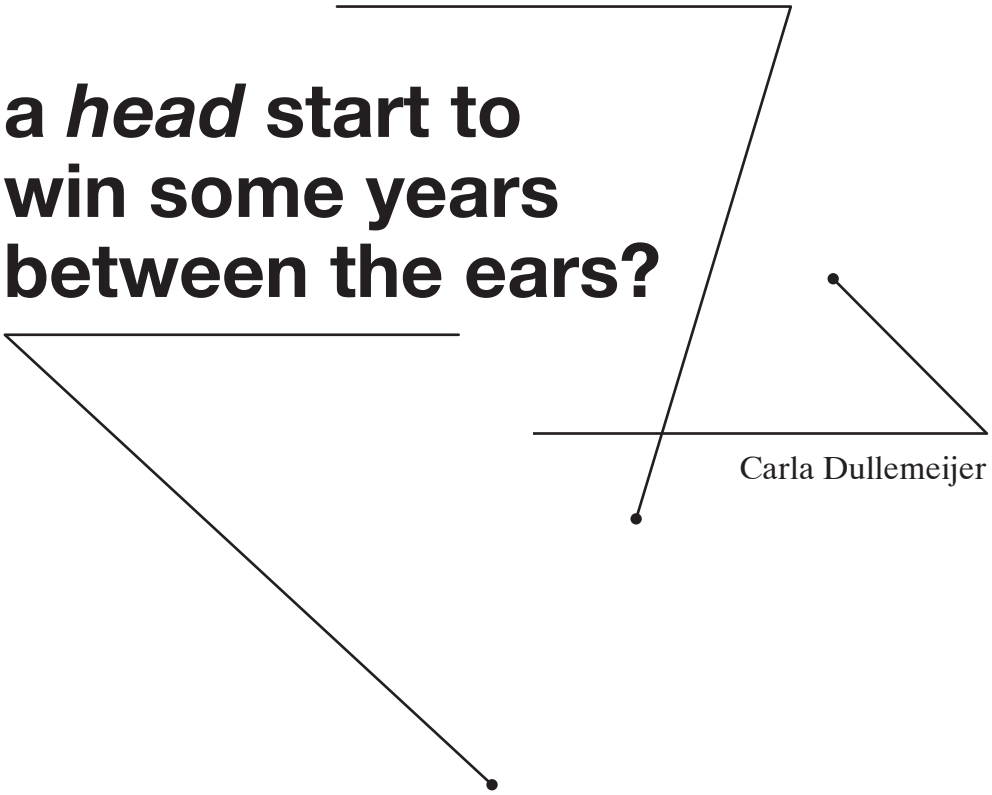
Very long-chain n-3 polyunsaturated fatty acids:



a *head* start to win some years between the ears?

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Abstract

Do very long-chain n-3 PUFA give someone a head start to win some years between the ears, in brain development as well as in brain ageing? This thesis will shed light on three areas related to this question: the role of very long-chain n-3 PUFA in 1) brain tissue development, 2) cognitive decline, and 3) macrovascular and microvascular blood supply in the head region. We first focused on the beginning of the life span, by investigating the effects of dietary very long-chain n-3 PUFA on the fatty acid composition of several brain lobes in juvenile pigs. We showed that a diet enriched with fish oil resulted in higher proportions of docosahexaenoic acid (DHA) in the frontal, parietal and occipital brain lobes compared with the temporal brain lobe. This suggests a region-specific incorporation of DHA in the developing brain. Subsequently, we focused on the end of the life span, by investigating the association of very long-chain n-3 PUFA with cognitive decline and blood supply in the head region in humans. We demonstrated that higher plasma proportions of very long-chain n-3 PUFA were associated with less decline in the cognitive domains sensorimotor speed and complex speed over a 3-year period, but not in memory, information-processing speed and word fluency, compared with lower plasma proportions of very long-chain n-3 PUFA. Furthermore, we showed that plasma very long-chain n-3 PUFA were not associated with changes in carotid intima-media thickness and common carotid distension over a 3-year period. This could suggest that the role of very long-chain n-3 PUFA in healthy populations may extend in particular to the smaller blood vessels instead of the large blood vessels. We subsequently hypothesized that microvascular disease may decrease the blood supply to the highly vascularised cochlea, which could result in age-related hearing loss. We showed that that higher plasma proportions of very long-chain n-3 PUFA were indeed associated with less age-related hearing loss compared with lower plasma proportions of very long-chain n-3 PUFA in older adults. This implies that the hypothesis of improved microcirculation, if proven correct, may have far-reaching consequences. This thesis provides an encouraging basis for future research into the role of very long-chain n-3 PUFA in both brain development and brain ageing.

Contents

Chapter 1

General introduction

Hypes and hopes about n-3 fatty acids

Very long-chain n-3 (or omega-3) polyunsaturated fatty acids have attracted considerable public interest during the past few years for their potential beneficial role in a variety of diseases and disorders. They are probably known best for their heart health benefits, but a growing amount of media attention also points towards the potential benefits that very long-chain n-3 polyunsaturated fatty acids may provide in cognitive performance. These benefits stretch from advantages in developing brains of infants and children to preventing cognitive decline at old age. The prospect of a nutrient that can improve cognitive performance or delay cognitive decline has obvious appeal for many. Bread, margarines, eggs, and other foods labelled as being rich in n-3 fatty acids are popping up in stores more and more. Claims such as 'for healthy hearts and minds' and 'boosts children's' brain power' are attractive, but nevertheless quite vague as well. Should we brush these claims aside as clever marketing strategies by those who know or should know better? Have scientific data been stretched and distorted to create an inappropriate hype about the benefits of n-3 fatty acids in cognitive performance? The statements elicit curiosity about the scientific basis for these hoped-for benefits. *Is there really a role for n-3 fatty acids in our heads or is it just between our ears?* Beyond the hype lies some interesting science...

Biochemistry and biosynthesis of n-3 fatty acids

Fatty acids are chains of carbon atoms with a carboxylic acid at the beginning of the chain, also known as the "alpha"-end, and a methyl group at the opposite end, the "omega"-end. The n-3 fatty acids are polyunsaturated fatty acids (PUFA), implying that they have at least two carbon-to-carbon double bonds within their chain. The position of the double bond nearest to the omega-end of the carbon atom chain is used to classify polyunsaturated fatty acids. Since "omega" is the last letter of the Greek alphabet, this word refers to the last carbon atom in the chain. The n-3 (or omega-3) polyunsaturated fatty acids are a family of polyunsaturated fatty acids in which the double bond closest to the omega-end of the carbon atom chain is on the third position (**Figure 1.1**). In this respect, they are structurally distinct from the more commonly encountered n-6 (or omega-6) family of polyunsaturated fatty acids, which have the terminal double bond on the sixth position.

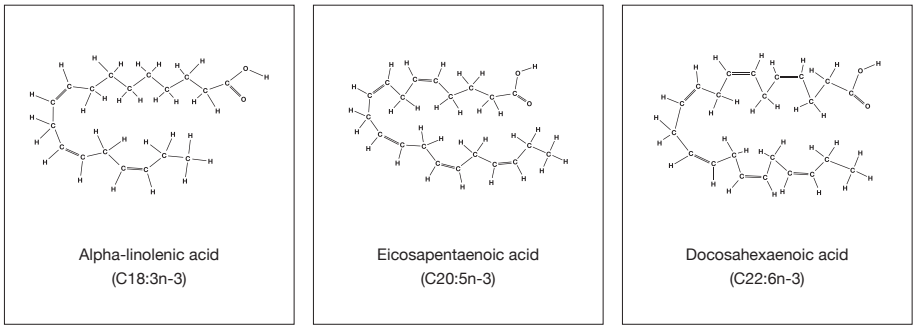

Figure 1.1: Structures of α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid.

Alpha-linolenic acid (C18:3n-3) is the so-called "parent" member of the n-3 fatty acid family. Mammalian cells cannot synthesize α-linolenic acid *de novo*, because mammals lack the delta-15 desaturase enzyme for insertion of a double bond at the third position. Therefore, α-linolenic acid is an essential fatty acid, which should be obtained from the diet. After consumption, α-linolenic acid can be metabolized in a series of desaturation and elongation steps into longer chain, more unsaturated derivatives, such as eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (n-3 DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) (**Figure 1.2**). However, the conversion of α-linolenic acid into its longer chain derivatives is not at all efficient in adult humans[1]. This means that consuming the very long-chain derivatives EPA and DHA themselves is the easiest way to increase amounts of these fatty acids in the human body.

Dietary sources of very long-chain n-3 PUFA

A good dietary source of very-long chain n-3 PUFA is fish. Fish can be classified into "fatty" fish, which store fat as triglycerides in the flesh (e.g. mackerel, herring, salmon, tuna) and "lean" fish, which store fat as triglycerides in the liver (e.g. cod). The consumption of fatty fish therefore provides the body with more very long-chain n-3 PUFA than consumption of lean fish. In this thesis, we will focus on the very long-chain n-3 PUFA from fish, a term that includes EPA, n-3 DPA and DHA.

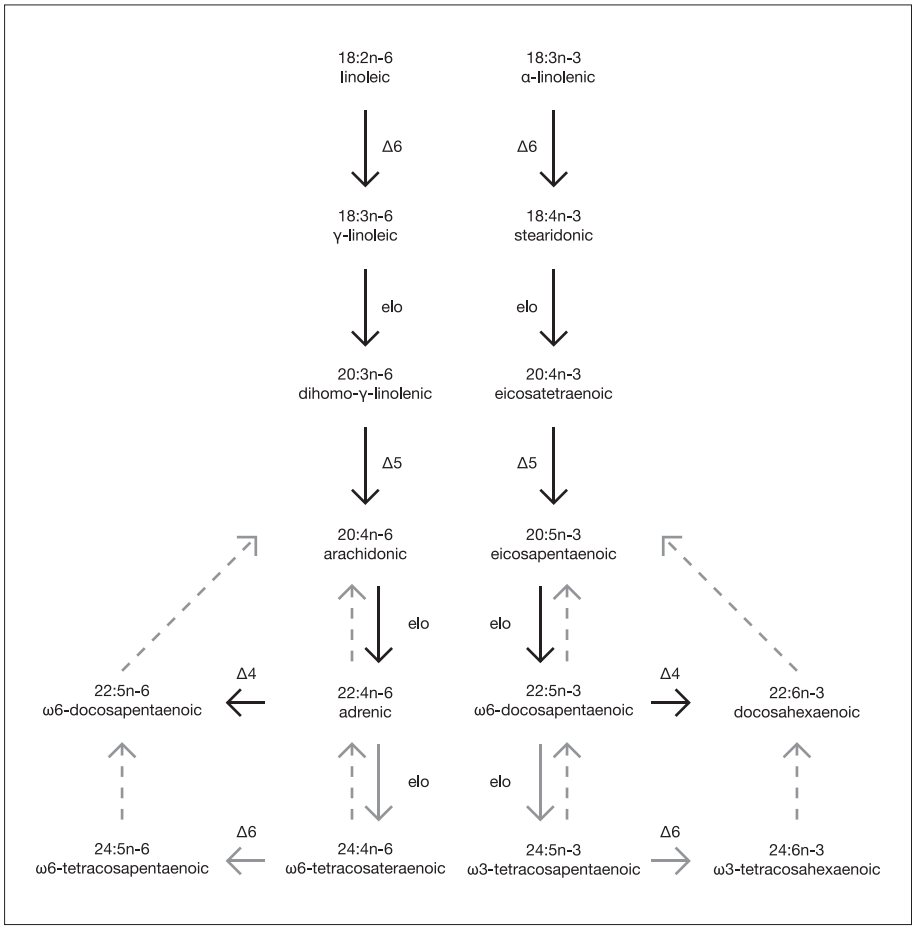

Figure 1.2: Metabolic pathways of long-chain PUFA. Elo=elongation. Bold arrows: the common pathway for the synthesis of n-6 and n-3 fatty acids. Grey arrows: alternative pathways. Broken arrows: retro-conversion.

Very long-chain n-3 PUFA in brain tissue

Compared with other organs, the brain has an unusually high content of lipids[2]. About 10% of the fresh weight and 50% of the dry weight of the brain is accounted for by lipids[3]. Almost two-thirds of these lipids in the brain consist of phospholipids[2]. Phospholipids are structural components of cell membranes, and many physical properties of cell membranes, such as permeability and fluidity, are greatly influenced by the fatty acid composition of these phospholipids[2]. The brain phospholipids are particularly rich in long-chain polyunsaturated fatty

acids, with DHA being the major very long-chain n-3 polyunsaturated fatty acid in the human brain[4, 5]. This abundance of DHA in the brain triggered the idea that DHA may be an essential nutrient for the development of the brain. In addition, the brain is reported to retain DHA as much as possible, even in n-3 fatty acid deficiency[6]. This reinforced the concept that DHA could be functionally important in the brain.

The brain growth trajectory is modelled as a sigmoid curve when brain weight is plotted against age. The period of rapid growth, illustrated by the steep section in such a curve, is commonly known as the 'brain growth spurt'[7]. In humans, the brain growth spurt occurs during the third trimester of fetal life, in which brain weight can increase 4-5 fold[8], and the first two years of childhood[9]. Humans are therefore categorized as perinatal brain developers[7]. Since rapid accretion of structural lipids is necessary for the developing fetus or infant in this period to enable the synthesis of brain tissue, the brain growth spurt may be a period of enhanced susceptibility to nutritional factors such as fatty acids.

However, experimental data on the developing brain often needs to be collected from animals, as brain tissue sampling is not feasible in living human subjects. When attempts are made to extrapolate results obtained in animal species to the human species, the timing of the brain growth spurt is a major factor to take into account[7]. A lot of research on the developing brain is conducted in rats, although rats are postnatal brain developers[7] and differ a lot from humans in size, life span, and nature of their diet. These characteristics may make them less valuable as models for humans. Pigs, on the other hand, are perinatal brain developers and omnivores like humans, and additionally resemble human subjects very closely in brain morphology and brain surface anatomy[7, 10].

Several studies in newborn piglets have investigated the effects of dietary n-3 PUFA on the fatty acid composition of the brain[11-20]. The piglets were fed formula milk either with or without n-3 PUFA directly after birth. The majority of these studies reported higher proportions of n-3 PUFA in the brain tissue after n-3 PUFA feeding compared with control feeding[11-14, 18-20]. This suggests that the fatty acid composition of the brain is indeed affected by dietary fatty acids during the period of rapid brain growth, which confirms the 'brain growth spurt' hypothesis of susceptibility to dietary factors.

However, this hypothesis should not be interpreted as if the period of rapid

brain growth spurt is the only period in which the brain is susceptible for dietary factors. The hypothesis by no means excludes other vulnerable periods in the life time[7]. For instance, it is unclear whether a surplus of dietary very long-chain n-3 PUFA in the period after the brain growth spurt – the childhood stage for humans or the juvenile stage for animals – also increases the levels of these fatty acids in the brain. In addition, the hypothesis of the brain growth spurt as index of vulnerability is a general statement and therefore limited, because the brain consists of many particular parts. Brain development is not a homogeneous process in time or space. Various parts of the brain develop at different time points and with different speeds[21], which may have consequences for the responsiveness of different brain regions to dietary factors.

In order to evaluate regional differences in the responsiveness of different brain regions to dietary very long-chain n-3 PUFA, we performed an intervention study in pigs. *Chapter 2* of this thesis describes this intervention study in which we studied the effect of feeding very long-chain n-3 PUFA on the fatty acid composition of the frontal, parietal, temporal and occipital brain lobes. To investigate whether the brain is still susceptible for fatty acids after the brain growth spurt, we performed this intervention study in juvenile pigs. These pigs were seven weeks old at commencement of the study and were therefore beyond the period of the brain growth spurt, which extends from about six weeks before birth to about five weeks afterwards in pigs[22, 23].

Very long-chain n-3 PUFA and cognitive performance

The role of very long-chain n-3 PUFA in brain development spurred the scientific interest into a possible role of these fatty acids in brain function at old age. The underlying idea was that if very long-chain n-3 PUFA indeed played an important role in the development of the human brain, they may also be beneficial for the maintenance of cognitive performance in older adults.

Although some older persons maintain very high levels of cognitive performance throughout life, most older people will experience a decline in certain cognitive abilities over time. This decline is usually not pathological, but is rather a phenomenon of normal ageing. For some older persons, however, decline goes beyond what may be considered “normal”. This form of cognitive deterioration is very progressive and can negatively affect memories, intellect, abilities to

recognize spouses or children, maintenance of basic personal hygiene, and can even complicate comprehensible speech. These serious forms of cognitive decline are caused by a variety of neuropathological conditions and dementia diseases. Mild cognitive decline is associated with an increased risk of developing dementia[24]. The estimated rates of conversion from mild cognitive impairment to dementia vary from 1 to 25% per year[24]. The long preclinical phase of dementia and the mild cognitive decline that precedes the onset of this disease have led to research efforts to establish if the start of mild cognitive decline could be delayed.

The most common approach to determine cognitive performance or decline in scientific research is the use of cognitive tests. An advantage of these tests is that they are relatively easy to apply in large-scale research settings. The Mini-Mental State Examination (MMSE)[25] is an example of a brief and easy, and therefore widely used, cognitive screening tool. The usefulness of such easy applicable cognitive screening tools depends on the purpose of the research and the composition of the study population. The MMSE was originally created to quantitatively estimate the severity of cognitive impairment[25]. A cut-off criterion of 23 points on the MMSE is generally accepted as indicating the presence ($\leq$23 points) or absence (>23 points) of cognitive impairment[26]. Validation studies have demonstrated that the sensitivity of the MMSE increases remarkably as the cognitive impairment in the study population increases[26]. The MMSE is therefore a useful, valid and sensitive tool in dementia patients to separate those with moderate from those with severe levels of dementia[26]. However, an important shortcoming of the MMSE relates to its lack of sensitivity in mild cognitive impairment and its failure to discriminate between subjects with mild cognitive impairment and "normal" subjects[26]. In addition, mild cognitive decline can be quite heterogeneous and may differentially affect specific cognitive domains. Cognitive screening tools with a cut-off criterion to classify cognitive impairment are not able to specify cognitive deficits in a specific cognitive domain. Therefore, if the research is focused on investigating mild cognitive decline in persons who do not have apparent serious dementia diseases yet, the use of sensitive cognitive tests on multiple cognitive domains may be more useful than a cognitive screening device.

Another aspect that needs to be taken into account with the use of cognitive

tests in longitudinal research is the follow-up time. To assess mild cognitive decline by means of cognitive tests in apparently healthy subjects requires sufficient follow-up time for significant cognitive deterioration to become evident. In addition, a sufficiently long test-retest interval between the initial and subsequent exposure to the cognitive tests is necessary to diminish potential practice effects and prevent that participants "study" for the cognitive tests by rehearsing answers given on a previous administration of the tests.

The association between very long-chain n-3 PUFA and performance on cognitive tests has been examined in several observational studies in older adults[27-34]. In these studies the exposure to very long-chain n-3 PUFA was quantified either by estimating the fish consumption of the participants[31, 33, 34], or by measuring the levels of very long-chain n-3 PUFA in blood[27-30, 32]. Both approaches are suitable to rank people according to their exposure to very long-chain n-3 PUFA. However, the estimation of fatty acid or fish consumption requires the use of a sufficiently detailed dietary questionnaire, and these methods may rely on accuracy of memories and awareness of food intake. Fatty acid proportions in blood are generally considered objective and valid estimates of dietary fatty acid intake[35] and have the advantage of taking individual absorption into account[36].

Some studies have reported that lower dietary intake or lower blood proportions of n-3 PUFA were indeed associated with cognitive impairment and cognitive decline[27-29, 31, 34]; although other studies have reported no such associations[30, 32, 33]. However, the majority of the studies in this field define a global measure of overall cognitive performance (28-30, 32-34), rather than report performance in specific cognitive domains separately. This domain-specific information is, however, of consequence, because cognitive deficits in a specific domain may not be detected by generalized measures of cognitive performance. Moreover, this information provides insight into the specific cognitive domains that may be associated with n-3 PUFA, which is valuable information when designing future randomized controlled trials on very long-chain n-3 PUFA and cognitive decline.

In *Chapter 3* we investigated the association between plasma very long-chain n-3 PUFA and cognitive decline in multiple cognitive domains over three years. Our study population consisted of 807 Dutch older adults with no apparent signs of serious dementia and the cognitive domains that we investigated were sensorimotor speed, complex speed, memory, information processing speed and word fluency.

Very long-chain n-3 PUFA and blood supply in the head region

Reduced blood supply to the brain or disrupted microvascular structures in the head region may occupy an initiating or intermediate position in the chain of events ending with cognitive failure. The brain needs a constant supply of oxygen and nutrients in order to function, and structural vascular damage can decrease the perfusion rate of the brain resulting in a drop in cerebral glucose and oxygen utilization. Consequently, cerebral metabolism can suffer a setback that may lead to suboptimal cognitive capacity.

The main supply of oxygenated blood to the brain is carried by the carotid arteries. The carotid arteries branch off from the aorta and extend upward on each side of the neck. Narrowing of these carotid arteries due to atherosclerosis can obstruct the blood flow to the brain. Observational studies have emphasized the role of vascular abnormalities such as atherosclerosis as risk factors that may aggravate the progression of cognitive decline[37, 38]. In addition, patients with atherosclerosis of the carotid arteries often have cerebral microemboli, which may cause cognitive impairment if they enter the cerebral circulation[39]. Atherosclerosis in the carotid arteries supplying the brain not only thickens the vascular wall, but also contributes to blood flow disturbances by narrowing the vessel lumen. This may eventually result in massively increased rigidity of the arterial wall and severe stenosis, which in turn can cause stroke (brain infarct), a loss of blood flow to the brain that continues long enough to cause permanent brain damage.

The arterial wall consists of three layers: intima, media, and adventitia. Atherosclerosis in its early stage is generally characterized by endothelial damage and gradual diffuse thickening of the intima. High-resolution B-mode ultrasonography is a non-invasive technology that yields images of the walls of arteries. On these images two echogenic lines representing the lumen-intima interface and the media-adventitia interface can be identified. The distance between these echogenic lines is referred to as intima-media thickness. The ultrasonic evaluation of the carotid intima-media thickness is a widely used and valid surrogate marker for subclinical atherosclerosis[40]. In addition, the ultrasonography technique can be used to assess arterial stiffness, which relates to the cushioning function of the arteries and is determined by the visco-elastic properties of the arteries. A parameter of arterial stiffness is carotid distensibility, the absolute change in lumen diameter during systole for each cardiac cycle.

Carotid intima-media thickness and arterial stiffness are both surrogate markers of atherosclerosis and have both been associated with the risk of stroke[41-43]. Previous studies have not provided consistent results on the role of very long-chain n-3 PUFA and carotid intima-media thickness. An inverse association between dietary intake of very long-chain n-3 PUFA and carotid intima-media thickness was reported in three cross-sectional studies[44-46], however, no effect of dietary very long-chain n-3 PUFA supplementation was detected on the progression of carotid intima-media thickness in patients with coronary artery disease or abnormally elevated concentrations of specific lipoprotein particles[47-49]. The role of very long-chain n-3 PUFA in arterial stiffness has been even less well studied[49, 50]. No prospective studies have investigated the association between plasma very long-chain n-3 PUFA and common carotid distension as a marker of arterial stiffness. In *Chapter 4* of this thesis we will evaluate whether plasma very long-chain n-3 PUFA are associated with changes in carotid intima-media thickness and common carotid distension over a period of three years in 808 Dutch older adults.

Besides the macrovascular lesions, disrupted microvascular structures in the brain may also play a role in cognitive failure[51]. The microvessels represent the finest branches of the vascular tree and, unlike arteries, form a three-dimensional vascular network. Animal studies have described a beneficial effect of dietary very long-chain n-3 PUFA on cerebral microcirculation[52, 53]. We hypothesized that the role of very long-chain n-3 PUFA in the microcirculation may not be restricted to the brain, but that these fatty acids may affect the microcirculation in other organs, such as the ear, as well. Since the cochlea is highly vascularised, it has been suggested that if microvascular disease decreases the blood supply to the cochlea, this may result in age-related hearing loss[54]. Up until now, very little scientific attention has been given to the possible role of fatty acids in age-related hearing loss. A study in two psychiatric hospitals in Finland showed that adults who consumed a low fat diet for a period of five years had better hearing levels throughout the entire audiometric range than adults who consumed a diet high in saturated fatty acids[55]. However, there are currently no published studies that have investigated the relationship between very long-chain n-3 PUFA and age-related hearing loss.

Chapter 5 of this thesis describes a study in which we investigated whether high-

er proportions of plasma very long-chain n-3 PUFA are associated with less age-related hearing loss over three years in 720 Dutch older adults, who were free from middle ear dysfunction or unilateral hearing loss.

Outline of this thesis

The role of very long-chain n-3 PUFA in cognitive performance has become a rapidly extending research field in the last decade. Simultaneously, it is a field that seems to hold great promises for a wide range of people and consequently receives a lot of media attention. In this first chapter, we explored the scientific background for the role of very long-chain n-3 PUFA in brain tissue and cognitive performance and elaborated on the previous work done in this field, without aiming at total comprehensiveness. Our intention was to identify some of the gaps in our current knowledge and highlight areas that need further research. The question *whether very long-chain n-3 PUFA give someone a head start to win some years between the ears* is a very complex question to answer. With this first chapter, we aimed to indicate that the currently available scientific literature on this subject is not consistent and that many aspects related to this question are still to be cautiously examined. Therefore, in addressing such a complex question, a little scientific modesty is called for as well as an honest recognition of the gap between a research publication and a textbook. Nevertheless, the thesis intends to make a valuable contribution by addressing several issues arising from this field, to provide some of the pieces that will hopefully contribute to the overall picture.

The effects of feeding dietary fish oil on brain tissue in juvenile pigs will be addressed in *Chapter 2*, in which we investigated the fatty acid composition of the frontal, parietal, temporal and occipital brain lobes to evaluate regional differences in the responsiveness of different brain regions to dietary very long-chain n-3 PUFA. *Chapter 3* of this thesis describes the associations between very long-chain n-3 PUFA and cognitive decline in various cognitive domains over a three year period in older adults. The role of very long-chain n-3 PUFA in the macrovascular as well as microvascular supply in the head region is going to be touched upon in *Chapters 4* and *5*. We evaluated whether plasma very long-chain n-3 PUFA are associated with changes in carotid intima-media thickness and common carotid distension (*Chapter 4*). In addition, based on the hypoth-

esis that very long-chain n-3 PUFA may affect the microcirculation, which could decrease the blood supply to the cochlea, *Chapter 5* describes the association between very long-chain n-3 PUFA and age-related hearing loss in older adults. The human observational analyses in this thesis (*Chapters 3*, *4* and *5*) are performed within a population of men and postmenopausal women who originally participated in a randomized controlled trial investigating the effect of folic acid supplementation on carotid intima-media thickness, cognitive performance, and hearing[56, 57]. For the purpose of this study, subjects were randomly assigned to either folic acid or placebo treatment for a period of three years. To take this intervention into account in our analyses, we performed stratified analyses by intervention group when necessary. In *Chapter 6*, we will elaborate more on the interplay between folic acid and very long-chain n-3 PUFA and the way that this interplay may have affected our analyses. In the last chapter (*Chapter 7*) we will summarize the results presented in this thesis and discuss our findings in the perspective of other studies.

Chapter 2

Differences in fatty acid composition between cerebral brain lobes in juvenile pigs after fish oil feeding

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Abstract

Very long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) from fish are suggested to play a role in the development of the brain. Fish oil feeding results in higher proportions of n-3 PUFA in the brains of newborn piglets. However, the effect of fish oil on the fatty acid composition of specific cerebral brain lobes in juvenile pigs is largely uninvestigated. This study examined the effect of a fish oil diet on the fatty acid composition of the frontal, parietal, temporal and occipital brain lobes in juvenile pigs (7-weeks-old). Pigs were randomly allocated to a semipurified pig diet containing either 4% (w/w) fish oil (n=19) or 4% (w/w) high oleic acid sunflower oil (HOSF diet, n=18) for a period of eight weeks. The fish oil diet resulted in significantly higher proportions (%) of docosahexaenoic acid (DHA) in the frontal (mean ± SD, 10.6 ± 1.2), parietal (10.2 ± 1.5) and occipital brain lobes (9.9 ± 1.3), but not in the temporal lobe (7.7 ± 1.6), compared with pigs fed the HOSF diet (frontal lobe, 7.5 ± 1.0; parietal lobe, 8.1 ± 1.3; occipital lobe, 7.3 ± 1.2, temporal lobe, 6.6 ± 1.2). Moreover, the proportion of DHA was significantly lower in the temporal lobe compared with the frontal, parietal and occipital brain lobes in pigs fed a fish oil diet. In conclusion, the brains of juvenile pigs appear to be responsive to dietary fish oil, although the temporal brain lobe is less responsive compared with the other three brain lobes. The functional consequences of these differences are a challenging focus for future investigation.

Introduction

Docosahexaenoic acid (DHA) is the most abundant very long-chain n-3 polyunsaturated fatty acid (n-3 PUFA) in the central nervous system[3] and is therefore often considered an essential nutrient for the development of the brain[58]. Evidence for an effect of dietary n-3 PUFA on cognitive development of human infants is still limited and inconclusive[59, 60]. However, evidence from animal studies suggests that higher brain concentrations of DHA are associated with better cognitive performance[9].

Investigating whether dietary n-3 PUFA increases the proportion of n-3 PUFA in the human brain is complicated, as brain tissue sampling is not feasible in living human subjects. However, in animals we can examine the effect of n-3 PUFA consumption on the n-3 PUFA proportions in the developing brain. Although no animal species perfectly parallels the human situation, pigs resemble human subjects very closely in brain morphology, brain surface anatomy and postnatal brain development and maturation[7, 10]. Therefore, pigs confer significant advantages over other animal species for modelling the effects of dietary n-3 PUFA on the fatty acid composition of human brain tissue.

Several studies have investigated the effects of dietary n-3 PUFA on the fatty acid composition of the brain in newborn piglets provided with formula milk with or without n-3 PUFA[11-20]. The choice for newborn piglets arises from the fact that the brain growth spurt in pigs extends from about six weeks before birth to about five weeks afterwards, and embraces a phase of rapid deposition of lipids[23]. However, it is largely unknown whether dietary n-3 PUFA still affect the fatty acid composition of the brain after this period of rapid brain growth in juvenile pigs.

Most studies in piglets that investigated the effects of dietary n-3 PUFA determined the fatty acid composition of the whole brain[11-14, 16-20] and many reported higher proportions of n-3 PUFA in the brain tissue after n-3 PUFA feeding compared with control feeding[11-14, 18-20]. However, a limitation of such whole-brain analyses is that they do not take into account that brain development is not a homogeneous process in time or space. Various parts of the brain develop at different time points and grow with different speeds[21], which may have consequences for the responsiveness of different brain regions to n-3 PUFA. Effects of dietary n-3 PUFA on the whole brain may therefore mask regional heterogeneity in the brain lobes regarding the n-3 PUFA proportions and the response to dietary n-3

PUFA. Each hemisphere of both the human and the pig brain can be divided into the frontal, parietal, temporal and occipital brain lobes and in human subjects these separate brain lobes are all involved in specific cognitive functions[61-64]. The separate brain lobes may be differentially affected by n-3 PUFA consumption, which could be of importance for future research concerning dietary n-3 PUFA and brain function.

We investigated the effect of feeding dietary fish oil in a semipurified pig diet for a period of eight weeks on the fatty acid composition of the frontal, parietal, temporal and occipital brain lobes in a randomized, controlled feeding trial in juvenile pigs aged seven weeks at commencement.

Methods

The experimental protocol complied with the *Guide for the Care and use of Laboratory Animals*[65] and was approved by the animal experiments committee of Amsterdam Medical Centre, the Netherlands. Some of the brain samples used in this study were derived from twenty-two pigs that were also involved in another study, designed to investigate effects of dietary n-3 PUFA on electrophysiological changes in isolated pig hearts[66]. However, the brain samples of these pigs were dissected before the electrophysiological experiments on the isolated pig hearts started, thus the study conditions were similar for all pigs in our experiment.

Animals and diets

Five-week-old male non-littermate pigs (Topigs 40 sow x Tempo boar), were purchased from a research piggery (V.O.F. van Beek, Lelystad, the Netherlands) (n=37) and were housed in groups of two animals per pigpen in the animal care facilities of University Medical Centre Utrecht, the Netherlands. After two weeks of habituation on a regular pig chow diet, the seven-week-old pigs were randomly assigned to a semipurified pig diet containing either 4% (w/w) fish oil, Marinol C-35 (n=19) or 4% (w/w) high oleic acid sunflower oil (HOSF diet, n=18) for a period of eight weeks. The source of the fish oil was anchovy. The fish oil and HOSF (Loders Croklaan, Wormerveer, the Netherlands) were blended into the pig diets (Research Diets Services, Wijk bij Duurstede, the Netherlands) every two weeks and stored in a cold room (4˚C). Each pig received 1 kg feed per day and all pigs consumed the complete amount of feed every day. We used block

randomization with a block size of four to ensure approximately equal group sizes. Pigs entered the study in a phased procedure; i.e. two pigs started the trial each week. All study investigators as well as the laboratory and technical staff were blinded for the treatment allocation. Both diets contained similar amounts of total fat, carbohydrates and proteins and contained various vitamins and other essential food components (**Table 2.1**). Both diets were enriched with all-rac-α-tocopheryl acetate (40 mg/kg diet) to diminish *in vivo* oxidation of unsaturated fatty acids. We analysed the fatty acid composition of both diets (**Table 2.2**) at three different time points during the intervention period.

Table 2.1: Macro- en micronutrient composition of the semipurified pig diets containing either fish oil (fish oil diet) or high-oleic acid sunflower oil (HOSF diet)

		HOSF diet (g/kg diet)	**Fish oil diet (g/kg diet)**
Carbohydrate	Corn starch	326.5	326.5
	Dextrose	326.5	326.5
	Cellulose	50.0	50.0
Protein	Casein	180	180
Fat	Sunflower oil	10.0	10.0
	HOSF oil	40.0	0
	Fish oil	0.0	40.0
Minerals	$CaCO_3$	12.5	12.5
	$CaHPO_4$	20.0	20.0
	NaCl	5.0	5.0
	MgO	2.0	2.0
	$KHCO_3$	15.0	15.0
	$NaHCO_3$	2.5	2.5
Premix*		10.0	10.0
Energy content (kJ/ kg diet)		15958	15958

HOSF, high oleic acid sunflower oil.*The premix contained (mg/kg diet): MnO_2 (70.0); $FeSO_4.7H_2O$ (400.0); $ZnSO_4.H_2O$ (300.0); $NaSeO_3.5H_2O$ (0.2); KI (0.5); $CuSO_4.5H_2O$ (100.0); $CoSO_4.7H_2O$ (2.5); thiamin hydrochloride (2.0); riboflavin (5.0); nicotinamide (30.0); calcium pantothenate (12.0); pyridoxine hydrochloride (3.0); cyanocobalamin (0.04); folic acid (1.0); biotin (0.1); ascorbic acid (50.0); choline chloride (1000.0); menadione (3.0); all-rac-a-tocopheryl acetate (40.0); retinyl acetate and retinyl palmitate (18.0); cholecalcipherol (0.045); corn starch carrier material (7962,62).

Tissue sampling and lipid analyses

After eight weeks of feeding, the fasted pigs received 350 mg ketamine (Nimatek, Animal Health BV, the Netherlands) and 80 mg azaperone (Stresnil, Janssen, the Netherlands) intramuscularly and were anaesthetized with 20 mg pentobarbital/kg (Nembutal, CevaSate Animale) intravenously. Blood was collected, plasma and erythrocytes were extracted and stored at -80°C until lipid extraction and fatty acid analysis. The cerebrum was excised and separated into the left and right cerebral hemispheres by a mid-saggital section. Samples from the frontal, temporal, parietal and occipital lobes of the left hemisphere of the cerebrum were dissected, frozen in liquid nitrogen and stored at -80°C until lipid extraction and fatty acid analysis. Total lipids were extracted from all tissues according to the method of Folch *et al.*[67]. We analysed the fatty acid composition in brain total lipids, plasma cholesteryl esters and erythrocyte phospholipids. The identity of the fatty acid peaks was shown by gas chromatography by comparing each peak's retention time to the retention times of fatty acids in synthetic standards of known composition. Fatty acid composition data are expressed as g/100g fatty acid methyl esters. The sum of all peak areas of the fatty acids identified was taken as 100%. The percentages fatty acid methyl esters from the diet samples were converted to grams fatty acids per 100g total lipid using lipid conversion factors (0.93 for fish oil and 0.96 for HOSF[68]) and converted into grams fatty acids per 100g diet using the total lipid content.

Statistical analyses

Normal distribution of the data was tested using Shapiro-Wilk tests, and final decisions regarding normal distribution of the data were based on visual inspection of normal probability plots. Equality of variances was tested using Levene's tests. Two-way analysis of variance was used to evaluate the effects of diet and brain lobe on the fatty acid composition of the brain lobes. When significant F-tests were obtained, Tukey's honest significant differences (HSD) tests were applied for the *post hoc* comparison. Statistical significance was defined as $P<0.05$. All statistical analyses were conducted using SAS version 9.1.3 (Statistical Analysis Software; SAS Institute, Cary, NC, USA).

Table 2.2: Fatty acid composition of the semipurified pig diets containing either fish oil (fish oil diet) or high oleic acid sunflower oil (HOSF diet). (Means and standard deviations from three diet samples for each diet, taken at three different time points during the intervention period)

	HOSF diet (g/kg diet)		**Fish oil diet (g/kg diet)**	
	Mean	SD	Mean	SD
Saturated fatty acids, total	7.2	0.3	10.9	0.2
16:0	3.3	0.2	5.8	0.1
18:0	2.4	0.1	2.0	0.1
n-9 MUFA, total	37.4	0.8	8.0	0.1
18:1n-9	37.3	0.8	7.3	0.1
n-6 PUFA, total	13.9	1.2	11.8	0.2
18:2n-6	13.9	1.2	10.8	0.3
20:4n-6 (AA)	ND		0.6	0.1
n-3 PUFA, total	0.1	0.0	18.2	0.2
18:3n-3	0.1	0.0	0.2	0.1
20:5n-3 (EPA)	ND		8.1	0.1
22:5n-3 (n-3 DPA)	ND		1.1	0.1
22:6n-3 (DHA)	ND		7.1	0.1
Other fatty acids, total	0.8	0.1	2.8	0.1

AA, arachidonic acid; ND, not detectable; DPA; docosapentaenoic acid.

Results

Thirty-seven pigs received the diets containing either fish oil or HOSF for a mean period of 57.2 days (± 2.8 (SD)). The baseline fatty acid patterns in plasma and erythrocytes in the pigs assigned to the fish oil diet and those allocated to the HOSF diet were well balanced (**Table 2.3**). Brain samples from all four cerebral brain lobes were collected from 14 pigs in the fish oil group and 16 pigs in the HOSF group. **Table 2.4** shows the fatty acid composition in total lipids of the frontal, temporal, parietal and occipital brain lobes of pigs fed the fish oil diet and the HOSF diet. DHA was the predominant n-3 PUFA and arachidonic acid was the predominant n-6 PUFA found in all brain lobes of the pigs.

Table 2.3: Fatty acid patterns in erythrocyte membranes and plasma cholesteryl esters of pigs at baseline and after eight weeks on a semipurified pig diet containing either fish oil (fish oil diet, n=14) or high oleic acid sunflower oil (HOSF diet, n=16)[1]

	HOSF diet (% of total fatty acids)				**Fish oil diet (% of total fatty acids)**			
	Baseline		*After 8 wk*		*Baseline*		*After 8 wk*	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Erythrocyte membranes								
18:1n-9	26.9	1.8	33.9	3.7	26.5	2.3	24.9	3.9
18:2n-6	14.5	1.2	11.3	2.8	14.3	1.3	11.7	1.9
20:4n-6 (AA)	4.3	0.4	3.8	1.2	4.4	0.6	2.9	1.2
18:3n-3	0.5	0.2	0.1	0.1	0.6	0.4	0.2	0.1
20:5n-3 (EPA)	0.2 (0.1-0.2)		0.1 (0.0-0.1)		0.2 (0.1-0.2)		4.5 (3.7-4.7)	
22:5n-3 (n-3 DPA)	1.1	0.1	0.6	0.5	1.1	0.1	1.8	0.5
22:6n-3 (DHA)	1.6	0.3	1.0	0.8	1.4	0.3	2.9	1.0
total n-6 PUFA	19.7	1.3	16.1	4.2	19.8	1.8	15.2	3.2
total n-3 PUFA	3.5	0.4	2.4	2.6	3.4	0.5	8.7	3.4
Plasma cholesteryl esters								
18:1n-9	26.2	3.6	38.8	5.8	25.3	2.1	20.4	6.8
18:2n-6	42.3	5.0	35.5	2.0	42.9	3.7	34.8	1.1
20:4n-6 (AA)	7.1	1.1	6.1	1.0	7.6	1.1	3.7	1.1
18:3n-3	1.0	0.2	0.1	0.1	1.1	0.2	0.2	0.2
20:5n-3 (EPA)	0.3	0.2	ND		0.4	0.2	13.4	4.5
22:5n-3 (n-3 DPA)	0.1	0.1	ND		0.1	0.1	0.3	0.2
22:6n-3 (DHA)	0.4	0.2	0.2	0.4	0.4	0.2	1.3	0.5
total n-6 PUFA	50.2	5.0	42.3	2.7	51.3	3.4	39.2	1.6
total n-3 PUFA	1.8 (1.4-2.3)		0.2 (0.1-0.2)		1.9	0.5	15.5	5.2

AA, arachidonic acid; DPA, docosapentaenoic acid; ND= not detectable.

[1] For details of diets and procedures, see Methods. Means and standard deviations or medians with interquartile ranges.

Pigs on the fish oil diet had higher proportions of DHA in the frontal, parietal and occipital brain lobes but not in the temporal lobe, compared with pigs fed the diet containing HOSF. The interaction between diet and brain lobe was significant for DHA ($p=0.03$), indicating that the differences in DHA proportions between the brain lobes were not completely similar for the pigs on the fish oil diet compared with the pigs on the HOSF diet. In the fish-oil-fed pigs, the

proportion of DHA was significantly lower in the temporal lobe compared with the other three brain lobes. However, in the pigs fed the HOSF diet, the proportion of DHA was significantly lower in the temporal lobe compared with the parietal lobe only, but not compared with the other two brain lobes. EPA and n-3 DPA together were hardly detectable in the brain lobes of the pigs on the HOSF diet and comprised about 1.7% of total fatty acids in the fish-oil-fed pigs.

Pigs on the fish oil diet had significantly lower proportions of the n-6 PUFA arachidonic acid (C20:4n-6), adrenic acid (C22:4n-6) and osbond acid (C22:5n-6) in all four brain lobes compared with the pigs fed HOSF. A *post hoc* Tukey HSD test on the differences between the brain lobes showed that the proportions of n-6 PUFA were different between the brain lobes in both intervention groups. The frontal lobe had significantly higher proportions of n-6 PUFA compared with the other three brain lobes.

Although the HOSF diet contained higher proportions of oleic acid (mean ± SD, 37.3 ± 0.8 g/kg diet) compared with the fish oil diet (7.3 ± 0.1 g/kg diet), there were no significant differences in the proportions of total n-9 MUFA between the HOSF diet group and the fish oil group. However, a *post hoc* Tukey HSD test on the differences between the brain lobes showed that the temporal lobe had higher proportions of n-9 MUFA compared with the frontal lobe in both intervention groups.

With regard to the saturated fatty acids it appeared that the temporal lobe had lower proportions of total saturated fatty acids compared with the frontal lobe in both intervention groups.

Discussion

This study shows that a diet enriched with fish oil affects the fatty acids composition of the brain, even when supplied after the period of rapid brain growth in juvenile pigs. Moreover, we showed regional differences between specific cerebral brain lobes. Pigs on the fish oil diet had significantly higher proportions of DHA in the frontal, parietal and occipital brain lobes, but not in the temporal lobe. This suggests that the temporal lobe is less responsive to dietary fish oil compared with the other three brain lobes.

An important issue with regard to the interpretation of these data is that both intervention diets contained low amounts of α-linolenic acid. Dietary α-linolenic

Table 2.4: Fatty acid composition in total lipids (% of total fatty acids) of frontal, parietal, temporal and occipital brain lobes of pigs fed a semipurified pig diet containing either fish oil (fish oil diet, n=14) or high oleic acid sunflower oil (HOSF diet, n=16) for eight weeks*

	HOSF diet				Fish oil diet				P-value†		
	Frontal mean ± SD	*Parietal mean ± SD*	*Temporal mean ± SD*	*Occipital mean ± SD*	*Frontal mean ± SD*	*Parietal mean ± SD*	*Temporal mean ± SD*	*Occipital mean ± SD*	*Diet*	*Brainlobe*	*Inter-action*
SFA	37.0 ± 1.3^{a}	36.4 ± 1.4^{ab}	35.6 ± 2.5^{b}	36.1 ± 1.7^{ab}	37.6 ± 1.1^{a}	36.4 ± 1.0^{ab}	35.8 ± 1.9^{b}	36.8 ± 1.1^{ab}	0.19	<0.01	0.79
16:0	15.3 ± 0.9^{a}	15.2 ± 1.1^{a}	13.9 ± 1.8^{b}	14.7 ± 1.3^{a}	15.8 ± 0.9^{a}	15.0 ± 0.9^{a}	14.0 ± 1.6^{b}	15.1 ± 0.9^{a}	0.37	<0.001	0.73
18:0	19.6 ± 0.7^{a}	19.0 ± 0.7^{b}	18.9 ± 1.2^{b}	19.0 ± 0.8^{ab}	19.8 ± 0.5^{a}	19.0 ± 0.5^{b}	19.0 ± 1.0^{b}	19.4 ± 0.7^{ab}	0.19	<0.01	0.74
n-9 MUFA	20.9 ± 1.7^{a}	21.3 ± 2.2^{ab}	22.6 ± 3.7^{b}	21.2 ± 2.5^{ab}	20.6 ± 1.7^{a}	21.8 ± 1.6^{ab}	23.1 ± 3.8^{b}	20.9 ± 1.9^{ab}	0.87	0.02	0.79
18:1n-9	17.2 ± 1.1	17.1 ± 1.4	17.9 ± 2.2	17.7 ± 1.7	17.5 ± 1.2	17.9 ± 1.2	18.8 ± 2.2	17.9 ± 1.4	0.06	0.08	0.77
22:1n-9	0.3 ± 0.1^{a}	0.3 ± 0.1^{ab}	0.4 ± 0.2^{b}	0.4 ± 0.1^{ab}	0.2 ± 0.1^{a}	0.3 ± 0.1^{ab}	0.3 ± 0.1^{b}	0.3 ± 0.1^{ab}	<0.01	0.01	0.75
24:1n-9	2.7 ± 0.4^{a}	3.0 ± 0.6^{ab}	3.5 ± 1.2^{b}	3.2 ± 0.7^{ab}	2.3 ± 0.4^{a}	2.9 ± 0.4^{ab}	3.2 ± 1.1^{b}	2.7 ± 0.6^{ab}	0.03	<0.001	0.74
n-6 PUFA	16.4 ± 1.0^{a}	15.2 ± 1.3^{b}	14.7 ± 2.2^{b}	15.0 ± 1.5^{b}	12.1 ± 0.5^{a}	11.0 ± 0.6^{b}	11.5 ± 1.5^{b}	11.2 ± 1.0^{b}	<0.001	<0.001	0.36
18:2n-6	0.5 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.4 ± 0.1^{b}	0.4 ± 0.1^{ab}	0.5 ± 0.1^{a}	0.4 ± 0.1^{ab}	0.4 ± 0.1^{b}	0.5 ± 0.1^{ab}	0.21	<0.01	0.37
20:3n-6	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.4 ± 0.1^{b}	0.5 ± 0.1^{ab}	<0.001	0.02	<0.001
20:4n-6	8.5 ± 0.5^{a}	7.9 ± 0.6^{b}	8.0 ± 1.1^{ab}	7.7 ± 0.7^{b}	7.1 ± 0.3^{a}	6.5 ± 0.4^{b}	6.8 ± 0.8^{ab}	6.6 ± 0.6^{b}	<0.001	<0.001	0.82
22:4n-6	4.0 ± 0.3^{a}	3.6 ± 0.3^{b}	4.2 ± 0.5^{c}	3.8 ± 0.4^{ab}	2.5 ± 0.2^{a}	2.3 ± 0.3^{b}	3.0 ± 0.4^{c}	2.4 ± 0.3^{ab}	<0.001	<0.001	0.51
22:5n-6	2.1 ± 0.5^{a}	1.9 ± 0.7^{a}	1.4 ± 0.6^{b}	1.8 ± 0.6^{ab}	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{b}	0.4 ± 0.1^{ab}	<0.001	<0.01	0.08
n-3 PUFA	7.6 ± 1.0^{ab}	8.2 ± 1.1^{a}	6.6 ± 1.2^{b}	7.3 ± 1.2^{ab}	12.3 ± 1.3^{a}	11.8 ± 1.5^{a}	9.5 ± 1.9^{b}	11.6 ± 1.4^{a}	<0.001	<0.001	0.03
18:3n-3	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
20:5n-3	ND	ND	ND	ND	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	-	-	-
22:5n-3	ND	0.1 ± 0.1	ND	ND	1.4 ± 0.2	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	-	-	-
22:6n-3	7.5 ± 1.0^{ab}	8.1 ± 1.3^{a}	6.6 ± 1.2^{b}	7.3 ± 1.2^{ab}	10.6 ± 1.2^{a}	10.2 ± 1.5^{a}	7.7 ± 1.6^{b}	9.9 ± 1.3^{a}	<0.001	<0.001	0.03

ND= not detectable. ^{a,b,c} Mean values with different superscript letters indicate significant differences between the brain lobes within each dietary intervention group, according to post hoc Tukey HSD tests.*For details of diets and procedures, see Methods. Mean values and standard deviations. †Two-way analysis of variance.

acid has been shown to support the synthesis and deposition of DHA in neural tissues, in the absence of a dietary source of DHA in pigs[11]. It is therefore possible that the insufficient amount of α-linolenic acid in combination with the absence of DHA in the HOSF diet may have caused a decrease in brain DHA.

We specifically addressed the effect of dietary fish oil, without this potentially disturbing effect of α-linolenic acid conversion into DHA. Our results suggest that the temporal lobe is less responsive to dietary fish oil with regard to DHA proportions compared with the other brain lobes, but that there are no differences in DHA proportions between the temporal, parietal and occipital brain lobes in the absence of dietary DHA. However, we have to keep in mind that our study design does not enable us to compare the effect of dietary fish oil on the fatty acid composition of the separate brain lobes with the effect of a normal pig chow diet containing sufficient amounts of α-linolenic acid.

The use of pigs as an animal model to study the effects of dietary fish oil on the fatty acid composition of the brain has distinct advantages. The pig brain resembles the human brain very closely in overall shape and structure. Both pigs and human subjects have brains with a highly convoluted surface (gyrencephalic brain), as opposed to rodents which have a smooth, lissencephalic brain. Moreover, pigs have a human-like distribution of grey and white matter in the brain, whereas rodents have little white matter[7, 10]. The timing of the brain growth spurt in relation to birth varies between animal species. However, pigs closely resemble the human brain development sequence as the brain growth spurt peaks at birth in both human subjects and pigs[7, 10]. Furthermore, the fatty acid composition of the pig brain corresponds well with the fatty acid composition of the human brain. The total proportion of saturated fatty acids in the pig's brains was approximately 36%, with the major saturated fatty acids being palmitic acid (16:0) and stearic acid (18:0). This is comparable to the human infant brain, where palmitic acid and stearic acid are the major saturated fatty acids comprising about 35-50% of total fatty acids in phosphoglycerides[4]. Oleic acid (18:1n-9) comprised about 18% of total fatty acids in both the pig brain as well as the human infant brain[4]. DHA and arachidonic acid are the two major polyunsaturated fatty acids in both the pig and the human brain[5, 69]. Moreover, the DHA:arachidonic acid ratio in the frontal lobe in the HOSF pigs was approximately 0.9, which is consistent with the DHA:arachidonic acid ratio of approximately 0.8 in the forebrain of human

infants[69].

Random allocation of the pigs to the two treatment groups was applied to prevent selection bias and to generate groups that were roughly comparable in terms of responsiveness to the diet and baseline proportions of n-3 PUFA in the brain tissue. As the pigs were non-littermates, were all fed the same pig chow diet before entering the study, and were randomly allocated to the intervention groups, it is highly unlikely that differences in fatty acid handling due to lineage or differences in the pre-treatment diet between the two groups would have occurred in our study that could have biased our results. Moreover, the fatty acid levels in plasma cholesteryl esters and erythrocyte membranes support there being no differences in n-3 PUFA proportions in these tissues at baseline between the two intervention groups.

A limitation for translating our findings to human subjects is that the dose of fish oil we applied was fairly high. The diets contained either 4% (w/w) fish oil or 4% (w/w) HOSF, which is 2.51 gram of fish oil or high oleic sunflower oil per 1000 kJ of metabolizable energy. In human studies, normal doses used are 2 - 10 grams of fish oil per day, which corresponds to approximately 0.2 - 1.0 grams of fish oil per 1000 kJ energy intake. Thus, based on energy intake, the dose of fish oil in this pig study was about 2.5 to 12.5 times higher than in human trials. However, in view of the magnitude of the effects seen in pigs, it is likely that lower doses of fish oil will also have a notable effect on brain fatty acid composition.

The proportions of saturated fatty acids, n-9 monounsaturated fatty acids (n-9 MUFA), n-6 PUFA and n-3 PUFA in the pig brains observed in this study are comparable to the proportions reported in the whole brain in other pig studies [12, 16, 70]. However, relatively little is known about the effects of dietary fish oil on the fatty acid composition of specific brain lobes in pigs. To the best of our knowledge, there is only one other study in pigs comparing the effect of dietary fish oil on the fatty acid composition of the four cerebral brain lobes. This study in newborn piglets showed no significant differences in DHA between the temporal lobe and the other brain lobes after two weeks of fish oil feeding, although the authors describe an apparent preservation of the temporal lobe with regard to the fatty acid composition in their discussion section[15]. However, our results are in agreement with a study in adult rats that showed lower proportions of DHA in the temporal lobe, compared with the parietal, frontal and occipital lobes after a diet with

preformed DHA[71]. Although this suggests that the temporal lobe could be less responsive to dietary fish oil compared with the other brain lobes, the number of animal studies comparing the four cerebral brain lobes is too limited to make definite inferences.

A hypothesis regarding the regional differences in fatty acid composition after dietary fish oil may be that the four cerebral brain lobes have different growth rates. It may be that regions associated with more primary functions (e.g. primary motor cortex, within the frontal lobe) develop earlier compared with regions that are involved with more complex and integrative tasks (e.g., temporal lobe)[72].

An issue of concern is the interpretation given to the data. If the insensitivity for dietary fish oil in the temporal lobe would be confirmed in future studies in pigs, the question arises what a relative enrichment of DHA in specific brain lobes means, in particular when considering human brain development and function. Are cognitive functions that are linked to the temporal lobe in humans less likely to be influenced by dietary fish oil than cognitive functions linked to other brain lobes? If indeed the proportion of DHA in the brain would influence cognitive performance, our data would suggest that increasing fish oil consumption will poorly affect cognitive functions in which the temporal lobe is involved, such as verbal and visual memory[73]. Moreover, we saw significantly lower levels of n-6 PUFA, arachidonic acid in particular, in all four brain lobes in the pigs on the fish oil diet compared with the HOSF pigs, which may also have consequences for cognitive development. However, the relation between structure and function in the brain is not very well defined. Moreover, as the evidence for effects of dietary fish oil on specific cognitive functions at present is still limited and inconclusive for infants and children[9, 60], these contentions are highly speculative.

The responsiveness of the developing brain for nutrients depends on whether a nutrient actually reaches the brain as well as on the timing of exposure. It is not well defined how long-chain PUFA accumulate in the brain, although it has been suggested that direct transport from the plasma is more important for brain accretion of DHA than local de-novo synthesis[74]. The transport of long-chain PUFA across cell membranes is suggested to be mediated by passive diffusion through the phospholipid bilayer[75] or facilitated by membrane- and cytoplasma-associated proteins[76]. However, there is little information regarding passive

diffusion of fatty acids through the blood-brain barrier and the blood cerebrospinal fluid barrier. On the other hand, a number of fatty acid transporter proteins have been identified in the brain, indicating that facilitated transport may be the major mechanism for long-chain PUFA transport into the brain[74]. DHA is mainly incorporated into phosphatidylethanolamine and phosphatidylcholine, whereas arachidonic acid is mainly incorporated into phosphatidylinositol and phosphatidylcholine[74]. In this study, we measured the fatty acid composition of total lipids, and not the individual lipid classes. The total lipid fraction includes any non-esterified fatty acids, which may have originated from hydrolysis of fatty acid which may occur during the post-mortem period. Another factor regarding the responsiveness of the developing brain for nutrients depends on the timing of exposure. Our study shows that dietary DHA is efficiently incorporated in the brain of juvenile pigs. This suggests that the juvenile pig brain is apparently still responsive to dietary differences in fatty acid composition, although the period of rapid lipid deposition which is associated with the brain growth spurt[77] has ended.

In summary, our study shows that increasing the dietary intake of EPA and DHA through fish oil feeding in juvenile pigs after their brain growth spurt resulted in higher proportions of DHA and lower proportions of n-6 PUFA in the frontal, parietal, and occipital brain lobes. The fatty acid composition of the temporal brain lobe appears to be less responsive to dietary differences in DHA. The effects of lower dietary dosages of fish oil on regional differences in brain n-3 PUFA as well as the consequences of these differences for cognitive development and performance should be the focus for future investigation.

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Chapter 3

N-3 fatty acid proportions in plasma and cognitive performance in older adults

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Abstract

Background: Very long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) are suggested to be related to cognitive performance in older adults. However, limited data exists on the association between n-3 PUFA and performance in specific cognitive domains.

Objective: We evaluated the association between plasma n-3 PUFA proportions and cognitive performance in five cognitive domains and determined whether plasma n-3 PUFA proportions predict cognitive change over three years.

Design: We used data from the FACIT trial, in which participants received folic acid or placebo capsules for three years. Fatty acid proportions in plasma cholesteryl esters at baseline were measured in 807 men and women aged 50-70 years. Cognitive performance for memory, sensorimotor speed, complex speed, information-processing speed and word fluency was assessed at baseline and after three years. The cross-sectional analyses were based on all 807 participants; the longitudinal analyses were based only on 404 participants in the placebo group.

Results: Higher plasma n-3 PUFA proportions predicted less decline in sensorimotor speed (multiple linear regression coefficient, z-score = 0.31, 95%CI: 0.06, 0.57) and complex speed (0.40, 95%CI: 0.10, 0.70) over three years. Plasma n-3 PUFA proportions did not predict 3-year changes in memory, information-processing speed or word fluency. The cross-sectional analyses showed no association between plasma n-3 PUFA proportions and performance in any of the five cognitive domains.

Conclusion: In this population, plasma n-3 PUFA proportions were associated with less decline in the speed-related cognitive domains over three years. These results need to be confirmed in randomized controlled trials.

Introduction

A decline in cognitive performance over time is observed in the general elderly population[78]. However, the extent and rate of this decline can vary considerably between individuals[78]. There is increasing scientific interest in the hypothesis that very-long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), as present in fish or fish oil, explain part of this inter-individual variation and are beneficial for the maintenance of cognitive performance of adults.

Several observational studies, conducted among older adults, have examined the association between n-3 PUFA and cognitive performance[27-34]. Some authors have reported that lower dietary intake or lower plasma or erythrocyte proportions of n-3 PUFA were indeed associated with cognitive impairment and cognitive decline[27-29, 31, 34]; others have reported no such associations[30, 32, 33]. Some of these studies also evaluated the association between fish consumption and cognitive performance and reported a lower risk of cognitive impairment[31] and slower cognitive decline[33, 34] or a trend toward a lower risk of cognitive decline[30] with increasing fish intakes. The inconsistency of these findings may be due to various factors: differences in sample size, heterogeneity of study populations, different methods of estimating n-3 PUFA intakes, or the wide variety of the applied cognitive tests.

Cognitive function in older adults declines with different rates in specific cognitive domains[79]. Domain-specific information is valuable, because it provides insight into the specific cognitive functions that may be associated with n-3 PUFA. However, most of the studies in this field define a global measure of overall cognitive performance[28-30, 32-34], rather than report performance in specific cognitive domains separately. Cognitive deficits in a specific domain may or may not be detected by such generalized measures of cognitive performance and this may explain the inconsistent results between studies.

Only two studies conducted in middle-aged persons have addressed the association between n-3 PUFA and cognitive performance in different domains[27, 31]. Beydoun *et al.* showed that higher proportions of plasma n-3 PUFA reduced the risk of decline in verbal fluency[27], whereas Kalmijn *et al.* showed that a higher intake of fatty fish and n-3 PUFA was associated with a lower risk of impaired psychomotor speed[31]. However, this latter study used dietary assessments to evaluate n-3 PUFA intake, a method that relies on accuracy of memories and aware-

ness of food intake; no plasma fatty acid proportions were available. Moreover, because this study was solely cross-sectional, it was not possible to evaluate the association between n-3 PUFA consumption and cognitive change over time.
The present study assessed whether plasma n-3 PUFA proportions predict changes in cognitive performance over three years in five cognitive domains in older adults living in the Netherlands.

Subjects and methods

Subjects

For this study we used data from the FACIT study, a randomized placebo-controlled trial investigating the effect of folic acid supplementation on cognitive performance, carotid intima-media thickness, and hearing, which was approved by the Wageningen University Medical Ethics Committee[80]. The baseline measurements, which included cognitive testing, were conducted between 2000 and 2001. In this study, 819 men and postmenopausal women aged 50-70 years were randomly assigned to either folic acid (n=406) or placebo (n=413) treatment for three years. Participants were originally included in this study if they had plasma total homocysteine levels ≥ 13 μmol/L and ≤ 26 μmol/L and serum vitamin B12 levels ≥ 200pmol/L.
In our analyses, we excluded eight participants from whom insufficient amounts of blood could be obtained, three participants who did not give permission for the fatty acid analyses and one participant who did not perform the cognitive tests, which resulted in a total of 807 participants.

Assessment of cognitive performance

Cognitive performance was assessed by using a concise battery of five cognitive tests. These tests are reported to be sensitive enough to detect small cognitive differences with ageing[79, 81-85]. These tests have no ceiling effect and are therefore presumed to be sensitive and robust in detecting cognitive impairment, even at middle age.

Concept Shifting Test

The Concept Shifting Test evaluates the ease of switching between two psychological concepts[85]. On each of four test cards, 16 small circles are grouped in a

larger circle. The small circles are either empty or contain a number or a letter and are randomly arranged in the larger circle. First, subjects were asked to cross out circles with numbers in chronologic order (subtask A), then circles with letters in alphabetical order (subtask B), and finally circles with either letters or numbers, in chronologic and alphabetical order (subtask C). The final task was to cross out empty circles (subtask O). The time needed to complete each of the four tasks was recorded.

Stroop Colour-Word Test

The Stroop Colour-Word Test is considered to be a general measure of cognitive flexibility and executive functioning[81]. Subjects were first asked to read the names of colours (subtask I) and subsequently to name colour blocks (subtask II). Finally, participants were asked to name the colour of the ink in which the words were printed, rather than reading the word (subtask III). The time needed to complete each of the three tasks was recorded.

Word Learning Test

The Word Learning Test evaluates the declarative memory, the part of the memory used for specific facts or experiences[82]. Fifteen monosyllabic words in a fixed sequence were visually presented in three subsequent trials, with a recall procedure immediately following each presentation (immediate recall). Twenty minutes after the last trial, participants were again asked to recall the memorized words (delayed recall). In each trial, the number of correctly recalled words was recorded.

Letter Digit Substitution Test

The Letter Digit Substitution Test evaluates the general speed of visual information processing[83]. Nine different letters are coupled with nine different numbers in a key on top of the form. Participants were asked to copy the corresponding number by each letter as quickly as possible. The number of correctly filled in numbers in 90 seconds was recorded.

Verbal fluency Test

The Verbal fluency Test measures the ability to recollect as many words in a

specific category as possible from memory[84]. Participants were asked to name as many animals as possible in 60 seconds. The number of different animal names was recorded.

To compare the results of the different cognitive tests, we transformed the raw test scores of all participants into z-scores ($z = [x - \bar{x}] / sd$) . For the cross-sectional analyses, we used the mean and standard deviation of the baseline scores to calculate the z-scores. For the longitudinal analyses, the mean and standard deviation at baseline and at follow-up were pooled to calculate the grand mean and standard deviation per test; this grand mean and standard deviation was used to calculate z-scores at both time points. This latter step was necessary, because both the follow-up scores and the baseline scores are included in the statistical model and therefore have to refer to the same z-distribution.
The z-scores were clustered into five cognitive domains which had been established *a priori*; they were denoted as sensorimotor speed, complex speed, memory, information-processing speed and word fluency[80]. Sensorimotor speed was calculated by averaging the z-scores on the O, A and B subtests of the Concept Shifting Test and subtest I of the Stroop Colour-Word Test. Complex speed was composed of subtest C of the Concept Shifting Test and subtest III of the Stroop Colour-Word Test. Signs of sensorimotor speed and complex speed scores were inverted so that they reflected above average performance if positive and below average performance if negative. Memory was calculated by averaging the z-scores of the total, maximum and delayed recall scores of the Word Learning Test. Information-processing speed was composed of the z-score of the Letter Digit Substitution Test and word fluency was composed of the z-score of the Verbal fluency Test.

The cognitive tests were derived from the Maastricht Aging Study, a prospective study on the determinants of cognitive ageing[86]. Additionally, we included the Mini-Mental State Examination (MMSE)[25]. The MMSE was not one of our outcome measures, but we used it to compare our study population with other study populations and to enable the exclusion of persons with suspected dementia (score: <24 points). We adopted this widely used cut-off point, because population studies investigating the MMSE as a screening test for dementia reported this cut-off point to be a good compromise between sensitivity and specificity[87, 88].

Trained research assistants administered the cognitive tests during a 40-min session using standard test protocols. All participants performed the tests after an overnight fast followed by a standardized breakfast without coffee or tea. Cognitive testing was repeated after three years using parallel versions of the tests used at baseline to prevent learning effects. We did not use a parallel version of the Verbal fluency Test, because a valid parallel version of this test is not available.

Assessment of plasma n-3 PUFA

Venous blood was collected after an overnight fast in one 10-ml Vacutainer tube containing EDTA as anticoagulant. The samples were centrifuged and the obtained plasma was stored within 2 hours at -80°C until analysed. Fatty acids in plasma cholesteryl esters were measured as described previously[89]. We calculated plasma n-3 PUFA by adding up the proportions of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA).

Assessment of potential confounders

Participants completed questionnaires on demographic variables, which were reviewed by a research assistant in the presence of the participant. Level of education was divided into three groups according to the highest level attained: primary education, junior vocational training, and senior vocational or academic training. We assessed alcohol and fish consumption during the preceding three months with a food-frequency questionnaire and estimated physical activity using the Physical Activity Scale for the Elderly. Height and weight were measured to calculate body mass index (in kg/m^2). The *ApoE* genotype was determined by the polymerase chain reaction-based restriction fragment length polymorphism method and restriction enzyme digestion with Hha 1. The apolipoprotein *ε4* allele is involved in neuropathology and is the major known genetic risk factor for Alzheimer's disease[90]. We divided *ApoE* genotype according to the absence of the apolipoprotein *ε4* allele or the presence of one or two apolipoprotein *ε4* alleles.

Statistical analyses

We assessed the association between n-3 PUFA and cognitive performance with multiple linear regression analysis, with possible confounders based on previously published associations and associations with exposure and outcomes (change

in the beta coefficient ≥ 10%) in the current dataset. We applied base-10 logarithmic transformation of n-3 PUFA proportions, because the distribution was skewed to the right. Logarithmic transformation disabled interpretation on a ratio scale and implied interpretation in terms of ten-fold multiplications.

Longitudinal analyses

We present the longitudinal association between n-3 PUFA and cognitive performance after three years only for the 404 participants that received placebo capsules, because the folic acid treatment has been shown to improve performance in memory, information-processing speed, and sensorimotor speed[80] and appeared to be an effect modifier in our dataset. We used one sample t-tests to determine if cognitive performance significantly changed over three years. We used multiple linear regression analysis to investigate the association between n-3 PUFA proportions (independent) measured at baseline, and cognitive performance in z-scores (dependent) in five different domains after three years, with adjustment for baseline cognitive performance scores. We examined whether potential confounders (age, sex, level of education, erythrocyte folate concentrations, alcohol intake, physical activity, smoking, body mass index, number of apolipoprotein *ε4* alleles, depression, diabetes mellitus and cardiovascular disease) were associated both with plasma n-3 PUFA and cognitive performance, and substantially attenuated the association when added to the model. The final model was adjusted for age, sex, level of education (three categories), erythrocyte folate concentrations and alcohol intake. We chose to correct for baseline cognitive performance in contrast to calculating change scores, because change scores do not control for baseline imbalances and may therefore lead to regression-to-the-mean[91]. Nevertheless, analysis of the change scores yielded similar results with regard to the p-values.

Cross-sectional analyses

We used multiple linear regression analyses to investigate the association between n-3 PUFA proportions (independent) and cognitive performance in z-scores (dependent) at baseline in the five different cognitive domains. We evaluated the same potential confounders as in the longitudinal analyses, and the final model was adjusted for age, sex, level of education (three categories), erythrocyte folate

concentrations, and alcohol intake.
Statistical significance was defined as $p<0.05$. The statistical analyses were conducted by using SAS version 9.1.3 (Statistical Analysis Software; SAS Institute, Cary, NC).

Results

The mean age of the participants was 60 years at baseline, 72% of the participants were male, 77% of the participants completed junior/senior vocational training or academic training, and the median alcohol intake was approximately one drink per day. The geometric mean plasma n-3 PUFA proportion was 1.57% of total fatty acids, with an interquartile range of 1.18-2.04% of total fatty acids. The median MMSE score at baseline was 29, and the range was 15-30 points (**Table 3.1**). Seven participants scored <24 out of 30 points on the MMSE. However, the exclusion of these seven participants did not substantially change the results; hence, we present all results including these subjects. There were no differences in baseline characteristics of participants between the cross-sectional and the longitudinal analyses. The fatty acid profiles of the participants are shown in **Table 3.2**.

Change in cognitive performance over three years (longitudinal analyses)

Four participants did not return for the cognitive measurements after three years, because they died (n=2) or suffered from severe illnesses (n=2). Sensorimotor speed, complex speed and information-processing speed declined significantly over three years. The mean (±SD) 3-year change in z-scores was -0.10 ± 0.46 for sensorimotor speed, -0.07 ± 0.53 for complex speed and -0.15 ± 0.51 for information-processing speed. Participants improved on memory over three years; the mean (±SD) 3-year change in z-scores was 0.34 ± 0.73 for memory, because of procedural learning effects (**Table 3.3**).
Higher proportions of plasma n-3 PUFA significantly predicted less decline over three years in cognitive performance scores in the domains of sensorimotor speed and complex speed (Table 3.3). The change in sensorimotor speed improved by a z-score of 0.31 (95% CI: 0.06-0.57) for each 1% change in log-transformed n-3 PUFA proportions. This translates to an improvement in z-score of 0.31 for every ten-fold multiplication in n-3 PUFA proportions and to an increase in z-score of

0.09 for every doubling in n-3 PUFA proportions. For example, this implies that a person with 2.0% n-3 PUFA of total fatty acid proportions in plasma has 9% less decline in sensorimotor speed than does a person with 1.0% n-3 PUFA of total fatty acids in plasma.

Table 3.1: Baseline characteristics of participants in cross-sectional and longitudinal analyses[1]

Characteristics	Cross-sectional analyses (n=807)	Longitudinal analyses (n=404)
Age (y)	60 ± 6[2]	60 ± 6
Sex (%), male/female	72 / 28	71 / 29
Level of education (%), low/middle/high[3]	22 / 38 / 39	19 / 41 / 41
Physical activity, PASE score	153 ± 69	152 ± 68
Body Mass Index (kg/m^2)	27 ± 4	27 ± 4
Diabetes mellitus (%)	3	4
Depression (%)	6	5
Alcohol consumption (g/d)[4]	13 (5-24)	13 (5-24)
Smoking (%), never/former/current	28 / 52 / 20	29 / 51 / 20
ApoE ε4 allele (%), 0 / 1 / 2[5]	68 / 29 / 3	69 / 28 / 3
EPA+DPA+DHA (%) cholesteryl esters[6]	1.57 (1.18-2.04)	1.56 (1.21-2.01)
MMSE score (points)[4]	29 (28-30)	29 (28-30)

[1] PASE, physical activity scale for elderly; ApoE, apolipoprotein E; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; MMSE, Mini-Mental State Examination. Percentages are rounded and may therefore not always add up to 100. Means (Student's t-test) and percentages (Chi-square test) were not significantly different between the two groups (p>0.05).

[2] Mean ± SD (all such values).

[3] Low, primary education; middle, junior vocational training; high, senior vocational or academic training.

[4] Values are median; interquartile ranges in parentheses.

[5] Data available for 803 participants in the cross-sectional analyses and 401 in the longitudinal analyses.

[6] Values are geometric mean; interquartile range in parentheses.

The change in complex speed improved with a z-score of 0.40 (95%CI: 0.10-0.70) for every ten-fold multiplication in n-3 PUFA proportions. This corresponds to an increase in z-score of 0.12 for every doubling in n-3 PUFA proportions. This implies that a person with 2.0% n-3 PUFA of total fatty acid proportions in plasma has 12% less decline in complex speed than does a person with 1.0% n-3 PUFA of total fatty acids in plasma. Thus, higher plasma n-3 PUFA proportions at baseline predicted less decline in scores of sensorimotor speed and complex

speed over three years. These associations were present after adjustments for baseline cognitive performance, age, sex, level of education, erythrocyte folate status and alcohol consumption. N-3 PUFA proportions at baseline did not predict changes in memory (regression coefficient [95% CI]: 0.17 [-0.23, 0.57]), information-processing speed (0.14 [-0.14, 0.42] or word fluency (0.36 [-0.10, 0.82]) over three years (Table 3.3). The results were similar when we evaluated the association between plasma DHA proportions and 3-year cognitive change in all five cognitive domains (data not shown). Furthermore, plasma proportions of n-6 PUFA (the sum of linoleic acid, gamma-linolenic acid and arachidonic acid) did not predict 3-year changes in any of the five cognitive domains (data not shown).

Table 3.2: Fatty acid contents in plasma cholesteryl esters in 807 participants

Fatty acids	Value
	% of total fatty acids
Saturated fatty acids, total	12.85 ± 1.14[1]
Stearic acid (18:0)	0.83 ± 0.20
Palmitic acid (16:0)	10.98 ± 0.90
Monounsaturated fatty acids, total	21.24 ± 3.17
Oleic acid (18:1n-9)	17.14 ± 2.25
Polyunsaturated fatty acids, total	64.54 ± 4.08
Linoleic acid (18:2n-6)	53.59 ± 5.00
Arachidonic acid (20:4n-6)	6.68 ± 1.42
Alpha-linolenic acid (18:3n-3)	0.59 ±0.14
Eicosapentaenoic acid (20:5n-3)	0.92 (0.69-1.32)[2]
Docosapentaenoic acid (22:5n-3)	0.00 (0.00-0.04)
Docosahexaenoic acid (22:6n-3)	0.60 ± 0.21

[1] Mean ± SD (all such values)
[2] Median; interquartile range in parentheses (all such values)

Cognitive performance (cross-sectional analyses)

Multiple linear regression analysis showed no significant linear associations between plasma n-3 PUFA proportions and performance in any of the five cognitive domains. Higher plasma n-3 PUFA proportions were not significantly as-

sociated with better performance in sensorimotor speed (regression coefficient [95% CI]: 0.01 [-0.26, 0.29]), complex speed (0.07 [-0.24, 0.38]), memory (0.20 [-0.15, 0.55], information-processing speed (0.04 [-0.32, 0.41]), or word fluency (0.18 [-0.20, 0.57]), after adjustments for age, sex, level of education, erythrocyte folate concentrations and alcohol consumption (**Table 3.4**). The results were similar when we evaluated the association between plasma DHA proportions and cognitive performance in all five cognitive domains (data not shown). Furthermore, plasma proportions of n-6 PUFA (the sum of linoleic acid, gamma-linolenic acid and arachidonic acid) were not significantly associated with cognitive performance at baseline in any of the five cognitive domains (data not shown).

Discussion

This study among older adults showed that higher plasma proportions of n-3 PUFA significantly predicted less decline in the sensorimotor speed and complex speed cognitive domains over three years than did lower proportions of n-3 PUFA. We observed no significant associations between plasma n-3 PUFA proportions and 3-year changes in memory, information-processing speed and word fluency. The cross-sectional analyses showed no significant associations between plasma n-3 PUFA proportions and performance in any of the five cognitive domains.

A major strength of our study is that we assessed cognitive performance longitudinally using an extensive cognitive battery under standardized test conditions. This battery is reported to be sensitive enough to detect small cognitive differences in this age range[79, 81-85], and it provided us with specific information on performance in various cognitive domains. These are two important advantages compared with commonly used dementia screening tools, such as the MMSE. A second strength is that we used plasma cholesteryl ester proportions of n-3 PUFA as a marker of dietary intake. Plasma cholesteryl ester proportions are generally considered an objective and valid estimate of the dietary intake of fatty acids during the prior weeks[35] and have the advantage of taking individual absorption into account[36]. Moreover, additional data on dietary intake of fish from a food-frequency questionnaire indicated that baseline dietary fish intake correlated well with dietary fish intake after three years (Spearman correlation coefficient:

0.79 ($p<0.001$)). Therefore, we assume that participants did not change their dietary intake of fish, and n-3 PUFA proportions in plasma were stable over this period. Finally, close follow-up of the subjects reduced the chance of missing values on outcome variables; only four participants (1%) in the placebo group did not return for the cognitive function measurements after three years.

Table 3.3: Results of the longitudinal analyses: change in cognitive performance over three years (left side of dotted line) and differences in 3-year cognitive change per ten-fold multiplication in plasma cholesteryl ester n-3 PUFA proportions (right side of dotted line) in 400 Dutch older adults[1]

	Performance at baseline	Performance after 3 years	3-year change in cognitive performance	p	Regression coefficient (95%CI)	p
Sensorimotor speed (z-score)[2]	0.02 ± 0.84[3]	-0.08 ± 0.82	-0.10 ± 0.46	<0.01	0.31 (0.06, 0.57)	0.02
Complex speed (z-score)[4]	0.00 ± 0.88	-0.07 ± 0.86	-0.07 ± 0.53	<0.01	0.40 (0.10, 0.70)	<0.01
Memory (z-score)	-0.21 ± 0.88	0.14 ± 0.96	0.34 ± 0.73	<0.01	0.17 (-0.23, 0.57)	0.41
Information-processing speed (z-score)[2]	0.03 ± 1.01	-0.14 ± 1.01	-0.15 ± 0.51	<0.01	0.14 (-0.14, 0.42)	0.32
Word fluency (z-score)	-0.08 ± 0.96	0.00 ± 0.95	0.07 ± 0.86	0.10	0.36 (-0.10, 0.82)	0.13

[1] Left side of dotted line: Change in cognitive performance over 3 years was tested with one-sample t-tests. Right side of dotted line: multiple linear regression analyses, adjusted for baseline cognitive performance, age, sex, level of education, erythrocyte folate concentrations and alcohol consumption.
[2] Data were available for 399 participants.
[3] Mean ± SD (all such values).
[4] Data were available for 395 participants.

A possible limitation of this study was that the participants did not represent a random sample of the Dutch older adult population. We performed observational analyses in a population that was selected specifically for participation in a randomized controlled trial. Hence, selective participation may have affected the generalizability of our results. Our participants had relatively high plasma homocysteine concentrations and low serum vitamin B12 concentrations. Therefore, it could be possible that they represent a group of people with relatively unhealthy dietary habits, such as a lower dietary intake of fish. However, the range of plasma

Table 3.4: Results of the cross-sectional analyses: differences in cognitive performance per ten-fold multiplication in plasma cholesteryl ester n-3 PUFA proportions in 807 Dutch older adults[1]

Cognitive domains	Regression coefficient (95% CI)	p
Sensorimotor speed (z-score)[2]	0.01 (-0.26, 0.29)	0.92
Complex speed (z-score)[3]	0.07 (-0.24, 0.38)	0.66
Memory (z-score)	0.20 (-0.15, 0.55)	0.27
Information Processing Speed (z-score)[4]	0.04 (-0.32, 0.41)	0.81
Word fluency (z-score)	0.18 (-0.20, 0.57)	0.34

[1] Multiple linear regression analyses, adjusted for age, sex, level of education, erythrocyte folate concentrations, and alcohol consumption.
[2] Data available for 806 participants.
[3] Data available for 801 participants.
[4] Data available for 804 participants.

n-3 PUFA proportions in our study population was comparable with that of other large studies in older adult populations[36, 92]. Furthermore, although we used an extensive test battery to assess cognitive performance, we cannot exclude the possibility that a more detailed cognitive assessment might have revealed associations undetected by the current tests.

We observed that higher plasma levels of n-3 PUFA significantly predict less decline in scores of sensorimotor speed and complex speed over three years; however, this finding was not confirmed in the cross-sectional analyses. One could argue that the inconsistency between the cross-sectional and the longitudinal findings may be explained by differences between the populations because the longitudinal analyses were performed in the placebo group only. However, there were no differences in baseline characteristics of the participants in cross-sectional and longitudinal analyses. Moreover, the cross-sectional analyses still yielded no associations when restricted to the placebo group only.

Because our analyses were performed in a population of normal ageing participants with no signs of cognitive disorder or dementia (with the exception of 7 participants with an MMSE score < 24), it is difficult to interpret whether an improvement of 9% in sensorimotor speed and 12% in complex speed for every doubling in plasma n-3 PUFA proportions is clinically relevant. Moreover, there is considerable debate in the literature about whether or not cognitive decline is the beginning of a broad spectrum of cognitive changes leading to dementia. To determine the relevance of our findings, we considered our results along with

those from other observational studies in comparable populations (**Table 3.5**). Our longitudinal associations are in line with earlier findings from Morris *et al.*, in which the annual rate of overall cognitive decline was reduced by 10% to 13% in persons who consumed one or more fish meals per week compared with those with less than weekly consumption[33]. Furthermore, two reports from the Zutphen Elderly study reported less cognitive decline with high fish consumption[30, 34], although these findings were not statistically significant in one of these studies (30). Two other studies have examined the role of n-3 PUFA status in cognitive decline in normal ageing persons without cognitive disorder or dementia. Heude *et al.* reported that lower proportions of n-3 PUFA in erythrocytes are associated with a higher risk of cognitive decline[29]. In contrast, Laurin *et al.* found higher proportions of EPA in plasma phospholipids in cognitive impaired subjects than in controls[32]. However, in the latter two studies, the number of participants experiencing cognitive decline was small. A recent study by Beydoun *et al.* showed that higher plasma concentrations of n-3 PUFA reduced the risk of decline in verbal fluency, but not psychomotor speed or memory[27]. Therefore, more observational studies in this population within a normal range of plasma n-3 PUFA proportions using an extensive battery of cognitive tests are welcome to verify the direction and size of the association. Subsequently, randomized controlled trials with n-3 PUFA supplementation should help to clarify the importance of the observed associations.

One way to describe the predictive value of n-3 PUFA in effecting cognitive changes is to express it in terms of another predictor in the linear regression model, such as the chronological age. For example, we can calculate the average number of years that participants are cognitively younger, by using the ratio between the regression coefficients associated with the plasma n-3 PUFA proportions and with age in years[79]. Thus, a doubling in plasma n-3 PUFA proportions in our statistical model gives a person a hypothetical 3-year decline in cognitive performance of someone 4.5 years younger for sensorimotor speed (0.09/-0.02= -4.5 years), or 12 years younger for complex speed (0.12/-0.01=-12 years). However, we have to keep in mind that these regression equations are derived from observational data, and therefore we can make no direct interferences on causality. Moreover, these numbers are reflective of our specific population only.

We have shown in a population of Dutch older adults that higher plasma

Table 3.5: Cross-sectional and longitudinal studies examining the association between n-3 PUFA and cognitive performance in older adults[1]

First author	Study population	N-3 PUFA	Cognitive tests	Results[2]
Cross-sectional studies				
Conquer *et al.*[28]	Cognitive impairment (n=36); no cognitive impairment (n=19); age: ~77-83 y	Plasma phospholipids	1 SD below age norm on 6 tests[3]	Lower n-3 PUFA proportions in cognitively impaired group
Kalmijn *et al.*[31]	Cognitive impairment (n=163), no cognitive impairment (n=1450); age: 45-70 y	FFQ	Lowest 10% on overall cognition, speed, flexibility, memory[4]	Higher n-3 PUFA and fish intake associated with lower risk of overall cognition impairment and speed
Kalmijn *et al.*[30]	Cognitive impairment (n=153), no cognitive impairment (n=323); age: 64-89 y	Dietary history method	MMSE (cut-off score ≤25)	N-3 PUFA and fish intake not associated with cognitive impairment
Laurin *et al.*[32]	Cognitive impairment (n=43), no cognitive impairment (n=79); age: ≥65 y	Plasma phospholipids	Diagnosis on Zaudig's criteria	No difference in n-3 PUFA proportions between groups
Van Gelder *et al.*[34]	Elderly men (n=210); age: 70-89 y	Dietary history method	MMSE	No difference in cognition between categories of fish consumers
Longitudinal studies				
Kalmijn *et al.*[30]	Cognitive decline (n=51), no cognitive decline (n=291); age: 64-89 y, follow-up: 3 y	Dietary history method	MMSE (drop >2 points)	N-3 PUFA and fish intake not associated with cognitive decline
Laurin *et al.* [32]	Incident cognitive impairment (n=16); no cognitive impairment (n=52); age: ≥65 y, follow-up: ~5 y	Plasma phospholipids	Diagnosis on Zaudig's criteria	N-3 PUFA proportions not different between groups. Higher EPA levels in cognitive impaired group
Heude *et al.*[29]	Cognitive decline (n=27), no cognitive decline (n=219); age: 63-74 y, follow-up: 4 y	Erythrocyte membranes	MMSE (drop ≥2 points)	Higher n-3 PUFA proportions associated with lower risk of cognitive decline
Morris *et al.*[33]	Community residents (n=3718); age: ≥65 y, follow-up: 6 y	FFQ	Sum of scores on 4 tests[5]	Higher fish intake, but not n-3 PUFA intake, associated with slower cognitive decline
Van Gelder *et al.*[34]	Elderly men (n=210); age: 70-89 y, follow-up: 5 y	Dietary history method	MMSE	Higher fish intake and n-3 PUFA intake associated with slower cognitive decline
Beydoun *et al.*[27]	Cognitive decline (n=140), no cognitive decline (n=2111); age: 50-65 y, follow-up: 6 y	Plasma phospholipids and cholesteryl esters	RCI<-1.645 on overall cognition, memory, psychomotor speed, verbal fluency	Higher n-3 PUFA proportions associated with lower risk of decline in verbal fluency

[1] EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; MMSE, mini-mental state examination; RCI, reliable change index.
[2] Results adjusted for confounding factors are shown if reported in original paper.
[3] WAIS-R, Digit symbol, California Verbal Learning test, Rey Osterrieth figure: copy and short delay, Benton Visual Naming test, Boston Naming test.
[4] Domains determined by cognitive test battery: Verbal Learning test, Concept Shifting task, Stroop Colour-Word test, Letter Digit Substitution test, Category fluency test.
[5] East Boston tests of immediate and delayed recall, MMSE, Symbol Digit Modalities Test.

proportions of n-3 PUFA are associated with less decline in 3-year cognitive performance in sensorimotor speed and complex speed, but not in memory, information-processing speed and word fluency. Further examination of the role of n-3 PUFA in cognitive performance in older adult populations in observational and intervention studies seems justified. These studies should preferably be conducted with sensitive cognitive outcome measurements that provide domain-specific information.

Chapter 4

The association between plasma very long-chain n-3 polyunsaturated fatty acids and progression of carotid stiffness and carotid intima-media thickness in older adults

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Submitted for publication

Abstract

Background: Very long-chain n-3 polyunsaturated fatty acids (PUFA) may play a role in the reduction of the risk of stroke. Carotid intima-media thickness and arterial stiffness are both predictors of stroke risk.

Objective: To investigate whether plasma very long-chain n-3 PUFA are associated with progression of carotid stiffness and carotid intima-media thickness.

Design: A longitudinal study was conducted in a group of 808 men and postmenopausal women aged 50-70 y. Fatty acid proportions were assessed in plasma cholesteryl esters. Ultrasonography of the common carotid artery was used to determine the change in carotid distension and carotid intima-media thickness at baseline and after three years.

Results: There were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in common carotid distension ($p=0.95$, ANCOVA) or common carotid intima-media thickness ($p=0.44$, ANCOVA) after three years, after adjustments for baseline value of the common carotid function marker, age, sex, current smoking, body mass index, alcohol consumption, physical activity, and serum cholesterol concentrations.

Conclusion: Due to the small progression of carotid intima-media thickness in our study population over the three year period, a beneficial association could potentially have been missed. However, in the context of other observational and intervention studies, there is currently no sufficient consistent evidence to support the hypothesis that very long-chain n-3 PUFA are associated with common carotid stiffness or common carotid intima-media thickness in a healthy population.

Introduction

Several cohort studies have reported that a high fish intake was associated with a lower risk of stroke. A meta-analysis of cohort studies showed that subjects who ate fish 1 to 3 times a month had a significantly lower risk of stroke than subjects who ate fish less than once a month[93]. The risk of stroke has been associated with atherosclerosis in the carotid arteries[94]. Carotid intima-media thickness and arterial stiffness, indicators of atherosclerosis, are both predictors of stroke[41, 43, 95, 96]. Although arterial stiffness and carotid intima-media thickness are not assumed to be a cause of stroke, they may serve as surrogate markers of atherosclerosis elsewhere in the arterial system[41].

Previous studies have not provided consistent results on the association of very long-chain n-3 polyunsaturated fatty acids (PUFA) from fish and carotid intima-media thickness. An inverse association between dietary intake of very long-chain n-3 PUFA and carotid intima-media thickness was reported in three cross-sectional studies[44-46], however, no effect of dietary very long-chain n-3 PUFA supplementation was detected on the progression of carotid intima media thickness in persons with coronary artery disease, hyperlipidaemia or hyperlipoproteinemia[47-49].

The role of very long-chain n-3 PUFA in arterial stiffness has been even less well studied. A small cross-sectional study reported lower aortic pulse wave velocity in inhabitants of a fishing village compared with inhabitants of a farming village, although this association was significant in males only[50]. Furthermore, an intervention study in elderly men with hyperlipidaemia also showed a favourable effect of n-3 PUFA supplementation on arterial elasticity general stiffness of the arterial circulatory system[49]. However, up until now, no prospective observational studies in apparently healthy persons have investigated the association between plasma very long-chain n-3 PUFA and common carotid distension as a marker of arterial stiffness.

The present study evaluates whether plasma very long-chain n-3 PUFA are associated with changes in common carotid intima-media thickness and common carotid distension over a period of three years in a population of older adults.

Methods

Study design

Data come from participants of the FACIT study, a randomized controlled trial investigating the effect of folic acid supplementation on common carotid intima-media thickness, cognitive performance and hearing (56, 57). This study included 819 men and postmenopausal women aged 50-70 years, who were randomly assigned to either folic acid (n=406) or placebo (n=413) treatment for a period of three years. Major exclusion criteria were plasma total homocysteine levels <13 μmol/L and >26 μmol/L, serum vitamin B12 levels <200 pmol/L, self-reported medical diagnosis of renal or thyroid diseases, and current use of B vitamin supplements or medications that influence common carotid intima-media thickness progression (i.e., lipid-lowering medications, hormone replacement therapy). The baseline measurements, which included measurements of common carotid intima-media thickness and common carotid distension, were conducted between 2000 and 2001. The FACIT study, on which the present article is based, was approved by the Medical Ethics Committee of Wageningen University, the Netherlands. All study participants provided written informed consent.

Ultrasonography of the common carotid arteries

High resolution B-mode ultrasonography of the common carotid arteries was performed in a dimmed and quiet room, using a conventional echo-imaging system (Ultramark® IX, ATL, Bothell, Washington) equipped with a 7.5-MHz linear-array transducer. Participants were measured in supine position with the head tilted 45° in the opposite direction of the carotid being scanned. Longitudinal images of the distal common carotid arteries were obtained at eight predefined angles marked on the Meijer's Arc: 90°, 120°, 150° and 180° when the right carotid was scanned and at 270°, 240°, 210° and 180° when the left carotid was scanned. Images were frozen on the top of the R-wave of the electrocardiogram and recorded on VHS-videotape for off-line analysis. Subsequently, the ultrasonography system was switched to M-mode at the angle that gave the best visualization of the arterial walls, as judged by the sonographer. M-mode images of the common carotid artery pulsation displaying approximately three seconds of the cardiac cycle were recorded on VHS-videotape for off-line analysis.

Carotid image analysis

The ultrasound images from the videotapes were analysed by the Vascular Imagine Center (Utrecht, the Netherlands) and were interpreted in batch fashion by a single reader. The batch reading implied that the four scans (duplicate baseline and duplicate follow-up scans) of every participant were read in a random order within a short time frame. Each batch contained scans of 25 participants. To determine mean intima-media thickness, the near and far wall of the distal 10 mm of the left and right common carotid arteries were measured using an automated edge detection program (Artery Measurement System). The B-mode images of all eight angles were analysed and averaged for each individual. To determine common carotid stiffness, we calculated arterial distensibility in diameter. Arterial distensibility in diameter was calculated as the absolute change in lumen diameter during systole for each cardiac cycle. The measurements over three cardiac cycles were averaged. The ultrasound examination was performed in duplicate at baseline and after three years, and we used the average of the duplicates for the statistical analyses.

Plasma n-3 PUFA proportions

Venous blood was collected after an overnight fast in one 10-ml Vacutainer tube containing EDTA as anticoagulant. The obtained plasma was stored within 2 hours at -80°C until analysis. Fatty acids in plasma cholesteryl esters were determined as described previously (89). We calculated plasma n-3 PUFA by adding up the proportions of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3 DPA), and docosahexaenoic acid (DHA).

Other measurements

At baseline, participants completed a questionnaire on general demographic variables and medical history. Height and weight were measured to calculate body mass index (kg/m^2). Brachial blood pressure was measured using an automated meter (Dinamap Compact Pro 100, General Electric, Waukesha, Wisconsin) and we used the mean of eight measurements. Physical activity was estimated using the Physical Activity Scale for the Elderly. Serum total cholesterol, LDL-cholesterol and HDL-cholesterol were determined on a Hitachi® 747 analyser (Roche Diagnostics, Basel, Switzerland). C-reactive protein, oxidized LDL, and

auto-antibodies against oxidized LDL were measured at baseline as described previously[97].

Statistical analyses

In our analyses, we excluded eight participants from whom insufficient amounts of blood could be obtained and three participants who did not give permission for the fatty acid analyses, resulting in a total of 808 participants for the cross-sectional analyses. Baseline characteristics of subjects in the different quartiles were compared with chi-square tests, one-way analysis of variance for normally distributed variables, or Kruskal-Wallis tests for non-normally distributed variables. We used quartiles of plasma very long-chain n-3 PUFA and analysis of covariance (ANCOVA) was used to evaluate the associations between quartiles of very long-chain n-3 PUFA and common carotid intima-media thickness and common carotid distension. Equality of variances of the dependent variables was tested using Levene's tests. We considered age, sex, body mass index, smoking status, alcohol consumption, physical activity, and serum cholesterol concentration as potential confounders. To investigate whether the folic acid treatment modified the association in the longitudinal analyses, we also performed stratified analysis by intervention group. Statistical significance for all analyses was defined as $P < 0.05$. The data analyses were performed with the Statistical Analysis System (SAS version 9.1.3; SAS Institute Inc, Cary, NC).

Results

Baseline characteristics of the study population according to quartiles of plasma very long-chain n-3 PUFA are shown in **Table 4.1**. There were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in the potential confounders age, sex, body mass index, smoking habits, and physical activity. However, compared with subjects in the lowest quartile of plasma very long-chain n-3 PUFA, subjects in the highest quartile had a higher consumption of alcohol. The proportions of individual fatty acids in plasma cholesteryl esters according to quartiles of plasma very long-chain n-3 PUFA are shown in **Table 4.2**.

Table 4.1: Baseline characteristics of the study population according to quartiles of plasma very long-chain n-3 PUFA[1]

	Plasma very long-chain n-3 PUFA				
	Quartile 1 (n=202)	*Quartile 2 (n=202)*	*Quartile 3 (n=202)*	*Quartile 4 (n=202)*	*P-value[2]*
Age (y)	60.1 ± 5.9	59.9 ± 5.7	60.3 ± 5.5	60.7 ± 5.5	0.49
Sex (male/female)	156 / 46	149 / 53	137 / 65	137 / 65	0.09
Body mass index (kg/m^2)	26.1 ± 3.5	26.6 ± 3.3	26.9 ± 3.9	26.8 ± 3.8	0.14
Education (low/middle/high)[3]	53 / 71 / 78	50 / 75 / 77	41 / 85 / 76	38 / 77 / 87	0.46
Smoking (never/ former/ current)	61 / 100 / 41	62 / 103 / 37	59 / 95 / 48	48 / 118 / 36	0.30
Alcohol consumption (g/day)	8.4 (1.7-15.9)	14.1 (4.5-21.7)	13.1 (5.2-26.5)	17.2 (7.4-27.2)	<0.001
Physical activity (PASE-score)	151 ± 71	162 ± 70	156 ± 72	144 ± 61	0.06
Total cholesterol (mmol/L)	5.66 ± 1.10	5.71 ± 1.11	6.00 ± 1.05	5.92 ± 1.16	0.01
LDL cholesterol (mmol/L)	3.90 ± 0.97	3.94 ± 0.97	4.16 ± 0.94	4.07 ± 1.02	0.03
HDL cholesterol (mmol/L)	1.22 ± 0.32	1.20 ± 0.34	1.23 ± 0.36	1.27 ± 0.41	0.23
Triglycerides (mmol/L)	1.29 ± 0.68	1.37 ± 0.82	1.44 ± 0.99	1.36 ± 0.85	0.38
C-reactive protein (mg/L)	1.0 (0.5-1.9)	1.2 (0.6-2.4)	1.3 (0.6-2.4)	1.2 (0.7-2.6)	0.10
sICAM-1 (µg/L)	138 (116-158)	143 (118-167)	140 (113-162)	130 (110-156)	0.30
Oxidized LDL (U/L)	51 (38-67)	51 (40-64)	55 (40-77)	50 (39-78)	0.47
IgG against oxidized LDL, OD_{450}	0.15 (0.09-0.25)	0.15 (0.09-0.28)	0.14 (0.08-0.21)	0.12 (0.08-0.19)	0.20
IgM against oxidized LDL, OD_{450}	0.13 (0.08-0.24)	0.13 (0.07-0.26)	0.14 (0.08-0.23)	0.16 (0.08-0.25)	0.64
Systolic blood pressure (mm Hg)	133 ± 16	132 ± 16	134 ± 17	133 ± 16	0.83
Diastolic blood pressure (mm Hg)	77 ± 9	77 ± 9	77 ± 8	77 ± 8	0.85
Mean arterial pressure (mm Hg)[4]	96.0 ± 10.4	95.2 ± 10.5	96.0 ± 10.3	95.8 ± 10.0	0.85
Lumen diameter (mm)	6.16 ± 0.68	6.13 ± 0.64	6.16 ± 0.72	6.10 ± 0.65	0.74

Abbreviations: PASE, physical activity scale for the elderly. sICAM-1, soluble intercellular adhesion molecule-1; LDL, low-density lipoprotein; OD_{450}, optical density at 450 nm. Values are means ± SD, medians with interquartile range in parentheses or n.

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04%.

[2] P-values for differences across quartiles were obtained by one-way analysis of variance for continuous, normally distributed variables, Kruskal-Wallis tests for continuous, non-normally distributed variables, or chi-square tests for categorical variables.

[3] Low=primary education; Middle=junior vocational training; High= senior vocational or academic training.

[4] Mean arterial pressure is diastolic blood pressure + 1/3 x (systolic blood pressure – diastolic blood pressure).

Table 4.2: Fatty acid contents (% of total fatty acids) in plasma cholesteryl esters according to quartiles of plasma very long-chain n-3 PUFA[1]

Fatty acids	Quartile 1 (n=202)	Quartile 2 (n=202)	Quartile 3 (n=202)	Quartile 4 (n=202)
Saturated fatty acids, total	12.2 ± 1.1	12.9 ± 1.1	13.0 ± 1.1	13.3 ± 1.0
Palmitic acid (16:0), %	10.5 ± 0.8	11.0 ± 0.9	11.1 ± 0.8	11.4 ± 0.8
Stearic acid (18:0), %	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
N-9 mono-unsaturated fatty acids, total	16.3 ± 2.2	17.0 ± 2.2	17.4 ± 2.3	17.7 ± 2.1
Oleic acid (18:1n-9), %	16.3 ± 2.2	17.0 ± 2.2	17.4 ± 2.3	17.7 ± 2.1
N-6 polyunsaturated fatty acids, total	64.7 ± 3.8	62.4 ± 3.9	61.2 ± 4.2	59.3 ± 4.1
Linoleic acid (18:2n-6), %	57.1 ± 4.1	53.7 ± 4.5	52.6 ± 4.8	50.9 ± 4.4
Arachidonic acid (20:4n-6), %	6.0 ± 1.4	7.0 ± 1.4	6.9 ± 1.4	6.8 ± 1.3
N-3 polyunsaturated fatty acids, total	1.6 ± 0.2	2.0 ± 0.2	2.3 ± 0.2	3.4 ± 0.9
α-Linolenic acid (18:3n-3), %	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Eicosapentaenoic acid (20:5n-3), %	0.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	2.0 ± 0.8
Docosapentaenoic acid (22:5n-3), %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Docosahexaenoic acid (22:6n-3), %	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.2

Values are means ± SD.

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04%.

In the longitudinal analyses, we were able to analyse the carotid intima-media thickness data of 790 of the 808 participants and the carotid stiffness data of 786 of the 808 participants. Twenty-two participants did not provide one or both carotid function measures after three years, because they died during the follow-up (n=12), were lost to follow-up (n=5), had unreadable carotid scans (n=2) or incomplete measurements to calculate carotid stiffness (n=3).

The annualized rate of change in mean common carotid intima-media thickness was 0.002 ± 0.018 (mean ± SD) mm/year. There were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in 3-year progression of mean common carotid intima-media thickness (**Table 4.3**, p=0.44, ANCOVA), after adjustments for baseline intima-media thickness, age, sex, current smoking, BMI, alcohol consumption, physical activity, and serum cholesterol concentrations. Similarly, no differences between the quartiles were found when common carotid intima-media thickness was based on the maximum thickness rather than the mean thickness of the arterial wall (Table 4.3, p=0.50, ANCOVA).

The annualized rate of change in common carotid distension was -0.019 ± 0.041 (mean ± standard deviations [SD]) mm/year. There were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in 3-year regression of common carotid distension (Table 4.3, p=0.95, ANCOVA), after adjustments for baseline carotid distension, age, sex, current smoking, BMI, alcohol consumption, physical activity, and serum cholesterol concentrations. There were also no significant differences between the quartiles for other measures associated with arterial stiffness, i.e. blood pressure, lumen diameter, pulse pressure or mean arterial pressure (data not shown). After stratifying the data by intervention group, comparable longitudinal results were found with regard to the association between plasma very long-chain n-3 PUFA, common carotid distension and common carotid intima-media thickness when they were performed separately for the placebo and the folic acid group (data not shown). Furthermore, the results were similar when we evaluated the association between plasma DHA proportions or EPA proportions and 3-year change in common carotid intima-media thickness or distension (data not shown).

In the cross-sectional analyses, we found that there were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in mean common carotid intima-media thickness at baseline (Q1 (Quartile 1): 0.781 (95%CI: 0.766 to 0.796); Q2: 0.786 (95%CI: 0.772 to 0.801); Q3: 0.787 (95%CI: 0.772 to 0.802); Q4: 0.783 (95%CI: 0.768 to 0.798; p=0.94 for ANCOVA), after adjustments for age, sex, current smoking, BMI, alcohol consumption, physical activity and total cholesterol concentrations. In addition, no significant differences between the quartiles of plasma very long-chain n-3 PUFA were found in common carotid distension at baseline (Q1: 0.650 (95%CI: 0.626 to 0.673); Q2: 0.631 (95%CI: 0.608 to 0.654); Q3: 0.609(95%CI: 0.586 to 0.632); Q4: 0.644 (95%CI: 0.621 to 0.667); p=0.07 for ANCOVA), after adjustments for the same confounders. Evaluating the association between plasma DHA or EPA proportions with common carotid intima-media thickness or distension yielded similar results (data not shown). In addition, we found no significant differences between the quartiles of plasma very long-chain n-3 PUFA in the inflammatory markers: serum concentrations of C-reactive protein, soluble intercellular adhesion molecule-1, oxidized low-density lipoprotein, and autoantibodies against oxidized low-density lipoprotein (Table 4.1).

Table 4.3: Common carotid stiffness and intima-media thickness after three years according to quartiles of plasma very long-chain n-3 PUFA [1]

	Plasma very long-chain n-3 PUFA				
	Quartile 1	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p* [2]
Arterial distension in diameter (mm)	0.574 (0.559; 0.589)	0.579 (0.564; 0.593)	0.575 (0.560; 0.589)	0.579 (0.564; 0.594)	0.95
Sample size	196	196	196	198	
Mean intima-media thickness (mm)	0.788 (0.780; 0.795)	0.784 (0.777; 0.792)	0.784 (0.776; 0.791)	0.792 (0.784; 0.799)	0.44
Sample size	197	197	197	199	
Maximum intima-media thickness (mm)	0.930 (0.922; 0.938)	0.928 (0.920; 0.936)	0.926 (0.918; 0.934)	0.935 (0.926; 0.943)	0.50
Sample size	197	197	197	199	

Results are adjusted means with 95% CI in parentheses or n.

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04%.

[2] P-values for differences across quartiles were obtained by analysis of covariance, adjusted for baseline value of the common carotid function marker, age, sex, current smoking, body mass index, alcohol consumption, physical activity, and serum cholesterol concentrations.

Discussion

The present longitudinal study in older adults does not support the hypothesis that very long-chain n-3 PUFA are associated with progression of common carotid stiffness or common carotid intima-media thickness, both markers of generalized atherosclerosis and predictors of vascular disease risk, over a period of three years.

We are confident that the lack of an association in our dataset is not due to the quality of our measurements as we took multiple methodological measures to minimize measurement error in our carotid distensibility and intima-media thickness measurements. We performed duplicate ultrasound examinations at baseline and after three years to decrease the variability of the measurements. These duplicate scans were performed by the same sonographer in 91% of the baseline scans and in 80% of scans after three years. In addition, one reader performed all offline ultrasound readings, and we applied batch reading with the reader blinded to the scan sequence to reduce measurement variation over time. Moreover, we used an automated edge detection program to reduce the chance of a drift in reading behaviour over time.

Despite the potential benefits of fish intake in de reduction of stroke risk[93], it is not clear whether very long-chain n-3 PUFA have a role in the pathogenesis of carotid atherosclerosis. The lack of an association between very long-chain n-3 PUFA and arterial stiffness in our study is in line with findings from a cross-sectional study in 470 Japanese adults that showed no significant correlation between dietary very long-chain n-3 PUFA consumption and aortic pulse wave velocity as a marker of arterial stiffness[50]. However, an intervention study among 563 elderly men with hyperlipidaemia showed a favourable effect of very long-chain n-3 PUFA supplementation compared with diet counselling for three years on arterial elasticity, as measured by pulse wave propagation time[49]. Moreover, another intervention study among 84 patients with dyslipidaemia showed that supplementation with EPA for 12 months compared with diet therapy attenuated the increase in arterial stiffness, as measured by pulse wave velocity[98]. These inconsistencies between the results from observational and intervention studies may be due to the different measures of arterial stiffness, the different study populations and possibly some amount of publication bias. The intervention studies measured pulse wave velocity, a maker of aortic stiffness, rather than carotid distension, a marker of carotid stiffness. In healthy persons, aortic stiffness and carotid stiffness correlate quite well[99]. However, the intervention studies were performed in high-risk patients with long-standing hyperlipidaemia[49] or dyslipidaemia[98]. It has been suggested that high-risk patients with more cardiovascular risk factors have "accelerated ageing" of the aortic wall compared with carotid wall[99]. Therefore, the aorta of these patients becomes disproportionally stiff with ageing compared with the carotid artery. Beneficial effects of fish oil on measures of aortic stiffness in high-risk populations may therefore not just be extrapolated to measures of carotid stiffness.

The absence of a significant association between very long-chain n-3 PUFA and common carotid intima-media thickness in our study does not agree with findings from three cross-sectional studies among Japanese adults[46], Alaskan Eskimo's[44], and a multiethnic group of US citizens[45]. This may suggest that the range of plasma very long-chain n-3 PUFA in our Dutch study population may have been too narrow to show statistically significant differences in progression of carotid intima-media thickness between the quartiles. However, our findings are in line with three intervention studies in patients with combined hyperlipopro-

teinemia, coronary artery disease and hypercholesterolemia that showed no significant effect of very long-chain n-3 PUFA supplementation for two years[47, 48] or three years[49] on carotid intima-media thickness. One of these studies did, however, report that very long-chain n-3 PUFA supplementations mitigated the progression of atherosclerosis in the coronary arteries[100]. The authors speculated that the anti-atherogenic effect of n-3 PUFA may be specific for certain vascular beds such as the coronary circulation, or that the effect on very long-chain n-3 PUFA supplementation is at least smaller in carotid than in coronary arteries[100]. However, their findings do not agree with an intervention study among 59 coronary heart disease patients, that showed no changes in the diameter of the coronary arteries after two years of fish oil treatment[101]. The absence of a significant association between high proportions of very long-chain n-3 PUFA and progression of common carotid intima-media thickness in the present study may be due to the relatively small rate of change in common carotid intima-media thickness (0.002 ± 0.018 mm/year, annualized mean ± SD) in our study population. The average carotid intima-media thickness progression rate is estimated to be 0.0147 mm/year, based on progression rates of control groups from published randomized controlled trials[102]. Although we have to keep in mind that this pooled estimate is derived mainly from studies in patients with either coronary artery disease, hypercholesterolemia or miscellaneous vascular abnormalities, the progression rate in our study population may have been too small to illustrate a subtle association between very long-chain n-3 PUFA and carotid intima-media thickness. Because we also showed no association between very long-chain n-3 PUFA and markers of inflammation, another possibility to consider is that the role of very long-chain n-3 PUFA in stroke risk is may not be in atherosclerosis as measured by intima-media thickness or stiffness, but may be as measured by other factors involved in vascular disease, such as haemostasis[103] or plaque stability[104].

In summary, the present study shows that plasma very long-chain n-3 PUFA are neither associated with change in carotid distension nor with change in carotid intima-media thickness in this study population of older adults. Due to the small progression of carotid intima-media thickness over the three year period, a beneficial association could potentially have been missed. However, in the context of other observational and intervention studies, there is currently no sufficient

consistent evidence to support the hypothesis that very long-chain n-3 PUFA are associated with common carotid stiffness or common carotid intima-media thickness in a general healthy population.

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Chapter 5

Plasma very long-chain n-3 polyunsaturated fatty acids and age-related hearing loss in older adults

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Submitted for publication

Abstract

Background: Age-related hearing loss is a common social and health problem in the older adult population. Up until now, very little scientific attention has been given to the potential role of fatty acids in age-related hearing loss.

Objective: To investigate whether plasma very long-chain n-3 polyunsaturated fatty acids (PUFA) are associated with age-related hearing loss over three years.

Design: Fatty acid proportions in plasma cholesteryl esters were measured in 720 men and postmenopausal women aged 50-70 y free from middle ear dysfunction or unilateral hearing loss. Hearing thresholds (in decibels, dB) at baseline and after three years were measured with pure-tone audiometry. Hearing loss was calculated as the increase in mean hearing thresholds in the low (0.5-kHz, 1-kHz, and 2-kHz) and high (4-kHz, 6-kHz, and 8-kHz) frequencies over three years..

Results: Subjects in the highest quartile of plasma very long-chain n-3 PUFA had less hearing loss in the low frequencies over three years, compared with those in the lowest quartile ($p<0.01$, ANCOVA, difference in mean adjusted hearing thresholds= -1.2 dB). There were no significant differences between the quartiles of plasma very long-chain n-3 PUFA in hearing loss in the high frequencies ($p=0.49$, ANCOVA). These associations are adjusted for baseline mean hearing thresholds, age, sex, level of education and alcohol consumption.

Conclusions: This study is the first to show an inverse association between plasma very long-chain n-3 PUFA and age-related hearing loss. These results are encouraging, but require confirmation from future studies.

Introduction

Age-related hearing loss is attributed to age-related degeneration of the cochlea as well as other physiologic, environmental, and pathological processes that occur during the lifespan[105]. It initially affects hearing sensitivity in the high-frequencies, affecting communication in noisy situations. Once the loss extends to the lower frequencies (2-4 kHz range), important for understanding the voiceless consonants, speech understanding in any situation is affected[105]. Age-related hearing loss is a common chronic condition, affecting 30-35% of the people aged 65-75 years and 40-50% of the people over 75 years[106].

The relationship between hearing loss and nutritional status is a relatively new area of investigation[106.] The hypothesis that nutrition may play a role in age-related hearing loss is based on evidence from two converging research areas: research showing that hearing loss is related to vascular disease[54, 107-109], and research showing that vascular systems rely on certain nutrients for optimal structure and function[106]. Very long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), as present in fish and fish oil, are suggested to protect against vascular diseases[110]. Since the cochlea is highly vascularised, it has been suggested that age-related hearing loss may be caused by a decrease in the blood supply to the cochlea[54].

Very little scientific attention has been given to the possible role of fatty acids in age-related hearing loss. A study in two psychiatric hospitals in Finland showed that adults who consumed a low fat diet for a period of five years had better hearing levels throughout the entire audiometric range than adults who consumed a diet high in saturated fatty acids[55]. However, there are currently no published studies that have investigated the relationship between very long-chain n-3 PUFA and age-related hearing loss. Therefore, the present study assesses whether plasma very long-chain n-3 PUFA are associated with age-related hearing loss over a period of three years in a population of Dutch older adults.

Subjects and methods

Subjects

We used data from participants of the FACIT study, a randomized controlled trial investigating the effect of folic acid supplementation on hearing, carotid intima-media thickness and cognitive performance[57]. In this study, 819 men and postmenopausal women aged 50-70 years were randomly assigned to either folic

acid (n=406) or placebo (n=413) treatment for a period of three years. Participants were recruited by using municipal and local blood bank registries from the Gelderland province in the Netherlands. Major exclusion criteria were plasma total homocysteine levels <13 μmol/L and >26 μmol/L, serum vitamin B12 levels <200pmol/L, renal or thyroid diseases, and current use of B-vitamin supplements. The baseline measurements, which included measurements of hearing, were conducted between 2000 and 2001. The original study on which the present article is based was approved by the Medical Ethics Committee of Wageningen University, the Netherlands. All study participants provided written informed consent.

In our analyses, subjects with middle ear dysfunction (defined as air-bone gap ≥15 dB on the audiogram of either ear) or unilateral hearing loss (≥20 dB difference in mean pure-tone hearing thresholds for 0.5 kHz, 1 kHz, and 2 kHz between the right and left ear) were excluded (n=91), as these hearing problems are unlikely due to age-related hearing loss. In addition, we excluded eight participants from whom insufficient amounts of blood could be obtained or who did not give permission for the fatty acid analyses, resulting in a total of 720 participants.

Audiometric measurements

Excessive cerumen, if present, was removed from the participants' ears prior to the audiometric measurements. The pure tone audiometric assessments were performed in an acoustical booth (Audiofon G, Audiovox, Hauppauge, New York), which muted sounds up to 42 dB and was placed in a quiet, isolated, carpeted room next to the college library. Participants were measured in seated position using an audiometer (Madsen Voyager 522, Madsen Electronics, Taastrup, Denmark), circum-aural earphones and a handheld response button system. We calibrated the audiometer according to the International Organization of Standardization standard 389 and we performed the audiometric testing by using a variation of the Hughson and Westlake method as described earlier[57]. Our outcomes measures are the mean pure-tone air conduction hearing thresholds in the low (0.5-kHz, 1-kHz, and 2-kHz) and high (4-kHz, 6-kHz, and 8-kHz) frequencies.

To enable exclusion of participants with possible middle ear dysfunction at the start of the study, we measured bone conduction hearing thresholds at 0.5 kHz,

1 kHz, 2 kHz, and 4 kHz by using contra-lateral masking. To enable exclusion of participants with unilateral hearing loss at the start of the study, we used contra-lateral masking when the difference in air conduction hearing threshold between the right and left ear was 50 dB or more. Subjects with a difference of 20 dB or more in mean air conduction hearing thresholds after contra-lateral masking between the right and left ear were excluded from the analyses.

Plasma n-3 PUFA proportions

Venous blood was collected after an overnight fast in one 10-ml Vacutainer tube containing EDTA. The obtained plasma was stored within 2 hours at -80°C until analysis. Fatty acids in plasma cholesteryl esters were determined as described previously[89]. We calculated plasma very-long chain n-3 PUFA by adding up the levels of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3 DPA), and docosahexaenoic acid (DHA).

Other measurements

At baseline, participants completed a questionnaire on general demographic variables and medical history. Height and weight were measured to calculate body mass index (kg/m^2). Level of education was divided into three groups according to the highest level attained: primary education, junior vocational training and senior vocational or academic training. Blood pressure was measured using an automated meter (Dinamap Compact Pro 100, General Electric, Waukesha, Wisconsin) and we took the mean of eight measurements. Serum total cholesterol, LDL-cholesterol and HDL-cholesterol were determined on a Hitachi® 747 analyser (Roche Diagnostics, Mannheim, Germany). Physical activity was estimated using the Physical Activity Scale for the Elderly[111].

Statistical analyses

Subjects were grouped according to quartiles of plasma very long-chain n-3 PUFA proportions. Equality of variances was tested using Levene's tests. Baseline characteristics of subjects in the different quartiles were compared with chi-square tests, one-way analysis of variance (normally distributed variables), or Kruskal-Wallis tests (non-normally distributed variables). Analysis of covariance (ANCOVA) was used to evaluate the association between quartiles of plas-

ma very long-chain n-3 PUFA and mean hearing thresholds in the low (0.5-kHz, 1-kHz, and 2-kHz) and high (4-kHz, 6-kHz, and 8-kHz) frequencies. Because we excluded participants with unilateral hearing loss, we averaged the hearing thresholds from both ears in our outcome measures. When significant F-tests were obtained, Tukey's honest significant difference tests were applied for the *post hoc* comparison. We evaluated age, sex, level of education, smoking, alcohol consumption and erythrocyte folate concentrations as potential confounders. To investigate whether the folic acid treatment was an effect modifier in the longitudinal analyses, we performed stratified analysis by intervention group. Statistical significance for all analyses was defined as $p<0.05$. The data analyses were performed with the Statistical Analysis System (SAS version 9.1.3; SAS Institute Inc, Cary, NC, USA).

Results

The mean age of our participants at baseline was 60 years and 72% of our study population was male. When grouped according to quartiles of plasma very long-chain n-3 PUFA, there were no differences between the four groups in age, sex, level of education, body mass index, smoking habits, physical activity, blood pressure and blood parameters (**Table 5.1**). Alcohol consumption was higher in the highest quartile of very long-chain n-3 PUFA, compared with the lowest quartile. The proportions of individual fatty acids in plasma cholesteryl esters according to quartiles of plasma very long-chain n-3 PUFA are shown in **Table 5.2**.

Hearing loss over three years (longitudinal results)

We were able to analyse the data of 705 of the 720 participants in our longitudinal analyses. Fifteen participants did not provide hearing data after three years, because they died during the follow-up (n=11), or were lost to follow-up (n=4). The mean increase in all participants in hearing threshold over three years was 1.4 dB (95% CI: 1.1 to 1.7) for the low frequencies and 4.8 dB (95% CI: 4.4 to 5.2) for the high frequencies. Subjects in the highest quartile of very long-chain n-3 PUFA had less hearing loss in the low frequencies over three years, compared with those in the lowest quartile (**Table 5.3**, $p<0.01$, ANCOVA). The difference in the adjusted mean hearing thresholds in the low frequencies between the highest and lowest quartile was -1.2 dB (95%CI: -2.2 to -0.1). There were no significant

differences between the quartiles of plasma very long-chain n-3 PUFA in mean hearing thresholds in the high frequencies after three years (p=0.49, ANCOVA). These longitudinal associations were adjusted for baseline mean hearing threshold values, age, sex, level of education and alcohol consumption.

Table 5.1: Baseline characteristics of participants according to quartiles of plasma very long-chain n-3 PUFA[1]

Characteristics	Quartile 1 (n=180)	Quartile 2 (n=180)	Quartile 3 (n=180)	Quartile 4 (n=180)
Age (y)	60.0 ± 6.0	59.8 ± 5.6	60.1 ± 5.4	60.5 ± 5.4
Sex (male/female)	140 / 40	133 / 47	122 / 58	122 / 58
Level of education (low/middle/high)[2]	47 / 62 / 71	44 / 66 / 70	39 / 74 / 67	34 / 68 / 78
BMI (kg/m^2)	26.1 ± 3.5	26.7 ± 3.3	26.8 ± 3.8	26.8 ± 3.8
Smoking (never / former / current)	56 / 89 / 35	55 / 94 / 31	50 / 87 / 43	40 / 107 / 33
Alcohol consumption (g/day)	8.6 (1.7 – 15.7)	13.6 (4.5 – 22.3)	13.3 (5.2 – 26.5)	17.2 (7.6 – 27.5)
Physical Activity (PASE score)	150 ± 71	163 ± 68	155 ± 73	145 ± 61
Self-reported hearing problems	16	21	17	19
Serum total cholesterol (mmol/L)	5.64 ± 0.99	5.76 ± 1.11	5.97 ± 1.02	5.91 ± 1.17
Serum LDL cholesterol (mmol/L)[3]	3.87 ± 0.87	3.96 ± 0.97	4.13 ± 0.91	4.07 ± 1.02
Serum HDL cholesterol (mmol/L)	1.23 ± 0.32	1.21 ± 0.34	1.23 ± 0.35	1.28 ± 0.40
Systolic blood pressure (mm Hg)[4]	132 ± 16	132 ± 16	134 ± 17	133 ± 15
Diastolic blood pressure (mm Hg)[4]	77 ± 8	77 ± 9	77 ± 8	77 ± 8

Values are means ± SD, medians (interquartile range) or n.

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04%.

[2] Low=primary education; Middle=junior vocational training; High= senior vocational or academic training.

[3] Data available for 718 participants.

[4] Data available for 716 participants.

Because we previously showed that the folic acid treatment slowed down the decline in mean hearing threshold in the low frequencies[57], we performed the longitudinal analyses also separately for the placebo group and the folic acid group. When considering the longitudinal analyses separately for the placebo and the folic acid group, it appeared that higher proportions of plasma very long-chain n-3 PUFA were still associated with less hearing loss over three years in the low frequencies in the placebo group. The difference in mean hearing threshold in the low frequencies between the highest and lowest quartile was -1.8 dB (95%CI -3.2 to -0.3). In the folic acid group, however, the association was not statistically significant.

Because the study by Rosen *et al.*[55] reported worse hearing thresholds in persons on a saturated fat diet compared with those on a low fat diet, we also evaluated the associations between plasma saturated fatty acids and mean hearing thresholds (data not shown). However, there were no significant differences between the quartiles of plasma saturated fatty acids in mean hearing thresholds in the low or high frequencies after three years (p=0.46 and p=0.29 for ANCOVA, respectively).

Hearing thresholds (cross-sectional results)

There were no significant differences between the four quartiles of plasma very long-chain n-3 PUFA in the mean hearing threshold at baseline in the low frequencies (Q1 (Quartile 1): 13.5 (95%CI: 12.1 to 14.8); Q2: 14.6 (95%CI: 13.3 to 15.9); Q3: 13.8 (95%CI: 12.5 to 15.1); Q4: 12.6 (95%CI: 11.3 to 13.9; p=0.21 for ANCOVA), after adjustments for age, sex, and education level. In addition, no significant differences between the quartiles were found in the mean hearing threshold in the high frequencies (Q1: 36.9 (95%CI: 34.5 to 39.3); Q2: 38.1 (95%CI: 35.7 to 40.5); Q3: 39.5 (95%CI: 37.1 to 41.9); Q4: 35.4 (95%CI: 33.0 to 37.9); p=0.12 for ANCOVA), after adjustments for the same confounders. Mean hearing thresholds in the low and high frequencies were not significantly different between quartiles of plasma saturated fatty acids (p=0.36 and p=0.54 for ANCOVA, respectively), although the results were in the expected direction. Mean hearing thresholds on the low and high frequencies tended to be higher for the highest quartile of saturated fatty acids, compared with the lowest quartile of saturated fatty acids (data not shown).

Table 5.2: Fatty acid contents (% of total fatty acids) in plasma cholesteryl esters according to quartiles of plasma very long-chain n-3 PUFA[1]

Fatty acids	Quartile 1 (n=180)	Quartile 2 (n=180)	Quartile 3 (n=180)	Quartile 4 (n=180)
Saturated fatty acids, total	12.2 ± 1.1	12.9 ± 1.2	13.0 ± 1.1	13.2 ± 1.0
Palmitic acid, 16:0	10.5 ± 0.8	11.0 ± 0.9	11.1 ± 0.9	11.3 ± 0.8
Stearic acid, 18:0	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
N-9 mono-unsaturated fatty acids, total	16.3 ± 2.2	17.0 ± 2.3	17.4 ± 2.3	17.8 ± 2.2
Oleic acid, 18:1n-9	16.3 ± 2.2	17.0 ± 2.3	17.4 ± 2.3	17.8 ± 2.2
N-6 polyunsaturated fatty acids, total	64.8 ± 3.9	62.4 ± 4.1	61.2 ± 4.3	59.3 ± 4.1
Linoleic acid, 18:2n-6	57.2 ± 4.2	53.8 ± 4.7	52.6 ± 4.9	50.9 ± 4.5
Arachidonic acid, 20:4n-6	6.0 ± 1.5	6.9 ± 1.4	6.9 ± 1.4	6.8 ± 1.3
N-3 polyunsaturated fatty acids, total	1.6 ± 0.2	2.0 ± 0.2	2.3 ± 0.2	3.4 ± 0.9
α-Linolenic acid, 18:3n-3	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Eicosapentaenoic acid, 20:5n-3	0.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	2.0 ± 0.8
Docosapentaenoic acid, 22:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Docosahexaenoic acid, 22:6n-3	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.2

Values are means (SD).

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04%.

Discussion

The present study in older adults shows that people in the highest quartile of plasma very long-chain n-3 PUFA had less hearing loss in the low frequencies over three years, compared with those in the lowest quartile. Plasma very long-chain n-3 PUFA appeared not to be associated with hearing loss in the high frequencies.

The hearing loss in our study population was likely to be age-related hearing loss of cochlear origin. By excluding participants with middle-ear dysfunction and unilateral hearing loss from our study population, we aimed to exclude participants with altered hearing thresholds due to conductive hearing loss or noise-induced hearing damage.

Rosen *et al.* were the first to investigate whether a change in diet affects hearing loss in older adults (40-60 y). They showed better hearing in subjects receiving a low-fat diet and worsening of hearing in subjects receiving a diet high in saturated fat[55]. In contrast, we did not show significant differences between quartiles

Table 5.3: Hearing thresholds in the low and high frequencies after 3 years according to quartiles of plasma very long-chain n-3 PUFA[1]

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	p [2]
All participants					
Sample size (n)	176	177	175	177	
Hearing threshold on low freq. (dB)	15.7 (15.1; 16.2)[a]	15.4 (14.8; 15.9)[ab]	14.6 (14.1; 15.2)[b]	14.5 (14.0; 15.1)[b]	<0.01
Hearing threshold on high freq. (dB)	42.4 (41.5; 43.3)	42.6 (41.8; 43.5)	41.7 (40.8; 42.6)	42.2 (41.3; 43.1)	0.49
Placebo group only					
Sample size (n)	91	90	90	91	
Hearing threshold on low freq. (dB)	16.1 (15.3; 16.8)[a]	15.5 (14.7; 16.2)[ab]	14.8 (14.1; 15.5)[ab]	14.3 (13.6; 15.1)[b]	<0.01
Hearing threshold on high freq. (dB)	42.5 (41.3; 43.8)	43.4 (42.2; 44.6)	42.5 (41.3; 43.7)	42.3 (41.1; 43.5)	0.58
Folic acid group only					
Sample size (n)	85	87	86	85	
Hearing threshold on low freq. (dB)	15.1 (14.3; 15.9)	15.4 (14.7; 16.2)	14.4 (13.6; 15.2)	14.7 (13.9; 15.5)	0.31
Hearing threshold on high freq. (dB)	42.0 (40.8; 43.3)	42.1 (40.8; 43.4)	40.9 (39.6; 42.2)	42.0 (40.7; 43.3)	0.51

Results are adjusted means (95% CI); dB=decibel.

[1] Plasma very long-chain n-3 PUFA quartiles 1: <1.18%; 2: 1.18-1.51%; 3: 1.52-2.04%; 4: >2.04% (all participants).

[2] ANCOVA adjusted for baseline mean hearing threshold, age, sex, level of education and alcohol consumption. Post-hoc comparison by Tukey's Honest Significant Differences test (means in a row with different superscript letters are significantly different).

of saturated fatty acids. However, one has to keep in mind that plasma saturated fatty acids can not provide direct information on absolute saturated fat intake. Although plasma saturated fatty acids are dependent on their absolute concentrations and relative proportions in food, they also include endogenously synthesized saturated fatty acids[36].

Our analyses are the first to show an inverse relationship between plasma very long-chain n-3 PUFA proportions and age-related hearing loss in older adults. This study has several strengths, including a longitudinal design, the use of plasma proportions of very long-chain n-3 PUFA as a valid estimate of dietary intake of fatty acids[35] and sound audiometric assessments under standardized test con-

ditions. Nevertheless, several issues should be addressed to enable a balanced interpretation of the findings.

First, plasma very long-chain n-3 PUFA were associated with hearing loss in the low frequencies, but not significantly with hearing loss in the high frequencies. The apex of the cochlea transduces the low-frequencies sounds, whereas the base of the cochlea is responsible for the transduction of the high-frequencies sounds. Since the inner ear is an end organ and is only supplied by one or sometimes two arteries[112], microvascular disease could affect the blood supply to the cochlea. As the apex of the cochlea is the farthest away from the blood supply, it may be most susceptible to changes in the microcirculation[113], and therefore particularly affect hearing thresholds on the low frequencies[107]. An improvement in microcirculation may explain the association of plasma very long-chain n-3 PUFA with low-frequency thresholds. Alternatively, more variation in the hearing threshold in the high frequencies compared with the low frequencies, may explain why we did not detect a significant difference between very long-chain n-3 PUFA quartiles and hearing thresholds on the high-frequencies with the current study sample size.

A second study-related issue that needs to be addressed is that half of our participants received folic acid supplementation for three years, because our study population originally participated in a randomized controlled trial. This folic acid treatment has been shown to slow down the decline in hearing in the low frequencies[57]. Our stratified analyses show that higher proportions of plasma very long-chain n-3 PUFA were still associated with less hearing loss in the low frequencies in the placebo group, but not in the folic acid group. Although the mean hearing thresholds in the folic acid group were not significantly different across the quartiles, the results were in the expected direction. This suggests that the treatment effect of folic acid overruled a potential association between plasma very long-chain n-3 PUFA and hearing loss in the folic acid group.

A third issue is the apparent inconsistency between the cross-sectional and the longitudinal results. Because our study population consisted of relatively young subjects (mean age: 60 yrs), it could well be that hearing disabilities are just beginning to develop in our participants. This corresponds with our findings that subjects with higher mean hearing thresholds at the start of the study also had a faster threshold increase compared with subjects with lower mean hearing thresholds at baseline.

Two other issues that require attention are the actual difference in hearing loss between the quartiles and the range of plasma very long-chain n-3 PUFA. The difference in hearing loss between the highest and the lowest quartile of plasma very long-chain n-3 PUFA appears to be small: -1.2 dB for the whole study population and -1.8 dB when only the placebo group was considered. However, since hearing loss increases over time and since our study population was relatively young, the difference between the quartiles could be greater in older populations. This is supported by our findings of a difference in hearing loss between the highest en lowest quartile of -0.8 dB in subjects of 50-60 yrs, and -1.5 dB in subjects of 60-70 yrs. Moreover, although the range of plasma very long-chain n-3 PUFA proportions in our study population was comparable with that of other large studies in older adult populations[36, 92], it could be possible that the differences in hearing loss between the quartiles become even more pronounced if this range would have been wider.

Finally, it is important to realize that our study population may not represent a random sample of the Dutch older adult population, because our subjects were selected on the basis for participation in a randomized controlled trial (e.g. moderate levels of homocysteine, no vitamin B12 deficiency, no lipid-lowering drugs). This may affect the generalizability of our results.

In summary, these analyses provide us with encouraging results on an inverse association between very long-chain n-3 PUFA and hearing loss. These results require confirmation from future observational studies to verify the direction, size and importance of the reported associations.

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Chapter 6

Interplay between very long-chain n-3 polyunsaturated fatty acids and folic acid

Adapted from: Dullemeijer C, Durga J, Brouwer IA, Verhoef P. Erythrocyte folate and plasma DHA in the FACIT study. The Lancet 2007; 370 (9583): 216

Abstract

Background: It has been suggested that some of the enhanced cognitive performance produced by 3-year supplementation with folic acid in the FACIT study could be mediated by increased docosahexaenoic acid concentrations.

Objective: To investigate the association of serum or erythrocyte folate with the proportions of docosahexaenoic acid in plasma cholesteryl esters.

Methods: Fatty acid levels in plasma cholesteryl esters and folate concentrations in serum and erythrocytes were measured in 808 FACIT participants (50-70 years) from blood samples obtained at the baseline visits.

Results: We saw no correlation between erythrocyte folate concentrations and plasma DHA concentrations (Spearman rank correlation coefficient Rho=0.04, p=0.21). In addition, serum folate concentrations were not correlated with plasma DHA concentrations (Rho=-0.01, p=0.68).

Conclusion: Although we have no data on DHA concentrations after folic acid supplementation, our data combined with convincing clinical trial evidence do not support the hypothesis that folic acid supplementation alters the proportion of docosahexaenoic acid in plasma. Therefore, there are no strong indications that the effects of folic acid on cognitive performance in the FACIT study were mediated by DHA.

Introduction

Folate and very long-chain n-3 polyunsaturated fatty acids (PUFA) are both nutritional factors that have received a lot of interest in the scientific nutrition community during the last decades. It has been suggested that folate and very long-chain n-3 PUFA may have a synergistic effect on cognitive performance. In a correspondence letter, Dr. Umhau suggests that some of the enhanced cognitive performance produced by the 3-year supplementation with folic acid in the FACIT study[56] could be mediated by increased docosahexaenoic acid (DHA) concentrations[114]. However, his speculations were derived largely from an experiment with animals[115] and a small cross-sectional study in humans[116].

The observational analyses in this thesis *(Chapters 3, 4 and 5)* are performed within the FACIT study in which the participants were randomly assigned to either folic acid or placebo treatment for three years. The primary aim of the FACIT study was to investigate health effects of folic acid supplementation. In *Chapter 3*, we evaluated the association between very long-chain n-3 PUFA and cognitive decline in the FACIT study population, and we performed stratified analyses by intervention group because treatment was an effect modifier. However, considering the speculations of Dr. Umhau it seemed justified to take a closer look at the interplay between folic acid and very long-chain n-3 PUFA in the FACIT dataset. Therefore, we used to FACIT dataset to test the hypothesis that serum or erythrocyte folate concentrations are associated with docosahexaenoic acid in plasma cholesteryl esters.

Subjects and methods

Subjects

Subjects were men and post-menopausal women aged 50-70 years who participated in the FACIT study, a placebo-controlled trial investigating the effect of folic acid supplementation on cognitive performance, carotid intima-media thickness and hearing, which was approved by the Wageningen University Medical Ethics Committee[56]. In this study, 819 participants were randomly assigned to either folic acid (n=406) or placebo (n=413) treatment for 3 years. Participants were originally included in this study if they had plasma total homocysteine concentrations ≥13 μmol/L and ≤26 μmol/L and serum vitamin B-12 concentrations ≥200 pmol/L.

Measurements of fatty acids and folate

The blood samples were collected during the baseline measurements, which were conducted between 2000 and 2001. Venous blood was collected after an overnight fast, directly processed and stored at -80°C. Fatty acids in plasma cholesteryl esters were measured as described previously[89]. Total very long-chain n-3 PUFA was calculated by adding up the proportions of eicosapentaenoic acid (EPA), n-3 docosapentaenoic acid (DPA) and DHA. Serum folate and erythrocyte folate were measured as described previously[117]. A food-frequency questionnaire was administered during the baseline measurements to estimate dietary folate intake during the past 3 months.

Statistical analyses

Serum and erythrocyte folate concentrations and the proportions of docosahexaenoic acid and total very long-chain n-3 PUFA in plasma cholesteryl esters were not normally distributed. Therefore, we used Spearman's rank correlation coefficients to determine the correlations.

Results

The original FACIT study sample consisted of 819 individuals. In our analyses, we were able to use the data of 808 participants, because 11 participants did not provide sufficient amounts of blood or did not give permission for the fatty acid analyses. The baseline characteristics of our study sample are shown in **Table 6.1**. The mean age of the participants was 60 years and 72% of the participants were male.

The Spearman correlation coefficients for the cross-sectional analyses are shown in **Table 6.2**. There were no significant correlations between erythrocyte folate concentrations and plasma DHA proportions (Spearman rho=0.04, p=0.21) or between serum folate concentrations and plasma DHA proportions (Spearman rho=-0.01, p=0.68). In addition, we found no significant correlations between dietary folate intake and plasma DHA proportions (Spearman rho=0.01, p=0.72). Furthermore, no significant correlations between folate concentrations and EPA or total very long-chain n-3 PUFA were seen.

Table 6.1: Baseline characteristics of the participants (n=808)

Characteristics	
Age (y)	60 ± 6
Sex (male/female)	72 / 28
Level of education (low/middle/high)[1]	22 / 38 / 39
Body Mass Index (kg/m^2)	27 ± 4
Smoking (never / former / current)	28 / 52 / 20
Alcohol consumption (g/day)	12.7 (4.5 – 23.5)
Serum folate (nmol/L)	12 (10-15)
Erythrocyte folate (nmol/L)	660 (515-820)
Dietary folate intake (µg per day)	194 (159-240)
Plasma docosahexaenoic acid (% of total fatty acids)	0.60 ± 0.21
Plasma eicosapentaenoic acid (% of total fatty acids)	0.92 (0.69-1.32)

Values are means ± SD, medians (interquartile range) or percentages.

[1] Low=primary education; Middle=junior vocational training; High= senior vocational or academic training.

Table 6.2: Spearman rank correlation coefficients between erythrocyte or serum folate concentrations or folate intake and plasma very long-chain n-3 PUFA proportions at baseline (n=808)

	Erythrocyte folate		**Serum folate**		**Folate intake**	
	rho	*p*	*rho*	*p*	*rho*	*p*
DHA	0.04	0.21	-0.01	0.68	0.01	0.72
EPA	0.05	0.18	-0.02	0.63	-0.01	0.98
Total n-3 PUFA	0.05	0.17	-0.02	0.66	-0.01	0.98

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; total n-3 PUFA, the sum of EPA, n-3 DPA and DHA.

Discussion

We observed no significant correlations between erythrocyte or serum folate concentrations and plasma DHA proportions in the FACIT study population. In addition, no significant correlations between folate concentrations and EPA or total very long-chain n-3 PUFA were seen.

A major strength of our approach is that our sample size was rather large. For large samples it is relatively easy to achieve significance, but one must pay attention to the strength of the correlation to determine if the relation explains

very much. In our case, the correlation was neither significant nor strong, which convincingly suggests that there is no relation between folate and very long-chain n-3 PUFA in our study population. A limitation of our approach is that we have no data available on DHA concentrations after folic acid supplementation in the FACIT study population. Since the FACIT study protocol did not include any dietary advice with regard to fish consumption, we do not expect the study participants to have changed their very long-chain n-3 PUFA intake over the 3-year study period. However, we can not exclude the possibility that folate supplementation influenced the metabolism of very long-chain n-3 PUFA in the body.

Two studies in rats suggest that a low folate status decreases the proportion of very long-chain n-3 PUFA in tissues[118], whereas intramuscular injection of folate increases the proportions of these fatty acids[115]. However, there is very little information available from human studies investigating the relation between folate and very long-chain n-3 PUFA. A small study among 22 aggressive and hostile men showed that erythrocyte folate concentrations were correlated with plasma DHA concentrations ($r=0.57$; $p<0.01$)[116]; however, a study among 44 patients with recurrent depression showed no significant associations between plasma homocysteine concentrations and any of the very long-chain n-3 PUFA in plasma or erythrocytes[119]. In addition, a placebo-controlled trial among 253 elderly participants showed that lowering homocysteine concentrations with folate, vitamin B12 and vitamin B6 supplementation for two years had no effect on the proportion of very long-chain n-3 PUFA in plasma phospholipids[120].

There are two mechanisms proposed by which folate may exert an effect on docosahexaenoic acid. The first mechanism involves a reduction of the generation of reactive oxygen species. By reducing homocysteine concentrations, folate may reduce the generation of reactive oxygen species and thus spare docosahexaenoic acid, which is a major target for lipid peroxidation[118]. The second proposed mechanism involves the ability of dietary folate to provide methyl groups to the liver, yielding phosphatidylcholine, which is a major route for the appearance of DHA in the plasma[116, 120]. Despite the plausibility of these mechanisms, our findings combined with convincing clinical trial evidence[120] do not support the hypothesis that folic acid supplementation alters the proportion of docosahexaenoic acid in plasma.

Chapter 7

General discussion

The research in this thesis was inspired by the high hopes that presently surround very long-chain n-3 PUFA regarding their role in brain development and cognitive performance. The central question "Do very long-chain n-3 PUFA give someone a head start to win some years between the ears?" is quite broad. The findings presented in this thesis shed light on three specific areas: the role of very long-chain n-3 PUFA in *1)* brain tissue development, *2)* cognitive decline, and *3)* macrovascular and microvascular blood supply in the head region. This final chapter aims to summarize the main findings of our research and to place our findings in the context of the current understanding of the role of very long-chain n-3 PUFA in these three areas. In addition, some methodological issues will be addressed as well as several suggestions for future research in these separate areas.

Very long-chain n-3 PUFA in brain tissue development in pigs

We hypothesized that separate brain lobes may be differentially affected by n-3 PUFA consumption, based on the research of Rice *et al.*[21] that showed that various parts of the brain develop at different time points and grow with different speeds. In addition, we investigated whether dietary very long-chain n-3 PUFA still affect the fatty acid composition of the brain after the period of rapid brain growth spurt, as previous studies were performed in newborn piglets only.

Key findings from this thesis

A diet enriched with fish oil resulted in higher proportions of DHA in the frontal, parietal and occipital brain lobes compared with the temporal lobe in juvenile pigs *(Chapter 2)*. In addition, the developing brain appeared to be responsive to a diet rich in fish oil, even after the brain growth spurt. These findings suggest that providing dietary very long-chain n-3 PUFA just after the brain growth spurt period may differentially affect specific brain lobes.

Methodological considerations

Our findings may suggest that the difference in DHA accretion between the

brain lobes points to specific regional requirements. This would imply that the frontal, parietal and occipital brain lobes, the lobes that predominantly appear to respond to dietary DHA in our experiment, have a larger requirement for DHA than the temporal brain lobe. Subsequently, one might speculate that cognitive functions that are linked to the frontal, parietal and occipital brain lobes may be more affected by dietary fish oil than the temporal lobe. Although these speculations are tempting, the specific relationship between regional fatty acid composition and functional effects is yet to be determined. Moreover, there are three important methodological considerations that need to be taken into account with the interpretation of our data.

The first methodological consideration relates to the incorporation of DHA in the brain tissue. Since our study set-up only permitted brain tissue sampling at the end of the intervention period, there was no possibility to evaluate the baseline brain fatty acid composition. Therefore, we cannot be sure whether the differences between the two intervention groups were caused by the intervention group incorporating DHA or the control group experiencing a loss in DHA. If our control group would have received a regular pig chow diet, we could have assumed that the fatty acid composition of the brain at the end of the intervention period in this group would have been comparable to the fatty acid composition of the brain at baseline. However, we have investigated the effects of dietary fish oil against a low α-linolenic acid-background diet. Under these "extreme" dietary conditions, the control (high oleic acid sunflower oil) group was expected to experience a loss of DHA, as this diet provided no dietary DHA and hardly any α-linolenic acid to support the synthesis of DHA. Our rationale for the low α-linolenic acid-background diet in both intervention groups was that we specifically wanted to address the effect of supplemental EPA and DHA, without a potentially disturbing effect of α-linolenic acid conversion into DHA[11]. As a consequence, however, we cannot be sure about the cause of the regional differences between the brain lobes. Our control diet resulted in lower proportions of DHA in the temporal lobe compared with the other lobes. This could either mean that the temporal lobe lost more DHA, or that the temporal lobe had less DHA to start with. If future research aims to shed light on the question whether an absence of dietary DHA and α-linolenic acid indeed differentially decreases brain DHA in the separate brain lobes, an extra control group should be added to the

research design that receives a regular pig chow diet with sufficient amounts of α-linolenic acid.

Our fish oil group was expected to incorporate DHA. This diet resulted in higher proportions of DHA in the frontal, parietal and occipital brain lobes compared with the temporal lobe, which could again either mean that the temporal lobe incorporated less DHA, or that the temporal lobe had less DHA to start with. A way to answer the question whether extra dietary DHA differentially increases brain DHA in the separate brain lobes would be to study the effect of fish oil while including sufficient amounts of α-linolenic acid in both the intervention and the control diet.

A second methodological issue that needs to be considered with the interpretation of our findings relates to the nature of the regional differences. The regional differences between the brain lobes that we found in our experiment are based on fatty acid analyses in total phospholipids. However, the fatty acid patterns in the brain may be phospholipid-specific, meaning that the fatty acid patterns may be different among the four major phospholipid classes: phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine[4]. These last two, phosphatidylethanolamine and phosphatidylserine are the phospholipid species that contain the highest concentrations of DHA[121]. It is therefore possible that the differences in brain DHA between the lobes may actually represent differences in phospholipid classes between the lobes. If a particular brain lobe has low levels of phosphatidylethanolamine and phosphatidylserine and corresponding low levels of DHA, this may imply that there is little margin for change. In addition, within each phospholipid class there may also be differences between the fatty acid composition of the gray matter and the white matter[5]. The fatty acid composition in a particular region of the brain may therefore not only be phospholipid-specific, but also tissue-specific. If the fatty acid composition of different classes of phospholipids and tissues is indeed specific for the different regions in the brain, analyses in total phospholipids may be of limited value. A strategy to take these considerations into account would be to study the effect of a diet rich in fish oil on the fatty acid composition of specific phospholipid classes from well-defined brain regions, with separate gray and white matter samples, in order to obtain meaningful information about regional fatty acid changes in the brain.

A third methodological consideration that holds for all animal studies is that the translation to the human situation remains problematic. Animal studies can only suggest the possibility that dietary fish oil causes small differences in brain DHA incorporation or loss between brain regions in humans, which may result in subtle effects on cognitive performance. Nevertheless, the great strength of animal studies is that they afford the opportunity for more flexibility in design and in the ability to control experimental variables than can be achieved in human studies. Moreover, pigs are the preferred animal model for brain research, because the pig brain and the human brain have important resemblances in the morphology of the brain. Pigs have a human-like relative distribution of grey and white matter in the brain, as oppose to rodents that have little white matter[10]. In addition, both pigs and humans have brains with a highly convoluted surface, whereas rodents have a smooth, lissencephalic brain surface[10]. In addition, the pig brain and the human brain have important resemblance in the stage of maturation. The brain growth spurt peaks at birth in both humans and pigs and changes in the composition of the piglet brain during development parallel the human situation[10]. The similarity in maturation of piglets to human infants allows for a precise comparison of the brain's response to dietary fatty acids at specific developmental stages. However, the use of pigs as an animal model also has some disadvantages. Pigs are more expensive than rodents, are more difficult to handle and to house and require more complex care. Moreover, anaesthetic considerations and surgical procedures are more difficult in pigs.

Implications for the field of very long-chain n-3 PUFA and brain development

Although there are some methodological considerations to take into account with the interpretation of our findings, the results from our study do support the hypothesis that there are regional differences in the brain in susceptibility for dietary very long-chain n-3 PUFA. This is in line with another study in newborn piglets that also reported an apparent preservation of the temporal lobe with regard to the fatty acid composition, as oppose to the other brain lobes[15]. Although there are also some indications of a region-specific distribution of DHA in the brain from research conducted using rodents[122, 123], the number of animal studies comparing the four cerebral brain lobes is too limited to make definite inferences.

In our study, we have investigated pigs in the period after the rapid brain growth spurt. However, it is clear that a considerable amount of DHA is incorporated in the human brain during the brain growth spurt. DHA amounts increase not only in an absolute way because of the growth in brain size, but also in a relative way[124].The relative concentration of DHA in the developing brain increases in a parabolic manner from about 25 weeks post-conception, when it is 3000 nmol/g brain, until about 2 years of age, when it reaches 10,000 nmol/g[124]. Because DHA accretion is greatest during the brain growth spurt, this period is generally considered to reflect a critical time during which deficiency of DHA may have long-term consequences for later brain function. From this point of view, it would also be interesting to investigate if separate regions of the developing brain are differentially affected by dietary very long-chain n-3 PUFA consumption during the period of brain growth spurt.

Future research

The effects of dietary fatty acids on the incorporation of fatty acids in the developing brain should preferably be investigated in an animal model that most closely matches the human situation, such as piglets. If it is possible to overcome the financial and practical considerations that are intrinsic to the use of this animal species, this choice would increase our chances of finding results that may eventually be extrapolated to humans.

To gain important information to understand the origin of the regional differences in fatty acid changes after fish oil supplementation, an intervention study in piglets may be considered that evaluates the incorporation of dietary very long-chain n-3 PUFA in the phospholipid-specific and tissue-specific (gray vs. white matter) regions of separate brain lobes. These effects should be determined for diets with a range of dose levels of DHA – including one that corresponds to a dose level in humans that can potentially be consumed – and sufficient amounts of α-linolenic acid. This would facilitate the translation of the findings to the human situation. Finally, if the long-term consequences for later brain function are the eventual area of interest, it would be interesting to perform such a study in piglets during the period of brain growth spurt. The results from a study as proposed above may provide some suggestions to guide future research into the mechanism by which very long-chain n-3 PUFA may be involved in brain development.

Very long-chain n-3 PUFA and cognitive decline in humans

Several observational studies have examined the association between n-3 PUFA and cognitive performance or decline in older adults. However, the results are still inconsistent. Specific cognitive functions can decline with different rates in older adults[79]. Many studies so far have not taken this aspect into account and the association between very long-chain n-3 PUFA and cognitive performance or decline is often studied with global measures of overall cognitive performance. Because of the great variation in decline between cognitive domains, it will be hard, if not impossible, to find a general association between very long-chain n-3 PUFA and cognitive decline with a consistent direction and size, if such a general association would exist at all. Therefore, we anticipated that measuring cognitive decline with extensive domain-specific cognitive tests will yield a greater understanding of which specific cognitive functions are associated with very long-chain n-3 PUFA. Our research described in *Chapter 3* is a step in that direction.

Key findings from this thesis

Higher plasma proportions of very long-chain n-3 PUFA predicted less decline in the cognitive domains sensorimotor speed and complex speed over three years in older adults, compared with lower plasma proportions of very long-chain n-3 PUFA. However, no significant associations were observed between plasma very long-chain n-3 PUFA proportions and changes in memory, information-processing speed and word fluency.

Methodological considerations

Our findings suggest that higher proportions of very long-chain n-3 PUFA in plasma specifically predict decline in the speed-related cognitive domains. However, an important issue in this field is the question whether the observed differences in cognitive decline among exposure groups are of relevance. The interpretation of changes in cognitive test scores is often difficult, and requires information on how much change occurs normally in cognitively healthy individuals. Change in cognitive test performance lacks the specificity of a uniform disease diagnosis by

clinicians and may therefore have less relevance for clinical practice. However, if cognitive decline is the beginning of a broad spectrum of cognitive changes leading to dementia, it is particularly useful to characterize these changes on a continuous scale rather than with a single cut-off point for disease.

Another important issue in this area is the use of different cognitive test batteries, which causes discrepancy between studies. At present, there is a bewildering range of cognitive tests available. The lack of gold standards for the validation of cognitive tests means that multiple tests are available to examine any given cognitive domain. Therefore, comparisons between studies that have used different batteries of cognitive tests must be made with caution, even though they may have been attempting to examine the same cognitive domains. This lack of common outcome measures precludes the combination of study results by meta-analysis, which diminishes the value of the individual studies and delays the scientific progress in this field.

Another consideration that is of importance when interpreting study results is that one could argue that change in cognitive performance is a superior outcome measure to cognitive test performance at a single time point. If the focus of interest is on cognitive decline then it is best to measure this decline directly. Cognitive test performance at a single time point is influenced by many factors other than cognitive decline itself. Some of these factors, such as age and education level, are easy to measure, but other factors may be unknown or difficult to identify and measure. Measurement of individual change in cognitive performance has an advantage in this situation because, to the extent that these influences on cognitive performance remain constant over the study period, the measured change over the study period will reflect true individual change.

An additional consideration to take into account is the range of very long-chain n-3 PUFA and the assessment method. A small range in the levels of very long-chain n-3 PUFA in a study population decreases the contrast between the exposure groups, which reduces the probability to find a significant association. In addition, very long-chain n-3 PUFA can be assessed by dietary intake methods or by biomarkers, such as measurements in blood or adipose tissue. In general, biomarkers of very long-chain n-3 PUFA will be more appropriate to reflect the status, because they take individual absorption, usage and storage into account. Moreover, they are objective estimates that do not rely on accuracy of memories

and awareness of food intake. However, some biomarkers of very long-chain n-3 PUFA are susceptible for short-term fluctuations in dietary intake. For instance, plasma cholesteryl esters reflect dietary intake over the past two weeks, whereas erythrocytes reflect dietary intake over the past two months[35]. In this respect, an assessment of dietary intake may give a better reflection of long-term very long-chain n-3 PUFA status. However, these methods rely on composition tables that assume constant nutrient content of foods and are based on chemical analyses with their own measurement error. Moreover, if the dietary intake is based on questionnaires, these questionnaires need to be validated for fish consumption.

Implications for the field of very long-chain n-3 PUFA and decline in cognitive domains
Few other observational studies in older adults have examined the association between very long-chain n-3 PUFA and cognitive performance in separate cognitive domains. A study of Beydoun *et al.* among older adults showed that higher plasma n-3 PUFA proportions reduced the risk of cognitive decline in verbal fluency, but not in psychomotor speed or delayed word recall[27]. Furthermore, Kalmijn *et al.* showed that a higher intake of fatty fish and n-3 PUFA was associated with a lower risk of impaired psychomotor speed, but not with memory or cognitive flexibility in older adults[31]. Although the currently available observational studies in this field seem inconsistent, a few randomized controlled trials investigating the effect of very long-chain n-3 PUFA on cognitive decline in separate cognitive domains have already been executed. A study in 218 individuals reported no effects of EPA and DHA supplementation for a period of 12 weeks on speed of information processing, reasoning, impulsivity and working memory[125]. Likewise, no effect of EPA and DHA supplementation for a period of 26 weeks was shown on attention, sensorimotor speed, memory, and executive function in a study in 302 older adults[126]. The largest and longest randomized controlled trial in this area up until now has been conducted in 868 elderly people who were supplemented with either EPA and DHA or olive oil for a period of two years[127]. Cognitive performance was measured by immediate and delayed recall, letter search / cancellation tasks, prospective memory, verbal fluency, digit span, symbol digit modalities tests, reaction time and spatial memory. The results of this trial are, however, still awaited for. In summary, the now published randomized controlled trials did not demonstrate statistically significant beneficial

effects from very long-chain n-3 PUFA supplementation on late life cognitive performance in separate cognitive domains. However, with these small sample sizes, differences in cognitive test batteries and so few studies it is impossible to draw definitive conclusions and we need to be cautious not to over-interpret these interesting but preliminary findings.

Future research

If significant scientific progress is to be made in the field of very long-chain n-3 PUFA and domain-specific cognitive decline, there are a number of aspects that need consideration in future observational studies.

First, the development and application of a standard core battery of cognitive tests is important to facilitate future comparisons across different studies. The included tests should ideally cover a wide range of cognitive function, from "normal" function to severe impairment. The tests should therefore be sensitive enough to detect small cognitive changes in healthy persons who do not have apparent serious cognitive complaints yet. Otherwise, misleading results may occur if the immeasurable levels of performance outside the test range are differentially distributed among the exposure groups. In addition, these floor or ceiling effects may cause associations that are artefacts of the test characteristics, because persons with the lowest or highest possible scores can only change in one direction. However, the key point is that the standard core battery of cognitive tests provides a basis for making comparisons between studies and allows the combination of study results by meta-analysis. Next to this, individual researchers should of course still be at liberty to add supplementary tests if they feel that it is appropriate for the purpose of their particular study.

Second, future observational studies in this field should preferably be longitudinal to enable measurement of individual change in cognitive performance directly. On the one hand, the interval between the assessments should be sufficiently long to be able to observe change in cognitive performance and to diminish potential practice effects. On the other hand, however, the intervals should not be too long in order to avoid sample attrition due to illness and mortality.

Third, future studies should preferably combine information from dietary intake data and biomarkers of very long-chain n-3 PUFA. This combination will enable researchers to check whether the objective estimates of dietary n-3 PUFA intake

from biomarkers correspond to the reported dietary intake data, which will reduce the influence of measurement error.

Indulging all these considerations imposes nearly impossible demands on future observational research. Especially, because observational studies usually also deal with time constraints and the need to restrain subject burden in order to secure sufficient sample size and prevent premature drop-out of participants. Arriving at the best compromise among all these aspects will therefore probably be the most challenging factor of future research in this field.

A next step in the advancement of this field – provided that these future observational studies will indeed show consistent associations between very long-chain n-3 PUFA and specific cognitive domains – would be to investigate whether the relationship is causal. In this case, the observational studies will offer us essential information for the design of future randomized controlled trials. First, they will provide important clues about which cognitive domains or tests should be included as outcome measures. Second, information on the differences in cognitive decline between the exposure groups and the accompanying variation in the measurements will be provided, which is indispensible information for a meaningful power calculation. Third, the sensitivity of the cognitive tests for small cognitive changes in combination with data on how much change normally occurs in cognitively healthy individuals will provide important considerations to base the length of the intervention period on. Fourth, observational studies may provide clues about whether there is a dose-response relationship to be expected.

In summary, agreement on and widespread adoption of a standard core battery of cognitive tests will facilitate future comparisons between observational studies. This standard core battery combined with sufficient follow-up time and extensive exposure information on very long-chain n-3 PUFA will lead to an improvement in the quality of observational studies. As quality improves, consistent findings will shine through and unambiguous replications will follow. If the findings from observational studies continue to be beneficial associations, placebo-controlled intervention studies using fish oil supplementation to delay cognitive decline may eventually be considered to determine whether a causal relationship exists.

Very long-chain n-3 PUFA and blood supply in the head region in humans

The research presented in *Chapter 4* and *5* of this thesis was set out to investigate a couple of questions related to the role of very long-chain n-3 PUFA in the macrovascular as well as the microvascular blood supply of the head region. We evaluated in *Chapter 4* whether plasma very long-chain n-3 PUFA were associated with 3-year changes in carotid intima-media thickness and common carotid distension in Dutch older adults, because narrowing of the carotid arteries can obstruct the blood flow to the brain and thereby aggravate the progression of cognitive decline[37, 38]. Subsequently, we hypothesized that very long-chain n-3 PUFA may play a role in the microcirculation. We investigated in *Chapter 5* whether plasma very long-chain n-3 PUFA are associated with age-related hearing loss over a period of three years in older adults, since microvascular disease may decrease the blood supply to the highly vascularised cochlea, which may result in age-related hearing loss.

Key findings from this thesis

Very long-chain n-3 PUFA were not associated with 3-year change in common carotid intima-media thickness and common carotid stiffness in a healthy older adult population *(Chapter 4)*. However, we did show an inverse association between plasma very long-chain n-3 PUFA and age-related hearing loss in this population *(Chapter 5)*.

Methodological considerations

In the last decade, it is increasingly recognized that the very long-chain n-3 PUFA can modulate mechanisms of development and progression of atherosclerosis[128]. However, our findings from *Chapter 4* do not support an association between very long-chain n-3 PUFA and the late stages of atherosclerosis, such as carotid intima-media thickness. A consideration to take into account is that our study population was relatively young and healthy. A beneficial association between very long-chain n-3 PUFA and carotid thickness or stiffness may have been missed due to the small progression of carotid intima-media thickness and

carotid stiffness over the three year period. However, it is also possible that the role of very long-chain n-3 PUFA in a healthy population extends in particular to the smaller blood vessels. An observational study examined the relation between intake of fish and the risk of stroke, and the reduced risk of thrombotic infarction that was reported was due to a reduced risk in the small cerebral arteries but not in the large carotid arteries[129]. Moreover, the beneficial impact of very long-chain n-3 PUFA on endothelial function of small arteries[130], endothelial modulation[131], arterial compliance[132] and blood viscosity[133] alone or in concert may also favourably affect the microvascular blood supply in the head region.

Implications for the field of very long-chain n-3 PUFA and blood supply in the head region

The absence of a significant association between very long-chain n-3 PUFA and common carotid intima-media thickness in our study does not agree with findings from three cross-sectional studies[44-46], although our findings are in line with three intervention studies that showed no significant effect of very long-chain n-3 PUFA supplementation for two years[47, 48] or three years[49] on carotid intima-media thickness. In addition, the lack of an association between very long-chain n-3 PUFA and arterial stiffness in our study was in line with findings from one cross-sectional study[50], but two intervention studies showed favourable effects of very long-chain n-3 PUFA on arterial elasticity[49] and arterial stiffness[98]. Despite the potential benefits of fish intake in de reduction of stroke risk[93], it is therefore still not clear whether very long-chain n-3 PUFA have a role in the pathogenesis of carotid atherosclerosis.

Nevertheless, several studies have shown that carotid intima-media thickness[134-136], plaques in the carotid arteries[136, 137], and peripheral arterial atherosclerotic disease[137, 138] are all associated with cognitive functioning in middle-aged and elderly populations. These results are compatible with the view that atherosclerotic disease may account for a portion of the cognitive impairment in the general population. It has been hypothesized that brain hypoperfusion[136], white matter lesions[139, 140], and cerebral microemboli[39] and subsequent silent cerebral infarcts[141] could be involved in the mechanism by which subclinical cardiovascular disease or atherosclerosis produces cognitive dysfunction. Moreover, modest consumption of fish has been associated with lower prevalence of silent brain infarcts and

white matter abnormalities[142].

Besides the macrovascular lesions, pathology of the cerebral microvessels may also affect cognitive processes. The cerebral microvascular system maintains the blood-brain barrier and sustains continuous nutrient, electrolyte and waste product trafficking between brain and blood. A number of factors influence the cerebral blood flow in the brain microvessels, leading to suboptimal nutrient transport through the blood-brain barrier: microturbulent flow, viscosity of the blood, and vascular resistance[51]. First, microturbulent flow, a disrupted flow pattern with random swirls, can develop when the usual shape of the vascular lumen is locally thickened or partially obstructed. This compromises the slow blood flow of the layer near the vessel wall, which leads to suboptimal nutrient transport through the blood-brain barrier[51]. Second, the viscosity of the blood is inversely related to cerebral blood flow velocity. A major factor influencing viscosity is the membrane fluidity of the erythrocytes. Inflexibility of the cell membrane can hinder the passage of erythrocytes through capillaries and therefore decrease the cerebral blood flow[51]. Third, changes in the vascular diameter directly lead to alterations in vascular resistance and cerebral blood flow. Severe vasoconstriction increases vascular resistance and causes a decrease in cerebral blood flow. A decreased cerebral blood flow, an accompanying drop in cerebral glucose and oxygen utilization, and compromised structural integrity of the cerebral microvessels are representative degenerative features of the vascular system of the ageing brain that may contribute to suboptimal cognitive performance in elderly[51]. There are several hypotheses about the potential beneficial role of very long-chain n-3 PUFA in the cerebral microvessels. For instance, very long-chain n-3 PUFA may modulate brain glucose transport in endothelial cells of the blood-brain barrier via changes in GLUT1 protein expression and activity[143]. However, other authors suggest that DHA increases the cerebral blood flow by vasodilatation of the microvessels[144]. In addition, a DHA-enriched diet has been shown to increase the cerebral blood volume without changing blood flow in mice, indicating vasodilatation of the microvessels[145]. Furthermore, microvascular parameters such as the condition of pericytes and endothelial mitochondrial counts improved in rats fed a diet rich in very long-chain n-3 PUFA, which suggests an improved condition of the blood brain barrier[146].

Future research

The brain is critically dependent on a stable supply of glucose and oxygen from the blood. Hence, cerebral blood flow and substrate transport across the blood brain barrier are the primary determinants of brain function. The endothelium plays a primary role at the blood side of the blood brain barrier and produces vasodilator and vasoconstrictor compounds that are critical for achieving optimal perfusion of brain regions. It has been hypothesized that very long-chain n-3 PUFA may improve cerebral endothelial function[147]. This in turn may result in optimized cerebral perfusion and blood brain barrier integrity and thereby facilitate the delivery of glucose and oxygen to brain regions as required for healthy brain function[147].

A future research direction in this field could be to explore this possibility. Dietary n-3 PUFA supplementation has already been shown to improve cerebral perfusion[148] and blood brain barrier function[146] in rats with experimentally-induced chronic cerebral hypoperfusion. To explore this hypothesis in humans, measures of cerebral blood flow could be added to observational studies or randomized controlled trials investigating the role of very long-chain n-3 PUFA in cognitive performance. However, this is only feasible if resources are available to assess cerebral blood flow, such as magnetic resonance imaging equipment or transcranial Doppler ultrasound machinery.

Final overall reflections

Do very long-chain n-3 PUFA give someone a head start in brain development as well as in brain ageing? This thesis has been wide ranging, but some clear points have emerged with regard to this question.

First, we examined the role of very long-chain n-3 PUFA at the beginning of the lifespan and we showed that a diet enriched with fish oil resulted in higher proportions of DHA in the brain of pigs. It has already been shown that the total amount of DHA in the brain increases dramatically during the brain growth spurt[124], but we now showed that the developing brain is also responsive to a diet rich in fish oil after the brain growth spurt period. In addition, our findings suggest that providing dietary very long-chain n-3 PUFA may differentially affect specific brain regions. The incorporation of DHA in the brain during brain development is an important element for a number of theories describing the

underlying biological mechanisms for the role of very long-chain n-3 PUFA in brain development. One of the main hypothesized mechanisms of action of very long-chain n-3 PUFA involves an effect on the fluidity of neuronal membranes, which would affect the properties of membrane-bound enzymes, receptors and transporters that may be involved in signal transduction.

Second, we examined the role of very long-chain n-3 PUFA near the end of the lifespan. We showed that higher plasma proportions of very long-chain n-3 PUFA were associated with less decline in the cognitive domains sensorimotor speed and complex speed, but not in memory, information-processing speed and word fluency, compared with lower plasma proportions of very long-chain n-3 PUFA. We speculate that the incorporation of dietary very long-chain n-3 PUFA in brain tissue, which is a very important aspect for brain development at the beginning of life, slowly looses importance for maintaining brain function near the end of the lifespan. Other mechanisms of action of very long-chain n-3 PUFA, which may be of minor importance during brain development, may become more important near the end of life. Beneficial effects of very long-chain n-3 PUFA on the blood supply to the brain could be an example of such an hypothesized underlying mechanism that gains importance during ageing. Since the blood vessels of healthy, young, developing organisms are probably still in perfect condition, it is not likely that potential favourable effects of very long-chain n-3 PUFA on the blood supply to the brain have any impact on brain development or function that is worth mentioning. However, near the end of life a potential beneficial role of very long-chain n-3 PUFA in the blood vessels could well be a contributing mechanism for the maintenance of optimal brain function.

Although we showed no inverse association between very long-chain n-3 PUFA and common carotid intima-media thickness or stiffness in older adults, this does not necessarily imply that there is no favourable role for very long-chain n-3 PUFA in the blood supply of the brain. There are many indications that very long-chain n-3 PUFA play an important role in vascular structure and function, and measurements of carotid thickness and stiffness may not have been the right outcome measures in this particular situation. It is for instance possible that the role of very long-chain n-3 PUFA in healthy populations extends in particular to the smaller blood vessels instead of the large blood vessels. The role of very long-chain n-3 PUFA in the microcirculation of the brain could therefore be an

interesting future direction of research. Although we did not directly investigate the role of very long-chain n-3 PUFA in the brain microcirculation, our findings that higher plasma proportions of very long-chain n-3 PUFA were associated with less age-related hearing loss compared with lower plasma proportions of very long-chain n-3 PUFA do suggest that this hypothesis, if proven correct, may have far-reaching consequences.

Concluding remarks

Do very long-chain n-3 PUFA give someone a head start to win some years between the ears? Although the lay press and commercial advertisements already purport that very long-chain n-3 PUFA are beneficial for cognitive performance, we have to realize that this fascinating question hides many more sub-questions. Obtaining an answer to this question is an ongoing process of adding pieces to complete the overall puzzle. With this thesis, we have contributed some of these pieces. However, as oppose to a regular jigsaw puzzle, we do not have the advantage of being able to look at the box to actually see what should be assembled from the assorted pieces. Nevertheless, as pointed out in this general discussion, knowledge about these separate pieces and working with each single piece enhances the ability to ask better questions to inform and improve future attempts.

We have seen that the literature on the role of very long-chain n-3 PUFA in brain development and brain ageing is not consistent and that many aspects related to the sub-questions are still to be examined. We should keep ourselves from cherry-picking only the success stories of outstanding associations. Alternatively, we should not just concentrate on the bad news of the role of very long-chain n-3 PUFA in cognitive performance and brain development and the inconsistencies. Given the starting character of this field, criticism and compliments will accrue simultaneously. How long it will take for these pluses and minuses to eventually balance out and how they will balance out, depends on the quality and comparability of collective future research efforts. An attitude of modesty and the willingness to look at things from various perspectives remains indispensable to keep ourselves from the pitfall of exaggerating the results of poorly designed studies and reputable but preliminary research.

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Summary in Dutch (Nederlandse samenvatting)

De langketenige n-3 (of omega-3) meervoudig onverzadigde vetzuren staan de laatste tijd flink in de belangstelling vanwege hun mogelijk gunstige gezondheidseffecten. Ze zijn waarschijnlijk het bekendst vanwege hun gunstige rol in de gezondheid van het hart, maar er gaat ook steeds meer aandacht naar een mogelijk gunstige rol van deze vetzuren in cognitief functioneren. De langketenige n-3 vetzuren, ook wel visvetzuren genoemd, zouden zowel een gunstige rol spelen tijdens de hersenontwikkeling van kinderen als bij het voorkómen van cognitieve achteruitgang bij ouderen.
Maar is er echt een gunstige rol voor langketenige n-3 vetzuren in ons hoofd weggelegd of zit het slechts "tussen onze oren"? In dit proefschrift gaan we dieper in op de vraag of langketenige n-3 vetzuren je *"een voorsprong geven om wat jaren tussen de oren te winnen"*. Met andere woorden, we hebben onderzocht of er mogelijk een gunstige rol is weggelegd voor langketenige n-3 vetzuren in zowel hersenontwikkeling als hersenveroudering.

Langketenige n-3 vetzuren in het hersenweefsel
Ongeveer 50% van de droge stof van de hersenen bestaat uit lipiden. Een van de belangrijkste langketenige meervoudig onverzadigde vetzuren in de hersenen is het langketenige n-3 vetzuur docosahexeenzuur (DHA). Deze grote hoeveelheid DHA in de hersenen was een eerste aanleiding voor het idee dat dit vetzuur mogelijk essentieel is voor de ontwikkeling van de hersenen. Daarnaast is gebleken dat de hersenen zoveel mogelijk DHA proberen vast te houden, zelfs bij een tekort aan n-3 vetzuren in de voeding. Dit versterkte het vermoeden dat DHA functioneel belangrijk zou kunnen zijn in de hersenen.
De groeispurt van de hersenen vindt bij mensen plaats vanaf de laatste drie maanden van de zwangerschap tot ongeveer twee jaar na de geboorte. Mensen worden daarom ook wel "perinatale hersenontwikkelaars" genoemd. In deze periode zijn er snel veel structurele lipiden nodig voor de hersenenontwikkeling. Daarom zou de groeispurt van de hersenen wel eens een periode van grotere ontvankelijkheid voor voedingsfactoren (zoals vetzuren) zou kunnen zijn.
Experimenteel onderzoek op het gebied van hersenontwikkeling wordt vaak gedaan in dieren, omdat het verzamelen van hersenweefsel in levende mensen

meestal niet uitvoerbaar is. Hoewel het altijd lastig blijft om de resultaten van dierexperimenteel onderzoek te vertalen naar de menselijke situatie, is de timing van de groeispurt van de hersenen een belangrijke factor bij de keuze van het diermodel. Veel experimenteel onderzoek op het gebied van hersenontwikkeling wordt gedaan bij ratten. Echter, ratten zijn zogenaamde "postnatale hersenontwikkelaars" en verschillen daarnaast ook veel van mensen in lichaamsgrootte, levensduur en voedingspatroon. Deze kenmerken maken ze minder geschikt als model voor mensen. Varkens zijn echter wel "perinatale hersenontwikkelaars", net zoals mensen. Bovendien zijn het omnivoren en vertonen ze veel gelijkenissen met mensen in hersenmorfologie en oppervlakteanatomie.

Verscheidende studies in pasgeboren biggen hebben onderzocht of het toevoegen van langketenige n-3 vetzuren aan de voeding resulteerde in grotere hoeveelheden van deze vetzuren in het hersenweefsel. De meesten van deze studies rapporteerden inderdaad hogere percentages n-3 vetzuren in het hersenweefsel van biggen die melk met n-3 vetzuren kregen vergeleken met biggen die controlemelk kregen. Het lijkt er dus op dat de vetzuursamenstelling van de hersenen inderdaad beïnvloed wordt door de vetzuren in de voeding tijdens de groeispurt van de hersenen. Echter, dit sluit niet uit dat er nog andere periodes tijdens de ontwikkeling zijn waarin de hersenen ontvankelijk zijn voor voedingsfactoren. Het is bijvoorbeeld vooralsnog onduidelijk of een extra hoeveelheid langketenige n-3 vetzuren in de voeding tijdens de periode ná de groeispurt van de hersenen ook resulteert in hogere percentages in de hersenen. Daarnaast bestaan de hersenen ook nog eens uit veel verschillende delen en is hersenontwikkeling geen homogeen proces in de tijd of ruimte. Verschillende delen van de hersenen ontwikkelen zich op verschillende tijdstippen en met verschillende snelheden. Het is vooralsnog onduidelijk of de verschillende hersenregio's ook een verschillende gevoeligheid hebben voor vetzuren.

Om te onderzoeken of er regionale verschillen zijn in de gevoeligheid van de verschillende hersenregio's voor langketenige n-3 vetzuren hebben we een interventiestudie uitgevoerd in jonge varkens. *Hoofdstuk 2* van dit proefschrift beschrijft deze studie waarin we hebben onderzocht of het toevoegen van langketenige n-3 vetzuren aan de varkensvoeding resulteerde in grotere hoeveelheden van deze vetzuren in de vier verschillende hersenlobben: de frontaalkwab, the parietaalkwab, de temporaalkwab en de occipitaalkwab. Bovendien hebben we met

deze studie onderzocht of het toevoegen van langketenige n-3 vetzuren aan de voeding tijdens de periode n ná de groeispurt van de hersenen ook resulteerde in hogere percentages in de hersenen De varkens waren zeven weken oud bij het begin van het onderzoek, wat betekende dat de groeispurt van de hersenen reeds voorbij was. De varkens werden willekeurig onderverdeeld in twee groepen: een groep die varkensvoer kreeg met visolie (19 varkens) en een groep die varkensvoer kreeg met zonnebloemolie (18 varkens). Na acht weken bepaalden we de hoeveelheid vetzuren in de verschillende hersenlobben van de varkens. De varkens die visolie hadden gekregen hadden significant hogere hoeveelheden DHA in de frontaalkwab, the parietaalkwab en de occipitaalkwab, maar niet in de temporaalkwab, vergeleken met de varkens die de voeding met zonnebloemolie hadden gekregen. Bovendien was het percentage DHA significant lager in de temporaalkwab vergeleken met de andere drie kwabben in varkens die de visolie kregen. Hieruit concludeerden we dat de hersenen van de jonge varkens nog steeds ontvankelijk blijken te zijn voor visolie in de voeding, zelfs na de groeispurt van de hersenen, hoewel de temporaalkwab minder ontvankelijk lijkt dan de andere drie hersenlobben.

Langketenige n-3 vetzuren en cognitief functioneren

De mogelijke rol van langketenige n-3 vetzuren tijdens de hersenontwikkeling heeft aangespoord tot meer wetenschappelijk interesse in een mogelijke rol van deze vetzuren tijdens hersenveroudering. Het onderliggende idee was dat als langketenige n-3 vetzuren inderdaad een belangrijke rol spelen bij de ontwikkeling van de hersenen, ze wellicht ook een rol spelen bij het behoud van cognitief functioneren in ouderen.

De meest voorkomende manier om cognitief functioneren in ouderen te meten voor wetenschappelijk onderzoek is het gebruik van cognitieve testen. Een voordeel van deze testen is namelijk dat ze relatief gemakkelijk te gebruiken zijn in grote onderzoeken. De MMSE (Mini-Mental State Examination) is een voorbeeld van een kort, gemakkelijk, en daarom veel gebruikt, cognitieve screeningsinstrument. De bruikbaarheid van dergelijke screeningsinstrumenten hangt af van het doel van het onderzoek en van de samenstelling van de onderzoekspopulatie. De MMSE is oorspronkelijk ontworpen als een screeningsinstrument om patiënten met dementie onder te verdelen in patiënten met matige en ernstige

vormen van dementie en meet globaal cognitief functioneren. Echter, een belangrijk minpunt van de MMSE is dat het niet zo goed onderscheid kan maken tussen mensen met milde cognitieve achteruitgang en "normale" mensen. Bovendien kan milde cognitieve achteruitgang heel heterogeen zijn, en verschillende cognitieve domeinen op een verschillende manier beïnvloeden. Als wetenschappelijk onderzoek gericht is op het onderzoeken van milde cognitieve achteruitgang in personen die nog geen klaarblijkelijke serieuze dementieklachten hebben, dan is het gebruik van gevoelige cognitieve testen op meerdere cognitieve domeinen waarschijnlijk nuttiger dan het gebruik van een globaal cognitief screeningsinstrument.

Een ander aspect dat van belang is bij het gebruik van cognitieve testen in longitudinaal onderzoek is de follow-up tijd. Als je milde cognitieve achteruitgang bij ogenschijnlijk gezonde mensen wilt meten heb je voldoende follow-up tijd nodig zodat cognitieve achteruitgang ook echt zichtbaar wordt. Bovendien moeten de intervallen tussen de testen lang genoeg zijn om te voorkomen dat deelnemers zich de testen herinneren en gaan "oefenen" aan de hand van de antwoorden van een vorige keer.

De relatie tussen langketenige n-3 vetzuren en prestaties op cognitieve testen is al onderzocht in verschillende studies in ouderen. In deze studies wordt de blootstelling aan langketenige n-3 vetzuren meestal gekwantificeerd door het schatten van de visconsumptie aan de hand van vragenlijsten of door het meten van de hoeveelheid langketenige n-3 vetzuren in het bloed. Beide methoden zijn bruikbaar om mensen te rangschikken naar hun blootstelling aan langketenige n-3 vetzuren. Echter, bij de schatting van de visconsumptie met behulp van vragenlijsten is een voldoende gedetailleerde vragenlijst nodig. Bovendien gaan sommige van deze methoden er vanuit dat mensen zich bewust zijn van hun voedselinname en zich nauwkeurig herinneren wat ze hebben gegeten. De vetzuursamenstelling van het bloed wordt over het algemeen gezien als een meer objectieve schatting van de voedingsinname van langketenige n-3 vetzuren.

Een aantal onderzoeken hebben laten zien dat een lagere inname van n-3 vetzuren of lagere percentages n-3 vetzuren in het bloed inderdaad geassocieerd was met cognitieve problemen of cognitieve achteruitgang, hoewel er ook een aantal zijn die een dergelijke relatie niet vonden. De meeste studies op dit gebied definiëren echter een globale maat van cognitief functioneren, in plaats van het

functioneren op specifieke cognitieve domeinen. Deze domeinspecifieke informatie is juist waardevol, omdat cognitieve achteruitgang in een specifiek domein misschien niet gedetecteerd wordt door een globale meting van het cognitief functioneren.

In *Hoofdstuk 3* van dit proefschrift hebben we bekeken of mensen met grotere hoeveelheden langketenige n-3 vetzuren in hun bloedplasma beter presteerden op verschillende cognitieve testen vergeleken met mensen die lagere hoeveelheden in hun bloed hadden. We hebben hierbij gebruik gemaakt van diverse cognitieve testen op vijf verschillende cognitieve domeinen: sensorimotorische snelheid, complexe snelheid, geheugen, informatieverwerkingssnelheid, en taalbeheersing. Deze testen hebben we aan het begin van het onderzoek afgenomen en nogmaals na drie jaar. De deelnemers waren 807 Nederlandse ouderen (50-70 jaar) die ogenschijnlijk geen tekenen van dementie vertoonden en die oorspronkelijk meededen aan het FACIT-onderzoek, een onderzoek waarin ze drie jaar lang oftewel foliumzuur oftewel placebocapsules kregen. Omdat de foliumzuurinterventie een effect bleek te hebben op geheugen, informatieverwerkingssnelheid en sensorimotorische snelheid, hebben we onze longitudinale analyses alleen gebaseerd op de 404 deelnemers uit de placebogroep. De cross-sectionele analyses zijn echter wel in alle 807 deelnemers uitgevoerd. Hogere percentages langketenige n-3 vetzuren in bloed plasma bleken geassocieerd te zijn met minder cognitieve achteruitgang in sensorimotorische snelheid en complexe snelheid, vergeleken met lagere percentages. Echter, de percentages langketenige n-3 vetzuren in bloedplasma waren niet gerelateerd aan veranderingen in geheugen, informatieverwerkinssnelheid of taalbeheersing. In de cross-sectionele analyses vonden we geen associaties tussen langketenige n-3 vetzuren in bloedplasma en functioneren op de vijf cognitieve domeinen. We concluderen hieruit dat langketenige n-3 vetzuren met name geassocieerd lijken te zijn met minder achteruitgang in de snelheidsgerelateerde cognitieve domeinen in onze specifieke onderzoekspopulatie. Hiermee lijkt toekomstig observationeel en interventieonderzoek naar de rol van langketenige n-3 vetzuren in cognitief functioneren van ouderen gerechtvaardigd. Echter, deze studies zouden bij voorkeur uitgevoerd moeten worden met gevoelige cognitieve uitkomstmaten die domeinspecifieke informatie verschaffen.

Langketenige n-3 vetzuren en de doorbloeding van en in het hoofd

Verminderde bloedtoevoer naar de hersenen of verstoorde microvasculaire structuren in het hoofd kunnen een initiërende of intermediaire rol spelen bij cognitieve achteruitgang. De hersenen hebben een constante toevoer van zuurstof en voedingsstoffen nodig om te kunnen functioneren, en structurele vasculaire schade kan deze toevoer verstoren. Daarom hebben we in het laatste deel van dit proefschrift onderzocht of de mogelijk gunstige rol van langketenige n-3 vetzuren in cognitief functioneren misschien te maken heeft met een gunstige rol van langketenige n-3 vetzuren in de bloedtoevoer van het hoofd. Hiervoor hebben we zowel de macrocirculatie als de microcirculatie bekeken.

De belangrijkste toevoer van zuurstofrijk bloed naar de hersenen gaat via de halsslagaders. Vernauwing van deze halsslagaders door bijvoorbeeld atherosclerose kan de bloedtoevoer naar de hersenen blokkeren. Atherosclerose is een risicofactor die cognitieve achteruitgang kan verergeren. Bovendien hebben patiënten met atherosclerose ook vaak last van cerebrale micro-emboli, die cognitieve schade kunnen veroorzaken als ze in de cerebrale circulatie terechtkomen. Tot slot kan atherosclerose van de halsslagaders ook bijdragen aan een verstijving van de slagaderwand waardoor uiteindelijk een herseninfarct met permanente hersenschade kan ontstaan.

De wand van de slagader bestaat uit drie lagen: de intima, de media en de adventitia. Het eerste stadium van atherosclerose wordt gekenmerkt door een geleidelijke verdikking van de binnenste laag, de intima. Met behulp van echografie is het mogelijk de dikte van de intima en de media samen te meten. Bovendien kan de echografietechniek ook gebruikt worden om de visco-elastische eigenschappen van de slagader te bepalen, de zogenoemde stijfheid van de slagader. Zowel de intima-media dikte als de stijfheid van de halsslagader zijn veelgebruikte markers voor subklinische atherosclerose en zijn beide gerelateerd aan het risico op een herseninfarct.

In *Hoofdstuk 4* van dit proefschrift hebben we onderzocht of de hoeveelheid langketenige n-3 vetzuren in het bloedplasma gerelateerd is aan veranderingen in intima-media dikte en stijfheid van de halsslagaders in 808 Nederlandse ouderen. Met behulp van echografie hebben we de intima-media dikte en de stijfheid van de halsslagaders gemeten, zowel aan het begin van het onderzoek als na 3 jaar. Er bleken echter geen significante verschillen te zijn in de veranderingen in

stijfheid of intima-media dikte van de halsslagaders tussen mensen met hogere en lagere hoeveelheden langketenige n-3 vetzuren in hun bloedplasma. Door de relatief kleine verandering over de driejarige periode in onze onderzoekspopulatie, zouden we mogelijk een gunstige associatie kunnen hebben gemist. Echter, in de context van andere observationele en interventiestudies is er op dit moment niet voldoende consistent bewijs om de hypothese te ondersteunen dat langketenige n-3 vetzuren geassocieerd zijn met stijfheid en intima-media dikte van de halsslagaders in gezonde ouderen.

Echter, naast macrovasulaire problemen kunnen ook microvasculaire structuren in de hersenen een rol spelen bij cognitieve achteruitgang. Onderzoek in dieren heeft al gunstige effecten gevonden van langketenige n-3 vetzuren op de cerebrale microcirculatie. Onze hypothese is dat de rol van deze langketenige n-3 vetzuren in de microcirculatie mogelijk niet alleen in de hersenen plaatsvindt, maar dat deze vetzuren ook de microcirculatie in andere organen, zoals in het oor, kan beïnvloeden. Omdat het slakkenhuis van het oor zeer goed doorbloed is, zouden microvasculaire verstoringen de bloedtoevoer naar het slakkenhuis kunnen verminderen, waardoor gehoorverlies kan ontstaan. Op dit moment is er nog heel weinig bekend over een mogelijke rol van vetzuren in gehoorverlies. Een onderzoek in twee psychiatrische ziekenhuizen in Finland heeft laten zien dat volwassen die vijf jaar lang een voeding met een lage hoeveelheid vet kregen een beter gehoor hadden dan volwassen die een voeding kregen die rijk was aan verzadigd vet. Echter, er zijn op dit moment nog geen studies bekend die onderzoek hebben gedaan naar de relatie tussen langketenige n-3 vetzuren en gehoorverlies in ouderen.

In *Hoofdstuk 5* van dit proefschrift hebben we onderzocht of mensen met grotere hoeveelheden langketenige n-3 vetzuren in hun bloedplasma beter hoorden en minder gehoorverlies hadden dan mensen met lagere hoeveelheden van deze vetzuren in hun bloedplasma. De gehoortesten zijn uitgevoerd in 720 Nederlandse ouderen (50-70 jaar) aan het begin en het einde van een driejarig onderzoek. Het bleek dat ouderen die grotere hoeveelheden langketenige n-3 vetzuren in hun bloedplasma hadden minder gehoorverlies hadden in de lage frequenties dan ouderen met lagere hoeveelheden van deze vetzuren. Er was echter geen associatie tussen langketenige n-3 vetzuren en gehoorverlies op de hoge frequenties. Dit is de eerste studie die een inverse relatie laat zien tussen langketenige n-3

vetzuren en gehoorverlies. Hoewel de resultaten van dit onderzoek veelbelovend zijn, moeten ze natuurlijk nog wel versterkt worden door toekomstige studies op dit gebied in andere onderzoekspopulaties.

Conclusie

Geven langketenige n-3 vetzuren ons nou een voorsprong om wat jaren tussen de oren te winnen? We moeten ons realiseren dat deze fascinerende vraag vele subvragen verbergt. Het verkrijgen van een antwoord op een dergelijke brede vraag is een voortdurend proces van het toevoegen van stukjes om de puzzel volledig te maken. Met dit proefschrift hebben wij enkele van deze stukjes toegevoegd. Hoewel het volledige plaatje nog niet duidelijk is, geeft kennis over deze losse stukjes en het werken met deze losse stukjes ons de mogelijkheid om nog betere vragen te stellen die toekomstige pogingen om de puzzel compleet te maken zullen verbeteren.

Dit proefschrift heeft drie specifieke deelgebieden aangestipt: de rol van langketenige n-3 vetzuren in *1)* hersenontwikkeling, *2)* hersenveroudering, en *3)* macrovasculaire en microvasculaire doorbloeding van het hoofd. We hebben gezien dat de literatuur over de rol van langketenige n-3 vetzuren in deze deelgebieden niet consistent is en dat er veel aspecten nog verder onderzocht moeten worden. Aangezien dit een vrij jong veld binnen de voedingswetenschap is zullen de positieve en negatieve berichten elkaar nog een tijdje afwisselen. Hoe deze plussen en minnen uiteindelijk tegen elkaar zullen afwegen, zal afhangen van de kwaliteit en vergelijkbaarheid van onze toekomstige onderzoeksprestaties.

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Dikke kus,

Carla

Curriculum vitae

Carla Dullemeijer was born on the 1st of September, 1980, in Breda, the Netherlands. She graduated in 2001 from Fontys Hogescholen in Tilburg, the Netherlands, with a Bachelor's degree in Biology and a second-grade teaching qualification. She obtained her Master's degree in Health Sciences, specialization in Biological Health Sciences, from Maastricht University, the Netherlands, in 2003.

In that same year, she joined Wageningen Center for Food Sciences as a scientific research assistant. During this period, she contributed to the coordination of a randomized placebo-controlled trial in 26 cardiology clinics across Europe, which investigated the effect of supplemental fish oil versus placebo on the incidence of ventricular tachyarrhythmia in patients with implantable cardioverter defibrillators.

In July 2005, she started as a PhD-fellow at the Wageningen Center for Food Sciences (currently known as Top Institute Food and Nutrition) and the Division of Human Nutrition, Wageningen University. Her PhD-research focused on the role of very long-chain n-3 polyunsaturated fatty acids in brain development, cognitive decline and the macro- and microvascular blood supply in the head region. During her PhD-period, Carla attended several courses, conferences and discussion groups, was a member of the VLAG graduate school PhD-Council and was involved in the education of both BSc and MSc undergraduate students. In her last year as a PhD-fellow, she was also a part-time trial coordinator for a period of five months as a maternity leave replacement at the Division of Human Nutrition of Wageningen University. Within this project, she was responsible for the project management and progress of a randomized, placebo-controlled trial in three centres in the Netherlands, which aimed to investigate the efficacy of vitamin B12 and folic acid supplementation in the prevention of fractures in elderly people.

Currently, she's employed as a postdoctoral researcher in epidemiology at the Division of Human Nutrition, Wageningen University, the Netherlands, on the project "EURRECA" (EURopean micronutrient RECcommendations Aligned). This project is funded by the European commission and aims to work together, within an network of 34 partners in 17 countries, on harmonizing micronutrient recommendations across Europe.

List of publications

Dullemeijer C, Durga J, Brouwer IA, Brummer R-JM, Kok FJ, Verhoef P. *The association between plasma very long-chain n-3 polyunsaturated fatty acids and progression of carotid stiffness and carotid intima-media thickness in older adults*.

Dullemeijer C, Verhoef P, Brouwer IA, Brummer R-JM, Kok FJ, Durga J. *Plasma very long-chain n-3 polyunsaturated fatty acids and age-related hearing loss in older adults.*

Brouwer IA, Raitt MH, **Dullemeijer C**, Kraemer DF, Zock PL, Morris C, Katan MB, Connor WE, Camm AJ, Schouten EG, McAnulty J. *Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators.* Eur Heart J 2009; in press.

Dullemeijer C, Zock PL, Coronel R, Den Ruijter H, Katan MB, Brummer R-JM, Kok FJ, Beekman J, Brouwer IA. *Differences in fatty acid composition between cerebral brain lobes in juvenile pigs after fish oil feeding.* Br J Nutr 2008; 100: 794-800

Van de Rest O, Geleijnse JM, Kok FJ, Van Staveren WA, **Dullemeijer C**, OldeRikkert MGM, Beekman ATF, de Groot CPGM. *Effect of fish oil on cognitive performance in older subjects: a randomized, controlled trial.* Neurology 2008; 71 (6): 430-8

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Dullemeijer C, Durga J, Brouwer IA, Verhoef P. *Erythrocyte folate and plasma DHA in the FACIT study [letter].* Lancet 2007; 370 (9583): 216

Dullemeijer C, Durga J, Brouwer IA, van de Rest O, Kok FJ, Brummer R-JB, van Boxtel MPJ, Verhoef P. *Very long-chain n-3 fatty acids an cognitive function in older adults [abstract].* Ann Nutr Metab 2007; 51 (1): 240-241

Brouwer IA, Zock PL, Camm AJ, Böcker D, Wever EF, Hauer RN, **Dullemeijer C**, Ronden JE, Katan MB, Lubinski A, Buschler H, Schouten EG, SOFA Study Group. *Effect of fish oil on ventricular tachyarrhythmia and death in patients with implantable cardioverter defibrillators: the Study on Omega-3 Fatty Acids and Ventricular Arrhythmia (SOFA) randomized trial.* JAMA 2006; 295: 2613-9

Brouwer IA, Zock PL, Camm AJ, Böcker D, Hauer RN, Wever EF, **Dullemeijer C**, Ronden J, Katan MB, Schouten EG. *SOFA: Study of omega-3 fatty acids and ventricular arrhythmia. A multicenter, randomized clinical trail [abstract].* Circulation 2006; 113 (8): E371

Dullemeijer C, Geleijnse JM, de Groot CPGM. *N-3 fatty acids in the elderly brain*. ISSFAL Newsletter 2004; 11: 26-31

Overview of completed educational activities

Discipline-specific activities: courses

History of Epidemiologic Ideas, 2008
Erasmus Summer Programme / NIHES, Rotterdam, the Netherlands

Survival analysis, 2008
Erasmus Summer Programme / NIHES, Rotterdam, the Netherlands

Methods of Public Health Research, 2008
Erasmus Summer Programme / NIHES, Rotterdam, the Netherlands

Regression analysis, 2007
Erasmus Summer Programme / NIHES, Rotterdam, the Netherlands

Good Clinical Practice, 2005
NUTRIM, Maastricht, the Netherlands

Discipline-specific activities: scientific meetings

8th ISSFAL meeting, 2008
International Society for the Study of Fatty Acids and Lipids, Kansas City, USA

10th European Nutrition Conference, 2007
Federation of European Nutrition Societies, Paris, France

N-3 fatty acids and Mental Health symposium, 2007
Graduate School VLAG, Wageningen, the Netherlands

The greatest discoveries and challenges in nutrition research, 2007
Division of Human Nutrition, Wageningen University, the Netherlands

7th ISSFAL meeting, 2006
International Society for the Study of Fatty Acids and Lipids, Cairns, Australia

Nutrition and Ageing symposium, 2006
Division of Human Nutrition, Wageningen University, the Netherlands

SeafoodPlus symposium, 2006
SeafoodPlus, Tromsø, Norway

Dietary influences on blood pressure, 2006
Graduate School VLAG, Wageningen, the Netherlands

European Society of Cardiology Congress, 2005
European Society of Cardiology, Stockholm, Sweden

Annual Epidemiology Symposium WEON, 2005
WEON, Wageningen, the Netherlands
6th ISSFAL meeting, 2004
International Society for the Study of Fatty Acids and Lipids, Brighton, UK
NWO Nutrition meetings, 2004-2005-2006-2008
NWO Nutrition community, Arnhem and Deurne, the Netherlands

General courses
Networking skills for PhD-students, 2008
Top Institute Food and Nutrition, Wageningen, the Netherlands
Career perspectives, 2008
Wageningen Graduate Schools, Wageningen, the Netherlands
Ph.D. Competence assessment, 2007
Wageningen Graduate Schools, Wageningen, the Netherlands
Philosophy and Ethics of Food Science and Technology, 2007
Graduate School VLAG, Wageningen, the Netherlands
Scientific writing, 2006
Centa language centre, Wageningen, the Netherlands
Debating skills for PhD-students, 2007
Wageningen Centre for Food Sciences, Wageningen, the Netherlands
PhD Introduction week, 2006
Graduate School VLAG, Bilthoven, the Netherlands
Scientific oral presentation skills, 2005
Wageningen Centre for Food Sciences, Wageningen, the Netherlands

Miscellaneous training activities
PhD Study Tour to universities and research institutes in the USA, 2007
Division of Human Nutrition, Wageningen University, the Netherlands
PhD Study Tour to universities and research institutes in the UK and Ireland, 2005
Division of Human Nutrition, Wageningen University, the Netherlands
Literature group "Journal Club", 2004-2007
Division of Human Nutrition, Wageningen University, the Netherlands

Literature group “N-3 Club” , 2004-2007

Division of Human Nutrition, Wageningen University, the Netherlands

Literature group “Oldsmobiles”, 2006-2008

Division of Human Nutrition, Wageningen University, the Netherlands

Programme 1 meetings WCFS / TIFN, 2003-2008

Wageningen Centre for Food Sciences / Top Institute Food and Nutrition

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