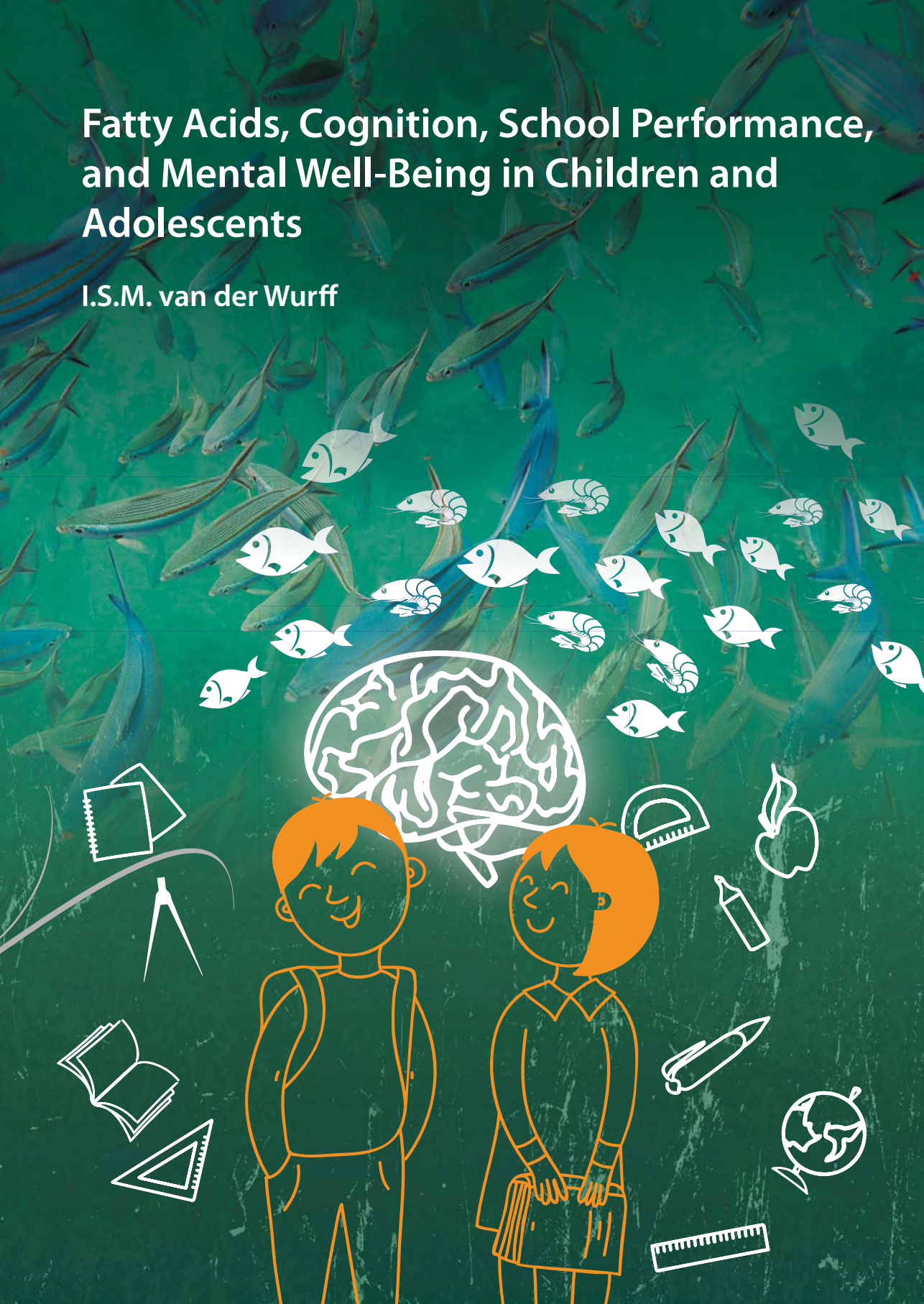
Fatty Acids, Cognition, School Performance, and Mental Well-Being in Children and Adolescents

I.S.M. van der Wurff



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Chapter 1

General introduction

General introduction

Long-chain polyunsaturated fatty acids (LCPUFA) are important constituents of the cell membrane. Several studies have shown that essential fatty acids and their longer chain derivatives, LCPUFA play a role in brain functioning. This notion has led to research that has studied the relation between LCPUFA and cognitive functioning in children and adults [1,2] as well as cognitive decline in elderly [3,4]. They have also been studied in relation to mental disorders such as ADHD and autism [5,6], and affective disorders such as depression and bipolar disorder [7,8]. In contrast to the many studies in which the influence of LCPUFA on cognition in infants, children, and elderly has been studied (for review see among others [1,2,9,10]), there are, as far as we are aware, no LCPUFA supplementation studies with a focus on cognition in adolescents. Moreover, even though the relation between LCPUFA and cognition in children has been studied extensively, there are, to our knowledge, no studies focusing on the relationship between LCPUFA and school performance in children. In adolescents there are a number of studies in which a positive association between fish consumption (most important source of LCPUFA) and school performance is shown [11–13].

LCPUFA have also attracted attention for their possible influence on depression. There are many studies investigating the influence of LCPUFA supplementation on depression in adults (for meta-analyses see [14–16]). However, as depression is especially common in adolescents [17], and it can have profound long lasting negative effects [18], it seems especially important to study the influence of LCPUFA in this age group. Unfortunately, the influence of LCPUFA supplementation on mental well-being (i.e., depression and self-esteem, as self-esteem is a core construct in mental health) in adolescents, has, to our knowledge, not yet been studied.

Taking all above into consideration, the aim of this thesis is to study the: (1) relationship between LCPUFA and school performance in children (2) influence of LCPUFA on cognitive functioning in typically developing adolescents, and (3) influence of LCPUFA on mental well-being in typically developing adolescents.

To study the relationship between LCPUFA and school performance in children, data from the historic Maastricht Essential Fatty Acid Birth cohort (MEFAB) were used. To study the influence of LCPUFA on cognition and mental well-being (i.e., both depressive feelings and self-esteem) in typically developing adolescents, Food2Learn, a double-blind randomized, placebo controlled repeated measures intervention study was designed.

The next paragraphs start with background information on fatty acids, LCPUFA in the diet, krill oil supplementation, LCPUFA in relation to brain functioning, and brain development. Next, information on school performance, cognition, and mental well-being and the role of LCPUFA in each is discussed. Then, the aim of this thesis and the studies included in this thesis are discussed. The chapter ends with the outline of this thesis.

Fatty acids

A fatty acid is a chain of 6 to 32 carbon atoms with 1 or 2 hydrogen atoms attached to every carbon atom, an acid group (COOH) on one end and a methyl group (CH_3) on the other (see Figure 1.1). A fatty acid chain can have no, one or more double bounds between carbon

atoms. If a fatty acid has no double bound, the fatty acid is a saturated fatty acid. A fatty acid that has one double is called a monounsaturated fatty acid (MUFA). A fatty acid with more than one double bond in the chain is a polyunsaturated fatty acid (PUFA). Note that fatty acids with 20 or more carbon atoms are called long-chain polyunsaturated fatty acids (LCPUFA).

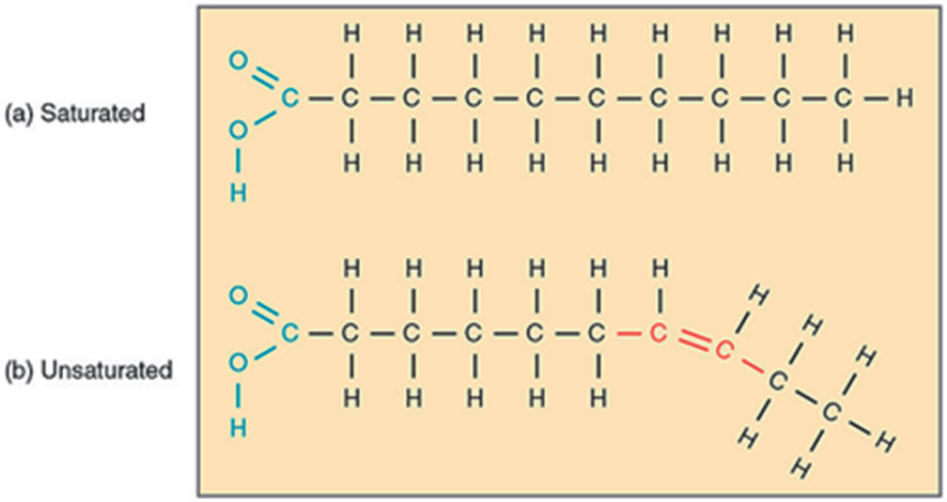

Figure 1.1: Basic formula of (a) a saturated fatty acid with no double bond, and (b) an unsaturated fatty acid with one double bond.

Fatty acids can vary in chain length (i.e., number of carbon atoms) and the number of double bonds, but also the location of the double bond can vary. The position of the first double bond counted from the methyl end (CH_3) gives the fatty acid its omega number. For example, a fatty acid with its first double bond on the third carbon from the methyl end is an omega-3 fatty acid, also noted as n-3 or ω-3 fatty acid. If the first double bond appears on the sixth carbon atom, it is an omega-6 fatty acid, also noted as n-6 or ω-6 fatty acid. There are various families of unsaturated fatty acids. This thesis focuses on the fatty acids from the n-3 and n-6 families.

All fatty acids have a specific name and most also an abbreviation (e.g., docosahexaenoic acid, DHA), but there is also a short hand notion based on the number of carbon atoms, number of double bonds, and the position of the first double bond. For example, for DHA, the short hand notion is C22:6n-3, which indicates that DHA has 22 carbon atoms, 6 double bonds, and that the first double bond is located at the third position. In the current thesis both the name or abbreviation and the number notation will be used.

The n-3 and n-6 fatty acids are essential fatty acids, or, in other words, the human body cannot make these fatty acids *de novo*. A lack of these fatty acids leads to deficiency symptoms such as impairment in visual acuity and skin lesions [19,20]. The human body cannot produce n-3 and n-6 fatty acids *de novo* because it lacks the enzymes to insert a double bond at the third or the sixth position. This inability to introduce a double bond at the third or the sixth position makes the parent n-3 and n-6 fatty acids α-linolenic acid (C18:3n-3, ALA) and linoleic acid (C18:2n-6, LA) essential fatty acids (i.e., they have to be taken in via the diet). Both ALA and LA can be converted in their longer chain more

unsaturated derivatives, such as DHA and AA, via chain elongations, desaturation and beta oxidation (see Figure 1.2). However, the conversion of ALA to EPA and conversion from ALA to DHA is an inefficient process. Conversion of ALA to EPA has been suggested to be around 8% (i.e., about 8% of ingested ALA will be converted into EPA) and the conversion from ALA to DHA below 1% [21,22]. Moreover, since the n-3 and n-6 fatty acids are metabolized by the same desaturase enzymes, competition for these enzymes exists between the families. Consequently, higher intake of the n-6 fatty acid LA leads to inhibition of the conversion of ALA to stearidonic acid (C18:4n-3), and higher intake of n-3 fatty acid ALA leads to inhibition of conversion of LA to gamma-linoleic acid (C18:3n-6, GLA). Moreover, the consumption of preformed EPA and DHA leads to a much higher increase in the concentrations of these fatty acids in the blood than what can ever be achieved by consumption of any n-3 precursor. It has therefore been suggested that at least DHA is a semi-essential nutrient (i.e., a nutrient that can be synthesized by the body, but not in amounts adequate for health and should therefore be consumed in the diet) [23,24]. Again, it is therefore essential that these parent fatty acids, ALA and LA, as well as their elongation products, predominantly EPA, DHA, and AA are consumed with the diet.

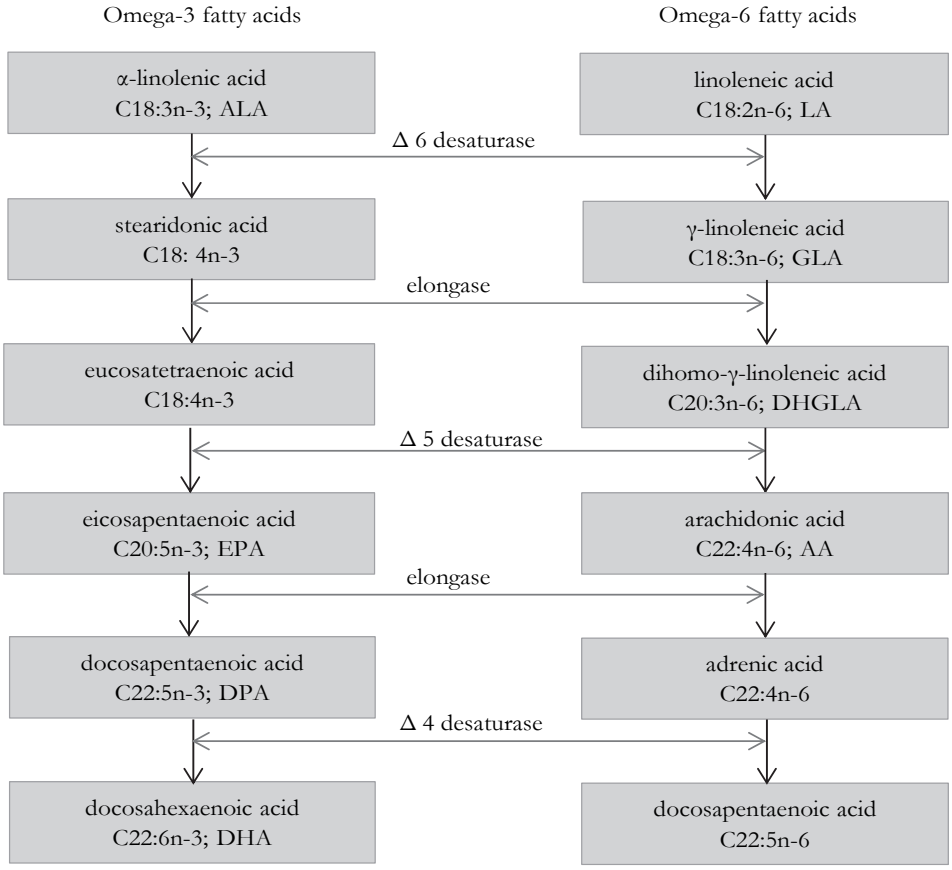

Figure 1.2: Schematic overview of n-3 and n6 fatty acid conversion.

LCPUFA in the diet

As noted earlier ALA, LA, EPA, DHA, and AA are (semi) essential nutrients and should be consumed via the diet. ALA can be found in seeds and nuts (e.g., flaxseeds, walnuts) and vegetable oils (e.g., canola oil, soybean oil). LA can be found in vegetable oils (e.g., corn, sunflower), meat and nuts and seeds. AA is found in meat and eggs. EPA and DHA are found mainly in fish and shellfish, supplements, human milk, and infant formulae.

The Western diet contains n-6 fatty acids in higher concentrations than the n-3 fatty acids. The ratio of n-6 to n-3 fatty acid intake in Western countries is about 20-30:1 [25]. Similarly, in the Dutch Consumption Survey, the average consumption of LA (11-17g per day) is also multiple times higher than the consumption of ALA (1166 - 2069mg per day; see Table 1.1) [26]. Moreover, the consumption of EPA + DHA is also very low in the Dutch population varying from 66 to 133mg per day (see Table 1.1). This is well below the amount of 450mg EPA + DHA as recommended by the Dutch Health Council. Fish consumption, the most important source of EPA and DHA, is also very low. Of the children between 7 and 18 years old, 6 to 9% consumes fish twice or more per week (recommended amount), for adults this varies between 11 and 28% [26].

Foetuses are dependent on supply of DHA and other LCPUFA via the placenta. The amount of LCPUFA the mother consumes, influences the amount of LCPUFA which is available to transfer via the placenta to the foetus [27]. After birth, supply of DHA via breast milk or infant formulae is needed for the infant to get sufficient amounts of the fatty acid. The amount of DHA in breast milk is dependent on the maternal diet and ranges between 0.17% and 1.0% of total fatty acids in breast milk [28,29].

The low intakes of n-3 fatty acids can possibly have health consequences, as they have many functions in the human body, varying from influence on vision, to regulation of gene-expression, to brain functioning .

Table 1.1: Median intake of ALA, LA and EPA + DHA per day of the Dutch population, data from [26]

	ALA (mg/day)		LA (g/day)		EPA + DHA (mg/day)	
Age	Male	Female	Male	Female	Male	Female
7-8	1237	1166	11	11	62	66
9-13	1489	1267	13	11	65	66
14-18	1745	1343	16	11	67	71
19-30	1972	1390	17	11	75	76
31-50	2069	1444	17	12	97	100
51-69	1930	1465	15	11	131	133

Krill oil supplementation

For those who are not able or willing to consume fish and, consequently, do not get DHA/EPA via that route in their diet, supplements are available. Supplements are often made of fish (i.e., fish oil), but another option is krill. The form of supplementation used in the studies in this thesis is krill oil.

In fish oil the fatty acids are mostly in the triglyceride form. In krill oil, approximately 30-65% of fatty acids are in phospholipid form. Phospholipids have amphiphilic

properties, and thus emulsifying properties that enhance absorption [30,31]. Moreover, DHA and AA in the phospholipid form seem to be accreted at higher rates in the brain as compared to DHA and AA in triglyceride form [32].

LCPUFA and brain functioning

LCPUFA have many functions in the body. With regard to influencing brain functioning and brain health, six key mechanism of n-3 LCPUFA have been identified [24].

(1) **LCPUFA are structural components of the cell membranes in the brain**. Especially AA and DHA are important structural components of brain cells and are abundant in the brain. For example, about 15% of total fatty acid composition of grey matter of the frontal cortex in adults is DHA, and around 10% is AA [33].

(2) **LCPUFA are needed for neurite growth**. For the outgrowth of a neurite from a neuron, the membrane surface needs to be increased first, and this process relies on DHA and AA. DHA and AA are needed to promote fusion of membrane phospholipid bilayer, and with that, the outgrowth of the neurite. Moreover, it has been shown that DHA stimulates neurogenesis and increases synaptogenesis and dendritic spines [34,35].

(3) **LCPUFA influence membrane fluidity**. Cell membranes are made up of a phospholipid layer with protein and cholesterol imbedded. The spatial arrangement of the membrane depends on the specific fatty acyl chains associated with the phospholipids, which are incorporated in the membrane. The spatial arrangement of a saturated fatty acid with no double bond is more compact than an unsaturated fatty acid with a double bond (i.e., with a curve in its tail; see Figure 1.1 and Figure 1.3). The flexibility or the fluidity of the membrane is influenced by the incorporation of PUFAs such as DHA. In other words, the more poly-unsaturated fatty acids incorporated in the cell membrane, the more fluid the membrane will be, and the better the movement through the lipid bilayer will be. The fluidity of the membrane also influences the activity of the enzymes which are bound to the membrane.

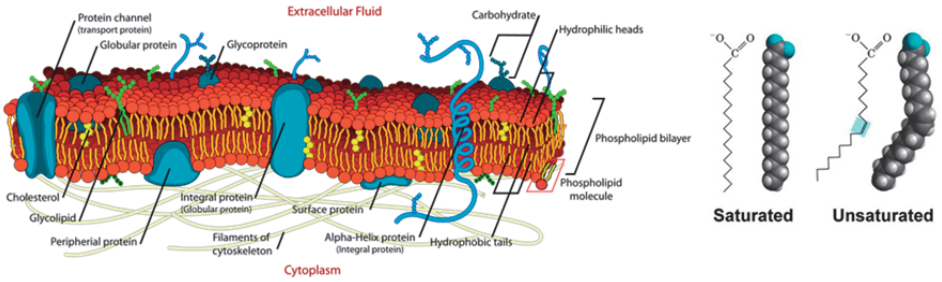

Figure 1.3: Schematic overview of a cell membrane including phospholipid molecules (left). Schematic overview of spatial arrangement of a saturated and an unsaturated fatty acid (right) taken without adaptation from [36]).

(4) **LCPUFA influence neurotransmitters**. The influence of DHA on membrane fluidity and increase in synapses also influence the functioning of proteins in the membrane, which in turn influence neurotransmitter functioning. Low n-3 LCPUFA levels lead to reduced activity of the proteins, which are needed to open the ion channels, allowing the signals of neurotransmitters to be transmitted. In that case, more

neurotransmitter is needed to depolarize the cell and transmit the signal. Thus, for effective neurotransmission, n-3 LCPUFA have to be present in the brain.

(5) **LCPUFA and endothelial: eicosanoids and blood brain barrier**. Eicosanoids can be formed from polyunsaturated fatty acids. Eicosanoids are cell signalling molecules which are involved in many processes in the body such as inflammation, immunity, platelet aggregation and smooth muscle contraction. There are many eicosanoids subfamilies (e.g., prostaglandins, leukotrienes, resolvins) and these subfamilies include many different metabolites. The eicosanoids, which are formed from the n-3 LCPUFA, are prostaglandins and leukotrienes from the 3 and 5 series. These eicosanoids reduce blood clotting, increase blood flow, and have anti-inflammatory and vasodilatory effects. Moreover, DHA can also inhibit the production of inflammatory products. N-3 LCPUFA have also an effect on glucose transport to and glucose uptake by the brain; this is a very important process, as glucose is the main source of energy for the brain. In rats it has been shown that decreased DHA leads to less glucose uptake across the brain blood barrier. Lastly, LCPUFA might also play a role in the endothelial function in the brain, it may improve the blood flow in the brain, improve the brain blood barrier integrity, and decrease inflammation in the brain.

(6) **Neuronal survival**. The presence of DHA in the brain protects the brain from neuronal loss. The exact mechanisms via which DHA exerts this protection against neuronal loss are not known. It has been suggested to be related to the incorporation of DHA in phosphatidylserine (i.e., a specific phospholipid which is part of the cell membrane), but it could also be related to the anti-inflammatory effects of DHA or its influence on neurogenesis.

Brain development

The development of the brain starts very early in pregnancy and does not stop for at least the 25 years thereafter [37]. Brain development starts early on in pregnancy with the closing of the neural tube, it continues throughout pregnancy, and a brain growth spurt occurs during the last trimester of pregnancy and the first two years postnatally. The physical size of the brain increases only up to about the age of seven. After that age, the weight of the brain does not change much anymore [38]. Even though the brain does not increase in weight anymore, the brain does undergo profound development up to at least the mid-twenties. The major changes that occur during this development are the myelination of axons and changes in synaptic density. The areas of the brain with many myelinated axons are called the white matter, and areas with mainly neuronal cell bodies and synapses with almost no myelinated axons are called grey matter.

The white matter mass increases continuously and linear with age throughout childhood, adolescence and early adulthood. The myelination occurs throughout the whole brain and enhances the neuronal conduction, speed, and, with that, neuronal communication [39]. In contrast, the grey matter mass development follows an inverted u-shape. There is a drastic increase in the formation of synapses (i.e., synaptogenesis) during the postnatal period, which leads to a synaptic density much higher than the adult levels. After the peak of synaptic density, a process of eliminations of over-abundant and weaker synapses (i.e., pruning) and strengthening of habitual used synaptic connections occurs [38]. However, the development of grey matter (i.e., the synaptogenesis and pruning) does not occur simultaneously in all regions of the brain: the time course is brain region specific

(see Figure 1.4). The parts of the brain associated with more basic functions (i.e., primary motor cortex) develop earlier than the brain regions associated with the more advanced complex functions (i.e., the prefrontal cortex and temporal lobe) [37]. The fact that the brain is still in development until at least the mid-twenties and the fact that pruning is thought to be an experience-based and life-long process indicate that the brain development can most likely be influenced by environmental influences such as nutrition, exercise and teaching, at least up to the mid-twenties.

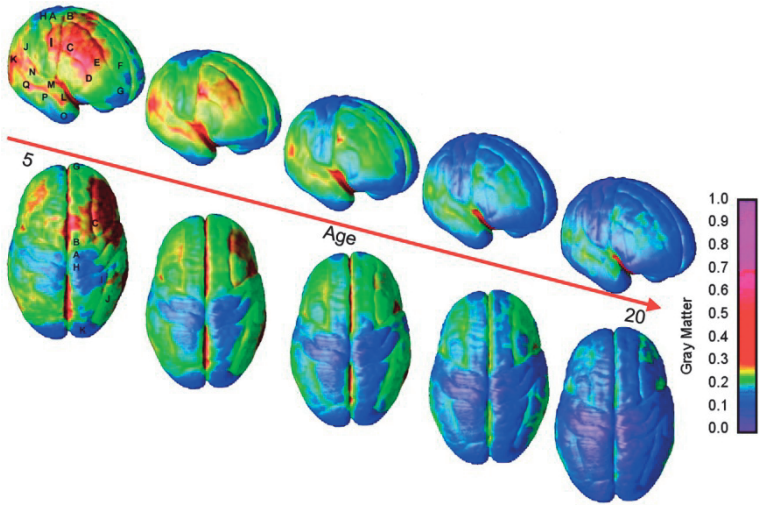

Figure 1.4: Cortical grey matter development from age 5 to age 20; the darker the colour (i.e., blue or purple), the more developed. Taken from [37] copyright (2004) National Academy of Sciences, U.S.A.

School performance

School performance is the most important outcome measure of education and can be measured in a variety of ways. It can for example be measured as grades on specific school subjects (e.g., English or Dutch), an average grade over all school subjects, or scores on a standardized test such as a spelling, reading, or arithmetic test. School performance can be affected by a large number factors including, but not limited to, sex, socio economic status, parental educational level, attitude towards school, motivation, personality, executive functions, time spent on homework, teacher quality, and school resources [40–43]. However, lifestyle factors and especially nutrition might also influence school performance. It has for example been suggested that providing adequate DHA may lead to small improvements in multiple cognitive functions, which combined could influence school performance such as reading and spelling [44]. Moreover, there is a number of studies showing a positive association between fish consumption and school performance in adolescents [11,12]. There are also a few studies investigating the relationship between LCPUFA and spelling, reading, math, and/or grade average in children [45–49]. The results of these studies are mixed, with some showing no effect of supplementation [45,49], and others showing an effect of supplementation or an association between LCPUFA and

school performance [46–48]. However, all but one study did not include blood values [47]. Moreover, we are not aware of any studies investigating the association between prenatal fatty acid exposure and school performance during childhood, while a programming effect might be possible.

To sum this up, there is some evidence that LCPUFA intake or concentrations measured in blood are associated with school performance in children and adolescents. However, most studies do not include blood measurement. Furthermore, there are no studies investigating the association between prenatal exposure to LCPUFA and later school performance.

Cognition

Cognition is a broad term that refers to mental processes involved in for example attention, memory, inhibition, and planning. Concurrent with the brain development, as discussed above, cognitive development occurs. For example, performance on tasks assessing inhibitory control, processing speed, working memory, and decision making continues to improve throughout childhood and adolescence [50–52]. During adolescence, specifically, the prefrontal cortex continues to develop. It can be expected that while specific regions of the brain develop, the cognitive functions which rely on that brain region also develop. The cognitive functions which rely mainly on the prefrontal cortex are the executive functions.

Executive functions are a specific group of top-down mental processes which are needed to be able to concentrate and pay attention and to show adaptive goal directed behaviour [53]. It is mostly agreed that there are three core executive functions: (1) shifting between tasks or mental sets (i.e., *shifting)*, (2) updating and monitoring of working memory representation (i.e., *working memory)*, and (3) inhibition of dominant response (i.e., *inhibition)* [54]. These executive functions are used to build higher order skills such as planning, decision making, reasoning, and problem solving. This makes the executive functions very important for school performance and cognitive development [43,55].

Executive functions and their development are already apparent very early in development. For example, infants aged 7.5-9 months perform worse on the delayed Piaget's *A not B task* than do infants aged 12 months [56]. In that task, a toy is hidden in one of two possible spots. After the hiding, the infant is not allowed to look for a certain period of time (i.e., number of seconds also called the delay period). The delay period is increased with each trial. After the delay period the infant is supposed to reach into the direction of the hidden toy. Piaget's A not B task is one of the earliest measures of intentional goal-directed behaviour. Most infants of 7.5-9 months old fail when there is a 1-5 second delay in being allowed to reach for the toy, while infants aged 12 months succeed even in trials with a 10 second delay.

The executive functions have been shown to develop and improve during childhood and adolescence. For example, in the study of Luna and colleagues, participants were asked to not look at a randomly appearing stimulus, but instead to look in the opposite directions (i.e., a measure of inhibition). Eight year old children were able to execute this inhibition task, but performance increased greatly up to age 14-15 years when adult level performances were reached [51]. Also working memory improves during childhood and adolescence. Crone and colleagues showed that children aged 8-12 years performed worse

on a working memory task (i.e., number correct and reaction time), especially the manipulation task, compared to 13-17 year olds and 18-25 years olds [57]. In another study of Crone *et al.*, a steep decrease in errors on a shifting task was shown when comparing 8-10 year olds with 12-14 year olds, and a smaller decrease was shown comparing 12-14 year olds with 16-18 year olds [58]. The higher order skills such as decision making also have been shown to improve during childhood and adolescence. It has, for example, been shown that older adolescents perform better on tests to assess the ability to make long term advantageous choices (i.e., ability to anticipate the future consequences of decisions), than children and younger adolescents [59,60].

The possible positive influences of LCPUFA, specifically DHA and EPA, on brain functioning has led to a large number of observational studies and supplementation trials in which the relationship between LCPUFA and cognition was investigated. The majority focus on either pregnant women and infants, children below the age of 12, participants with developmental disorder such as ADHD or autism spectrum disorder, adults, or elderly with cognitive decline, Alzheimer's disease, or dementia. These studies have been summarized in a multitude of reviews, for example [1,2,9,44,61–64]. Conclusions of these reviews are opaque. Some conclude that there is evidence for an effect of LCPUFA on cognition [1,2,44,61], others conclude that there is only proof for prenatal effects [63,64] on later life cognition, and others conclude that there is no effect [62].

Adolescents, however, have received rather limited attention. There are, to our knowledge, only three observational studies available in adolescents in which the association between fish consumption (i.e., the most important dietary source op EPA and DHA) and school performance, cognitive performance, and vocabulary was studied [11-13]. Åberg and colleagues reported an association between high fish consumption at age 15 and better cognitive performance at age 18 [13]. Kim *et al.* showed that adolescents who regularly consumed fish had higher school performance scores compared to their peers who did not consume fish [12]. Lastly, de Groot *et al.* revealed a quadratic (i.e., inverted u shape) association between fish consumption (never, < 1 per week, 1-2 times per week, > 2 times per week) and average end term grade and vocabulary score [11].

All in all, observational studies in adolescents point to a positive relationship between fish consumption and both cognitive performance and school performance. However, observational studies cannot prove causality, and intervention studies or long term follow up studies in for example a birth cohort are needed.

Mental well-being

In this thesis, mental well-being refers to both self-esteem and depressive feelings. *Self-esteem* is a core construct of mental health and refers to the subjective evaluation of ones worth as a person [65]. *Depressive feelings* are characterized by sadness, loss of interests and pleasure, feelings of guilt or low self-worth, distributed sleep and/or appetite, feelings of tiredness, and poor concentration [66]. Both depressive feelings and low self-esteem are common in adolescents and have profound and long term effects such as lower educational attainment and negative mental health outcomes [17,18,67,68].

LCPUFA have attracted a lot of attention in relationship to depression, as many of the biological changes associated with depression (alteration in immune function, increased

levels of inflammation, blood flow abnormalities, and decreased glucose metabolism) are processes in which LCPUFA are involved. Thus, it seems possible that LCPUFA could reduce or counteract the biological chances associated with depression.

There are many studies looking at the relationship between fish consumption and depression, or between LCPUFA blood values and depression. But also studies comparing LCPUFA blood concentration between depressed and non-depressed participants, and LCPUFA supplementation interventions in depressed adult individuals. These studies have been summarized in a vast number of reviews and meta-analyses, for example [14–16,69–71]. All these reviews come to the conclusion that there is some proof that n-3 fatty acids can be effective in depression.

Depression often has its onset during adolescence. Adolescents are therefore an important population to study the influence of LCPUFA on depression. There are a number of observational studies in which the association between intake of LCPUFA [72,73], or LCPUFA concentration in blood [74], or LCPUFA in adipose tissue [75,76] and depression in adolescents is studied. However, there are, to our knowledge, no studies in which the influence of LCPUFA supplementation on depressive feelings in adolescents is studied. Moreover, to our knowledge, there are no earlier studies investigating the influence of LCPUFA on self-esteem, another important aspect of mental health.

To summarize, observational studies in adolescents do point to a positive relationship between LCPUFA and depression (i.e., higher n-3 LCPUFA, less depression). LCPUFA supplementation studies in adults also indicate a positive effect of n-3 LCPUFA on depression. However, as observational studies cannot indicate causality, a supplementation study investigating the effect of LCPUFA supplementation on depression in adolescents is needed.

Aim and research questions

The aim of this thesis is threefold. Firstly, to investigate the association between LCPUFA, measured during pregnancy, at birth, and at age 7, and school performance in children at age 7. Secondly, to investigate the influence of LCPUFA supplementation in the form of krill oil supplementation on cognition of developing adolescents attending general secondary education in the Netherlands. Thirdly, to investigate the influence of LCPUFA supplementation in the form of krill oil supplementation on mental well-being (i.e., depressive feelings and self-esteem) in typically developing adolescents attending general secondary education in the Netherlands.

The hypothesis was that higher n-3 LCPUFA levels during pregnancy, at birth and at age 7 would be associated with higher school performance scores at age 7. Furthermore, a second hypothesis was that a year of krill oil supplementation would lead to improved cognitive scores in adolescents, which would possibly also translate to better school performance scores. Moreover, it was hypothesized that a year of krill oil supplementation would lead to lower scores with respect to depressive feelings in the same population.

Design

The current thesis uses data collected in two studies: the Maastricht Essential Fatty Acids Birth Cohort (MEFAB) and Food2Learn.

MEFAB cohort

MEFAB was founded in 1989 and was set-up to investigate how fatty acid concentrations changed during pregnancy, how these changes related to fatty acids concentration of the neonate, and the association between essential fatty acid status during pregnancy and birth outcomes. MEFAB included multiple follow-up moments: at age 7-8 (only those born before 1994; MEFAB 2), age 4 (only those born 1994-1995; MEFAB 3), at age 12 (MEFAB 4), and age 20-25 (MEFAB 5). In MEFAB, a vast array of data was collected (for an overview see **Chapter 2)**.

Food2Learn

Food2Learn was a double-blind randomized, placebo controlled repeated measures intervention in adolescents (age 13-15 years) attending lower general secondary education in the Netherlands. Data on cognition, mental well-being, and school performance were collected. In **Chapter 4**, the study design of Food2Learn is presented in full detail.

Outline of this thesis

In **Chapter 2** and **3** the focus is on the MEFAB cohort. In **Chapter 2,** an overview of the MEFAB cohort can be found. Furthermore, an elaboration on why the MEFAB cohort was set up, which data were collected at which time-point, and the main findings from MEFAB 1 to MEFAB 4 are summarized. In **Chapter 3,** the association between fatty acid concentrations during pregnancy, at birth, and at age 7 and school performance scores at age 7 is presented.

In **Chapter 4** to **8** the focus is on the study Food2Learn. In **Chapter 4,** the design of Food2learn and the rationale behind the research design is presented. In **Chapter 5,** the baseline association between DHA + EPA concentration measured in blood (i.e., the Omega-3 Index) and cognitive measures from the Food2Learn study is described. In **Chapter 6,** the association between LCPUFA levels in blood of the Food2Learn at baseline and depression and self-esteem are reported. In **Chapter 7** and **8,** the focus is on the results of one year of krill oil supplementation. In **Chapter 7,** the influence of one year of krill oil supplementation on cognitive achievements are shown. In **Chapter 8,** the effect of one year of krill oil supplementation on both self-esteem and depression is reported.

Chapter 9 is a review study in which the recruitment, adherence and drop-out rates in omega-3 polyunsaturated fatty acid supplementation trials in children and adolescents was investigated.

Finally, in **Chapter 10,** an overview of the results reported in **Chapter 2** to **9** is given. The results are put in perspective of other studies, the limitations of the studies mentioned in this thesis are given as well as directions for future research.

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Chapter 2

Maastricht essential fatty acid birth cohort

Adapted from: van der Wurff, I. S. M., de Groot, R. H. M., Stratakis, N., Gielen, M., Hornstra, G. and Zeegers, M. (2015). Maastricht essential fatty acid birth cohort. Lipid Technology, 27: 59–62.

Abstract

The Maastricht Essential Fatty Acid Birth cohort (MEFAB) was established in 1989 to study the changes in fatty acid concentration during pregnancy and how this related to the fatty acid concentrations of the neonate. The original sample contains data of 1203 subjects. Some participants whom participated in the original MEFAB study also participated in follow-up studies at age 4, 7 or 12; 41%, 52% and 35% of eligible participants, respectively. Collected data include maternal fatty acid concentrations at multiple points during pregnancy and at birth and at age 7 for the child, but also anthropometric measurements during development, assessments of cognitive development, asthma, atopy and cardiovascular risk factors. Data of MEFAB have been used in 37 articles in peer-reviewed journals and 4 doctoral theses have been completed. Data of MEFAB is upon request available for new research questions

Why was the cohort set up?

From the 1970s onwards essential fatty acids (EFA) and especially their longer-chain, more unsaturated derivatives, the long-chain polyunsaturated fatty acids (LCPUFA) have been a topic of research and discussion because of their potential beneficial effects on numerous health conditions. In the past decades beneficial associations have been found with for example heart disease, metabolic syndrome, cognitive decline, asthma, immune support, visual development and depression (for a review see for example [1]). In the late 1980's it was serendipitously found that the walls of umbilical arteries and the veins demonstrate biochemical signs of EFA deficiency [2]. This led to the question whether the suboptimal supply of EFA during foetal life would exert an influence on development. Moreover, it was not known how maternal availability of LCPUFA during pregnancy related to the concentrations of the infants and whether a particular period in pregnancy or a specific fatty acid had a distinct importance. It was hypothesized that the maternal EFA and LCPUFA status was inadequate to support optimal tissue concentrations in the developing foetus. Because of the important biological activities of these fatty acids and the inability of the foetus to produce the n-3 LCPUFA in sufficient amount itself, this insufficient supply could have important implications for later health and functioning.

The Maastricht Essential Fatty Acid Birth cohort (MEFAB) was established in 1989 to study the changes in fatty acid concentration during pregnancy and how this related to the fatty acid concentrations of the neonate. Furthermore the association between LCPUFA status of mothers during pregnancy and of their infants at birth in relation to various birth outcomes (weight, length and head circumference) was studied. For this purpose 42 different fatty acids were determined in maternal plasma in early, middle and late pregnancy. Furthermore, these fatty acids were determined in the blood vessels of the umbilical cord and plasma of the child. Thus the association between the status of mother and the status of the child could be determined. Later three follow-up studies were added at age 4, 7 and 12 where further assessments of cognitive development, asthma/atopy, growth and cardiovascular disease risks were performed. MEFAB was originally founded by Professor Gerard Hornstra at the department of Human Biology, Maastricht University. After his retirement in 2003, the NUTRIM School for Nutrition, Toxicology and Metabolism at Maastricht University took over the MEFAB cohort. A multidisciplinary steering committee continues the follow-up of this cohort for its 25th year now.

Who is in the cohort?

Pregnant women attending one of 3 antenatal clinics in the South of Limburg (a province located in the South of the Netherlands) at the time of their first antenatal visit between 1989 and 1995 were asked to participate in the study. Women were eligible to participate if they were less than 16 weeks pregnant and did not suffer from any cardiovascular, neurological, renal or metabolic condition. A total of 1334 pregnant women were screened for this study. 131 women were either excluded or dropped out before partus, leaving 1203 subjects available for analyses (90%).

How often have they been followed-up

For the flow diagram see Figure 2.1. The pregnant women were followed during pregnancy and after pregnancy and their neonates were studied at birth (MEFAB 1). Between 1997 and 2000, when the children were approximately 7 years old a follow-up study was executed to investigate the longer term associations between their prenatal and perinatal exposure to EFA and LCPUFA and selected aspects of their mental and physical development (MEFAB 2). For this follow-up singletons born before 1994 of whom an umbilical blood sample was available, were eligible for participation. Parents of children born between January 1994 and September 1995 were asked to let their children participate in a follow-up when they were approximately 4 years old (MEFAB 3). The main focus of MEFAB 3 was cognitive development. Parents of children born between 1990 and 1993 who participated in both MEFAB 1 and MEFAB 2 were asked to let their child participate in MEFAB 4 between 2002 and 2005. The focus of MEFAB 4 was body composition and obesity. Exclusion criteria for MEFAB 4 were any chronic illnesses or depression in the child. Currently a 25-year follow-up is in preparation, which will focus on cardio metabolic risk factors and brain functioning.

Ethics

Pregnant woman who entered the study gave written consent for their participation and for the use of the plasma, red blood cells and/or umbilical cord of their neonate (MEFAB 1). For both MEFAB 2 and MEFAB 3 both parents (if possible) gave written consent for their own participation and for that of their child in the study. For MEFAB 2 consent was obtained for questionnaires, blood sampling, and for the execution of cognitive function tests, motor function tests, physical fitness tests and peak flow measurements. Some parents only gave consent for either the tests or the questionnaires. For MEFAB 3 parents gave their informed consent for both the questionnaires and the cognitive development test or for the questionnaires only. For MEFAB 4 both parents and children gave their informed consent for participation in the study and for the use of the questionnaires, blood sampling and body composition measurements. The initial study and all follow-up studies were approved by the Ethics committee of the University hospital Maastricht/Maastricht University.

Attendance

The number of available maternal blood samples during pregnancy and immediately after delivery differed per time point and varied between 1057 (79%) and 1123 (84%).

For MEFAB 2 750 children were eligible to participate. Of these 728 (97%) were traced and 691 (92%) could be invited. A total of 364 parents did not reply or did not consent, 21 children dropped out during the study. The final study population eventually consisted of 306 children (41%), with blood samples of 265 children (39%). For MEFAB 3 246 children were eligible. Of these children, 1 had passed away, 38 could not be localized, 36 parents

only consented to the questionnaires, 39 parents did not consent at all and 4 children dropped out, leaving 128 (52%) children in the study population. For MEFAB 4 305 children were eligible (participated both in MEFAB 1 and 2), 105 (35%) of these children participated (See Figure 2.1).

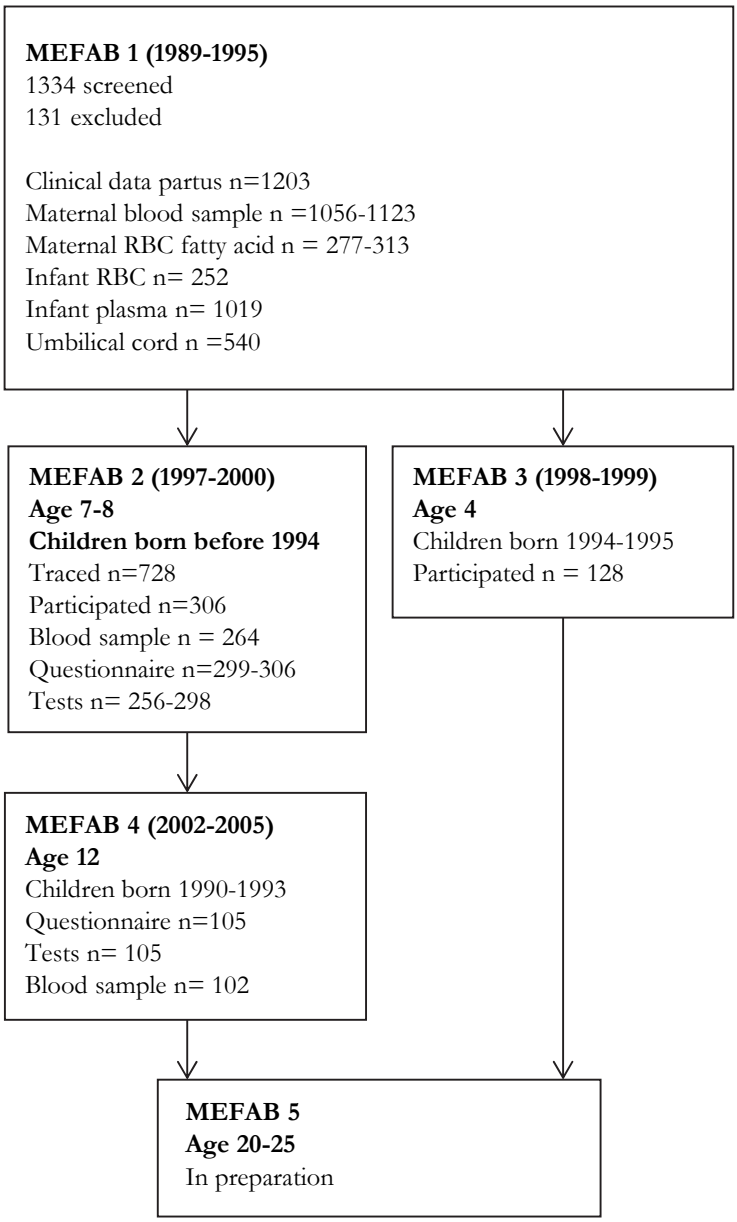

Figure 2.1: Flow-chart of MEFAB

What has been measured?

In Table 2.1 all measurements from MEFAB 1 to MEFAB 4 are listed. Fatty acid composition of maternal plasma phospholipids was assessed in blood collected at study entry (approximately 14 weeks of pregnancy), 22 and 32 weeks of pregnancy and immediately after delivery. Additionally, fatty acid measurements were performed in umbilical cord plasma phospholipids of their neonates and in phospholipids isolated from the walls of umbilical arteries and veins. The standardized methods used to measure plasma fatty acid concentrations and the other study parameters at baseline and during follow-ups have been described in detail by Al *et al.* [3]. During the follow-up of the children, anthropometric parameters (height, weight, skin fold thickness measurements, and waist circumference) were collected at age 0.5, 1, 2, 3, 4 (data received from local health services) and at age 7.

Children who participated in MEFAB 2 donated blood for the determination of the plasma lipoprotein profile, haematology profile, clotting profile, apolipoprotein E (ApoE) and cholesteryl ester transfer protein (CETP) and polymorphisms and plasma leptin, C reactive protein, glucose, insulin and proinsulin concentrations. Children who participated in MEFAB 2 also underwent a Bruce treadmill test to measure physical fitness. They also performed extensive peak flow measurements. For children who participated in MEFAB 3 information on children's neurodevelopment was collected at age 4 (Groningen Developmental Scale, motor scale of the McCarthy Scales of Children's Mental Abilities) for those who participated in MEFAB 2 this information was collected at age 7 (Kaufman Assessment Battery for Children, Maastricht Motor Test, Revised Amsterdam Child Intelligence Test, Child Behavior Checklist).

Mothers of the children who underwent the neurodevelopment test completed the RAVEN test to measure intelligence. Participants in MEFAB 4 again donated blood in which serum leptin concentration, polymorphism of obesity related genes coding for PPARγ2, glucocorticoid receptor and ciliary neurotrophic factor were determined. Furthermore they filled out a questionnaire about attitude towards eating and a questionnaire about their physical activity.

What has been found? Key findings and publications

At this moment 37 articles using MEFAB data have been published in peer reviewed journals and 4 doctoral theses have been completed (for all articles see Supporting Table 2.1).

MEFAB 1

Results concerning MEFAB 1 have been summarized by Al and Hornstra [4–6]. In this paper we will focus on the findings of the follow-up studies; however all articles can be found in Supporting Table 2.1. In the current paper we will shortly summarize the most important findings from MEFAB 1, for more in depth information the reviews can be consulted. The maternal plasma concentration of phospholipid-associated essential PUFA increased during pregnancy by on average 40%, however the non-essential fatty acids

increased more than 65%. Furthermore, it was shown that absolute and relative amount of docosahexaenoic acid (DHA) in the maternal plasma phospholipids were significantly lower in multigravidae compared to primigravidae. Strong correlations between maternal and foetal EFA and LCPUFA were observed. Presence of trans fatty acids in the cord tissue was associated with proportional lower amount of essential PUFA. Preterm infants had a significantly lower LCPUFA status compared to term neonates, but this appeared a physiological phenomenon. Significant positive associations were found between the maternal intake of n-3 fatty acids plus arachidonic acid (AA) and birth length (BL), whereas the intake of linoleic acid (LA) was negatively related to head circumference (HC) [7]. In addition, a positive association was observed between maternal DHA concentrations early in pregnancy and birth weight (BW) and HC [8].

Table 2.1: Data available at the different parts of the MEFAB cohort.

MEFAB 1 1989-1995	*Maternal*	Social economic status (based on postal code). Clinical data during pregnancy and delivery from hospital records. Fatty acid profile of phospholipids (PL) during pregnancy (< 16, 22, 32 weeks) and at delivery or maternal fatty acid composition of erythrocyte PL at same time points.
	Neonatal	Birth outcome (weight, length and head circumference) and conditions at birth (Apgar score, and pH and pCO2 of cord blood). Fatty acid profile of cord plasma PL, umbilical artery wall PL, umbilical vein wall PL or cord blood erythrocyte PL (multiple possible).
MEFAB 2 1997-2000	*Maternal*	Medical questionnaire related to pregnancy and birth and family anamnesis. Intelligence (RAVEN), Fatty acid profile of maternal plasma PL at follow up.
	Children	Growth (weight and height) during early childhood. Body composition based on skin folds (body fat, fat mass, fat free mass). Early development, medical history and medical examination of children at age 7. Atopy questionnaire. Mental processing (Kaufman ABC). School results (spelling, reading, arithmetic and vocabulary). Motor functions (Kaufman motor ABC and Maastricht motor test). Child Behaviour Checklist. Neurological examination. Physical fitness at age 7 (blood pressure heart rate, exercise test). Peak flow measurement at rest and at physical exhaustion. *Blood sample*: Haematology profile. Clotting profile. Plasma lipoprotein profile, ApoE, CETP polymorphisms. Leptin and C reactive protein in plasma. Fatty acid composition of children's plasma PL. Fasting plasma glucose, insulin and proinsulin concentrations. *Subsample*: Language (Revision Amsterdam child intelligence test) and mental fatigue score.
MEFAB 3 1998-1999	*Parental*	Maternal intelligence measured with Progressive Matrices test of Raven. Maternal and paternal education (8 point scale).
	Children	Kaufman Assessment Battery for Children Groningen Developmental Scale McCarthy Scales of Children's Mental Ability (motor scale only).
MEFAB 4 2002-2005	*Parental*	Weight and height Attitude towards eating (Three factor eating Questionnaire).
	Children	Weight, height and BMI at age 1, 7, 12. Body composition (body fat, fat mass and fat free mass). Attitude towards eating (Three factor eating Questionnaire). Physical activity (Baecke questionnaire). *Blood sample*: Serum leptin concentration, polymorphism of obesity related genes coding for PPARγ2, glucocorticoid receptor and ciliary neurotrophic factor.

In contrast, maternal concentrations in late pregnancy and/or at delivery of AA and dihomo γ-linolenic acid (DGLA, the direct precursor of AA) were negatively related to BW and BL. This suggests that maternal DHA content may program foetal growth in a positive way and that the maternal AA may be involved in foetal growth limitation. Interestingly, significant negative associations were observed between BW and umbilical plasma this is caused by a limited DHA transfer from mother to foetus, resulting in lower DHA concentrations in larger foetuses [9]. This would imply that relationships with function variables are less convincing for neonatal than for maternal fatty acid concentrations.

Metabolic syndrome

In MEFAB 2 the association between early life time exposure to LCPUFA and markers of the metabolic syndrome (e.g., obesity and insulin resistance) was studied. Rump *et al.* showed that umbilical cord plasma phospholipid concentrations of γ-linolenic acid and DGLA were negatively related to plasma insulin concentrations and the calculated insulin resistance at age 7 [10]. Gamma-linoleic acid was also negatively related to body fatness, pro-insulin levels and leptin concentrations at age 7. Interestingly, in the study of De Vries *et al.* it was shown that maternal DGLA concentrations throughout pregnancy were positively associated with an increased BMI at age 7 [11].

Neural development

Another focus of MEFAB 2 (and MEFAB 3) was the association between early LCPUFA exposure (as reflected by umbilical plasma and red cell AA and DHA concentrations) and later brain function, cognition and behaviour. Neither at age 4 [12] nor at age 7 [13] were significant associations found between LCPUFA exposure during pregnancy and cognition. Bakker *et al.* did observe that movement quality (a measure of brain-muscle interaction and a low score of which may predict ADHD and later learning problems) at age 7 was positively associated with plasma phospholipid DHA levels measured at birth, but were unrelated to DHA concentrations at follow-up [14]. This suggests that prenatal LCPUFA availability may be more important for later motor function than childhood dietary LCPUFA intake. In contrast, associations with early AA levels tended to be negative, but were not significant. When looking at behaviour, research has found that higher levels of DHA at birth are associated with lower levels of internalizing behaviour at age 7 [15]. Relationships with DHA status at follow-up were, again, not significant. This could imply that the perinatal exposure to higher DHA levels may reduce the risk for depression in adult life.

Other measurements

No clear and consistent associations were found between prenatal AA status and lung function or plasma inflammation markers at age 7 [16].

What are the main strengths and weaknesses?

One of the main strengths of the MEFAB study and database, is that it is, as far as we know, world-wide the only prospective mother-child cohort containing extensive data on maternal plasma fatty acid composition at various time points during pregnancy and at delivery. In addition, fatty acid data are available from umbilical plasma as well as from the walls of umbilical veins and arteries. This enables the assessment of perinatal (umbilical plasma) and earlier (umbilical vessel walls) fatty acid exposure of the developing foetus. MEFAB is one of the few fatty acid related cohorts that is being followed up for a long period of time and has presently the potential to contact participants 25 years after birth. Another strength of the cohort is the fact that all common essential and nonessential fatty acids have been measured, including the non-essential longer-chain, more unsaturated derivatives. Since some of these latter fatty acids (20:3n-9 and 22:3n-9) are essential fatty acid status markers, MEFAB data can be used to relate later functions to pre and perinatal EFA and LCPUFAs statuses. Furthermore, fatty acid data are available from different time points during pregnancy; consequently, later functional conditions can be related to fatty acid exposures during early or later fetal development. Alongside the measurements of fatty acids, a vast array of other measurements were taken at different follow-ups which are now available to study the influence of pre- and perinatal LCPUFA exposure to a large number of health outcomes. Data collection in the MEFAB cohort started around 25 years ago, follow-ups provide cost-effective opportunities to examine the long-term impact of maternal LCPUFA status in early, mid- and late pregnancy on future offspring health and function. Such data may assist in determining critical development periods during which dietary fat modulation has the potential to influence later function and health. Weaknesses include the loss to follow-up over time. However, this issue affects many observational studies and clinical trials with a long follow-up, especially those covering such an extended time period as MEFAB. This leads to a relative small cohort, findings should thus be interpreted with caution. Another weakness is the lack of data on other dietary factors, which might exert a confounding role in the association of prenatal LPUFA status with later health. Lastly, the study was executed in the South of Limburg, a province of the Netherlands, which has a predominantly Caucasian population. Therefore results are not generalizable to more multicultural populations. But the homogeneity of the study population does add to the validity of the studies.

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Supporting Table 2.1: Overview of all articles published on the MEFAB cohort.

Main author(s)	MEFAB	Research question and main finding
Houwelingen & Hornstra [17]	1	How does the maternal status of trans fatty acid change over pregnancy and how does this relate to the foetal status of trans fatty acid and essential fatty acids? How do birth outcome relate to the trans fatty acids exposure? The degree of intrauterine trans fatty acid exposure is related to maternal values of trans fatty acids. The amount of C18:1 trans isomer did not increase during pregnancy. Birth weight (BW) and head circumference (HC) are significantly correlated with C18:1 trans fatty acid.
Al [3]	1	What is the relationship between EFA status of mothers and their new-borns and pregnancy-induces hypertension (PIH)? Altered EFA status was observed late in the pregnancy in PIH, indicating that EFA is unlikely to contribute to the pathogenesis of PIH.
Al [18]	1	What is the course of maternal EFA status during an uncomplicated pregnancy? How do the maternal and foetal EFA status relate? Total amount of fatty acids, individual fatty acids and fatty acid families increased significantly. The relative amount of LA did not change, while the relative amounts of AA decreased. Overall maternal EFA status steadily declined during pregnancy. Correlation between maternal and umbilical relative amounts for LA, AA and DHA were high.
Al [19]	1	How are the EFA composition of the maternal diet, maternal EFA status and EFA status of healthy infants related? Maternal dietary fat consumption was consistent during pregnancy. A high maternal LA intake had a lowering effect on maternal and neonatal n-3 fatty acid status. Neonatal EFA status was strongly related to maternal EFA status.
Houwelingen [20]	1	What is the EFA status of foetus during intrauterine development? The relative amount of LA slightly increased during development, the same was true for both relative and absolute amounts of DHA. AA decreased both absolute and relative. EFA profile of foetal samples were equal with postnatal results of infants with the same gestational age.
Foreman-van Drongelen [21]	1	Does multiple pregnancy lead to lower EFA status of the infants born? Levels of n-3 and n-6 were lower, while EFA deficiency indicating n-9 PUFA were significantly higher in infants born after multiple pregnancy compared to first born infants.
Al [22]	1	Do succeeding pregnancies affect maternal and neonatal DHA status? The maternal DHA status was significantly lower in multigravidae (MG) compared to primigravidae (PG). The relative amount of DHA in umbilical artery and vein vessel walls was significantly lower in MG- than in PG-neonates.
Badart-Smook [7]	1	Are dietary factors in pregnancy related to foetal growth? Maternal intake of n-3 fatty acids plus AA and of riboflavin was positively associated with foetal growth. Linoleic acid intake was negatively associated with foetal growth.
Zeijdner [23]	1	Is maternal EFA supply the limiting factor to the neonatal EFA status? A slightly lower EFA status was found in maternal and umbilical plasma for multiplets compared to singletons. Correlation between maternal and umbilical plasma EFA levels were comparable for multiple and singleton pregnancies.
Otto [24]	1	To study EFA status in plasma and erythrocyte phospholipids in woman before conception and during the first 10 weeks of pregnancy and to assess diet during that period. Maternal plasma and erythrocyte phospholipid DHA levels started to increase very early in pregnancy, which could not be explained by diet alone.
Otto [25]	1	How does maternal plasma and erythrocyte LCPUFA and especially DHA develop post-partum in relation to lactation and dietary LCPUFA intake. After delivery LA, AA, EPA and docosapentaenoic acid% increased over time, the pattern of change did not differ significantly between lactating and non-lactating. The DHA level declined significantly in both lactating and non-lactating women, but more in lactating women.

Main author(s)	MEFAB	Research question and main finding
Rump [9]	1	Are there relationships between EFA composition of cord and maternal plasma phospholipids and birth weight in term neonates? Lower EFA status in umbilical cord plasma and a larger decrease in maternal plasma LCPUFA's was associated with higher weight-for-gestational age at birth. AA and DHA were both negatively related to weight SD scores.
Rump [26]	1	Is there a relationship between plasma leptin concentration and the DHA content of plasma phospholipids during early pregnancy and post-partum? Total amounts of phospholipids associated fatty acids was related to plasma leptin concentration before and during early pregnancy but not in late pregnancy and post-partum.
Ghys [12]	3	What is the relationship between LCPUFA status at birth and cognitive development at 4 years of age. No significant association was found between DHA or AA status at birth and cognitive development at age 4.
Rump & Hornstra [27]	1	Overview of the distribution of n-3 and n-6 PUFA in the plasma phospholipid fraction of pregnant women on a Western diet and their neonates. Distribution is reported for first, second and third trimester of pregnancy, at delivery and from umbilical vein at birth.
Rump [10]	2	Does fatty acid composition of cord blood relate to childhood body composition and glycaemic control? Cord plasma phospholipid γ-linolenic acid and DGLA concentrations were negatively related to insulin concentrations and insulin resistance (calculated) at age 7. This association remained significant after adjustment for birth weight. γ-linolenic acid concentration was negatively related to body fatness and proinsulin and leptin concentrations at several ages.
Rump [28]	2	Do the gene variations in ApoE and CETP interact with regard to their influence on lipoprotein levels? Children with ApoE E2E3 had lower concentration LDL cholesterol and ApoB than those with Apo E4E3 or Apo E3E3. The associations were only significant in children who were homozygous or heterozygous for the absence of the Taq-IB polymorphism at the CETP gene locus. The difference in HDL level between the CETP genotype groups was only significant in E2E3 carriers.
Rump [29]	2	What is the relation between frequently used indicators of cardiorespiratory fitness, sex, and body composition in young prepubescent children? Result showed that when oxygen uptake measurement is not possible, calculated total work or maximal power output (expressed per kilogram fat free mass) seem to provide better indicators of aerobic power than endurance time.
Bakker [13]	2	What is the relationship between LCPUFA status at birth and at 7 years of age and cognitive performance at 7 years of age. No significant associations were found between DHA and AA status at birth and cognitive performance at 7 years of age. The LCPUFA levels at 7 years of age were also not associated with cognitive outcomes.
Otto [30]	1	What is the relationship between postpartum depression and the change in DHA concentrations in maternal plasma phospholipids after delivery and during lactation? Slower postpartum normalization of functional DHA status (22:6n-3/22:5n-6 ratio) is related to higher occurrence of depressive symptoms.
Vlaardingerbroek & Hornstra [31]	1	Are erythrocyte phospholipids as reliable as plasma phospholipids to reflect the EFA status of an individual? The relative concentrations of erythrocytes and plasma phospholipids fatty acids were strongly correlated, but not in early pregnancy. The changes in fatty acid concentrations and absolute amounts during pregnancy were comparable between plasma and erythrocytes, although this decreased at the end of the pregnancy. Neonatal erythrocyte values also correlated strongly with maternal values early in the pregnancy.

Main author(s)	MEFAB	Research question and main finding
Hornstra [32]	1	What is the influence of trans fatty acids on foetal development? BL and HC were significantly and negatively associated with C18:1 trans concentration in umbilical plasma (HC), umbilical arterial walls (BL and HC) or umbilical venous walls (BL).
Vogels [33]	4	What is the effect of early development, parental and genetic variables, and behavioural determinants on overweight at age 12? Rapid increase in weight during first year, BMI of the father and high dietary restraint score of the mother was significantly associated with overweight at age 12. No genetic relations were observed. Overweight was positively associated with dietary restraint of the child, body fat percentage was negatively correlated with child's activity score.
Krabbendam [15]	2	Is there a relationship between LCPUFA status at birth and later development of problem behaviour at age 7? Association between LCPUFA status at birth and later development of problem behaviour was studied. Higher levels of DHA at birth were associated with lower levels of internalising problem behaviour at age 7, this association was only present in infants fed infant formula.
Rutters [34]	4	Are genetic, behavioural, parental and psychological factors involved and to what extend in the development of body weight at age 12-13? Genetic distribution was not different between lean and overweight children. Overweight children and the parents of overweight children showed higher dietary restraint and disinhibition scores. Leptin appeared to play a role in the development of body weight during puberty.
Slinger [35]	2	What is the association between insulin resistance and physical fitness, leptin concentration, body composition and family history of diabetes in young children? Plasma leptin concentration was independently associated with the development of insulin resistance. There was an association between physical fitness and insulin resistance which was mediated by the sum of the skin folds..
Dirix [8]	1	Are there associations between BW, BL or HC and the relative content of DHA, AA, DGLA and 18:1 trans in maternal plasma phospholipids during early, middle and late pregnancy and at delivery? Maternal DHA (especially in early pregnancy) was positively associated with BW and HC. AA status in late pregnancy and at delivery was negatively associated with BW and BL. Maternal DGLA contents at delivery was negatively associated with BW and BL.
Dirix [16]	2	Is prenatal AA associated with immune-related clinical conditions and plasma markers at age 7? After correction for covariates no significant associations between AA and atopy, lung function and plasma inflammation markers were found.
Dirix [36]	1	Are there associations between term birth dimensions and prenatal exposure to some of the LCPUFA reflected by neonatal status at birth. A negative association between BW and umbilical plasma DHA was shown. AA concentration in umbilical plasma and arterial and venous vessel walls was also negatively associated with BW and BL. BL was negatively associated with arterial vessel wall AA concentration only. C18:1 trans in cord erythrocytes was negatively associated with BW.
Dirix [37]	1	Is there an association between foetal brain functioning and the early essential poly unsaturated fatty acid status? Positive trends were observed between short term memory measures before 38 weeks of pregnancy and mead acid and Mead acid + dihomo Mead acid, and between long term memory and levels of Osbond acid.
Bakker [14]	2	What is the relationship between motor function at age 7 and AA and DHA levels in umbilical venous plasma phospholipids and in plasma phospholipids at age 7? Umbilical plasma DHA concentrations were significantly and positively related with Maastricht Motor Test both total and quality score. DHA and AA were not significant related to quantitative movement scores.

Main author(s)	MEFAB	Research question and main finding
Rutters [38]	4	What is the relationship between sleep duration and BMI from Tanner stages 1 to 5 (stages of puberty)? Changes in BMI during puberty were inversely related to changes in sleep duration, independent of confounders.
Veldwijk [39]	2	Is there an association between BMI and cognitive ability in young children in primary school? Study did not find any associations between BMI and cognitive ability in school aged children.
De Vries [11]	2	What is the association between maternal PUFA status during pregnancy and offspring adiposity at age 7? Maternal DGLA throughout gestation was associated with increased BMI and some other measures of adiposity at age 7.

Chapter 3

Association between prenatal and current exposure to selected LCPUFAs and school performance at age 7

Adapted from: van der Wurff, I. S. M., Bakker, E. C., Hornstra, G., Kirschner, P. A., Gielen, M., Godschalk, R. W. L., Kremers, S., Zeegers, M.P. & de Groot, R. H. M. (2016). Association between prenatal and current exposure to selected LCPUFAs and school performance at age 7. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 108 (2016): 22-29.

Abstract

Introduction: Long-chain polyunsaturated fatty acids (LCPUFAs) are important for brain functioning and might, thus, influence cognition and school performance. However, research investigating LCPUFAs relationships with school performance is limited. The objective of this study was to determine the association between levels of the LCPUFAs docosahexaenoic acid (DHA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and n-6 docosapentaenoic acid (Osbond acid, ObA) at study entry, 22 weeks of pregnancy, 32 weeks of pregnancy, at partus, in umbilical cord plasma and child's plasma at age 7 and school performance scores at age 7.

Methods: Data from the Maastricht Essential Fatty Acid Birth cohort (MEFAB) were used for this study. Fatty acid levels of plasma phospholipids were measured in maternal blood plasma at study entry, 22 weeks of pregnancy, 32 weeks of pregnancy and partus. Childs fatty acid levels of plasma phospholipids were measured a in umbilical cord blood plasma, and in blood plasma of the child at age 7. Scores on national standardised tests for spelling, reading and arithmetic at age 7 were obtained via the school (scores were available for 149, 159 and 155 children, respectively). Associations between LCPUFA levels and school performance scores were analysed with categorical regression analyses with correction for covariates (smoking, maternal education, sex, breastfeeding, maternal intelligence, birth weight and BMI at age 7).

Results: Significant ($p < .001$) associations between DHA level at age 7 and both reading ($\beta = 0.158$) and spelling ($\beta = 0.146$) were found. Consistent significant negative associations were observed between all maternal DHA plasma levels and arithmetic scores at age 7 (all $p < .001$, all $\beta < -0.019$). Additional significant negative associations were observed between maternal LCPUFA plasma levels at study entry and both reading and spelling scores at age 7; these associations were less consistent.

Conclusion: Plasma DHA levels at age 7 were positively associated with reading and spelling scores at age 7. Consistent significant negative associations between maternal plasma DHA levels and arithmetic scores of the child at age 7 were found. Although this is an observational study, which cannot proof causality, the consistent negative associations observed between maternal plasma DHA levels and the arithmetic scores of the children at age 7 calls upon prudence when considering DHA supplementation during pregnancy.

Introduction

Long-chain polyunsaturated fatty acids (LCPUFAs) are important constituents of all cell membranes. In this way they are involved in, among others, neuronal membrane fluidity, neurotransmission, signal transduction, brain blood flow and blood-brain barrier integrity [1,2]. With respect to brain functioning, four LCPUFAs are of major importance: docosahexaneoic acid (DHA) and arachidonic acid (AA) are important components of the neuronal membrane [3] , eicosapentaenoic acid (EPA) influences a large number of brain processes [4], and n-6 docosapentaenoic acid (Osbond acid, ObA) is a deficiency marker of DHA [5] . Because these LCPUFAs seem to be essential for brain functioning they might in turn affect school performance. Therefore, the associations between LCPUFA levels in maternal plasma at study entry (< 16 weeks of pregnancy), 22 weeks of pregnancy, 32 weeks of pregnancy and at partus, in umbilical cord blood plasma, and in blood plasma of the child at age 7 and school performance at age 7 were investigated in this study. The maternal plasma levels were used as a proxy for the foetal exposure, as a number of studies have shown that maternal LCPUFA plasma levels correlate highly with the LCPUFA levels of foetuses [6–8].

In earlier studies the association between fish intake (primary source of DHA and EPA) [9–15], LCPUFAs intake calculated based on answers to questions regarding fish consumption [10,12,15] and/or LCPUFA concentrations in blood [12,14–19] and cognition has been studied, but the results of these studies do not show consistent patterns. Similarly, results from randomized controlled trials are mixed. Some supplementation trials studying the influence of maternal n-3 LCPUFA supplementation during pregnancy and/or lactation and/or n-3 supplementation to infants after birth on cognition of the child have shown positive results [20–29], others have found null results [16,23,30] and some found negative results [31–35].

To the best of our knowledge, the association between LCPUFA intake or levels during early life and school performance at age 7 has not yet been addressed. This is unfortunate since children are not judged on their cognition but, rather, on their school performance. Although no studies are available about this association in children, some studies in adolescents have been executed. De Groot *et al.* showed an inverted u-shaped association in 700 adolescents (age 12–18) between reported fish intake and vocabulary. Thus higher fish consumption was associated with a higher vocabulary score, however fish intake higher than twice a week was associated with a lower vocabulary score. A similar trend (non-significant) trend, was found for the average school grades of Dutch, English and mathematics [36]. Likewise, Kim *et al.* found a strong positive association between the number of meals containing fish per week and average end-term grade across 16 school subjects in adolescents age 15 years [37]. These studies suggest a possible relation between school performance and LCPUFA intake during adolescence, a period of life which is characterised by development of the prefrontal cortex [38,39] .

Brain development is also prominent during pregnancy and childhood. Considering the observed positive association between fish consumption and school grades in adolescents, one could infer that a relationship between LCPUFA levels during pregnancy and childhood and school performance is also possible. Brain development starts very early in pregnancy with neural tube formation and continues throughout pregnancy with in the last trimester and the first 2 years of postnatal life a brain growth spurt. In this growth-spurt

DHA rapidly accumulates in the brain [40,41]. DHA availability is in this period therefore very important, as the foetus is largely dependent on supply of DHA and other LCPUFAs via the placenta [42–44]. After birth supply of DHA via breast milk or infant formula is needed to provide sufficient amounts [45]. Lastly, during childhood, DHA might also play a role in brain development and maturation since brain development continues until late adolescence [38].

Overall, the exact influence of LCPUFA exposure during development on school performance remains unclear. Therefore, the objective of the current study is to investigate the associations between pre-, peri-, and postnatal exposures to DHA, EPA, AA and ObA and school performance at age 7. Exposure data were inferred from LCPUFA concentrations in phospholipids from maternal plasma collected at study entry, 22 weeks of pregnancy, 32 weeks of pregnancy, at partus, in umbilical cord plasma, and in plasma phospholipids of the children at age 7. It is hypothesised that higher LCPUFA exposure during pregnancy, in umbilical cord plasma and child's plasma at age 7 are associated with higher school performance scores in children aged 7.

Participants and methods

Design

Data from the Maastricht Essential Fatty Acid Birth (MEFAB) cohort were used for this study. Founded in 1989, MEFAB was originally set-up to investigate the association between essential fatty acid status during pregnancy and birth outcomes (MEFAB 1, for an overview see [46,47]). Later, the children and their parents were asked to participate in various follow-up studies (MEFAB 2, 3 and 4, for an overview see [48]).

For the current study data from MEFAB 1 and 2 were used to study the association between selected LCPUFA levels (DHA, AA, EPA an ObA) in maternal non-fasted plasma phospholipids collected at study entry (before 16 weeks of pregnancy; μ=10.93 weeks), at 22 weeks and 32 weeks of pregnancy and at delivery and school performance scores at age 7. Furthermore, LCPUFA levels of the children were determined in phospholipids from umbilical cord plasma and from non-fasted venous plasma at age 7, these levels were also used to study the associations between selected LCPUFAs and school performance at age 7. The study was approved by the Ethics Committee of the University Hospital Maastricht/ Maastricht University.

Participants

The eligible study population consisted of 750 Caucasian children aged 7–8 years born between December 1990 and January 1994. After 7 years, 728 of these children were traced. Of them 3 were deceased and 34 moved abroad and therefore did not participate in the follow-up. In addition, 133 parents did not reply to the invitation. The parents of 231 children refused to give consent for participation in the follow-up, primarily mentioning lack of time and concerns about the burden on their child as reason for refusal. As a result,

the parents of 327 children gave their consent for participation. During the MEFAB 2 study period, 21 children dropped out leaving a final study population of 306 children.

Unfortunately, not all schools attended by the participants administered the standardised school performance tests. In addition, some schools administered the tests at another age or were not willing to provide the results. The school performance scores were obtained for 149, 159 and 155 children for reading, spelling and arithmetic, respectively. For the flow diagram, see Figure 3.1.

Blood and fatty acid analyses

Fatty acid profiles of maternal and umbilical plasma phospholipids were determined by gas-liquid chromatography as described by Al *et al.* [46]. The analysis of the blood samples at age 7 was slightly different, total lipids were extracted with the method of Blight and Dyer [49]. The gas-chromatographic methodology enabled the quantitative measurement of forty-two fatty acids [46]. Focus of the current paper is on DHA, AA, EPA, and ObA, as previously indicated. Fatty acid data are presented as relative levels (% of weight of total phospholipid-associated fatty acids).

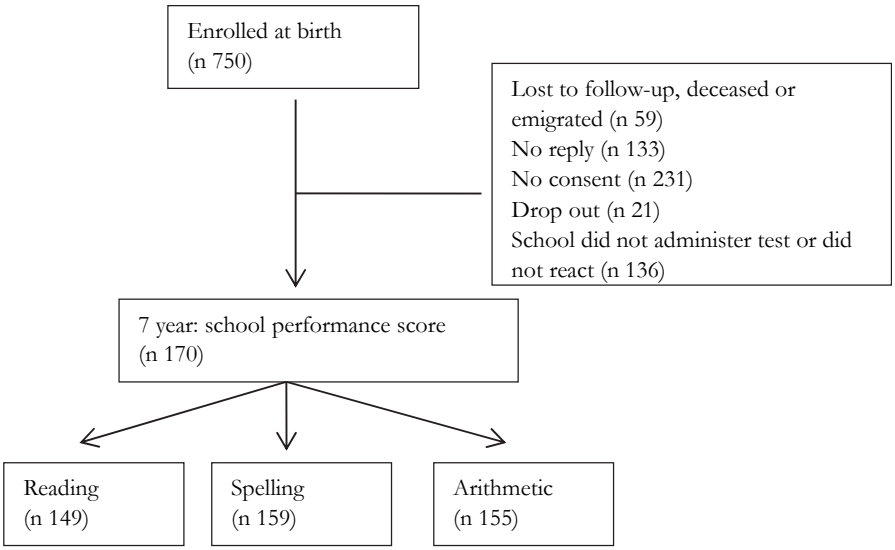

Figure 3.1: Flow diagram of children enrolled in the study and followed up until 7 years of age.

Measurements of academic achievement

To assess school performance at age 7, scores from the standard Dutch Cito-system (Central institute of test development) for spelling, reading and arithmetic were obtained from the school [50]. Results on the Cito tests are given as a letter (A through E). This letter is based on the scores of a normative group of children on the tests. If the score of a child is in the same range as the 25% best scoring children in the normative group the child

will receive an A. The 25% above average is indicated by a B, 25% below average is indicated by a C, the 15% far below average are indicated by a D and the 10% worst scores are indicated by E. To achieve even groups, group D and E have been combined for the current study.

Covariates

A number of factors known to be important determinants of cognitive development were included as covariates in the analyses: breastfeeding (yes/no)[51] , birth weight in grams [52,53], the sex of the child [54] [54], maternal intelligence[55], maternal education (low/high) [55,56], maternal smoking during pregnancy (yes/no) [57,58], and the child's BMI at 7 years of age [59]. Maternal intelligence was based on the Raven's Standard Progressive Matrices (RAVEN) with a maximum score of 60 [60]. Body weight was measured to the nearest 100 g on a SECA electronic digital scale, while children wore light underwear only. Height was measured to the nearest millimetre using a wall mounted stadiometer (Holtain LTD, Crymych, UK) as described in detail by Gerver and de Bruin [61]. BMI (kg/m2) was calculated and classified for boys as underweight (BMI ≤ 14.03), healthy weight (BMI 14.04–17.91) or overweight (BMI ≥ 17.92) and for girls as underweight (BMI ≤ 13.85), healthy weight (BMI 13.86–17.74) or overweight (BMI ≥ 17.75) [62].

Statistical analyses

Because of missing values, multiple imputations (i.e., fully conditional specification technique) were used to analyse the data [63]. Categorical regression analysis[64] was used to estimate the association between DHA, AA, EPA or ObA levels measured at study entry, 22 weeks of pregnancy, 32 weeks of pregnancy, partus, in umbilical cord blood plasma, and in blood plasma of the child at age 7 and school performances at age 7. All covariates were entered in the initial model. When the Pratt-coefficient of relative importance for a given covariate was <.05 or the p-value was > .1 that covariate was excluded from the analysis. The beta and p-value of the fatty acid levels at specific time points in the adjusted model are reported The beta value as reported is the normal standardized regression coefficient of the optimally transformed variables. CATREG computes quantifications for each category by optimizing a least squares criterion. For example, if one looks at the association between maternal DHA level at study entry and arithmetic score (A, B, C or D), the categories A, B, C, and D get the values 0.80, −0.50, −1.55 and −2.02, respectively. Here the beta for DHA is −0.170. This implies that an increase of 1SD in DHA would lower the predicted score of the transformed arithmetic scale with 0.170. For example, a difference between C and D, which is −2.02 + 1.55 = 0.47, can be quantified in 0.47/0.170 = 2.76 standard deviations of DHA. This is 2.76 * 0.82 = 2.26%wt/wt of total FA.

Due to the large number of analyses p <. 01 was considered statistically significant, unless noted otherwise. Analyses were carried out using SPSS statistics version 22 (IBM).

Quality check

As check for selection bias characteristics of participants who participated in MEFAB 1 only and those who also participated in MEFAB 2 were compared. Data was first checked for normality. If scale data was normally distributed an independent t-tests was performed, if not a Mann Withney U test was performed. For the nominal and ordinal data a Chi-squared test was performed. These analyses were performed for all fatty acids at all time-points, birthweight, birth length, head circumference, gestational age, age mother, maternal intelligence, maternal education sex child and smoking during pregnancy. This procedure was also followed to check for differences between participant of MEFAB 2 of whom school performance scores were available and those whose scores were not available. Lastly the procedure was repeated to check whether there was a difference between mothers who suffered from adverse pregnancy events (intrauterine growth restriction, preeclampsia or pregnancy induced hypertension) and those who did not suffer from adverse pregnancy events. For these analyses $p < .05$ was considered statistically significant.

Results

For 170 children, one or more school performance scores were available. The characteristics of these participants and their mothers can be found in Table 3.1.

Table 3.1: Participants' characteristics.

	Value (mean+/- SD or n)	n
Infant		
Sex (male/female)	94/75	169
Gestational age (d)	278 ± 12.3	138
Birth length (cm)	50.1 ± 2.4	158
Birth weight (g)	3340 ± 511	169
Head circumstance (cm)	34.3 ± 1.77	143
Birth order (first child/other)	109/60	169
In uterine growth restriction (normal/too small/too big for gestational age)	135/16/16	167
Preeclampsia		2
Pregnancy induced hypertension		4
Breastfed (yes/no)	81/82	163
Duration breastfeeding[1] (mo.)	4.8 ± 5.1	81
BMI at age 7	15.5 ± 1.7	161
Underweight, normal, overweight/obese[2]	21/125/15	161
Mother		
Maternal smoking (yes/no)[3]	38/130	168
Maternal age (yrs.)	30.1 ± 4.1	169
Maternal education (low /high)	105/56	161
Maternal intelligence (RAVEN)	46.6 ± 6.5	162

[1] Mean of participants who were breastfed [2] Defined as for boys: underweight (BMI ≤ 14.03), healthy weight (BMI 14.04-17.91), overweight (BMI ≥ 20.63) and for girls as underweight (BMI ≤ 13.85), healthy weight (BMI 13.86-17.74) or overweight (BMI ≥ 17.75) [3] Maternal smoking at study entry.

Plasma phospholipid fatty acid levels

Of the 170 children, 140 donated non-fasted blood to determine the plasma phospholipid fatty acid levels at follow-up. The levels of these fatty acids at age 7 can be found in Table 3.2. This table also contains the maternal fatty acid levels at study entry (mean pregnancy duration 10.93 weeks), at 22 and 32 weeks of pregnancy, and at partus, as well as the umbilical plasma fatty acid levels.

School performances scores

School performance scores for reading, spelling and arithmetic were available for 149, 159 and 155 children respectively. Table 3.3 shows the frequency of the available school achievement scores, as well as the percentage of students in this school performance quartile.

Table 3.2: Selected fatty acids levels (% w/w, mean ± s.d. and range (smallest-to largest value) of maternal plasma at study entry (μ=10.93), 22 and 32 weeks pregnancy, and at delivery, umbilical cord plasma at birth and children's blood plasma phospholipids at age 7.

Fatty acid (%wt/wt of total FA)	Maternal plasma PL at entry study (n= 152)	Maternal plasma PL at 22wks. (n= 152)	Maternal plasma PL at 32wks. (n= 148)	Maternal plasma PL at partus. (n= 149)	Umbilical plasma PL (n=157)	Plasma PL at 7 years (n =140)
DHA 22:6n-3	3.96 ± 0.82 2.34-6.34	4.07 ± 0.81 2.59-7.14	3.95 ± 0.73 2.51-7.02	3.82 ± 0.71 2.08-5.60	6.04 ± 1.34 3.43-10.12	2.99 ± 0.80 1.61-6.73
EPA 20:5n-3	0.51 ± 0.39 0.16-4.04	0.36 ± 0.16 0.15-1.25	0.37 ± 0.23 0.14-1.40	0.33 ± 0.18 0.09-1.63	0.23 ± 0.12 0.0-0.73	0.66 ± 0.40 0.2-2.79
AA 20:4n-6	9.63 ± 1.50 5.95-15.10	8.55 ± 1.23 5.67-12.16	8.12 ± 1.15 5.49-11.16	8.30 ± 1.38 5.23-12.58	16.5 ± 1.7 11.04-20.28	9.15 ± 1.18 6.22-12.69
ObA 22:5n-6	0.35 ± 0.12 0.08-0.98	0.45 ± 0.15 0.14-0.98	0.47 ± 0.14 0.19-0.93	0.51 ± 0.16 0.19-0.94	0.85 ± 0.26 0.03-1.77	0.28 ± 0.09 0.00-0.54

PL = phospholipids

Table 3.3: Number of participants (and percentage of total) at every level for the school performances scores on the CITO test at 7 years of age.

	Level				
	A	B	C	D	n
Reading (%)	58 (39)	40 (27)	42 (28)	9 (6)	149
Spelling (%)	95 (60)	32 (20)	20 (13)	12 (8)	159
Arithmetic (%)	82 (53)	44 (28)	22 (14)	7 (5)	155

Achievement scores were divided in quartiles based on the scores in a normative group of children with respectively A representing very good (highest quartile in the normative group), B good, C moderate, D weak (lowest quartile).

Associations between fatty acids and school performance

For arithmetic scores at age 7 the analyses showed significant negative associations with maternal DHA levels throughout the pregnancy, and with umbilical plasma DHA level (all

p < .001) (see Figure 3.2). The association with children's plasma DHA level at age 7 was not significant (p = .20).

For reading and spelling, the associations with DHA levels throughout pregnancy and at partus and in umbilical cord blood plasma were non-significant. Significant positive associations were found for spelling (p < .001), and reading scores (p < .001), and children's plasma DHA level at age 7.

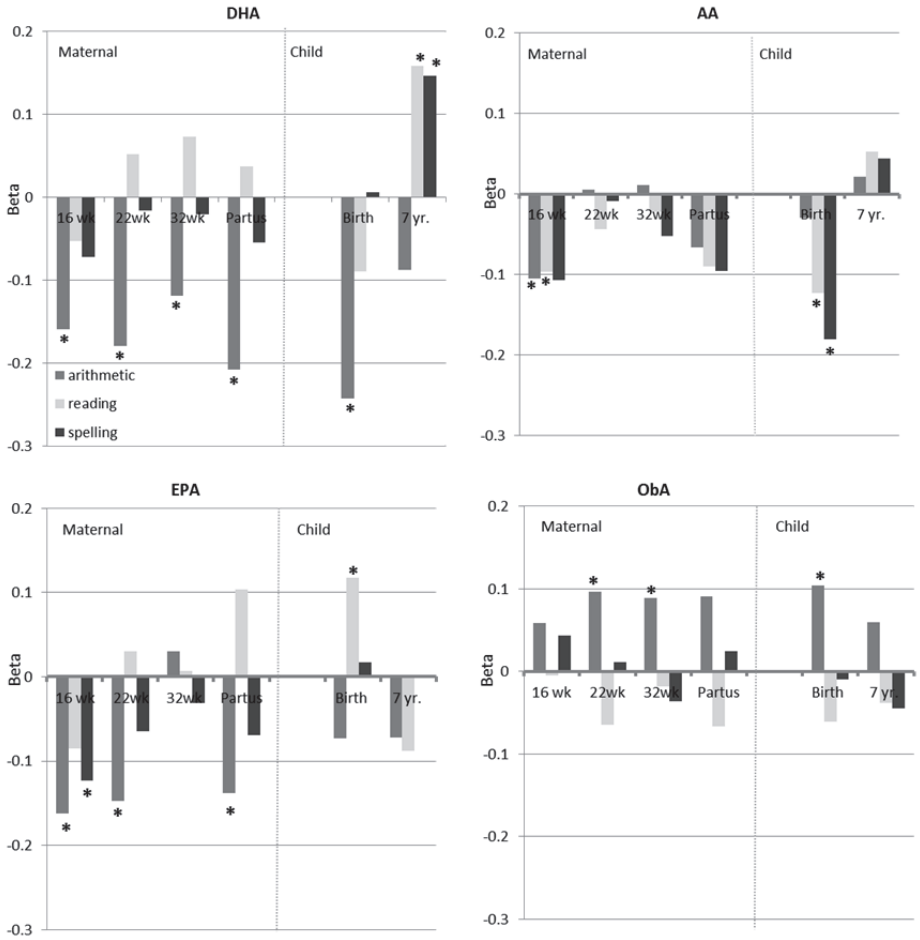

Figure 3.2: Results of adjusted model CATREG analyses: Beta of fatty acid (DHA, AA, EPA, ObA) at specified time point for arithmetic, reading or spelling scores. *p value of fatty acid < .01 Full models included the following covariates: breastfeeding (yes/no), birth weight, sex of child, maternal intelligence (RAVEN score) maternal education, maternal smoking during pregnancy (yes/no), child's BMI at age 7 (underweight, healthy, overweight). Covariates were excluded if p value > .1 or Pratt coefficient of relative importance < 0.05. The first fatty acid assessment (here named 16 weeks) was done on blood collected at study entry mean= 10.93 weeks pregnant.

Maternal AA level at study entry was negatively associated with both arithmetic ($p = .008$) and reading ($p = .005$) scores. In addition, AA level in umbilical cord blood plasma was negatively associated with reading ($p = .001$) as well as spelling ($p < .001$).

Maternal EPA levels at study entry, at 22 weeks of pregnancy, and at partus showed significant negative associations with arithmetic scores (all $p < .001$). The level of EPA at study entry was also negatively associated with spelling scores at age 7 ($p < .001$). Furthermore, there was a positive association between EPA level in umbilical cord plasma and reading scores at age 7 ($p = .005$).

Lastly, maternal ObA levels at 22 and 32 weeks of pregnancy and level in umbilical cord blood plasma were positively associated with arithmetic scores ($p = .006$, $p = .004$ and $p = .003$, respectively). All these associations were positive, and since a higher ObA level reflects a lower DHA status [5], this finding confirms the negative relationship between DHA status and arithmetic scores.

Quality check results

The group of children whose school performance scores were available and those whose scores were not available were compared, and some group differences were seen. Of special interest was the difference in the maternal RAVEN score, which was higher in those with school performance scores available. However, upon further inspection of the data, three extreme outliers were identified in the group without school performances available (RAVEN scores of 8, when the average score was 40). When these outliers were removed the group difference faded out. When the results of children whose mothers suffered adverse pregnancy outcomes were compared to those who had a healthy pregnancy, only a difference on birthweight was seen ($\mu = 3166.9$ vs $\mu = 3383.9$, $p = .027$), which is to be expected as this included children whom were small for gestational age. The data-analyses were corrected for birthweight; this difference is thus not expected to influence results.

Discussion and conclusion

In line with our hypothesis a positive association between DHA level at age 7 and spelling and reading scores at age 7 was shown. In contrast, mostly significant negative associations were found between maternal DHA levels throughout pregnancy and arithmetic score at age 7. This was supported by ObA, a functional deficiency marker of DHA [5], which showed significant positive associations with arithmetic.

The positive associations between DHA level at age 7 and spelling and reading scores are mostly in line with previous studies in adolescents. The negative associations between DHA levels and arithmetic scores in the current study are mostly in contrast to studies in adolescents that showed a positive association between fish consumption (most important source of DHA) and school grades [36,37]. De Groot *et al.* did, however, show a negative association when fish consumption was more than twice a week [36]. Though, the studies of De Groot *et al.* and of Åberg *et al.*, assessed fish intake, which is only a proxy for n-3 LCPUFAs status in the blood [65]. Furthermore, these studies in adolescents looked at grade

average over a number of school subjects; this could have led to averaging out negative associations in one school subject with positive associations in another school subject.

While studies investigating the association between LCPUFA concentrations in blood and school performance in children are, to our knowledge, not available, studies that look at the association between LCPUFA blood levels and cognitive measures are available. Some of these studies looking at the association between LCPUFA levels and cognitive measures showed negative associations [31,32,34,35,66,67], while others showed positive [17,19] or no associations [14,16]. Intervention studies also show mixed results: positive [20–29], negative [31–35] or neutral [16,23,30]. These mixed results have been speculated to be due to the large methodologically difference between the studies: differences in cognitive tests used moment, sort and dosage of supplementation [68]. It is however important to keep into mind that cognition and school performance are related, but not the same [69,70]. School performance is dependent on more factors such as motivation, personality and time spend on homework [71]. Thus a positive association between LCPUFA and cognition, does not mean that LCPUFA and school performance are positively associated.

Previous reported results thus remain mixed, with positive, negative and neutral associations being reported between LCPUFAs levels in blood and cognitive measures, and the association with school performance mostly unstudied. In the current study negative associations between maternal plasma levels during pregnancy and arithmetic scores at age 7 are shown, while the association between DHA level at age 7 and reading and spelling scores at age 7 was positive. The opposing associations between LCPUFA levels and arithmetic and LCPUFA levels and spelling/reading are surprising.

Since a positive association for spelling and reading scores at age 7 was found and this was not found for arithmetic scores, one could speculate that this is because these skills are located in different brain region. The brain does not develop uniformly, every regions has its own development curve [38]. Arithmetic is related to executive functioning [72], which is mainly located in the pre-frontal cortex, a region of the brain which develops late in the development. Language is located mainly in the parietal lobes, a brain region which develops earlier in the development [38].

Though a number of studies observe negative associations between LCPUFA levels and cognitive measurement, most fail to explain these negative associations. No biochemical explanation for the negative associations were found in the literature, with the exception of the possible detrimental effects of high levels of toxic substances present in some fish species (e.g., mercury, cadmium, PCBs and dioxins). However, not all studies that looked at these substances showed unfavourable neurological development (for review see among others Oken [73]). Therefore, it seems time that the research community pays attention to, and further explores the possible unfavourable association between LCPUFA levels and cognition and school performance.

This observational study had a number of limitations. Unfortunately, school performance scores were only available for about 150 children, resulting in 21% of the original sample being analysed on school performance. The non-availability of the school performance scores was due to the fact that some schools did not administer the tests (such tests are not obligatory), administered the tests at another time point than age 7, or the school did not respond to the request for the test scores. These missing data could have influenced the results, especially since the number of children with a score in the lower

quartiles was limited. However, no difference between children whose school performance scores were available and children whose school performance scores were not available were seen on a number of covariates. Maternal and child plasma LCPUFA levels are furthermore similar to those found in other studies (e.g., [74,75]). Maternal LC n-3 PUFA intake from the women in the current study did not change during pregnancy [76] and the maternal LC n-3 PUFA intake was similar to other pregnant populations [77].

Maternal plasma LCPUFA concentrations were measured frequently during gestation which is a valuable aspect of this study. Plasma phospholipids were used as a measure of fatty acids level as they are a good indicator of longer term dietary fatty acid intake [78] and as foetal blood values are correlated with maternal blood values [6–8], this is a good measure of foetal fatty acid exposure. Furthermore, participants were followed up until age 7, which allows the study of long-term associations of early life time exposure to LCPUFAs. Finally, even with the more conservative p of .01, results were significant, pointing to robust associations.

This study is of associative nature and the sample size is rather limited, only randomized-controlled trials can confirm or refute the findings of the current observational study. However, with the negative associations in mind and the fact that such intervention studies, with a long-term follow-up, are difficult to design and covariates might influence the results as well, a randomized controlled trial might not be feasible. Thus, the results of the current study are an useful proxy, which can be used for hypothesis formation.

To summarize, this study is the first, to our knowledge, to investigate the association between prenatal, perinatal and current LCPUFA exposure and school performance scores in children aged 7. Interestingly, consistent significant negative associations were observed between pre- and perinatal exposures to DHA and arithmetic scores at age 7. These results are in line with a few studies regarding cognition that also show negative associations [31–35,67].

In contrast, the association between DHA level at age 7 and spelling and reading scores was positive. To conclude, although this is an observational study not implying causality, the consistent negative associations observed between maternal plasma DHA levels during pregnancy and the arithmetic scores of the children at age 7 calls upon prudence when considering DHA supplementation during pregnancy.

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Chapter 4

A protocol for a randomised controlled trial investigating the effect of increasing Omega-3 index with krill oil supplementation on learning, cognition, behaviour and visual processing in typically developing adolescents.

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Abstract

Introduction: The influence of n-3 long-chain polyunsaturated fatty acids (LCPUFA) supplementation on brain functioning is debated. Some studies have found positive effects on cognition in children with learning difficulties, elderly people with cognitive impairment and depression scores in depressed individuals. Other studies have found null or negative effects. Observational studies in adolescents have found positive associations between fish consumption (containing n-3 LCPUFAs) and academic achievement. However, intervention studies in typically developing adolescents are missing.

Objective: The goal of this study is to determine the influence of increasing Omega-3 Index on cognitive functioning, academic achievement and mental well-being of typically developing adolescents.

Methods and data analysis: Double-blind, randomised, placebo controlled intervention; 264 adolescents (age 13–15 years) attending lower general secondary education started daily supplementation of 400 mg eicosapentaenoic acid and docosahexaenoic acid (EPA + DHA) in cohort I (n=130) and 800 mg EPA + DHA in cohort II (n=134) or a placebo for 52 weeks. Recruitment took place according to a low Omega-3 Index (< 5%). The Omega-3 Index was monitored via a finger prick at baseline and after 3, 6 and 12 months. The supplement dose was adjusted after 3 months (placebo analogously) to reach an Omega-3 Index of 8–11%. At baseline, 6 and 12 months, a neuropsychological test battery, a number of questionnaires and a standardised math test (baseline and 12 months) were administered. School grades were collected. In a subsample, sleep quality and quantity data (n=64) and/or eye-tracking data (n=33) were collected.

Ethics and dissemination: Food2Learn is performed according to Good Clinical Practice. All data collected are linked to participant number only. The results will be disseminated on group level to participants and schools. The results will be presented at conferences and published in peer-reviewed journals. The study is approved by the Medical Ethical Committee of Atrium-Orbis-Zuyd Hospital and is registered at the Netherlands Trial Register (NTR4082).

Trial registration numbers: NTR4082 and NCT02240264; Pre-results.

Introduction

There is a debate whether long-chain polyunsaturated fatty acids (LCPUFA) improve cognitive performance. LCPUFAs from the n-3 family such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are involved in many aspects of brain functioning, for example, neuronal membrane fluidity and neurotransmission [1,2]. There is evidence that consumption of Omega-3 LCPUFAs plays a protective role in age-related cognitive decline [3] and Alzheimer's disease [4]. In premature babies, it has been shown that higher Omega-3 LCPUFAs consumption is associated with advanced cognitive development [5]. Furthermore, in children, it might lead to improvement of symptoms associated with autism and attention deficit hyperactivity disorder (ADHD) [6]. Although previous research is not conclusive, it does suggest that in certain sensitive periods Omega-3 LCPUFAs might contribute to optimising cognitive performance and/or cognitive development. Adolescence has received limited attention in studies on n-3 LCPUFAs. The brain and prefrontal cortex continue to develop until the late 20s [7–11]. It is thus crucial to study the role of Omega-3 LCPUFA in adolescence as they play an important role in brain development and functioning. For example, DHA is an important structural component of the neural cell membrane, influencing membrane fluidity and signal transduction [12,13]. Moreover, higher DHA intake has been associated with changes in the functional activity of the prefrontal cortex in boys aged 8–10 years [14]. Even though EPA is only present in very small amounts in the brain,15 it is also involved in important brain processes such as neurite outgrowth, regulation of gene expression and in anti-inflammatory, antithrombotic and vasodilatory processes which might assist brain functioning [15]. Studies on the relationship between measured LCPUFA status or supplementation and cognitive functioning in adolescents are, to the best of our knowledge, not available. Observational studies investigating the association between fish intake and cognitive functioning in adolescents are, however, available. Kim *et al.* [16] showed that adolescents aged 15 years who regularly consumed fish had significantly better academic performance than their non-fish consuming or less fish consuming peers. Åberg *et al.* [17] demonstrated that high fish consumption in boys at age 15 years was associated with better cognitive performance at age 18 years. Finally, de Groot *et al.* [18] studied 700 adolescents aged 12–18 years for whom fish consumption data, end-term grades (for Dutch, English and Math) and score on the Amsterdam Vocabulary Test was collected. Contrast analyses demonstrated a u-shape association between fish consumption and vocabulary ($p = .01$) and a nearly significant association with average end-term grades ($p = .07$). Thus, higher fish intake was associated with more advanced vocabulary and an almost significant higher average end-term grade. However, eating more fish than the recommended amount (> 2 fish portion/week) was not associated with a beneficial outcome. Studies looking at the measured LCPUFA status and depression in typically developing adolescents are also limited. We are only aware of the study by Mamalakis that showed a negative association between EPA measured in adipose tissue and score on the Beck Depression Inventory (i.e., higher EPA, fewer depressive symptoms). Furthermore, a positive association between dihomogamma linolenic acid (DGLA, C20:3n-6) measured in adipose tissue and score on the Centre for Epidemiologic Studies Depression Scale (CES-D) (i.e., higher DGLA, more depressive symptoms) was shown [19]. This was, however, only true after correction for adiponectin. Although self-esteem has often been associated with depression [20], we found no studies

taking self-esteem into account when studying the association between LCPUFA and depression in adolescents. While observational studies point to a beneficial association between fish intake (the main source of DHA and EPA) and school grades in adolescents and since causality cannot be proven by observational studies, intervention studies are needed. Furthermore, little is known about the effects of Omega-3 fatty acids on depression and self-esteem in typically developing adolescents. This paper describes the design of Food2Learn, a double-blind, randomised, placebo controlled intervention in which the influence of an increase in the Omega-3 Index with 1-year of krill oil supplementation on cognition, academic achievement and behaviour in typically developing adolescents is investigated. In addition, we try to counteract design issues such as bioavailability, baseline Omega-3 Index and study population which could have caused neutral results in earlier trials [21].

Bioavailability

The bioavailability of DHA and EPA depends mostly on the form in which they are bound and the food matrix with which the DHA and EPA are taken. Most previous trials have chosen unemulsified ethyl-ester or triglycerides and have advised participants to consume capsules with breakfast, both of which lead to lower absorption of the LCPUFAs [21–23]. In Food2Learn, krill oil is used as supplementation. Krill oil contains 30–65% of the EPA and DHA in the phospholipid form. Phospholipids have amphiphilic properties and therefore emulsifying properties that enhance absorption [24–26] and are also better absorbed by the brain than triglycerides [27]. Another factor important for DHA and EPA absorption is the presence of fat in the meal. Some studies report a trifold higher LCPUFA absorption when LCPUFA capsules are taken with a high fat meal, compared to a low fat meal [23]. This is hypothesised to be caused by the stimulating effect of fat on pancreatic lipase [22]. Therefore, participants in Food2Learn are asked to consume the capsules with dinner, the fattiest meal of the day [28].

Baseline Omega-3 Index

In earlier studies, participants were often recruited without researchers knowing the baseline Omega-3 fatty acid status of participants. This has led to participants with a wide range in statuses being included, while one can expect that effects of supplementation are more likely in participants with a low Omega-3 fatty acid status. This wide range in statuses can lead to similar end Omega-3 statuses in the placebo and treatment groups and thus similar outcome measures. To increase the chance of observing any effects of supplementation, participants for Food2Learn are recruited on the basis of a low Omega-3 Index (< 5%) [29]. The Omega-3 Index is defined as EPA plus DHA in erythrocytes and is based on a standardised analytical method [21]. Additionally, to counteract interpersonal variability in the uptake/ metabolism of the LCPUFA supplementation, a dose adjustment based on the individual Omega-3 Index blood levels after 3 months of supplementation was applied. This dose adjustment should ensure that participants in the active treatment group achieve the target range of 8–11%. This target range is an estimate based on the Omega-3 Index associated with the lowest mortality risk in coronary heart disease [29].

Study population

Richardson showed that LCPUFA supplementation was especially beneficial in the 20% lowest performing children [30]. In the Netherlands, secondary education is divided into three levels: pre-university, higher general and lower general secondary education (LGSE). Approximately 38% of all adolescents attend LGSE [31]. LGSE is further divided into four sublevels. For the current study, students from the highest sublevel, the theoretical learning pathway (TLP), were recruited. Approximately 40% of students attending LGSE are in the TLP.

Objective and hypotheses

The primary objective of Food2Learn is to study the effect of an increase in the Omega-3 Index due to 1-year of krill oil supplementation in 13–15 year-old typically developing adolescents from LGSE on cognitive performance. The secondary objective is to study the effect of an increase in the Omega-3 Index due to 1 year of krill oil supplementation in 13-15 year-old typically developing adolescents from LGSE on academic achievement and behaviour. Furthermore, the relationship between Omega-3 Index and cognitive performance, academic achievement and behaviour in typically developing adolescents will be further explored. The hypothesis is that 1 year krill oil supplementation and a higher Omega-3 Index will lead to improved cognition, academic achievement scores and behaviour scores in typically developing Dutch adolescents attending the LGSE. The third objective is to study the effect of an increase in the Omega-3 Index due to 1 year of krill oil supplementation in 13–15-year-old typically developing adolescents from LGSE on sleep quality and quantity, and visual processing.

Methods

Study design

Food2Learn is a double-blind, randomised, placebo-controlled trial, with repeated measurements (at baseline, 3 months, 6 months and 12 months) to study the effect of an increase in the Omega-3 Index due to 1-year of krill oil supplementation on cognitive performance, academic achievement and behaviour of second year LGSE students. Informed consent was obtained from all participants and their parent(s) and/or guardian(s).

This trial is registered at The Netherlands Trial Register (NTR4082) and at ClinicalTrials.gov (NCT02240264). The Items from the WHO Trial Registration Data Set can be found in the online supplementary files.

Participants

Inclusion and exclusion criteria

All second year students attending high school at the LGSE TLP level with a baseline Omega-3 Index < 5% were eligible to participate in the study. Students were excluded if they had (1) a baseline Omega-3 Index of > 5%, (2) an allergy to fish or shellfish or (3) haemophilia.

Determination of participation

Participants could discontinue participation at any time during the study. If a participant indicated that he/she wished to stop supplementation, the reason for discontinuation was asked and the participant was asked to continue with the test sessions according to the intention to treat principle, but both were voluntary.

Recruitment and screening

One hundred and twenty-three schools in the southern provinces of the Netherlands were contacted to participate in Food2Learn. Eventually, 17 school boards gave approval. The target group was then approached in a classroom setting. A video explaining the study was shown, the capsules and the finger prick were shown, and any questions were answered. Students received an information letter and were asked to discuss the study with their parents. If requested, a voluntary additional information evening was held. If the student wanted to participate, the informed consent form had to be signed by the student and parent(s) and/or guardian(s) and handed in to the researchers at site. All students who handed in an informed consent form received a finger prick to determine the Omega-3 Index; no other pre-screening methods were used. If the participant completed the whole study, he/she received a voucher for the cinema. In cohort I of Food2Learn, the needed number of participants was not enrolled; therefore, a second cohort was added to the study. Currently, cohort II is in progress.

Randomisation and blinding

On entry, participants received a participant number. An independent researcher was responsible for treatment allocation. The participants were stratified by sex and were randomly allocated to the placebo or krill oil group by an independent researcher. Equal numbers of participants were randomised to the active treatment and the placebo groups. The group allocation sequence was computer generated and executed by an independent researcher. Researchers at site were only told which box number had to be given to which participant number and were not aware how boxes were divided over placebo/krill oil group. To prevent the risk of unblinding, each participant received a personalised box with capsules. The packaging and the capsules themselves were identical for the active treatment and placebo groups. The capsules were coloured black to hide the redness of krill oil. Furthermore, a vanilla odour was added to hide any possible fishy smell/taste. Neither the researchers at the site nor the participants were informed of group allocation prior to the

completion of the whole study (cohorts I and II). Preliminary unblinding of the trial was to be executed only by an independent researcher in the case of severe adverse effects.

Procedure—baseline

Before starting supplementation, participants underwent a neuropsychological test battery, filled out questionnaires and performed a standardised math test (see outcome measure, Figure 4.1 and Table 4.1); these tests were executed at school in a classroom setting. Three cognitive tests were administered in a group setting (10 students max): The Letter Digit Substitution Test (LDST), D2 test of Attention (D2) and Digit Span Forward (DSF) and Backward (DSB). These tests were led by one researcher via a standardised protocol, while one or two other researchers (depending on group size) made sure that participants understood the tests and complied with the protocol. All tests were first explained and a practice version was always completed by the participants. After this group test session, all participants filled out the questionnaires individually, during which participants were called one by one to perform the Stroop Test and Concept Shifting Test (CST) individually under supervision of one researcher. After data collection, participants completed a standardised computerised math test, in silence, in a classroom setting.

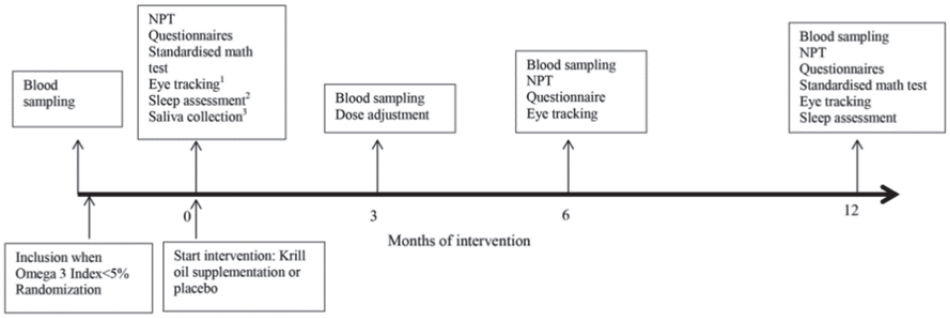

Figure 4.1: Timeline of study
NPT = neuropsychological tests, at all time points equal: The Letter Digit Substitution Test, D2, digit span forward and backward, Concept Shifting Test and Stroop Test.
[1] Eye tracking was done in a subsample of participants [2] Sleep was measured in a subsample of participants in the second year with a combination of ActivPal and self-reported sleep diary, furthermore participants filled out the Adolescent Sleep-Wake Scale and the Adolescents Sleep Hygiene Scale. [3] Saliva was collected to determine Apoe status

Table 4.1: questions and questionnaires at different time-points

Question / questionnaire	Before	Baseline	3 months	6 months	12 months
Weight	X				
Length	X				
Level of parental education	X				
Nationality		X			
Country of birth		X			
Language spoken at home		X			
School carrier (focus chosen, skipping or staying behind a year)		X			
Medical: medicine use, diagnoses, glasses/contacts		X		X	X
Allergies		X			
Use of vitamins and fish oil		X			
Pubertal Development Scale		X			X
Alcohol and cigarette use		X			X
Fish Questionnaire		X		X	X
Centre for Epidemiologic Studies Depression Scale		X		X	X
The Rosenberg Self-Esteem Scale		X		X	X
Motivated Strategies for Learning Questionnaire		X		X	X
Self-reported capsule adherence			X	X	X
Self-reported side-effects				X	X
Self-reported group allocation					X

Procedure—3 months follow-up

After 3 months of supplementation, participants again received a finger prick to determine the Omega-3 Index. Blood analyses and dose adjustments were executed by a researcher who was not actively involved in the study. When the increase in the Omega-3 Index in a participant of the active group was insufficient, the supplementation dose was adjusted in this participant and in one participant in the control group with the same number of capsules. This was carried out to ensure blinding of the researchers. However, the Omega-3 Indexes of the participants of cohort I were found to be significantly lower than the target range at 3 months. Therefore, it was decided that the dose of all participants would be increased to eight capsules per day. Furthermore, it was decided to increase the starting dose of the cohort II to eight capsules per day to ensure that the target range would be achieved.

Procedure—6 months and 12 months follow-up

The procedure completed at baseline was repeated again after 6 months. This process was again repeated after 12 months of intervention. The standardised math test was only completed at baseline and after 12 months (for an overview, see Figure 4.1).

Intervention

After baseline neuropsychological testing, participants started with supplementation. The krill oil contained at least 40 g phospholipids per 100 g krill oil. The fatty acid profile of these phospholipids was at least 14 g EPA and 6 g DHA. The remaining composition of krill oil was mainly triglycerides (32%), free fatty acids (6–7%) and small amounts of cholesterol, cholesterol esters, ash and a trace amount of astaxanthin (80 μg/100 g). One krill oil capsule contained a total of 0.5 g krill oil, which supplied 65 mg EPA and 35 mg DHA. For the placebo capsules, a fatty acid mixture was chosen that reflects the fatty acid composition of the average European diet (26.0% C16:0, 4.6% C18:0, 35.8% C18: 1–9, 16.7% C18:2–6, 2.1% C18:3–3, 0% C20:4–6 and 14.8% other compounds) [32]. The placebo contained a mix of olive oil, corn oil, palm oil and medium chain triglycerides in the following ratio 4:4:9:3. The placebo contained no marine n-3 fatty acids. In cohort I, participants were instructed to start with an intake of four capsules per day containing in total 260 mg EPA and 140 mg DHA, almost the daily recommended amount of 450 mg of EPA + DHA per day as set by the Dutch health council [33]. In cohort II, the starting dose was increased to eight capsules, as cohort I showed that the initial dose of 400 mg DHA + EPA did not lead to a sufficient increase in the Omega-3 Index. Eight capsules provide 520 mg EPA and 280 mg DHA per day. To increase adherence to the protocol, participants could receive a daily text message reminder. Furthermore, participants who had an insufficient increase in their blood Omega-3 Index after 3 months of supplementation and an equal number of participants from the placebo, as noted by the independent researcher, received a phone call to try to increase adherence. Participants were asked to return all left-over capsules at the end of the study or when they decided to drop out, so adherence could be calculated on the basis of the number of capsules returned.

Outcome measures—cognitive performance measurements

The cognitive tests were selected on the basis of their usability as determined in other studies with adolescents reference scores are thus available. These tests have also previously been shown to increase activation of the frontal cortex, the area of the brain associated with the accumulation of DHA [14], furthermore the prefrontal cortex is the brain area most in development during adolescence [9]. Tests used were: the LDST to measure speed of information processing, D2 as a measure for selective attention, DSF and DSB as measures of short-term memory and working memory, CST as a measure for cognitive shifting and Stroop task as a measure for cognitive inhibition. For an explanation of the tests, see online supplementary appendix A.

Academic achievement

Math test

All students complete a standardised math test at baseline and at the end of the study. This math test is based on the end terms as set by the Dutch government. The test is computer-based and participants have to take the test without a calculator [34].

School performance
All schools provide school grades (ranging from 1.0=very bad to 10.0=excellent) of participants for the subjects Dutch, English and Mathematics. These subjects are chosen as they are considered core subjects and are therefore compulsory for all students.

Behaviour measurements

Absenteeism
Schools provide the number of hours the participants were absent and the reason for absence.

Motivation
The Motivated Strategies for Learning Questionnaire consists of two parts: a motivation part and a learning strategies part [35]. In this study, only the motivation part is used. The motivation section assesses student goals and value beliefs concerning school, their beliefs about their skills to succeed in school and their test anxiety. The internal consistency for the section measuring motivation is very good (α between 0.62 and 0.93) [36].

Mood
Mood is assessed with the Dutch version of the Centre for Epidemiologic Studies Depression Scale (CES-D) [37], a tool sensitive for detecting depressive symptoms and distinguishing between depressed and non-depressed individuals [38,39]. The CES-D has a high internal reliability in adolescents (α=0.88) [40]. The 20 questions questionnaire assesses the presence of depressive symptoms in the past week. Total sum scores are calculated (range 0-60), with higher scores indicating more depressive symptoms.

Self-worth/self-esteem
The Rosenberg Self-Esteem Scale (RSE) is a measure of self-esteem [41]. The internal reliability in adolescents is high (α=0.88) [42,43] and construct validity has been shown [43]. This test consists of 10 questions requiring participants to indicate their level of agreement with a series of statements about themselves. Higher scores indicate a higher self-esteem.

Other measurements

Fish consumption is measured with a short, validated self-reported questionnaire [44]. The pubertal phase is assessed with the Pubertal Development Scale [45]. This questionnaire assesses the pubertal status of adolescents by asking to what extent a number of bodily changes related to puberty are present. Furthermore, nationality, country of birth of the participants and parents and/or guardians, language spoken at home, use of medicine, diagnosis related to learning (e.g., ADHD or autism), allergies, whether the student wears glasses or contact lenses, whether he/she takes vitamins or fish oil supplements, drinking and smoking behaviour were assessed via a questionnaire. Finally, questions about school career such as whether the participant has ever skipped or repeated a year are asked. At follow-up, additional questions with regard to adherence, side effect and group allocation are asked. For an overview, see Table 4.1.

Biological measurements

Blood analysis
Blood samples are collected with a finger prick. First, the finger of the participants is disinfected with alcohol. Then a prick with an automated one-time use lancet is administered at the fingertip after which blood drops are collected on specially prepared filter paper. Erythrocyte fatty acid compositions are analysed according to the HS-Omega-3 Index methodology as described previously [29,46]. Fatty acid methyl esters are generated by acid transesterification and analysed by gas chromatography using hydrogen as the carrier gas. Fatty acids are identified by comparison with a standard mixture of fatty acids. Results are given as the Omega-3 Index, which is EPA + DHA expressed as a percentage of total identified fatty acids after response factor correction and a correction for the fact that whole blood was used instead of erythrocytes [46]. Furthermore, the concentrations of 26 other fatty acids are determined. The coefficient of variation for EPA plus DHA typically is 5%. Analyses are quality controlled according to DIN ISO 15189.

ApoE4- analyses
Apolipoprotein E (APOE, protein; Apoe, gene) is a protein which plays an important role in lipid homeostasis. The Apoe gene has four polymorphisms: ε2, ε3 and ε4. Studies have suggested that ε4 carriers have an increased risk of late-onset Alzheimer's disease and accelerated brain atrophy and that healthy carriers of the ε4 allele have poorer performance on neuropsychological tests [47]. Furthermore, the effect of an Omega-3 supplement on cognitive performance might be modulated by the presence of ε4 polymorphisms [48]. However, not all studies show an interaction between Apoe status and cognitive performance [49–51]. To elucidate whether Apoe status interacts with cognitive performance in typically developing adolescents consuming LCPUFA supplementation, all participants donate 2 mL saliva for the determination of Apoe status. The analyses of the Apoe gene will be executed by the departments of Molecular Genetics and Clinical Genetics of the Maastricht University Medical Center+, according to the procedure as described by the Duke University Clinical Molecular Diagnostics Laboratory [52]. Shortly, PCR amplification followed by Sanger DNA sequencing is used to determine the genotype of two single nucleotide polymorphisms in the APOE gene Rs7412 and Rs429 358 and the associated APOE genotype. With the participant's and parents' approval (indicated in the informed consent form), samples will be kept for a maximum of 15 years.

Sub studies

All students entering the main study in cohort II were asked to participate in the sleep study. Pupils of four schools (due to logistic constraints) in both cohorts were asked to participate in the eye tracking study. Participation in both sub studies was voluntary and pupils could participate in the main study regardless of participation in the sub studies.

Sub study I: eye tracking
A subsample of participants (n=33) participated in an eye tracking study at baseline, 6 months and 12 months. Eye tracking is a method in which eye movements of participants are recorded and can be used to calculate measures such as number of blinks, number of

fixations and total duration of fixations. Eye movement measures can help reveal underlying cognitive processes such as amount of visual processing, what participants are processing and the mental effort expended. In Food2Learn, eye tracking is used to measure the effect of krill oil supplementation on cognitive processing in the form of visual processing and mental effort (for more information, see online supplementary appendix B).

Sub study II: sleep

In cohort II, a subsample (n=64) is included in a study investigating the relation between increasing Omega-3 fatty status and sleep quality and duration. Montgomery *et al.* [53] showed that DHA supplementation in children led to increased sleep duration and fewer waking episodes per night. Since sufficient sleep is essential for good health, cognitive performance and school performance [54–56], and insufficient sleep is common in adolescents[57,58], this was included as a sub study. Participants wore an ActivPAL3 (Paltechnologies, Glasgow, UK) accelerometer for seven consecutive days at baseline and seven consecutive days after 12 months of intervention. With the help of algorithms, ActivPal data can be used to determine sleep duration and quality. Furthermore, participants filled out a diary in which they noted their bedtime and wake-up time during this week. Finally, they filled out the Adolescent Sleep-Wake Scale and the Adolescent Sleep Hygiene Scale [59].

Power calculation

Originally, the sample size calculation was based on a power of 0.8 and a medium effect size of 0.3 and analysis of variance (ANOVA) as the analysis method. However, new insights led to the conclusion that not ANOVA but a mixed methods analysis would be more appropriate. The power analyses were thus redone for this. Also, the new power calculation took into account that there were multiple measurement moments and that dropout is possible. Multiple calculation in RMASS software with standardised input numbers such as an average effect size of d=0.25 at 6 month follow-up and an equal or 10% higher effect size at 12 months follow-up and a dropout of 25% per measurement moment (and thus a total dropout rate of 43%), an error variation varying from 0.4 to 0.5 and a intercept variation of 0.3 to 0.5 with a slope variation of 0.0 (fixed effects), showed that between 183 and 285 participants at baseline should be sufficient to achieve a power of 0.8. The power calculation for the sleep sub study suggested that 42 students (21 in the placebo group and 21 in the krill oil group) were sufficient. This number of students is based on power calculation with a power of 0.8, $\alpha = 0.05$ and the ability to detect a 20 min difference in sleep duration. For the eye tracking study, no power calculation was executed. As a study looking at the effect of krill oil supplementation on cognitive processing measured with eye tracking had never been executed before, we did not believe that a reliable power calculation would be possible.

Data analysis plan

Data are scored by one of the researchers and both D2 and CST tests are checked a second time by another researcher; 10% of the LDST and digit span are also checked by a second researcher. All data are entered twice and any discrepancies between the two data entries

are checked and corrected. Data analysis will be performed by both the intention-to-treat procedure and related to the blood Omega-3 Index. The main effects of the intervention (krill oil condition in intention-to-treat analyses) and of the Omega-3 Index on the change from baseline with regard to cognitive test scores (primary), scores on questionnaires (secondary) and school grades average (z-score; secondary) will be estimated using mixed models that account for the correlation of repeated measurements within participants. All estimates will be adjusted for drinking behaviour, smoking behaviour, level of parental education, age, sex, body mass index, pubertal status, cohort number and time trends (baseline, 6 months, 12 months) if necessary. Furthermore, moderation analyses for sex and APOE status will be executed and, if necessary, separate group analyses will be executed. Finally, an interaction between treatment condition and time trend will be used to estimate the difference between the groups regarding time trend effects. After these analyses, secondary sensitivity analyses will be executed with treatment adherence instead of intervention condition. These analyses will also be run with the sub scores of the neuropsychological tests as outcome variables.

Study monitoring

The study is monitored by the METC, NWO (Dutch Scientific Organisation) and the director clinical trials research and development of AkerBiomarine. The METC and NWO receive a yearly update. NWO had one site visit. The director clinical trials research and development visits the research site twice every year and checks whether all data are complete and the research is executed according to Good Clinical Practice. The risk of participating in the study is judged to be very low as no severe adverse effects of krill oil are known; however, any adverse effects will be registered and the METC will be notified.

Ethics and dissemination

Any major amendments to the protocol will be submitted to the Medical Ethics Committee for approval. A signed and dated informed consent form is required from all participants signed by the participant and parent(s)/guardian(s). All collected data will be linked to the participant number and only the principal investigators will have access to the non-anonymised data. The principal investigators have access to all trial data. The results of the study will be disseminated on group level to participants and schools. The results of this study will also be presented at international conferences and published in peer-reviewed journals.

Included participants

In total, 288 students provided informed consent. No blood sample was obtained for four participants. One participant suffered severe hyperventilation after the blood sampling and was excluded. Two participants withdrew their consent before the study started. Fifteen participants had an Omega-3 Index of > 5%. Thus, 266 participants were included in the supplementation study; baseline characteristics can be found in Table 4.2.

Table 4.2. Baseline participant characteristics and blood values of selected fatty acids.

Participant characteristics	Mean ± SD or N [%]	N	Fatty acid (%wt/wt of total FA)	Mean ± SD (N=261)
Age (years)	14.10 ± 0.49	266	Omega-3 Index	3.83 ± 0.60
Male/Female	127/139 [47.7/52.3%]	266	DHA 22:6n-3	2.58 ± 0.49
Smoking no/yes[1]	239/26 [90.2/9.8%]	265	EPA 20:5n-3	0.39 ± 0.16
BMI	19.92 ± 3.00	248	AA 20:4n-6	11.19 ± 1.25
Underweight, healthy weight, overweight[2]	28 (10.5%) 182 (68.4%) 39 (14.7%)		ObA 22:5n-6	0.43 ± 0.10
Alcohol units per week[3]	0.46 ± 1.77	266		
LPE[4]	5.07 ± 1.52	248		

[1] Smoking was defined as anybody who indicated to smoke more than 0 cigarettes per week. [2] BMI cut-off points: Boys 14 years : underweight < 16.40, healthy weight 16.41-22.61, overweight > 22.62. Girls 14yr: underweight < 16.87, healthy weight 16.88-23.33, overweight > 23.34. [3] Alcohol units per week was operationalised as number of day per week that alcohol is consumed times units per consumption moment. A unit of alcohol was defined as one standard unit e.g., one wineglass of wine,[4] Highest level of parental education. Parents/guardians were asked to report both parents'/guardians educational level. Socioeconomic status was defined as the highest educational level of the parents/guardians.

Discussion

To the best of our knowledge, Food2Learn is the first intervention study in which the influence of an increase in the Omega-3 Index due to 1 year of krill oil supplementation on cognition, academic achievement and behaviour in typically developing adolescents is assessed. Adolescence is a period of brain maturation in general, and especially of the higher order cognitive skills, as well as social and emotional behaviour. Furthermore, adolescence is a critical phase in the school career of students.

All in all, adolescence is an important life period to study the association between LCPUFAs and cognition/school performance. Food2Learn is also one of the first studies which has recruited the participants based on a low Omega-3 Index and which makes use of a personalized dose adjustment based on individual response to supplementation. The sub studies will shed light on areas that have not received much attention of the LCPUFA research community yet: sleep quantity and quality and visual processing and mental workload. The first students were enrolled in March of 2014; the final data will be collected in August 2016. The first longitudinal results of Food2Learn are expected in the second semester of 2016.

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Appendix A: Cognitive Tests

Letter Digit Substitution Test

The LDST is a measure of speed of information processing. A nine letter/digit key is noted at the top of a page, underneath rows with only letters are printed. Participants are asked to fill out as many corresponding numbers as possible in 60 seconds. The total number of correctly filled out numbers in 60 seconds is used as a measure of speed of information processing. Three version of the LDST are used which have been randomised over schools and over test moments.

D2 Test of Attention

The D2 test is a paper-pencil task to measure selective attention. Participants are presented with 14 rows each consisting of 47 stimuli. Stimuli are the letters d and p with a varying number of dashes (between 1 and 4), below, above, or on both sides. Participants are instructed to only cross out the d with two dashes (d2) and ignore all other stimuli. Participants must process as many stimuli as possible in 20 seconds after which they have to continue with the next row without pausing. The following measures per row and in total are noted after completion: total number of stimuli processed, number of correctly crossed out d2's, number of d2's not crossed out, number of stimuli wrongly crossed out. The main outcome variable is the concentration performance, this is calculated as the total number of correctly cancelled stimuli minus the total number incorrectly cancelled stimuli and is used as a measure of concentration.

Digit Span Forward and Backward

The Digit Span Forward (DSF) is a measure for short-term memory. The Digit Span Backward (DSB) activates the executive component directly and shows the dynamic relationship between passive storage and active manipulation of information held in the memory. The DSF consists of 10 sequences varying in length (3-8 digits, each length twice). Digits are announced by researcher at a rate of 1 digit per second, after completing of the sequence participants write down the sequence. The DSB is similar to the DSF, except for the fact that it consists of 10 digit sequences varying in length (2-7 digits, each length twice) and after completion of the sequence by the researcher, students are asked to write down the sequence backwards. The longest digit span is noted, this is the longest length sequence of digits for which the participant had correctly written down at least one digit sequences. For every test moment a different set of digit sequences is used.

Concept Shifting Test

The Concept Shifting Test (CST) is a measure for cognitive shifting. The test consists of 4 parts. All parts consist of a sheet of paper with 16 small circles that are grouped in one large circle. In task A, the small circles are randomly filled with numbers, in task B the circles are filled with letters and in test C the circles are filled with both. Students are asked

to cross out the items in the correct order (A: 1-2-3-4; B: A-B-C-D; C: 1-A-2-B). Lastly, there is Test Zero, which consists of empty circles, where students are asked to cross out the circles as fast as possible as a measure of basic motor speed. For all tests the time taken to complete and the number of errors is noted. The extra time which is needed to complete task C compared to task A and B is attributed to shifting. Three versions of the CST were used and these versions were randomised over participants and test moments.

Stroop test

The Stroop test provides a measure for cognitive inhibition. The Stroop test consists of three cards containing 40 stimuli each: colour names printed in black (Task 1), coloured patches (Task 2) and colour names printed in congruent or incongruent colour (Task 3). For task 1 participants are asked to read the name out loud, for task 2 participants have to name the colour of the patches, and for task 3 participants have to name the ink colour the word is printed in. Task 3 is a measure of mental flexibility and ability to inhibit a dominant response (reading). An interference score is calculated (Time task 3 – (Time task 1 – Time task 2)) this is a measure for inhibition. Three versions of the Stroop are used and the versions are randomised over participants and test moment.

Appendix B: Eye-tracking

In Food2Learn, eye tracking is used to measure the effect of krill oil supplementation on cognitive processing in the form of perceptual processing and mental effort. Eye movement is recorded with a SMI RED 250 system with a temporal resolution of 250Hz. The eye tracking apparatus is adapted to the participant's individual features by the use of a 9-point calibration and 4-point validation at the start of the procedure. After calibration and validation, participants read aloud a 1-page text and then study this text for 3 minutes. After this participants are asked to recall as much information as possible, which is scored on surface information and detailed information, this is a measure of immediate recall. During the reading and recall, eye movement is continuously recorded. Basic parameters and paragraph related parameters will be calculated. Basic parameters include number of blinks, number of fixations, total duration of fixations, average fixation duration, average dispersion of fixation positions, average saccadic amplitude and average saccadic velocity. Paragraph related parameters will include time spent on each paragraph (number and duration of fixations) time elapsed until first looking a paragraph and amount of times going back to a paragraph.

Chapter 5

Association between blood Omega-3 Index and cognition in typically developing Dutch adolescents

Adapted from: van der Wurff, I., Von Schacky, C., Berge, K., Zeegers, M., Kirschner, P. A., & de Groot, R. (2016). Association between blood omega-3 index and cognition in typically developing Dutch adolescents. Nutrients, 8(1), 13.

Abstract

The impact of omega-3 long-chain polyunsaturated fatty acids (LCPUFAs) on cognition is heavily debated. In the current study, the possible association between omega-3 LCPUFAs in blood and cognitive performance of 266 typically developing adolescents aged 13–15 years is investigated. Baseline data from Food2Learn, a double-blind and randomized placebo controlled krill oil supplementation trial in typically developing adolescents, were used for the current study. The Omega-3 Index was determined with blood from a finger prick. At baseline, participants finished a neuropsychological test battery consisting of the Letter Digit Substitution Test (LDST), D2 test of attention, Digit Span Forward and Backward, Concept Shifting Test and Stroop test. Data were analysed with multiple regression analyses with correction for covariates. The average Omega-3 Index was 3.83% (SD 0.60). Regression analyses between the Omega-3 Index and the outcome parameters revealed significant associations with scores on two of the nine parameters. The association between the Omega-3 Index and both scores on the LDST ($\beta = 0.136$ and $p = .039$), and the number of errors of omission on the D2 ($\beta = 0.053$ and $p = .007$). This is a possible indication for a higher information processing speed and less impulsivity in those with a higher Omega-3 Index.

Introduction

In recent decades, an increasing interest in the health benefits of long-chain polyunsaturated fatty acids (LCPUFAs) has been developed. Aside from its influence on cardiovascular health, it has also attracted attention because of its association with mental health (ADHD, autism, dyslexia) [1], cognitive functioning of healthy individuals [2–4] and cognitive decline in the elderly [5–7]. LCPUFAs and especially docosahexaenoic acid, 22:6n-3 (DHA), and eicosapentaenoic acid 20:5n-3 (EPA) are involved in many aspects of brain functioning such as neuronal membrane fluidity, neurotransmission, signal transduction, brain blood flow, and blood-brain barrier integrity [8,9]. The interest in the possible positive influence of LCPUFAs on brain functioning has led to a large number of both observational and experimental studies (for a review see [10,11]). These studies have, however, mainly focused on either diseased populations of infants, children, adults, and the elderly. Studies in typically developing adolescents are limited. The current study addresses this deficit. Adolescence is a period in which LCPUFAs could be of special importance. During adolescence, the brain, especially the prefrontal cortex, undergoes development which continues until after age 20 [12,13]. The development of the prefrontal cortex is of utmost importance, since this development lays the basis for higher order cognitive functions that have been associated with academic achievements [14]. Moreover, the prefrontal cortex is a brain region especially enriched in DHA [15], and higher DHA intake has been associated with changes in the functional activity of the prefrontal cortex in boys aged 8–10 [16]. To our knowledge, three observational studies looking at the association between fish intake (the most important source of omega-3 LCPUFAs) and cognitive functioning in adolescents have been executed. Kim and colleagues showed that adolescents aged 15 years who regularly consumed fish had significantly better academic performance than peers who never or hardly ever consumed fish [17]. Åberg *et al.* demonstrated that high fish consumption in boys at age 15 was associated with better cognitive performance at age 18 [18]. Lastly, de Groot *et al.* studied 700 Dutch high school students aged 12–18 years. Fish consumption data, end term grades in Dutch, English and Math, scores on the Amsterdam Vocabulary Test, and scores on the Youth Self-Report (a self-reported measure for attention problems) were collected [19]. Results revealed that 13.6% of the Dutch adolescents never ate fish, 63.1% ate fish but too little to meet at least half of the recommended amount, 16.9% reached half of the recommended amount, and 6.4% met national guidelines (fish twice per week). Analysis of the variance showed significant differences between the four fish consumption groups (never, < 1 per week (e.g., 1 time per month), 1 to 2 times per week, > 2 times per week) in vocabulary, and a trend for significance was found for the average end term grade. Significant quadratic associations (u-shape association) between fish consumption, vocabulary ($p = .01$), and average end term grades ($p = .001$) were shown. Higher fish intake was associated with a more advanced vocabulary and an almost significantly higher average end term grade. However, eating more fish than the recommended amount (> 2 fish portions/week) seemed to no longer be beneficial. Overall, the observational studies in adolescents point to a beneficial association between fish intake (the main source of the omega-3 LCPUFAs DHA and EPA) and school grades. Fish consumption is the most important dietary source of LCPUFAs but not the only source [20].

Moreover, there is a large interpersonal variability in the uptake of LCPUFAs [21]. Thus, to be sure about the association between LCPUFAs and cognition in adolescents, measurement of LCPUFAs in blood is needed. Therefore, the main objective of this study is to investigate the association between the Omega-3 Index (EPA + DHA in erythrocytes as percentage of total fatty acids measured [22]) measured in blood and cognitive performance in typically developing adolescents of lower general secondary education (LGSE). Cognition is a very broad term that includes both lower order simple responses and higher order processes. The higher order processes are also called the executive functions, and it is generally agreed that there are three core executive functions namely: (i) inhibition and interference; (ii) working memory; and (iii) cognitive flexibility [23]. These executive functions are used to build higher order skills such as reasoning and problem solving. Therefore, the executive functions are important for academic success and cognitive development [23]. These executive functions are located in the prefrontal cortex, the brain area most in development during adolescence [24]. The cognitive tasks used in the current study are standard tasks of cognitive/executive functioning for this age group and have previously been shown to increase activation of the frontal cortex, the area of the brain associated with the accumulation of DHA [16]. In addition to the main objective, two sub-objectives will be addressed. A number of earlier studies have shown differences in the LCPUFA status between typically developing participants and participants with disorders such as ADHD, autism, and dyslexia [25,26]. However, to our knowledge, whether LCPUFAs are associated with cognition in participants with learning disorders differently than in those without learning disorders has not yet been assessed. The second objective of the current study is, therefore, to explore whether the association between the Omega-3 Index and cognitive ability is different between adolescents with and without learning disorders. Social economic status, often operationalized as educational level, has been shown to be associated with diet quality (i.e., people with a higher SES have better diet quality) [27]. Moreover, in adults, higher social economic status has been found to be associated with higher fish consumption [28]. However, even though it is known that students from lower general secondary education (LSGE) levels have a less healthy diet and lifestyle than students from the higher levels [29], how much fish students from the LSGE consume has, to our knowledge, not yet been assessed. Therefore, the third objective of this study is to explore the fish consumption of second year students of the LSGE.

Materials and methods

Design

This study was part of a larger randomized controlled clinical trial (Food2Learn) studying the influence of omega-3 LCPUFA supplementation on cognitive performance, mental well-being, and academic achievement scores in adolescents attending LGSE. Baseline data of Food2Learn were used to study the association between the Omega-3 Index measured in whole blood and cognition. Food2Learn has been approved by the Medical Ethical Committee of Atrium-Orbis-Zuyd Hospital (now Zuyderland), Heerlen, The Netherlands (NL45803.096.13). Food2Learn has been registered at the Netherlands Trial Register (NTR4082), which is connected to Clinicaltrials.gov (registered as NCT02240264.)

Procedure and participants

Participants were recruited from 17 schools in the south of the Netherlands. For students who wanted to participate, an informed consent form had to be signed by themselves as well as by both parents and/or guardians. After informed consent was received, students underwent a finger prick to measure their Omega-3 Index. Inclusion criteria for Food2Learn were: 1 Omega-3 Index < 5%, as it was expected that omega-3 fatty acid supplementation will be especially beneficial for participants with a very low baseline Omega-3 Index [22]; and 2 attending the second year of LSGE because Richardson *et al.* showed that omega-3 supplementation was especially beneficial in the 20% lowest performing students [30]. Therefore, the choice for students at one of the lowest educational levels in The Netherland's LGSE was made. In the Netherlands, secondary education is divided into three levels: pre-university, higher general secondary education, and LGSE. Approximately 38% of all adolescents follow LGSE [31]. LGSE is further divided up into four sublevels. For this study, students from the highest sublevel, the theoretical learning pathway (TLP), were recruited. Approximately 40% of students attending LGSE are in the TLP [31]. No other inclusion criteria were applied, thus, all second year students of the LSGE with an Omega-3 Index < 5% could participate.

After inclusion, participants underwent a neuropsychological test battery in a small group setting (10 students max) consisting of: Letter Digit Substitution Task (LDST), D2 test of Attention (D2), Digit Span Forward (DSF), and Backward (DSB). In addition, they filled out a number of questionnaires to collect important background information. The tests were led by one researcher via a standardized protocol, while one or two other researchers (depending on the group size) were monitoring to ensure that participants understood the tests and complied with the protocol. Before continuing with the real tests, students received a practice version of the tests, feedback was given, and the students confirmed they understood the tests. After this group test session, all participants filled out a questionnaire individually (data not used in the current study), during which participants were called one by one to perform the individual neuropsychological tests: Stroop Test and Concept Shifting Test (CST) under the supervision of one researcher.

Dependent variable—blood analysis

Whole blood was obtained from a finger prick with an automated lancet and directly transferred to a filter paper (Whatman 903, General Electric, Frankfurt, Germany) pre-treated with a stabilizer. Filter papers were shipped immediately to Omegametrix, Martinsried, Germany for analysis. Whole blood fatty acid compositions were analysed according to the HS-Omega-3 Index methodology [32]. Fatty acid methyl esters are generated by acid transesterification and analysed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Duisburg, Germany) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA, USA) using hydrogen as a carrier gas. Fatty acids are identified by comparison with a standard mixture of fatty acids. Results are given as EPA plus DHA expressed as a percentage of total identified fatty acids after response factor correction.

Since the Omega-3 Index is defined as EPA + DHA in erythrocytes, it was calculated using a sliding correction factor. The coefficient of variation for EPA plus DHA typically is 5%. Analyses are quality-controlled according to DIN ISO 15189.

Independent variables—cognitive measures

Letter Digit Substitution Task
The LDST is a paper-pencil task used to measure speed of information processing [33]. A nine letter/digit key is noted at the top of a page. Below this key, rows of letters are printed, and participants are asked to write the corresponding number in the box underneath the letter as quickly as possible. The number of correctly filled in numbers in 60 s is used as a measure of speed of information processing.

D2 Test of Attention
The D2 test is a paper-pencil task used to measure selective attention [34]. Participants are presented with 14 rows each consisting of 47 stimuli. Stimuli are the letters d and p with a varying number of dashes (between 1 and 4), below, above, or on both sides. Participants are instructed to only cross out the d with two dashes (2 above, 2 below or one on both sides) and ignore all other stimuli. Participants have to process as many stimuli as possible in 20 s per line after which they have to continue with the next row without pausing. The following measures per row and in total are noted after completion: total number of stimuli processed, number of correctly crossed out d2's, number of d2's not crossed out, and number of stimuli wrongly crossed out (thus, non d2). The total number of stimuli processed is used as a measure for information processing speed. The number of target stimuli not crossed out (i.e., errors of omission) and non-target stimuli crossed out (i.e., errors of commission) are used as a measure for inattention and impulsivity, respectively.

Digit Span Forward and Backward
The DSF is a measure for short-term memory that primarily activates the phonological loop. The DSB activates the executive component directly and shows the dynamic relationship between passive storage and active manipulation or transformation of information held in the memory [35] and is thus a measure for working memory (the ability to hold information in the mind and work with it). The DSF consists of 12 sequences of digits varying in length from three to eight digits (each length twice). Digits are announced by the researcher at a rate of approximately one digit per second. After completion of the digit sequence, participants are asked to write down the sequence. The DSB is similar to the DSF, except for the fact that it consists of 12 digit sequences varying in length from two to seven digits (each length twice) and after completion of the sequence by the researcher, students are asked to write down the sequence backwards, starting with the last number announced. The longest sequence of numbers of which participants had correctly written down at least one of the two rows was used as a measure for working memory.

Concept Shifting Task
The CST is a measure for cognitive shifting [36]. Cognitive shifting is the ability to adapt to changes in the environment by switching from one mental set to another [37]. The task consists of four parts. All parts consist of a sheet of paper with 16 small circles grouped in one large circle. In task A, the small circles are randomly filled with numbers, in task B the circles are filled with letters, and in task C the circles are filled with both. Participants are asked to cross out the items in the correct order (A: 1 to 12; B: A to P; C: 1–A–2–B to 8-H). Lastly, there is Task Zero, which consists of empty circles, where participants are

asked to cross out the circles as quickly as possible. Task Zero is administered twice, and the average of these times is used to correct for basic motor speed in the other tasks. For all tasks, the time taken to complete and the number of errors are noted. The average of motor-speed corrected time needed for A and B was subtracted from the motor speed corrected time needed for C and used as a measure for shifting.

Stroop test

The Stroop test provides a measure for cognitive inhibition. Cognitive inhibition is the ability to inhibit an overlearned response in favour of a more unusual one [38]. The Stroop task, as used in Food2Learn, consists of three cards containing 40 stimuli each: colour names printed in black (Task 1), coloured patches (Task 2), and colour names printed in congruent or incongruent colour (Task 3). For Task 1, participants are asked to read the name out loud. For Task 2, participants name the colour of the patches, and for Task 3, participants name the ink colour the word is printed in. Task 3 is a measure of mental flexibility and the ability to inhibit a dominant response (reading). The time needed for Task 2 was subtracted from the time needed for Task 1, the result of this sum was subtracted from the time needed for Task 3. The result of this sum was used as a measure for inhibition.

Additional measures

Students filled out a questionnaire to assess covariates. The following covariates were assessed as they are known to correlate with cognition: BMI (weight/length2, self-reported) [39], sex [40], age[38] , alcohol consumption [41], smoking [42], and parental level of education [43]. Alcohol consumption was assessed with two questions: the number of days/week the participant generally drank alcohol and the number of units the participant drinks on a day that (s)he drinks alcohol. Alcohol consumption was defined as the number of alcohol units/time multiplied by the number of drinking days/week, and the measurement was used as a continuous measure. Smoking was assessed with the question: "How many cigarettes do you smoke per week?". If the participant indicated consuming cigarettes, (s)he was classified as smoker. Parental level of education was filled out by the parents on an ordinal eight-point scale [44]. Parental level of education was defined as the parent with the highest level of education, which is an indication for social economic status [45]. Additionally, fish consumption was assessed with a short, validated, and self-reported questionnaire [3]. Different kinds of fish were divided based on their DHA content: low (fish fingers, prawns, pickled herring, cod, mussels, plaice, tuna, tilapia); medium (trout, raw herring, smoked eel, smoked salmon, canned salmon); and high (smoked herring, herring and tomato sauce, mackerel, canned sardines, salmon). The consumption (never, once a month, two to three times a month, once a week or more than once a week) was used to calculate the fish consumption score. For the low DHA fish 0, 1, 2, 4, 8 points; for the medium DHA fish, 0, 2, 4, 8, 16 points; and for the high DHA fish, 0, 3, 6, 12, 24 points. The score for fish consumption could thus vary between 0 and 48 points. Lastly students were asked to indicate whether they had a disorder which could influence learning (examples were given) and who had made that diagnosis.

Quality control

To ensure the quality of the data, all tests were scored by two independent researchers. Any discrepancies were solved by discussion. Furthermore, in order to prevent typing mistakes, all data were entered in the database twice, after which the two files were automatically compared. Any discrepancies between the two data files were checked and corrected by a third researcher.

Statistical analyses

Data were checked for normality and if necessary, transformation was applied. Data were analysed with linear regression or generalized linear regression (Poisson) for count data and data with a skewed distribution. For all analyses, first, a model with all covariates (i.e., smoking, alcohol consumption units per week), BMI, age, level of parental education, sex, and diagnosis) was built; Model A. In Model B, the Omega-3 Index was added. In a separate analysis, potential moderation between the Omega-3 Index and diagnosis was tested. If results were significant, a sub-group analysis for typically developing adolescents and those who had indicated to have some sort of learning disorder (autism, dyslexia, ADHD, etc.) were executed in the same way (diagnosis was not entered as a covariate). For all analyses, a p-value below .05 was considered to be significant. All analyses were carried out using SPSS statistics version 22.

Results

Participants

A total of 286 students consented to participate in the study. Of these, four dropped out before blood sampling due to personal reasons and 16 had an Omega-3 Index > 5%. Thus, the associations between the Omega-3 Index and cognition of 266 participants (127 boys, 139 girls; M_{age} = 14.1 years) are discussed in this paper. Characteristics of the participants can be found in Table 5.1. Omega-3 Index and LCPUFAs as determined in blood can be found in Table 5.2. Scores on the cognitive tests can be found in Table 5.3. In this sample, 69 participants indicated having a disorder which can impact learning; 14 indicated having Attention Deficit Hyperactivity Disorder (ADHD) or Attention Deficit Disorder (ADD); 45 indicated having dyslexia or dyscalculia; eight reported an autism spectrum disorder; and two indicated a depression. In the total sample of 266 adolescents, 13.8% indicated never consuming fish, 77% indicated eating fish very irregularly (i.e., less than half of the recommend amount of 450 mg DHA + EPA per day), 8.4% consumed at least half of the recommended amount (once a week), and 1% indicated consuming fish more than once a week. There was a significant difference in fish consumption between boys and girls ($p = .024$), with boys consuming more fish than girls. However, this did not result in significant differences in the Omega-3 Index ($p = .561$). The total score on the fish questionnaire correlated significantly with both the Omega-3 Index ($n = 216$, $r = 0.294$, $p < .001$) and DHA concentration ($n = 216$, $r = 0.287$, $p < .001$).

Cognitive performance

Analyses revealed a significant association between the Omega-3 Index and score on the LDST (β= 0.136, p = .039). The addition of the Omega-3 Index to the model increased the r^2 with 0.017 (Table 5.4), i.e., an additional 1.7% of the variance was explained. Furthermore, a significant association between the Omega-3 Index and errors of omission on the D2 was shown (β = 0.053, p = .007) (Table 5.5). The analysis for errors of omission also showed a significant moderator effect (p = .005). No other significant associations between the Omega-3 Index and any of the other cognitive measures were found.

Table 5.1: Participant characteristics.

	All Participants		With diagnosis[1]		Without diagnosis[2]		
	Mean ± SD or N [%]	N	Mean ± SD or N [%]	N	Mean ± SD or N [%]	N	P[5]
Age (years)	14.10 ± 0.49	266	14.26 ± 0.51	69	14.05 ± 0.47	196	0.002
Male/Female	127/139 [47.7/52.3%]	266	36/33 [52.2/47.8%]	69	93/103 [47.5/52.5%]	196	0.499
Smoking no/yes[3]	239/26 [90.2/9.8%]	265	59/10 [85.5/14.5%]	69	179/16 [91.8/8.2%]	195	0.132
BMI	19.92 ± 3.00	248	20.34 ± 3.61	65	19.77 ± 2.74	183	0.187
Alcohol units per week[4]	0.46 ± 1.77	266	0.69 ± 2.85	69	0.39 ± 1.19	196	0.218
LPE	5.07 ± 1.52	248	5.21 ± 1.40	66	5.02 ± 1.56	182	0.371

[1] Diagnosis was defined as a diagnosis possible to influence learning; this was indicated by students themselves and included (but not limited to) dyslexia, dyscalculia, depression, autism and ADHD. [2] Without diagnosis was defined as all students who did not indicate to have a diagnosis. [3] Smoking was defined as anybody who indicated to smoke more than 0 cigarettes per week. [4] Alcohol units per week was operationalized as number of day per week that alcohol is consumed times units per consumption moment. [5] Comparison between those with and those without diagnoses. ANOVA was used for age, BMI, LPE and alcohol units per week, Chi Square for smoking and sex. Significant difference p < .05 are noted in bold.

Table 5.2: Fatty acid blood

Fatty acid (%wt/wt of total FA)	All participants N = 261 Mean ± SD	With diagnosis[1] N=68 Mean ± SD	Without diagnosis[2] N=193 Mean ± SD	P[3]
Omega-3 Index	3.83 ± 0.60	3.79 ± 0.61	3.84 ± 0.60	0.537
DHA 22:6n-3	2.58 ± 0.49	2.56 ± 0.50	2.59 ± 0.49	0.667
EPA 20:5n-3	0.39 ± 0.16	0.38 ± 0.13	0.39 ± 0.16	0.356
AA 20:4n-6	11.19 ± 1.25	11.49 ± 1.34	11.08 ± 1.20	**0.022**
ObA 22:5n-6	0.43 ± 0.10	0.43 ± 0.11	0.44 ± 0.10	0.725

[1] Diagnosis was defined as a diagnosis possible to influence learning; this was indicated by students themselves and included (but not limited to) dyslexia, dyscalculia, depression, autism and ADHD. [2] Without diagnosis was defined as all students who did not indicate to have a diagnosis.[3] Comparison between those with and those without diagnoses. Significant difference p < .05 are noted in bold

Table 5.3: Scores on the cognitive tests

Measures	All participants N = 261 Mean ± SD	With diagnosis[1] N=68 Mean ± SD	Without diagnosis[2] N=196 Mean ± SD	P[3]
LDST (number)	34.47 ± 5.46	33.52 ± 6.51	34.80 ± 5.02	0.094
D2- correct (number)	163.13 ± 22.95	160.04 ± 24.24	164.22 ± 22.45	0.194
D2 – error of omission (number)	11.83 ± 10.73	11.25 ± 8.07	12.04 ± 11.53	0.598
D2 – error of commission (number)	1.31 ± 10.73	1.54 ± 1.96	1.22 ± 1.43	0.161
D2 – Total (number)	417.33 ± 56.46	408.93 ± 55.11	420.29 ± 56.77	0.151
Shifting score (sec)	11.70 ± 6.83	11.69 ± 6.50	11.71 ± 6.96	0.980
Inhibition score (sec)	31.35 ± 8.50	34.85 ± 9.19	30.12 ± 7.91	**0.000**
Digit span Forward (digits)	5.58 ± 0.88	5.26 ± 0.87	5.70 ± 0.85	0.616
Digit Span Backward (digits)	4.56 ± 0.98	4.51 ± 0.93	4.58 ± 1.00	**0.000**

[1] Diagnosis was defined as a diagnosis possible to influence learning; this was indicated by students themselves and included (but not limited to) dyslexia, dyscalculia, depression, autism and ADHD. [2] Without diagnosis was defined as all students who did not indicate to have a diagnosis.[3] Comparison between those with and those without diagnoses. Significant differences ($p < .05$) are noted in bold

Table 5.4: Results of multiple linear regression analyses between Omega-3 Index and score on the LDST in the complete sample.

Predictor variable	B (standardized)[1]	P[2]
Model A ($r^2 = 0.058$, $df = 7$, $p = 0.051$)		
Smoking	0.028	0.679
Alcohol consumption	0.031	0.649
BMI	0.089	0.171
Age	0.047	0.477
Sex	0.177	**0.007**
Highest LPE	-0.056	0.387
Diagnosis	-0.104	0.113
Model B ($r^2 = 0.075$, $df = 8$, $p = 0.019$)		
Smoking	0.031	0.643
Alcohol consumption	0.045	0.500
BMI	0.080	0.218
Age	0.036	0.584
Sex	0.172	**0.008**
Highest LPE[3]	-0.084	0.203
Diagnosis	-0.094	0.147
Omega-3 Index	0.136	**0.039**

[1] Standardized beta refers to how many standard deviations the dependent variable will change per standard deviation change in the predictor variable. Smoking, sex and diagnosis were not standardized as they are dichotomous variables. [2] Significant results ($p < .05$) are printed in bold. [3] LPE= level of parental education.

Table 5.5: Results of generalized linear model analyses between Omega-3 Index and number of error of omission on the D2 test in the complete sample.

Predictor variable	B (standardized)[1]	P[2]
Model A (χ^2 = 47.90 df =7, p < 0.001)		
Smoking	0.066	0.310
Alcohol consumption	0.036	**0.030**
BMI	0.043	**0.026**
Age	0.036	0.068
Sex	-0.047	0.226
Highest LPE	-0.087	**0.000**
Diagnosis	-0.071	0.109
Model B (χ^2 = 51.852, df = 8, p < 0.001)		
Smoking	0.062	0.349
Alcohol consumption	0.030	0.078
BMI	0.043	**0.028**
Age	0.041	0.037
Sex	-0.052	0.181
Highest LPE[3]	-0.077	**0.000**
Diagnosis	-0.083	0.063
Omega-3 Index	-0.053	**0.007**

[1] Standardized beta refers to how many standard deviations the dependent variable will change per standard deviation change in the predictor variable. Smoking, sex and diagnosis were not standardized as they are dichotomous variables. [2] Significant results (p < .05) are printed in bold. [3] LPE= level of parental education.

Table 5.6: Results of generalized linear model analyses between Omega-3 Index and number of error of omission on the D2 test in the typically developing participant sample.

Predictor variable	B (standardized)[1]	P[2]
Model A (χ^2 = 42.11 df = 6, p < 0.001)		
Smoking	0.032	0.685
Alcohol consumption	0.036	0.277
BMI	0.091	**0.000**
Age	0.002	0.914
Sex	-0.136	**0.003**
Highest LPE	-0.085	**0.000**
Model B (χ^2 = 55.642, df = 7, p < 0.001)		
Smoking	0.029	0.714
Alcohol consumption	0.027	0.410
BMI	0.089	**0.000**
Age	0.015	0.515
Sex	-0.138	**0.002**
Highest LPE[3]	-0.067	**0.003**
Omega-3 Index	-0.083	**0.000**

[1] Standardized beta refers to how many standard deviations the dependent variable will change per standard deviation change in the predictor variable. Smoking and sex were not standardized as they are dichotomous variables.[2] Significant results (p < .05) are printed in bold.[3] LPE= level of parental education.

Sub-group analyses

When participants were divided into those without learning disorders and those who indicated having one or more learning disorders, differences between the two groups arose. Those with a diagnosis were significantly older (Table 5.1, $p = .002$, 14.26 ± 0.51, and 14.05 ± 0.47, respectively) than those without a diagnosis. Furthermore, they had a slightly higher AA status (Table 5.2, 11.08 ± 1.20, and 11.49 ± 1.34, respectively). With regard to the test scores, there was a significant difference in average score between those with and those without a diagnoses in inhibition as measured with the Stroop test ($p < .001$, 34.85 ± 9.19, and 30.12 ± 7.91, respectively) and on the digit span backwards ($p < .001$, 5.26 ± 0.869, and 5.7 ± 0.851, respectively).

When a moderation term was added to the regression analysis, a moderation effect was seen for the number of errors of omission ($p = .005$), i.e., the association between the Omega-3 Index and score on the D2—the errors of omission were different between those with and those without diagnosis. Therefore, a separate group regression analysis was executed. This analysis showed no significant associations in adolescents with one or more learning disorders between the Omega-3 Index and errors of omission ($p = .073$). For typically developing adolescents, a significant association between the Omega-3 Index and errors of omission was seen (Table 5.6), students with a higher Omega-3 Index had a lower number of errors.

Discussion

The main aim of this study was to investigate the association between the Omega-3 Index measured in blood and cognitive performance of 14-year-old Dutch adolescents. The Omega-3 Index was significantly associated with information processing operationalized as LDST score. This indicates that a higher Omega-3 Index was associated with better information processing speeds. Every 1% increase in the Omega-3 Index was associated with an increase of 1.23 digits on the LDST. Also, students with a higher Omega-3 Index had fewer errors of omission on the D2 test of attention, an indicator of inattention/impulsivity (i.e., they paid more attention than students with a lower Omega-3 Index). An increase of 1% in the Omega-3 Index was associated with a decrease of 0.94 stimuli forgotten to cross out. Associations with all other cognitive measures were not significant. To our knowledge, this is the first study assessing the association between the Omega-3 Index measured in blood and cognition in typically developing adolescents from the general population. There are a number of observational studies of adolescents that found positive associations between fish consumption, the most important source of omega-3 LCPUFAs, and school grades [17–19]. However, even though cognition/executive functioning and school performance are correlated, they are not equal. School performance depends on additional factors such as time spent on homework [46] and personality [47]. Although we are not aware of studies looking at the association/relationship between LCPUFA status and cognition in adolescents, multiple studies of children are available. For example, Portillo-Reyes *et al.* found an improvement in processing speed in their supplementation study (180 mg DHA and 270 mg EPA per day for three months) of marginally malnourished children age 8–12 years [48]. Parletta *et al.* also found a positive

effect of supplementation (750 mg EPA + DHA per school day for 40 weeks) on a non-verbal cognitive test [49]. However, there are also a number of studies that do not show an association or relationship between omega-3 LCPUFAs and cognition in children [50–52]. Overall, results remain mixed, and a number of possible explanations for these differences have been proposed [52,53]. For example, it has been suggested that an effect of LCPUFAs on cognition might be more likely to be demonstrated in underperforming children and adolescents, as shown in the study of Richardson *et al.* [30]. We tried to address this in the current study by recruiting students from one of the lowest educational levels in the Netherlands. Additionally, it has been suggested that LCPUFAs might only be beneficial in certain periods of life when the brain is developing, the so-called windows of opportunity. We tried to address this by including adolescents because the brain undergoes profound development in adolescence [12]. A number of earlier studies have shown a positive relationship between LCPUFAs and cognition in people with learning disorders [30,54–56]. Therefore, a moderator analysis was executed to check whether the association between the Omega-3 Index and score on the cognitive test was different between those with and those without a learning disorder. If a moderator effect was shown, separate analyses for adolescents who indicated to have a learning disorder versus typically developing adolescents were executed. There was a significant association between the covariate diagnosis and score on errors of commission and on the interference score. The moderator effect could, however, only be shown for errors of omission. Firstly, the number of students with a diagnosis was relatively low (n = 69), which could have led to a reduced statistical power. Secondly, the self-reporting of diagnosis and the fact that many adolescents did not know who made the diagnosis could have led to attenuation of the associations. Thus, the measure of diagnosis might not be accurate. However, when the test scores of those with and those without a diagnosis were compared, students with a diagnosis score lower on the test of interference (Stroop). This would suggest that the assessment of a learning disorder is accurate, since it has been shown before that patients with ADHD and other psychiatric problems have impaired performance on this test [57]. Moreover, the variation in the Omega-3 Index (inherent to our pre-selection of participants with an Omega-3 Index < 5%) was relatively low (SD = 0.61), which makes the appearance of associations less likely. In contrast, even though this spread was also low (SD = 0.60) in typically developing adolescents, a significant association between Omega-3 Index and cognitive measures could be shown. This could be explained by the fact that the number of students with a diagnosis was only 69; therefore, the power to detect an association was not sufficient [58]. The Omega-3 Index (3.83%) in this sample was relatively low (well below the recommended range of 8%–11% [22]). This could be due to the exclusion of participants with a high Omega-3 Index, although if these were included the mean was still only 3.89 (SD 0.67). The low Omega-3 Index in this sample is no surprise since 13.9% of the students did not consume any fish and 77% consumed fish rarely, as measured by the fish consumption questionnaire. This frequency of fish consumption is somewhat lower than the consumption of the adolescents in the sample of de Groot *et al.* [19]. However, the study of de Groot *et al.* was carried out with students in higher general secondary education or pre-university education with a somewhat higher social economic status (assessed by level of education of the parents) than the students in the current study. The number of students that never consume fish is also in line with the results from the National Dutch Consumption Survey, which indicates that 11% of boys and 18% of girls never consume

fish [59]. Similarly in our sample, girls also consumed significantly less fish than boys. However, the number of adolescents who consumed fish twice or more a week was in only 1% in this sample, while in the survey 9% of the boys and 7% of the girls consumed the recommended amount of fish.

The main strength of the current study is that the Omega-3 Index was measured in blood. Furthermore, standardized and validated cognitive tests that assess several aspects of executive functioning were used. The main limitation of the study is that it is an observational study and can, therefore, not prove causality. Also, the variation in the Omega-3 Index was rather small. Furthermore, no Bonferroni correction for multiple statistical was applied, with correction significant results were not present anymore, which weakens the certainty of the associations found. However, the data presented here are part of a large intervention study, which will elucidate the effect of LCPUFA supplementation on cognition, mood, and academic achievement in adolescence. Furthermore, the supplementation study will achieve a higher Omega-3 Index and a larger spread in the Omega-3 Index, which could lead to more significant results (a number of associations were borderline significant).

In conclusion, this study has revealed a positive association between the Omega-3 Index measured in blood from typically developing adolescents and two of the nine cognitive measures. The results of the supplementation study will further elucidate the effect of LCPUFA supplementation on cognition. If a positive effect of LCPUFA supplementation on cognition is shown, this could help improve cognitive functioning and possibly the school performance of adolescents in a relatively inexpensive way.

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Chapter 6

Exploring the association between whole blood long-chain polyunsaturated fatty acid levels and depression and self-esteem in adolescents of lower general secondary education

Adapted from: van der Wurff, I.S.M., Von Schacky, C., Bergeland, T, Leontjevas, R., Zeegers, M.P., Kirschner, P. A., & de Groot, R.H.M. (under revision). Exploring the association between whole blood long-chain polyunsaturated fatty acid levels and depression and self-esteem in adolescents of lower general secondary education.

Abstract

Purpose: Depression is common in adolescents and long-chain polyunsaturated fatty acids (LCPUFA) are suggested to be associated with depression. However, research in adolescents is limited. Furthermore, self-esteem has never been studied in relation to LCPUFA. The objective here was to determine associations of depression and self-esteem with eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), Omega-3 Index (O3I), n-6 docosapentaenoic acid (ObA), n-3 docosapentaenoic acid (DPA), and arachidonic acid (AA) concentrations in blood of adolescents attending lower general secondary education (LGSE).

Methods: Baseline cross-sectional data from a krill oil supplementation trial in adolescents attending LGSE with an O3I < 5% were analysed using regression models built with the BayesFactor package in R. Fatty acids and O3I were determined in blood. Participants filled out the Centre for Epidemiologic Studies Depression (CES-D) scale and the Rosenberg Self-Esteem Scale.

Results: Depression (CES-D ≥ 16) was found in 29.4% of the respondents. Of all fatty acids, we found extreme evidence (BayesFactor [BF] > 100) for a weak negative association between ObA and depression score (-.15; 95% credible interval [CI] -.28 to -.04; $BF_{10} = 252$), and substantial evidence for a weak positive association between ObA and self-esteem score (.09; 95% CI, -0.03 to 0.20; $BF_{10} = 4$).

Conclusion: No evidence was found for associations of DHA, EPA and O3I with depression or self-esteem scores in LGSE adolescents with O3I < 5%. The associations of higher ObA status with lower depression and higher self-esteem scores warrant more research.

Introduction

Depression affects approximately 350 million people worldwide and is especially common in adolescence [1,2]. It has been suggested that 14-25% of adolescents will experience at least one depressive episode before the age of 18 [3]. Adolescent depression has been associated with many adverse short-term outcomes such as poor social relationships, lower concentration and lower school performance [4] and long-term outcomes such as negative physical and mental health outcome and increased risk for adult depression [5–7]. Sub-threshold depression (i.e., depressive symptoms are present but criteria for a major depression are not met) is also common, with a prevalence between 1 and 23% in adolescents [8] and life-time prevalence up to age 17 varying between 5.3 and 12% [9]. Sub-threshold depression has also been related to poorer quality of life, socio-emotional dysfunction, and increased risk for the development of major depression [8,10].

Depression is a complex and heterogeneous disorder and its aetiology is not completely understood. Many factors have been related to the commencement of depression: heritability, childhood adversity, acute stressful live events, and chronic stress among others [11–15]. These factors can lead to changes in brain structures, production of proinflammatory factors, neuroinflammation, alteration of the immune system, dysfunction and elevation of homocysteine levels, blood flow abnormalities, and a decrease in glucose metabolism [16–21].

Long-chain polyunsaturated fatty acids (LCPUFA) have long been suggested to be associated with depression. LCPUFA are important constituents of all cell membranes and are involved in many aspects of brain functioning such as neuronal membrane fluidity, brain blood flow, signal transduction and neurotransmission[22,23].The body cannot produce the LCPUFA *de novo*, they have to be consumed via the diet. Moreover, LCPUFA have been suggested to modulate neuroendocrine factors, have anti-inflammatory properties and may in this way help counteract or reduce the neurobiological changes associated with depression [24]. Multiple observational studies have shown an association between higher fish consumption (source of n3-LCPUFA) and a decreased risk for depression in adults [25–27]. Furthermore, it has been shown that blood and brain eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3)) concentrations are lower in people with a depression compared to non-depressed controls [28–31]. The observational studies, thus, point to a possible role of LCPUFA in depression occurrence. This notion has been supported by a number of intervention trials [32–35], but not all [36,37].

Studies in adolescents either focus on adolescents in treatment for depression[38,39], on dietary intake of LCPUFA [40,41], or on adolescents and adults together [42]. There is only one study, to the best of our knowledge, which investigates LCPUFA status and depressive symptoms in adolescents from the general population [43]. Mamalakis *et al.* showed a negative association between EPA measured in adipose tissue and depression score measured by the Centre for Epidemiologic Studies Depression (CES-D) scale after correction for adiponectin, indicating that a lower EPA status was associated with more depression. Furthermore, they showed a positive association between dihomo-gamma linolenic acid (DGLA, C20:3n-6) in adipose tissue and depression score measured with the Beck Depression Inventory, again after correction for adiponectin, indicating that a lower DGLA status is associated with less depression. Lastly, depression occurs more often in adults and adolescents with a lower socioeconomic status [44,45]. However, the association

between the Omega-3 Index (O3I) measured in blood and depression in adolescents with a lower education level has, to our knowledge, not yet been studied.

The main aim of this study is to estimate the association between the O3I and depression in in adolescents with a lower education level. The O3I is defined as the amount of EPA plus DHA in erythrocytes and is based on a standardized analytical method [46]. The suggested target range for O3I is 8 -11% which is based on the lowest mortality risk in coronary heart disease [47]. However, most people do not reach this target range. Stark and colleagues showed that the majority of countries with blood data on EPA + DHA had low to very low levels, with just a handful of countries or regions in which the target range is achieved (i.e., Norway, Japan, Greenland and Alaska)[48].

Thus, little is known about the association between omega-3 fatty acids and depression in healthy adolescents of a lower educational level. We investigated the association between the O3I, measured in blood and depression in a sample of second year students of lower general secondary education (LGSE). We expected that a higher O3I is associated with less depressive feelings. Secondary, associations between DHA, EPA, DPA, AA, and ObA measured in blood and depression were explored. We focussed on AA, DHA and EPA because they are implicated to play a role in mental health [49], whereas ObA is considered to be a functional shortage indicator of DHA [50] and DPA as the major intermediate between DHA and EPA. Therefore, it was expected that higher DHA, EPA and DPA were associated with lower depression scores and that higher AA and ObA were associated with higher depression scores in adolescents. Furthermore, exploratively, we estimated the associations of the fatty-acids with self-esteem. Self-esteem is a core construct of mental health as it represents a person's overall evaluation of his or her own worth [51]. Low self-esteem has been associated with poor health behaviour and many forms of mental illness, including an association between low self-esteem and major depression in adolescents [51–53] and even negative long term physical and mental health outcomes[52,54]. However, to our knowledge, studies are lacking that explore an association between fatty acids and self-esteem.

Methods

This study used baseline data from a large double-blind, randomised, placebo controlled intervention study (Food2Learn) to study the effect of one year of krill oil supplementation on cognitive performance, academic achievement, and mental well-being of students in the second year of LGSE. Full details about the cohort and measurements have been reported previously[55]. Food2Learn was approved by the Medical Ethical Committee of Atrium-Orbis-Zuyd Hospital Heerlen, the Netherlands (NL45803.096.13). Each participant as well as parent(s) and/or guardian(s) provided written informed consent for participation in the study. Food2Learn is registered at The Netherlands Trial Register (NTR4082) and at Clinicaltrials.gov (NCT02240264).

Data collection

Students of 17 schools offering LGSE in the Netherlands participated in Food2Learn. In the Netherlands, secondary education is divided into three levels: pre-university, higher

general and lower general secondary education (LGSE). Approximately 38% of all adolescents in the Netherlands attend LGSE, which is again subdivided into four levels; for the current study, students from the highest sublevel, the theoretical learning pathway (TLP), were recruited. Approximately 40% of students attending LGSE are in the TLP [56]. We approached students in the second year of the LGSE-TLP, who are between 13 and 15 years of age. LGSE-TLP students at participating schools were approached in a classroom setting and the study was explained orally by a research assistant who also used video material to support the presentation. Students then received an information letter and were asked to discuss their participation with their parent(s) and/or guardian(s). If students wanted to participate, they had to hand in an informed consent form signed by both themselves and their parent(s) and/or guardian(s). After informed consent was received the student received a finger prick to determine the O3I. Only students with a low O3I (defined as O3I < 5%) could participate in the study as this was a selection criterion of the main Food2Learn study. At baseline, participants filled out a number of questionnaires to determine mood status and self-esteem and to collect information with respect to a number of covariates.

Blood analyses

A finger prick was administered by a trained researcher or research assistant with an automated one-time use lancet; blood was collected on specially prepared filter paper. Whole blood acid compositions were analysed according to the HS-Omega-3 Index® methodology as described previously [47,57]. Fatty acid methyl esters were generated by acid transesterification and analysed by gas chromatography using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids. Results are given as the O3I, which is EPA + DHA expressed as a percentage of total identified fatty acids after response factor correction and a correction for the fact that whole blood was used instead of erythrocytes. Typically, the coefficient of variation for EPA plus DHA is 5%. Analyses are quality-controlled according to DIN ISO. Furthermore, 26 other fatty acids were determined. We focussed on AA, DHA, EPA, ObA, and DPA.

Questionnaires

Centre for Epidemiologic Studies Depression Scale

Depressive feelings were assessed with the Dutch version of the CES-D scale [58], one of the most commonly used screening tools for depression that has been shown to be able to distinguish between depressed and non-depressed individuals in both clinical and community populations [58]. The questionnaire consists of 20 questions assessing whether six symptoms of depression were experienced by the participant in the last week. The CES-D has shown a high internal reliability in adolescents (α=.88) [59]. The measured symptoms include depressed mood, guilt/worthlessness, helplessness/hopelessness, psychomotor retardation, loss of appetite, and sleep disturbance. For each question, the participants has to indicate whether a symptom occurred seldom or never (< 1 day), sometimes or a few times (1–2 days), often (3–4 days), or most of the time/always (5–7 days). Each answer is scored as 0, 1, 2, or 3 respectively and a total sum score is calculated. The score of the CES-D can thus vary between 0 and 60, with a higher score indicating

more depressive feelings. It has been suggested that depression should be considered as a continuum of increasing severity [9]. Therefore, the CES-D score was taken as a continuous variable in the current study. A CES-D score of 16 or higher is, in general, accepted as an indication of depression [60], although some have suggested that a higher cut-off point might be more appropriate in adolescents i.e., a score of 22 or higher [61]. We used a score of > 16 in descriptive analyses to indicate the number of adolescents with possible depression in our sample.

The Rosenberg Self-Esteem Scale

Self-esteem was measured with the Dutch version of the Rosenberg Self-Esteem Scale (RSE). The internal reliability for the RSE has been found to be high in adolescents (α=.88) [62,63] and construct validity with other measures of self-esteem has been shown [63]. The RSE consists of 10 questions scored on a 4-point response system (from strongly disagree =0 to strongly agree = 3) requiring participants to indicate their level of agreement with a series of statements about themselves. The total score of the RSE can vary between 0 and 30, with a higher score indicating higher self-esteem. The RSE score was also taken as a continuous variable.

Additional measures

Students filled out a questionnaire to assess covariates. The following variables were assessed as they are known to be associated with depression: BMI (weight/length^2, self-reported) [64,65], sex [66,67], age in years [68], alcohol consumption (number of days per week that alcohol was consumed times units consumed per consumption moment) [69], smoking (yes/no, yes if the participant had indicated to smoke more than 0 cigarettes per week) [70] , parental level of education (subdivided in 8 levels from primary school to university) [71], pubertal status conform according to the categorisation suggested by Petersen *et al.* (subdivided in 5 levels from prepubertal to postpubertal) [72] (association between pubertal status and depression [73,74]), and diagnosis which might influence learning (yes/no, e.g. autism, dyslexia, ADHD) [75,76].

Data analyses

Twelve participants had a maximum of two missing data points for the CES-D, these missing points were imputed by the person average score of the other CES-D items (if necessary reversed scored) as explained by Bono *et al.* [77]. For the RSE the same procedure was used. All continuous variables (all fatty acids and BMI) were standardized.

We estimated the effect sizes for the association between the fatty acids and depression score/self-esteem using Bayesian statistical approach. This approach makes it possible to incorporate prior knowledge about relationships (called a prior), and, using a Bayes factor, to compare different models including the model representing the null hypotheses. Bayes Factor$_{10}$ (BF) indicates how many times the alternative hypothesis (H_1, e.g., DHA is related to depression) is more likely compared to the null hypothesis (H_0, e.g., DHA is not related to depression) or another hypothesis. It is, in general, accepted that a BF_{10} between 0.33 and 3 indicates that data do not favour either H_0 or H_1, the data are insensitive. A BF_{10} for the comparison of H_1 to H_0 of less than 0.33 indicates evidence in favour of the H_0 and a

BF_{10} of more than 3 indicates evidence for the H_1 [78]. We considered the degree of evidence in favour of a model in accordance with Jeffrey's classification: BF_{10} of 1 for no evidence; 1 to 3 (resp. when BF_{10} is 1/3 to 1 there is evidence in favour of null hypothesis) for anecdotal evidence in favour of alternative hypothesis; 3 to 10 (1/10 to 1/3) for substantial evidence; 10 to 30 (1/30 to 1/10) for strong evidence; 30-100 for very strong evidence, (1/100 to 1/30); > 100 (< 1/100) for extreme evidence [79]. Next to estimating BF_{10} indices, we also used a top-down approach in which the full model is compared with a model with all factors but one (i.e., inclusion of all fatty acids but DHA). BF_{omit} indicates whether removing the factor from the model is deleterious, it is suggested that a $BF_{omit} < 3$ indicates that the factor is beneficial for the model and a $BF_{omit} > 3$ that removing the factor is better for the model [80]. Because of the same denominator, BF_{10} indices of different models with all possible combinations of covariates and predictors can be compared. For estimated effect-sizes, a 95% credibility interval is provided that describes intervals where the parameters fall within a 95% probability given the observed data.

Analyses were executed in the R statistical environment (R studio version 3.3.2) with the package BayesFactor (version 0.9.12-2). We used the standard settings of the package: the package considers the regression parameters to be distributed normally around zero, with negative effects and positive effects being equally likely, and smaller effects being more likely than larger effects. For the descriptive comparisons of characteristics between those with and without depression, ANOVA and Chi-square analyses were carried out. To explore the associations between each respective fatty acid or the O3I and depression respectively self-esteem, regression models were built predicting CES-D and RSE from the fatty acids.

Next to the analyses with each fatty acid as individual predictor, two regression models were additionally built (one for depression and one for self-esteem) with all fatty acids as predictors in one analysis, which allows adjustment of the effect of a fatty acid for the effects of other fatty acids. All regression models were corrected for covariates ,which were selected by comparing BFs of regression models with all possible combinations of covariates and the fatty acid. For estimation of the effect size 10.000 Monte Carlo iterations were run.

Results

In total, 286 students provided informed consent. For three participants no blood sample was obtained. Additionally, one participant was excluded because of severe hyperventilation after blood sampling , one participant withdrew consent before the study started, and one student quit during the first test session and was therefore excluded. Fourteen participants had an O3I > 5% and were therefore, based on the protocol of the main Food2Learn study, excluded. Nine additional students did have an O3I above 5% but were not excluded before the start of the study, they were however excluded from analyses.

In total 257 students started the study, however baseline data on depression were available for 252 participants and self-esteems scores for 255 participants. Characteristics of all participants are presented in Table 6.1. A total of 74 (29.4%) students (with CES-D score available) scored ≥16 on the CES-D indicative of a depression. When using the stricter criterion of ≥22, a total of 43 students (17.1%) could be classified as possibly

having depression. Comparing those with a depression (CES-D ≥ 16) and those without a depression (CES-D ≤ 15) there was extreme evidence (BF_{10} = 1.1E+4) that girls had more often a depression (42.3% with CES-D ≥ 16) than boys (15.6%). There was substantial evidence (BF = 5.64) that those with a depression had a higher BMI (M = 20.84, SD = 3.10) than those without depression (M = 19.64, SD = 2.93) and extreme evidence (BF_{10} = 3.7E+3) that those with a depression were further in puberty (80% were advanced or postpubertal) than those without depression (51.7% advanced or postpubertal). There was extreme evidence

(BF_{10} = 1.7E+28) that self-esteem was significantly lower in those with a depression (M = 15.4, SD = 5.64) compared to those without (M = 23.88, SD = 3.96). Also when looking at the association between self-esteem and depression, their association was strong (self-esteem regressed on depression, regression coefficient = -.77 [95% credibility interval -.85 to -.68]; BF_{10} = 7.76E43).

When comparing models predicting CES-D from potential confounders (namely smoking, alcohol, age, sex, BMI, LPE, pubertal status and diagnosis), the models with the highest BF_{10} included the variables smoking, sex and BMI. We, therefore, included these variables as covariates in the main analyses. For RSE, the best models contained smoking, sex and diagnosis, or smoking and sex. We, therefore, only included smoking, sex and diagnosis as covariates in the analyses for RSE. Excluding participants with missing data, data of 230 participants was available for CESD and data of 249 participants for RSE.

Table 6.1: Participants' characteristics

	N	Mean	SD	Fatty acid (%wt/wt of total FA, except for O3I)	N	Mean	SD
Age (years)	257	14.11	0.50	Omega-3 Index	257	3.78	0.56
Male/Female	257	124/133 [48.2/51.8%]	-	DHA 22:6n-3	257	2.55	0.46
Smoking no/yes[1]	255	231/24 [89.9/9.3%]	-	EPA 20:5n-3	257	0.38	0.15
BMI	240	19.98	3.00	DPA 22:5n-3	257	1.22	0.19
Alcohol units per week[2]	256	0.47	1.80	AA 20:4n-6	257	11.15	1.27
LPE	240	5.05	1.5	ObA 22:5n-6	257	0.44	0.10

O3I = Omega-3 Index, LPE = level of parental education

[1] Smoking was defined as anybody who indicated to smoke more than 0 cigarettes per week.

[2] Alcohol units per week was operationalized as number of day per week that alcohol is consumed times units per consumption moment.

Individual models that predicted CES-D scores from a fatty acid showed negative regression coefficients with 95% credibility intervals without zero for AA (regression coefficient = -.13 [95% credibility interval -.25 to -.02]; BF_{10} = 2.65), DPA (-.13 [-.24 to -.02]; BF_{10} = 1.01), and ObA (-.16 [-.28 to -.04]; BF_{10} = 251.95) (see Table 6.2). This indicates that higher levels of these fatty acids in blood are associated with lower depression scores. However, BF_{10} indicated evidence in favour of the association for ObA only (extreme evidence, BF_{10} > 100). The BF_{10} for O3I and DHA were below .33, which indicates that there is more evidence for the H_0 i.e., no association between O3I and DHA and depression. When all fatty acids were taken in one model as predictors of CES-D (BF_{10} for the model = 1.2E+5), the regression coefficient for ObA became smaller and its 95%

CI contained a zero score (-.09. [-.22 to .03]). The regression coefficient of DPA was almost identical to that in the individual model -.11 [-.25 to .03]), but 95% CI for DPA, as well as for all other fatty acids, contained zero. The BF_{omit} indicated that the model will be improved when DHA was omitted ($BF_{omit} > 3$). For other acids, BF_{omit} indices were not higher than 3 or lower than 0.33, thus removing them would not improve or worsen the model.

Table 6.2: Bayesian analyses, fatty acids as predictor for score on the CESD, both separate and combined in one model.

One fatty acid				All fatty acids in 1 model			
Predictor fatty acid	Regression coefficient	95% credibility interval[1]	BF_{10} [2]		Regression coefficient	95% credibility interval	BF_{omit}[3]
Omega-3 Index	-0.016	-0.14;0.11	0.14				
DHA	-0.04	-0.16;0.08	0.15	DHA	-0.02	-0.15;0.11	3.12
EPA	0.06	-0.06;0.18	0.34	EPA	0.09	-0.05;0.22	1.42
AA	-0.13	-0.25;-0.02	2.65	AA	-0.03	-0.18;0.11	2.79
DPA	-0.13	-0.24;-0.02	1.01	DPA	-0.11	-0.25;0.03	0.84
ObA	-0.16	-0.28;-0.04	251.95	ObA	-0.09	-0.22;0.03	1.20

One fatty acid model, is a model in which only one predictor of interest is entered (Omega-3 Index, DHA, EPA, AA, DPA, or ObA). All fatty acids model is a model in which all fatty acids (DHA, EPA, AA, DPA, and ObA) are entered, i.e. for example the association between DHA and self-esteem is corrected for the other fatty acids (EPA, AA, DPA, ObA). All analyses were adjusted for BMI, smoking (yes/no), and sex.
[1] Credibility intervals are analogous to confidence intervals in traditional statistics. [2] BF_{10} refers to the evidence for the model with the specific fatty acid compared to a model with only covariates.[3] BF_{omit} indicates whether the model improves with the omission of that specific fatty acid. BF_{omit} numbers above 3 indicate that keeping the variable in the model is not preferable.

Table 6.3: Bayesian analyses, fatty acids as predictor for score on the RSE, both separate and combined in one model.

One fatty acid				All fatty acids in 1 model			
Predictor fatty acid	Regression coefficient	95% credibility interval[1]	BF_{10}[2]		Regression coefficient	95% credibility interval	BF_{omit}[3]
Omega-3 Index	-0.05	-0.18;0.08	0.18				
DHA	-0.03	-0.15;0.10	0.15	DHA	0.001	-0.13;0.13	3.12
EPA	-0.09	-0.20;0.03	0.32	EPA	-0.08	-0.21;0.05	1.59
AA	0.008	-0.12;0.10	0.14	AA	-0.06	-0.20;0.08	2.04
DPA	-0.02	-0.09;0.14	0.16	DPA	0.07	-0.07;0.20	1.76
ObA	0.09	-0.03;0.20	4.15	ObA	0.07	-0.05;0.20	1.63

One fatty acid model, is a model in which only one predictor of interest is entered (Omega-3 Index, DHA, EPA, AA, DPA or ObA). All fatty acids model is a model in which all fatty acids (DHA, EPA, AA, DPA and ObA) are entered, i.e., for example the association between DHA and self-esteem is corrected for the other fatty acids (EPA, AA, DPA, ObA). All analyses were adjusted for smoking (yes/no), sex and diagnosis (yes/no).
[1] Credibility intervals are analogous to confidence intervals in traditional statistics. [2] BF_{10} refers to the evidence for the model with the specific fatty acid compared to a model with only covariates.[3] BF_{omit} indicates whether the model improves with the omission of that specific fatty acid. BF_{omit} above 3 indicate that keeping the variable in the model is not preferable.

Bayesian analyses for regressions that predicted the RSE score showed no 95% CIs that did not include zero (see Table 6.3). When looking at the Bayes factors, there was only substantial evidence for an association between ObA and RSE ($BF_{10} = 4.15$), for all other

fatty acids and the O3I, BF_{10} was below 0.33 which indicates more evidence for the H_0, i.e., no association between fatty acids and self-esteem. BF_{omit} indices for DHA exceed the threshold values (> 3), which indicates that removing this from the model would improve the model that contains all fatty acids.

Discussion

Analyses showed Bayes factors that indicated extreme evidence for a weak negative association between ObA levels and depression score, and substantial evidence for a weak positive association between ObA and self-esteem score. In other words, more ObA measured in blood corresponded with lower depression and higher self-esteem scores. For DHA and O3I, our data provided evidence for absence of associations with both depression and self-esteem scores. For DPA and AA, there was anecdotal evidence for an association with depression score and evidence for absence of an association with self-esteem score. For EPA, the evidence for the association with depression score was indecisive and there was evidence for no association with self-esteem score. When the associations of fatty acids were adjusted for each other effects, all effect-sizes for tested associations decreased and 95% credible intervals for regression coefficients contained a zero-score.

The evidence for the relation of depression score with DHA, EPA and O3I in the current study was in favour of the null hypothesis (i.e., no association) and the standardized effect sizes were very small. Earlier studies in adolescents that used the frequentist hypothesis testing showed non-significant associations between fatty acids and depression score [41,81]. However the disadvantage of the frequentist hypothesis testing approach is that a non-significant result can either imply that there is evidence for the null hypothesis (i.e., there is no association) or the data are insensitive in distinguishing the theory from the null hypothesis (i.e., nothing follows from the data) [82]. Bayesian analyses provide either evidence for the null hypothesis, evidence for the alternative hypothesis or indicate that evidence is insensitive. With the Bayesian analyses, we showed that there is evidence for an absent association of depression with respectively DHA, EPA, and O3I (i.e., H_0 is more likely than H_1).

Our findings are in contrast to an earlier studies in adolescents from the general population in which an negative association between depression measured with the Beck Depression Inventory (BDI) and EPA in adipose tissue of adolescents was found (i.e., more EPA, lower depression score; analyse was controlled for other fatty acids but no correction for multiple testing) [43]. However, some researchers have suggested that the CES-D, like used in our study, is better at discriminating depression at lower levels than the BDI and the CES-D, thus, might be a better measure of depression in the general population [83,84]. Furthermore, an association between adipose fatty acids and brain fatty acids has, to our knowledge, not been established, while the association between blood levels and brain levels has been established in animal studies [85]. Blood levels of fatty acids might, therefore, be a better measure for assessing the association between fatty acids and depressive feelings.

It is remarkable that the negative associations of ObA with depression and self-esteem score became less convincing and the relationship between ObA and depression score

became weaker after correction for other fatty acids. We are not aware of earlier studies reporting the association between ObA and depression score, although studies looking at other omega-6 fatty acids mostly showed positive associations (i.e., more omega-6 fatty acids, higher depression scores) [86,87]. ObA can, in the case of DHA deficiency, take the place of DHA in the brain [88]. Possibly the replacement of DHA by ObA does not changes the brain functionality and therefore a negative association between ObA and depression score in the case of DHA deficiency can be found. This surprising negative association between ObA and depression score does merit more research.

Our data provided evidence for absence of associations of depression score with DHA, EPA and O3I in this adolescent sample, while meta-analyses that included studies in adults have shown that higher fish consumption, higher LCPUFA consumption or LCPUFA supplementation are associated with less depressive feelings [49,89–91]. One of the possible explanations for an absent relationship in the current study could be that the design of this trial led us to preselect participants with an O3I < 5%. In other fatty acid studies, it has been suggested that positive associations with O3I are only visible at higher O3I levels (i.e., 8% and up). For example, in cardiovascular health, an O3I of > 8% is associated with the greatest risk reduction and the study of Markhus *et al.* showed a linear relation between O3I and depressive symptoms during pregnancy only when the O3I was > 5.1% [47,92]. Such O3I values seem rare in the specific population of students of LGSE as we only had to exclude 23 out of 286 student for having an O3I > 5%, with the highest O3I being around 6.09%. This means that none of the adolescents reached the target O3I of 8-11% [47], and only 8% had a O3I > 5%. So, such a low O3I seems to be reality in this specific target group. Furthermore, whenever an effect of omega-3 fatty acids in trials is found it is mostly a small effect, which was also true in this sample where the effect sizes ranged between 0.001 and 0.15. Such a small effect could easily be offset by other environmental factors. This might especially be the case in adolescence, a period of life characterised by profound changes in brain development, but also social and emotional behaviour [93,94]. Another important issue to consider is that most of the studies looking at depression that find significant associations find very small effect sizes and do not use Bayesian statistics. In the current study we did find some regression coefficients with 95% CI that did not include 0 (e.g. AA and DPA predicting depression score). 'Frequentist' regressions with SPSS software showed comparable regression coefficients that were significant (not shown). However, Bayesian analyses did not reveal evidence for these relationships. This could also be the case for other studies that found significant associations.

This study showed that depressive feelings are common in adolescents of LGSE; in our sample 29.4% of the adolescents scored 16 or higher on the CES-D which is indicative for a depression. When we used a stricter cut-off point (as some suggest for adolescents) of 22 or higher 17.1% had scores indicative of depression. Although the percentage of adolescents with depression in this sample seems high, it is similar to percentages found in earlier studies in adolescents. Munhoz *et al.*, for example, showed that 17% of the Brazilian adolescents had scores indicative for major depression [95]. Grant and colleagues (2015) also showed numbers comparable to the current study with 22% of the boys and 34.4% of girls showing depressive symptoms [96], and Mamalakis *et al.* (2006) observed a similar mean CES-D score (mean of 14.9 compared to 12.2 in our sample) [43]. Furthermore, it has long been known that, from age 13-14 onward, there is a female preponderance of

depression [97]. We also showed that girls in this sample had a higher incidence of depression than boys. In the current study, 42.3% of the girls had an indication for depression while, for boys, this was 15.6%.

There are a multitude of reasons as to why depression is so common in this sample of adolescents with a lower education level. Both genetic and environmental factors influence the susceptibility of developing depression [98]. Environmental factors which have been associated with depressive feelings are among others early childhood adversities (e.g., abuse, chaotic households, loss of a parent) and negative life events (e.g., parental divorce, financial problems or housing problems) which are more common in a population with a lower educational level [12,99–101] than in a population with a higher educational level. Furthermore adolescents and especially adolescents from lower education levels often have a less favourable lifestyle [102], a suboptimal diet, high intakes of processed foods (e.g., cake, cookies, crisps) and lower than recommended intake of many vitamins and minerals [103–105]. Childhood adversities, negative life events, unhealthy diet, and vitamin and mineral deficiency all have been associated with depression [99,106–110].

This observational study has a number of limitations. The low O3I of the participants and low spread in the O3I could have led to low statistical power. However, as we explained above, the low O3I seems to be the reality in this specific target group. Moreover this was a cross-sectional study and can thus not be used for proving causal relationships, experimental studies are needed for more insight in the effects of fatty acids in depression and self-esteem in this specific target group.

One of the main strengths of the current study is the use of finger prick blood samples to determine the fatty acids status of the adolescents. Moreover, the population was rather homogenous and this study is one of the first specifically focusing on adolescents of a lower education level. Furthermore, we used the Bayesian approach that, in contrast to the frequentist approach, can be used to provide evidence for the null hypothesis and to compare different models without issues concerning multiple testing.

To summarise, our data in Dutch LGSE adolescents with a low O3I in blood revealed high depression rates and provided extreme evidence for a weak association between ObA levels and depression score (more ObA, lower depression score) and substantial evidence for a weak association between ObA and self-esteem score (more ObA, higher self-esteem). This surprising result calls for more research into the role of ObA in depression. Results of the intervention study Food2Learn should shed light on the effect of krill oil supplementation (high in omega-3 fatty acids) on depressive feelings in adolescents of the LGSE.

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Chapter 7

Effect of 1 year krill oil supplementation on cognitive achievement of Dutch adolescents: a double-blind randomized controlled trial.

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Abstract

Background: Long-chain polyunsaturated fatty acids (LCPUFA) are important for brain development and function, maybe especially adolescence. Observational studies demonstrated an association between fish consumption (source of LCPUFA) and cognition in adolescents, but intervention trials are lacking.

Objective: To investigate the effect of one year krill oil (source of LCPUFA) supplementation on cognitive performance of adolescents with a low Omega-3 Index (O3I).

Design: Double-blind, randomized, placebo controlled krill oil supplementation trial with repeated measurements (baseline (T0), 3 months (T1), 6 months (T2) and 12 months (T3)) in adolescents attending lower the second year of general secondary education (LGSE). Participants were randomized to 400mg eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from krill oil per day in cohort I or placebo, and 800mg EPA + DHA per day in cohort II or placebo. O3I was monitored by a finger prick at all time points. At T0, T2 and T3 participants executed a neurocognitive test battery. Covariate corrected mixed models were run with either condition (krill or placebo) or O3I as predictors.

Results: Krill oil supplementation lead to a small but significant increase in mean O3I, but few participants increased their levels as intended. There was no significant effect of supplementation on any of the neurocognitive tests, nor was there a relationship between O3I and neurocognitive test scores.

Conclusions: One year of krill oil supplementation led to an increase in O3I, but did not improve neurocognitive test scores. The increase in O3I was in most participant small, probably due to non-adherence. Possibly the increase in O3I was too small to demonstrate an effect. More research on the influence of LCPUFAs on cognition in adolescents is needed.

Introduction

The influences of long-chain polyunsaturated fatty acids (LCPUFAs) on health and cognition have been studied extensively. Studies have focused on varying outcomes ranging from cardiovascular health, diabetes, cognitive decline and cognitive development [1–4]. The extensive research into LCPUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is not surprising as they are involved in many processes such as neuronal membrane fluidity, signal transduction, blood-brain barrier integrity and inflammation [5,6]. The possible positive influence of LCPUFAs on brain functioning has led to many observational and experimental studies. However, these studies have mostly focused on infants, pregnant women, children and elderly, while LCPUFAs might be especially important in adolescents.

Adolescence is characterized by profound brain development, with especially the prefrontal cortex continuing to mature into the late twenties [7,8]. In this period of brain development, the basis is laid for executive functions (e.g., shifting, updating and short-term memory), among others. Optimal development of the prefrontal cortex is very important as the executive functions have been related to academic achievements [9]. Considering brain development during adolescence, it is important to take the role of omega-3 LCPUFA into account, especially since the omega-3 LCPUFA DHA and EPA are important structural components of neuronal cell membranes and influence membrane fluidity and signal transduction [6]. Moreover, the prefrontal cortex is very rich in DHA [10] and an earlier study with DHA supplementation in children aged 8-10 years showed higher functional activity in the prefrontal cortex [11].

Although a positive effect of LCPUFA on adolescent brain and cognition seems likely based on biological mechanisms, LCPUFA supplementation studies on cognition in healthy adolescents are, to the best of our knowledge, lacking. There are three observational studies in adolescents that show an association between fish consumption (important source of LCPUFA) and cognition and/or school performance [12–14].

All in all, observational studies and biological mechanisms point to a possible positive relationship between LCPUFAs and cognitive performance in adolescents. We aimed to investigate the causal relationship between LCPUFA supplementation from krill oil and cognitive performance in adolescents in their second year of lower general secondary education (LGSE) who have a low Omega-3 Index (O3I).

Methods and participants

Food2Learn was approved by the Medical Ethical Committee of Atrium-Orbis-Zuyd Hospital Heerlen, the Netherlands (NL45803.096.13) and is registered at both the Netherlands Trial Register (NTR4082) and at Clinicaltrials.gov (NCT02240264). Below is a brief description of the procedure, design and methods, more details have been reported previously [15].

Study design

Food2Learn is a double-blind, randomized, placebo controlled intervention trial with repeated measurements (baseline (T0), 3 months (T1), 6 months (T2) and 12 months (T3)) to study the effect of krill oil supplementation on among others cognitive performance of second year high school students in the Netherlands. The goal was to increase the Omega-3 Index (O3I) of participants in the active treatment group to 8-11%. This target O3I was an estimate based on the O3I associated with the lowest mortality risk in coronary heart disease [16].

Recruitment procedure

Seventeen schools in the south of the Netherlands participated in the study. For the current study, all second year students of the LGSE theoretical learning pathway (TLP) at the participating schools were approached in a classroom setting to participate. A research assistant explained the study and handed out an information letter and informed consent forms. The students were asked to discuss the information with their parent(s) and/or guardian(s). When they wanted to participate, the informed consent form had to be signed by the adolescent as well as by both parent(s) and/or guardian(s). When the informed consent form was received, a finger prick was executed to determine the O3I in blood. As it was expected that any effect of krill oil supplementation would be more likely in participants with a low O3I, only those students with an O3I < 5% were eligible to participate in the study. Together with the informed consent, parents and students filled out a short questionnaire with personal contact information, weight, height and level of parental education.

Participants

All students attending LGSE TLP level at the participating school with an O3I < 5 % were eligible to participate. Exclusion criteria were an O3I > 5%, an allergy to fish or shell fish, or hemophilia. A total of 286 participants gave informed consent of whom 267 were randomized. Participants were recruited in two cohorts, cohort I from November 2013 to February 2014 and cohort II from November 2014 to February 2015. Data were collected from February 2014 to April 2015 and from February 2015 to April 2016, respectively.

Randomization and blinding

All participants received a participant number upon entering the study and were allocated to a condition by an independent researcher. Participants were stratified by sex and an equal number of participants were allocated to krill and placebo condition; group allocation sequence was computer generated. Both researchers at the site and participants and parents were blind to treatment condition. The packing of the boxes and placebo and krill oil capsules were visually exactly the same. Furthermore, capsules were coloured black and a vanilla odour was added to ensure blinding.

Intervention

The intervention started after baseline neuropsychological testing. In cohort I, participants were instructed to take four capsules (krill or placebo) daily with their dinner, the fattest meal of the day [17]. Four krill oil capsules contained 260mg EPA and 140mg DHA which was nearly the recommended amount of 450mg DHA + EPA per day as suggested by the Dutch Health Council [18]. After three months of supplementation a personalized dose adjustment was planned to account for interpersonal difference in metabolism. However, at the three month point, no participant achieved the target O3I of 8-11%. Therefore, all participants were instructed to increase the daily dosage to eight capsules per day (both krill and placebo). Furthermore, it was decided that Cohort II would immediately start with eight capsules. Eight capsules of krill oil contained 520mg EPA and 280mg DHA per day.

Data collection

Blood analyses
Whole blood was obtained from a finger prick at T0, T1, T2 and T3 with an automated one-time use lancet and directly transferred to a filter paper (Whatman 903, General Electric, Frankfurt, Germany) pre-treated with a stabilizer. Filter papers were shipped immediately to Omegametrix, Martinsried, Germany for analysis. Whole blood fatty acid compositions were analysed according to the HS-Omega-3 Index methodology as described previously [15,16,19].

Cognitive measurements
As the neuropsychological tests have been described in more detail in [15], a brief description follows. Five neuropsychological tests were administered at T0, T2 and T3. Three were administered in a small group setting (maximum 10 students), namely: The Letter digit substitution task (LDST), the D2 test of attention (D2), and the Digit Span backward and forward (DSB and DSF). After these group tests were administered the Stroop Interference test and the Concept Shifting Task (CST) were administered individually. The LDST is a measure for speed of information processing [20,21], D2 is used to measure selective attention [22], DSB is a measure for working memory, DSF is a measure for short term memory, the Stroop Interference Test is a measure for cognitive inhibition, and lastly CST is a measure for cognitive shifting [23].

Other measurements
After the neurocognitive tests, participants filled out a number of questionnaires. Among others pubertal phase was assessed with the Pubertal Development Scale [24]. Students also filled out if they smoked cigarettes and how many, and if they drank alcohol and if so how often and how much. They also indicated whether they had a diagnosis which could influence learning (i.e., autism, dyslexia, or ADHD). At T2 and T3 questions were asked about adherence (how often did you forget the capsules?) and whether they experienced any side effects (open ended question). Lastly, at T3 students were asked to guess in which group they were allocated and to indicate how certain they were about their guess.

Statistical analyses

Sample size calculation was executed in R Mass. To investigate the effect of the intervention on cognitive scores mixed model analyses were executed in R statistical environment (R studio version 3.2), and all other analyses were executed with SPSS statistics version 24 (IBM).

Sample size

Sample size calculation was executed in R Mass software. Based on an effect size of d=0.25 at 6 months and an effect size equal or 10% larger at 12 months, a drop-out rate of 25% per measurement moment (thus 43% in total), an error variation between 0.4 and 0.5, and an intercept variation of 0.3 to 0.5 with fixed effects it was concluded that a sample of 183-285 participants would be sufficient to achieve a power of 0.8.

Group comparisons, treatment guess and adherence

Baseline comparisons on fatty acid concentrations, neurocognitive test scores and participant characteristics (i.e., age, BMI, alcohol, sex, smoking, level of parental education, pubertal status, school, and cohort) were done with ANOVA analyses for the continuous variables and Chi square tests for the categorical variables. Participants in the krill oil group were compared with participants in the placebo group, those who completed the study with those who dropped-out, and those who actively finished the study (i.e., taking placebo or krill oil) with those who quit taking the capsules (both those that still participated in neuropsychological testing and those that quit completely). Moreover, fatty acid concentrations measured in blood at all time points in participants in the krill oil group were compared with fatty acid concentrations measured in blood in participants in the placebo group with an ANOVA.

Treatment guess was compared for those in the krill oil group with the treatment guess in those in the placebo group with a Chi Square test. Students were asked to return capsules which they did not take as a measure of adherence. As an additional measure of adherence, the average increase in O3I between T0 and T2 and between T0 and T3 were studied. Moreover, the number of participants who had a decrease in O3I, had an increase up to 2.5%, and had an increase of more than 2.5% were noted

Imputing and recoding covariates

Data on drinking (units per week) and smoking (yes/no) were collected at T0 and at T3 and imputed for T2, an average score between 0 and 12 months was used for drinking, and a cut-off score of 0.5 cigarette a week was used to code yes/no for smoking (> 0.5 as yes). Level of parental education was coded as low (vocational education and training and below) and high (university of applied sciences and higher).

Cognitive measurements

Intention-to-treat analyses were conducted using linear mixed models that accounted for repeated measurements in subjects. Models allowed a comparison between groups (intervention versus control condition) and within groups (baseline data compared to intervention at other time points). Besides time trends (baseline as a reference) and treatment X time interactions, all estimates were adjusted for drinking behavior, smoking

behavior, level of parental education, age at baseline, pubertal status at baseline, sex, body mass index, diagnosis related to learning, and cohort number.

Furthermore, moderation analyses for sex and, if relevant, sub group analyses for males and females were executed. Secondary analyses were executed with: (1) O3I as exploratory analyses and (2) only participants who completed the full study (i.e., had measurements on and T0, and T2, and T3).

For D2 Total, D2 Correct, LDST, Shifting and Interference the R package nlme with standard settings was used [25]. D2 F1 (errors of omission on D2) and D2 F2 (errors of commission) had skewed distributions and, therefore, the generalized linear mixed-effects (Poisson) model option and the bootstrapping method were used to determine the 95% confidence interval in the package lme4 [26]. Lastly, digit span data were analyzed with the package ordinal with the function for cumulative link mixed models [27]. In these ordinal analyses one quadrature point and flexible thresholds were used. In all cases a $p <. 05$ was considered to be statistically significant.

Results

A total of 286 students provided informed consent, 17 did not meet inclusion criteria, 1 declined participation and 2 were not included due to other reasons yielding 267 respondents who were randomized into the study. Due to logistic reasons 9 participants with a baseline O3I > 5% were not excluded before the start of the trial. These participants were excluded from data analyses. One participant quit during baseline testing and was excluded from analyses. Thus data were available for 257 participants for at least one time point. During the study 82 students (31.9%) withdrew completely from the study and 53 (20.6%) stopped active participation (i.e., they were tested, but did not take capsules, see Figure 7.1). Baseline characteristics for the placebo and the krill oil group can be found in Table 7.1, Table 7.2, and Table 7.3. There were slightly more girls in the krill oil group than boys compared to the placebo group (54 girls in krill oil group 79 girls in placebo group; χ^2 (1) = 8.56; p = .004). Furthermore, those randomized into the krill oil group had a slightly lower DHA concentration at baseline compared to those in the placebo group (F (1,255) = 3.977; p = .047). There were no other differences between the placebo and krill oil group in participant characteristics (all p > .175) or baseline cognitive test scores (all p > .126).

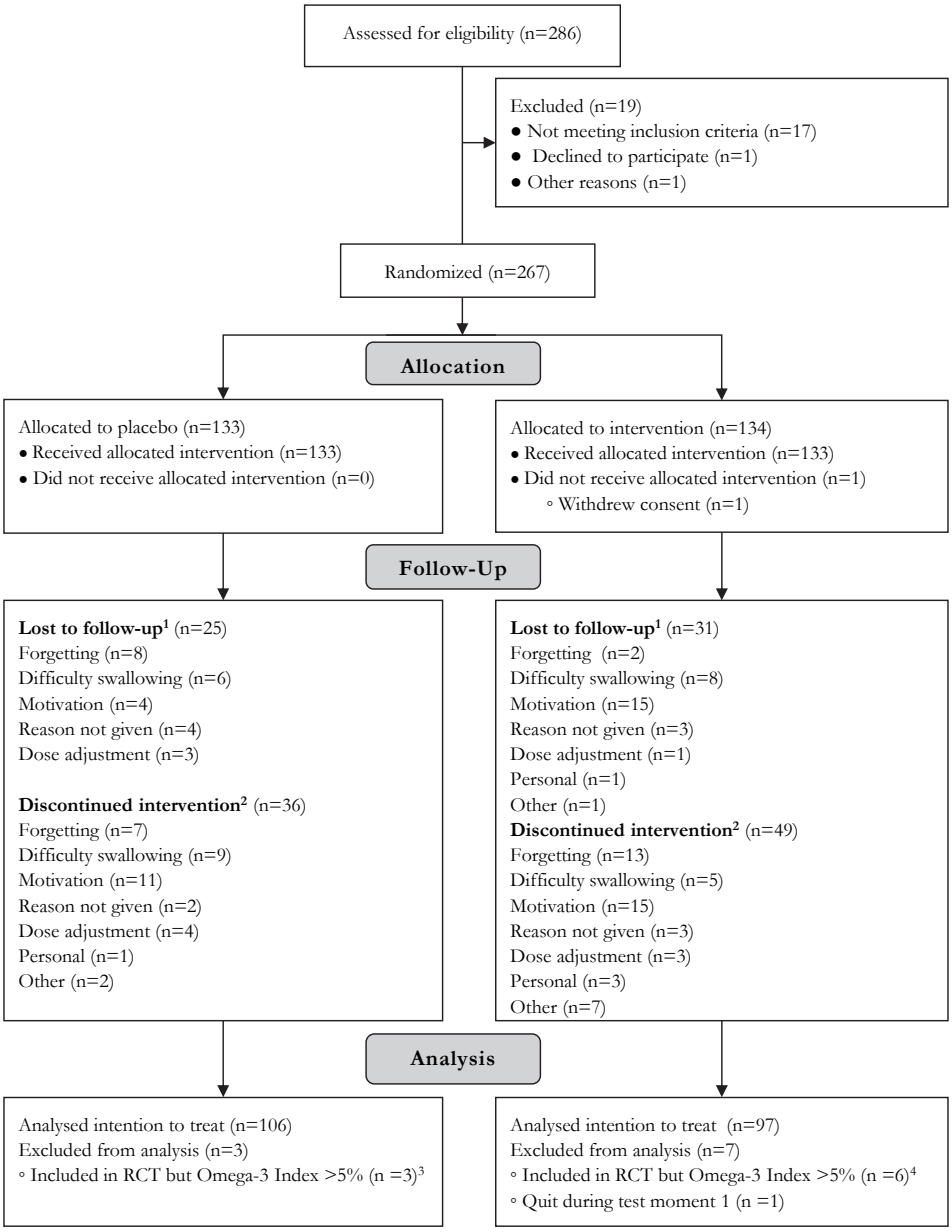

Figure 7.1: Flow chart

Flow chart adapted from Consort Guidelines [45]
[1] Participants whom quite taking capsules and quite participation in testing. [2] Participants whom quite taking capsules but did participate in testing. [3] Three participants with an O3I > 5% were included in the placebo group of these one was lost to follow up (reason forgetting), one discontinued the intervention (reason dose adjustment) and one finished the intervention, they were all excluded from the analyses. [4] Six participants with an O3I > 5% were included in the intervention group of these one was lost to follow up (reason motivation), two discontinued the intervention (reason motivation/ personal)and three finished the intervention, they were all excluded from the analyses.

Table 7.1: Participant characteristics at baseline

	Placebo	N	Krill	N	P[3]
Age (years)	14.07 ± 0.48	130	14.15 ± 0.51	127	0.175
Male/female N (%)	51/79 (39/61)	130	73/54 (57/43)	127	0.003
Smoking[1] (no/yes)	117/12 (91/9)	129	114/12 (90/10)	126	0.952
BMI	20.13 ± 3.05	123	19.81 ± 2.96	117	0.414
Alcohol units per week[2]	0.34 ± 1.16	129	0.60 ± 2.28	127	0.240
Level of parental education	5.02 ± 1.58	124	5.09 ± 1.43	116	0.686

[1] Smoking was defined as anybody who indicated to smoke more than 0 cigarettes per week; [2] Alcohol units per week was operationalized as number of days per week that alcohol is consumed times units per consumption moment; [3]ANOVA was used for age, BMI, LPE and alcohol units per week, Chi Square for smoking, and sex. Significant differences ($p < .05$) are noted in bold.

Fatty acids concentrations

Concentrations of fatty acids in blood can be found in Table 7.2. The intervention group had a significant higher EPA, DPA, DHA concentrations, a significantly higher O3I (all $p <. 001$), and significant lower concentrations of AA and ObA (all $p < .038$) compared to the placebo group at T1, T2 and T3. In the supplementary Table S7.1 fatty acid concentrations on T1 separate for cohort 1 and cohort 2 can be found.

Treatment guess

After the one year supplementation period 63 participants (65.6%) in the placebo group and 45 participants (50.6%) in the krill oil group correctly guessed their original treatment allocation. The percentage that correctly guessed treatment allocation did differ significantly with more participants in the placebo group correctly guessing their group allocation ($\chi^2 = 4.313$; $p = .038$).

Drop-out and adherence

There was a baseline difference between those who finished the study completely with supplementation and those who quit taking capsules for school and cohort. In some schools more children quit supplementation than in other schools ($\chi^2 = 11.329$, $p = .001$). Moreover, there was a difference in drop-out between cohorts, more students stopped taking capsules in cohort II (χ^2 (1) = 10.875, $p = .001$) compared to cohort I. All other participant characteristics, fatty acids and neurocognitive test scores were not significantly different between the groups (all $p > .066$)

Table 7.2: Fatty acids in blood at different time points intention to treat

	Baseline			3 months			6 months			12 months [1]		
Fatty acid (%wt/wt of total FA)	placebo (n 130)	krill (n 127)	p	placebo (n 124)	kril112 (n 118)	p	placebo (n 116)	krill (n 108)	p	placebo (n 104)	krill (n 95)	p
AA 20:4n-6	11.19 ± 1.36	11.12 ± 1.18	0.667	10.97 ± 1.18	10.26 ± 1.13	< 0.001	11.02 ± 1.49	10.28 ± 1.41	< 0.001	11.15 ± 1.30	10.72 ± 1.49	0.029
EPA 20:5n-3	0.38 ± 0.14	0.38 ± 0.15	0.807	0.43 ± 0.15	0.93 ± 0.58	< 0.001	0.41 ± 0.14	0.95 ± 0.69	< 0.001	0.40 ± 0.12	0.75 ± 0.58	< 0.001
ObA 22:5n-6	0.43 ± 0.11	0.45 ± 0.10	0.155	0.41 ± 0.17	0.32 ± 0.12	< 0.001	0.42 ±0.09	0.32 ± 0.11	< 0.001	0.38 ± 0.12	0.34 ± 0.13	0.038
DPA 22:5n-3	1.22 ± 0.20	1.22 ± 0.17	0.908	1.29 ± 0.23	1.58 ± 0.34	< 0.001	1.30 ±0.20	1.54 ± 0.35	< 0.001	1.30 ± 0.19	1.47 ±0.31	< 0.001
DHA 22:6n-3	2.60 ± 0.44	2.49 ± 0.46	0.047	2.61 ± 0.52	3.25 ± 0.73	< 0.001	2.69 ±0.53	3.40 ± 0.90	< 0.001	2.72 ± 0.54	3.20 ± 0.84	< 0.001
O3I	3.83 ± 0.54	3.71 ± 0.55	0.097	3.88 ± 0.64	5.10 ± 1.29	< 0.001	3.95 ± 0.64	5.29 ± 1.61	< 0.001	3.98 ± 0.63	4.86 ± 1.43	< 0.001

[1] Please note that 5 participants did participate in testing but did not have a blood sample available. [2] Note this includes both the participants from Cohort 1 who took 400mg EPA + DHA per day and participants from Cohort 2, who took 800mg EPA + DHA per day, separated analyses can be found in the supplemental material.

Table 7.3: Scores on the cognitive tests at different test moments in intention to treat analysis

	Baseline			6 months			12 months		
	Placebo Mean ± SD	Krill Mean ± SD	P	Placebo Mean ± SD	Krill Mean ± SD	P	Placebo Mean ± SD	Krill Mean ± SD	P
LDST (number)	35.02 ± 5.73	33.97 ± 5.20	0.126	37.56 ± 6.13	36.44 ± 6.46	0.184	39.79 ± 5.78	38.39 ± 5.81	0.085
D2 total (number)	421.42 ± 57.69	416.22± 55.72	0.464	468.88 ± 61.71	461.41 ± 57.68	0.350	506.08 ± 65.88	494.22 ± 57.00	0.175
D2 correct (number)	164.82 ± 24.34	162.65 ± 22.21	0.456	187.91 ± 28.47	184.87 ± 25.59	0.403	206.88 ± 34.09	201.29 ± 28.17	0.208
D2 error of commission (number)	11.97 ± 9.55	11.69 ± 12.07	0.839	10.0 ± 9.62	9.44 ± 7.81	0.631	9.14 ± 10.59	8.22 ± 6.90	0.469
D2 error of omission (number)	1.38 ± 1.72	1.24 ± 1.49	0.484	1.12 ± 1.74	1.06 ± 1.35	0.793	0.85 ± 1.64	0.68 ± 1.10	0.388
Shifting score (s)	11.69 ± 6.51	11.73 ± 7.21	0.965	10.30 ± 5.59	9.99 ± 7.06	0.715	10.26 ± 5.69	9.98 ± 7.20	0.760
Interferences score (s)	31.64 ± 8.74	30.61 ± 7.85	0.322	28.01 ± 6.60	27.83 ± 6.14	0.834	26.77 ± 6.76	25.92 ± 5.31	0.321
Digit Span Forward (digits)	5.55 ± 0.83	5.61 ± 0.89	0.526	5.37 ± 0.80	5.53 ± 1.05	0.211	5.68 ± 0.98	5.64 ± 0.90	0.783
Digit Span Backward (digits)	4.58 ± 0.98	4.53 ± 1.00	0.689	4.71 ± 0.81	4.69 ± 0.91	0.816	4.83 ± 0.96	4.65 ± 1.11	0.224

LDST = letter digit substitution task

Furthermore, when we compared activate participants (those who took capsules) with participants who only took part in neuropsychological testing (without taking capsules), and those participants that quit completely were compared the same patterns were seen. There was a difference between cohort (χ^2 = 13.139, p = .001) and between pubertal status at baseline with those participating without taking capsules having a slightly lower pubertal status (M = 3.27 versus M = 3.52 for active participants and M = 3.65 for those who dropped-out completely; F (2,235) = 4.890; p = .023). All other participant characteristics, fatty acids and neurocognitive test scores were not significantly different between the groups (all p > .059).

Students that were taking krill oil at T2 had an average increase, compared to T0, in the O3I of 2.02% (SD 1.58). At T2 compared to T0, 5 (6.8%) of the active participants in the krill group had a decrease in their O3I, 44 (60.3%) had an increase between 0 and 2.5% and 24 (32.9%) had an increase of > 2.5%. At T3 compared to T2 there was an average decrease in the O3I of 0.70% in active krill oil participants. When looking at the O3I at T3 compared to T2 35 (70%) of active participants in the krill oil group had a decrease in their O3I and were thus most likely non-adherent to the protocol, 15 (30%) had an increase between 0.14% and 1.96%.

Participants were asked to return capsules at the last test moment. Of the active participants (i.e., those taking capsules) at T3, 56 handed in capsules and 37 counted the left-over number of capsules at home. On average participants handed in or counted at 628.82 ± 395.23 left-over capsules. So on average participants did not take the capsules 78.6 days of the approximately 180 days between T2 and T3.

Neuropsychological tests

The random intercept models with time moment (T0, and T2, and T3), condition (krill oil or placebo), the interaction term (time moment X condition) and covariates showed that group allocation (krill or placebo) did not predict the score on any of the neurocognitive tests (**see Table 7.4**). There was however a clear time effect (i.e., students improved over time) on all neurocognitive tests with exception of the digit span forward and backward. Moreover, there was an interaction effect for condition X time moment T3 for the number of target stimuli processed on the D2 (D2 correct). This indicates that the increase in number of correctly processed stimuli between T2 and T3 was slightly higher in the placebo group compared to the krill oil group, namely 4.67 stimuli more.

Table 7.4: Multilevel analyses of cognitive test scores predicted by condition (intention to treat) and according to Omega-3 Index

			Estimate (SE)	95%CI		Estimate (SE)	95%CI
D2- Total	Test moment	T2	48.05 (3.60)	**[41.06; 55.03]**	T2	47.92 (3.01)	**[42.08; 53.75]**
		T3	88.39 (3.80)	**[81.02; 95.76]**	T3	84.90 (3.07)	**[78.95; 90.85]**
	Condition	Krill	-9.67 (7.93)	[-25.09; 5.74]	O3I	-0.20 (1.63)	[-3.36; 2.96]
	Interaction	T2 × krill	-0.33 (5.25)	[-10.51; 9.84]			
		T3 × krill	-8.04 (5.44)	[-18.59; 2.51]			
D2- Correct	Test moment	T2	23.05 (1.55)	**[20.04; 26.06]**	T2	23.02 (1.30)	**[20.51; 25.53]**
		T3	43.50 (1.64)	**[40.32; 46.68]**	T3	41.64 (1.32)	**[39.08; 44.20]**
	Condition	Krill	-1.54 (3.69)	[-8.71; 5.62]	O3I	-0.48 (0.70)	[-1.85; 0.88]
	Interaction	T2 × krill	-0.67 (2.26)	[-5.06; 3.71]			
		T3 × krill	-4.67 (2.35)	**[-9.23; -0.13]**			
D2- F1	Test moment	T2	-0.13 (0.04)	**[-0.21; -0.04]**	T2	-0.14 (0.04)	**[-0.22; -0.07]**
		T3	-0.24 (0.05)	**[-0.32; -0.14]**	T3	-0.27 (0.04)	**[-0.34; -0.20]**
	Condition	Krill	-0.09 (0.10)	[-0.30; 0.13]	O3I	0.01 (0.02)	[-0.03; 0.05]
	Interaction	T2 × krill	-0.002 (0.06)	[-0.15; 0.12]			
		T3 × krill	-0.05 (0.07)	[-0.18; 0.09]			
D2-F2	Test moment	T2	-0.24 (0.13)	[-0.48; 0.01]	T2	-0.13 (0.10)	[-0.33; 0.05]
		T3	-0.53 (0.14)	**[-0.80; -0.27]**	T3	-0.56 (0.12)	**[-0.76; -0.34]**
	Condition	Krill	-0.21 (0.16)	[-0.51; 0.07]	O3I	-0.04 (0.05)	[-0.14; 0.05]
	Interaction	T2 × krill	0.16 (0.19)	[-0.21; 0.53]			
		T3 × krill	-0.05(0.22)	[-0.50; 0.36]			
	Test moment	T2	2.49 (0.48)	**[1.57; 3.42]**	T2	2.42 (0.39)	**[1.66; 3.18]**
LDST		T3	4.74 (0.50)	**[3.76; 5.72]**	T3	4.56 (0.40)	[3.78; 5.33]
	Condition	Krill	-0.19 (0.81)	[-1.75; 1.38]	O3I	-0.07 (0.74)	[-0.46; 0.32]
	Interaction	T2 × krill	-0.24 (0.70)	[-1.59; 1.11]			
		T3 × krill	-0.31 (0.72)	[-1.71; 1.08]			
Shifting	Test moment	T2	-1.60 (0.81)	**[-3.17; -0.02]**	T2	-1.80 (0.64)	**[-3.03; -0.55]**
		T3	-1.23 (0.85)	[-2.87; 0.41]	T3	-1.87 (0.66)	**[-3.14; -0.59]**
	Condition	Krill	-0.09 (0.90)	[-1.83; 1.66]	O3I	0.07 (0.27)	[-0.46; 0.60]
	Interaction	T2 × krill	-0.24 (1.18)	[-2.53; 2.06]			
		T3 × krill	-1.42 (1.22)	[-3.78;0.95]			
Interference	Test moment	T2	-4.04 (0.72)	**[-5.43; -2.65]**	T2	-3.47 (0.58)	**[-4.60; -2.35]**
		T3	-5.42 (0.75)	**[-6.87; -3.96]**	T3	-5.41 (0.59)	**[-6.55; -4.26]**
	Condition	Krill	-0.97 (0.97)	[-2.84;0.91]	O3I	-0.02 (0.28)	[-0.56; 0.52]
	Interaction	T2 × krill	1.10 (1.05)	[-0.93;3.12]			
		T3 × krill	-0.19 (1.08)	[-2.28;1.90]			

D2 = D2 test of attention, D2 correct = number of target stimuli processed, D2F1 = errors of omission on D2, D2F2 = errors of commission on D2, LDST = letter digit substitution task, O3I = Omega-3 Index, T2 = test moment 6 months, T3 = test moment 12 months

All models included test moment, condition (krill/placebo) plus covariates (alcohol consumption, smoking behavior, age at baseline, puberty status at baseline, BMI, highest level of parental education, sex, cohort number and diagnosis). Significant 95 % CI are printed in bold. The model with condition also includes the time moment X condition interaction factor. Note that a lower score on D2F1, D2F2, Shifting and Interference is a better performance.

Table 7.5: Multilevel analyses of digit span forward and digit span backward, predicted by either condition (intention to treat) or Omega-3 Index

		Odds ratio (SE)	95%CI		Odds ratio (SE)	95%CI
DSF						
Test moment	T2	0.46 (1.32)	**[0.26;0.77]**	T2	0.54 (1.25)	**[0.35; 0.84]**
	T3	1.15 (1.33)	[0.66;2.02]	T3	1.16 (1.25)	[0.75; 1.78]
Condition	Krill	0.86 (1.41)	[0.44;1.69]	O3I	1.08 (1.11)	[0.88; 1.32]
Interaction	T2 × krill	1.84 (1.50)	[0.83;4.06]			
	T3 × krill	1.21 (1.51)	[0.54;2.71]			
DSB						
Test moment	T2	1.38 (1.30)	[0.83;2.30]	T2	1.49 (1.23)	[0.99; 2.22]
	T3	1.61 (1.32)	[0.93;2.77]	T3	1.61 (1.24)	**[1.05; 2.45]**
Condition	Krill	0.87 (1.37)	[0.47;1.61]	O3I	1.00 (1.10)	[0.83; 1.21]
Interaction	T2 × krill	1.14 (1.46)	[0.55;2.39]			
	T3 × krill	0.97 (1.49)	[0.44;2.12]			

DSF = digit span forward, DSB = digit span backward, O3I = Omega-3 Index, T2 = test moment 6 months, T3 = test moment 12 months.
All models included test moment, condition (krill/placebo) or Omgea-3 Index plus covariates (alcohol consumption, smoking behavior, age at baseline, puberty status at baseline, BMI, highest level of parental education, sex, cohort number and diagnosis). The model with condition also includes the time moment X condition interaction factor. Please note that for odds ratios significance is indicated by a 95% CI which does not contain 1.

The random intercept with time moment (T0, and T2, and T3), O3I and covariates did not show an effect of O3I on neurocognitive test scores either. There was however a clear time effect (i.e., students improved over time) on all neurocognitive tests with exception of the digit span forward and backward.

All above analyses were run with all participants, whether they had data available for one, two or three time points. To ensure the accuracy of the data a secondary exploratory analysis was executed with only those participants that had data available at all three test moments. The results of these analyses were similar to the results of the intention to treat analyses (see Supplementary Table S7.2 and S7.3).

Side effects

At the end of T2 and T3 we asked whether the participant had experienced any side effects. The question was open ended. At T2 27 participants indicated to have a side effect. Fourteen of these 'side effects' were positive; that is they mentioned to have better focus or better grades at school. The negative side effects which were reported were minor (3x stomach ache, 2x nauseous, 2x tired, 1x more hay fever, throwing up, hunger swings, more nervous, mood swings, dizziness, gaining weight, 'a weird feeling in my stomach' and throat ache). One participant learned during the trial that (s)he was allergic to fish. At T3, there were 29 participants with side effects, of whom 14 were positive. The negative side effects were again minor (4x head ache, 3x nauseous, 2x throat ache, 2x stomach ache, 2x less hunger, 1x each for tired, drier skin, sleeplessness, less focus, gaining weight, bad breath, belches, skin rash and 'getting ill from it')

Discussion

This study did not show an effect of one year of krill oil supplementation on a number of cognitive tests in typically developing adolescents of the lower general secondary education level in the Netherlands with a low baseline O3I. Moreover, sensitivity analyses did not show the association of higher O31 and neurocognitive test score. Note that our analyses of both depression and self-esteem questionnaire data showed similarly no effect of supplementation. However, the goal of the study was that participants in the krill oil group would achieve a predetermined O3I of 8-11% and that two distinct groups with regard to O3I would be achieved (thus no or minimal overlap in O3I between krill and placebo group). Unfortunately, neither goal was achieved. At 6 months follow-up the average O3I in the krill oil group was 5.29%, at 12 months 4.80. This was an average increase of 1.58% and 1.14%, respectively, compared to baseline. Moreover between 6 months and 12 months there was actually in average decrease in O3I in participant in the krill oil group who said to take the capsules. Moreover, only 3, 10, and 2 participants achieved the target O3I of > 8% at T1, T2, and T3, respectively. Thus, the average O3I of participants in the krill oil group was at all time points below the intended range of 8-11% and it seems likely that participants were non-adherent to the protocol. The O3I was also low if compared to, for example, unselected adult individuals from the German and European population, who have an average O3I of 7.15% and 6.96% respectively [28].

Mixed model analyses did not show an effect of krill oil supplementation on cognitive test scores (all $p > .161$), or a relationship between O3I and cognitive measures in the whole sample. It did show an interaction effect between condition and time moment T3 for the number of correctly processed stimuli on the D2 indicating that the increase in the correctly processed stimuli between T2 and T3 was slightly higher in the placebo group compared to the krill oil group. However, this effect was rather small (4.67), the placebo group scored higher on the D2 correct at every time point (albeit not significant) and no relationship in the O3I analyses were found, it is therefore not considered to be notable finding.

The findings that one year of krill oil supplementation did not improve cognitive test scores or a relationship between O3I and cognitive measures are in contrast with many supplementation studies in children [29–33], although not all studies in children show effects on cognition [34–40]. McNamara and colleagues did find increased functional activation of the dorsolateral prefrontal cortex after 8 weeks of DHA supplementation of boys 8-10 years, but this did not translate into a difference in performance task [11]. Bauer *et al.* found similar results in adults, with participants in the DHA supplementation group showing an increase in the functional activity in the right precentral gyrus, but no effect on cognitive measures [41]. Thus the fact that in this study no effect of krill oil supplementation on scores of cognitive tests could be shown does not preclude that there was increased activity in a some brain regions. It however remains to be seen what the real life avail would be of an increased brain activation when this does not lead to improved performance on the relatively short-term (up to 1 year). In a previous paper we reported on the baseline data of the current study and showed a positive association between higher O3I and better performance on the LDST in the whole sample and less D2 errors of omission [42]. It is unclear as to why that relationship could not be replicated in the current sample.

The small increase in the O3I is likely caused by the high number of students that quit taking capsules and the high non-adherence in active participants. It was tried to increase adherence to the protocol by sending participants a daily text message and having motivational talks. However, the study might just have been too long in duration (main reason for drop-out was loss of motivation) and the number of capsules the students had to take was too high (another important reason for drop-out was inability to take capsules). Both long study duration and high number of capsules have been suggested to influence adherence and drop-out [43]. This reasoning is also supported by the low drop-out and high adherence rates in the study of Tamman and colleagues. They studied a similar population but the study only lasted 16 weeks and students only had to take two capsule a day. They reported an adherence of 88% and a drop-out of 4.5% [44]. It is however important to note that if LCPUFA supplementation is found to be beneficial, long-term daily high dose intake of capsules might be needed to achieve and sustain effects, which seems to be extra problematic in adolescents.

The strength of the current study is that the O3I was measured in blood. So a reliable measure for adherence was available and analyses could be executed based on blood levels. Moreover, participants were preselected based on a low O3I as it can be expected that any effects of supplementation would be more pronounced in those with a low baseline O3I.

The main limitation of the current study is the fact that there was a relative high drop-out rate and adherence difficulties. However, the blood values and cognitive test scores for the majority of those that quit taking capsules were available. Moreover, participants that quit taking capsules did not differ significantly from those that continued the study actively on any of the relevant baseline measurements and thus selection bias seems unlikely

To summarize, the current study did not show an effect of one year of krill oil supplementation on cognitive measure of adolescents attending LGSE with a low baseline O3I. However, due to adherence problems and drop-out , it cannot be concluded that a relationship between krill oil supplementation and cognition does not exist. More studies on the influence of krill oil supplementation on cognition in adolescents are needed, moreover these studies should focus on achieving a set target O3I in the active group.

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Supplemental Table S7.1: Fatty acid concentrations at T1 separated for cohort 1 and cohort 2

	Cohort 1		Cohort 2	
Fatty acid (%wt/wt of total FA)	Placebo	Krill	Placebo	Krill
AA 20:4n-6	11.02 ± 1.16	10.16 ± 0.95	10.92 ± 1.21	10.61 ± 1.26
EPA 20:5n-3	0.45 ± 0.15	0.86 ± 0.35	0.40 ± 0.14	0.99 ± 0.71
ObA 22:5n-6	0.37 ± 0.09	0.27 ± 0.06	0.46 ± 0.21	0.36 ± 0.14
DPA 22:5n-3	1.30 ± 0.19	1.54 ± 0.23	1.29 ± 0.26	1.61 ± 0.41
DHA 22:6n-3	2.72 ± 0.49	3.34 ± 0.64	2.49 ± 0.52	3.18 ± 0.78
O3I	4.02 ± 0.62	5.12 ± 0.97	3.74 ± 0.65	5.09 ± 1.49

O3I =Omgea-3 index

Supplemental Table S7.2: Multilevel analyses of cognitive test scores predicted by condition and Omega-3 Index, participant whom participated in all test moments

			Estimate (SE)	95%CI		Estimate (SE)	95%CI
D2- Total	Test moment	T2	45.22 (3.73)	**[38.01; 52.43]**	T2	47.13 (3.10)	**[41.12; 53.14]**
		T3	87.59 (3.81)	**[80.22; 94.96]**	T3	84.45 (3.07)	**[78.51; 90.39]**
	Condition	Krill	-4.67 (8.85)	[-21.85; 12.50]	O3I	-0.12 (1.63)	[-3.28; 3.03]
	Interaction	T2 × krill	4.08 (5.43)	[-6.41; 14.58]			
		T3 × krill	-7.15 (5.45)	[-17.69; 3.40]			
D2- Correct	Test moment	T2	21.72 (1.61)	**[18.60; 24.84]**	T2	**22.74 (1.35)**	**[20.13;22.74]**
		T3	42.94 (1.65)	**[39.75; 46.13]**	T3	**41.47 (1.33)**	**[38.89;44.05]**
	Condition	Krill	0.38 (4.11)	[-7.60; 8.35]	O3I	-0.52 (0.71)	[-1.90;0.86]
	Interaction	T2 × krill	1.24 (2.35)	[-3.29; 5.78]			
		T3 × krill	-3.99 (2.36)	[-8.55; 0.56]			
D2- F1	Test moment	T2	-0.16 (0.04)	**[-0.24; -0.07]**	T2	**-0.16 (0.04)**	**[-0.23; -0.08]**
		T3	-0.25 (0.05)	**[-0.34; -0.16]**	T3	**-0.27 (0.04)**	**[-0.35; -0.20]**
	Condition	Krill	-0.15 (0.11)	[-0.36; 0.09]	O3I	0.02 (0.02)	[-0.02; 0.06]
	Interaction	T2 × krill	0.04 (0.07)	[-0.09; 0.17]			
		T3 × krill	-0.03 (0.07)	[-0.17; 0.11]			
D2-F2	Test moment	T2	-0.14 (0.13)	[-0.40; 0.11]	T2	-0.03 (0.11)	[-0.23; 0.19]
		T3	-0.46 (0.15)	**[-0.76; -0.19]**	T3	**-0.48 (0.13)**	**[-0.70; -0.26]**
	Condition	Krill	-0.24 (0.18)	[-0.56; 0.07]	O3I	-0.04 (0.05)	[-0.15; 0.07]
	Interaction	T2 × krill	0.19 (0.21)	[-0.19; 0.55]			
		T3 × krill	-0.03 (0.23)	[-0.44; 0.40]			
LDST	Test moment	T2	2.29 (0.50)	**[1.32; 3.26]**	T2	**2.43 (0.41)**	**[1.64; 3.22]**
		T3	4.64 (0.51)	**[3.65; 5.63]**	T3	**4.58 (0.40)**	**[3.80; 5.36]**
	Condition	Krill	-0.62 (0.87)	[-2.30; 1.07]	O3I	-0.07 (0.20)	[-0.47; 0.33]
	Interaction	T2 × krill	0.19 (0.73)	[-1.23; 1.60]			
		T3 × krill	-0.06 (0.73)	[-1.48; 1.36]			
Shifting	Test moment	T2	-1.73 (0.86)	**[-3.40; -0.07]**	T2	**-1.92 (0.68)**	**[-3.23; -0.60]**
		T3	- 1.30 (0.87)	[-2.98; 0.39]	T3	-1.96 (0.68)	**[-3.27; -0.66]**
	Condition	Krill	-0.06 (0.97)	[-1.96; 1.83]	O3I	0.15 (0.28)	[-0.38; 0.69]
	Interaction	T2 × krill	-0.11(1.26)	[-2.54; 2.33]			
		T3 × krill	-1.41 (1.26)	[-3.85; 1.04]			
Interference	Test moment	T2	-3.85 (0.76)	**[-5.32; -2.39]**	T2	**-3.39 (0.61)**	**[-4.58; -2.21]**
		T3	-5.26 (0.77)	**[-6.75; -3.77]**	T3	**-5.38 (0.61)**	**[-6.55; -4.20]**
	Condition	Krill	-0.38 (1.05)	[-2.42; 1.66]	O3I	-0.01 (0.29)	[-0.57; 0.54]
	Interaction	T2 × krill	0.90 (1.11)	[-1.24; 3.05]			
		T3 × krill	-0.45 (1.11)	[-2.59; 1.70]			

D2 = D2 test of attention, D2 correct = number of target stimuli processed, D2F1 = errors of omission on D2, D2F2 = errors of commission on D2, LDST = letter digit substitution task, O3I = Omega-3 Index, T2 = test moment 6 months, T3 = test moment 12 months

All models included test moment, condition (krill/placebo) plus covariates (alcohol consumption, smoking behaviour, age at baseline, puberty status at baseline, BMI, highest level of parental education, sex, cohort number and diagnosis). Significant 95 % CI are printed in bold. The model with condition also includes the time moment X condition interaction factor. Note that a lower score on D2F1, D2F2, Shifting and Interference is a better performance**.**

Supplemental Table S7.3: Multilevel analyses of DSF and DSB predicted by condition and Omega-3 Index, participant whom participated in all test moments

		Adjusted model			Adjusted model	
		Odds ratio (SE)	95%CI		Odds ratio (SE)	95%CI
DSF						
Test moment	T2	0.52 (1.34)	[0.29; 0.91]	T2	0.58 (1.26)	[0.37; 0.92]
	T3	1.28 (1.34)	[0.72; 2.28]	T3	1.26 (1.26)	[0.80; 1.96]
Condition	Krill	1.03 (1.46)	[0.49; 2.17]	O3I	1.03 (1.36)	[0.84; 1.27]
Interaction	T2 × krill	1.44 (1.53)	[0.30; 3.32]			
	T3 × krill	1.06 (1.53)	[0.46; 2.44]			
DSB						
Test moment	T2	1.31 (1.32)	[0.76; 2.24]	T2	1.55 (1.24)	[0.94; 2.21]
	T3	1.53 (1.33)	[0.87; 2.68]	T3	1.58 (1.25)	[1.02; 2.43]
Condition	Krill	0.82 (1.41)	[0.42; 1.60]	O3I	0.99 (1.10)	[0.82; 1.20]
Interaction	T2 × krill	1.15 (1.49)	[0.52; 2.51]			
	T3 × krill	1.03 (1.51)	[0.46; 2.30]			

DSF = digit span forward, DSB = digit span backward, O3I = Omega-3 Index, T2 = test moment 6 months, T3 = test moment 12 months.

All models included test moment, condition (krill/placebo) or Omgea-3 Index plus covariates (alcohol consumption, smoking behaviour, age at baseline, puberty status at baseline, BMI, highest level of parental education, sex, cohort number and diagnosis). The model with condition also includes the time moment X condition interaction factor. Please note that for odds ratios significance is indicated by a 95% CI which does not contain 1.

Chapter 8

Effect of 1 year krill oil supplementation on depression and self-esteem of Dutch adolescents: a randomized controlled trial.

Adapted from: van der Wurff, I.S.M., Von Schacky, C., Bergeland, T, Leontjevas, R., Zeegers, M.P., Kirschner, P. A., & de Groot, R.H.M. (to be submitted). Effect of 1 year krill oil supplementation on depression and self-esteem of Dutch adolescents: a randomized controlled trial.

Abstract

Background: Depression is common in adolescents and long-chain polyunsaturated fatty acids (LCPUFA) are suggested to be associated with depression. However, experimental research in adolescents is limited. Furthermore, self-esteem has never been studied in relation to LCPUFA.

Methods: A one-year double-blind, randomized, placebo controlled krill oil supplementation trial with repeated measurements at baseline (T0), 3 months (T1), 6 months (T2), and 12 months (T3) in adolescents. Students from cohort I started with 400mg eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) or placebo, at T1 this was increased to 800mg EPA + DHA per day (or placebo). Cohort II started with this higher dose (or placebo). Omega-3 Index (O3I) was monitored via a finger prick during the study. At T0, T2 and T3 participants filled out the Centre for Epidemiologic Studies Depression Scale (CES-D) and the Rosenberg Self Esteem questionnaire (RSE). Mixed models adjusted for covariates were run for both intention-to-treat analyses and O3I as predictor of CES-D and RSE scores.

Results: Krill oil supplementation led to a small but significant increase in the O3I, however the O3I remained relatively low probably due to non-adherence. Both intention-to-treat and O3I analyses did not show effects on CES-D and on RSE.

Limitations: Depressive feelings were assessed with a questionnaire. Drop-out and non-adherence were high.

Conclusion: The trial did not reveal evidence for an improvement of depressive feelings, and self-esteem after one year of krill oil supplementation. The lack of evidence might be explained by the very low increase in the O3I in the krill oil group as a result of drop-outs and non-adherence. The study thus does not preclude that an effect of krill oil supplementation on depressive feelings in adolescent is possible. More research with higher LCPUA doses and strategies to increase adherence in adolescents is needed.

Introduction

According to the World Health Organisation approximately 322 million people (4.4 % of the world population) are affected by depression [1]. Relatively uncommon in childhood (prevalence in last 6-12 months of 0.4-2.5%) [2], depression prevalence increases sharply during adolescence [3]. About 14-25% of adolescents experience at least one episode of depression before age 18 [4]. Adolescent depression has serious social, mental and physical consequences, for example increased risk of reoccurrence of depression, increased risk of anxiety, and risk of suicide [5]. But it has also been associated with lower educational attainment, lower wages, poorer social relationships, and poorer self-rated health [6,7]. Moreover, sub-threshold depression (i.e., depressive symptoms present, but not enough to reach a diagnosis of major depression) has also been associated with negative outcomes, such as the development of major depression and reduced quality of life [8,9]. Sub-threshold depression is common in adolescence with point prevalence between 0.24 and 14%, and life-time prevalence (i.e., life-time being up to age of measurement during adolescences) between 1 and 22.9% [8,9]. Numbers vary in different populations (ages, gender, country), but also due to the manner in which depression is assessed and according to criteria for (subthreshold) depression used.

Depression is a complex and incompletely understood multi-factorial disorder, and probably has a heterogeneous aetiology [10]. Depression has been characterized by biological changes such as increased levels of pro-inflammatory cytokines [11,12], alterations in immune function [13], elevation of plasma homocysteine levels [14], changes in brain structure [15,16], blood flow abnormalities [17], and decreased glucose metabolism [18], and a low Omega-3 Index (O3I) [19,20].

Long chain polyunsaturated fatty acids (LCPUFA) have long attracted attention for their possible positive effect on depression. LCPUFA have many important functions in the body which are biological plausible ways in which LCPUFA intake could prevent or cure depression. For example LCPUFA can modulate neuroendocrine factors, have anti-inflammatory properties, play a role in neurogenesis and in neuroplasticity [21,22], all factors which could reduce or counteract biological changes associated with depression. Observational studies, mostly show that blood and brain concentrations of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are lower in people with depression compared to people without depression [20,23,24]. LCPUFA supplementation intervention trials in adult depressed participants mostly show positive results (for meta-analyse see among others [25–27]).

Depression mostly has its onset during adolescence and adolescents' depression can have profound long lasting effects [5], it is therefore an important group to study with regard to depression. There are a number of observational studies investigating the relationship between LCPUFA and depression in adolescents [20,28–31]. Most, but not all, of these observational studies show a relationship between LCPUFA and depression (i.e., higher intake or higher concentrations in blood, less depression [20,28–30]). However, LCPUFA supplementation studies investigating depression in adolescents from the general population are not available yet.

The main aim of this study was to assess the influence of one year of krill oil supplementation, a source of the LCPUFA DHA and EPA, on depressive feelings in adolescents of lower general secondary education. We hypothesised that one year krill oil supplementation would lead to lower depressive feelings.

Furthermore we explored the influence of the krill oil supplementation on self-esteem. Self-esteem is a core construct in mental health and refers to the subjective evaluation of

ones worth as a person [32]. Low self-esteem has been suggested to be a risk factor for the development of many mental illnesses including depression [32]. However to our knowledge, studies exploring the effect of LCPUFA supplementation (here krill oil supplementation) are missing.

Methods

This study was approved by the Medical Ethical Committee of Atrium-Orbis-Zuyd Hospital Heerlen, the Netherlands (NL45803.096.13). Food2Learn is registered at the Netherlands Trial Register (NTR4082) and at Clinicaltrials.gov (NCT02240264). Full details about the designs and methods of the study have been reported previously [33]. Below a shortened version follows. Note that in the current manuscript only the data with regard to mental well-being are presented.

Design

Food2Learn was a double-blind, randomized, placebo controlled intervention trial with repeated measurements (baseline (T0), 3 months (T1), 6 months (T2), and 12 months (T3)) to study the effect of one year of krill oil supplementation on cognitive performance, academic achievement and mental well-being of second year high school students attending lower general secondary education (LGSE) in the Netherlands. After informed consent was received, a finger prick was executed to determine the Omega-3 Index (O3I). Only students with an O3I < 5% were included in the study. Goal of the study was to increase the Omega-3Index (O3I) of participants in the krill oil group to 8-11%. This target O3I was based on the O3I associated with the lowest mortality risk in coronary heart disease [34].

Intervention

The intervention started after baseline testing. In Cohort I participants were asked to take four placebo or krill oil capsules, containing 260mg EPA and 140mg DHA with their dinner, as this is the fattest meal of the day and this helps with absorption. After three months of supplementation a personalized dose adjustment was planned to account for interpersonal difference in metabolism. However, at T1 only 3 participants achieved the target O3I of 8-11%, therefore all participants (both krill and placebo) were asked to increase the daily dosage to eight capsules per day, containing 520mg EPA and 280mg DHA per day. Furthermore, it was decided that Cohort II would start with eight capsules.

Blood analyses

Whole blood was obtained from a finger prick at T0, T1, T2, and T3. Whole blood fatty acid compositions were analysed according to the HS-Omega-3 Index methodology [34,35]. Results are given as EPA plus DHA expressed as a percentage of total identified fatty acids after response factor correction. Since the O3I is defined as EPA + DHA in erythrocytes, it was calculated using a sliding correction factor.

Questionnaires

Centre for Epidemiologic Studies Depression Scale

Depressive feelings were assessed with the Dutch version of the Centre for Epidemiologic Studies Depression Scale (CES-D) [36]. Although developed for adults, the CES-D has been shown to have a high reliability in both clinical and non-clinical samples of children and adolescents ($\alpha = 0.88$) and also reasonable sensitivity ($\alpha = 0.76$) [37]. The questionnaire consists of 20 questions which assess six symptom areas of depression. The score on the questionnaire can vary between 0 and 60, with higher scores indicating more depressive feelings. Originally a cut-off score of ≥16 was suggested as indication for depression, although some have suggested a higher cut-off score of ≥22 for adolescents [38]. Moreover, it has also been suggested that the CES-D score should be considered as a continuum of increasing severity [8]. For the current study we used both the standard cut-off score of ≥16 and the adolescents' cut-off score of ≥22 for descriptive purposes, while for the main analyses we used CES-D score as a continuous score.

Rosenberg Self-Esteem Questionnaire

Self-esteem was assessed with the Dutch version of the Rosenberg Self-Esteem Questionnaire (RSE) [39]. The reliability of the RSE has been shown to be high ($\alpha = 0.72$-0.88) [40]. The RSE consists of 10 questions about the evaluation of themselves. The total score of the RSE can range from 0 to 30 with higher scores indicating higher self-esteem. For the current study the RSE score was used as a continuous score.

Additional measures

At T0, T2, and T3 a number of variables were measured as they are known to be associated with depression: BMI (weight/length^2 , self-reported at baseline) [41,42], sex [43,44], age in years [45], alcohol consumption (number of days that alcohol was consumed times number of standardized units of alcohol that were consumed per moment) [46], and number of cigarettes smoked per week (if student indicates to smoke more than 1 cigarette per week this was coded a smoking yes, otherwise smoking no) [47]. Moreover parental level of education (subdivided in 8 levels ranging from primary school to university level) as a proxy for socioeconomic status [48], pubertal status as assessed by the Pubertal Development Scale (subdivided in 5 levels from prepubertal to postpubertal) [49], and diagnosis which might influence learning (yes/no, e.g., autism or ADHD) [50] were assessed .

Statistical analyses

Sample size calculation

Sample size calculation for multilevel analyses were executed in RMass, and showed that a sample of 183-285 participants would be sufficient [51]. This sample size is based on an effect size of 0.25 at 6 months and an effect size equal or 10% larger at 12 months, a drop-out rate of 25% per measurement moment (thus 43% in total), an error variation between 0.4 and 0.5 and an intercept variation of 0.3 to 0.5 with fixed effects.

Imputing and recoding covariates

Data on drinking and smoking were only collected at T0 and T3, and the average score between 0 and 12 months was imputed for drinking at T2. A cut-off score of 0.5 cigarette per week was used to code yes/no for smoking at T2 (i.e., more than 0.5 cigarette per week was coded as yes). Level of parental education was recoded to low (vocational education and training and below) and high (university of applied sciences and higher).

Some participants had a maximum of two missing items on the CES-D (11, 15, and 7 participants at T0, T2, and T3 respectively) or the RSE (12, 6, and 6 participants at T0, T2, and T3 respectively). The missing items were imputed by the person average score on the other items as suggested by Bono *et al.* [52].

Group comparison, treatment guess and adherence

Group comparisons and treatment guess analyses were executed in SPSS statistics version 24 (IBM).

The following baseline comparisons were executed: krill oil group versus the placebo group, participants who completed the study versus participants who dropped-out, participants who completed the study actively (i.e., taking capsules) versus those who quit taking capsules (both those who dropped out completely and those who still participated in testing). Baseline comparisons were executed with ANOVA for continuous variables and Chi square test for categorical variables. We compared groups on fatty acid concentrations, CES-D score, RSE, score and participants' characteristics (BMI, age, sex, alcohol consumption, smoking, level of parental education, pubertal status, school and cohort). We also compared the fatty acid concentrations measured in blood on all time points in participants in the krill oil group and the placebo group.

Treatment guess was compared between participants in the krill oil group and participants in placebo group with a Chi Square test.

We asked participants to hand in left-over capsules and intended to use this as an adherence measure. As an additional measure for adherence we noted the average increase in O3I between T0 and T2, and between T0 and T3. Lastly, we documented how many participants had a decrease in their O3I, had an increase up to 2.5%, and had an increase of more than 2.5%.

Effect on depressive feelings and self-esteem

Intention-to-treat analyses were conducted using linear mixed models that accounted for repeated measurements in subjects. These analyses were executed in R statistical environment (R studio version 3.3.2) with the package nlme (version 3.1-131) using the standard settings [53].

Models allowed a comparison between groups (krill oil versus placebo condition) and within groups (T0 data compared to intervention at T2 and T3). Besides time trends (baseline as a reference) and treatment X time interactions, all estimates were adjusted for drinking behaviour, smoking behaviour, level of parental education, age at baseline, pubertal status at baseline, sex, BMI at baseline, diagnosis related to learning, and cohort number. Furthermore, moderation analyses for sex was executed and if necessary a sub group analyses (boys and girls separate) were executed. Secondary analyses were executed with O3I instead of group allocation. A $p < .05$ was considered to be statistically significant.

Results

A total of 286 students provided informed consent, of which 267 were randomized into the study. 17 participants did not meet inclusion criteria, one retracted consent, one had other reasons. After randomization, one participant withdrew consent before starting supplementation and one participant quit during the first test moment; both were excluded from the analyses. Additionally, due to logistic issues, 9 participants with an O3I > 5% were not excluded before the start of the study, they were excluded from the analyses. So, for 256 participants data were available for at least one time moment.

During the study 53 (20.6%) students withdrew completely from the study and 82 (32%) stopped active participation (i.e., they were tested, but did not take capsules). Main reported reasons for discontinuing the study were lack of motivation, difficulty swallowing capsules, and difficulties remembering to take capsules (see Figure 8.1).

Baseline characteristics for the placebo and the krill oil group can be found in Table 8.1, Table 8.2 and Table 8.3. There were somewhat more girls in the krill oil group than boys (54 girls, 72 boys) compared to the placebo group (79 girls, 51 boys; $\chi^2 = 8.224$, $p = .004$). There were no other differences between the placebo and krill oil group in participant's characteristics (all $p > .175$), blood values (all $p > .055$) or baseline depressive feelings or self-esteem scores ($p = .259$ and $p = .237$, respectively)

At baseline 74 students (29.5%) had a score ≥16 on the CES-D (i.e., indicative for depression), at T2 48 (21.3%), and at T3 54 (26.5%). Using a stricter cut-of criterion of ≥ 22, 18.3%, 12.4% and 18.6% reached the cut off point for depression at T0, T2, and T3, respectively.

Table 8.1: Participants' characteristics at baseline

	Placebo	N	Krill	N	P[3]
Age (years)	14.07 ± 0.48	130	14.15 ± 0.51	126	0.185
Male/female N (%)	51/79 (39/61)	130	72/54 (57/43)	126	0.004
Smoking[1] (no/yes)	117/12 (91/9)	129	113/12 (90/10)	126	0.935
BMI	20.13 ± 3.05	123	19.84 ± 2.96	116	0.455
Alcohol units per week[2]	0.34 ± 1.16	129	0.61 ± 2.29	126	0.234
Level of parental education	5.02 ± 1.58	124	5.10 ± 1.43	115	0.652

[1] Smoking was defined as anybody who indicated to smoke more than 0 cigarettes per week [2] Alcohol units per week was operationalized as number of days per week that alcohol is consumed times units per consumption moment [3]ANOVA was used for age, BMI, LPE and alcohol units per week, Chi Square for smoking, and sex. Significant differences ($p < .05$) are noted in bold.

Blood fatty acids

Concentrations of blood fatty acids at T0, T1, T2, and T3, can be found in Table 8.2. Compared to the placebo group, the krill oil group had significant higher EPA, DPA, DHA concentrations, a higher O3I (all $p < .001$), and a significant lower concentration of AA and ObA (all $p < 0.38$) at T1, T2, and T3. Note that participants in cohort 1 started with a different supplementation dosage than cohort 2. In the supplementary Table S8.1 fatty acid concentrations on T1 separate for cohort 1 and cohort 2 can be found.

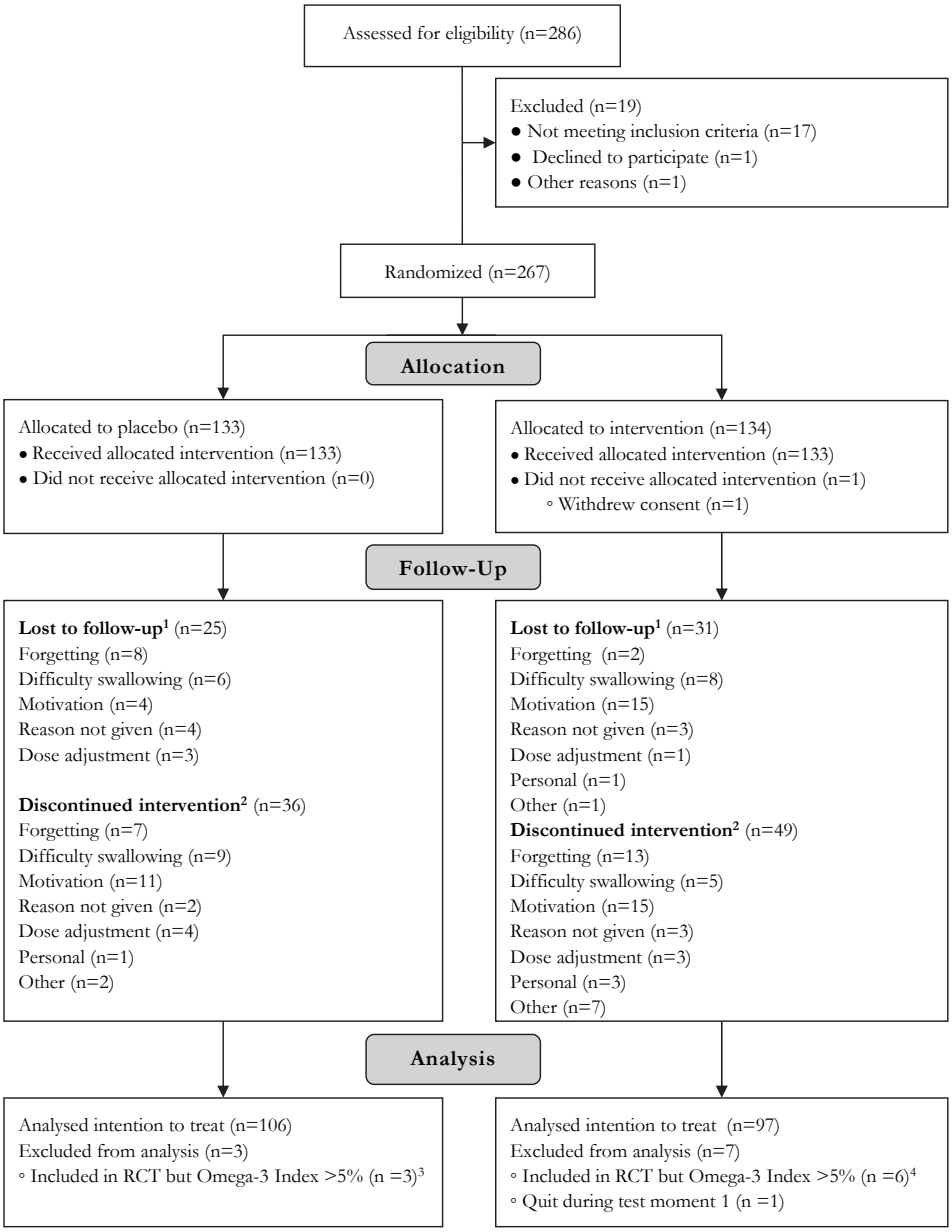

Figure 8.1: Flow charts
Flow chart adapted from Consort Guidelines [54]
[1] Participants who quit taking capsules and quite participation in testing.[2] Participants who quit taking capsules but did participate in testing. [3] Three participants with an O3I > 5% were included in the placebo group of these one was lost to follow up (reason forgetting), one discontinued the intervention (reason dose adjustment), and one finished the intervention, they were all excluded from the analyses. [4] Six participants with an O3I > 5% were included in the intervention group of these one was lost to follow up (reason motivation), two discontinued the intervention (reason motivation/ personal), and three finished the intervention, they were all excluded from the analyses

Table 8.2: Fatty acids in blood at different time points in intention to treat analysis

	Baseline			3 months			6 months			12 months		
Fatty acid (%wt/wt of total FA)	placebo (n 130)	krill (n 126)	p	placebo (n 124)	krill (n 118)	p	placebo (n 116)	krill (n 108)	p	placebo (n 104)	krill (n 95)	p
AA 20:4n-6	11.19 ± 1.36	11.12 ± 1.18	0.657	10.97 ± 1.18	10.26 ± 1.13	< 0.001	11.02 ± 1.49	10.28 ± 1.41	< 0.001	11.15 ± 1.30	10.72 ± 1.49	0.029
EPA 20:5n-3	0.38 ± 0.14	0.38 ± 0.15	0.860	0.43 ± 0.15	0.93 ± 0.58	< 0.001	0.41 ± 0.14	0.95 ± 0.69	< 0.001	0.40 ± 0.12	0.75 ± 0.58	< 0.001
ObA 22:5n-6	0.43 ± 0.11	0.45 ± 0.10	0.154	0.41 ± 0.17	0.32 ± 0.12	< 0.001	0.42 ±0.09	0.32 ± 0.11	< 0.001	0.38 ± 0.12	0.34 ± 0.13	0.038
DPA 22:5n-3	1.22 ± 0.17	1.22 ± 0.19	0.858	1.29 ± 0.23	1.58 ± 0.34	< 0.001	1.30 ±0.20	1.54 ± 0.35	< 0.001	1.30 ± 0.19	1.47 ±0.31	< 0.001
DHA 22:6n-3	2.60 ± 0.44	2.49 ± 0.46	0.055	2.61 ± 0.52	3.25 ± 0.73	< 0.001	2.69 ±0.53	3.40 ± 0.90	< 0.001	2.72 ± 0.54	3.20 ± 0.84	< 0.001
O3I	3.83 ± 0.54	3.72 ± 0.55	0.106	3.88 ± 0.64	5.10 ± 1.29	< 0.001	3.95 ± 0.64	5.29 ± 1.61	< 0.001	3.98 ± 0.63	4.86 ± 1.43	< 0.001

O3I = Omega-3 Index

Table 8.3: Scores on the Centre for Epidemiologic Studies Depression Scale and the Rosenberg's Self-Esteem Scale separated for krill and placebo group

	CES-D		p	RSE		p
	Krill M ± SD (min-max)	Placebo M ± SD (min-max)		Krill M ± SD (min-max)	Placebo M ± SD (min-max)	
Baseline	11.52 ± 9.00 (1-53)	12.91 ± 10.51 (0-43)	0.259	21.89 ± 5.61 (1-30)	21.01 ± 6.22 (4-30)	0.237
6 months	9.98 ± 8.72 (0-48)	11.21 ± 9.71 (0-41)	0.319	22.69 ± 5.34 (0-30)	21.93 ± 5.48 (8-30)	0.298
12 months	10.86 ± 8.92 (0-49)	13.60 ± 11.95 (0-49)	0.066	23.00 ± 4.96 (2-30)	21.74 ± 6.36 (2-30)	0.120

CES-D Centre for Epidemiologic Studies Depression Scale, RSE = Rosenberg's Self-Esteem Scale

Depressive feelings

The random intercept model with time point (T0 as reference), condition (krill or placebo), the interaction term (time moment x condition) and covariates showed that group allocation did not predict score on the CES-D (see Table 8.4). Interaction analysis for sex and condition did not shown an interaction effect ($p = .161$).

The random intercept model with time point (T0 as reference), O3I, and covariates showed that O3I did not predict depression score. Interaction analysis for sex and O3I did show an interaction effect ($p = .03$). Subgroup analyses showed that for girls there was a possible significant relationship between higher O3I and higher depression score ($b = 1.08$, $SE= 0.56$, $p = .053$, 95% CI [0.01; 2.15]), indicating that girls with a higher O3I might have more depressive feelings. For boys no relationship between O3I and depression score was shown ($b = -0.37$, $SE= 0.36$, $p = .311$, 95% CI [-1.06; 0.33]). Closer inspection of the data showed that there were 5 participants whom had a Z-score on the CES-D of > 3.29, which is considered to be an extreme outliers and not a reliable value [55]. These 5 participants were all girls, when these girls were excluded from the analyses, the interaction term became non-significant ($p = .168$).

Self-esteem

The random intercept model with time point (T0 as reference), condition (krill or placebo), the interaction term (time moment x condition) and covariates showed that group allocation did not predict score on the RSE. The interaction analysis for sex X condition did not show an interaction effect ($p = .671$)

The random intercept model with time point (T0 as reference), O3I, and covariate showed that O3I did not predict self-esteem score. The interaction analysis for sex X O3I did not show an interaction effect either ($p = .661$)

Treatment guess

After the one year supplementation period 63 participants (65.6%) in the placebo group and 45 participants (50.6%) in the krill oil group correctly guessed their original treatment allocation. The percentage that correctly guessed treatment allocation did differ significantly with more participants in the placebo group correctly guessing their group allocation ($\chi^2 = 4.313$; $p = .038$).

Table 8.4: Multilevel analyses of score on the Centre for Epidemiologic Studies Depression Scale by condition (intention to treat) and according to Omega-3 Index.

Condition			Omega-3 Index		
	b (SE)	95%CI		b (SE)	95%CI
T2	-1.50 (0.82)	[-3.09; 0.08]	T2	-1.64 (0.66)	[-2.93; -0.36]
T3	0.95 (0.86)	[-0.71; 2.61]	T3	0.18 (0.67)	[-1.12; 1.49]
Krill	-0.65 (1.32)	[-3.20; 1.91]	Omega-3 Index	0.38 (0.34)	[-0.28; 1.03]
T2 x krill	0.37 (1.18)	[-1.92; 2.66]			
T3 x krill	-1.04 (1.22)	[-3.41; 1.32]			
Alcohol	-0.07 (0.19)	[-0.44; 0.30]	Alcohol	-0.06 (0.19)	[-0.44; 0.31]
Smoking[1]	2.66 (1.22)	[0.29; 5.03]	Smoking[1]	2.72 (1.24)	[0.31; 5.13]
Age	0.47 (1.28)	[-2.02; 2.96]	Age	0.31 (1.28)	[-2.18; 2.80]
Pubertal status[2]			Pubertal status[2]		
Beginning	-5.42 (4.46)	[-14.07; 3.23]	Beginning	-6.04 (4.44)	[-14.67; 2.60]
Mid	-1.78 (4.32)	[-10.17; 6.61]	Mid	-2.26 (4.31)	[-10.63; 6.12]
Advanced	-0.82 (4.43)	[-9.41; 7.78]	Advanced	-1.34 (4.42)	[-9.93; 7.26]
Post	8.83 (6.23)	[-3.25; 20.92]	Post	8.03 (6.21)	[-4.04; 20.10]
BMI	0.49 (0.20)	[0.11; 0.87]	BMI	0.50 (0.20)	[0.12; 0.89]
LPE [3]	0.46 (1.17)	[-1.81; 2.73]	LPE [3]	0.39 (1.17)	[-1.88; 2.67]
Sex [4]	4.83 (1.60)	[1.72; 7.94]	Sex [4]	4.97 (1.60)	[1.86; 8.07]
Cohort [5]	1.47 (1.17)	[-0.80; 3.75]	Cohort [5]	1.45 (1.16)	[-0.80; 3.70]
Diagnosis [6]	2.43 (1.17)	[0.16; 4.69]	Diagnosis [6]	2.63 (1.16)	[0.37; 4.89]

LPE = level of parental education, T2 = test moment 6 months, T3 = test moment 12 months.
[1] No smoking as reference, [2] Pre-pubertal as reference, [3] Low level of parental education as reference,
[4] Boy as reference, [5] Cohort 1 as reference, [6] No diagnosis as reference.

Drop-out and adherence

There we no baseline differences between those who finished the study completely with supplementation and those who quit taking capsules for age, BMI, drinking behaviour, sex, smoking behaviour, level of parental education, or pubertal status (all $p > .460$), any of the fatty acids (all $p > .066$), depression score ($p = .861$) or self-esteem score ($p = .987$). There was a difference in drop-out between cohorts, more students stopped taking capsules in cohort 2 ($\chi^2 = 11.329$, $p = .001$). Moreover, in some schools more students dropped out than in other schools ($\chi^2 = 28.299$, $p = .029$).

When we compared those that actively participated (with taking capsules), with those who only participated in neuropsychological testing (without taking capsules), and those that quit completely, we saw the same patterns. No difference for age, BMI, drinking behaviour, sex, smoking behaviour, school or, level of parental education, pubertal status (all $p > 0.059$), any of the fatty acids (all $p > .102$), or score on the CES-D, or RSE ($p = .165$ and $p = .297$). A difference between cohorts ($\chi^2 = 13.139$, $p = .001$) did again exist.

Table 8.5: Multilevel analyses of score on the Rosenberg's Self-Esteem Scale by condition (intention to treat) and according to Omega-3 Index.

Condition			Omega-3 Index		
	b (SE)	95%CI		b (SE)	95%CI
T2	0.64 (0.42)	[-0.19; 1.46]	T2	0.55 (0.35)	[-0.12; 1.22]
T3	0.47 (0.45)	[-0.40; 1.34]	T3	0.62 (0.35)	[-0.06; 1.30]
Krill	0.25 (0.77)	[-1.25; 1.75]	Omega-3 Index	-0.03 (0.18)	[-0.38; 0.32]
T2 x krill	-0.18 (0.62)	[-1.38; 1.01]			
T3 x krill	0.23 (0.64)	[-1.00; 1.47]			
Alcohol	0.11 (0.10)	[-0.09; 0.31]	Alcohol	0.12 (0.10)	[-0.08; 0.32]
Smoking[1]	-1.58 (0.67)	[-2.87; -0.29]	Smoking[1]	-1.75 (0.67)	[-3.05; -0.45]
Age	-0.91 (0.78)	[-2.42; 0.61]	Age	-0.90 (0.78)	[-2.41; 0.62]
Pubertal status[2]			Pubertal status[2]		
Beginning	4.71 (2.71)	[-0.54; 9.97]	Beginning	4.89 (2.70)	[-0.35; 10.14]
Mid	3.34 (2.63)	[-1.76; 8.44]	Mid	3.42 (2.62)	[-1.67; 8.51]
Advanced	3.13 (2.69)	[-2.09; 8.34]	Advanced	3.25 (2.68)	[-1.97; 8.47]
Post	2.13 (3.79)	[-5.23; 9.49]	Post	2.38 (3.78)	[-4.98; 9.74]
BMI	-0.23 (0.12)	[-0.46; 0.005]	BMI	-0.24 (0.12)	[-0.47; -0.005]
LPE [3]	0.17 (0.71)	[-1.21; 1.54]	LPE [3]	0.13 (0.71)	[-1.25; 1.51]
Sex [4]	-3.33 (0.97)	[-5.22; -1.44]	Sex [4]	-3.41 (0.97)	[-5.30; -1.52]
Cohort [5]	0.31 (0.71)	[-1.07; 1.69]	Cohort [5]	0.33 (0.70)	[-1.04; 1.70]
Diagnosis [6]	-0.88 (0.68)	[-2.20; 0.44]	Diagnosis [6]	-0.91 (0.68)	[-2.22; 0.40]

[1] No smoking as reference, [2] Pre-pubertal as reference, [3] Low level of parental education as reference,
[4] Boy as reference, [5] Cohort 1 as reference, [6] No diagnosis as reference.
T2 = test moment 6 months, T3 = test moment 12 months.

Looking at those students that were active participants at T2, there was an average increase, compared to T0, in the O3I of 2.02% (SD 1.58%). Compared to T0 5 (6.8%) of the active participants had an decrease in their O3I, 44 (60.3%) had an increase between 0 and 2.5% and 24 (32.9%) had an increase of >2.5%. At T3, compared to T2, there was an average decrease in the O3I of 0.70% in active krill oil participants. When comparing T3 to T2, 35 (70%) of active participants in the krill oil group had a decrease in their O3I and were thus most likely not compliant with the protocol, the other 15 (30%) had an increase varying from 0.14 to 1.96%.

Participants were asked to return capsules at the last test moment. Of the active participants (i.e., those taking capsules) at test moment 12 months, 56 handed in capsules and 37 counted the left-over number of capsules at home. On average participants had 628.82 ± 395.23 capsules left over. This corresponds to 78.6 days with not taking capsules in the approximately 180 days between T2 and T3.

Discussion

The current trial in adolescents attending LGSE who had a low baseline O3I did not show improvements of depressive feelings and self-esteem in the krill oil supplementation condition compared to placebo, nor did it lead to a higher self-esteem score. Sensitivity

analyses did not show the association of higher O3I with less depression score or better self-esteem score. Note that our analyses of neurocognitive test scores showed similarly no effect of supplementation. However, the goal of the study was that participants in the krill oil group would achieve a predetermined O3I of 8-11% and that two distinct groups with regard to O3I would be achieved (thus no or minimal overlap in O3I between krill and placebo group). Unfortunately, neither goal was achieved. The average O3I in the krill oil group was 5.29% at T2 and 4.80% at T3, which indicates an average increase in O3I of 1.58% and 1.14% compared to T0, respectively. However, between T2 and T3 there was actually in average decrease in O3I in participant in the krill oil group who said to take the capsules. Moreover, only 3, 10, and 2 participants achieved the target O3I of >8% at T1, T2, and T3, respectively. Thus, the average O3I in the krill oil group was at all time-points well below the intended range of 8-11% and the participants were uncompliant with the protocol. Even if we compare the average O3I of our sample to unselected adult individuals from the European and German population, whom have an average O3I of 6.96% and 7.15% respectively, the O3I in our sample after supplementation is still very low. It has been argued that positive effects of O3I only exist at higher levels of O3I. For example, in the study of Markhus and colleagues, there was only a linear relation between O3I and pregnancy depressive feelings when the O3I was above 5.1% [56]. Moreover, in cardiovascular health an O3I > 8% has been associated with the greatest risk reduction [57].

Depressive feelings were very common in this sample of adolescents attending LGSE. When using cut-off score of ≥16 on the CES-D, 29.5%, 21.3% and 26.5% had scores indicative of depression at T0, T2, and T3 respectively. When using the higher cut-off score of ≥22, still 18.3%, 12.4% and 18.6% of participants had a score indicative of depression at T0, T2, and T3 respectively. Surprisingly, there were only 6 students who indicated to have a diagnosed depression. These numbers of students with a possible depression might seem high, but other studies in adolescents have shown similar rates. For example, Oddy *et al.* showed using the Beck Depression Inventory for Youth, that 21.2% of the sample had mild to severe depressive symptoms [31]. In the study of Grant and colleagues 21.7% of boys and 34.4% of girls had scores indicative of mild to extremely severe depression [58]. Lastly, Mamalakis *et al.* showed in their sample an average CES-D score of 14.9 [30], which is very comparable to the average scores in the current study.

To our knowledge there are no earlier LCPUFA supplementation studies in which the effect on depressive feelings in adolescents is studied. LCPUFA supplementation studies in adults mostly show positive effects of supplementation (for meta-analyses see among others [25,27,59]. It is moreover uncertain if and how the results from the studies on depression in adults can be compared to studies in adolescents. For example it has been suggested that juvenile-onset and adult-onset depression are distinct with different causes and different outcomes [60,61]. Moreover, in adolescence the brain is still in development and this is accompanied by profound physical, social, emotional and cognitive development [62]. All these factors could influence depression, but depression in itself also disrupts these developmental processes which can have severe long term negative effects, which possibly are more profound than the long-term effects of depression in adulthood. Lastly, the use of the LCPUFA in the body might also be different in adolescents compared to adults. For example, up to 18 years old the amount of DHA in the brain rises and then it plateaus until the age of 88 year [63].

As mentioned previously the increase in O3I in the krill oil group was smaller than anticipated and smaller than aimed for. We aimed for an O3I of between 8-11%, but unfortunately only 3,10 and 2 participants achieved that at T1, T2, and T3, respectively. This low number of participants whom achieved the target range had to do with the large number of participants that quit supplementation and the non-adherence of the active participants. We did utilize a multitude of techniques to improve adherence: we sent participants a daily text message reminder, had motivational telephone talks, and handed out a tip sheet with tips on how to remember to take the capsules (i.e., example of tips were: put the capsules on the kitchen table, put a reminder alarm in your phone, or but a sticky note with a reminder somewhere). The study did last for 1 year, which might simple be too long, this is echoed in the fact that most participants quit because of lack of motivation/ inability to remember to take the capsules. Moreover, the students had to take 8 capsules per day, which many students indicated to be too much and was another reason why many students dropped-out (inability to take capsules). Long study duration and high number of capsules have been suggested to influence drop-out and adherence [64]. It is however important to realize that if LCPUFA supplementation is found to be beneficial for depressive feelings, a high dose of capsules might need to be taken for a prolonged period of time. Adherence with medication, and thus most likely supplements as well, has already been found to be problematic in those with depression [65], but might even be more challenging in adolescents.

The limitation of the current study is the fact self-rated questionnaires to assess depression were used instead of clinician interviews. Self-rated questionnaires to assess depression could lead to false positive, or false negatives. However, the CES- D has been validated and in the main analyses we did not use cut-off scores but used the continuous score on the CES-D. Another limitation of the current study is the fact, as discussed, that there was a rather high dropout rate and there were difficulties with achieving adherence to the protocol. However, blood samples and test scores were available of the majority of participants that quit taking capsules, so they could be included in sensitivity analyses. Moreover, participants that dropped-out did not differ significantly from those that continued the study actively on relevant baseline characteristics.

To summarize the current study did not reveal an effect of one year of krill oil supplementation on depression score and on self-esteem score in adolescents attending LGSE with a low baseline O3I. However, due to the lack of adherence and high drop-out rate, we feel we cannot preclude that a relationship between krill oil supplementation and depression score or self-esteem score in adolescents does not exists. More studies in this specific age group with high rates of depressive symptoms are needed. Moreover specific attention should be payed to achieve adherence, decrease drop-out and achieve a set target O3I.

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Supplemental Table S8.1: Fatty acid concentrations at T1 separated for cohort 1 and 2

	Cohort 1		Cohort 2	
Fatty acid (%wt/wt of total FA)	Placebo	Krill	Placebo	Krill
AA 20:4n-6	11.02 ± 1.16	10.16 ± 0.95	10.92 ± 1.21	10.61 ± 1.26
EPA 20:5n-3	0.45 ± 0.15	0.86 ± 0.35	0.40 ± 0.14	0.99 ± 0.71
ObA 22:5n-6	0.37 ± 0.09	0.27 ± 0.06	0.46 ± 0.21	0.36 ± 0.14
DPA 22:5n-3	1.30 ± 0.19	1.54 ± 0.23	1.29 ± 0.26	1.61 ± 0.41
DHA 22:6n-3	2.72 ± 0.49	3.34 ± 0.64	2.49 ± 0.52	3.18 ± 0.78
O3I	4.02 ± 0.62	5.12 ± 0.97	3.74 ± 0.65	5.09 ± 1.49

O3I =Omega-3 Index

Chapter 9

A review of recruitment, adherence and drop-out rates in omega-3 polyunsaturated fatty acid supplementation trials in children and adolescents

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Abstract

Introduction: The influence of *n*-3 long-chain polyunsaturated fatty acids (*n*-3 LCPUFA) supplementation on health outcomes has been studied extensively with randomized controlled trials (RCT). In many research fields, difficulties with recruitment, adherence and high drop-out rates have been reported. However, what is unknown is how common these problems are in *n*-3 LCPUFA supplementation studies in children and adolescents. Therefore, this paper will review *n*-3 LCPUFA supplementation studies in children and adolescents with regard to recruitment, adherence and drop-out rates.

Methods: The Web of Science, PubMed and Ovid databases were searched for papers reporting on RCT supplementing children and adolescents (2–18 years) with a form of *n*-3 LCPUFA (or placebo) for at least four weeks. As a proxy for abiding to CONSORT guidelines, we noted whether manuscripts provided a flow-chart and provided dates defining the period of recruitment and follow-up.

Results: Ninety manuscripts (reporting on 75 studies) met the inclusion criteria. The majority of the studies did not abide by the CONSORT guidelines: 55% did not provide a flow-chart, while 70% did not provide dates. The majority of studies provided minimal details about the recruitment process. Only 25 of the 75 studies reported an adherence rate which was on average 85%. Sixty-five of the 75 studies included drop-out rates which were on average 17%.

Conclusion: Less than half of the included studies abided by the CONSORT guidelines (45% included a flow chart, while 30% reported dates). Problems with recruitment and drop-out seem to be common in *n*-3 LCPUFA supplementation trials in children and adolescents. However, reporting about recruitment, adherence and dropout rates was very heterogeneous and minimal in the included studies. Some techniques to improve recruitment, adherence and dropout rates were identified from the literature, however these techniques may need to be tailored to *n*-3 LCPUFA supplementation studies in children and adolescents.

Introduction

Fatty acids, and especially the omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), are being researched extensively for a wide array of health outcomes varying from, but not exclusive to, cardiovascular diseases, depression and cognition [1–3]. As in every health related field, randomized controlled supplementation trials are the gold standard to demonstrate efficacy of n-3 LCPUFA [4]. For these trials, voluntary participants are needed, however recruitment of participants can be challenging, especially when it involves research in children and adolescents (< 18 years)[5]. It has been reported that less than 31% of British studies funded by two funding bodies between 1994 and 2002 achieved their original recruitment target number [6]. Similarly, others have reported that up to 60% of the randomized controlled trials (RCT) fail to meet their participant target or need an extension [7] and this percentage might be even higher in paediatric and adolescent studies [8,9]. However, even after the recruitment phase, difficulties with conducting research do not end, because drop-out and non-adherence are also common. Drop-out in RCT is normal and attrition rates can vary enormously from 0 up to 65% [10–12]. Compliance and adherence are often used interchangeably, but are not the exactly same. Compliance is the extent to which the behaviour of a person coincides with the advice given by a doctor or researcher. The term compliance has received criticism because of its paternalistic connotation [13] and because it implies patient passivity [14]. As a more neutral term, adherence has been suggested, which presumes that the person agrees with the advice given by a doctor or researcher [14]. We choose to use the term adherence in the current manuscript. Adherence issues, are common, with non-adherence ranging anywhere from 3.5 to 80% [15,16]. One must also be aware that there is no one single definition of adherence. This means that somebody who is considered non-adherent in one study, might be considered adherent in another (e.g., one study defined a participant as non-adherent when the participant took less than 75% of the prescribed medicine or supplements, while another used a cut-off level of < 80%). As low recruitment rates, high drop-out and high non-adherence are common and have serious consequences [6,17,18], it is important to study factors which possibly affect recruitment, drop-out, and adherence rates. In 2013, we started a one-year long double blind randomized n-3 LCPUFA supplementation trial in healthy Dutch adolescents called Food2Learn [19]. We experienced difficulties in the recruitment, drop-out and adherence of the study participants. Furthermore, many other n3- LCPUFA supplementation studies have had the same difficulties (personal communication). However, a review of recruitment, adherence and drop-out rates in nutrition interventions and in specific n-3 LCPUFA supplementation studies in children and adolescents does not, to our knowledge, exist. Therefore, the aim is to execute a thorough review to summarize n-3 LCPUFA supplementation studies in children (2–12 years) and adolescents (12–18 years) with regard to recruitment effort, drop-out and adherence rates.

Materials and methods

The Web of Science, PubMed and Ovid databases were searched up to 2 March 2017. We searched for human clinical trials including children aged between 2 and 18 years. We used

the search terms: "Omega-3", "DHA", "EPA", "LCPUFA" and "PUFA" in combination with "RCT", "randomized controlled trial", "supplementation", "trial" or "fish oil" and "child*)", "adolescent", "school", "preschool" or "toddler". Furthermore, a myriad of reviews were checked for additional studies [20–40] and reference lists of all articles were hand checked for additional references. Moreover, a search of the Cochrane library was also conducted to identify reviews regarding n-3 LCPUFA supplementation. The studies included in the Cochrane reviews were checked for inclusion in the current study [41–56]. Lastly, for all included articles, the "cited by" option of Web of Science was checked (this option gives all articles that cite that specific article).

Studies were eligible for inclusion if they met the following criteria: (1) participants were aged between 2 and 18 years; (2) the study was a randomized placebo controlled n-3 LCPUFA supplementation trial; (3) the trial had at least 10 participants per treatment arm; (4) supplementation duration was at least 4 weeks; and (5) studies were published in English.

All papers were scanned by the first author, and the following information was extracted and entered in a database:

Participants' characteristics: Age range of participants, percentage of girls, healthy participants or those with a diagnosed disease, and country in which the study was executed;

Study characteristics: Number of participants, number of measurement moments (i.e., how often did participants have to come to the research facility/how often did they have to fill out questionnaires), number of measurements, treatment condition, placebo condition, form of supplementation, if capsules were used then how many, if supplementation was taken under supervision, if supplement was taken in multiple dosages or once a day, whether an incentive was provided, duration of the study, manner in which adherence was assessed, adherence rate, whether fatty acids were determined in blood, and percentage of people who quit the treatment (hereafter called drop-out); and

Recruitment characteristics: Invited/responded or screened, started, finished as well as method of recruitment, recruitment setting, and study period.

The recruitment characteristics were defined as follows:
Invited: The total number of potential participants invited to participate;
Responded: The total number of potential participants who responded to the invitation or the number of participants that were screened for participation in the study; and
Started: The number of participants who were assessed as eligible and began supplementation.

Furthermore, efficiency percentages were calculated, namely: started/invited (dividing the number of people who started by the number of people who were invited times 100), started/ responded (dividing the number of people who started by the number of people who responded times 100) and started/finished (dividing the number of people who finished by the number of people who started times 100).

As a proxy for adherence to the CONSORT guidelines, we noted whether the article included a flow-chart and whether the article provided the dates defining the period of recruitment and follow-up.

Statistics

All extracted data were entered in SPSS (IBM SPPS statistics for Windows, version 24, Armonk, NY, USA). SPSS was used to calculate averages and SDs for the participant' characteristics, study characteristics and recruitment characteristics. For comparison reasons outpatient clinics and hospitals were combined into one setting, which was named "hospital setting". Countries were furthermore grouped in regions for comparisons (Europe, USA/Canada, Asia, Middle East, Australia, Africa and South America). When a study mentioned multiple adherence rates, these were combined into one adherence rate for the whole study.

Results

Study characteristics

The original search led to 2163 hits. Upon first screening, 1656 articles were excluded, additional screening of the whole articles led to a further exclusion of 173 articles. Additional checking of the reference lists of reviews, included articles and forward checking led to 15 more studies being included (see Figure 9.1, adapted from [57]). Thus, in total, 90 papers, describing 75 studies, were included in this review. The characteristics of these studies can be found in Table 9.1. Fifteen studies focussed on healthy children. The other 60 studies focused on children with a disorder or disease, with attention deficit hyperactivity disorder (ADHD) being the most studied disorder (n = 21) (see Table 9.1). The majority of studies focussed on children (defined as aged between 2 and 12 years, n = 38) or both children and adolescents (n = 31). A minority of studies focussed only on adolescents (n = 6) (see Table 9.1). Duration of study varied from 4 to 52 weeks, with the majority of studies lasting 26 weeks or less (n = 59, 79%, see Table 9.2. Number of measurement moments (i.e., how often did participants have to come to the research facility/how often did they have to fill out questionnaires) varied from 2 to 16 with a mean of 3.7 (SD 2.7), the number of different measurements per moment varied from 1 to 19 with a mean of 4.9 (SD 3.7).

Recruitment

Most of the studies included in this review did not report the number of children or adolescents that were invited to participate in the study, as only 11 out of 75 studies mentioned the number of participants that were invited. The total number of people invited to participate varied from 46 to 3562 (Mean (M) = 804.5, SD = 1083.28). The percentage of invited participants that eventually started the study ranged from 2.4% to 87% (see Table 9.3).

Forty out of 75 studies mentioned the number of participants that responded to the invitation or were screened for the study and this varied from 30 to 1556, with 12% to 100% of these people actually starting the study.

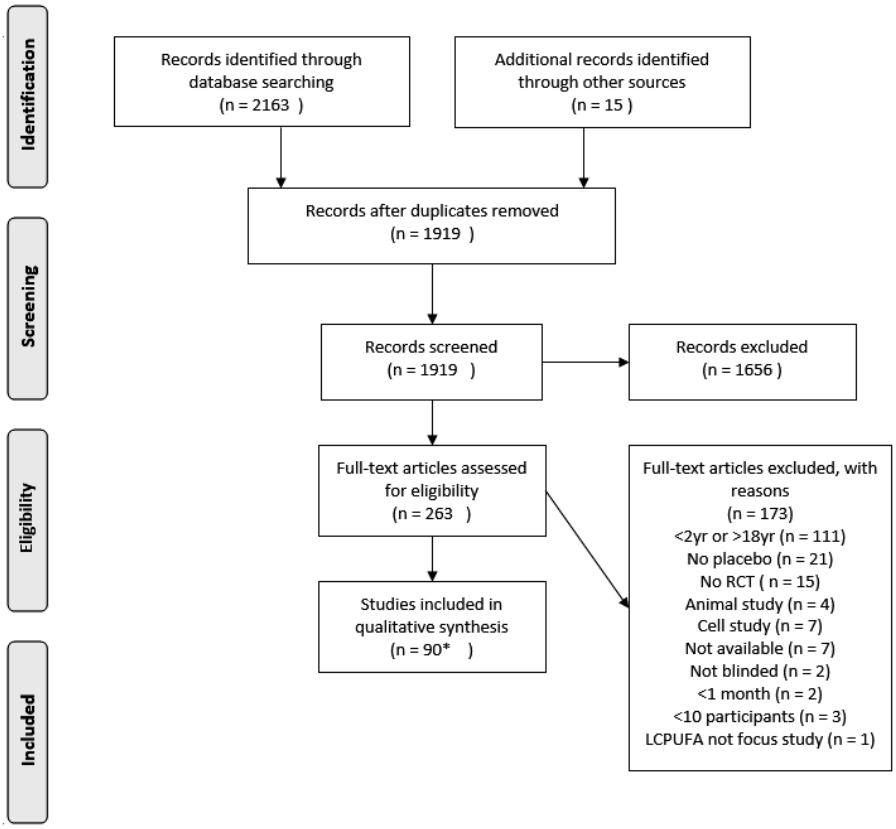

Figure 9.1. Flow diagram of study selection: 90 manuscripts were found reporting on 75 studies.

Most studies did not specify the exact method(s) of recruitment, mostly just mentioning the recruitment setting. Most studies recruited their participants from a hospital or outpatient clinic setting (n = 33). Other settings from which participants were recruited were schools (n = 23) and the community (n = 15). Nine studies reported multiple settings for recruitment; one study recruited participants from a summer camp for children with ADHD and other disorders; one study recruited from an online registry; one study recruited participants from those who participated in earlier studies; and eight studies did not mention the recruitment setting.

Looking at the efficiency percentages for started/invited, started/responded or started/finished for studies including those with an illness (averages 43.5%, 63.5%, and 83.1%, respectively) and those without (averages 36.2%, 53.1%, and 84.2%, respectively), there was a clear difference for started/invited and started/responded but not for started/finished. A comparison for average rates between studies including only children

(38.8%, 62.2%, and 85.4%, respectively), only adolescents (15.5%, 56.4%, and 87.8%, respectively) or both (53.4%, 52.7%, and 79.6%, respectively) showed notable differences. For different recruitment settings, there were mainly clear differences for started/invited. However, for all rates, the school setting had the highest average rate: hospital (17.7%, 56.8%, and 82.9%, respectively), community (29.9%, 57.1%, and 81.7%, respectively), and school (46.0%, 64.6%, and 86.8%, respectively). Lastly, when looking at these average efficiency percentages for the different continents, we also saw clear differences: Europe (35.3%, 64.3%, and 87.8%, respectively), North America (6.6%, 48%, and 77.7%, respectively), Asia (49.5%, 48.7%, and 92.6%, respectively), Africa (NA, 28%, and 91.1%, respectively), Middle East (33.2%, 64.6%, and 79%, respectively), Australia (72.9%, 69.5%, and 70.7%, respectively), and South America (54%, 93.2%, and 90.9%, respectively).

Supplementation

Most studies used capsules as the form of supplementation (n = 57), however there were also some other approaches (see Table 9.2). The number of capsules that participants were instructed to take also varied widely from 1 to 12 capsules a day, with some studies basing the dose per body weight of the participant (see Table 9.2). Moreover, a huge range of different placebos was used (see Table 9.2).

Adherence

The included studies mentioned a wide variety of methods to measure adherence: capsule count (or product weighting) (n = 30), diaries or tick-off forms (n = 13), interviews face to face/via phone/via e-mail (n = 11), taking the capsules under supervision (n = 8), and blood values (n = 5) (see Table 9.4). Thirteen studies used more than one method to assess adherence. Furthermore, 23 studies did not specify how or whether they assessed adherence. The way in which adherence was reported in the studies also varied greatly. Some studies mentioned percentages of capsules taken, the average number of capsules taken per day, blood values, or just mentioned that adherence was good or mentioned how many students were excluded due to non- adherence.

Twenty-five studies mentioned a specific percentage of adherence, which varied from 60% to 97%, mean 85% (SD 10.1). In addition, the levels of capsules that needed to be taken to be considered as being adherent differed per study, varying from 65% to 90%. Other studies defined adherence as the number of days of not taking capsules.

Table 9.1: Characteristics of studies

Reference	Age range or mean (SD)	Gender (%female)	Population: healthy, disorder or disease	Country
[58]	3–15	44	Acute lymphoblastic leukaemia	Egypt
[59]	6–12	25	ADHD	Iran
[60]	7–15	NR	ADHD	Iran
[61]	11–12	31	ADHD	Canada
[62]	8–14	0	ADHD	Netherlands
[63]	6–16	41 (after intervention)	ADHD	Israel
[64]	7–12	20	ADHD	Sweden
[65]	6–12	38	ADHD	Iran
[66]	6–12	NR	ADHD	Japan
[67,68]	8–18	15	ADHD	Sweden
[69]	12–16	0	ADHD	UK
[70,71]	6–13	23	ADHD	Australia
[72]	6–12	27	ADHD	Sri Lanka
[73]	7–13	41	ADHD	Israel
[74,75]	7–12	23	ADHD	Australia
[76]	6–13	13	ADHD	USA
[77]	8–13	25	ADHD	Israel
[78]	6–12	22	ADHD	USA
[79]	6–12	22	ADHD	Germany
[80,81]	6–13	34	ADHD	Israel
[82]	7–12	43 (after intervention)	ADHD or lower IQ	China
[83]	6–14	15	ADHD	Australia
[84]	6.9–11.9	NR	ADHD	Canada
[85]	8–16	48	Aggressive behaviour	Mauritius
[86]	6–14	42	Asthma	USA
[87]	8–12	56	Asthma	Australia
[88]	10–12	31	Asthma	Taiwan
[89]	10.2 (2.5) fish oil, 11.9 (3.1) control	48	Bronchial asthma	Japan
[90]	3–8	11	Autism	USA
[91]	5–8	NR	Autism	USA
[92]	2–5	26	Autism	Canada
[93]	3–10	17	Autism	USA
[94]	6–17	48% placebo, 46% flax oil	Bipolar disorder	USA
[95]	7.3–9.5	54	CF	Italy
[96]	5–16	47	Crohn's disease	Italy
[97]	5–12	33	DCD	UK
[98]	10.6	43	Dyslexia	Finland
[99]	15–18	100	Dysmenorrhea	USA
[100]	4–12	NR	Epilepsy	Egypt
[101]	7–9	53	Healthy	South-Africa
[102]	8–14	50	Healthy	Indonesia
[103]	9–12	51	Healthy	Japan

Reference	Age range or mean (SD)	Gender (%female)	Population: healthy, disorder or disease	Country
[104]	9–10	50	Healthy	Sweden
[105]	10–12	49	Healthy	UK
[106]	8–10	52	Healthy	UK
[107]	5–7	NR	Healthy	Canada
[108]	8–10	0	Healthy	USA
[109]	6–10	46	Healthy	Australia/ Indonesia
[110]	3–13	46	Healthy	Australia
[111]	8–14	51	Healthy	Spain
[112]	4	47	Healthy	USA
[113]	10–12	100	Healthy	Turkey
[114]	13–16	50	Healthy	UK
[115]	9–12	47	Healthy	Thailand
[116]	8–13	47	Hyperlipidaemia	Italy
[117]	14 (2)	31	Hypertriglyceridemia and low LDL	USA
[118–121]	6–11	49	Iron deficiency	South-Africa
[122]	8–12	15	Literacy problems	UK
[123]	8–12	58	Malnourished	Mexico
[124]	5–14	56	Migraine	Iran
[125]	7–14	NR	MDD	USA
[126]	6–12	NR	MDD	Israel
[127]	10–18	59 (after intervention)	Metabolic syndrome	Iran
[128]	9–17	47	NAFL and obesity	Turkey
[129,130]	11–15	14	NAFL and overweight	Poland
[131–133]	6–16	58	NAFL	Italy
[134]	10.8 (2.8)	48	NAFL and overweight	Italy
[135]	8–18	0	NAFL	Canada
[136,137]	14–17	56	Obesity	Sweden
[138,139]	13–15	0	Overweight	Denmark
[140]	9–18	NR	Overweight + insulin resistance	Mexico
[141]	5–10	NR	PKU	Italy
[142]	6–18	18	Tourette's Disorder	USA
[143–145]	10 (7)	45	Type-1 hyperphenylalaninemia, HPA	Italy
[146,147]	6–10	47	Underperforming	UK

ADHD = attention deficit hyperactivity disorder; NAFL = non-alcoholic fatty liver; MDD = major depressive disorder; DCD = Developmental Co-ordination Disorder; CF = cystic fibrosis, NR = not reported.

Table 9.2: Treatment characteristics per study

Reference	Treatment per day unless otherwise stated	Placebo	Form of supplementation	Number of capsules	Duration [b] (weeks)
[108]	DHASCO [a]: 400 or 1200 mg DHA	Corn oil	Capsules	6	8
[106]	800 mg FO: 400 mg DHA, 56 mg EPA	Olive oil	Chewable capsules	2	16
[113]	670 mg FO	Olive oil	Capsules	2	16
[110]	2400 mg FO and 600 mg evening primrose oil: 174 mg DHA, 558 mg EPA, 60 mg GLA.	Palm oil	Capsules	6	28.6
[104]	174 mg DHA,558 mg EPA, 60 mg GLA	Palm oil	Capsules	6	12 + 12 (open)
[102]	1260 mg DHA rich oil: 652 mg DHA, 101 mg EPA	Placebo oil (656 mg LA, 87 mg ALA)	Capsules	6	12
[101]	Fish flour: 892 mg of DHA per week	Placebo spread contained bread flour	Margarine	NA	14.9
[107]	14–21 mg DHA, 20–30 mg AA	Placebo supplement	Sachets to mix into foods	2–3 sachets	30
[103]	FO: 3600 mg DHA, 840 mg EPA per week	50% soybean oil, 50% rapeseed oil (4200 mg LA per week)	Bread and sausages	NA	12
[114]	541 mg FO: 116 mg DHA, 165 mg EPA	Sunflower oil	Capsules	2	12
[112]	DHASCO-S [a]: 400 mg DHA	High oleic sunflower oil	Capsules	2	16
[115]	FO: 1 g DHA, 200 mg EPA	Soybean oil	Chocolate milk	NA	15.6
[109]	88 mg DHA, 22 mg EPA	Unclear	Drink	NA	52
[105]	500 mg DHASCO-S [a]: 200 mg DHA, 4 mg EPA	Vegetable oil (15 mg ALA, 250 mg LA)	Capsules	5	8
[111]	FO in dairy drink 120 mg DHA, 60 mg EPA	Whole milk	Milk drink	NA	20
[117]	4 g FO: 1.5 g DHA, 1.86 g EPA	Corn oil	Unclear	Unclear	8 + 8 with 4 w wash-out in between
[100]	3 mL dose of 1200 mg FO: 240 mg DHA, 36o mg EPA.	Corn oil	Liquid oil	NA	12
[88]	FO: 125 mg DHA, 230 mg EPA	Corn oil	Capsules	Dependent on bw	16
[96]	3 g O3FA	Olive oil	Capsules	Dependent on bw	52
[92]	1.875 mL FO: 0.75g of DHA + EPA. If well tolerated dose ×2 after 2 wks.	Olive oil and medium chain triglycerides.	Liquid oil	NA	24
[65]	165 mg DHA, 635 mg EPA, 100 mg other O3FA	Olive oil	Capsules	NS	8
[67,68]	174 mg DHA, 558 mg EPA, 60 mg GLA	Olive oil	Capsules	6	12 + 12 (open)
[97]	FO and EPO: 174 mg DHA, 558 mg EPA, 60 mg GLA	Olive oil	Capsules	6	26
[79]	120 mg DHA, 600 mg EPA	Olive oil	Capsules	2	16
[66]	DHA-rich fish oil: 3600 mg DHA 700 mg EPA per week.	Olive oil	Milk and bread	NA	12

Reference	Treatment per day unless otherwise stated	Placebo	Form of supplementation	Number of capsules	Duration [b] (weeks)
[122]	480 mg DHA, 186 mg EPA, 96 mg GLA, 864 mg LA, 42 mg AA, 8 mg thyme oil	Olive oil	Capsules	NR	12
[76]	480 mg DHA, 80 mg EPA, 40 mg AA, 96 mg GLA	Olive oil	Capsules	8	16
[143–145]	LCPUFA supplementation: varying dosage	Olive oil	Capsules	1 per 4 kg of bw	52
[94]	Flax seed oil: 0.55 to 6.6 g ALA	Olive oil	Capsules	Varying up to 12	16
[89]	FO: DHA 7.3 ± 11.5 mg/kg of bw, EPA 17.0 ± 26.8 mg/kg of bw	Olive oil	Capsules	Dependent on bw: 6–12	43.6
[83]	PCSO-524 [c]: 16.5–22 mg DHA, 21.9–29.2 mg EPA	Olive oil, lecithin and coconut oil	Capsules	Dependent on bw: 3–4	14
[86]	Drink containing FO (1.6 g DHA, 3 g EPA) and borage oil (3.0 g GLA)	Control drink with high oleic safflower oil	Drink	NA	12
[70]	EPA-rich FO: 108 mg DHA, 1,109 mg EPA or DHA-rich FO: 1,032 mg DHA, 264 mg EPA	Safflower oil	Capsules	4	16 + 16 + 16
[90]	FO: 1.1 g DHA + EPA	Safflower oil	Pudding packet	2 pudding packs	12
[123]	FO: 180 mg DHA, 270 mg EPA	Soybean oil	Capsules	3	12
[135]	2 g FO: 1200 mg DHA + EPA	Sunflower oil	Capsules	4	24
[87]	FO: 1.2 g O3FA	Sunflower oil	Capsules, salad dressing and margarine	4	24
[72]	FO and EPO oil: 592.74 mg O3FA	Sunflower oil	Capsules	2	26
[58]	1 g FO:120 mg DHA, 180 mg EPA	Sunflower oil	Capsules	Unclear	24
[61]	100–400 mg DHA, 500–100 mg EPA	Sunflower oil	Capsules	Dependent on bw: 2–4	16
[129,130]	AO: 450–1300 mg O3FA (DHA: EPA in 3:2 proportion)	Sunflower oil	Capsules	Dependent on bw	24
[116]	AO: 500 mg DHA or FO:500 mg DHA + EPA	Wheat germ oil	Capsules	1	16
[131–133]	AO: 250 or 500 mg DHA	Germ oil	Capsules	1	26.1
[134]	AO: 250 mg DHA	Germ oil	Capsules	NR	26
[95]	Algae triacylglycerol 100 mg DHA/kg/day 1st month then 1 g DHA/day	Germ oil	Capsules	4	52
[93]	AO: 200 mg DHA	Corn oil + soy bean oil	Capsules	1	26
[146,147]	AO: 600 mg DHA	Corn oil + soy oil	Capsules	3	16
[138,139]	4.9 g FO: 892 mg DHA, 191 mg EPA	6:1:1 mix of palm shortening, soy oil, and rapeseed oil	Bread	NA	16
[141]	2.5–4 g FO (12% DHA, 18% EPA)	Blackcurrant seed oil (45.7% LA, 18% GLA, 14% ALA)	Capsules	Dependent on bw: 5-8	26
[62]	650 mg DHA, 650 mg EPA	Normal margarine (1 g LA)	Margarine	NA	16

Reference	Treatment per day unless otherwise stated	Placebo	Form of supplementation	Number of capsules	Duration [b] (weeks)
[99]]	FO: 720 mg DHA, 1080 mg EPA	1800mg lactose	Capsules	2	8+8
[125]	200 mg DHA, 1400 mg EPA, 400 mg other O3FA	Placebo capsule	Capsules	2	12
[136,137]	FO and EPO: 290 mg DHA, 930 mg EPA, 100 mg GLA	Placebo	Capsules	10	12 + 12 with 6w wash-out in between
[91]	FO: 1.1 g DHA + EPA	Identical placebo	Pudding packet	2 pudding packs	6
[128]	1000 mg PUFA	Placebo	Capsule	1	52
[63]	2 g sage oil: 1 g ALA	Lactose placebo	Capsules	2	8
[60]	240 mg DHA, 360 mg EPA	Placebo	Capsules	2	8
[64]	FO: 2.7 mg DHA, 500 mg EPA	Placebo	Capsules	1	15
[127]	2.4 g omega-3	Vitamin E or placebo	Tablets	NR	8
[84]	100 mg DHA, 250 mg EPA, 25 mg phospholipids	Sunflower oil	Capsules	According to bw: 1–2	16
[124]	1 g FO: 120 mg DHA, 180 mg EPA	Placebo capsule	Capsules	1	At least 8wks
[78]	Algae oil: 345 mg DHA	Placebo capsule	Capsules	1	16
[59]	241 mg DHA, 33 mg EPA, and 180 mg omega-6	Identical placebo	Capsules	1	10
[118–121]	FO: 155 mg DHA, 29 mg EPA	Placebo	Capsules	2	15
[142]	Varying 500–6000 mg O3FA	Placebo	Capsules	Varying up to 12	20
[140]	Salmon oil: 360 mg DHA, 540 mg EPA	Placebo (corn starch, lactose, magnesium stearate and polyvinyl pyrrolidone)	Capsules	NR	4
[85]	300 mg DHA, 200 mg EPA, 400 mg ALA, 100 mg of DPA	Drink without omega-3	Drink	NA	24
[77]	FO: 96 mg DHA, 153 mg EPA or n-3 LC-PUFA containing PLs: 95 mg DHA, 156 mg EPA	Rapeseed oil	Chocolate flavoured spread	NA	13.1
[73]	240 mg LA, 60 mg ALA, 95 mg mineral oil	Vitamin C capsules	Capsules	1	7
[69]	FO and EPO: 174 mg DHA, 558 mg EPA, 60 mg LA.	Medium chain triglycerides	Capsules	6	12
[126]	200 mg DHA 400 mg EPA, or 180 mg DHA, 380 mg EPA	Olive oil or safflower oil	Capsules	1–2	16
[74,75]	FO and EPO: 174 mg DHA, 558 mg EPA, 60 mg GLA	Palm oil	Capsules	6	30
[98]	500 mg ethyl-EPA	Triglycerides and cellulose	Capsules	NR	12.9
[80,81]	1–15 wk: 120 mg EPA + DHA 16–30 wk: 60 mg EPA + DHA	Cellulose	Capsules	4	15 + 15
[82]	321 mg DHA, 42.2 g EPA per 100 g egg	Ordinary egg	Egg	1	13.1

bw: body weight, FO: fish oil, NA: not appropriate, NR: not reported

[a] DHASCO is an algal triglyceride DHA; [b] Some studies gave duration in months or number of days supplementation was received, we recalculated the duration to weeks; [c] PCSO-524 is an lipid extract of the New Zealand green-lipped mussel;

Looking at the adherence percentage between studies in healthy and diseased children, there seemed to be a slightly lower average adherence in diseased children (M = 83.7%, SD = 11.9), compared to healthy children (M = 87.6%, SD = 7.1). When we looked at the different age groups recruited, there seemed to be a lower average adherence in the child only group (M = 82.5%, SD = 9.5), compared to adolescents (M = 89.2%, SD = 1.1) or the combined group (M = 88.5%, SD = 11.2). The difference in average adherence in different recruitment settings was less clear; hospital (M = 86%, SD = 10.6), community (M = 89.5%, SD = 7.8), and school (M = 83.9%, SD = 11.6). The average adherence rate also differed between continents with a lower average rate in Australia and USA/Canada: Europe (M = 87.5%, SD = 9.3), USA/Canada (M = 78.7%, SD = 6.5), Asia (M = 92%, SD = 1.4), Africa (M = 95%, SD = 0.5), Australia (M = 79.7%, SD = 13.5), and South America (M = 94.5%, just one study). There seemed to be a tendency for higher average adherence when capsules were used (M = 88.2%, SD = 8.0) instead of food (M = 74.8%, SD = 14.3) or drinks (M = 81.5%, SD = 9.2), or other forms of supplementation (M = 80.3%, SD = 18.0). Some studies mentioned that participants took capsules under supervision, but they did not show a higher mean adherence (M = 82.8%, SD = 15.2) than those that did not have supervision of capsule intake (M = 86.2%, SD = 8.3). Seven studies, that reported adherence, reported that participants consumed capsules more than once a day while 12 studies, that reported adherence, mentioned that the capsules were only taken once a day. There was no difference in average adherence between those two methods of supplementation (M= 87.3%, SD = 9.4 vs. M = 87.6%, SD = 7.8). Fifteen studies, reporting adherence, mentioned talking to parents or participants either via telephone or face to face (or via e-mail) during the study about the supplementation to increase adherence [61,67,69,70,72,90,91,107,111,113,131,134–136,143]. The studies that included a phone call did not have a higher average adherence rate (M = 81.5%, SD =9.5) than those that did not include a phone call (M = 86.2%, SD = 10.3). There were three studies that provided some form of incentive [98,107,146], however only one of these studies reported an adherence percentage. Forty-six studies mentioned that they took either blood or cheek samples, but only five studies mentioned that they used blood as an adherence measure [61,85,86,117,134]

Table 9.3: Recruitment effort and recruitment rates

Reference	Invited	Responded/ screened	Started	Finished	Started/ invited %	Started/ responded %	Started/ finished %	Recruitment method	Recruitment setting	Study period
[141]	NS	NS	21	21	-	-	100	NS	Department of Paediatrics	NS
[66]	46	40	40	40	87	100	100	Parents of summer camp participants were asked.	Summer camp for children with psychiatric disorders	NS
[98]]	107	107	61	61	57		100	Teachers nominated children with reading difficulties	School	Autumn 2005–January 2006
[131–133]	NS	NS	60	60	-	-	100	NS	Hospital	NS
[115]	NS	NS	180	180	-	-	100	NS	School	NS
[116]	NS	NS	36	36	-	-	100	NS	Hospital	8 month period
[146,147]	1376	675	362	359	26	54	99	Parents of underperforming children received a letter inviting their children to take part in the formal screening assessments.	School	NS
[105]	NS	NS	90	88	-	-	98	Via advertising in newspapers and schools	Community and schools	NS
[88]	NS	298	197	192	-	66	98	Participants with asthma diagnosis were recruited from elementary schools through parent conferences	Schools	NS
[82]	1556	1556	179	171	12	12	96	Children were screened from students in two township primary schools	Schools	NS
[62]	NS	372	79	76	-	21	96	Via hospital and advertising at schools.	Hospital and schools	NS
[72]	NS	422	98	94	-	23	96	NS	Outpatient treatment program	NS
[114]	NS	408	196	189	-	48	96	NS	School	NS
[58]	NS	100	70	65	-	70	93	NS	Hospital	NS
[117]	NS	NS	42	39	-	-	93	NS	Hospital	NS
[118–121]	NS	926	321	294	-	35	92	Parents were invited to an information meeting.	School	November 2009–November 2010
[127]	NS	NS	90	83	-	-	92	NS	Cardiovascular Research Centre	NS
[60]	NS	NS	75	69	-	-	92	NS	Outpatient ADHD clinic	2007

Reference	Invited	Responded/ screened	Started	Finished	Started/ invited %	Started/ responded %	Started/ finished %	Recruitment method	Recruitment setting	Study period
[103]	NS	230	179	166	-	78	92	Via advertisements	Community	NS
[85]	NS	938	200	184	-	21	92	Via parents who themselves had participated in a study.	Participants earlier study	November 2009–December 2011
[123]	NS	59	55	50	-	93	91	Parents were invited to a meeting at which the study procedures were explained and a written informed consent from the tutors and a verbal assent from their children were obtained.	School	NS
[95]	NS	NS	41	37	-	-	90	NS	Hospital	NS
[101]	NS	NS	183	164	-	-	90	NS	School	NS
[138,139]	3652	NS	87	78	2	-	90	Subjects were recruited via addresses obtained from the Danish Civilian Person Register.	Community	NS
[111]	NS	NS	119	107	-	-	90	NS	School	NS
[99]	NS	NS	42	37	-	-	88	NS	School	NS
[134]	NS	118	58	51	-	49	88	NS	Hospital	May 2012–September 2014
[113]	NS	44	33	29	-	75	88	Via public flyers	Community	NS
[135]	NS	30	24	21	-	80	88	NS	Hospital	NS
[87]	NS	NS	45	39	-	-	87	NS	NS	Over period of 16 mo.
[108]	NS	48	38	33	-	79	87	NS	NS	NS
[112]	NS	405	202	175	-	50	87	NS	NS	NS
[86]	NS	NS	43	37	-	-	86	NS	Outpatient clinic	NS
[65]	NS	NS	120	103	-	-	86	NS	Hospital	NS
[97]	189	129	117	100	62	91	86	Letters of invitation were sent to parents of children who were identified by teachers.	School	NS
[78]	NS	250	63	54	-	25	86	Via advertisements	Community	NS

Reference	Invited	Responded/ screened	Started	Finished	Started/ invited %	Started/ responded %	Started/ finished %	Recruitment method	Recruitment setting	Study period
[79]	NS	334	110	95	-	33	86	Via health professionals, teachers, leaflets handed out to support groups, leaflet distributed at community centres and advertisements in a free of charge regional newspaper.	Community, Health professionals, schools, support groups.	NS
[64]	NS	NS	109	92	-	-	84	NS	Hospital and secondary treatment centres	January 2005–June 2007.
[129,130]	NS	86	76	64	-	88	84	NS	Hospital	2008-2011
[92]	NS	101	38	32	-	38	84	NS	Hospital	December 2010–December 2013
[107]	NS	NS	37	31	-	-	84	NS	NS	NS
[143–145]	NS	NS	24	20	-	-	83	NS	NS	Recruited over 6 month
[125]	NS	178	23	19	-	13	83	Via advertisements and clinician referrals.	Community and referral	July 2011–May 2014
[109]	NS	932	780	643	-	84	82	Via advertisement at schools and media advertisement.	Schools	Auguet 2003–April 2005
[136,137]	108	47	31	25	29	66	81	NS.	Outpatient clinic	NS
[73]	NS	~300	78	63	-	26	81	Via advertisement on radio health program, in health newspapers and in ADHD clinics.	Community and ADHD clinic	January 2007–June 2007
[80,81]	NS	247	200	162	-	81	81	Advertisements in newspapers, on the Internet and medical centres.	Community	NS
[91]	863	118	57	45	7	48	79	E-mail invitations to in registry and longitudinal study of families of children affected by ASD.	Online registry	18 September 2012–31 December 2012
[67,68]	NS	NS	75	59	-	-	79	NS	Hospital	October 2004–August 2006
[128]	NS	NS	138	108	-	-	78	NS	Outpatient clinic	March 2010–June 2012
[122]	NS	NS	41	32	-	-	78	NS	School	NS
[106]	NS	511	450	348	-	88	77	Via school	Schools	NS

Reference	Invited	Responded/ screened	Started	Finished	Started/ invited %	Started/ responded %	Started/ finished %	Recruitment method	Recruitment setting	Study period
[89]	NS	NS	30	23	-	-	77	NS	Hospital	January 1994–March 1995
[142]	NS	NS	33	25	-	-	76	Via community, hospital and through patient association.	Community and referral	NS
[69]	NS	138	76	57	-	55	75	School and parent group circulated screening information to all potential eligible families	Schools and parent groups	NS
[83]	NS	351	144	108	-	41	75	NS	NS	NS
[77]	250	102	83	60	33	81	72	Newspaper advertisement	Community	July 2004–January 2005
[126]	NS	NS	28	20	-	-	71	NS	Hospital	NS
[93]	NS	143	48	34	-	34	71	Via recruitment flyers across campus and sent to autism support groups.	Campus, autism support groups	NS
[61]	NS	NS	37	26	-	-	70	NS	ADHD clinic	NS
[90]	NS	32	27	19	-	84	70	NS	Outpatient autism clinic	5 November 2008–25 June 2009
[84]	NR	NR	37	26	-	-	70	NS	NS	NS
[104]	NS	162	154	105	-	95	68	Via teachers who informed families	School	December 2009–July 2011
[76]	NS	193	50	33	-	26	66	NS	Community	NS
[74,75]	NS	201	167	109	-	83	65	NS	NS	Start March–May 2004
[70]	NS	199	96	57	-	48	59	Via media releases, television interviews, newspaper advertisements, school newsletters, and flyers.	Community and School	June 2007–June 2009
[110]	560	447	408	227	73	91	56	Via information sessions and school newsletters.	Schools	December 2010–May 2011
[94]	NS	NS	51	24	-	-	47	NS	Hospital	November 2001–March 2005
[63]	NS	NS	40	17	-	-	43	NS	ADHD clinic	NS

Reference	Invited	Responded/ screened	Started	Finished	Started/ invited %	Started/ responded %	Started/ finished %	Recruitment method	Recruitment setting	Study period
[59]	NS	NS	40	NS	-	-	-	NS	Outpatient ADHD clinic	June 2009–March 2010
[124]	NS	NS	25	NR	-	-	-	NS	Hospital	NS
[140]	142	NS	76	NS	54	-	-	From previous sample children with insulin resistance were identified and invited	Community	NS
[102]	NS	NS	233	NS	-	-	-	Via school	School	NS
[100]	NS	NS	70	NS	-	-	-	NS	Hospital	NS
[96]	NS	NS	38	NS	-	-	-	NS	Hospital	NS

NS = not specified.

Drop-out

Sixty-five of the 75 included studies mentioned a drop-out rate or included numbers which made it possible to calculate the drop-out rate. The average drop-out was 17% (SD 13%), but it varied between 0% and 58% (see Table 9.4). There was no clear difference in average drop-out rate between studies in healthy (mean = 16.5%, SD = 11.5) and diseased populations (M = 17.9%, SD = 13.7). There was a difference in average drop-out with regard to the recruited age group: children M = 15%, SD = 11.1), adolescents (M = 12.3%, SD = 7.3) or both (M = 21.5%, SD = 14.9); with a higher average drop-out rate in the combined age group.

There was also no clear difference in mean drop-out between recruitment setting: hospital (M = 15%, SD = 11.1), community (M = 18.2%, SD = 8.6) or school (M = 15.3%, SD = 13). Differences could be seen in the average drop-out according to the continent on which the study was executed: Europe (M = 13.3%, SD = 9.8), USA/Canada (M = 23.1%, SD = 11.6), Asia (M = 6.8%, SD = 7.4), Africa (M = 11.6%, SD = 3.9), Middle East (M = 20.1%, SD = 16.7), Australia (M = 34.9%, SD = 7.8), and South-America (M = 9.1%, just one study). When looking at different forms of supplementation, no clear differences in average drop-out rate could be seen: capsules (M = 17.5%, SD = 12.9), food (M= 14.1%, SD = 14.9), drinks (M = 19%, SD = 13.7), and others (M = 17.9%, SD = 8.4). Eight studies who reported drop-out rate mentioned that capsules were taken under supervision, this seemed to lead to somewhat lower average drop-out rate (M = 13%, SD = 15.6), compared to the 57 studies in which participants did not take the capsules under supervision (M = 17.9%, SD = 15.6). Sixteen studies that reported drop-out rate divided the capsules over multiple intake moments (M = 17.2%, SD = 9.3). This did not seem to increase or decrease the average drop-out rate if compared to those studies that specified one intake moment (M = 17.1%, SD = 13.6). Fourteen studies that noted drop-out rate reported that they contacted the participants during the study. Studies that did so seemed to have a slightly higher average drop-out rate (M = 20.4%, SD = 11.4) than studies that did not contact participants during the study (M = 16.5%, SD = 14.4). Of the studies that reported giving participants an incentive, two mentioned a drop-out rate, this was on average 15.3% (SD 20.4). Studies that did not state an incentive had an average drop-out rate of 17.4% (SD 12.7). Of the 65 studies that mentioned a drop-out rate, 50 specified a reason for drop-out (six did not have drop-out, and nine did not specify the drop-out). Fifty-two different reasons for drop-out were mentioned, with the most common reasons mentioned being lost to follow-up, poor or no adherence or inability to take supplement.

Table 9.4:. Adherence and drop out characteristics per study.

Reference	Adherence assessment	Adherence (mean or nr. of part. non-adherent	Blood FA determined?	Drop-out rate (%) Treatment	Placebo
Healthy					
[114]	Supervision and tick-off form	Active: 88.4%, Placebo: 88.5%	Y	3.1	6.1
[115]	Supervision	NR	Y	0	0
[108]	NR	NR	Y	Low DHA: 20; High DHA: 7.1	17
[112]	Capsule count	Nearly 100%	Y	7.1	5.6
[101]	Supervision	Active: 94.8%, Placebo: 94.5%	Y	11	9.8
[106]	Pill diary by teacher or parent	Active: 68.4%, Placebo: 66.7%	Y	24	21
[102]	NR	NR	Y	NR	NR
[103]	NR	> 90%.	Y	6.7	7.8
[109]	Sachet count and diary (Australia)/Supervision (Indonesia)	Australia: 73-84% Indonesia: 85-87%	Y	27 3.6	34 5.3
[111]	Interview	small increase in DHA in supplemented group	Y	NR	NR
[107]	Diary	*n* = 6	Y	NR	NR
[105]	Parental signing of diary card	> 80%.	N	NR	NR
[113]	NR	NR	N	5.9	19
[110]	Supervision	Phase 1: 59%, Phase 2: 61%	N	47	42
With disorder or illness					
[141]	NR	NR	Y	0	0
[140]	Pill count	Active: 93%, Placebo: 96%	Y	NR	NR
[82]	Supervision	count of consumed eggs showed good compliance and % of adherence to treatment was 100%	Y	5.6	3.2
[127]	Pill count	pill count revealed no essential irregularities	Y	13.3	Vit. E: 0, Placebo: 10
[84]	NR	NR	NR	NR	NR
[80,81]	Pill count	*n* = 14	N	20	18
[83]	Pill count, compliance diary and telephone call	96.7%	N	23	23
[95]	NR	*n* = 2 DHA supplementation induced a median plasma DHA enrichment of 5% suggesting adherence	Y	14	5
[138,139]	Interview	90%	Y	NR	NR
[79]	Capsule count	*n* = 1	Y	13	11
[86]	Diary and blood values	80–85%	Y	21.1	11.1
[77]	Phone calls and product weighting	*n* = 6	Y	Phospholipids: 38, Fish oil: 25	24
[118–121]	Supervision	95.4%	Y	6.9	9.9
[61]	Blood	NR	Y	CO	CO
[94]	Capsule count and diary	➢ 75%	Y	42	64

Reference	Adherence assessment	Adherence (mean or nr. of part. non-adherent	Blood FA determined?	Drop-out rate (%) Treatment	Placebo
[70]	Capsule count	EPA: 83%, DHA: 86% , LA: 85%	Y	CO	CO
[87]	Capsule count	75%	Y	NR	NR
[76]	Diary	88%	Y	28	40
[143–145]	NR	NR	Y	17	17
[131–133]	Capsule count and interview	excellent in all groups	Y	NR	NR
[62]	Product weighting	*n* = 1	Y	0	5.1
[135]	Capsule count and interview	NR	Y	0	25
[78]	Capsule count	Active: 96.7%, Placebo: 100%	Y	15	13
[93]	Capsule count	excellent	Y	21	38
[136,137]	Capsule count	*n* = 1	Y	CO	CO
[90]	Parent interview	Active: 69% , Placebo: 75%	Y	36	23
[69]	Capsule count	FA changed in the expected direction.	Y	24	30
[89]	NR	NR	Y	27	14
[64]	Capsule count	NR	Y	30	19
[116]	Capsule count	DHA: 96.5%, DHA + EPA: 96.9%, Placebo: 96.7%	Y	DHA: 0, DHA + EPA: 0	0
[117]	Blood value	NR	Y	CO	CO
[129,130]	Capsule count	95.5%	Y	21	11
[128]	Capsule count	NR	Y	NR	NR
[134]	Blood values	*n* = 5	Y	14	10
[98]	NR	According to parents children took the capsules carefully	Y	NR	NR
[88]	Supervision and capsule count	Pill count: 91%	Y	0	0
[92]	NR	there was no overlap between the distributions of plasma levels between groups at week 24	Y	21	11
[65]	Capsule count	*n* = 5	N	NR	NR
[97]	Capsule count and diary	Period 1: 88.7%, Period 2: 85.5%	N	17	12
[91]	Parents e-mail	Active: 69%, Placebo: 83%	N	28	14
[85]	Parent interview and blood values	Average number of drink per week 6.5.	N	10	22
[73]	Capsule count	Active 7.88 capsules left; Placebo: 14 capsules left	N	18	21
[125]	NR	89–97%	N	10	23
[142]	NR	NR	N	18	31
[67,68]	Parent interview	Period 1: 93.4%, Period 2: 93.3%	N	CO	CO
[126]	NR	*n* = 5	N	NR	NR
[122]	Capsule count	Active: 90.4%, placebo 86.6%	N	23	21
[74,75]	Capsule count and diary	*n* = 2	N	CO	CO
[63]	Capsule count	NR	N	60	55

Reference	Adherence assessment	Adherence (mean or nr. of part. non-adherent	Blood FA determined?	Drop-out rate (%) Treatment	Placebo
[59]	NR	NR	N	NR	NR
[124]	NR	NR	N	NR	NR
[100]	NR	NR	N	NR	NR
[96]	NR	Compliance was optimal	N	NR	NR
[66]	NR	NR	N	0	0
[146,147]	Diary	At school: 75%	N	0.6	1.1
[72]	NR	NR	N	2	6.1
[58]	NR	*n* = 5	N	8.6	5.7
[60]	NR	NR	N	NR	NR
[123]	Diary and capsule count	NR	N	0	20
[104]	Interview	Active: 94%, Placebo: 92%, Period 2: 91%	N	CO	CO
[99]	NR	*n* = 1	N	CO	CO

CO: cross-over study, NR: not reported.

Discussion

We conducted a thorough review to examine recruitment, adherence and drop-out rates in n-3 LCPUFA supplementation studies in children and adolescents, in order to identify strategies which can be implemented to improve those rates. Even though the CONSORT guidelines clearly state what data need to be included in the report of a RCT, the majority of the included studies did not provide a flow-chart (55% did not) or the dates defining the period of recruitment and follow-up (70% did not).

Recruitment

The majority of studies provided minimal details about the recruitment process. The low number of studies that reported the number of participants that they invited and screened is, however, not uncommon in research studies as similar numbers were reported by Toerien *et al.* who studied 129 studies in six major journals [148]. The literature does give some suggestions for methods that could increase recruitment; for example, telephone calls to those who do not reply, an opt-out system (participants contact the researchers if they do not want to participate, please do note that this is not legal in all countries), including incentives, making trials open, and in person recruitment [149]. The use of clinical referral is also suggested to be related to higher recruitment rates, as most patients will have a trusting relationship with their doctor [150]. When we looked at the research setting (hospital, community, school), though, the mean started/invited rate and mean started/responded rate seemed to be slightly higher in the school setting. However, in the studies that looked at diseased populations, the average percentage efficiency of started/invited and started/responded was higher than studies looking at healthy populations (M = 43.5% vs. M= 36.2% and M = 63.5 vs. M = 53.1%, respectively). It has been shown that in adolescents, giving monetary incentives does improve response rates and has a positive effect on their willingness to participate in studies [151]. However, the

provision of monetary incentives might be considered unethical in children/adolescents [152,153]. One might thus consider a form on non-monetary incentive, for example in Food2Learn participants received a cinema voucher [19]. In the current review, there were only three studies that provided an incentive and these studies did not have remarkably higher recruitment rates. Hence, more studies that do provide incentives are needed to elucidate whether or not incentives improve recruitment. Moreover, there are myriad reasons as to why somebody would or would not participate in a study. There are participant characteristics which in adults have been associated with a higher chance of non-participation such as younger age, being male, lower social economic status, and lower education level [154,155]. However, in the limited number of studies on recruitment in children, no association between age or sex has been seen, although the education level of parents was associated with higher enrolment rates [5].

Beliefs about the effectiveness of the treatment may also play a role. Examples of reasons as to why adolescents did not participate informally given in Food2learn included: (1) the belief that n-3 LCPUFA are not effective in improving health; (2) the belief that they already consume sufficient amount of n-3 LCPUFA/already eat healthy; (3) the belief that participation will take too much time/effort; and (4) lack of interest in research in general. These factors should be taken into account during the research process and it seems wise to include explanations that most people do not get enough n-3 LCPUFA in their diet as well as elaborating on the possible health benefits of n-3 LCPUFA specific to the age group being assessed.

Adherence

Just 25 studies mentioned a specific adherence percentage, which varied between 60% and 97% with a mean of 85%. Moreover, most studies included in the current review used indirect adherence assessment methods (i.e., diaries, interviews, and capsules counts) which are all subject to problems with reporting bias and errors or intentional manipulation [156]. More direct methods such as the determination of fatty acid levels in the blood seems to be the most reliable method to assess adherence, which was done in only five studies. However, it should be noted that taking blood samples in younger children might not be acceptable for all parents or ethical committees and could therefore lead to lower recruitment numbers.

In the current review, there was no difference in mean adherence in those studies where participants received a telephone call to try and increase adherence compared to those in which participants received no telephone call (M = 81.5% vs. M = 87.7%). There were only three studies that provided an incentive and only one of these studies provided an adherence percentage, which was 75%. There seemed to be a higher average adherence of capsules (M = 88.2%) compared to other forms of supplementation (M = 74.8%, M = 81.5%, M = 80.3%, for food, drink and other forms, respectively). Lastly, there was no difference in the mean adherence between those who took capsules multiple times a day compared to those who took capsules only once a day (M = 87.3% vs. M = 87.6%). It is however important to remember that all these findings are based on only 25 studies that mentioned an adherence rate.

Other studies suggest factors that are associated with higher adherence in children and adolescents, these include: sociodemographic factors (i.e., older children and older

adolescents are less likely to be adherent, and boys are less likely to be adherent), disease associated factors (i.e., if the disease also has positive symptoms the person is less likely to be adherent), the belief and attitude that a person has towards the treatment (i.e., those that belief that the treatment will be effective are more likely to be adherent), their mood (i.e., those with depression are less likely to be adherent) and the social context (i.e., those who are supported by family and friends are more likely to be adherent)[157,158]. Methods to increase adherence rates have also been suggested. Methods that have been employed to increase adherence include: educating participants about adherence, making medicine (or supplementation) more palatable, providing incentives/tokens, and involving parents or schools [159,160]. However, one must take into consideration that the vast majority of studies looking at which methods can help increase adherence have been executed in a medical setting with patients requiring medications and these results do not by definition translate to nutritional interventions in healthy participants or those with diagnosed disorders such as ADHD.

Some suggestions for improving adherence for n-3 LCPUFA supplementation studies may include: providing sufficient information about the importance of adherence (i.e., explaining the importance of adherence to get valid results), getting parents involved, and providing appropriate incentives [159,160].

Drop-out

In the current review, the average drop-out was 17% (range 0–58%). Three studies mentioned some form of incentive [98,107,146] and they reported a slightly lower average drop-out than those that did not use (or did not report) an incentive (M = 15.3% vs. M = 17.4%). There were differences in average drop-out rates between continents, with drop-out rates being higher in Australia (M = 34.9%), USA/Canada (M = 23.1%), and the Middle East (M = 20.1%) compared to Europe (M= 13.3%), Africa (M = 11.6%) and Asia (M = 6.8%). We can only speculate about explanations for this difference (e.g., individualistic vs. collective societies) and do point out that these differences have to be interpreted with caution as the number of studies per continent did differ greatly. A number of methods to decrease drop-out in studies involving adults has been suggested. They include emphasizing the benefit of participation, flexible scheduling of appointments, regular positive communication from the research team to the participants (e.g., birthday and Christmas cards, newsletters, etc.), a consistent research staff so participants can build a bond with the researchers, and appropriate incentives [150,161–163]. Other strategies that have been suggested include decreasing the complexity of the treatment and limiting the number of follow-up visits to the bare minimum [164]. Furthermore, a combination of multiple strategies is suggested to be most effective in increasing retention [161,164]. All these methods to decrease drop-out have been studied in adults; more research on methods to decrease drop-out in children and adolescents in RCT is warranted.

Suggestions for decreasing drop-out in n-3 LCPUFA supplementation trial include: keeping in regular contact with the participants, providing flexible appointment possibilities, providing incentives for participants and providing reminders. With regard to the supplement, one should keep the regime as simple as possible e.g., one (concentrated) capsule per day [150,161–164].

Strengths and limitations

Limitations of the current review include the fact that many of the included studies did not report all data on recruitment, dropout and (assessment of) adherence. Due to the incomplete reporting of data, results should be viewed with caution. The main advantage of the current review is the fact that we included all studies investigating n-3 LCPUFA supplementation in children/adolescents regardless of whether they were healthy children/adolescents or children/adolescents with a disease or disorder.

Conclusions

The conclusions drawn are based on minimal reporting from the included studies in this review. Less than half of the included studies abided by the CONSORT guidelines. Problems with recruitment and drop-out seem to be common in n-3 LCPUFA supplementation trials in children and adolescents. However, since the reporting about recruitment, adherence and dropout rates was very heterogeneous and minimal in the included studies, we cannot provide specific suggestions to improve LCPUFA supplementation studies in children and adolescents.

Recommendations

It is important for future studies to report on recruitment effort and rate, adherence (including the method of assessing adherence) and drop-out rates according to the CONSORT Guidelines.

Suggestions from other scientific areas to increase recruitment, adherence and minimize drop-out include: the provision of sufficient information about the importance of adherence (i.e., explaining the importance of adherence to get valid results), getting parents involved, provision of appropriate incentives, emphasizing the benefit of participation, being flexible with the scheduling of appointments, the research team engaging in regular positive communication with the participants, having a consistent research staff member so participants can build a bond with the researchers and to keep the supplementation regime as simple as possible.

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Chapter 10

General discussion

This thesis investigated the influence of long-chain polyunsaturated fatty acids (LCPUFA) on brain functioning in children and adolescents. To do so three aspects related to brain functioning were studied: school performance, cognition, and mental well-being.

To study the association between LCPUFA, measured during pregnancy, at birth, and at age 7, and school performance in children at age 7, data from the Maastricht Essential Fatty Acid Birth (MEFAB) cohort were used. The data used from the MEFAB cohort were the maternal plasma fatty acid concentrations during pregnancy (i.e., at study entry (before 16 weeks of pregnancy), at 22, and 32 weeks of pregnancy), maternal plasma fatty acid status at birth, cord fatty acid concentration, child plasma fatty acid concentration at age 7, and scores on standardized school tests (spelling, reading, arithmetic).

To investigate the influence of n-3 LCPUFA supplementation on cognition, Food2Learn was designed. Food2Learn was a double-blind randomized, placebo controlled repeated measures intervention trial in adolescents attending lower general secondary education. Participants received either krill oil (source of n-3 LCPUFA) or a placebo for one year. During that year blood samples (via a finger prick) were taken at baseline, 3, 6, and 12 months. At baseline, 6, and 12 months participants executed a number of neurocognitive tests, and filled out a number of mental well-being questionnaires. To study the influence of n-3 LCPUFA on mental well-being, defined as depression and self-esteem, data from the mental well-being questionnaires from Food2Learn were used. Below the results of both studies will be discussed in a general perspective. This discussion starts with an overview of the dissertation and the main findings.

Dissertation overview and main findings

Chapter 1 provides a general and theoretical introduction to this dissertation. Chapter 2 provides an overview of the MEFAB cohort, why it was designed, which data were collected and when, moreover the main findings of MEFAB are reported. In Chapter 3, data from the MEFAB cohort was used to assess the association between LCPUFA concentrations during pregnancy, at birth, birth, and age 7, and school performance at age 7. A clear negative association between maternal docosahexaenoic acid (DHA) concentration during pregnancy, at birth, and child arithmetic score at age 7 was shown. Moreover, child DHA concentration at age 7 was positively associated with reading and spelling at age 7. In Chapter 4, the design of Food2learn and the rationale behind the research design are presented. In Chapters 5 and 6, baseline results of Food2Learn are presented. In Chapter 5 it is reported that a higher baseline Omega-3 Index (O3I) was associated with higher information processing speed, and less inattention/impulsivity. In Chapter 6 it was shown with Bayesian analysis that there was highly significant negative association between Osbond acid (C22:5n-6, ObA) levels and depression score (i.e., higher ObA, less depression). Moreover, a highly significant positive association between ObA and self-esteem (i.e., more ObA, higher self-esteem) is shown. In Chapter 7 and 8 the effects of one year of krill oil supplementation on cognitive measures and mental well-being measures, respectively are reported. There was no significant effect of supplementation on either the cognitive measures or mental well-being measures. The most likely explanation was the non-adherence of participants with the protocol. While executing Food2Learn, there were difficulties with recruiting participants, but also with drop-out and adherence. Other researchers have experienced similar problems (personal communication).

Given that a review about recruitment, adherence and drop-out in n-3 LCPUFA supplementation studies in children and adolescents had not been reported, such a review was thus conducted. Results of this review are reported in Chapter 9. In each respective chapter, the studies and their results are discussed extensively. In the discussion below, the results are put into a broader and more general perspective.

Discussion of this dissertation

The following discussion focusses on the three main outcome measurements discussed in this dissertation: school performance, cognition, and mental well-being. The discussions for each of these outcome measures are presented below and includes the findings from the studies included in this thesis, but also compares the studies to earlier similar studies identified via a scoping review and puts the results in a broader perspective. Note that for cognition separate discussions for observational studies and intervention studies are included. Thereafter, the strength and the limitations of both MEFAB and Food2Learn are discussed. Then recommendations for future research are suggested and implications for the general public are discussed. Lastly, the conclusions of this dissertation are presented.

LCPUFA and school performance

School performance is the most important outcome measure of education and has been associated with long term educational attainment, health outcomes, socio economic status and mental well-being [1]. There is a large number of factors which can influence school performance such as personal characteristics, cognitive abilities, family characteristics, and school environment [1]. However, lifestyle factors including nutrition might also influence school performance. It has, for example, been suggested that providing adequate docosahexaenoic acid (C22:6n-3, DHA) may lead to small improvements in multiple cognitive functions, which combined could influence school performances such as reading and spelling [2].

To investigate the association between LCPUFA concentrations and school performance, the data collected in the MEFAB cohort were used. First, the association between fatty acid levels measured in maternal blood at study entry (before 16 weeks of pregnancy), at 22 and 32 weeks of pregnancy, and at delivery, and school performance scores for arithmetic, reading, and spelling at age 7 were investigated. Second, the association between LCPUFA concentrations determined in umbilical cord plasma and blood plasma at age 7, and school performance at age 7 was investigated. Results of these analyses showed, in contrast to the hypotheses, a clear negative association between maternal DHA levels throughout pregnancy and at birth, and arithmetic scores at age 7. This association was supported by the fact that ObA, a functional deficiency marker of DHA, showed a positive association with arithmetic score. In contrast, DHA concentration at age 7 was positively associated with reading and spelling scores at age 7.

As noted in the discussion in Chapter 3, there are to our knowledge no other studies investigating the association between prenatal LCPUFA exposure and school performance in later life. While the main focus of this study was on the potential prenatal programming effect of the LCPUFA, also associations between LCPUFA concentration at age 7 and

school performance at age 7 were investigated. The mixed associations which were shown in MEFAB (i.e., negative association between prenatal DHA and arithmetic, positive association between DHA at age 7 and reading/ spelling), made us curious as to what is known about the relationship between LCPUFA and school performance in children/adolescents. Therefore, a scoping review was executed. Web of Science was searched with the following search terms: 'LCPUFA', 'EPA', 'DHA', 'Omega-3', 'school performance', 'academic achievement', or 'grade'. Moreover, a number of reviews was checked for additional references [2–6]. Studies were included if (1) participants were between 4 and 18 years old; (2) an objective measurement of LCPUFA in the body (e.g., blood, cheek cells, adipose tissue) was included, or the study was a LCPUFA supplementation study; (3) a measure of school performance such as grades, or spelling, reading or math ability was included. Spelling, reading and math scores were only acceptable if they were assessed with a standardized test. Finally, the study had to be published in English to be included in the review. Information on study population, test used to assess school performance, the LCPUFA measurement method and outcomes were extracted.

In the scoping review five studies in which the relationship between LCPUFA and school performance was investigated were found (see Table 10.1). One was an observational study [7], the other four concerned LCPUFA intervention studies [8–11]. Looking at the school performance measure assessed in the studies: five investigated reading [7,9–12] three spelling [9,10,12], two math [7,12], and only one actual school grades [8] (see Table 10.1). Note that these numbers include our own study [12].

Of the five studies that looked at reading, four showed positive associations between n-3 LCPUFA concentration and reading, or an effect of n-3 LCUPFA supplementation on reading performance, albeit in a subgroup or a borderline effect [7,9,11,12] One study investigating the effect of n-3 LCPUFA supplementation on reading did not show a significant effect [10]. It is however important to note that the study that did not show a significant effect was executed in a very wide age range and included children of <6 years which are most probably not able to read yet. Summarizing, preliminary conclusions point to a positive effect of, or association with n-3 LCPUFA and reading performance in children.

Three studies included in the review assessed spelling [9,10,12]. One showed no effect of n-3 LCPUFA supplementation on spelling [10], one showed a positive effect of n-3 LCPUFA supplementation on spelling [9], and one showed a positive association between DHA and spelling [12]. It is, however, important to note that the study of Perletta *et al.* that did not show a significant effect [10] was executed in a very wide age range and included children of <6 years which are most probably unable to execute a spelling test. Moreover, do note that the study of Parletta *et al.* was executed in an Aboriginal population. Aboriginals do not communicate through written text, but through storytelling and pictures. The children do not practice spelling and reading (i.e., communication through written text) at home, this could influence results. The study did show a positive association on the picture based Draw-A-Person test (see LCPUFA and cognition – supplementation studies section). The study that did show an effect of n-3 LCPUFA supplementation on spelling noted that the result was caused by a decline in spelling ability in the placebo group. In sum, this suggests that the evidence for an effect of n-3 LCPUFA, or an association with n-3 LCPUFA and spelling are rather limited and that clear conclusions about the effectiveness of n-3 LCPUFA supplementation on spelling cannot be drawn.

The two studies that reported on math performance showed contradicting results [7,12]. Sorensen *et al.* showed a positive correlation between DHA and eicosapentaenoic acid (C20:5n-3, EPA) and math performance[7], while in MEFAB no significant association between either DHA or EPA at age 7 and math performance was shown [12]. However, the children in the Sorensen *et al.* study were older, and possibly in another brain development stage in which the brain regions responsible for math performance (i.e., prefrontal cortex) might be sensitive to LCPUFAs, while this was not the case in MEFAB. Earlier research suggests that brain development might be characterized by specific 'sensitive periods' [13,14]. The scientific proof for n-3 LCPUFA effects on math performance is thus still very limited and more research is needed.

Table 10.1: Observational studies and RCT investigating the relationship between LCPUFA and school performance in children.

Author, kind of study, n (reference)	Age population	Academic test	Outcome
Sorensen Observational[1] n 747 [7]	8-11	Danish standard test for reading and math	Positive correlation between DHA + EPA and math performance, and the percentage of sentences that were correct out of the total number of sentences read. For girls DHA + EPA positively correlated with reading speed and number correct on sentence reading test.
MEFAB[2] Observational n 170 [12]	7-8	Standard Dutch Cito tests for spelling, reading and arithmetic	Plasma DHA level at age 7 was positively associated with reading and spelling. Maternal plasma DHA levels during pregnancy were negatively associated with child arithmetic scores at age 7
Portillo-Reyes RCT n 55 [8]	8-12	Average score on the subjects Spanish, Mathematics, History and Geography, Science, and Civic Education	No difference between LCPUFA supplementation group and placebo group.
Dalton RCT n 183 [9]	7-9	Reading and Spelling test	There was a significant intervention effect for spelling, due to a significant decline in the scores in the placebo group. For reading a borderline significant (0.065) effect was seen.
Richardson RCT n 362 [11]	7-9	Word reading achievement sub-test of the British Ability Scale	Preplanned subgroup analyses showed that for children with baseline reading < 20th percentile improvement was greater in active treatment, for those in < 10th percentile the treatment effect was slightly greater.
Parletta RCT n 408 [10]	3-13[3]	Wide range achievement test – subtest word reading and spelling	No treatment by group interaction for reading and spelling.

[1] The study of Sorensen was an intervention assessing the effect of a Nordic Diet school lunch on among other cognition. Here only the baseline results were used. [2] Chapter three of the current thesis. [3] Only two out of 408 participants were three years old, it was therefore decided to include this study despite inclusion criteria.

Portillo-Reyes *et al.* was the only study investigating the effect on school grades [8], albeit the average over school five subject. They did not show an effect of n-3 LCPUFA supplementation on average score. However, taking the average of multiple subjects can lead to the averaging out of the negative association in one school subject by the positive association in another subject. Therefore this finding has to be interpreted with caution.

All in all, there is some evidence that n-3 LCPUFA might influence school performance in children, specifically for reading, and possibly for spelling. Furthermore, research on math performance and actual school grades is very limited; these areas need more research. Lastly, MEFAB seems to be the only study in which the relationship between prenatal LCPUFA exposure and later school performance has been assessed. Especially considering the consistent negative associations between DHA during pregnancy, and at birth and arithmetic scores at age 7 found in MEFAB, more research is needed.

LCPUFA and cognition – observational studies

Cognitive abilities are very important for school performance. LCPUFA have many functions in the body and can influence brain function in a multitude of manners (see chapter 1). It, therefore, should not come as a surprise that a multitude of observational studies have investigated the relationship between LCPUFA and cognition. There are, however, to our knowledge, no studies investigating the association between LCPUFA and cognition in adolescents. Therefore, Food2Learn was designed and conducted.

The baseline results of Food2Learn reported in Chapter 5 showed that a higher O3I was associated with better performance on the letter digit substitution task (LDST). This indicates that a higher baseline O3I in the adolescents participating in Food2Learns was associated with better information processing speed. Moreover, a higher O3I was also associated with fewer errors of omission on the D2 test of attention, which is a measure of inattention/impulsivity.

Food2Learn was, to our knowledge, the first study in which the association between fatty acid concentrations measured in blood and cognitive measures in adolescents was assessed. There are, however, studies available in children, investigating the link between O3I and cognition. It was therefore decided to compare the findings of Food2learn to those of earlier observational studies investigating the relationship between LCPUFA and cognition in children. To do so a second scoping review was executed.

Web of Science was searched with the following search terms: 'LCPUFA', 'EPA', 'DHA', 'Omega-3', 'cogniti*, 'child*', or 'adolescen*'. Moreover, a number of reviews was checked for additional references [2–6]. Studies were eligible for inclusion if (1) participants were between 4 and 18 years old; (2) the study included an objective measurement of LCPUFA in the body (e.g., blood, cheek cells, adipose tissue); (3) the study included a measure of cognition; (4) the study was of observational nature; (5) the study was published in English. Information on study population, test used to assess cognition, the LCPUFA measurement method and outcomes was extracted. Our goal was to see whether higher O3I was associated with positive outcomes, i.e., whether there is some sort of cut-off point above which a positive association between O3I and cognitive measures is apparent. Therefore, the measured DHA and eicosapentaenoic acid (EPA) concentrations reported in the studies were recalculated to the O3I. The O3I is defined as the sum of DHA + EPA in erythrocytes, expressed as a percent of total erythrocyte fatty acids, but there are non-validated conversion formulae available for measurements from other blood fractions (i.e., determined in whole blood or plasma) [15].

The review led to eight studies which met inclusion criteria (see Table 10.2) [7,16–23]. Unfortunately, the studies were very heterogeneous in study population (i.e., age and

nutritional status) and cognitive test used (i.e., specific tests or test battery such as Kaufman). This heterogeneity makes comparison of the studies rather difficult.

Of the eight studies included in the review, six studies included data that made it possible to calculate an O3I [7,16–21](see Table 10.2). The calculated O3I in those six studies varied between 3.37% [17] and 4.57% [7], which is rather similar to the baseline O3I measured in Food2Learn (i.e., 3.89% including all participants, also participants with O3I > 5%). The O3I in all studies were thus rather low and well below the recommended 8-11% for cardiovascular health and the target level we originally aimed for to reach with our supplement. As the O3I were so low and so similar, it was not possible to determine whether a O3I cut-off point, above which a positive association with cognition is apparent, exists.

Table 10.2: Observational studies in children investigating the association between measured LCPUFA concentrations and cognition.

Author (reference)	Age population	Blood fraction	Calculated O3I	Outcome
Jumbe [16] n 130	4-6	WB	4.36	In an adjusted model DHA was positively associated with score on Dimensional Change Card Sorting task (measures executive functioning).
Eilander [17] n 541	6-10	E	3.37	No association between either DHA or EPA and any of the cognitive measures (fluid reasoning, short term memory, retrieval ability and cognitive speediness).
Bakker [18] n 306	7	PP	3.63	No significant association between DHA at age 7 and score on Kaufman Assessment Battery for Children score (measures intelligence and achievement).
Montgomery [19] n 493	7-9	WB	3.63	Significant positive association between DHA + EPA and score for recall of digits forward (working memory), no significant associations for digit recall backward.
Sorensen [7] n 747	8-11	WB	4.57	Positive correlation between EPA + DHA and processing speed and concentration performance measured on D2 test. For boys, a higher DHA + EPA was associated with lower D2 error% and impulsivity error%. For girls, a higher DHA + EPA was associated with higher inattention errors.
Food2Learn [20][1] n 267	14-16	WB	3.83	Positive association between O3I and score on the LDST (information processing), negative association with number of errors of omission on D2 (inattention/impulsivity).
Boucher [21] n 151	10-13	PP	3.43	No association was shown between current DHA levels and score on the digit span forward (immediate memory), and the California Verbal Learning Task (short and long-term memory)
Kirby [23] n 411	8-10	CC[2]	NA	A positive correlation between DHA and non-verbal IQ on the Kaufman brief intelligence test was shown. Moreover, a positive correlation between DHA/EPA ratio and working memory. No significant association between either DHA or EPA, and working memory, Wechsler individual achievement test, test of everyday attention, or matching familiar figures tasks.
Haapala [22] n 444	6-8	PP[3] TG CE	NA	Higher EPA and DHA measured in plasma TG was associated with higher score on Raven's coloured progressive matrices (non-verbal reasoning), but only in overweight children.

ALA = alfa linolenic acid, CC = cheek cell, CE = cholesteryl esters, DHA = docosahexaenoic acid, E = erythrocytes EPA = eicosapentaenoic acid, NA = not appropriate, PP = plasma phospholipids, WB = whole blood

[1] Chapter 4 of this thesis [2] For cheek cells no conversation factor is available. [3] Fatty acids concentrations were reported as mol%

When looking at the cognitive tests used in the studies included in the review, studies could be divided in those using tests of general ability (i.e., the Kaufman, and the Raven) and those using tests assessing individual cognitive domains (i.e., attention or memory) . Of the studies included in the review, three studies assessed a general ability [17,18,22], five studies assessed one or more specific cognitive domain(s) [7,16,19–21], and one assessed both [23]. Interestingly, four out of five studies assessing a specific cognitive domain showed a positive association between LCPUFA concentration and the cognitive measure [7,16,19,20]. Moreover, the only study in which no significant association was shown, borderline significant associations between plasma DHA level and score on the digit span forward were apparent [21]. Furthermore, a borderline significant association between plasma DHA level and recognition on the California verbal learning task was also found.

In contrast, of the three studies [17,18,22] assessing a general ability only one showed a positive association, namely Haapala *et al.* [22]. In the study of Haapala and colleagues an association between DHA and EPA measured in plasma triacylglycerols and score on Raven's coloured progressive matrices was shown [22]. However, this association was only apparent in a relative small subgroup of 58 children with overweight/obesity, no association was shown for the 386 healthy children. Moreover, the association was only apparent for plasma triacylglycerols, which reflects the fatty acid intake of the last few hours, and not for cholesteryl esters and phospholipids, which reflect fatty acid intakes over the last days [24]. All in all, it seems very unlikely that such a short term fatty acid intake would relate to such a broad general ability.

Kirby and colleagues included both a general test (Kaufmann) and cognitive domain specific tests [23]. They showed a significant correlation between DHA and score on the Kaufmann non-verbal subscale. Moreover, they showed a correlation between DHA/EPA ratio and score on the digit recall, a measure of working memory. However, the correlations were weak $r < 0.15$ and they executed a large number of statistical test (> 300 statistical tests) without correction of the p-value for multiple testing. These results need to be interpreted with caution given the potential for false positive results.

As noted above there are no observational studies investigating the association between LCPUFA measured in blood and cognitive performance in adolescents. There are however two studies that investigated the association between fish consumption, which is the most important source of LCPUFA, and cognition, or score on a vocabulary test. Åberg and colleagues reported an association between high fish consumption at age 15 and better cognitive performance at age 18 [25]. De Groot *et al.* showed an inverted u-shape association between fish consumption and score on a vocabulary test (i.e., higher fish consumption was associated with higher vocabulary score up to 1-2 times fish per week) [26]. These observational studies assessing fish consumption do point to a possible positive association between LCPUFA and cognition in adolescents.

All in all, observational studies, including the baseline result of Food2Learn, do point to a positive relationship between LCPUFA concentration in blood and performance on individual cognitive domains. Although, the O3I in the studies included was very low (all < 5%), very cautiously there is an indication that a higher O3I is related to better cognitive performance. However, observational studies cannot provide evidence for causality, therefore placebo controlled randomized controlled trials (RCT) are needed.

LCPUFA and cognition – supplementation studies

The observational studies discussed above point to a possible positive relationship between LCPUFA and cognition in children. Moreover, the baseline results of Food2Learn and observational studies investigating the association between fish consumption and cognition do point to a similar positive association in adolescents. However, to prove causality, placebo controlled RCTs are needed. Therefore, Food2Learn was designed to elucidate the effect of LCPUFA supplementation on, among others, cognitive performance in typically developing adolescents. One year of krill oil supplementation in Food2Learn did not lead to higher scores on any of the cognitive measures (reported in Chapter 7). However, due to issues with drop-out and non-adherence in Food2Learn, it cannot be precluded that a relationship between krill oil supplementation and cognition in adolescents does not exist.

To compare our findings to those of earlier studies, investigating the effect of LCPUFA supplementation on cognition in children/adolescents, a third scoping review was executed. Web of Science was searched with the following search terms: 'LCPUFA', 'EPA', 'DHA', 'Omega-3', 'cogniti*, 'child*' or 'adolescen*'. Reviews on this topic were also checked for additional studies to include [2-6]. Studies were eligible for inclusion if (1) participants were between 4 and 18 years old; (2) participants received supplementation with DHA and/or EPA; (3) the study included a measure of cognition; (4) the study was a randomized placebo controlled trial; (5) the study was published in English. Information on study population, supplementation, test used to assess cognition, LCPUFA measurement method if included and outcomes were extracted. To compare the results of studies included in the review to the results of Food2Learn, the measured fatty concentrations, if reported, were converted to the O3I with the formulae as suggested by Stark and colleagues [15].

After reviewing the literature, 11 placebo controlled RCTs investigating the influence of LCPUFA supplementation on cognition could be included [6,8–11,27–32] (see Table 10.2 and Table 10.3). Unfortunately, no studies in adolescents were located. The age of participants included in the studies varied from 3 to 13 years. Studies were very heterogeneous (i.e., they differed on population, duration, form of supplementation etc.) which makes comparison very difficult.

Table 10.2: LCPUFA supplementation studies in children investigating the effect on cognition and reporting DHA and EPA levels measured in blood.

Author, n[1]	Population (age & any specifics)	Blood collected? What fraction?	Calculated O3I Before[1]	Calculated O3I after[2]	Dosage per day	Duration (in weeks)	Effect on cognition?
Baumgartner [27] n 321	6-11 iron deficient	Y, total pp fraction of erythrocyte membranes	3.19[3]	6.30[3]	240mg DHA 46mg EPA [4]	15	N
Ryan [6] n 202	4	Y, capillary whole blood [5]	2.08	4.83	400mg DHA	16	N
Muthayya [28] n 598	6-10 marginally nourished	Y, erythrocyte membranes in the pp fraction	3.38[6]	5.57[6]	86mg DHA (+771mg ALA)[7]	52	N
Food2Learn n 267	13-15	Y, Whole blood	3.72	4.86	280mg DHA 520mg EPA	52	N
Dalton [9] n 183	7-9 some underweight, wasted or stunted	Y, EPA reported log transformed	NA	NA	Fish flour: 892mg DHA per week, 410mg EPA[8]	14.9	Y
Kennedy [29] n 90	10-12	N	NA	NA	Low dose: 400mg DHA, 4mg EPA High dose: 1000mg DHA, 20mg EPA	8	N
Kirby [30] n 450	8-10	Cheek cells[9]	NA	NA	400mg DHA, 56mg EPA	16	N
McNamara [31] n 38	8-10	Y, EPA not reported	NA	NA	400 or 1200mg DHA	8	Y
Parletta [10] n 408	3-13[10]	N	NA	NA	174mg DHA 558mg EPA	28.6	Y
Portillo-Reyes [8] n 55	8-12 malnourished	N	NA	NA	180mg DHA 270mg EPA	12	Y
Richardson [11] n 362	7-9	N	NA	NA	600mg DHA	16	Y, in sub sample
Osendarp [32] n 780	6-10 Some marginally nourished	Y, FA concentrations reported in microgram/ml	NA	NA	88mg DHA 22mg EPA	52	N

ALA = alpha linolenic acid, DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid, N= no, PP = plasma phospholipids. [1] Note that the studies of Baumgartner, Muthayya, and Osendarp were 2 by 2 designs, there were thus 4 groups [2] Data presented here are for the intervention group receiving LCPUFA supplementation. [3] Average of groups receiving placebo + DHA + EPA and iron + DHA + EPA [4] Recalculated, participants received 420mg DHA and 80mg EPA per day 4 days a week. [5] Providing a blood sample was not compulsory for participation, 93 participants provided a blood sample. [6] Average of groups receiving high micronutrient + high n-3 group and low micronutrient + high n-3 group [7] Recalculated, participants received 900mg ALA and 100mg DHA per day 6 days a week. [8] Recalculated 127mg DHA, 55mg EPA per day. [9] For cheek cells no conversion formulae is available. [10] Only two out of 408 participants were three years old, it was therefore decided to include this study despite inclusion criteria.

Firstly, studies were compared on the increase of O3I. Of the 11 studies included in this review, seven reported that fatty acid concentrations were measured [6,9,27,28,30–32]. Unfortunately, the recalculation to the O3I was not possible for four [9,30–32] of the seven studies reporting the fatty acid values. One study only reported the log transformed EPA concentration [9], one did not report EPA concentration at all [31], one determined fatty acid concentration in cheek cells (no recalculation factor is available) [23], and one reported the fatty acid concentrations as micrograms per millilitre [32]. When looking at the three studies that allowed for recalculation to the O3I, they showed an increase in the O3I of 3.11, 2.75, and 2.19%, after 15, 16, and 52 weeks of supplementation, respectively (see Table 10.2).

The average increase in O3I was higher in all three studies than the average increase in O3I of 1.14% after 12 months in the krill oil group in the intention to treat analysis of Food2Learn. It is noteworthy that all three studies had a shorter duration and a lower supplementation dosage of DHA + EPA compared to Food2Learn and they still had a higher increase in O3I. This does point to an adherence problem in Food2Learn (for more on this see the strength and limitation section). However, similar to our results none of the three studies showed an effect of the supplementation on any cognitive measures in the main analyses in the overall sample. This would suggest that n-3 LCPUFA supplementation is not effective in increasing cognitive performance in children.

Although the increase of O3I was higher in the three studies compared to Food2Learn, the O3I was still relatively low after supplementation (6.3%, 4.83%, and 5.57%) for all three studies, at least well below the recommended target range of 8-11%, where we originally aimed for in our Food2Learn study (see chapter 4). Moreover, similar to Food2Learn, in the three studies there also seemed to be an overlap in the O3I between the placebo and the LCPUFA supplemented group. Such an overlap in O3I between placebo and treatment group could have caused the non-significant treatment effects [33], as analyses were not executed according to O3I, but according to group allocation. It is interesting to note that Ryan *et al.* did execute secondary preplanned analyses according to blood values and did show an association between higher DHA concentration and higher score on the Peabody Picture Vocabulary scale [34]. Additionally, Muthayya and Baumgartner [27,28] made use of general ability tests (i.e., subtests of Kaufman, Wechslers Intelligence Scale and Rey auditory Verbal scale), while it has been suggested that domain specific tests are more suitable to pick up the subtle effects of nutrition interventions [35]. Also note that many of the participants included in the studies of Muthayya and Baumgartner did have nutrient deficiencies and some were undernourished in general. Both nutrient deficiencies and undernourishment can influence cognitive performance negatively [36,37] and possible counteract any positive effects of n-3 LCPUFA supplementation.

The second aspect which could be considered in relation to the effectiveness of LCPUFA supplementation on cognition is daily supplementation dosage. The supplementation dosage of DHA + EPA varied immensely in the 12 included studies, varying from 86 to 100mg DHA per day, and from 0 to 558mg EPA per day. From Figure 10.1 it can be deduced that a supplementation dosage of > 400 mg DHA + EPA per day does seem to lead to positive effects on cognition. While studies with a lower supplementation dosage, with the exception of Dalton *et al.*[9], did not find a significant cognitive health benefit.

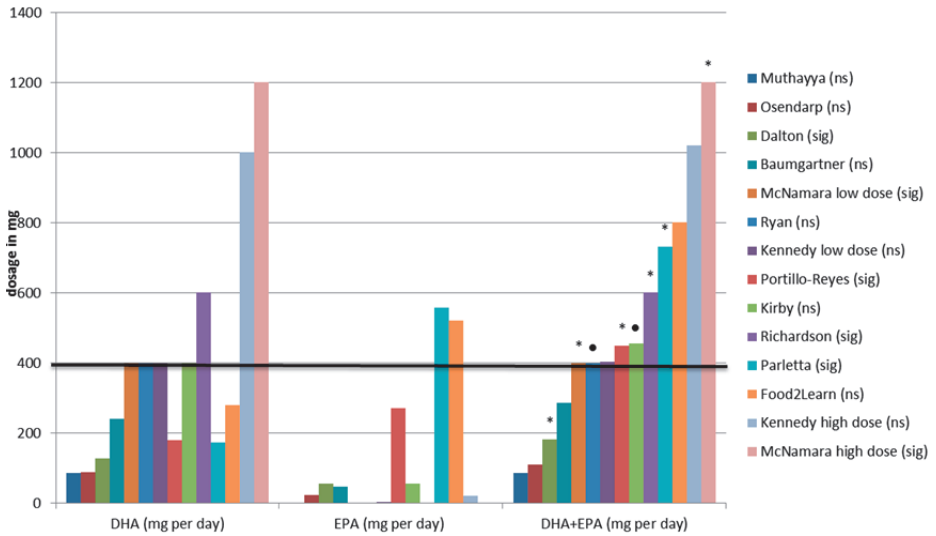

Figure 10.1: LCPUFA supplementation studies that studied cognition in children. Line indicates dosages of above 400mg DHA + EPA per day. Studies indicated with a * are those studies in which positive effects of supplementation are reported, studies with ● are those with significant positive effects in secondary analyses.

Of the non-significant studies with dosages above 400mg, two did show significant effects/associations in secondary analyses. Kirby and colleagues did show significant effects of supplementation on visual attention and impulsivity in those that were compliant with the protocol [30]. Furthermore, Ryan *et al.* did show an association between DHA measured in blood and score on the Peabody Picture Vocabulary Test in a preplanned secondary analysis [6]. The one study that did show significant effects with a dose below 400mg/day was the study of Dalton *et al.* in which fish flour was used as supplementation source [10]. Fish flour also includes other nutrients (e.g., protein) which could, especially for the undernourished children included in the study of Dalton *et al.*, provide additional cognitive benefits.

The study of Kennedy *et al.* was the only study with a dosage above 400mg DHA + EPA per day which did not show significant effects on cognition [29]. This study did have a relatively short supplementation duration of 8 weeks. The study did not include blood values. It is thus unsure whether the supplementation was successful and whether there was no overlap in fatty acid status between the three groups (i.e., placebo, 400mg DHA, 1000mg DHA per day) Moreover the study did only include 88 participants and was, according to the authors themselves, underpowered.

All in all, there seems to be some evidence that a dosage above 400mg DHA + EPA per day does lead to improved cognition in children. In contrast, in the studies that included data which made it possible to calculate the O3I [27,28,34], higher O3I in the supplemented group did not lead to better cognitive performance. However, due to the limited number of studies for which determination of the O3I was possible, the overlap between the placebo and intervention group, and the relative low O3I even after supplementation makes it difficult to draw conclusions. Moreover, possibly there is a cut-off point above which a positive effect could be seen, similar to the fact that O3I > 8% has been associated with the lowest mortality risk in coronary heart disease [38]. None of the

studies which included data, which made it possible to calculate the O3I, achieved such a high O3I.

It is important to note that the included studies are very heterogeneous and thus difficult to compare. The studies differed on among others: the study population (i.e., age, nutritional status), composition of supplementation (i.e., EPA/DHA ratio, but also inclusion of other fatty acids or nutrients), and duration of supplementation. For the assessment of cognition, a wide variety of cognitive tests were used, often with multiple subtests, assessing all different aspects of cognition. Additionally, the number of (sub)tests used varied from one to 21. In the studies with multiple tests, chance findings might be a problem.

It is also unsure whether and how the results in children translate to adolescents. In Food2Learn, which was plagued by drop-out and non-adherence, it was not possible to show an effect of one year of krill oil supplementation on cognition in adolescents. Taking into account that Food2Learn was the first LCPUFA supplementation study in adolescents assessing cognition, and the limitations of Food2Learn, more studies in adolescents should be executed. These studies should include blood values, supplement with > 400mg DHA + EPA per day and try to achieve higher O3I at the end of treatment (also see suggestion for future research).

LCPUFA and mental well-being

Fish consumption and LCPUFA consumption have long received attention for their possible influence on mental well-being, specifically depression. However, the focus has mostly been on adults. This is unfortunate, as depression is very common in adolescence. Furthermore, adolescent depression has been associated with important long term negative consequences, such as reoccurrence of depression, increased risk of anxiety, risk of suicide, poorer social relationships, poorer self-rated health, lower grades, and lower educational attainment [39–43]. Adolescent depression is, thus, very important to investigate both from an individual adolescent's perspective, but also from a public health perspective. Therefore, Food2Learn, the first n-3 LCPUFA supplementation intervention trial in typically developing adolescents from the general population was designed and conducted.

Baseline results of Food2Learn (reported in Chapter 6) indicated that there was extreme evidence (i.e., Bayesian analysis indicates how strong the evidence is for the alternative hypothesis) for a weak negative association between ObA levels and depression score as assessed with Bayesian analysis, i.e., higher ObA levels less depression. Note that Bayesian analysis gives an indication of how strong the evidence is for the alternative hypothesis e.g., extreme evidence or substantial evidence. Moreover, there was substantial evidence for a weak positive association between ObA levels and self-esteem, i.e., higher ObA better self-esteem. Thus, higher ObA measured in blood was associated with lower depression and higher self-esteem. At baseline, there was evidence for the null hypothesis (i.e., no association) when analysing the relationship between depression and DHA, EPA, and O3I.

There are a few observational studies investigating the association between LCPUFAs and depressive symptoms in adolescents [44–47]. There is, however, only one study in which similarly to our study the association between LCPUFA status and depressive symptoms and adolescents in the general population was investigated. Mamalakis and

colleagues measured fatty acid concentrations in adipose tissue and measured depression score with both the Center for Epidemiologic Studies Depression Scale (CES-D) and the Becks Depression Inventory (BDI)[48,49]. They showed a negative association between EPA status and score on the CES-D (i.e., more EPA, less depression), but only after correction for covariates. Moreover, they also showed a positive association between dihomo-gamma linolenic acid (C20:3n-6, DGLA) and score on the BDI (i.e., less DGLA, less depression), again only after correction. However, these associations just reached significance (p=.04 and p=.05), among a total of twenty analyses, one can therefore not exclude that these are chance finding. This is also supported the associations that were found with one of the depression questionnaires and not the other, while both are validated in adolescents [50]. Moreover, EPA and DHA are only present in very small amounts in adipose tissue [51] and an association between adipose fatty acids and brain fatty acids has, to our knowledge not been established, while the association between blood and brain fatty acids levels has been established in animal studies [52] .

One year of krill oil supplementation in Food2Learn did not lead to lower depression scores, or higher self-esteem score (reported in Chapter 8). Moreover, a higher O3I was not related with score on either depression or self-esteem questionnaire. It is however important to note that due to non-adherence and drop-out the increase in O3I was limited.

As noted, there are, to our knowledge, no earlier studies in which the influence of LCPUFA supplementation on depressive feelings in adolescents has been studied. There are a large number of LCPUFA supplementation in adults, with some showing a positive effect of LCPUFA supplementation, but most certainly not all studies (for meta-analyses see[53–57]. It is, however, uncertain if and to what extend results from studies in depression in adults can be translated to depressive feelings in adolescents. Adolescence is very different from adulthood as it is characterized by profound brain, physical, social, emotional and cognitive development [58]. Furthermore, there is also discussion about possible differences in causes and outcomes of juvenile-onset and adult-onset depression [59,60]. Thus, if supplementation of LCPUFA is an effective treatment for depression in adults, this does not automatically mean LCPUFA are effective for treatment of depression in adolescents.

To summarize, Food2Learn is the first LCPUFA supplementation study investigating mental well-being (depression and self-esteem) in adolescents. Baseline results showed an association between ObA and both depression and self-esteem. The intervention, although having major limitations, did not show an effect of one year of krill oil supplementation and an increase in O3I on either depression or self-esteem. All in all, more research investigating the effect of both n-3 and n-6 LCPUFA in adolescent depression is needed, as this is an age group with a high prevalence of depression and in which depression has serious negative long-term consequence both on a personal and a public health level.

Strengths and limitations of MEFAB and Food2Learn

In this section, the strengths and limitations of MEFAB and Food2Learn are elaborated upon. The names of the studies are printed in bold in this section so it is easier to recognize to which study the strength or limitation is related.

The main strengths of the studies in the current thesis are that for both **MEFAB** and **Food2Learn** blood values were available. Many of the studies investigating the effect of LCPUFA supplementation on cognition or school performance included in the reviews in this discussion did not include measurements of LCPUFA levels in the body (i.e., blood, cheek cells, adipose tissue). When there is no biological measure of LCPUFA, it is impossible to know whether the supplementation was actually successful. One cannot be sure that participants were actually compliant with the protocol. Moreover, there are large interpersonal differences in metabolism which can influence tissue EPA and DHA levels. For example, in one study participants received 0.5g DHA + EPA per day for 8 weeks [61]. After 8 weeks, there was an average increase of 2.43%, but the 'increase' ranged from -0.03% to +7.16%. In other words, there was 13-fold difference in response to the supplementation. This difference could have been caused by, among others, differences in metabolism, adherence, or other personal factors. But whatever the cause, it leads to different O3I while the LCPUFA dose is the same. These differences could influence results and it is thus important to measure O3I. When no blood levels are taken, one is also not aware of the baseline DHA/EPA levels, while it can be expected that any effect of DHA/EPA supplementation is more pronounced in participants with low baseline levels. In addition, a wide range of starting O3I can lead to similar end O3I in both placebo and treatment group and thus similar outcomes. These issues were tackled in **Food2Learn** by measuring blood levels at the start and three time points during the year of supplementation and by including only participants with an O3I below 5%. Furthermore, it was planned to execute a personalized supplementation dosage adjustment after three months of supplementation to deal with interpersonal metabolism. Unfortunately, none of the participants had achieved the set target O3I of 8 to 11%. Therefore, all participants received an increase in dosage. However, it is very likely that participants in Food2Learn were non-compliant. It is therefore still recommended to measure blood levels in these kinds of nutritional trials and to do dosage adjustments during a study to control for individual differences in response to supplementation.

Most earlier studies asked participants to take capsules at breakfast time or did not specify the time at which capsules needed to be taken. The absorption of EPA and DHA is dependent on the food matrix with which it is taken. It has been reported that LCPUFA absorption is three times higher when LCPUFA capsules are taken with a high fat meal compared with a low fat meal [62]. **Food2Learn** participants were therefore specifically asked to consume the krill oil capsules with dinner, the fattiest meal of the day [63].

It has been suggested that the neurocognitive tests used should be selected based on the most plausible cognitive outcome and/or brain regions to be influenced by the nutrient to be tested [35,64]. For **Food2Learn** tests that tap into the three core executive functions (i.e., shifting, updating of working memory, inhibition) were selected. The executive functions were chosen as they are mainly located within the (pre)frontal cortex, the brain region which is still in development during adolescence [65] and has been shown to have increased activity after DHA supplementation [31]. The selected tests are thus the tests which are most likely sensitive to LCPUFA supplementation in adolescents.

MEFAB is one of a few birth cohorts with measurements during pregnancy and a very long follow-up (up to age 23). The longitudinal nature is the only way to relate the early life exposure to later life outcomes, as was done in Chapter 3 by relating LCPUFA status during pregnancy to school performance at age 7. Moreover, birth cohorts are the only way

to assess the possibility of early life programming; that is, whether exposure early in life can be related to health and disease in later life [66].

The main limitation of **Food2Learn** is that it had rather high drop-out rates and difficulties with achieving adherence. Approximately 20% of the students withdrew completely from the study and 32% stopped active participation (i.e., they stopped taking supplements, but continued testing). The main reasons for stopping supplementation were lack of motivation, difficulties swallowing capsules, and difficulties remembering to take capsules. The drop-out seems high, but Food2Learn is not the only LCPUFA study with such high drop-out, as reported in Chapter 9. Therefore, during the study a number of techniques were used to try to increase adherence. A daily text message reminder was sent out, and motivational telephone talks were held. Moreover, a tip sheet was handed out at the start of the study with tips on how to remember to take capsules and how to make swallowing capsules easier (i.e., examples are: put the capsules at a clearly visible place, take the capsules with a tablespoon of custard, yoghurt or apple sauce, and put an alarm in your phone). The methods which were utilized to increase adherence, and decrease drop-out are also in line with the suggestions from the literature, as summarized in Chapter 9. Despite these efforts, adherence was not increased. Between test moments two and three there was actually a decrease in the average O3I of participants in the krill oil group that did not indicate to us to have stopped taking capsules. Moreover, only 3, 10, and 2 participants achieved the set target O3I range of 8-11% after 3, 6, and 12 months of supplementation, respectively. Fortunately, the blood values of 199 participants were available, which did make it possible to do the analyses according to blood O3I status.

One of the limitations of **MEFAB** is the fact that such a longitudinal study of prenatal exposure, does not take into account what the children themselves consume during their life. This intake during life could possibly bias results.

Lastly, only 21% of the original **MEFAB** participants had school performance scores available. These missing data could have influenced results, especially since the number of children with lower scores was limited. However, when those with school performance scores available and those without were compared, no differences on covariates were shown, indicating that the participants with school performance data available was a representative sample of the overall cohort.

In summary, a great effort was made to reduce these limitations. Unfortunately, these limitations seem to be inherent to this type of research. It is however important to report these studies to the scientific community and inform them about the limitations, so future studies can try to tackle these limitations.

Suggestions for future research

Based on the findings from the studies reported in this thesis and other studies there were reviewed, a number of suggestions for future research are described below.

Objectively measured LCPUFA concentration

As described in the *strengths and limitations* section, there are large interpersonal differences in the metabolism of LCPUFA and the uptake of LCPUFA. However, there are many studies that do not include objectively measured LCPUFA concentrations albeit in a blood

fraction, cheek cells or adipose tissue. For example, when looking LCPUFA supplementation studies in children with a focus on cognition reported in Table 10.2 and 10.3 only 4 out of 12 studies did not include any measure of LCPUFA concentration in the body. When the LCPUFA concentrations are not measured, one is not aware whether participants actually need additional LCPUFA (i.e., if a participant has an O3I of 8.4% at baseline, additional EPA/DHA is probably not necessary). Additionally, one is also not aware whether supplementation was effective and/or whether participants were compliant. Lastly, one does not know whether there was actually a difference in LCPUFA concentrations between the placebo and treatment group after the supplementation period. It is suggested that future research always includes an objective measure of LCPUFA concentrations at least at baseline and at the end of the intervention. Moreover, future studies should always report the value of all measured fatty acids in % weight of total fatty acids in their manuscript (and absolute concentrations were possible), to make comparisons between studies possible.

High dose supplementation

The recommended amount of DHA + EPA/day varies widely across countries and organizations (summarized in [67]), with recommendations for healthy adults varying from 90mg/day suggested by the US National Academy of Sciences to 1g per day suggested by the European Society of Cardiology. The recommendation of the Dutch Health Council of 450mg per day falls somewhere in the middle of these reported recommendations. Note that these recommended amounts are in general based either on the prevention of deficiency or the prevention of cardiovascular diseases [67]. Moreover, in the Netherlands no specific recommendations for children are available. It has been suggested that the brain can incorporate about 4mg DHA per day [68]. However only 0.5% of circulating DHA is delivered to the central nervous system [68]. It thus seems that to achieve such level of incorporation a relative high intake of DHA is needed. When looking at the studies investigating the influence of LCPUFA supplementation in children, the majority of studies that supplemented > 400mg DHA + EPA/day had significant effects. It is thus suggested that in future studies in children and adolescents, supplementation is at least 400mg DHA + EPA/day.

Long term supplementation

It has been suggested that red blood cells achieve a new steady state after about 4-6 months of DHA supplementation [51]. The amount of DHA produced *de novo* in the brain is negligible, thus brain DHA levels are maintained via the blood supply. The incorporation of DHA into the brain is rather slow at a rate of about 4mg/day, while the total brain contains about 5g of DHA [68]. So any full effect of brain enrichment with DHA, would only be expected after an extended period of supplementation (i.e., at least 4-6 months of supplementation [69]). The majority LCPUFA supplementation studies in children with a focus on cognition reported in Table 10.2 and 10.3 had a duration of 16 weeks (i.e., 4 months) or less. Only 4 of the 12 studies had a duration of more than four months. Of these four studies, only one showed positive effects. However two of the studies used a very low dosage of < 110 mg DHA + EPA per day [28,32], and as noted effects are not

expected at such low dosages. The third study with no effect was Food2Learn and as noted before this study had high drop-out and low adherence. All in all, it is suggested that future studies use supplementation durations of at least four months, but preferably longer.

Personalized dose adjustment

As noted above there are large interpersonal differences in the metabolism of LCPUFA. It thus seems odd to give all participants in one trial the same dosage, as not everybody needs the same dosage to achieve a certain target concentration in the body. Moreover, all participants have a different baseline status, thus again to achieve a target concentration everybody needs a different dosage. It thus seems wise to make use of personalized supplementation dose. As a person's LCPUFA metabolism is not known, one can use personalized dose adjustments. For example, start all participants on the same dosage, but monitor the blood fatty acid concentrations and adjust the dosage based on blood concentrations after 3-4 months of supplementation and repeat if necessary.

Adolescents

Food2Learn was the first LCPUFA supplementation study investigating cognition and mental well-being in adolescents from the general population. Adolescence is an extremely important period of life to investigate the influence of LCPUFA on both cognition and depressive feelings. It is a period of life characterized by profound brain development, as well as emotional, social, and physical development. For a child/adolescent to achieve her/his optimal (intellectual) potential there needs to be an optimal environment, which includes optimal nutrition [35]. LCPUFA intake could be an especially important aspect of nutrition as they can both structurally influence the brain, but can also affect the levels and activity of neurotransmitters. Moreover, LCPUFA have also been related to depressive feelings, which in turn can influence cognitive performance. All in all, biological mechanisms point to an important role of LCPUFA in adolescence for both cognition and depressive feelings. As Food2Learn was the first study in adolescents, and taking the limitations of Food2Learn into account, more omega-3 supplementation studies in adolescents should take place.

Recruitment, adherence and drop-out

In executing Food2Learn, problems with recruiting sufficient participants were experienced, but also in achieving adherence and retention into the study. This led us to review how other LCPUFA supplementation studies in children and adolescents dealt with recruitment, but also to what extent they experienced adherence and drop-out (reported in Chapter 9). It was difficult to draw conclusions from the review, because of the minimal reporting on recruitment, adherence and drop-out in the included studies. However, additional literature research did lead to a number of suggestions to improve recruitment, adherence, and drop-out (see Chapter 9). It is suggested that future studies take recruitment, adherence, and retention into account when designing them. For example, there is evidence that simple supplementation regimes lead to less drop-out. Thus, one

could provide one concentrated capsule per day, or provide capsules during lunchtime at school under supervision of a responsible teacher.

Analyses: intention to treat, per protocol, and according to blood

Intention to treat analysis (ITT) is the analysis in which all participants are included in the group to which they were originally randomized. Regardless of whether the participant actually took the treatment, deviated from the protocol, or dropped out for the analysis they are included in the original group they were randomized to. Or as others have put it: "once randomized, always analysed" [70,71]. The main advantage of ITT is that it preserves sample size and thus statistical power and it is the 'gold standard' of analysing RCTs. It is also more ecological valid, in that it takes into account that there will always be people who do not adhere to the protocol and drop-out [70]. However, there are also many arguments to prefer per protocol (PP) analysis. PP analysis is a comparison of treatment groups that only include participants that actually completed the treatment that they were originally assigned to. The estimated effect of treatment assessed with ITT is conservative, as this also includes those that were not compliant and dropped out. PP gives a true estimate of the efficacy of the treatment. However, PP analysis does not necessarily depict the real-life situation (i.e., people will be non-adherent and quit treatment). As there are both advantages and disadvantages to both ITT and PP analyses and the results of both analyses depict a different perspective (i.e., ecological valid effect, and true effect), it is recommended to do both analyses. Moreover, if reliable blood values are available, analyses according to blood values should always be included, because of the interpersonal difference in baseline fatty acid concentrations and difference in metabolism.

Publishing non-significant and negative results

In MEFAB negative associations were shown, and in Food2Learn non-significant results were shown. It has long been known that there is a publication bias, in that studies with positive significant results are published faster and more often [72]. However it has been suggested that negative / non-significant results actually offer more information gain, in that they can counteract possibly false beliefs about a previously thought positive intervention [73]. Moreover, for Food2Learn there were many difficulties and limitations, but if these results are published, others can learn from the difficulties and limitations and design new trials which possibly counteract these. All in all, trials with both negative and neutral results can contribute uniquely to the field and should therefore be published.

Implications for the general public

One might wonder: how do the results of this thesis translate to 'the real world'? What would recommendations be for an individual or a parent? It seems important to convey that nutrition, and in the light of this thesis specifically LCPUFA consumption, possibly influence cognition and school performance. Moreover, individuals and parents need to be aware, that the LCPUFA have to be consumed via the diet and that most people consume

far too little DHA and EPA. It is thus advised that individuals eat two portions of fatty fish a week to get sufficient amounts of DHA and EPA in their diet, as suggested for cardiovascular health benefits. If fatty fish consumption is not possible (i.e., due to dislike or unavailability), long-term, probably lifelong, n-3 LCPUFA supplementation of at least 400mg EPA + DHA per day is advised. The effect of n-3 LCPUFA supplementation on cognition might remain equivocal, the positive effect for cardiovascular health is rather robust and no serious adverse effects of fish consumption or n-3 LCPUFA supplementation are known. Therefore, we belief that two servings of fatty fish per week or 400mg EPA + DHA per day in the form of supplements is a sensible advice.

Furthermore, taking the results of MEFAB into account, a programming effect of LCPUFA seems plausible. It is thus important that pregnant women, and those planning to become pregnant know that the nutrition a child receives before birth (i.e., the food the mother eats) can impact the development of that child in later life.

Concluding remarks

In the present thesis, the relationship between LCPUFA and cognition, school performance and mental well-being in children and adolescents was investigated. A negative association between prenatal exposure to DHA and arithmetic scores at age 7 was shown. Moreover, a positive association between DHA concentrations at age 7 and spelling and reading scores at age 7 was reported. An association between baseline O3I and some cognitive measures in adolescents was found. However, one year supplementation with krill oil in our study did not influence any of the cognitive measures. Additionally, an association between baseline ObA concentration and lower depression and higher self-esteem in the same adolescents was shown. Again, one year supplementation with krill did not influence either depressive feelings or self-esteem in our study. Unfortunately, the majority of adolescents did not adhere to the study protocol and did not achieve the set target O3I of 8-11%.

To conclude, our findings do not provide conclusive results on the relationship between LCPUFA and school performance, cognition and mental well-being in children and adolescents. The findings showed negative associations, non-significant associations and effects and positive associations. More omega-3 supplementation studies in adolescents are needed and the studies should pay specific attention to achieve adherence, decrease drop-out and achieve a sufficient high O3I.

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Summary

A number of studies show that essential fatty acids and their longer chain derivatives (long-chain polyunsaturated fatty acids, LCPUFA), play a significant role in brain functioning, because they are important constituents of the cell membrane. A sufficient LCPUFA status has been related to better cognitive functioning of both children and adults, as well as less cognitive decline in elderly. The relationship between LCPUFA and school performance has been studied to a lesser extent. In adults, a beneficial LCPUFA status has been related to less depression.

Because the brain of children and adolescents is still profoundly developing, it can be presumed that LCPUFA might be especially important, as building blocks, in these periods of development. The goal of this dissertation was to study the relationship between LCPUFA and *school performance* in children, and to study the influence of LCPUFA supplementation on *cognition* and *mental well-being* in adolescents.

In **Chapter 1** a general and theoretical introduction to this dissertation is provided, information on the following topics is given: fatty acid metabolism, importance of intake of LCPUFA, in what foods LCPUFA are present, and the advantages of krill oil supplementation (the form of supplementation used in the studies in this dissertation) over fish oil supplementation. Next, six possible mechanisms in which LCPUFA can possibly influence brain functioning are elaborated upon. Then the brain development from birth throughout childhood and adolescence is explained. To finish, theoretical background information on the outcome variables investigated in this dissertation (i.e., school performance, cognition, and mental well-being) is provided.

In this dissertation data, collected in two studies, were used to answer the goals described above. In **Chapter 2** an overview of one of these studies, the Maastricht Essential Fatty Acids Birth Cohort (MEFAB cohort) is given. The MEFAB cohort was set up in 1989 to study the change in fatty acid concentrations during pregnancy and how this relates to the fatty acid concentrations of the neonate. Moreover, the association between the fatty acid concentrations and a number of birth outcomes (e.g., weight, length and head circumference) was assessed. The MEFAB cohort has three follow-up studies at age 4, 7 and 12 of the child. Data on cognitive development, asthma/atopy, growth, and cardiovascular disease risk factors among others, were collected. Besides the description of the design, the main earlier findings of the MEFAB cohort are discussed.

In **Chapter 3** data from the MEFAB cohort is used to assess the associations between maternal LCPUFA concentrations during pregnancy, at birth, and child's LCPUFA concentration at age 7, and school performance at age 7. Analyses with correction for covariates showed a negative association between maternal DHA concentration during pregnancy, maternal and child's DHA concentration at birth, and child's arithmetic score at age 7. Moreover, maternal Osbond acid (ObA) levels at 22 and 32 weeks of pregnancy, and ObA level in umbilical cord blood plasma were positively associated with arithmetic score at age 7. This last finding confirms the negative relationship between DHA status and arithmetic scores, as ObA is a deficiency marker of DHA (i.e., higher ObA levels reflect lower DHA levels). In contrast, child's DHA concentration at age 7 was positively associated with both the reading and spelling score at age 7.

Chapter 4 concerns the design of the second study, Food2Learn. In Chapter 4 the unique characteristics and the rationale behind the research design are presented. Food2Learn was a double-blind, randomised, placebo controlled intervention trial in adolescents (age 13–15 years) attending lower general secondary education. The goal of

Food2Learn was to determine the influence of increasing Omega-3 Index (O3I) on cognitive functioning, academic achievement and mental well-being of typically developing adolescents. Unique characteristics of Food2Learn are among others the fact that: participants were recruited based on a low O3I at baseline, students were asked to take krill oil capsules at dinner time to increase LCPUFA absorption, a personalized krill oil dose adjustment was planned, and the study focused on students of a lower educational level.

In **Chapters 5** and **6**, baseline results of Food2Learn are presented. In **Chapter 5** the associations between baseline O3I of the participants of Food2Learn and scores on the cognitive measures are reported. Analyses revealed a significant positive association between the O3I and score on the Letter Digit Substitution Task (i.e., measure for information processing speed). Moreover, a significant negative association between the O3I and errors of omission on the D2 (i.e., measure for inattention/impulsivity) was shown. Thus, participants with a higher O3I had a higher information processing speed, and less inattention/impulsivity

In **Chapter 6** the associations between baseline O3I and other fatty acids (DHA, eicosapentaenoic acid (EPA), arachidonic acid (AA), docosapentaenoic acid (DPA), and ObA) and both depressive feelings and self-esteem are reported. Bayesian analyses showed a weak negative association between ObA and depression score, and a weak positive association between ObA and self-esteem score. In other words, more ObA measured in blood corresponded with less depression and more self-esteem. There was no evidence for an association between DHA, EPA, and O3I, and depression and/or self-esteem.

In **Chapter 7** and **8** the effects of one year of krill oil supplementation on respectively cognitive measures (chapter 7) and mental well-being measures (chapter 8) are reported. There was no significant effect of krill oil supplementation on either the cognitive measures or mental well-being measures. The most likely explanation for these non-significant effects, is the high amount of drop-out and non-adherence with the protocol. Due to these problems, it cannot be concluded that a relationship between krill oil supplementation and cognition, and mental well-being does not exist.

While executing Food2Learn, there were difficulties with recruiting participants, drop-out, and adherence. It was however unknown how common those problems are in LCPUFA supplementation studies in children and adolescents. Therefore, a systematic review focussing on recruitment, adherence, and drop-out rates in LCPUFA supplementation studies in children and adolescents was executed in **Chapter 9**. Problems with recruitment and drop-out were common in LCPUFA supplementation trials in children and adolescents. Techniques to improve recruitment, adherence and dropout rates were identified in the literature and are reported in Chapter 9 as well.

In **Chapter 10**, the main results of the studies in this dissertation are discussed, and using an additional literature review, evaluated and put into a broader perspective.

To conclude, MEFAB and Food2Learn do not provide conclusive evidence for a relationship between LCPUFA and school performance, cognition and mental well-being in children and adolescents. However, associations between LCPUFA concentrations and both school performance, and cognition were found. Future studies should pay attention to, among other things, objectively measuring LCPUFA concentrations, long-term high dose LCPUFA supplementation, personalised dose adjustment, and focus on adolescence, as this is a period of profound brain development. The relevance for society of both MEFAB and Food2Learn can be translated into the advice to consume either two servings

of fatty fish per week or, based on the literature review in chapter 10, take a supplement with at least 400mg DHA + EPA per day, as this amount has by all means a positive effect on general health, and possibly a positive effect on brain functioning as well.

Samenvatting

Verschillende studies laten zien dat essentiële vetzuren en hun langere-keten derivaten (meervoudig onverzadigde vetzuren, long-chain polyunsaturated fatty acids [LCPUFA]) een grote rol spelen in het breinfunctioneren, omdat zij een belangrijke bouwsteen van het celmembraan zijn. Een gunstige LCPUFA status wordt in verband gebracht met beter cognitief functioneren van kinderen en volwassenen, alsmede minder cognitieve achteruitgang bij ouderen. De relatie tussen LCPUFA en schoolprestatie is in mindere mate onderzocht. Verder is er bij volwassenen een duidelijk verband tussen een gunstige LCPUFA status en minder depressie gevonden.

Omdat met name het brein van kinderen en adolescenten nog volop in ontwikkeling is, wordt verondersteld dat LCPUFA mogelijk extra belangrijk zijn, als bouwsteen, in deze periode van groei. Het doel van dit proefschrift was de relatie tussen LCPUFA en *schoolprestaties* onder kinderen, en de invloed van LCPUFA-suppletie op *cognitie* en *mentaal welbevinden* bij adolescenten te onderzoeken.

Hoofstuk 1 geeft een algemene en theoretische introductie op dit proefschrift, waarbij de volgende onderwerpen aan bod komen: het vetzuurmetabolisme, het belang van LCPUFA-inname, in welke voedingsmiddelen de LCPUFA voorkomen en de voordelen van krilloliesuppletie (de suppletievorm die in de studies in dit proefschrift wordt gebruikt) ten opzichte van visoliesuppletie. Daarna zijn zes mogelijke werkingsmechanismen genoemd via welke de LCPUFA het breinfunctioneren zouden kunnen beïnvloeden. Vervolgens is de hersenontwikkeling gedurende de kindertijd en de adolescentie beschreven.

Hoofdstuk 1 eindigt met de theoretische achtergrond van de uitkomstvariabelen die in dit proefschrift onderzocht werden; schoolprestaties, cognitie en mentaal welbevinden.

Voor dit proefschrift werden in twee separate wetenschappelijke studies gegevens verzameld die gebruikt zijn voor beantwoording van bovenstaande doelstellingen. In **hoofdstuk 2** is het onderzoeksdesign van één van deze studies beschreven; het Maastricht Essential Fatty Acids Birth (MEFAB) cohort. Het MEFAB-cohort is opgericht in 1989 om de veranderingen in vetzuurconcentraties tijdens de zwangerschap te bestuderen en hoe deze verband houden met de vetzuurconcentraties van de foetus. Ook is de associatie tussen de vetzuurconcentratie en een aantal geboorte-uitkomsten (bv. gewicht, lengte en hoofdomtrek) onderzocht. Het MEFAB-cohort heeft drie follow-up momenten, op 4-, 7- en 12-jarige leeftijd van het kind. Gegevens over onder andere cognitieve ontwikkeling, astma / atopie, groei, en risicofactoren voor hart- en vaatziekten zijn op die momenten verzameld. Naast de beschrijving van het design komen in hoofdstuk 2 tevens de reeds gedane bevindingen van het MEFAB-cohort aan de orde.

In **hoofdstuk 3** is, op basis van gegevens uit het MEFAB-cohort, de LCPUFA-concentraties van de moeder gemeten tijdens de zwangerschap en bij de geboorte van het kind, en de LCPUFA-concentraties van het 7-jarige kind, gerelateerd aan de schoolprestaties van het kind op 7-jarige leeftijd. Na correctie voor covariaten, werden er negatieve associaties aangetoond tussen enerzijds DHA-concentraties bij de moeder tijdens de zwangerschap en bij zowel moeder als kind bij de geboorte, en anderzijds de rekenscore van het kind op 7-jarige leeftijd. Bovendien waren de concentraties Osbondzuur (ObA) van de moeder bij 22 en 32 weken zwangerschap en het niveau in navelstrengbloedplasma positief geassocieerd met rekenscores op 7-jarige leeftijd. Deze laatste bevinding bevestigt de negatieve relatie tussen DHA-status en rekenscores, gezien een hogere ObA-concentratie een lagere DHA-concentratie weerspiegelt. De DHA-concentratie gemeten op 7-jarige leeftijd was daarentegen positief geassocieerd met lezen en spellen op 7-jarige leeftijd.

Hoofdstuk 4 betreft het design van de tweede studie, Food2Learn. Bovendien zijn in hoofdstuk 4 de unieke kenmerken en de redenering achter het onderzoeksdesign gepresenteerd. Food2Learn was een dubbelblind, gerandomiseerde, placebogecontroleerde interventie onder adolescenten (13-15 jaar) die op VMBO-TL zaten. Het doel van Food2Learn was de invloed van het verhogen van de Omega-3 Index (O3I) op cognitie, schoolprestaties en mentaal welbevinden van adolescenten te bepalen. Enkele unieke kenmerken van Food2Learn zijn: het feit dat deelnemers werden geworven op basis van een lage O3I, deelnemers gevraagd werden de krilloliecapsules bij de avondmaaltijd in te nemen om de LCPUFA-absorptie te verhogen, een gepersonaliseerde aanpassing van de krilloliedosis gepland was, en dat de studie gericht was op leerlingen van een laag onderwijsniveau.

In hoofdstukken 5 en 6 zijn de baseline-resultaten van Food2Learn gepresenteerd. In **hoofdstuk 5** zijn de associaties tussen baseline O3I van de deelnemers in Food2Learn en scores op de cognitieve maten onderzocht. Analyses lieten een significante associatie zien tussen de O3I en score op de Letter Digit Substitution Task (maat voor informatie-verwerkingssnelheid). Bovendien was er een significante associatie tussen de O3I en een D2-uitkomstmaat voor onoplettendheid / impulsiviteit. Deelnemers met een hogere O3I hadden dus een hogere informatieverwerkingssnelheid en waren oplettender.

In **hoofdstuk 6** zijn de associaties tussen enerzijds baseline O3I en andere vetzuren (DHA, EPA, arachidonzuur (AA), docosapentaeenzuur (DPA) en ObA), en anderzijds depressieve gevoelens en zelfvertrouwen gerapporteerd. Bayesiaanse analyses toonden een zwakke negatieve associatie tussen ObA en depressie, en een zwakke positieve associatie tussen ObA en zelfvertrouwen. Oftewel, een hogere ObA-concentratie in het bloed was gerelateerd aan minder depressieve gevoelens en een beter zelfvertrouwen. Er was echter geen bewijs voor een associatie tussen enerzijds DHA, EPA en O3I en anderzijds depressie en/of zelfvertrouwen.

In **hoofdstuk 7** en **8** zijn de effecten van één jaar krilloliesuppletie op respectievelijk uitkomstmaten ten aanzien van cognitie (hoofdstuk 7) en mentaal welbevinden (hoofdstuk 8) gerapporteerd. Er was geen significant effect van krilloliesuppletie op uitkomstmaten ten aanzien van cognitie en mentaal welbevinden. De meest waarschijnlijke verklaring voor deze niet-significante effecten is de grote hoeveelheid deelnemers die tussentijds hun studiedeelname beëindigden, of het protocol niet nakwamen (d.w.z. de capsules niet slikten). Vanwege deze moeilijkheden kan echter ook niet worden geconcludeerd dat er geen relatie bestaat tussen enerzijds krilloliesuppletie en anderzijds cognitie en mentaal welbevinden.

Tijdens het uitvoeren van Food2Learn werden moeilijkheden ondervonden met het werven van deelnemers, deelnemers die studiedeelname beëindigden (drop-out genoemd) en deelnemers die zich niet aan het protocol hielden (adherence genoemd). Het was echter onbekend hoe vaak zulke problemen zich voordoen bij andere LCPUFA-suppletiestudies onder kinderen en adolescenten. Daarom werd in **hoofdstuk 9** een systematische review uitgevoerd naar wervings-, adherence- en drop-out-percentages in LCPUFA-suppletiestudies bij kinderen en adolescenten. Problemen met werving en drop-out kwamen ook veel voor in andere LCPUFA-suppletiestudies onder kinderen en adolescenten. Technieken om de wervings-, adherence- en drop-outpercentages te verbeteren werden in de literatuur geïdentificeerd en zijn eveneens gerapporteerd in hoofdstuk 9.

Tenslotte zijn in **hoofdstuk 10** de belangrijkste bevindingen van de bovenstaande studies bediscussieerd en met behulp van additioneel literatuuronderzoek geëvalueerd en in een breder perspectief geplaatst.

Concluderend, MEFAB en Food2Learn leveren geen onomstotelijk bewijs voor een relatie tussen enerzijds LCPUFA, en anderzijds schoolprestaties, cognitie en mentaal welbevinden bij kinderen en adolescenten. Wel zijn er associaties gevonden tussen LCPUFA concentraties en zowel schoolprestaties als cognitie. Vervolgonderzoek zou zich daarom onder andere moeten richten op het objectief meten van LCPUFA concentraties in bloed, langdurige suppletie met een hoge dosis LCPUFA, gepersonaliseerde dosis-aanpassing, en als doelgroep specifiek de adolescentenpopulatie. De maatschappelijk relevantie van zowel MEFAB als Food2Learn kan vertaald worden in het advies om twee keer per week vette vis te consumeren dan wel, gebaseerd op de resultaten van de literatuurreview in hoofdstuk 10, minimaal 400mg DHA + EPA in de vorm van een supplement te nemen. Deze hoeveelheid heeft in ieder geval een positief effect op de algehele gezondheid en mogelijkerwijs een positief effect op breinfunctioneren.

Dankwoord

Daar zit ik dan op een grijze woensdag in december de laatste woorden van mijn dissertatie te typen. Wie had dat verwacht? Toen ik begon aan de opleiding Voeding en Gezondheid, was onderzoeker – zo iemand in een witte jas – het laatste wat ik wilde worden. Langzamerhand veranderde dat en groeide mijn interesse voor het onderzoek, zeker na zowel mijn masterscriptie én stage in een academische setting. Dan solliciteer je op een PhD-functie in Zuid-Limburg, kom je te laat op het sollicitatiegesprek, omdat je verdwaalt en krijg je in het magazijn van IKEA te horen dat je mag beginnen.

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Nu is de tijd gekomen om letterlijk de laatste punt te zetten achter dit hoofdstuk van mijn leven. Nogmaals, bedankt!

Inge van der Wurff
6 december 2017.

Curriculum vitae

Inge van der Wurff was born on the 18th of April 1989 in Maastricht, the Netherlands. In 2007, after receiving her gymnasium diploma from the Porta Mosana College in Maastricht, she started the study Nutrition and Health at Wageningen University. In 2010 Inge finished the bachelor Nutrition and Health and in 2013 the Master Nutrition and Health. During her master study she visited Karolinska University, Stockholm, Sweden for three months to study the influence of food particle size on the cumulative intake curve and eating behavior. After completing her master degree, Inge worked as an education assistant at Wageningen University, where she, among others, developed an e-module on good sensory practice and simple sensory tests. In 2013, she started as a PhD student at the Open University under supervision of Prof. Dr. Renate de Groot (Open University of the Netherlands), Prof. Dr. Paul Kirschner (Open University of the Netherlands) and Prof. Dr. Maurice Zeegers (Maastricht University). In her research she explored the relationship between long-chain polyunsaturated fatty acids, cognition, school performance and mental well-being in children and adolescents. During her PhD, Inge visited the University of Wollongong, Australia for three months to conduct additional research under supervision of Prof. Dr. Barbara Meyer. The results of her research have already been published in five scientific publications, three additional ones are currently under revision. Inge presented her work at four international conferences. To visit these conferences she received three travel grants, namely for the International Society for Nutritional Psychiatry Research conference, the International Society for the Study of Fatty Acids and Lipids conference, and the Mind-Body Interface International Symposium. Above, Inge won a new investigators award from the International Society for the Study of Fatty Acids and Lipids. Inge will continue her work as an assistant professor at the Open University of the Netherlands.

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