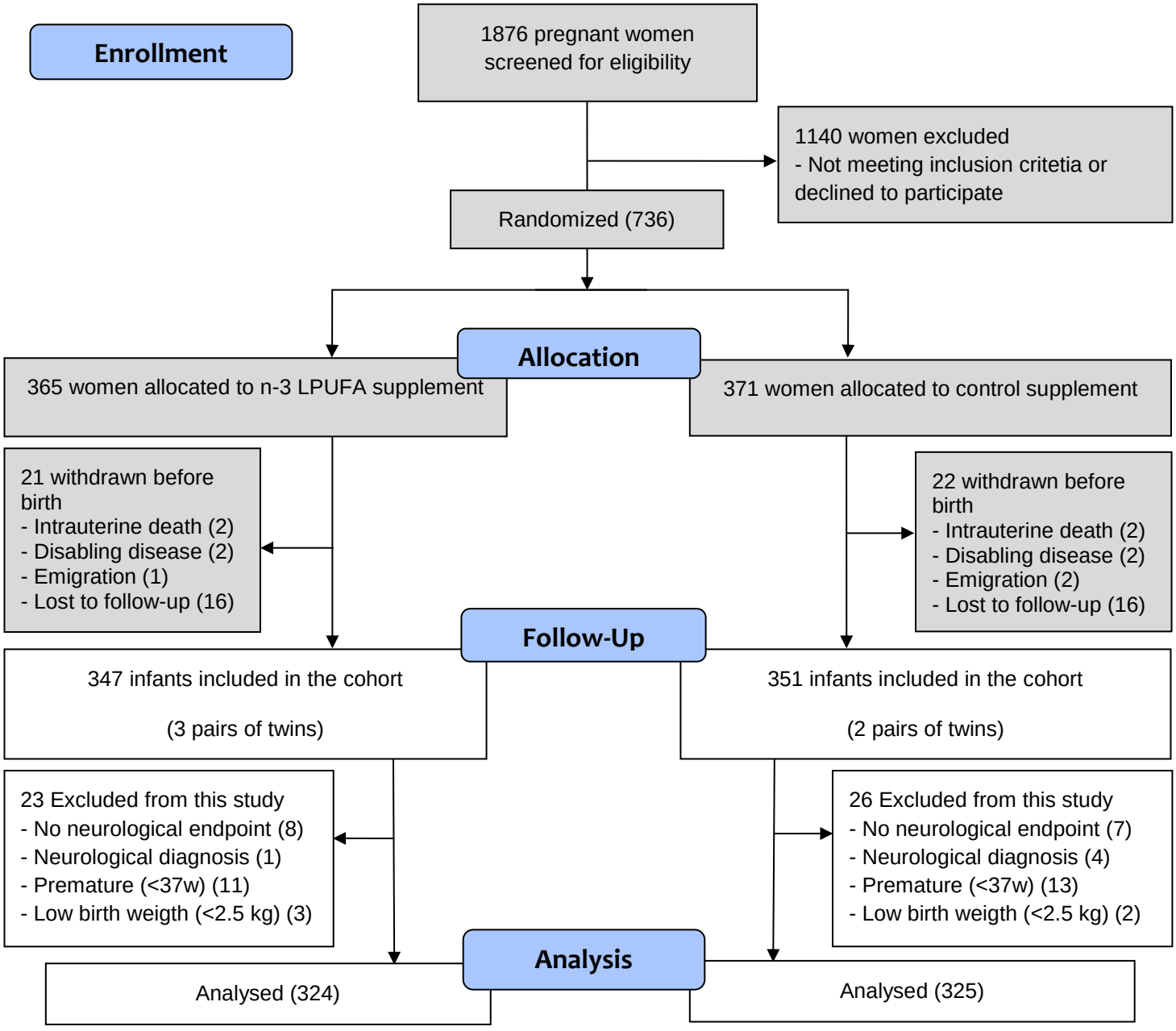


TABLE 9: Characteristics of the $COPSAC_{2010}$ birth cohort and their pregnant mothers

	All	**Randomization**	
		n-3 LCPUFA	**Control**
	N=649	**50% (324)**	**50% (325)**
Socioeconomics			
Maternal age at Birth in years, mean (SD)	**32.2 (4.3)**	32.3 (4.3)	32.2 (4.4)
Maternal Asthma, % (N)[a]	**25 (164)**	24 (76)	27 (88)
Social circumstances, mean (SD)[b]	**0 (1)**	0.004 (0.97)	-0.01 (1.01)
Smoking, % (N)	**7 (45)**	6 (18)	8 (27)
Alcohol, ≥ 1 unit / week, % (N)	**14 (93)**	13 (41)	16 (52)
Fish intake pre randomization, g/day, median (ITR)[c]	**26.3 (19-34)**	26.7 (19-34)	25.6 (19-33)
EPA intake pre randomization, g/day, median (ITR)[c]	**0.13 (0.09-0.17)**	0.12 (0.09-0.17)	0.13 (0.09-0.17)
DHA intake pre randomization, median (ITR)[c], g/day	**0.32 (0.23-0.41)**	0.31 (0.22-0.41)	0.33 (0.23-0.41)
Whole blood EPA+DHA level pre- randomization, % of fatty acids, mean (SD)[d]	**4.65 (1.21)**	4.64 (1.27)	4.66 (1.15)
Adherence			
>80% capsule consumption, % (N)	**71 (461)**	71 (229)	71 (232)
Whole blood EPA+DHA level post- randomization, % of fatty acids, mean (SD)[e]	**5.41 (1.97)**	6.64 (1.93)[f]	4.22 (1.09)[f]
Birth			
Gestational age in weeks, median (IQR)	**40 (39-41)**	40 (39-41)	40 (39-41)
Birth order, % first born (N)	**45 (359)**	43 (140)	46 (150)
APGAR score at 5 min, mean (SD)	**9 (3)**	9 (3)	9 (3)
Intra-partum Antibiotics % (N)	**30 (191)**	30 (97)	29 (94)
Antibiotics to the Child % (N)	**2 (16)**	3 (9)	2 (7)
Caesarean Section % (N)	**20 (129)**	21 (67)	19 (62)
Emergency % (N)	**10 (67)**	12 (38)	9 (29)
Elective % (N)	**9 (65)**	9 (29)	10 (33)
Child			
Gender, Male % (N)	**51 (332)**	49 (159)	53 (173)
Caucasian % (N)	**96 (622)**	96 (311)	96 (311)
Season of Birth			
Winter, % (N)	**31 (200)**	29 (94)	33 (106)
Spring, % (N)	**27 (172)**	26 (85)	27 (87)
Summer, % (N)	**21 (139)**	22 (70)	21 (69)
Fall, % (N)	**21 (138)**	23 (75)	19 (63)
Neurodevelopmental outcomes			
Completed BSID-III test, % (N)	**92 (600)**	92 (299)	93 (301)
Milestone registration, n	**94 (609)**	94 (305)	94 (304)
One year language test, % (N)	**49 (320)**	47 (151)	52 (169)
Two year language test, % (N)	**72 (470)**	72 (234)	73 (236)
ASQ test, % (N)	**65 (423)**	64 (206)	67 (217)

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval, ITR=inter-tertile range, IQR (inter-quartile range)

[a] History of doctor diagnosed asthma

[b] PCA component that consist of household income, maternal age and maternal educational level at the age of 2

[c] Calculated from a total of 567 available food frequency questioners (data from manuscript accepted for publication in NEJM)

[d] Calculated from a total of 570 available pre-randomization whole blood fatty acid analysis

[e] Calculated from a total of 637 available post-randomization whole blood fatty acid analysis

[f] $P<0.0001$

The Maternal dietary intake is not the subject of this thesis and is only presented briefly in the baseline table (**Table 9**) and not used in any of the analysis. Nevertheless, it is of interest to see dietary habits of the mothers acquired using a food frequency questionnaire which was completed by the participating women assessing dietary intake in the 4 weeks prior to randomization[32,83,84]. **Table 10** shows the dietary intake for energy, protein, carbohydrates, fat, fish and fish products. There is no difference between the two groups and the median daily fish and fish products intake was 26g (inter-tertile range, 19-33), EPA 0.13g (0.09-0.17) and DHA 0.32g (0.23-0.41), respectively.

Table 10: Maternal dietary intake during pregnancy week 20-24

		Randomization		
	All N=567	**n-3 LCPUFA N= 278**	**Control N=289**	**p value**
	Median (ITR)			
Energy (kJ)	8940 (8023-9946)	8988 (8117-9927)	8876 (7867-10030)	0.90
Protein (% of energy)	16.1 (15.2-17.0)	16.1 (15.5-17.0)	16.0 (15.1-16.9)	0.43
Carbohydrate (% of energy)	49.9 (47.9-51.9)	49.8 (47.8-51.9)	50.0 (48.0-51.8)	0.98
Fat (% of energy)	31.0 (28.8-33.1)	31.0 (28.7-33.3)	31.0 (29.0-33.0)	0.74
SFA	12.1 (10.9-13.6)	12.1 (18.8-13.6)	12.2 (10.9-13.5)	0.92
MUFA	10.0 (9.3-10.9)	9.9 (9.2-10.9)	10.0 (9.4-10.7)	0.45
PUFA	4.8 (4.5-5.1)	4.8 (4.5-5.1)	4.8 (4.5-5.2)	0.29
n-3 PUFA	0.9 (0.8-1.0)	0.9 (0.8-1.0)	0.9 (0.8-1-0)	0.31
n-6 PUFA	3.3 (3.1-3.6)	3.3 (3.1-3.5)	3.4 (3.1-3.6)	0.19
Fish and fish products (g)	26.3 (19.0-33.5)	26.7 (19.3-34.0)	25.6 (18.7-33.1)	0.82
EPA (g)	0.13 (0.09-0.17)	0.12 (0.09-0.17)	0.13 (0.09-0.17)	0.71
DHA (g)	0.32 (0.23-0.41)	0.31 (0.22-0.41)	0.33 (0.23-0.41)	0.52

Table 11 shows the full fatty acid composition of maternal whole blood before and after the trial. There is no difference between the two groups before the randomization, but post randomization there is a significant change in the fatty acid profile of the LCPUFA- mothers compared to the control group.

Table 11. Composition of maternal blood fatty acids constitution before and after the trial[a]

	Pre randomization		Post randomization		
Fatty acid	**n-3 LCPUFA N=275**	**Control N=295**	**n-3 LCPUFA N=315**	**Control N=322**	
	Mean ± SD		**Mean ± SD**		**P value**
SFA	38.3 ± 3.0	38.2 ± 2.7	39.5 ± 2.4	38.8 ± 2.5	<0.001
MUFA	23.0 ± 2.6	23.0 ± 2.7	22.2 ± 2.7	23.3 ± 2.9	<0.001
PUFA	34.8 ± 3.3	34.9 ± 3.3	34.5 ± 3.1	33.8 ± 3.1	0.003
n-6 PUFA	28.6 ± 2.8	28.7 ± 2.7	26.0 ± 2.6	28.2 ± 2.6	<0.001
18:2 n-6	17.9 ± 2.9	17.9 ± 2.6	16.7 ± 2.3	17.4 ± 2.4	<0.001
20:3 n-6	1.8 ± 0.3	1.8 ± 0.4	1.4 ± 0.3	1.7 ± 0.4	<0.001
20:4 n-6 (AA)	7.4 ± 1.1	7.5 ± 1.2	6.9 ± 1.3	7.7 ± 1.4	<0.001
22:4 n-6	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	<0.001
n-3 PUFA	6.2 ± 1.5	6.2 ± 1.3	8.5 ± 2.2	5.6 ± 1.2	<0.001
18:3 n-3 (ALA)	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.25
20:5 n-3 (EPA)	0.7 ± 0.4	0.7 ± 0.3	2.2 ± 1.1	0.7 ± 0.4	<0.001
22:5 n-3	1.0 ± 0.2	0.9 ± 0.2	1.4 ± 0.3	0.9 ± 0.2	<0.001
22:6 n-3 (DHA)	3.9 ± 0.9	4.0 ± 0.9	4.4 ± 1.0	3.5 ± 0.8	<0.001
EPA+DHA	4.6 ± 1.3	4.7 ± 1.2	6.6 ± 1.9	4.2 ± 1.1	<0.001
Ratio n-6/n-3 PUFA	4.8 ± 1.1	4.8 ± 0.9	3.3 ± 1.1	5.3 ± 1.1	<0.001

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, SFA=saturated fatty acid, MUFA=monounsaturated fatty acid, PUFA=polyunsaturated fatty acid, LA=linoleic acid, AA=arachidonic acid, ALA=alpha-linolenic acid, EPA=eicosapentaenoic acid, DHA= docosahexaenoic acid.

[a]Data are expressed as mean ±SD in % of all fatty acids in whole blood

Primary Outcome

600 children in the study population completed the BSID-III test. The children in the n-3 LCPUFA group showed a trend of higher BSID-III cognitive scores compared to the children in the control group: adjusted mean difference, 1.47; 95% CI [-0.13, 3.08], p=0.07. The analysis showed a significant gender interaction (p=0.03), indicating different effects of n-3 LCPUFA supplementation for boys compared to girls. Among boys, n-3 LCPUFA supplementation resulted in a significantly higher BSID-III cognitive score: adjusted mean difference, 3.04; 95% CI [0.97, 5.12], p=0.004 whereas no effect was observed among girls: -0.63 (-3.11, 1.84), p=0.61 (**Table 12**). As can be seen in **Figure 8**, the supplementation shifted the composite score of the boys from the n-3 LCPUFA group as a whole towards higher scores.

Table 12. Effect of the n-3 LCPUFA intervention on the composite cognitive score of the BSID-III test[a]

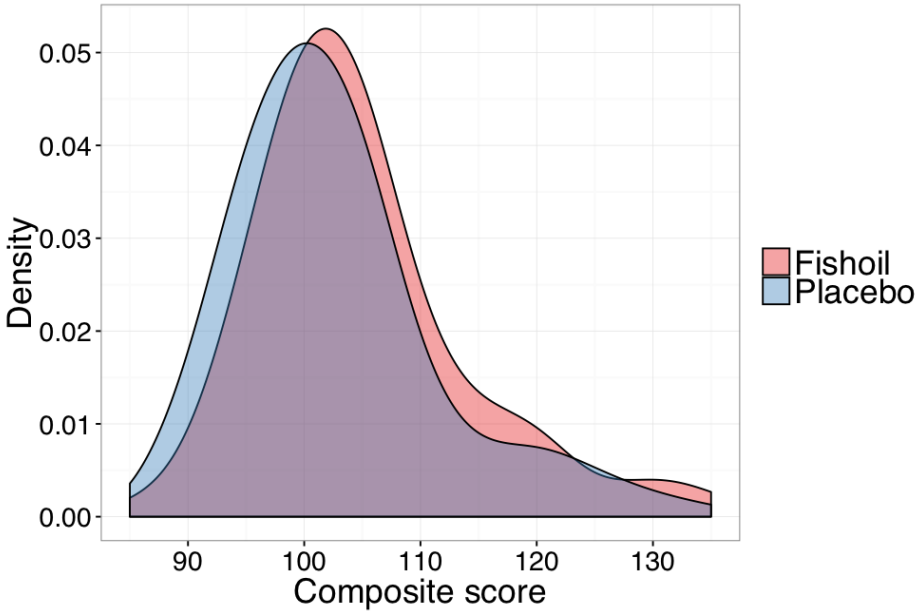
	N=299	N=301	N=600		N=527	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
Composite score	105.3(9.7)	104.4(9.9)	0.95(-0.62, 2.52)	0.24	1.47(-0.13, 3.08)	0.07
Female	105.8(10.2)	106.2(10.6)	-0.43(-2.82, 1.95)	0.72	-0.63(-3.11, 1.84)	0.61
Male	104.8(9.1)	102.7(9.0)	2.14(0.11, 4,16)	**0.039**	3.04(0.97, 5.12)	**0.004**

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval

[a]Data are expressed as mean (SD) with effect being differences in means (95% CI). Treatment x gender interaction, p=0.03 (adjusted)

[b]Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation.73 mothers are missing pre-intervention whole blood EPA+DHA results

Figure 8: Density plot showing the effect of n-3 LCPUFA intervention on composite score of the BSID-III cognitive test among boys.



Analyzing data without excluding any children yielded comparable results (**Table 13**).

Table 13: Effect of n-3 LCPUFA intervention on the composite cognitive score of the BSID-III test including children born before 37 gestational weeks, children with low birth weight (<2500 g) and children with a neurological diagnosis [a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**		**Adjusted[b]**	
	N=312	N=317	N=629		N=554	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
Composite score	105.2(9.6)	104.3(9.8)	0.92(-0.60, 2.44)	0.24	1.41(-0.15, 2.97)	0.08
Female	105.7(10.1)	106.1(10.5)	-0.48(-2.78, 1.83)	0.68	-0.56(-2.95, 1.83)	0.65
Male	104.7(9.1)	102.6(8.8)	2.10(0.13, 4,07)	**0.037**	2.98(0.97, 4.98)	**0.004**

Abbreviations: N-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval

[a] Data are expressed as mean (SD) with effect being differences in means (95% CI).Treatment x gender interaction, p=0.03 (adjusted)

[b] Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation.75 mothers are missing pre-intervention whole blood EPA+DHA results

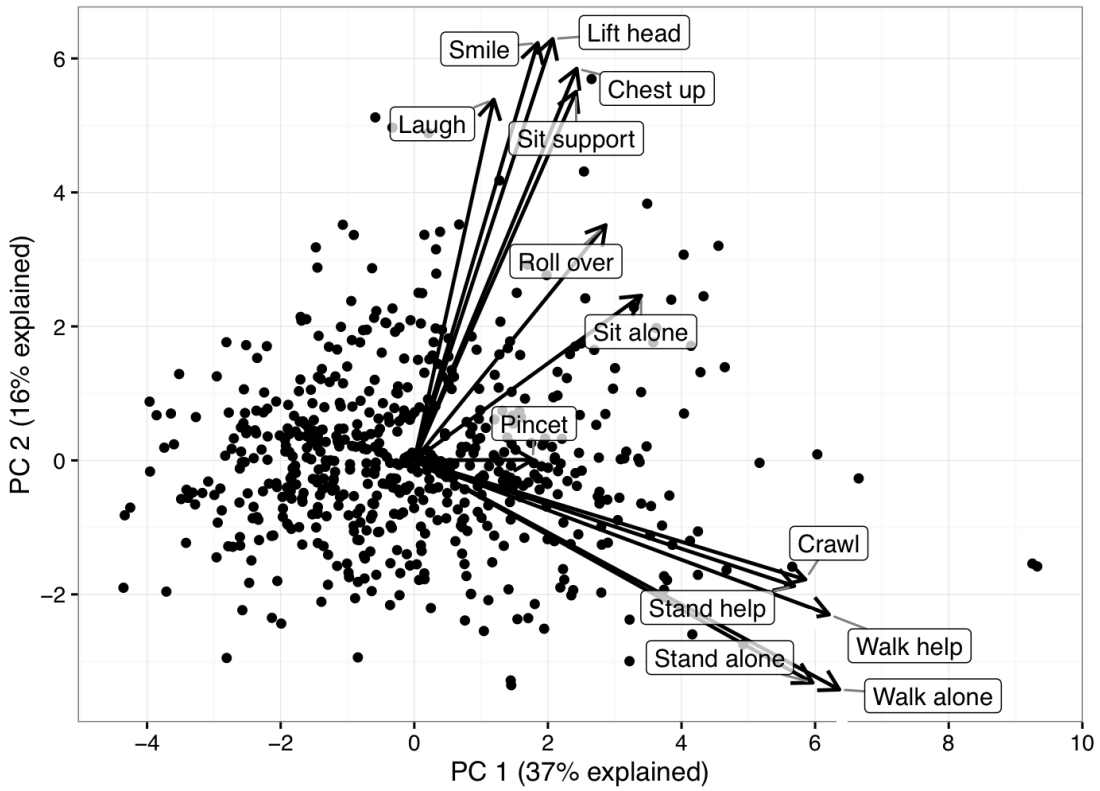
We did not find significant interaction between n-3 LCPUFA and vitamin D supplementation on the primary outcome (p=0.96).

Secondary Outcomes

Milestones

The pPCA approach including all the milestones in one model showed that the first two principal components (PC1 and PC2) explained 37% and 16% of the total variation in the milestone achievements (**Figure 9**).

Figure 9. Biplot from principal component analysis of all the 13 milestones.



There was no difference between the groups measured in PC1, but PC2 showed a lower score (that is younger age at milestone achievement) for the n-3 LCPUFA group compared to the control (p=0.03, **Table 14**). The significant effect for PC2 was driven by the boys (p=0.03), and there was also a trend of effect among boys for PC1 (p=0.08) with no effect among girls.

Table 14: Outcomes from milestone principal component analysis[a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**		**Adjusted[b]**	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
	N=305	N=304	N=609		N=541	
PC1	-0.07(1.88)	0.07(2.01)	-0.14(-0.45, 0.17)	0.36	-0.13(-0.46, 0.20)	0.44
Female	0.19(2.03)	0.05(2.00)	0.13(-0.32, 0.59)	0.57	0.12(-0.38, 0.61)	0.64
Male	-0.34(1.66)	0.09(2.03)	-0.43(-0.84, 0.01)	0.05	-0.40(-0.86, 0.05)	0.08
PC2	0.15(1.33)	-0.08(1.22)	0.23(0.03, 0.43)	**0.03**	0.23(0.01, 0.44)	**0.04**
Female	0.11(1.31)	-0.05(1.40)	0.16(-0.15, 0.47)	0.31	0.11(-0.22, 0.44)	0.52
Male	0.19(1.35)	-0.11(1.02)	0.30(0.03, 0.57)	**0.03**	0.32(0.04, 0.61)	**0.03**

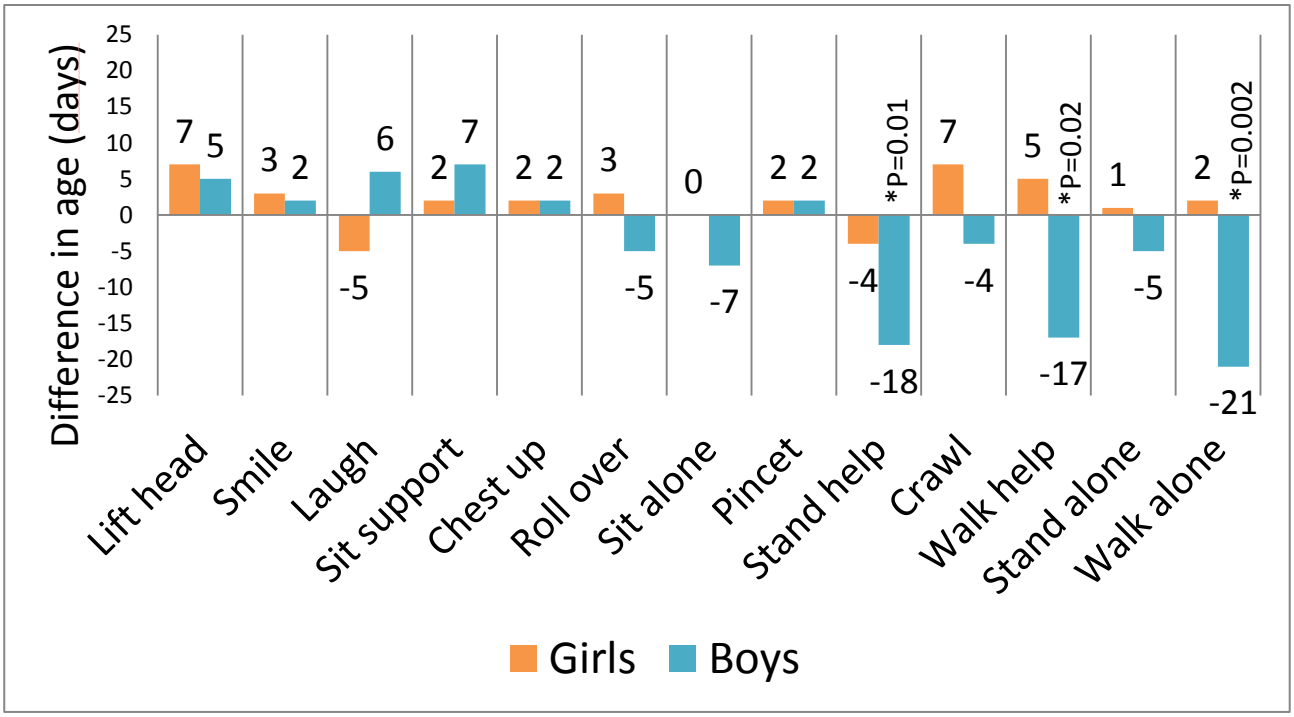
Abbreviations: N=number, SD=standard deviation, CI=confidence interval, PC1=principal component 1, PC2=principal component 2

[a] Data are expressed as mean (SD) with effect being differences in means (95% CI). Treatment x gender interaction PC1 p=0.08 and PC2 p=0.82 (adjusted)

[b] Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation. 68 mothers are missing pre-intervention whole blood EPA+DHA blood results.

The gender specific effect of n-3 LCPUFA supplementation observed in the pPCA analysis was confirmed by the conventional statistical approach. The individual milestones were not significantly affected by maternal n-3 LCPUFA supplementation in pregnancy (all p values >0.06, data not shown) in combined analyses of boys and girls. However, gender stratified analysis showed that the n-3 LCPUFA supplemented boys achieved the late motor milestones at a younger age compared to the boys in the control group: e.g. the milestone "walk alone" demonstrated a difference of 21 days (95% CI [-40.7, -10.7], p< 0.001), whereas no effects of the intervention were seen among the girls in any of the individual milestones (**Figure 10**).

Figure 10: The effect of n-3 LCPUFA intervention on age of milestone achievement for boys and girls[a]



[a] The effect is illustrated as the difference (in days) in milestone achievement, using the control group as the reference. Significant difference is indicated with asterisk (*) and p-value.

Language development

Language development at 1 year of age assessed as word production showed an overall positive effect from the n-3 LCPUFA supplementation (p=0.03). Gender stratified analysis showed no effect on the girls, but a possible positive effect on the boys in the n-3 LCPUFA group (p=0.14). There was no effect on word production at 2 years of age (**Table 15)**.

Table 15: Outcomes from the 1 and 2 year language tests[a]

	n-3 LCPUFA	**Control**	**Unadjusted**	**Adjusted[b]**
	Median (IQR)		P value	P value
1 year language test				
	N=151	N=169	N=320	N=317
Word Production	3.0(1-7)	2.0(0-6)	**0.03**	**0.03**
Female	3.0(1-8)	3.0(1-7)	0.19	0.30
Male	3.5(1-6)	1.0(0-6)	0.07	0.14
2 year language test				
	N=234	N=236	N=470	N=411
Word production	251(149-357)	250(96-377)	0.59	0.82
Female	278(182-378)	304(139-419)	0.62	0.23
Male	223 (97-327)	165 (71-328)	0.28	0.23

Abbreviations: IQR = inter quartile range

[a] Effect is expressed as median, with (IQR). Treatment x gender interaction 1year p=0.7 and 2 year 0.08 (adjusted)

[b] Adjusted for preintervention EPA+DHA blood levels and vitamin D-RCT allocation. 3 mothers are missing preintervention EPA+DHA blood results in the 1 year analysis and 59 in the 2 year analysis.

ASQ – 3 scores

ASQ-3 scores measuring the child´s general development at 3 years of age did not differ among the intervention groups (**Table 16**).

Table 16: Outcomes from Ages and stages Questionnaire[a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**
	Median (IQR)		P value
	N=206	N=217	
Communication	50 (50-55)	50 (50-55)	0.27
Female	50 (50-55)	50 (50-55)	0.41
Male	50 (50-55)	50 (50-55)	0.61
Gross motor skills	60 (55-60)	60 (55-60)	0.36
Female	60 (55-60)	60 (55-60)	0.96
Male	60 (55-60)	60 (55-60)	0.17
Fine motor skills	55 (50-60)	55 (50-60)	0.41
Female	60 (50-60)	55 (50-60)	0.25
Male	50 (45-60)	50 (40-60)	0.77
Problem solving	55 (50-60)	55 (50-60)	0.61
Female	55 (50-60)	55 (50-60)	0.95
Male	55 (50-60)	55 (50-60)	0.59
Social skills	55 (50-60)	55 (50-60)	0.82
Female	55 (55-60)	55 (55-60)	0.12
Male	55 (50-60)	55 (50-60)	0.72

Abbreviations: IQR = inter quartile range,
[a] Effect is expressed as median, with (IQR)

Safety

The safety-profiles of the n-3 LCPUFA and control supplementation showed no difference between the groups, except for that there was a trend (p=0.1) of lower infection rate in the mothers of n-3 LCPUFA group during the third trimester (**Table 17**).

Table 17: Safety assessment in the $COPSAC_{2010}$ cohort.

	Randomization % (N)		**P value**
Adverse Events	**n-3 LCPUFA**	**Control**	
	50% (365)	**50% (371)**	-
Any maternal			
Death	0% (0)	0% (0)	-
Intrauterine death	1% (2)	1% (2)	0.99
Gestational diabetes	2% (6)	3% (10)	0.32
Preeclampsia	5% (17)	4% (15)	0.69
Days hospitalized after birth, mean (SD)	2.9 (2.7)	2.8 (2.8)	0.51
Mother hospitalized >5 days	10% (34)	10% (35)	0.99
Emergency caesarean section	14% (52)	11% (41)	0.20
Antibiotics in third pregnancy trimester	18% (65)	17% (63)	0.78
Infection in third pregnancy trimester	28% (96)	33% (118)	0.10
Any infant			
Death	0% (0)	0% (0)	-
Extremely preterm (<28 weeks) birth	0% (1)	0% (1)	0.99
Very preterm (28 to <32 weeks) birth	1% (2)	1% (3)	0.66
Moderate to late preterm (32 to <37 weeks) birth	3% (12)	4% (15)	0.58
Child Hospitalized after Birth	12% (40)	11% (39)	0.88
Any Congenital Malformation	5% (20)	6% (24)	0.56

5. Discussion

5.1. Neurological development in the COPSAC2010 Cohort

The $COPSAC_{2010}$ children achieved gross motor milestones at a later age than children in the WHO Multicenter Growth study, but at a similar age as the Norwegian children in the same study [85] (additional results **Figure E2**), which might indicate a culture-specific care and behaviour and heritability. All the developmental outcomes in the study were within normal range.

Overall the 1 and 2-year language scores in the $COPSAC_{2010}$ cohort were lower than in other cohort studies[86] and slightly lower than in an another Danish birth cohort[81]. It is known that Danish children generally score lower on both the 1 and 2-year language tests[87] and it has been suggested that the delay is related to the nature of Danish sound structure, which presents Danish children with a harder task of segmentation[87]. However, it could also be affected by cultural differences and early attendance to daycare in Denmark[87].

The mean cognitive score from the BSID-III test was slightly above average (104.8, SD 9.8), and no children scored less than 85. The average score was similar to other cohort studies of healthy children[21,54] and the lack of participants with scores of less than 85 might be due to a selection bias in the cohort. It is unlikely to be due to exclusion criteria of the study (prematurity, low birthweight or neurological disorders), since explorative analysis, including those children did not show difference in the range of the cognitive score in the BSID-III test (additional results, **Table E1**).

None of the neurodevelopmental outcomes was influenced by breastfeeding duration. The duration of breastfeeding that has previously been associated with both increased cognitive function at school age[88] and adult IQ[13,89]. More than 90% of the children in the cohort were breastfed for more than 1 month (and over 60% of the children were breastfed fully for 4 months) which is much higher than in previous studies[13,89] showing positive effect of breastfeeding, where 20% of the mothers breastfeed for 1 month or shorter[89] . In our study, all children were followed closely from birth and we were able to consider multiple social factors to strengthen the reliability of our results. Thereby our study could point towards effects occurring later in life or perhaps more likely that a successful long breastfeeding period may be determined and thereby confounded by social factors. Alternatively, the lack of association could be due to an improved composition of infant formula

feeding over the last decades. Lastly, the reason for our negative finding might be due to the heterogeneity of breastfeeding duration in the $COPSAC_{2010}$ cohort.

Milestones were achieved at an earlier age in boys than in girls, which is in contradiction to a previous large multicenter study[85], but in agreement with another Danish study[73]. However, both language and cognitive scores were higher for girls than boys, which is in agreement with a number of other studies[7,54,90,91] and might be explained by the fact that girls on average mature faster than boys[92]. We found higher language scores at 1 year, associated with smoking during pregnancy, but did not find any associations with the other neurodevelopmental scores (including the 2 year language scores), which may indicate a spurious finding. Maternal smoking during pregnancy has in previous studies been shown to be a potential confounder of psychomotor development, both as a positive[53] and negative factor[91]. In contrast to previous studies[7,81,93], we did not find significant associations between any of the neurodevelopmental scores and maternal education. The effects of gestational age, maternal age and being first born were in concordance with previous studies[53,94,95].

We were unable to see any associations between the neurodevelopmental outcomes and persistent wheeze or eczema. These results are contradicting a previous study showing that children with allergic sensitization and especially eczema at 12 months, had lower motor scores at 18 months of age[21]. The inconsistencies of these finding might be explained by the age of assessment or the method of evaluation of the neurodevelopmental outcome. Furthermore, our close clinical follow up of the children assures early diagnosis and immediate initiation of the relevant treatment, thus minimizing negative effects of manifestation of these diseases. Lastly, we did not find any association between neurodevelopmental outcomes and the numbers of days the children were sick during their first year of life. I have not been able to find any other study investigating that outcome in otherwise healthy children.

5.2. Effects of the n-3 LCPUFA Supplementation

Maternal n-3 LCPUFA supplementation during third trimester of pregnancy positively affected neurological development in the offspring. We have demonstrated that boys in particular benefit

from the n-3 LCPUFA supplementation. When translated to an entire population the effect size is of an important magnitude.

To our knowledge only one other study, the Domino trial[54] was powered to analyze gender specific effects of n-3 LCPUFA supplementation during pregnancy. They found no overall effect of the intervention on cognitive score at age 1 ½ years, but in line with our study found a gender-specific effect with fever boys from the intervention group presenting with a score indicating delayed cognitive development (<85), but that effect was not seen among the girls. In contrast with their finding, we found that the entire group of boys of the n-3 LCPUFA supplemented mothers were skewed towards higher scores, not only the ones with the lowest scores (**Figure 8**). In the previous trial the children were younger when tested (1 ½ years vs 2½ years). Furthermore, the Domino trial had an overrepresentation of premature children (13.2%), whereas we excluded all children born before gestational week 37.

Furthermor, two RCT's, which examined the effect of DHA enriched feeds (breastmilk and/or formula) given to preterm infants found opposing results on BSID cognitive scores among boys and girls. In one of them the DHA-supplemented boys had higher cognitive scores (and no effect on the girls) [96], whereas the other found that the DHA-supplemented girls had higher cognitive scores than the control group (and no effect on the boys) [97]. One other RCT [56,57] saw a positive effect of n-3 LCPUFA supplementation (during pregnancy and lactation) on overall IQ scores at the age of 4, but that effect was no longer maintained at 7 years of age, but that study did not examine the effect in gender subgroups.

The different responses to the supplementation between genders might be due to gender-specific differences in the essential fatty acid metabolism that is their ability to endogenous synthesis of DHA. There is accumulating evidence pointing to an effect of sex hormones on fatty acid composition[98]. Animal experiments and human trials indicate that estrogen stimulates the conversion of essential fatty acids to their longer chain metabolites, whereas testosterone inhibits. These studies have shown that females have a higher rate of endogenous synthesis of DHA from the precursor fatty acid ALA [47,98] whereas testosterone decreases delta-6 desaturase activity, which could result in decreased rate of the endogenous synthesis among males compared to females. This could imply that boys were more sensitive to DHA intake and thus more dependent on n-3 LCPUFA supplementation to optimize their neurodevelopment compared to girls.

Our study is the only pregnancy n-3 LCPUFA RCT examining a possible effect on a broad range of childhood milestone achievements. We demonstrate that boys from the n-3 LCPUFA supplemented

mothers achieved motor milestones at a younger age, with no effect on the girls, which supports the effect seen on the cognitive scores. Previous trials including motor developmental measures as an outcome did not show any difference between the groups [54,58,59,61]. However, a large Danish population-based cohort of pregnant women and their children observed a benefit of higher maternal fish consumption during pregnancy on attainment of developmental milestones at both 6 and 18 months [53].

We found a positive effect of the intervention on word production at 1 year, which seemed to be driven by an increased word production among the boys. This effect did not persist till age 2 years, where the boys from the n-3 LCPUFA group still presented an increased word production, which was however non-significant. The DOMInO trial also demonstrated gender-specific effects on language scores, where the girls in the intervention group were more likely to have delayed language development.

Tests used to evaluate the children's neurodevelopment must be in accordance with the maturational stage of the children, and it is well established that boys develop slower than girls do. It is possible that BSID-III at 2 ½ years of age is better to detect differences between the boys than the girls, thus explaining the gender differences observed in cognitive score.

5.3. Strengths and Limitations

It is a significant strength, that the study was conducted in an unselected population-based cohort, which increases external validity. The study is further strengthened by the longitudinal design, allowing neurodevelopmental assessment at multiple time points from birth until 3 years of age.

The fact that all the assessors were well-known to the children may also have increased test compliance and feasibility. The Randomized Controlled trial is among the largest RCTs on n-3 LCPUFA supplementation in pregnancy and neurodevelopment.

The large sample size along with high adherence and follow-up rates, enabled sufficient power to analyze the effects of gender separately.

The specificity of the clinical endpoints is high as the children were followed in a comprehensive clinical program including a daily symptom diary and standardized diagnoses of clinical endpoints.

Another advantage of the design is the comprehensive information on factors possibly influencing neurodevelopment including information on breastfeeding duration, introduction of infant formula, and weaning foods obtained prospectively by interviews.

The PCA approach was a valid method to reduce milestone and language scores to fewer uncorrelated variables, which reduces the risk of multiple testing.

The BSID-III cognitive test was performed by highly trained personnel assuring consistency in the testing procedures. All tests were validated by video recordings, which improve reliability of the score for each child.

A possible limitation of the study is the post-randomization exclusion of 7% of the children, which could have biased the true effect of the n-3 LCPUFA supplementation. However, a similar effect was found in a *post-hoc* analysis of the primary neurodevelopmental outcome of the intervention study (BSID-III cognitive score) with all children, who completed the test (n=629) including premature and small for gestational age children as well as children with neurological diagnoses.

The main limitation of our study is the lack of assessment of parental IQ and parental stimulation of the child. Another possible limitation is the use of parent report for both the milestone registration and language development and the retrospective registration of milestones for some children, increasing the risk of recall bias. However, studies have shown an excellent correlation between gross motor milestones evaluated by a paediatric neurologist and parental recollection of the age of milestone achievement two years later[99]. Another study comparing the accuracy of direct language assessment and parent reports in 2-year-old toddlers showed that parent language report is a valid and efficient tool for assessing productive language abilities and comparable with those of direct language measures [100].

Finally, the number of research personal, who performed the cognitive test is a possible disadvantage. However, the same person trained them, and all sessions were video recorded allowing for subsequent review of the scoring by one person (EB), where no differences in scores were found.

6. Conclusion and Perspectives

In **Paper I** we found that neurodevelopment in the first years of life was not influenced by breastfeeding and thus contradicting previous reports. This could point towards residual confounding in previous studies, enhanced infant formulas over the last decades or the homogenous character of breast feeding pattern in our cohort. The study showed that all neurodevelopmental outcomes where affected by gender with motor milestones being achieved at an earlier age among boys, whereas higher language and cognitive scores were found among girls. A younger age of milestone achievement was observed with lower maternal age, higher gestational age and paternal leave; a higher one-year language score was associated with maternal smoking in pregnancy and a higher two-year language score with being first born. We did not find any association between neurodevelopmental outcomes and persistent wheeze, eczema or number of sick days.
In **Paper II** we found that supplementation with n-3 LCPUFA in third trimester of pregnancy enhanced the neurodevelopment of their male children. The boys from the supplemented group had higher cognitive scores and achieved motor milestones at a younger age. These findings may be used as a guideline for pregnant women, where a simple and safe supplementation may optimize the neurodevelopment of young boys.

Follow-up of this cohort is needed to assess, whether the effect of the n-3 LCPUFA supplementation remains beyond early childhood.

It will also be interesting to investigate if the effect of n-3 LCPUFA supplementation is modulated by the maternal and/or child *FADS genotype,* which has been shown to have a sex-specific effect on neurodevelopment in children[101].

Both studies confirm that when assessing neurodevelopment of young children it is important to take into consideration the differences of the genders, and preferably have enough power to be able to analyze data separately for boys and girls.

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Appendix A – Additional Results

Figure E1. Box-plot of the window of achievement of developmental milestones

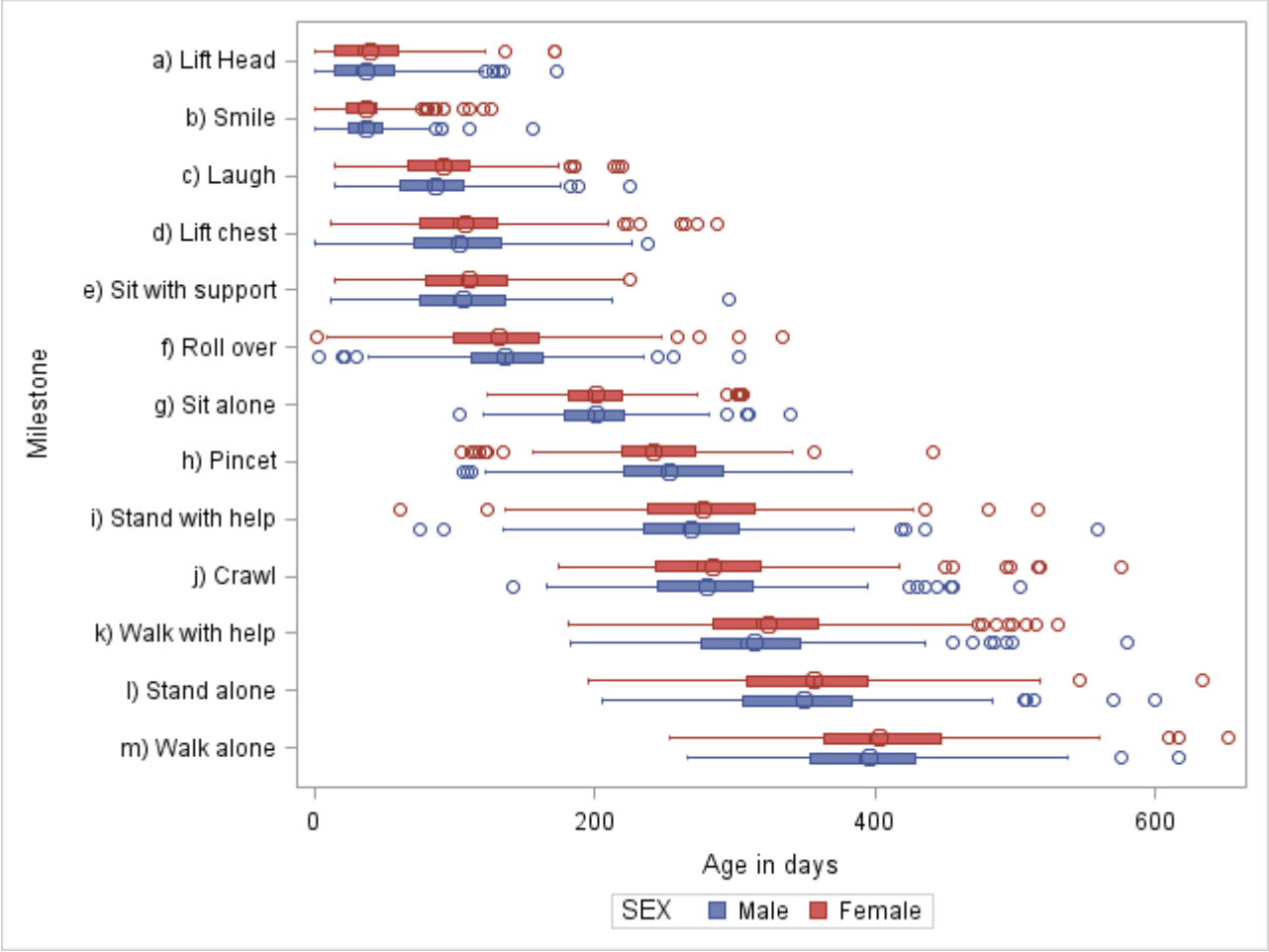

Figure E2: Comparison between the age of milestone achievement in COPSAC and WHO multicenter growth study

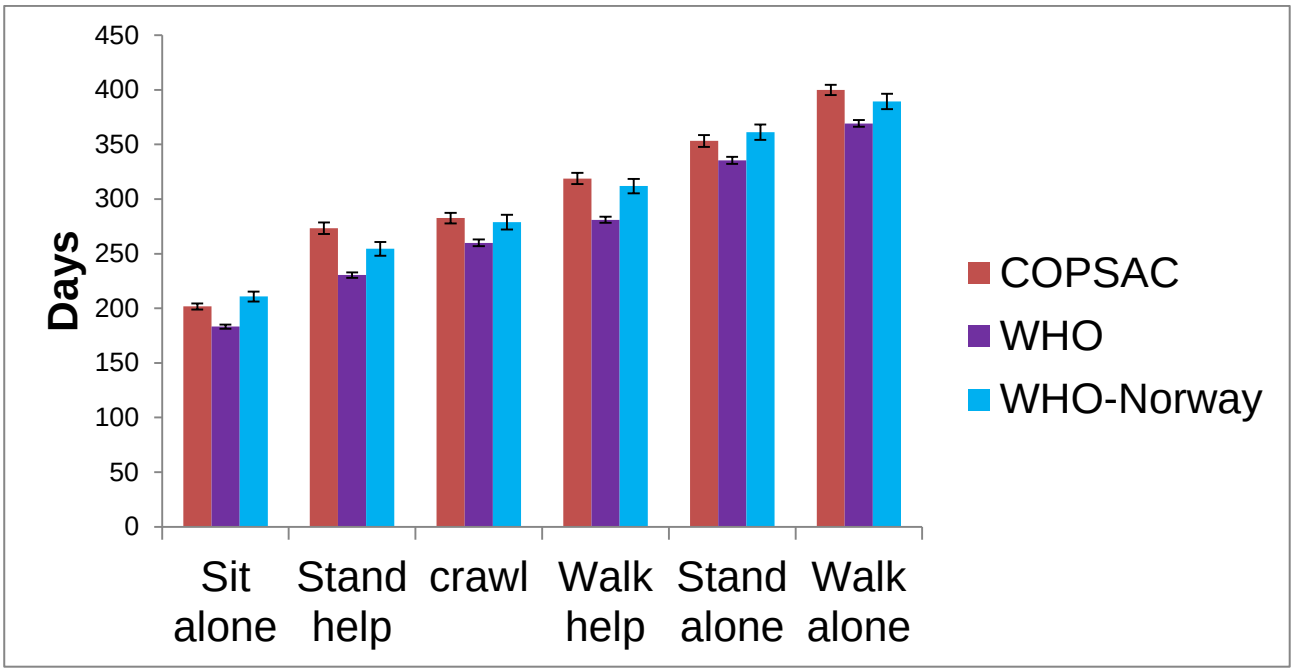

Table E1: Comparison of composite cognitive score of the BSID-III test including children born before 37 gestational weeks, children with low birth weight (<2500 g) and children with a neurological diagnosis

	N	Mean	Min	max
Study Group	600	104.8	85	145
All children that completed the BSID-III test	630	104.8	85	145

Figure E3. The graph shows the background distribution of chi-squared values from the cognitive score of the BSID-III test, and the blue lines represent the differences in the Chi-squared scores statistic by removing each examiner. The z-values obtained by removing individual examiner does not reach statistical significance.

Appendix B – Paper I, II

Paper I

Given the COPSAC cohort, it is an opportunity to evaluate factors influencing the development - although the foucus on airways is somewhat subdued. Some previous knowledge is confirmed, some is questioned and discussed. The factors chosen are questionable for evaluation and why something is important as parental development is omitted.

Given the COPSAC kohorte, er det en mulighed for at evaluere faktorer, der påvirker udviklingen - selv om foucus på luftvejene er noget afdæmpet. Nogle forkundskaber er bekræftet, nogle er afhørt og diskuteres. De valgte faktorer er tvivlsom til evaluering og hvorfor noget er vigtigt, da forældrenes udvikling er udeladt.

Paper II

As fish oil contains other bioactive compounds than n-3 LCPUFA there could have been a better distinction between fish oil and n-3 LCPUFA in general. Under intervention in the abstract, it could say, “fish oil capsules” not only “capsules”. And the conlusion should be about “fish oil capsules” not only n-3 LCPUFA. More explanation about why this study is necasary as there are many similar studies. Not clear if the adherence was taken into account in the analyses, for example if any per-protocol analysis has been performed. The amount of fish oil given could have been discussed in relation to other studies.

Paper I

Determinants of Neurological Development in Early Childhood

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Authors contributions:
The guarantor of the study is Professor Hans Bisgaard, MD, DMSc, who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript, and approved the final manuscript as submitted.

MD Elín Bjarnadóttir and MD, PhD Jakob Stokholm where responsible for acquisition, analysis and interpretation of data and writing of the manuscript and approved the final manuscript as submitted. Bjarnadóttir wrote the first draft of the manuscript.

MD, PhD, DMSc Bo Chawes, MD Jonathan Thosen, MD Anna-Rosa Cecilie Mora-Jensen, MD Maja Deleuran, MD, PhD Klaus Bønnelykke and MSc, PhD Lotte Lauritzen have all contributed to data interpretation and have made critical revision of the manuscript and approved the final version as submitted.

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Ethics committee approval: The study was conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the Ethics committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2008-41-2599).

Abstract

Background: The objective of this study was to identify possible pre- and postnatal factors influencing neurodevelopment of the young child.

Methods: We used data from the first 3 years of life of the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother-child cohort. Of the 700 children enrolled, this study excluded those with a neurological diagnosis, born <37 weeks of gestation and birth weights <2500g, resulting in 650 children analyzed. Neurodevelopment was assessed as age of achievement of early milestones, language scores at 1 and 2 years and cognitive score at 2 ½ years of age.

Findings: Early age at milestone achievement was associated with male gender (p=0.05), lower maternal age (p=0.02), higher gestational age (p<0.001) and paternity leave (p=0.01). A higher 1-year language score was associated with female gender (p=0.02) and maternal smoking during pregnancy (p=0.01) and a higher 2-year language score with female gender (p<0.001) and being first born (p=0.01). A higher cognitive score was associated with female gender (p=0.02). Milestones, language or cognitive scores were not influenced by breastfeeding, persistent wheeze, eczema, and number of sick days (p>0.05 in all tests).

Interpretation: Early age of milestones were affected by male gender, maternal age, gestational age and paternity leave. A higher 1-year language score was associated by female gender, maternal smoking, and 2-year language score by female gender and being first born and higher cognitive score was associated with female gender. Particularly, neurodevelopmental scores were unrelated to breast-feeding, persistent wheeze, eczema, and number of sick days.

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Council for Independent Research and The Capital Region Research Foundation have provided core support for COPSAC.

Abbreviations

BSID-III = Bayley Scales of Infant and Toddler Development, Third Edition
CDI= MacArthur Communicative Development Inventory
CI= Confidence interval
$COPSAC_{2010}$ = COpenhagen Prospective Studies on Asthma in $Childhood_{2010}$
PCA = Principal Component Analysis
PC = Principal Component
WHO = World Health Organization

Introduction

Early life exposure may play a role shaping neurological development[1] and be an important indicator of the child's subsequent life achievements[2–5]. Neurological development, including cognitive function such as intelligence and language as well as fine and gross motor skills, depends on the pre- and postnatal development of the brain, which is affected by both genetic and environmental factors. During the last trimester of fetal life and the first two years of childhood, the brain undergoes a period of rapid growth[6] where it reaches 80% of its adult weight. Throughout this period the brain development is particularly vulnerable to the pre- and perinatal environment including not just biological factors like lack of oxygen or extreme prematurity, but also other factors like socio-demographic determinants[7], stress and nutrition[2,5]. Higher IQ later in life has been associated with a longer duration of breastfeeding[8]. Delayed neurodevelopment in childhood has been associated with chronic inflammatory disease such as asthma, eczema and allergic sensitization in infancy [9] and conversely delayed neurodevelopmental scores have also been shown to predate later development of such disorders[10], though in general the studies have shown ambiguous results[11,12].

The aim of this study was to identify prenatal and early life factors associated with achievement of developmental milestones, and scores in language and cognitive tests in the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother-child cohort. We explored whether early neurological development was affected by duration of breastfeeding, gender, being a first born, paternity leave, smoking during pregnancy, gestational age, maternal age, age at start daycare, maternal education and furthermore examined potential effects of persistent wheeze, eczema and number of sick days in the first three years of life.

Methods

Study design and participants

The $COPSAC_{2010}$ is a population-based clinical prospective mother-child cohort study of 738 pregnant women and their children recruited during 2008-2010 which was previously described in details[13]. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the Ethics committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2008-41-2599). Both parents gave informed consent prior to enrollment of the children.

The children were enrolled in the study at 1 week of age, excluding anyone with severe congenital abnormality. The children were followed in the COPSAC clinical research units with 11 planned visits during their first three years of life. Children with a neurological diagnosis, born <37 weeks of gestation and birth weights <2500g where excluded from the present study.

Neurodevelopmental assessment

Milestones

At the child's first visit (1 week of age) the parents received a registration form, with thorough instructions, based on The Denver Development Index[14] and WHO (World Health Organization) milestones registration[15]. Dates of achievement of 13 predefined milestones were registered prospectively by the parents. Implementation of the prospective milestone registration started after the first 500 children were born, and some of the milestones of these children were therefore registered retrospectively.

Language development

Language development was assessed with the Danish version of The MacArthur Bates Communicative Developmental Inventory (CDI)[16]. The assessment was performed by a web-based

questionnaire filled out by parents around the child's 1st (CDI-WG: Words and gesticulation) and 2nd year birthdays (CDI-WS: Words and sentences). The 1-year questionnaire evaluates language comprehension, early word production and gestural communication and the 2-year questionnaire assesses vocabulary, grammatical skills, syntax and morphology.

Cognitive development

Cognitive development was assessed at 2½ years of age, using the cognitive part of the third edition of the Bayley Scales of Infant and Toddler Development (BSID-III)[17]. 10 examiners performed examinations under video surveillance, and inter-examiner consistency was all checked by the first author inspecting of every video recordings blinded for the children's score. During the examinations, the examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on its performance, the child was given a composite score, which was standardized by use of a normalization material of age corrected means of 100 and a standard deviation of 15 (range 50-150).

Further methodological details regarding neurodevelopment are described in the online repository.

Predictors of neurodevelopmental outcomes

Information regarding factors potentially influencing the child´s neurodevelopment was collected prospectively during the scheduled visits to the COPSAC clinics. This included information regarding pregnancy and birth (i.e. gender, ethnicity, anthropometric data, congenital disease, gestational age, maternal smoking during pregnancy and maternal age at delivery), home environment (i.e. older siblings, language spoken), socioeconomic and educational status, paternal leave and age at start in daycare.

Duration of breastfeeding

Information on breastfeeding was obtained longitudinally by interviews and investigated as duration of exclusive breastfeeding and total breastfeeding period. Breastfeeding was evaluated as both continuous variables and grouped: Exclusive breastfeeding (0-1 month, 1-4 months, 4-6 months, >6 months) and total breastfeeding (0-1 month, 1-4 months, 4-6 months, 6-12 months, >12months).

Clinical predictors of neurodevelopment

Respiratory and skin symptoms were recorded by the parents in daily diaries from birth to 3 years. Persistent wheeze was diagnosed according to a previously validated quantitative algorithm[18,19] requiring all of the following: (1) recurrent troublesome lung symptoms (verified diary recordings of ≥5 episodes of troublesome lung symptoms lasting ≥3 days within 6 months or continuous troublesome lung symptoms >4 weeks); (2) typical symptoms of asthma, e.g. exercise induced symptoms, prolonged nocturnal cough, persistent cough outside common cold; (3) need for intermittent bronchodilator; and (4) response to a 3-month trial of inhaled corticosteroids and relapse upon cessation[18].

Eczema diagnosis was based on the criteria of Hanifin and Rajka, which requires the presence of 3 of 4 major criteria and ≥3 of 23 minor signs[20].

Diagnoses of persistent wheeze and eczema at any time point before age 3 were used as dichotomized end-points.

Sick days: Infections, categorized as common cold, pneumonia, pharyngitis, otitis, fever, gastro-intestinal infection were monitored in the daily diaries. The number of days with either troublesome lung symptoms and/or infection in the child's first year was used as a continuous variable to define "number of sick days in first year of life".

Statistical analysis

All variables were tested for normal distribution and differences in the population characteristics were determined by Chi-square test, Student's t-test, or Wilcoxon rank-sum test. Missing data was treated as missing observations, except in the principal component analysis (PCA) of milestones.

Principal component analysis (PCA) was used to extract underlying latent components (principal components, PCs), which describe the systematic part of the variation across the original milestones and language variables in fewer uncorrelated variables. For the milestone data we used probabilistic PCA (R) to generate the PCs, as this analysis can analyze full data set with missing values, assuming that the missing values are at random. No transformation of data was needed for language data as there were no missing values. Linear or logistic regression analysis was used where appropriate to determine significant predictors of neurodevelopmental test scores. All the *a priori* factors identified as possible predictors of neurodevelopment where included in the model (gender, parity, smoking during pregnancy, gestational age, maternal age at delivery, maternal educational level, breastfeeding duration, paternal leave, and age at start in daycare). Analyses of clinical predictors (persistent wheeze, eczema and sick days) were adjusted for gender.

To examine correlations between neurological endpoints, a heatmap was drawn based on spearman correlations between all variables, using pairwise complete observations. For presentation, the variables were clustered using hierarchical clustering of euclidean distances, and drawn using the R package "pheatmap"[21]

A significance level of 0.05 was used in all types of analysis. All estimates were reported with 95% confidence intervals (CI). The data processing was conducted using SAS version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA) and R version 3.3.0 (R core team 2016), with the packages "ggplot2", "ggbiplot2" and "ggrepel"[22].

Results

Baseline characteristics

A total of 700 children were included in the $COPSAC_{2010}$ cohort at birth with a clinical follow-up rate of the cohort of 98% at age 1 year; 95% at age 2 years; and 94% at age 3 years. 34 children were excluded from the neurodevelopmental analysis: 5 because of a neurological diagnosis, 24 because they were born prematurely and 5 because of low birth weight. Furthermore, 16 children did not complete any of the neurological tests, leaving 650 eligible children with neurodevelopmental assessment. **Figure 1** shows the flow of participants throughout the study.

Baseline characteristics of the participants is shown in **Table 1**. The children were solely breastfeed for 104 days (SD 60) and the total length of breastfeeding was 245 days (SD 155). During the first year of life, the children had a median of 48 (IQR, 28-79) days with either an infection and/or troublesome lung symptoms. By the age of 3 years, persistent wheeze had been diagnosed in 19% (123 children), and eczema in 25% (165 children). There were no significant differences in the baseline characteristics between the children, who completed the neurological endpoints and the ones, who did not.

Neurodevelopment

Milestones

At least one of the 13 milestones were registered by 610 of the families and **Figure 2** shows the age of milestone achievement in these children. In the PCA all the milestones were positively correlated in the first principal component (PC1), which explained 37% of the variation and was driven primarily by the late gross-motor milestones (crawling, walking and standing). The second principal component (PC2) explained 16% of the variation and was primarily driven by the early milestones (smiling, lifting the head, and sitting with support) and the later milestones in opposite directions

(**On-line Figure E1**). PC1 and PC2 were used as the main milestone variables in our predictor analysis.

Early age at milestone achievement was associated with male gender (PC1, p=0.05), lower maternal age at birth (PC1, p=0.02), as well as higher gestational age (PC1, p<0.001; PC2, p=0.01) and paternity leave (PC2, p=0.01) (**Table 2**).

Analysis of the original milestone variables by conventional statistics confirmed these findings as 9 out of the 13 milestones showed early attainment correlated with high gestational age (p-values: <0.001 to 0.017), and boys achieved 9 out of 13 milestones at a younger age than the girls (1-8 days difference) (data not shown).

Language development

Complete language data at 1 and 2 years of age was obtained from 323 and 470 participants, respectively. The overall results from the language tests are shown in **Table 3**.

The 1-year language scores were all positively correlated in the PCA model, where PC1 explained 53% of the overall variation in the dataset (**On-line Figure E2**), which was therefore used as a combined measure of 1-year language development. A higher 1-year language score was associated with female gender (p=0.02) and maternal smoking during pregnancy (p=0.01) (**Table 4**). Analysis of word production as the endpoint (data not shown) confirmed that maternal smoking during pregnancy was the strongest predictor for the 1-year language development (mean difference=5.3 words (95% CI= 2.4, 8.2), p<0.001).

The 2-years language scores were all positively correlated in the PCA model, where PC1 explained 60% of the variation in the dataset (**On-line Figure E3**). A higher 2-year language score was associated with female gender (p<0.001) and being first born (p=0.01) (**Table 4**). Analysis of word

production as the endpoint (data not shown) confirmed that female gender was the strongest predictor for 2-year language development (mean difference=63 words (34-91), p<0.001).

Cognitive score

601 children completed the BSID-III test. The mean composite score was 104.9 (SD 9.8). There were no differences in scores among the 10 persons performing the tests. Nevertheless, we included test person as a possible confounding factor in our analysis. A higher cognitive score was only significantly associated with female gender (p=0.02); girls scored 1.88 (95% CI: 0.30-3.47) points higher than boys (**Table 4**).

Neurological development and breastfeeding period

None of the neurological endpoints was significantly associated with the continuous variables of exclusive or total breastfeeding duration. The same was found when categorizing the exclusive and total breastfeeding periods as <1month, 1-4 months, 4-6 months, >6 months (**Table 5**).

Neurological development and clinical predictors in the first years of life

None of the neurological endpoints was significantly associated with any of the clinical predictors: persistent wheeze 0-3yrs, eczema 0-3yrs or number of sick days in the first year of life (**Table 6**).

Correlation between neurodevelopmental endpoints

A heat map with all the original milestones, 1- and 2-year language scores and the BSID-III composite score (**On-line Figure E4**), showed that all the language scores and all the milestone were inter-correlated and that the composite score of the BSID-III was highly correlated with the 2-year language scores.

Discussion

Principal Findings

We found that all neurodevelopmental scores in early childhood was markedly gender determined with boys achieving motor milestones at an earlier age whereas girls had higher language scores and a higher cognitive composite score. None of the neurodevelopmental scores was influenced by persistent wheeze, eczema, or number of sick days in the first years of life. Particularly, neither milestone, language nor cognitive scores were influenced by breastfeeding duration.

Strengths and Limitations

It is a significant strength, that the study was conducted in an unselected population-based cohort, which increases external validity. Furthermore, the longitudinal design, following the children from birth until age 3 allowed neurodevelopmental assessment at 11 visits to the research clinic. The fact that all the assessors were well-known to the children may also have increased test compliance and feasibility.

The specificity of the clinical endpoints is high as the children were followed in a comprehensive clinical program including a daily symptom diary and standardized diagnoses of clinical endpoints. Another advantage of the design is the comprehensive information on factors possibly influencing neurodevelopment including information on breastfeeding duration, introduction of infant formula, and weaning foods obtained prospectively by interviews.

The PCA approach was a valid method to reduce milestone and language scores to fewer uncorrelated variables, which reduces the risk of multiple testing.

The main limitation of our study is the lack of assessment of parental IQ and parental stimulation of the child. Another possible limitation is the retrospective registration of milestones for some

children, increasing the risk of recall bias. However, studies have shown an excellent correlation between gross motor milestones evaluated by a pediatric neurologist and parental recollection of the age of milestone achievement two years later[23].

Finally, the number of research personal, who performed the cognitive test is a possible disadvantage. However, the same person trained them, and all sessions were video recorded allowing for subsequent review by the first author, where no differences in scores were found.

Interpretation

The $COPSAC_{2010}$ children achieved gross motor milestones at a later age than children in the WHO Multicenter Growth study, but at a similar age as the Norwegian children in the same study [24], which might indicate a culture-specific care behavior. All the developmental outcomes in the study were within normal range.

Overall the 1 and 2-year language scores in the $COPSAC_{2010}$ cohort were lower than in other cohort studies[25] and slightly lower than in an another Danish birth cohort[26]. It is known that Danish children generally score lower on both the 1 and 2-year language tests[27] and it has been suggested that the delay is related to the nature of Danish sound structure, which presents Danish children with a harder task of segmentation[27]. However, it could also be affected by cultural differences and early attendance to daycare in Denmark[27].

The mean cognitive score from the BSID-III test was slightly above average (104.8, SD 9.8), and no children scored less than 85. The average score was similar to other cohort studies of healthy children[9,28] and the lack of participants with scores of less than 85 might be due to a selection bias in the cohort or the exclusion criteria including prematurity and neurological disorders in our study.

None of the neurodevelopmental outcomes was influenced by breastfeeding duration. More than 90% of the cohort were breastfed for more than 1 month, all children were followed closely from birth and we were able to take multiple social factors into account to strengthen the reliability of our results. The duration of breastfeeding has previously been associated with both increased cognitive function in school age[29] and adult IQ[8]. Thereby our study could point towards effects occurring later in life or perhaps more likely that a successful long breastfeeding period may be determined and thereby confounded by social factors. Alternatively, the lack of association could be due to an improved composition of infant formula feeding over the last decades.

Milestones were achieved at an earlier age in boys than in girls, which is in contradiction to a previous large multicenter study[24], but in agreement with another Danish study[30]. Both language and cognitive scores were higher for girls than boys, which is in agreement with a number of other studies[7,28,31,32] and might be explained by the fact that girls on average mature faster than boys[33]. We found higher language scores at 1 year associated with smoking during pregnancy, but did not find any associations with the other neurodevelopmental scores, which may indicate a spurious finding. Maternal smoking during pregnancy has in previous studies been shown to be a potential confounder of psychomotor development, both as a positive[34] and negative factor[32]. In contrast to previous studies[7,26,35], we did not find significant associations between any of the neurodevelopmental scores and maternal education. The effects of gestational age, maternal age and being first born were in concordance with previous studies[34,36,37].

Lastly, we were unable to see any associations between the neurodevelopmental outcomes and persistent wheeze or eczema. These results are contradicting a previous study showing that children with allergic sensitization and especially eczema at 12 months, had lower motor scores at 18 months of age[9]. The inconsistencies of these finding might be explained by the age of assessment or the

method of evaluation of the neurodevelopmental outcome. Furthermore, our close clinical follow up of the children assures early diagnosis and immediate initiation of the relevant treatment, thus minimizing negative effects of the disease.

Conclusion

Early life neurodevelopment was not influenced by breastfeeding, contradicting previous reports. This could point towards residual confounding in previous studies or enhanced infant formulas over the last decades. This study confirms all neurodevelopmental outcomes being affected by gender with motor milestones being achieved at an earlier age among boys, whereas higher language and cognitive scores were found among girls. A lower age of milestone achievement was observed with lower maternal age, higher gestational age and paternal leave; a higher one year language score was associated with maternal smoking in pregnancy and a higher two year language score with being first born. We did not find any association between neurodevelopmental outcomes and persistent wheeze, eczema or number of sick days.

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Table 1. Baseline characteristics of the $COPSAC_{2010}$ cohort and the children that completed the different neurological test outcomes.

	All n=700	Milestones n=610	1 y language n=320	2y language n=470	Bayley n=601
Birth cohort					
Boys, % (n)	51% (360)	51% (297)	50% (160)	51% (236)	51% (293)
Mother's age at birth, mean ±SD, years	32.3 ±4.4	32.3 ± 4.3	32.3 ± 4.2	32.3 ± 4.2	32.3 ± 4.3
Caucasian % (n)	96% (669)	96% (588)	97% (323)	98% (463)	96% (580)
Pregnancy and birth					
Gestational age, mean ±SD, weeks	39.9 ± 1.7	40.1 ± 1.3	40.1 ± 1.3	40.1 ± 1.2	40.1 ± 1.2
Weight 1 week, mean ± SD, kg	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5
Length 1 week, mean ± SD, cm	52.1 ±2.2	52.1 ± 2.1	52.2 ± 2.1	52.1 ± 2.2	52.1 ± 2.1
Head circumference at 1 week, mean ± SD, cm	35.7 ±1.4	35.7 ± 1.3	35.7 ± 1.3	35.7 ± 1.3	35.7 ± 1.4
Apgar score at 5 min., mean ±SD	9.9 ±0.34	9.9 ± 0.33	9.9 ± 0.23	9.9 ± 0.33	9.9 ± 0.33
Exposure					
Birth order, % first born, (n)	46% (323)	45% (279)	44% (145)	43% (205)	46% (277)
Smoking during pregnancy, % (n)	7.7%(54)	7.2% (44)	6.0% (20)	6.1% (29)	7.4% (43)
Duration of solely breastfeeding, mean ± SD, days	104 ± 60	104 ± 59	105 ± 59	107 ± 58	105 ± 59
Duration of total breastfeeding, mean ± SD, days	245 ± 155	246 ± 147	248 ± 140	251 ± 142	248 ± 152
Age at start in daycare, mean ± SD, months	10.8 ± 3.1	10.8 ± 3.1	10.9 ± 3.2	10.7 ± 3.1	10.8 ± 3.0
Paternity leave >4 weeks, % (n)	53% (354)	54% (323)	55% (179)	54% (249)	53% (313)
Socioeconomic variables					
Maternal educational level (% Low: Medium: High) [1]	8:64:28	7:65:28	7:64:30	7:64:29	8:65:28
Annual household income (% Low: Medium: High) [2]	10:53:37	10:52:38	9:51:40	10:51:40	10:52:38
Clinical predictors[3]					
Persistent wheeze, % (n)	19% (123)	18% (108)	19% (61)	18% (83)	18% (108)
Eczema, % (n)	25% (165)	26% (153)	26%(85)	26% (121)	26%(154)
Days sick in first year of life, median (25^{th}-75^{th} percentile)	48(28-79)	49 (28-80)	47 (28-74)	50 (28-82)	48 (28-80)

[1]*: Low (elementary school or college graduate), Medium (tradesman or medium length), and High (university candidate)*
[2]*: Low (<*55.000 *Euros/year), High (>110.000 Euros/year)*
[3] *Prevalence of persistent wheeze and eczema in the 660 children who have full follow up to 3 years. Days sick in first year of life is given for the 522 children with a full diary registration (365 days)*

Table 2. Multivariate analysis of factors influencing overall age of milestone development[1]

Predictor	**PC 1** Effect (95% CI)	**PC 2** Effect (95% CI)
Gender (Female/Male)	0.32 (0.00, 0.63) **P=0.05**	0.02 (-0.20, 0.22) P=0.96
First born (Y/N)	-0.04 (-0.38, 0.30) P=0.83	-0.11 (-0.34, 0.11) P=0.33
Paternity leave (Y/N)	-0.01 (-0.33, 0.31) P=0.96	-0.28 (-0.50, -0.06) **P=0.01**
Smoking during pregnancy (Y/N)	0.15 (-0.48, 0.78) P=0.64	0.30 (-0.13, 0.72) P=0.17
Gestational age (weeks)	-0.23 (-0.36, -0.11) **P<0.001**	-0.11 (-0.20, -0.03) **P=0.01**
Maternal age at birth (years)	0.05 (0.01, 0.09) **P=0.02**	0.02 (-0.01, 0.05) P=0.22
Age start daycare (months)	-0.02 (-0.07, 0.03) P=0.37	-0.02 (-0.05, 0.01) P=0.26
Mother's education	P=0.60	P=0.56
-High	0.18 (-0.20, 0.55)	-0.06 (-0.31, 0.19)
-Medium (ref)	0	0
-Low	0.16 (-0.46, 0.79)	-0.22 (-0.64, 0.20)

Abbreviation: CI= confidence intervals, PC= Principal Component from PCA in On-line Figure E1
[1]The estimate for each predictor is adjusted for all the other predictors using a multiple linear regression model. The effects are expressed as either differences in means (categorical variables) or β (continuous variables) with (95% CI).

Table 3. Language development at 1 and 2 years of age assessed by MacArthur Bates Communicative Developmental Inventory and cognitive scores at 2½ years of age assessed by Bayley III[1].

1 year language test n=320	
Age at test (months), mean ± SD	12.2 ± 0.3
Starting to talk (labeling and/or imitation) (%)	47.4
Early gestures (n out of 18), mean ± SD	11.2 ± 2.6
Girls - early gestures	11.6 ± 2.6
Boys – early gestures	10.8 ± 2.6
Late gestures (n out of 45), mean ± SD	8.9 ± 5.2
Phrases understood (n out of 26), mean ± SD	9.8 ± 5.2
Vocabulary comprehension (n of words from a list of 409), median (25^{th} -75^{th} percentile)	39 (20-66)
Vocabulary production (n of words from a list of 409), median (25^{th} -75^{th} percentile)	3 (1-7)
2 year language test n=470	
Age at test (months), mean ± SD	24.2 ± 0.3
Vocabulary production (n of words from a list of 725), median, (25^{th} – 75^{th} percentile)	250 (121-364)
Girls – vocabulary production	287 (162-401)
Boys – vocabulary production	197 (79-327)
Use of abstract words (n from a list of 5), median (25^{th}- 75^{th} percentile)	5 (4-5)
Uses grammar (%)	67.2
Girls – uses grammar	73.7
Boys – uses grammar	61.0
Irregular words (n from a list of 29), median (25^{th} – 75^{th} percentile)	2.0 (0-6)
Girls – irregular words	3 (1-7)
Boys – irregular words	2 (0-5)
Overregularized words (n from a list of 61), median (25^{th} – 75^{th} percentile)	0 (0-2)

Length of longest sentences (n of morphemes), mean ± SD	3.6 ± 1.6
Girls – longest sentence	4.0 ± 1.5
Boys – longest sentence	3.2 ± 1.5
Sentence complexity (n of complex from a list of 33 pairs), median (25^{th} – 75^{th} percentile)	3 (0-7)
Girls – sentence complexity	4 (1-9)
Boys – sentence complexity	1 (0-5)
Bayley test at 2 ½ years n=601	
Age at test (months), mean ± SD	30.5 ± 0.92
Cognitive composite score (standardized), mean ± SD	104.9 (9.80)
Girls – composite score	106.0 (10.4)
Boys – composite score	103.8 (9.9)

[1] Genders are presented separately where there is a statistical difference in the outcomes ($p<0.05$)

Table 4. Factors influencing 1 and 2 years language development and Bayley scores at 2 ½ years of age[1]

	Language 1 year	**Language 2 years**	**Bayley composite score**
Predictor	PC1 Effect (95% CI)	PC1 Effect (95% CI)	Composite score Effect (95% CI)
Gender (F/M)	0.50 (0.08, 0.92) P=**0.02**	0.87 (0.50, 1.23) **P<0.001**	1.88 (0.30, 3.47) **P=0.02**
First born (Y/N)	0.21 (-0.23, 0.66) P=0.37	0.48 (0.09, 0.86) **P=0.02**	1.08 (-0.65, 2.82) P=0.22
Paternity leave (Y/N)	0.01 (-0.42, 0.44) P=0.97	0.09 (-0.29, 0.46) P=0.65	0.66 (-0.97, 2.29) P=0.43
Smoking during pregnancy (Y/N)	1.19 (0.28, 2.09) **P=0.01**	0.49 (-0.29, 1.28) P=0.22	-0.89 (-4.05, 2.26) P=0.58
Gestational age (weeks)	0.05 (-0.12, 0.23) P=0.56	0.01 (-0.14, 0.16) P=0.91	0.44 (-0.21, 1.08) P=0.18
Maternal age at birth (years)	-0.05 (-0.10, 0.01) P=0.10	-0.04 (-0.09, 0.01) P=0.10	-0.08 (-0.29, 0.13) P=0.47
Age start daycare (months)	0.04 (-0.03, 0.10) P=0.29	0.03 (-0.03, 0.09) P=0.37	-0.03 (-0.29, 0.24) P=0.85
Mother's education	P=0.23	P=0.37	P=0.15
-High	-0.44 (-0.93, 0.06)	-0.01 (-0.44, 0.42)	1.93 (-0.01, 3.87)
-Medium	0	0	0
-Low	-0.04 (-0.94, 0.85)	-0.52 (-1.24, 0.21)	0.36 (-2.83, 3.56)

Abbreviation: CI= confidence intervals; PC= Principal Component from PCA in On-line Figure E2

[1]The estimate for each predictor is adjusted for all the other predictors using a multiple linear regression model. The effects are expressed as either differences in means (categorical variables) or β (continuous variables) with (95% confidence intervals).

Table 5. Correlations between neurological development and exclusive and total duration of breastfeeding[1].

	Age at milestone (PC1)	Age at milestone (PC2)	Language at 1 year (PC1)	Language at 2 years (PC1)	Bayley composite score
Exclusively breastfeeding (months)	-0.01 (-0.09, 0.08) P=0.86	-0.05 (-0.11, 0.01) P=0.08	-0.05 (-0.17, 0.06) P=0.39	0.00 (-0.10, 0.09) P=0.93	0.05 (-0.37, 0.48) P=0.80
<1 month (N=137)	REF	REF	REF	REF	REF
1-4 months (N=166)	0.21 (-0.26, 0.67) P=0.38	0.15 (-0.16, 0.47) P=0.33	-0.06 (-0.69, 0.58) P=0.86	-0.48 (-1.03, 0.08) P=0.09	0.39 (-2.00, 2.78) P=0.75
4-6 months (N=322)	-0.18 (-0.60, 0.23) P=0.39	-0.12 (-0.41, 0.16) P=0.40	-0.30 (-0.88, 0.27) P=0.30	-0.11 (-0.60, 0.39) P=0.67	0.49 (-1.65, 2.64) P=0.65
6+ months (N=32)	0.71 (-0.11, 1.53) P=0.09	-0.43 (-0.99, 0.13) P=0.13	-0.22 (-1.26, 0.81) P=0.67	0.01 (-0.86, 0.88) P=0.98	0.14 (-3.93, 4.21) P=0.95
Total breastfeeding (months)	-0.03 (-0.07, 0.00) P=0.07	-0.03 (-0.04, 0.00) P=0.09	0.03 (-0.02, 0.08) P=0.22	0.04 (-0.00, 0.08) P=0.05	0.06 (-0.12, 0.24) P=0.54
<1 month (N=46)	REF	REF	REF	REF	REF
1-4 months (N=77)	0.25 (-0.54, 1.03) P=0.54	-0.10 (-0.63, 0.42) P=0.70	-0.22 (-1.29, 0.85) P=0.68	-0.09 (-1.07, 0.89) P=0.86	-1.63 (-5.66, 2.38) P=0.42
4-6 months (N=91)	-0.12 (-0.88, 0.64) P=0.76	0.12 (-0.39, 0.62) P=0.64	-0.34 (-1.36, 0.67) P=0.50	0.20 (-0.74, 1.14) P=0.68	-0.68 (-4.56, 3.20) P=0.73
6-12 months (N=340)	0.27 (-0.40, 0.94) P=0.43	-0.16 (-0.61, 0.29) P=0.49	-0.54 (-1.47, 0.37) P=0.24	0.37 (-0.44, 1.23) P=0.40	-1.32 (-4.81, 2.17) P=0.46
12+ months (N=102)	-0.29 (-1.06, 0.49) P=0.47	-0.10 (-0.62, 0.42) P=0.70	0.35 (-0.69, 1.39) P=0.51	0.79 (-0.17, 1.75) P=0.11	0.77 (-3.21, 4.75) P=0.70

Abbreviation: PC= Principal Component from PCAs in On-line Figure E1 and E2

[1]The estimate for each predictor is adjusted for all other predictors using a multiple linear regression model. The effects are expressed as either differences in means (categorical variables) or β (continuous variables) with (95% confidence intervals), using the neurodevelopmental score as the outcome.

Table 6. Correlations between neurological development and persistent wheeze, eczema and sickness in the first years of life[1]

	Age at milestone (PC1)	Age at milestone (PC2)	Language at 1 year (PC1)	Language at 2 years (PC1)	Bayley composite score
Persistent wheeze (yes/no)	0.17 (-0.25, 0.59) p=0.54	0.01 (-0.29, 0.27) p=0.95	0.34 (-0.21, 0.90), p=0.23	0.001 (-0.47, 0.48) p=0.98	-0.55 (-2.61, 1.51) p=0.60
Eczema (yes/no)	0.02 (-0.34, 0.39) p=0.90	0.09 (-0.15, 0.34) p=0.46	-0.28 (-0.77. 0.21) p=0.26	-0.15 (-0.56, 0.26) p=0.48	-0.58 (-2.38, 1.22) p=0.53
Sick days in first year of life (weeks)	-0.03 (-0.06, 0.002) p=0.07	0.002 (-0.02, 0.02) p=0.81	0.04 (-0.01, 0.08) p=0.09	0.02 (-0.01, 0.05) p=0.52	-0.05 (-0.20, 0.09) p=0.47

Abbreviation: PC= Principal Component from PCAs in On-line Figure E1 and E2

[1]The estimate for each predictor is adjusted for gender using a multiple linear regression model. The effects are expressed as either differences in means (categorical variables) or β (continuous variables) with (95% confidence intervals), using the neurodevelopmental score as the outcome.

Figure 1. Flow chart of the study participants through the trial and main outcome measures

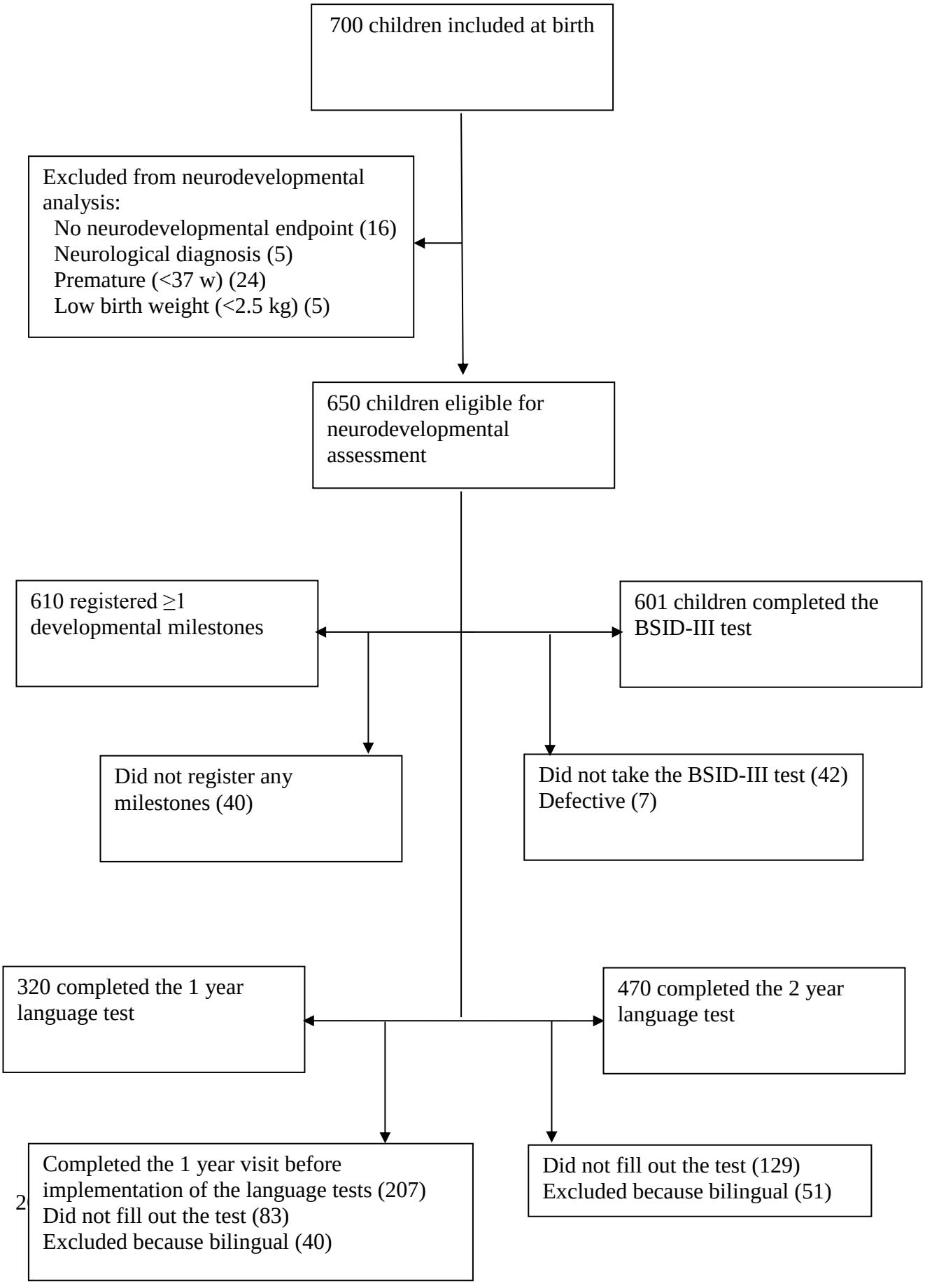
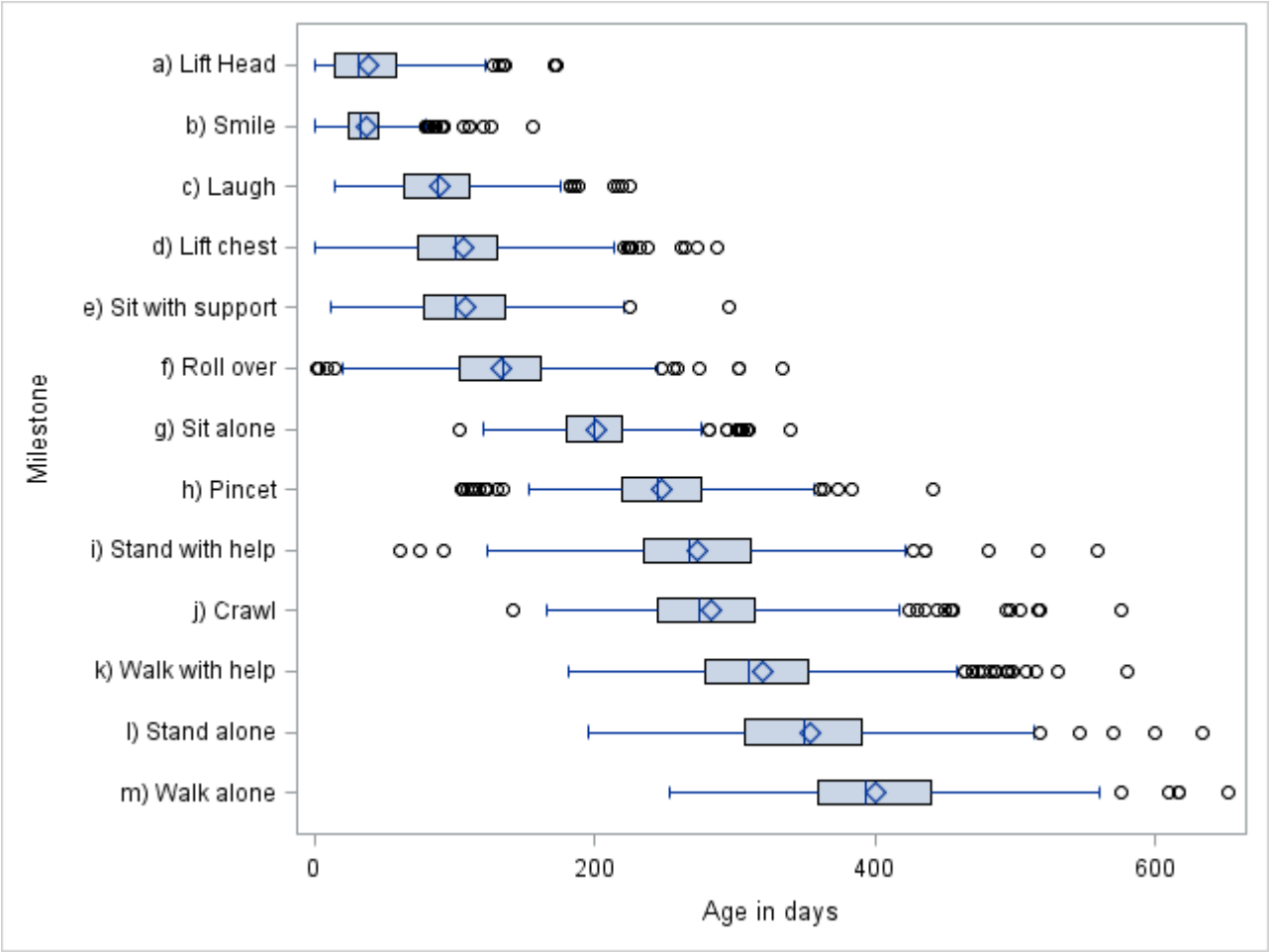


Figure 2. Box-plot of the window of achievement of developmental milestones



On-line Repository Figures

Figure E1. Biplot from principal component analysis of all the 13 milestones. Principal component 1 and 2 (PC1 & PC2) explained 37% and 16% of the overall variation in the data, respectively. PC1: Overall later milestone development. PC2: Late early milestones, early late motor milestones.

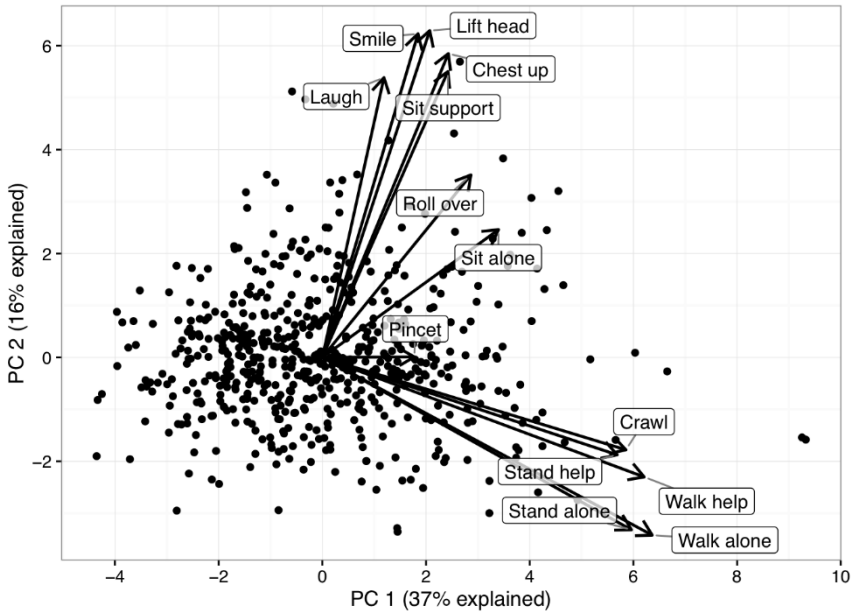


Figure E2. Biplot from principal component analyses of all the 1-year language development scores. Principal Component 1 (PC1) explained 53% of the variation in the dataset

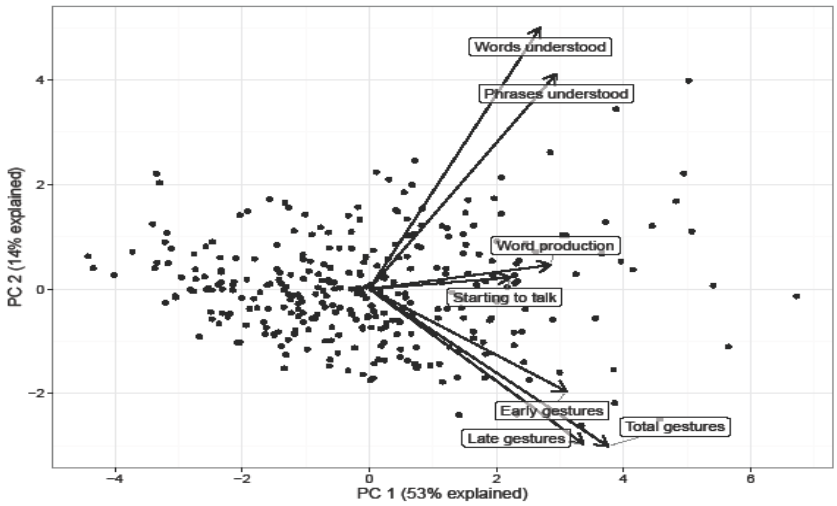


Figure E3. Biplot from principal component analyses of all the 2-year language development scores. Principal Component 1 (PC1) explained 58% of the variation in the dataset.

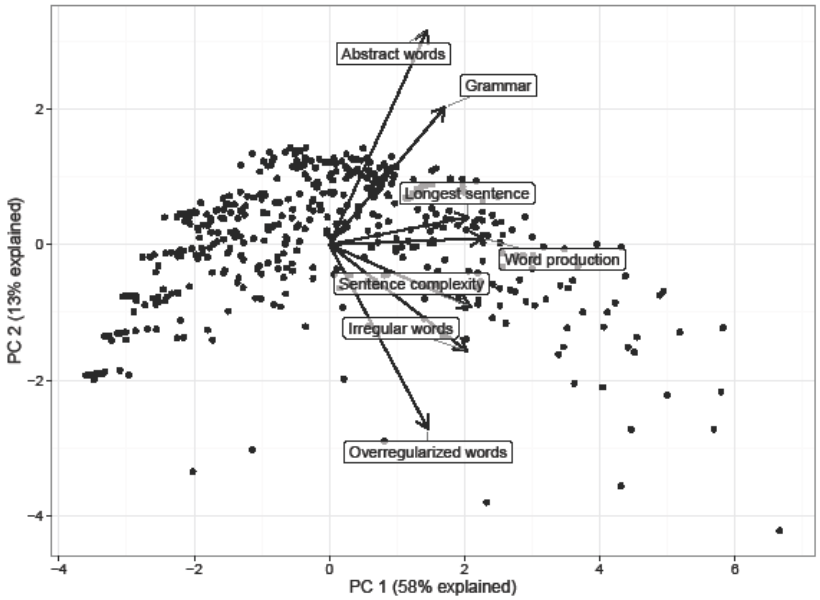
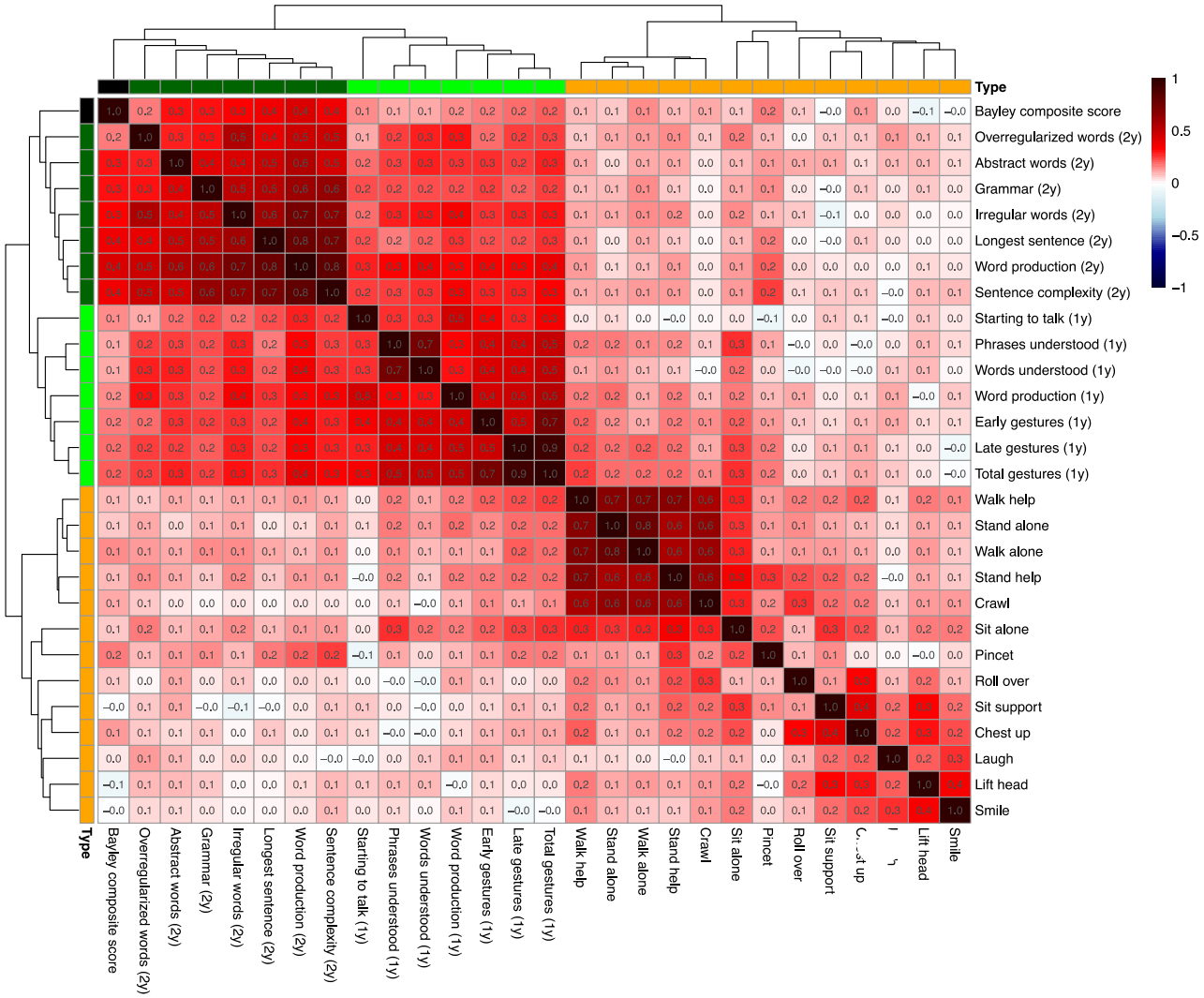


Figure E4. Heatmap based on spearman correlations showing the correlation between the neurological endpoints



On-line repository Methods

METHODS

Neurodevelopmental assessment

Milestones

At the child's 1 week visit the parents received a registration form, with thorough instructions, based on The Denver Development Index [1] and WHO (World Health Organization) milestones registration [2]. Dates of achievement of 13 predefined milestones were registered by the parents and reviewed at each visit to the research clinic. The registration form contained a description and an illustration of the milestones. Any difficulties in remembering the specific date were registered as "missing". The clinical staff carefully reviewed the forms with the parents in order to standardize the registration, and thus minimize differences in interpersonal interpretations. Implementation of milestone registration started after the first 500 children were born, thus some of the milestones were registered retrospectively.

Language development

Language development was assessed with the Danish version of The MacArthur Bates Communicative Developmental Inventory (CDI), which is a well-recognized and validated tool to assess monolingual children´s lexical growth by a standardized parent reporting system [3]. The test was performed as a web-based questionnaire filled out by parents around the child's 1 year (CDI-WG: Words and gesticulation) and 2 years birthdays (CDI-WS: Words and sentences). The 1-year test evaluates language comprehension, early word production and gestural communication. Language comprehension was assessed by counting the number of words that the parents think the child understands from a list of 409 words which are commonly found in the vocabulary of Danish

children around 1 year of age. The assessment of word production was based on the same list and counts the number of words the child actually says or does a lingual imitation of. Gestural communication was assessed by questions regarding the use of gestures typical of early and later communicative development (numbers used out of 18 and 45, respectively). The 2-year test assesses vocabulary, grammatical skills, syntax and morphology. Vocabulary was assessed by counting the number of words the child pronounces from a list of 725 common words. Grammatical skills were determined from the use of past, future, abstract, plural, possessive and past tense and the number of irregular and over-regularized words. Syntax and morphology was assessed according to whether the child combines words and the mean length of the three longest utterances (M3L: calculated from number of morphemes per utterance in the 3 longest sentences the child has said in the previous two weeks). The CDI was not performed in the first 209 participants as it was implemented in the cohort after they had completed their 1 year visit. Children who were considered bilingual (regularly in contact with another language than Danish at home) where excluded from the language development analysis (40 from the 1 year test and 51 from the 2 years test). Language tests completed when the child was more than 3.5 months older than intended were excluded (a total of 9 children).

Cognitive development

Cognitive development was assessed at 2½ years of age, using the cognitive part of the 3rd edition of the Bayley Scales of Infant and Toddler Development (BSID-III) [4]. The test was given on an individual basis lasting 20–60 minutes. The scale includes items that assess abilities such as sensorimotor development, exploration and manipulation, object relatedness, concept formation, memory, and other aspects of cognitive processing. The examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on its performance, the child was given a composite score, which was standardized by use of a normalization material

of age corrected means of 100 and standard deviation of 15 (range 50-150). Ten examiners were involved in performing the test and they were all trained by a single expert in the Bayley test procedure (first author). The sessions were video-recorded and the Bayley expert continuously reviewed the videos and supervised the test-persons in order to achieve consistency in all aspects of the testing procedure.

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Paper II

TITLE: n-3 Polyunsaturated Fatty Acid Supplementation during Pregnancy and Neurodevelopment during Childhood. A Randomized Controlled Trial

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ABSTRACT

Importance

Prenatal n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) supplementation may enhance neurodevelopment, but previous trials have shown ambiguous results.

Objective

To determine whether n-3 LCPUFA supplementation during 3rd trimester of pregnancy affects neurodevelopmental outcomes during early childhood.

Design

A single-center, double-blind, randomized controlled trial of pregnant women recruited at pregnancy week 24 into the Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (Copsac$_{2010}$) mother-child cohort.

Setting

The COPSAC$_{2010}$ cohort was followed prospectively at the research unit from pregnancy with in-depth clinical neurodevelopmental assessments during childhood.

Participants

An unselected population of 736 women were included in the study, excluding women not speaking fluent Danish or with a Vitamin D intake >600 IU/day, or any endocrine, heart, or kidney disorders. Neurodevelopmental outcomes were assessed in 649 children.

Intervention

The pregnant women received capsules containing 2.4 g/day n-3 LCPUFA or matched olive oil from pregnancy week 24 until one week after birth. The intervention was kept double-blinded until the youngest child turned 3 years (March 2014).

Main Outcome and Measure

The primary outcome was cognitive development at 2½ years of age assessed by the Bayley Scales of Infant and Toddler Development. Secondary outcomes were milestone development in the first year of life, language development (MacArthur Bates CDI) at 1 and 2 years of age, and general development (Ages & Stages Questionnaire) at 3 years of age.

Results

The primary outcome, Bayley cognitive testing, was completed in 600 children with a trend of higher score in the n-3 LCPUFA group (n=299) compared to the control group (n=301): adjusted mean difference, 1.47 [95% CI, -0.13, 3.08], p=0.07) and with a significant gender interaction (p=0.03). No difference was seen among girls, but boys from the n-3 LCPUFA supplemented mothers scored higher compared to boys in the control group: adjusted mean difference, 3.04 [0.97, 5.12], p=0.004).

Conclusion and Relevance

Supplementation with n-3 LCPUFA in 3rd trimester of pregnancy improved cognitive scores in boys, but not in girls. Prescription of dietary fish-oil supplements to pregnant women may therefore optimize neurodevelopment of male offspring.

Trial Registration: ClinicalTrials.gov: Identifier: NCT00798226

INTRODUCTION

The central nervous system is highly enriched with n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), specifically docosahexaenoic acid (DHA). n-3 LCPUFA is accumulated in the central nervous system during the brain growth spurt from the second half of pregnancy throughout the first two years of life [1–3] presumably dependent on the dietary intake of n-3 LCPUFA [4,5]. Epidemiological studies have suggested that maternal intake of DHA rich seafood during pregnancy is associated with improved neurodevelopmental outcome in the offspring [6,7].

A number of randomized controlled trials (RCTs) [8–15] and a recent meta-analysis [16] have analyzed the relationship between n-3 LCPUFA intake during pregnancy and neurodevelopment of the young child. One of these studies [8] suggested a possible gender specific effect, but the results are ambiguous and inconclusive.

Therefore, we conducted a RCT of n-3 LCPUFA supplementation during third trimester of pregnancy to women enrolled in the Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) mother-child cohort under the primary hypothesis that the supplementation would enhance the cognitive development of their children. Secondary, we investigated a possible effect of the supplementation on milestone achievement, language development and general development till age 3 years. The results where furthermore stratified by gender.

METHODS

Study Design

The study was a single-center, double-blind, placebo-controlled RCT of n-3 LCPUFA supplementation to pregnant women living in the eastern part of Denmark with in-depth neurodevelopmental assessments of their offspring at the research center during the first 3 years of life [17]. The study was approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2008-41-2599). Written and oral informed consent was obtained before enrolment from both parents.

Study Participants

An unselected population of women was recruited at 24 weeks of pregnancy into the $COPSAC_{2010}$ pregnancy cohort, excluding women who did not speak fluent Danish, had vitamin D intake >600 IU/day or any endocrine, heart, or kidney disorder. Their children were invited into the $COPSAC_{2010}$ birth cohort at one week of age. Children born before 37 gestational weeks, children with low birth weight (<2500 g) and children with a neurological diagnosis, which was believed to affect their development were excluded from the present study[18,19] .

Study Intervention

The pregnant women were randomized 1:1 to either supplementation with four 1 g-capsules of fish oil per day, providing 2.4 g/day n-3 LCPUFA (55% eicosapentaenoic acid (EPA) and 37% DHA, Incromega TG3322 from Croda, East Yorkshire, England) or four identically looking control capsules with olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid from Pharmatec A/S, Norway). The dose of the n-3 LCPUFA was estimated to increase the daily intake of n-3 LCPUFA 6-fold relative to the normal daily

intake of pregnant Danish women [20]. The olive oil supplement was estimated to contribute 3% of normal daily intake of linoleic acid. The women were instructed to take the capsules from the day of randomization until one week after delivery and to return any unused study capsules. Allocation was done by a simple randomization procedure using a computer-generated list of random numbers prepared by an external investigator with no other involvement in the trial. The study intervention was maintained double blinded until the youngest child in the cohort turned 3 years.

A subgroup (n=623) of the mothers also participated in a nested, factorial designed, double-blind, RCT of 2400 IU/day Vitamin D_3 supplementation during third trimester of pregnancy (ClinicalTrials.gov:NCT00856947)[21].

Adherence

Adherence to the n-3 LCPUFA intervention was assessed by comparing the number of capsules provided for the intervention period with the number returned. In addition, maternal whole-blood % EPA+DHA levels (relative percent of total fatty acids) were assessed at the time of randomization and at completion of the RCT [21,22].

Outcome Assessment

Primary Outcome

Persistent wheeze/asthma was the primary outcome measure of the n-3 LCPUFA intervention, which is reported separately (Accepted for publication in NEJM). The current manuscript describes the effect on neurological development from 0-3 years of age, which was a predetermined secondary endpoint of the trial.

The primary childhood neurodevelopmental outcome was the composite cognitive score from the Third Edition of the Bayley Scales of Infant and Toddler Development (BSID-

III) assessment evaluated at 2½ years of age. The cognitive scale includes items assessing sensorimotor development, exploration and manipulation, object relatedness, concept formation, memory, and other aspects of information processing [23]. The examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on objective performance, the child was given a composite score, which was standardized according to a normalization material of age corrected means of 100 and a standard deviation of 15 (range 50-150). All tests were performed by trained clinical personnel with a longstanding experience conducting pediatric examinations. Video recordings of every test were reviewed by the first author to further standardize the test.

Secondary Outcomes

Milestone achievement in the first year of life was monitored prospectively by the parents using a registration form based on the Denver Developmental Index and WHO milestones. Word production/language development at 1 and 2 years of age was assessed by The MacArthur Bates Communicative Developmental Inventory (CDI). General development at 3 years of age was assessed by the Ages & Stages Questionnaire (ASQ-3).

Statistics

The trial was powered according to the primary outcome of persistent wheeze/asthma. Therefore, the statistical power of the RCT on neurodevelopment was calculated *post-hoc* based on the eligible children for such sub-analysis. A total of 649 children were included in the neurodevelopmental outcome assessment and 600 of these completed the BSID-III cognitive test. The power calculation based on 600 children demonstrated

over 80% power (α=0.05, two-tailed) to detect a 5 points difference[8] between the two intervention groups for boys and girls, separately.

The effect of the n-3 LCPUFA intervention on the composite BSID-III cognitive score was assessed using linear regression models. Probabilistic principal component analysis (pPCA) was used to generate principal components for the milestone data assuming that missing values were random. These were used to assess if there was an overall effect on milestone development with subsequent analysis by linear regression analysis of individual milestones for interpretation. Intervention effects on language development were assessed using Poisson regression and the effect on ASQ-3 general development was analyzed using Wilcoxon signed-rank test.

Effect modification of gender was done *a priori* by inclusion of a treatment-gender interaction term, because previous studies have suggested a gender specific effect of n-3 LCPUFA supplementation [8,24] . The results were presented for all children and stratified by gender with p-values for both main effects and treatment-gender interaction.

Analyses were also performed with adjustment for maternal pre-intervention whole blood EPA+DHA levels as a continuous variable and the vitamin D RCT allocation, and are reported with 95% confidence intervals [CI]. All P values are 2-sided, and the level of significance was set at 0.05. Missing data was treated as missing observations, except in the milestone pPCA. The data processing was conducted using SAS version 9.3 for Windows (SAS Institute Inc., Cary, NC, USA) and R version 3.3.0 (R core team 2016), with the packages "ggplot2", "ggbiplot2" and "ggrepel".

Additional methodological details are outlined in Supplement 2 and in the $COPSAC_{2010}$ design paper [25].

RESULTS

Participants

A total of 736 population-based women were randomized at pregnancy week 24 to either n-3 LCPUFA or control supplementation. 43 women (6%) were withdrawn from the study before the child was born and 49 children (7%) were excluded from the present study (15 did not have any neurodevelopmental outcome; 5 had a neurological diagnosis; 24 were born preterm; and 5 had low birth weight), leaving 649 children in the final study group (**eFigure 1**).

Recruitment for the trial began November 10, 2008, and ended November 17, 2010, and the youngest child was born March 17, 2011. The follow-up rate among the cohort children was 97% (N=676) in the 3 year double-blind period.

Table 1 depicts the baseline characteristics of the pregnant women and their children showing as well as compliance and rates of follow-up assessments in the two treatment groups. Adherence is illustrated by 461 (71%) of the women taking >80% of the prescribed capsules with no difference between the 2 groups, demonstrating a similar prevalence in both groups and higher levels of post-randomization whole blood levels of EPA+DHA in women receiving n-3 LCPUFA vs. control: 6.64% of all fatty acids (SD 1.91) vs. 4.21% (SD 1.08), $p<0.0001$. **eTable 1** shows the full fatty acid composition of maternal whole blood before and after the trial. The maternal dietary intake has previously been reported (Accepted for publication in NEJM).

Primary Outcome

600 children in the study population completed the BSID-III test. The children in the n-3 LCPUFA group showed a trend of higher BSID-III cognitive scores compared to the

children in the control group: adjusted mean difference, 1.47; 95% CI [-0.13, 3.08], p=0.07. The analysis showed a significant gender interaction (p=0.03), indicating different effects of n-3 LCPUFA supplementation for boys compared to girls. Among boys, n-3 LCPUFA supplementation resulted in a significantly higher BSID-III cognitive score: adjusted mean difference, 3.04; 95% CI [0.97, 5.12], p=0.004 (**Figure 1**), whereas no effect was observed among girls: -0.63 (-3.11, 1.84), p=0.61 (**Table 2**).

Analyzing data without excluding any children yielded comparable results (**eTable 2**). We did not find significant interaction between n-3 LCPUFA and vitamin D supplementation on the primary outcome (p=0.96).

Secondary Outcomes

The pPCA approach including all the milestones in one model showed that the first two principal components (PC1 and PC2) explained 37% and 16% of the total variation in the milestone achievements (**eFigure 2**). There was no difference between the groups measured in PC1, but PC2 showed a lower score (that is younger age at milestone achievement) for the n-3 LCPUFA group compared to the control (p=0.03, **eTable 3**). The significant effect for PC2 was driven by the boys (p=0.03), and there was also a trend of effect among boys for PC1 (p=0.08) with no effect among girls. The gender specific effect of n-3 LCPUFA supplementation observed in the pPCA analysis was confirmed by the conventional statistical approach. The individual milestones were not significantly affected by maternal n-3 LCPUFA supplementation in pregnancy (all p values >0.06) in combined analyses of boys and girls. However, gender stratified analysis showed that the n-3 LCPUFA supplemented boys achieved the late motor milestones at a younger age compared to the boys in the control group: e.g. the

milestone “walk alone” demonstrated a difference of 21 days (95% CI [-40.7, -10.7], p< 0.001), whereas no effects of the intervention were seen among the girls in any of the individual milestones (**Figure 2**).

Language development at 1 year of age assessed as word production showed an overall positive effect from the n-3 LCPUFA supplementation (p=0.03). Gender stratified analysis showed no effect on the girls, but a possible positive effect on the boys in the n-3 LCPUFA group (p=0.14). There was no effect on word production at 2 years of age (**eTable 4)**.

ASQ-3 scores measuring the child´s general development at 3 years of age did not differ among the intervention groups (**eTable 5**).

Safety

The safety-profiles of the n-3 LCPUFA and control supplementation showed no difference between the groups, except for that there was a trend (p=0.1) of lower infection rate in the mothers of n-3 LCPUFA group during the third trimester (**eTable 6**).

DISCUSSION

Maternal n-3 LCPUFA supplementation during pregnancy positively affected the BSID-III score for cognition in the offspring at age 2½ years with an increase of more than 3 points among boys, but no effect among girls. The children in the n-3 LCPUFA group furthermore achieved milestones at a younger age, specifically motor milestones in boys. A transient positive effect of the n-3 LCPUFA supplementation was seen on language development at 1 year of age, but there was no effect on language at 2 years of age or on the 3-year ASQ-3 general developmental scores.

Strengths and Limitations

This study is among the largest RCTs on n-3 LCPUFA supplementation in pregnancy. The study is strengthened by the unselected recruitment strategy from the general population, which increases the external validity of our findings. The study is further strengthened by the longitudinal design, allowing neurodevelopmental assessment at multiple time points from birth until 3 years of age.

The BSID-III cognitive test was performed by highly trained personnel assuring consistency in the testing procedures. All tests were validated by video recordings, which improve reliability of the score for each child. Furthermore, the nine scheduled visits to our clinical research site until age 3 years improved co-operation during tests and assured a high quality of the data obtained from both parents and children.

The large sample size along with high adherence and follow-up rates, enabled sufficient power to analyze the effects of gender separately.

A possible limitation of the study is the post-randomization exclusion of 7% of the children, which could have biased the true effect of the n-3 LCPUFA supplementation.

However, a similar effect was found in a *post-hoc* analysis of our primary outcome (BSID-III cognitive score) with all children, who completed the test (n=629) including premature and small for gestational age children as well as children with neurological diagnoses.

Interpretation

Maternal n-3 LCPUFA supplementation during third trimester of pregnancy had a positive effect on neurological development in the offspring with a particular benefit among boys. We saw a parallel right shift in the Gaussian distribution, i.e. the intervention did not seem to have a selective effect in the group with the lower score (**Figure 1**). The absolute increase in cognitive BSID-III score of 3 points in the n-3 LCPUFA supplementation group indicates a potentially clinical relevant effect size.

To our knowledge only one previous study[8] was powered to analyze gender specific effects of n-3 LCPUFA supplementation during pregnancy. That study showed no overall effect of the intervention on BSID-III cognitive score at age 1½ years in either boys or girls but, in line with our findings, found a gender-specific effect with fewer boys in the n-3 LCPUFA intervention group having scores indicating delayed cognitive development (<85), but not among girls. Furthermore, two RCT's examining the effect of n-3 LCPUFA enriched feeds (breastmilk and/or formula) given to preterm infants also showed gender specific effects on BSID cognitive scores[26,27].

The differential responses to the supplementation between genders might be due to sex-specific differences in essential fatty acid metabolism. Testosterone is known to decrease delta-6 desaturase activity which could result in decreased rate of endogenous synthesis of DHA from the precursor fatty acid alpha linoleic acid among males [28]. This

could imply that boys were more sensitive to DHA intake and thus more dependent on n-3 LCPUFA supplementation to optimize their neurodevelopment compared to girls.

Our study is the only pregnancy n-3 LCPUFA RCT examining a possible effect on a broad range of childhood milestone achievements. We demonstrated that boys in the n-3 LCPUFA intervention group achieved motor milestones at a younger age, which supports the effects seen on the cognitive scores. Our milestone results are in contrast to previous trials including motor developmental outcomes[8,12,13,15] but in line with a large Danish population-based cohort of pregnant women and their children demonstrated a benefit of higher maternal fish consumption during pregnancy on attainment of developmental milestones at both 6 and 18 months [7].

Apart from cognitive score, we also observed a positive effect of the intervention on the language test at 1 year, which seemed to be driven by an increased word production among the boys. This effect did not persist till age 2 years, where the boys still presented an increased word production, which was however non-significant. In support of such transient effect, another RCT resulted in a positive effect of n-3 LCPUFA supplementation during pregnancy and lactation on overall IQ scores at the age of 4 years[11], which was no longer significant at 7 years of age.[10]

Conclusion

Supplementation with n-3 LCPUFA in third pregnancy trimester in an unselected group of Danish women enhanced the neurodevelopment of their male children. These findings may be used as a guideline for pregnant women, where a simple and safe supplementation may optimize the neurodevelopment of young boys. Follow-up of this cohort is needed to assess, whether these effects remain beyond early childhood.

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Contributors' Statements*:* The guarantor of the study is HB, from conception and design to conduct of the study and acquisition of data, data analysis, and interpretation of data. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input. EB has written the first draft of the manuscript. All authors have agreed that the accuracy and integrity of any part of the work has been appropriately investigated and resolved and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

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Figure 1: Density plot showing the effect of n-3 LCPUFA intervention on composite score of the BSID-III cognitive test among boys.

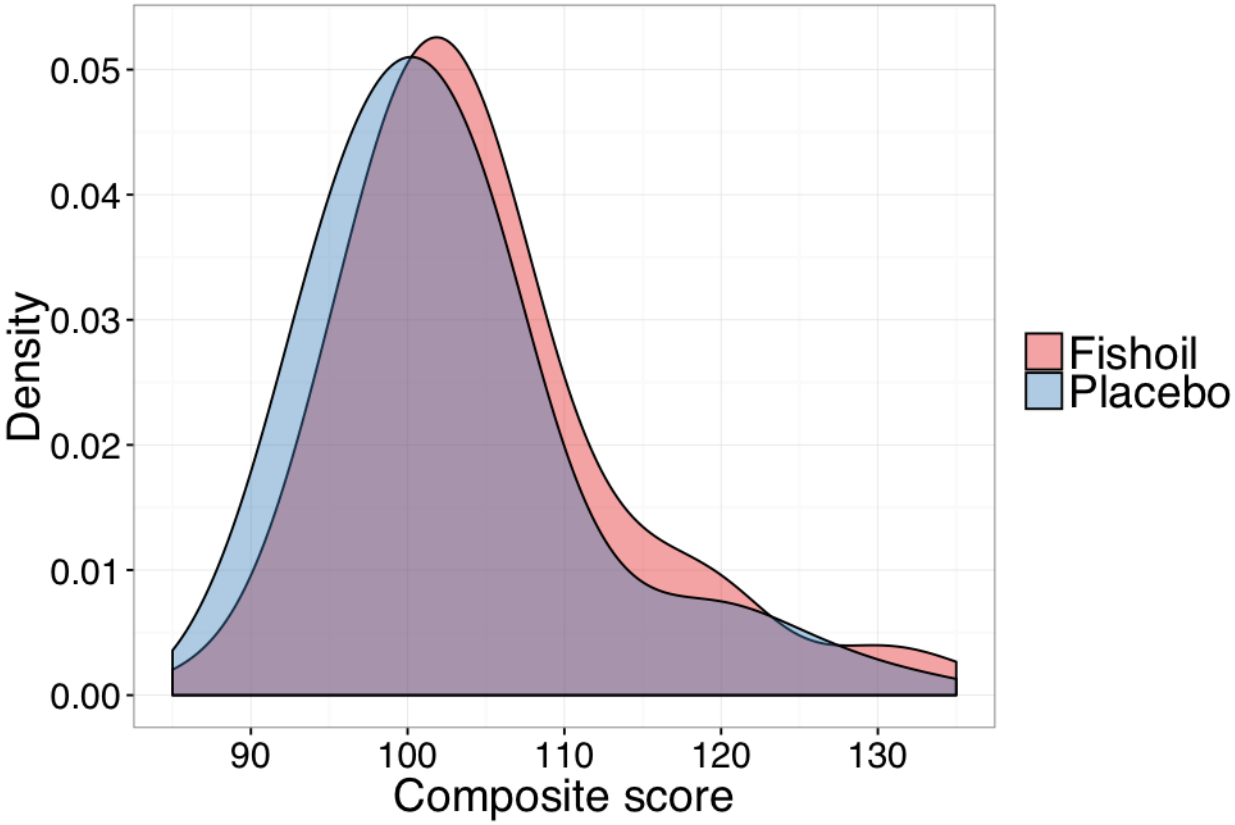
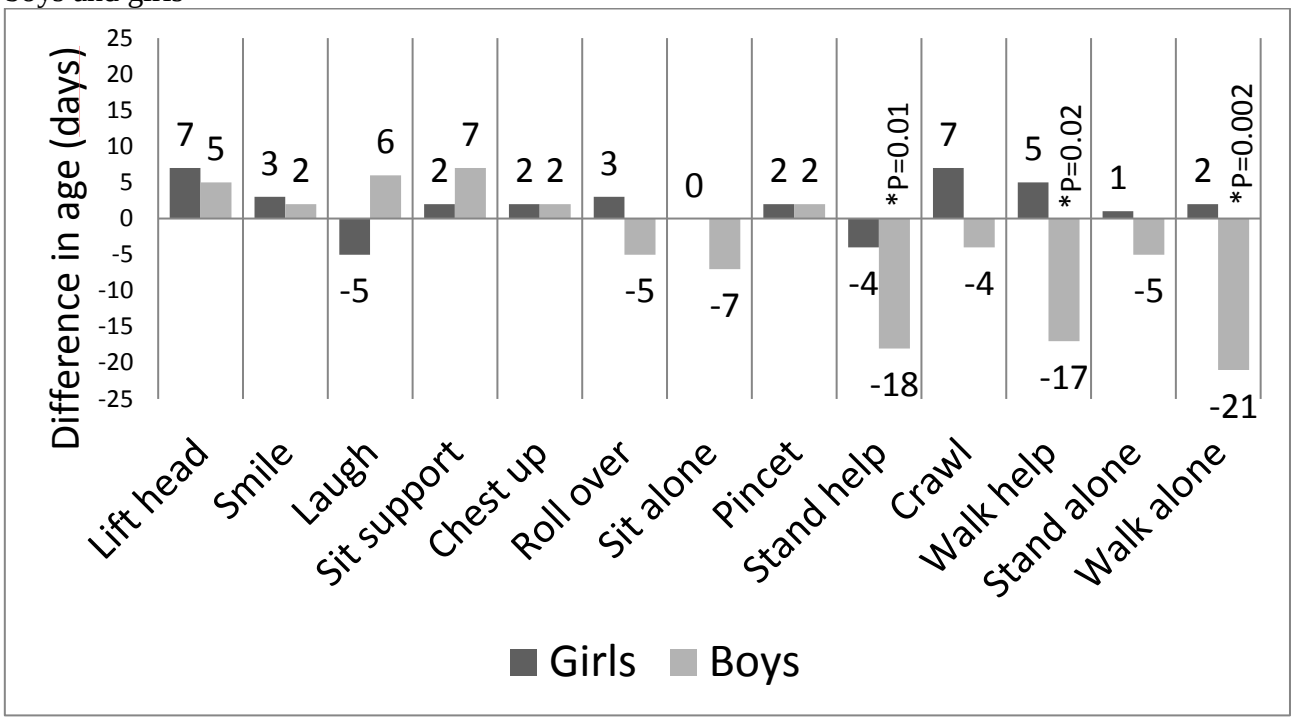


Figure 2: The effect of n-3 LCPUFA intervention on age of milestone achievement for boys and girls[a]



[a] The effect is illustrated as the difference (in days) in milestone achievement, using the control group as the reference. Significant difference is indicated with asterisk (*) and p-value.

TABLE 1: Characteristics of the $COPSAC_{2010}$ birth cohort and their pregnant mothers.

	All	**Randomization**	
		n-3 LCPUFA	**Control**
	N=649	**50% (324)**	**50% (325)**
Socioeconomics			
Maternal age at Birth, mean (SD), years	**32.2 (4.3)**	32.3 (4.3)	32.2 (4.4)
Maternal Asthma, % (N)[a]	**25 (164)**	24 (76)	27 (88)
Social circumstances, mean (SD)[b]	**0 (1)**	0.004 (0.97)	-0.01 (1.01)
Smoking, % (N)	**7 (45)**	6 (18)	8 (27)
Alcohol, ≥ 1 unit / week % (N)	**14 (93)**	13 (41)	16 (52)
Fish intake pre randomization, median (ITR)[c], g/day	**26.3 (19-34)**	26.7 (19-34)	25.6 (19-33)
EPA intake pre randomization, median (ITR)[c], g/day	**0.13 (0.09-0.17)**	0.12 (0.09-0.17)	0.13 (0.09-0.17)
DHA intake pre randomization, median (ITR)[c], g/day	**0.32 (0.23-0.41)**	0.31 (0.22-0.41)	0.33 (0.23-0.41)
Whole blood EPA+DHA level pre-randomization, mean (SD)[d], % of fatty acids	**4.65 (1.21)**	4.64 (1.27)	4.66 (1.15)
Adherence			
>80% capsule consumption, % (N)	**71 (461)**	71 (229)	71 (232)
Whole blood EPA+DHA level post-randomization, mean (SD)[e], % of fatty acids	**5.41 (1.97)**	6.64 (1.93)[f]	4.22 (1.09)[f]
Birth			
Gestational age, median (IQR), weeks	**40 (39-41)**	40 (39-41)	40 (39-41)
Birth order, % first born (N)	**45 (359)**	43 (140)	46 (150)
APGAR score at 5 min, mean (SD)	**9 (3)**	9 (3)	9 (3)
Intra-partum Antibiotics % (N)	**30 (191)**	30 (97)	29 (94)
Antibiotics to the Child % (N)	**2 (16)**	3 (9)	2 (7)
Caesarean Section % (N)	**20 (129)**	21 (67)	19 (62)
Emergency % (N)	**10 (67)**	12 (38)	9 (29)
Elective % (N)	**9 (65)**	9 (29)	10 (33)
Child			
Gender, Male % (N)	**51 (332)**	49 (159)	53 (173)
Caucasian % (N)	**96 (622)**	96 (311)	96 (311)
Season of Birth			
Winter, % (N)	**31 (200)**	29 (94)	33 (106)
Spring, % (N)	**27 (172)**	26 (85)	27 (87)
Summer, % (N)	**21 (139)**	22 (70)	21 (69)
Fall, % (N)	**21 (138)**	23 (75)	19 (63)
Neurodevelopmental outcomes			
Completed BSID-III test, % (N)	**92 (600)**	92 (299)	93 (301)
Milestone registration, % (N)	**94 (609)**	94 (305)	94 (304)

Continued	**All**	**Randomization**	
		n-3 LCPUFA	**Control**
One year language test, % (N)	**49 (320)**	47 (151)	52 (169)
Two year language test, % (N)	**72 (470)**	72 (234)	73 (236)
ASQ test, % (N)	**65 (423)**	64 (206)	67 (217)

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval, ITR=inter-tertile range, IQR (inter-quartile range)

[a] History of doctor diagnosed asthma

[b] PCA component that consist of household income, maternal age and maternal educational level at the age of 2

[c] Calculated from a total of 567 available food frequency questioners (data from manuscript accepted for publication in NEJM)

[d] Calculated from a total of 570 available pre-randomization whole blood fatty acid analysis

[e] Calculated from a total of 637 available post-randomization whole blood fatty acid analysis

[f] P<0.0001

Table 2. Effect of the n-3 LCPUFA intervention on the composite cognitive score of the BSID-III test[a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**		**Adjusted[b]**	
	N=299	N=301	N=600		N=527	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
Composite score	105.3(9.7)	104.4(9.9)	0.95(-0.62, 2.52)	0.24	1.47(-0.13, 3.08)	0.07
Female	105.8(10.2)	106.2(10.6)	-0.43(-2.82, 1.95)	0.72	-0.63(-3.11, 1.84)	0.61
Male	104.8(9.1)	102.7(9.0)	2.14(0.11, 4,16)	**0.039**	3.04(0.97, 5.12)	**0.004**

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval

[a] Data are expressed as mean (SD) with effect being differences in means (95% CI). Treatment x gender interaction, p=0.03 (adjusted)

[b] Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation.73 mothers are missing pre-intervention whole blood EPA+DHA results

Supplementaty Online Content

n-3 Polyunsaturated Fatty Acid Supplementation during Pregnancy and Neurodevelopment during Childhood. A Randomized Controlled Trial

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eMETHODS

Maternal whole-blood LCPUFA levels
Whole blood samples were collected by veni-puncture in the presence EDTA at time of randomization and at completion. Samples (500 µL) were aliquoted to cryovials and mixed with 50µL 0.1% 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene; Sigma-Aldrich, St. Louis, MO, USA) in ethanol. Samples were then purged with nitrogen, and frozen and stored at -80 °C for a maximum of one year. Fatty acid composition was determined as described previously [1]. Briefly, fatty acid methyl esters were prepared by direct transesterification using 14% boron trifluoride in methanol (Pierce Chemicals, Rockford, IL, USA) with hexane containing butylated hydroxytoluene (50 µg/mL) and an internal standard (22:3n-3 ethyl ester; Nu-Check Prep, Elysian, MN). Fatty acid methyl esters were recovered and analysed using fast gas chromatography. The data was quantified and then expressed as the percentage of each fatty acid with the total fatty acid sum. Maternal EPA+DHA relative percentage of blood fatty acids pre- and post-intervention was calibrated for the blood sample storage time at –80° C prior to analysis using the regression coefficient of EPA+DHA vs. storage time standardizing for the mean storage time. Maternal whole-blood levels of LCPUFA including EPA+DHA were maintained double-blinded until the youngest child in the cohort turned 3 years.

Maternal dietary intake before n-3 LCPUFA RCT
Maternal dietary intake was assessed from a semi-quantitative food frequency questionnaire consisting of 360 items as previously reported (Accepted for publication in NEJM)

Secondary neurodevelopmental outcomes
The milestone development was monitored prospectively by the parents using a registration form based on The Denver Developmental Index [2] and WHO milestone registration [3]. The parents were instructed to register the date where the child mastered 13 predefined milestones. Implementation of milestone registration started after the first 500 children were born, thus some of the milestones were registered retrospectively.
Language assessment was performed as a web-based questionnaire filled out by the parents around the child's 1 year (CDI-WG: Words and gesticulation) and 2 years (CDI-WS: Words and Sentences) birthdays. The CDI, is a well-recognized and validated tool to assess monolingual children's lexical growth [4]. The 1-year test evaluates language comprehension, early word production and gestural communication and the 2-year test assesses vocabulary, grammatical skills, syntax and morphology. The outcome word production was chosen *a priori* as the primary outcome from both the 1-and 2-year language tests. For the 1-year test word production is assessed by counting the number of word that the child says or does a lingual imitation of from a list of 409 words which are commonly found in the vocabulary of Danish children at that age. For the 2-year test word production was assessed by counting the number of word the child pronounces from a list of 725 common words. The CDI was not performed in the first 209 participants as it was implemented in the cohort after they had completed their 1 year visit. Children who were considered bilingual (regularly in contact with another language than Danish at home) where excluded from the language development analysis.
At three years of age the parents filled out the Danish version [5] of the ASQ-III questionnaires [6] which is a brief measure of the child's current skills and development in the areas of communication, gross motor skills, fine motor skills, problem solving, personal-social skills. The questionnaire consists of 30 items (six in each category) and each item is scored depending upon whether the child performs the item consistently (10 points), sometimes (5 points), or not yet (0 points). Scores for each area where then summed. The ASQ-III was not performed in the first 124 participants as they were too old when the testing was implemented (>3 years and 3 months).

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eTable 1. Composition of maternal blood fatty acids constitution before and after the trial[a]

	Pre randomization		Post randomization		
Fatty acid	**n-3 LCPUFA N=275**	**Control N=295**	**n-3 LCPUFA N=315**	**Control N=322**	
	Mean ± SD		**Mean ± SD**		**P value**
SFA	38.3 ± 3.0	38.2 ± 2.7	39.5 ± 2.4	38.8 ± 2.5	<0.001
MUFA	23.0 ± 2.6	23.0 ± 2.7	22.2 ± 2.7	23.3 ± 2.9	<0.001
PUFA	34.8 ± 3.3	34.9 ± 3.3	34.5 ± 3.1	33.8 ± 3.1	0.003
n-6 PUFA	28.6 ± 2.8	28.7 ± 2.7	26.0 ± 2.6	28.2 ± 2.6	<0.001
18:2 n-6 (LA)	17.9 ± 2.9	17.9 ± 2.6	16.7 ± 2.3	17.4 ± 2.4	<0.001
20:3 n-6	1.8 ± 0.3	1.8 ± 0.4	1.4 ± 0.3	1.7 ± 0.4	<0.001
20:4 n-6 (AA)	7.4 ± 1.1	7.5 ± 1.2	6.9 ± 1.3	7.7 ± 1.4	<0.001
22:4 n-6	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	<0.001
n-3 PUFA	6.2 ± 1.5	6.2 ± 1.3	8.5 ± 2.2	5.6 ± 1.2	<0.001
18:3 n-3 (ALA)	0.6 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.25
20:5 n-3 (EPA)	0.7 ± 0.4	0.7 ± 0.3	2.2 ± 1.1	0.7 ± 0.4	<0.001
22:5 n-3	1.0 ± 0.2	0.9 ± 0.2	1.4 ± 0.3	0.9 ± 0.2	<0.001
22:6 n-3 (DHA)	3.9 ± 0.9	4.0 ± 0.9	4.4 ± 1.0	3.5 ± 0.8	<0.001
EPA+DHA	4.6 ± 1.3	4.7 ± 1.2	6.6 ± 1.9	4.2 ± 1.1	<0.001
Ratio n-6/n-3 PUFA	4.8 ± 1.1	4.8 ± 0.9	3.3 ± 1.1	5.3 ± 1.1	<0.001

Abbreviations: n-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, SFA=saturated fatty acid, MUFA=monounsaturated fatty acid, PUFA=polyunsaturated fatty acid, LA=linoleic acid, AA=arachidonic acid, ALA=alpha-linolenic acid, EPA=eicosapentaenoic acid, DHA= docosahexaenoic acid.

[a] Data are expressed as mean ±SD in % of all fatty acids in whole blood

eTable 2. Effect of n-3 LCPUFA intervention on the composite cognitive score of the BSID-III test including children born before 37 gestational weeks, children with low birth weight (<2500 g) and children with a neurological diagnosis[a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**		**Adjusted[b]**	
	N=312	N=317	N=629		N=554	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
Composite score	105.2(9.6)	104.3(9.8)	0.92(-0.60, 2.44)	0.24	1.41(-0.15, 2.97)	0.08
Female	105.7(10.1)	106.1(10.5)	-0.48(-2.78, 1.83)	0.68	-0.56(-2.95, 1.83)	0.65
Male	104.7(9.1)	102.6(8.8)	2.10(0.13, 4,07)	**0.037**	2.98(0.97, 4.98)	**0.004**

Abbreviations: N-3 LCPUFA=n-3 long-chain polyunsaturated fatty acid, N=number, SD=standard deviation, CI=confidence interval

[a] Data are expressed as mean (SD) with effect being differences in means (95% CI).Treatment x gender interaction, p=0.03 (adjusted)

[b] Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation.75 mothers are missing pre-intervention whole blood EPA+DHA results

eTable 3 Outcomes from milestone principal component analysis[a]

Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**		**Adjusted[b]**	
	Mean (SD)	Mean (SD)	Effect (95% CI)	P value	Effect (95% CI)	P value
	N=305	N=304	N=609		N=541	
PC1	-0.07(1.88)	0.07(2.01)	-0.14(-0.45, 0.17)	0.36	-0.13(-0.46, 0.20)	0.44
Female	0.19(2.03)	0.05(2.00)	0.13(-0.32, 0.59)	0.57	0.12(-0.38, 0.61)	0.64
Male	-0.34(1.66)	0.09(2.03)	-0.43(-0.84, 0.01)	0.05	-0.40(-0.86, 0.05)	0.08
PC2	0.15(1.33)	-0.08(1.22)	0.23(0.03, 0.43)	0.03	0.23(0.01, 0.44)	0.04
Female	0.11(1.31)	-0.05(1.40)	0.16(-0.15, 0.47)	0.31	0.11(-0.22, 0.44)	0.52
Male	0.19(1.35)	-0.11(1.02)	0.30(0.03, 0.57)	0.03	0.32(0.04, 0.61)	0.03

Abbreviations: N=number, SD=standard deviation, CI=confidence interval, PC1=principal component 1, PC2=principal component 2

[a] Data are expressed as mean (SD) with effect being differences in means (95% CI). Treatment x gender interaction PC1 p=0.08 and PC2 p=0.82 (adjusted)

[b] Adjusted for pre-intervention whole blood EPA+DHA levels and vitamin D RCT allocation. 68 mothers are missing pre-intervention whole blood EPA+DHA blood results.

eTable 4. Outcomes from the 1 and 2 year language tests[a]

	n-3 LCPUFA	Control	Unadjusted	Adjusted[b]
	Median (IQR)		P value	P value
1 year language test				
	N=151	N=169	N=320	N=317
Word Production	3(1-7)	2(0-6)	0.03	0.03
Female	3(1-8)	3(1-7)	0.19	0.30
Male	3.5(1-6)	1(0-6)	0.07	0.14
2 year language test				
	N=234	N=236	N=470	N=411
Word production	251(149-357)	250(96-377)	0.59	0.82
Female	278(182-378)	304(139-419)	0.62	0.23
Male	223 (97-327)	165 (71-328)	0.28	0.23

Abbreviations: IQR = inter quartile range
[a] Effect is expressed as median, with (IQR). Treatment x gender interaction 1year p=0.7 and 2 year 0.08 (adjusted)
[b]Adjusted for preintervention EPA+DHA blood levels and vitamin D-RCT allocation. 3 mothers are missing preintervention EPA+DHA blood results in the 1 year analysis and 59 in the 2 year analysis.

eTabel 5. Outcomes from Ages and stages Questionnaire[a]

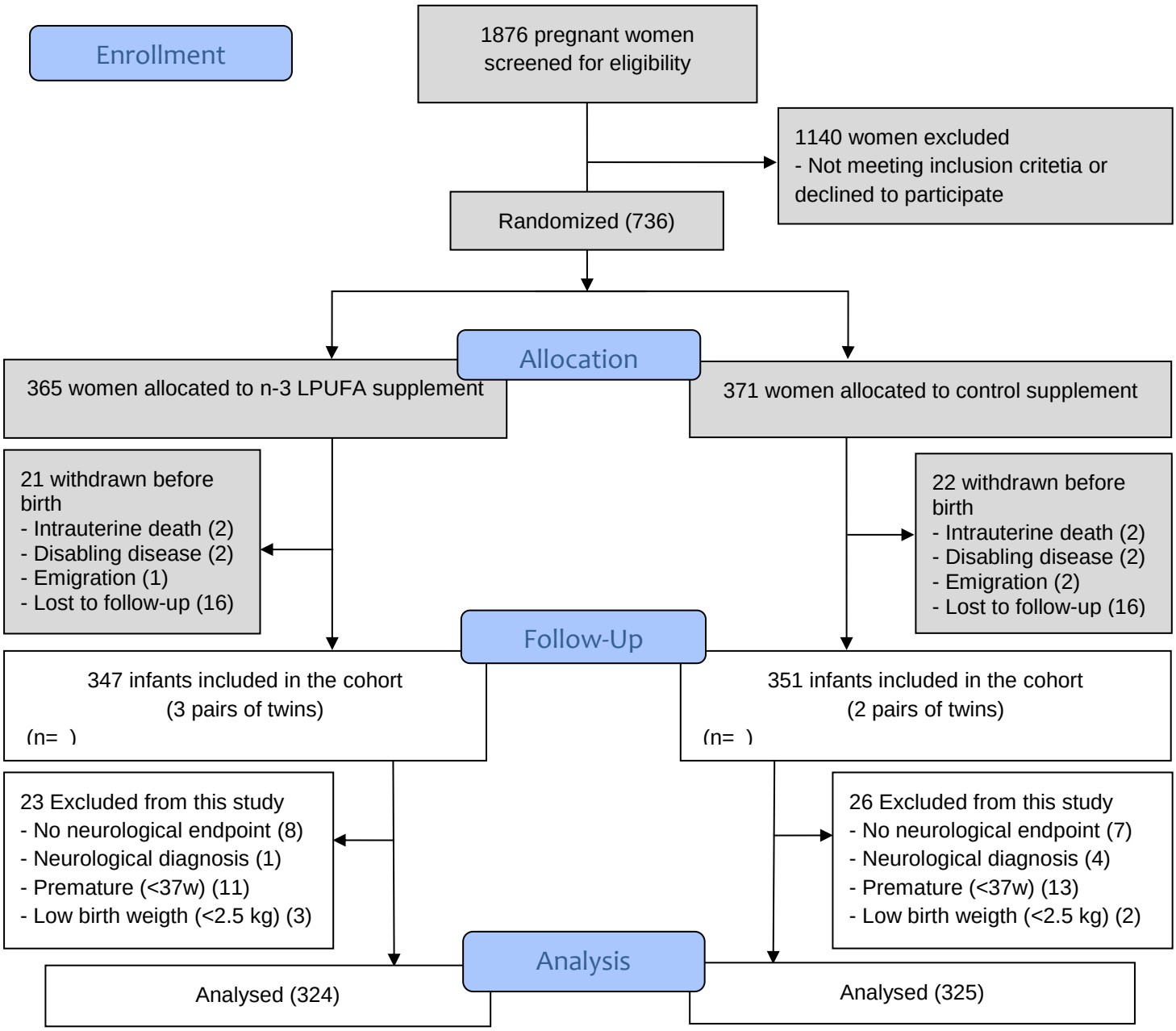
Outcome	**n-3 LCPUFA**	**Control**	**Unadjusted**
	Median (IQR)		P value
	N=206	N=217	
Communication	50 (50-55)	50 (50-55)	0.27
Female	50 (50-55)	50 (50-55)	0.41
Male	50 (50-55)	50 (50-55)	0.61
Gross motor skills	60 (55-60)	60 (55-60)	0.36
Female	60 (55-60)	60 (55-60)	0.96
Male	60 (55-60)	60 (55-60)	0.17
Fine motor skills	55 (50-60)	55 (50-60)	0.41
Female	60 (50-60)	55 (50-60)	0.25
Male	50 (45-60)	50 (40-60)	0.77
Problem solving	55 (50-60)	55 (50-60)	0.61
Female	55 (50-60)	55 (50-60)	0.95
Male	55 (50-60)	55 (50-60)	0.59
Social skills	55 (50-60)	55 (50-60)	0.82
Female	55 (55-60)	55 (55-60)	0.12
Male	55 (50-60)	55 (50-60)	0.72

Abbreviations: IQR = inter quartile range,
[a] Effect is expressed as median, with (IQR)

eTable 6. Safety assessment in the $COPSAC_{2010}$ cohort

	Randomization % (N)		P value
Adverse Events	**n-3 LCPUFA**	**Control**	
	50% (365)	**50% (371)**	-
Any maternal			
Death	0% (0)	0% (0)	-
Intrauterine death	1% (2)	1% (2)	0.99
Gestational diabetes	2% (6)	3% (10)	0.32
Preeclampsia	5% (17)	4% (15)	0.69
Days hospitalized after birth, mean (SD)	2.9 (2.7)	2.8 (2.8)	0.51
Mother hospitalized >5 days	10% (34)	10% (35)	0.99
Emergency caesarean section	14% (52)	11% (41)	0.20
Antibiotics in third pregnancy trimester	18% (65)	17% (63)	0.78
Infection in third pregnancy trimester	28% (96)	33% (118)	0.10
Any infant			
Death	0% (0)	0% (0)	-
Extremely preterm (<28 weeks) birth	0% (1)	0% (1)	0.99
Very preterm (28 to <32 weeks) birth	1% (2)	1% (3)	0.66
Moderate to late preterm (32 to <37 weeks) birth	3% (12)	4% (15)	0.58
Child Hospitalized after Birth	12% (40)	11% (39)	0.88
Any Congenital Malformation	5% (20)	6% (24)	0.56

eFigure 1. Flow chart of enrollment and allocation of the COPSAC$_{2010}$ pregnancy cohort and follow-up of the COPSAC$_{2010}$ birth cohort



eFigure 2. Bi-plot from principal component analysis of all the 13 milestones. Principal component 1 and 2 (PC1 and PC2) explain 37 and 16 % of the overall variation in the data, respectively