# The mind's ratio

## *How the balance between ω-6 and ω-3 fatty acids affects the risk of late-life dementia*

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## List of abbreviations

### Special characters

| | |
|---|---|
| α | Alpha |
| β | Beta |
| γ | Gamma |
| ω-3 | Omega-3 fatty acid |
| ω-6 | Omega-6 fatty acid |
| µmol/L | Micromole per liter |

**A**

| | |
|---|---|
| AD | Alzheimer’s disease |
| AgeCoDe | German Study on Ageing, Cognition, and Dementia |
| ALA | Alpha-linolenic acid |
| APOE | Apolipoprotein E |
| APP | Amyloid precursor protein |
| ARA | Arachidonic acid |
| ARIC | Atherosclerosis Risk in Communities Study |
| ATH | Atherosclerosis |
| Aβ | Amyloid beta |

**B**

| | |
|---|---|
| BMI | Body mass index |

**C**

| | |
|---|---|
| CAD | Coronary artery disease |
| CERAD | Consortium to Establish a Registry for Alzheimer's Disease |
| CHARGE | Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CNS | Central nervous system |
| cPLA2 | Cytosolic phospholipase A2 |
| CRP | C-reactive protein |
| CSF | Cerebrospinal fluid |
| CVD | Cardiovascular disease |

**D**

| | |
|---|---|
| D5D | Delta-5 desaturase |
| D6D | Delta-6 desaturase |
| DASH | Dietary Approaches to Stop Hypertension |
| DGE | German Nutrition Society |

| | |
|---|---|
| DGLA | Dihomo-gamma-linolenic acid |
| DHA | Docosahexaenoic acid |
| DNA | Deoxyribonucleic acid |
| DPA | Docosapentaenoic acid |
| DSM-IV | Diagnostic and Statistical Manual of Mental Disorders, 4th Edition |

**E**

| | |
|---|---|
| EFA | Essential fatty acids |
| ENCODE | Encyclopedia of DNA Elements |
| EOAD | Early-onset AD |
| EPA | Eicosapentaenoic acid |

**F**

| | |
|---|---|
| FA | Fatty acid |
| FADS | Fatty acid desaturase |
| FDR | False discovery rate |
| FFQ | Food frequency questionnaire |
| FINGER | Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability |
| FU | Follow-up |

**G**

| | |
|---|---|
| g | Gram |
| GLA | Gamma-linolenic acid |
| GP | General practitioner |
| GWAS | Genome-wide association study |

**H**

| | |
|---|---|
| h | Hour |
| HCl | Hydrochloric acid |
| HGDP-CEPH | Human Genome Diversity Cell Line Panel |
| HR | Hazard ratio |
| hs-CRP | High-sensitivity C-reactive protein |
| HUFA | Highly unsaturated fatty acid |

**I**

| | |
|---|---|
| ICD-10 | International Classification of Diseases, 10th Revision |
| IFN-γ | Interferon-γ |
| IGAP | International Genomics of Alzheimer’s Project |

**K**

| | |
|---|---|
| Kb | Kilobase |

**L**

| | |
|---|---|
| LA | Linoleic acid |
| LD | Linkage disequilibrium |
| LDL | Low-density lipoprotein |
| LOAD | Late-onset AD |
| LTB | Leukotriene B |

**M**

| | |
|---|---|
| MAF | Minor allele frequency |
| MAPT | The Multidomain Alzheimer Preventive Trial |
| MeDi | Mediterranean diet |
| mg/day | Milligram per day |
| MIND | Mediterranean-DASH diet Intervention for Neurodegenerative Delay |
| MMSE | Mini-Mental-State-Examination |
| mRNA | Messenger ribonucleic acid |
| MUFA | Monounsaturated fatty acid |
| MWD | Modern Western Diet |

**N**

| | |
|---|---|
| NF-κB | Nuclear transcription factor kappa B |
| ng | Nano gram |
| NS | Not significant |

**O**

| | |
|---|---|
| OR | Odds Ratio |

**P**

| | |
|---|---|
| PA | Palmitic acid |
| PNNS | The French National Nutrition and Health Program |
| PRS | Polygenic risk score |
| PSEN | Presenilin |
| PUFA | Polyunsaturated fatty acid |

**R**

| | |
|---|---|
| RBC | Red blood cell |
| RCT | Randomised controlled trials |
| RERI | Relative excess risk due to interaction |
| RR | Relative risk |

**S**

| | |
|---|---|
| SA | Stearic acid |
| SFA | Saturated fatty acids |

| | |
|---|---|
| SIDAM | Structured Interview for Diagnosis of Dementia of Alzheimer type, Multi-infarct Dementia, and Dementia of other Aetiology |
| SNP | Single nucleotide polymorphism |
| SPM | Specialized pro-resolving mediator |
| SPSS | Superior Performing Statistical Software |

**T**

| | |
|---|---|
| TAG | Triglyceride |
| TFA | Trans-fatty acid |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-α |

**U**

| | |
|---|---|
| UK | United Kingdom |
| USA | United States of America |

**V**

| | |
|---|---|
| VaD | Vascular dementia |

**W**

| | |
|---|---|
| wt% | Weight percentage |

# 1 Introduction

Dementia health care costs are rapidly rising due to the increasing number of people with dementia worldwide. There were around 50 million people living with dementia worldwide in 2020 (WHO, 2020) and this number is expected to increase to 66 million by 2030 and 115 million by 2050 (Prince *et al.*, 2013). Intensive research has clearly shown that dementia is caused by multiple factors, making it challenging to provide therapy. Understanding the complex interactions between genetic, lifestyle and environmental factors is crucial to developing efficient preventive measures and recommendations that help to significantly reduce the risk of dementia. Alzheimer's disease (AD) is the predominant form of dementia (50-75 %) and the vast majority of patients develop clinical symptoms at ages older than 65 years (late-onset AD, or LOAD), while 2-10 % of patients have an earlier onset of the disease (early-onset AD, or EOAD) (Van Cauwenberghe *et al.*, 2016). AD is pathologically defined by severe neurodegeneration with synaptic and neuronal loss, aggregation of amyloid β (Aβ) in extracellular senile plaques, and the formation of intraneuronal neurofibrillary tangles consisting of hyperphosphorylated tau protein (Lane *et al.*, 2018). The disease is clinically characterized by progressive worsening of episodic memory and cognitive functions, leading to an inability to perform activities of daily living, a loss of autonomy, increased dependence and ultimately required full-time medical care (Van Cauwenberghe *et al.*, 2016; Lane *et al.*, 2018). It has become widely recognized especially in older individuals that a large proportion of dementia is caused by a mix of AD and vascular pathology. In addition, epidemiological studies indicate that AD and cerebrovascular diseases share the same risk factors (Fotuhi *et al.*, 2009; Iadecola, 2010). Therefore, a clinical diagnosis alone cannot diagnose pure AD or pure vascular dementia, so the term dementia used in this study refers to dementia of the AD type with a possible strong vascular component.

Beside the genetic contribution to the risk of dementia, lifestyle and environmental factors represent a large field of life that have a major impact on dementia risk, but whose precise influence is only partially understood. Therefore, there is ongoing research on the amount of risk conferred by non-modifiable risk factors versus modifiable risk factors and how they interfere with each other (Licher *et al.*, 2019; Lourida *et al.*, 2019; Livingston *et al.*, 2020). In this context, nutrition is an important modifiable factor whose contribution can be an

important key in the prevention of non-communicable diseases. This involves understanding different food components and their importance for health, but also the impact of major changes in the diet and dietary patterns on human health. The Modern Western Diet (MWD) is a rather new dietary pattern in the history of humans and found primarily in North America and Central Europe, but is spreading globally leading to a rapid shift in diet structure (Drewnowski and Popkin, 1997). This type of diet is characterised by a significant increase in animal products, salt and sugar, as well as a significant change in the fatty acid (FA) composition of the diet (Cordain *et al.*, 2005; Blasbalg *et al.*, 2011). The MWD and Western way of life have already been associated with many diseases (Carrera-Bastos *et al.*, 2011; Simopoulos, 2011; Chilton *et al.*, 2014; Lands, 2014; Imamura *et al.*, 2015; Melo *et al.*, 2019). One possibility to assess the health risk conferred by diet is to find a reliable biomarker that is easy to obtain and independent from fairly imprecise lifestyle questionnaires. This current work investigates how the balance between certain FAs measured in blood serum phospholipids, along with consideration of further lifestyle and genetic factors can be used as an index to assess its effect on the risk of dementia/AD in community dwelling elderly aged 75 and older. It is further discussed how these results can easily be integrated into an individualized risk profile and used for personalised dietary recommendations to reduce the risk of dementia by promoting a healthier diet and lifestyle.

### 1.1 Non-modifiable factors and their contribution to the risk of dementia

Beside the genetic contribution to dementia, there are further non-modifiable risk factors such as age, family history, sex, and race and ethnicity that increase the risk of late-life dementia (Eid *et al.*, 2019). Age is the greatest of these risk factors, as the percentage of people with AD increases dramatically with age. Overall, about 80 % of dementias are in people aged ≥75 years (Livingston *et al.*, 2017). The reason behind this remains largely unclear; the literature suggests that the cause is due to dysregulation and disruption of the critical pathways of the inflammatory system, lipid homeostasis, and protein degradation and synthesis (Eid *et al.*, 2019). However, it is unlikely that AD occurs as a result of normal aging, as there are distinctive differences in those aged individuals who develop AD compared to those who do not, such as atrophy of specific brain regions and changes in cognition that occur much more rapidly in AD compared to normal aging (Eid *et al.*, 2019). Therefore, while aging remains a significant risk factor for late-life dementia,

it alone is not sufficient to cause dementia/AD. Furthermore, sex has an influence on the probability to develop dementia/AD later in life. The estimated lifetime risk for AD at age 45 is approximately 20 % for women and 10 % for men (Alzheimer Association, 2019). A publication by the Framingham Heart Study suggests that because men have a higher rate of death from cardiovascular disease (CVD) in middle age, selective survival of men with a healthier cardiovascular risk profile beyond age 65 may account for an apparent lower risk for dementia than women of the same age (Chêne *et al.*, 2015). This effect known as “survival bias”, can lead to a lowered measure of risk derived from epidemiological studies for men to develop AD, as these studies tend to include the healthiest men (Alzheimer Association, 2019). Since a lower level of education is also a risk factor for dementia, it is possible that the lower average level of educational seen in women born in the first half of the 20th century when compared to men of the same time period could account for a higher risk of AD and other dementias present in women today (Alzheimer Association, 2019). Evidence also suggests that these sex differences may also be rooted in inflammatory mechanisms and/or mitochondrial dynamics, as well as hormonal differences (Eid *et al.*, 2019). A recent study indicates that this increased risk for women is only at earlier stages of the disease, and that older individuals of both sexes have the same risk of developing dementia/AD (Eid *et al.*, 2019).

Genetic studies have made important discoveries on the genetic landscape of dementia/AD over roughly the last 30 years. While the majority of dementia/AD occurs on a sporadic basis, rare autosomal dominant forms of AD exist, predominantly presenting as EOAD caused by fully penetrant mutations in the *Amyloid precursor protein (APP), Presenilin 1 (PSEN1)*, and *Presenilin 2 (PSEN2)* genes in which symptoms typically develop between 30 and 50 years of age (Lane *et al.*, 2018). The identification of these genes has provided valuable insights into the pathological process of one of the two hallmarks underlying AD, the amyloidogenic pathway of the amyloid cascade hypothesis (Hardy and Higgins, 1992). APP is a transmembrane protein that is proteolytically processed by α-, β-, and γ-secretases following two pathways, the non-amyloidogenic or the amyloidogenic pathway. The non-amyloidogenic pathway is initiated by α-secretase, whereas the sequential cleavage of APP by β- and γ-secretases represents the amyloidogenic pathway that produces the suggested pathological forms of Aβ (Van Cauwenberghe *et al.*, 2016; Lane *et al.*, 2018). *PSEN1* and *PSEN2* are coding proteins

that are essential components of the γ-secretase complex, which catalyses the cleavage of APP among other membrane proteins. Mutations in *PSEN1* and *PSEN2* impair the γ-secretase mediated cleavage of APP in Aβ fragments, resulting in an increased ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$, either through an increased $A\beta_{1-42}$ production or decreased $A\beta_{1-40}$ production, or a combination of both (Van Cauwenberghe *et al.*, 2016).

For many years, *APOE* (*Apolipoprotein E*) was the only major gene known to increase disease risk. *APOE* plays several roles in the nervous system, such as the transport of cholesterol and other lipids, and is involved in neuronal growth, the repair response to tissue injury, nerve regeneration, immunoregulation, and the activation of lipolytic enzymes (Van Cauwenberghe *et al.*, 2016). Furthermore, *APOE* binds to Aβ and influences the clearance of soluble Aβ and the Aβ aggregation (Karch and Goate, 2015). The *APOE* gene contains three major allelic variants (ε2, ε3, and ε4), encoding for different isoforms (ApoE2, ApoE3, and ApoE4) that differ in two sites of the amino acid sequence (Van Cauwenberghe *et al.*, 2016). The *APOE* ε4 allele increases risk in familial and sporadic EOAD and LOAD, but it is not sufficient to cause disease. The risk effect is estimated to be threefold for heterozygous carriers (*APOE* ε34) and 15-fold for ε4 homozygous carriers (*APOE* ε44), and has a dose-dependent effect on onset age (Van Cauwenberghe *et al.*, 2016). Conversely, the *APOE* ε2 allele is thought to have a protective effect and delay onset age (Van Cauwenberghe *et al.*, 2016). Of the general population, only 20-25 % carry one or more ε4 alleles, whereas 40-65 % of AD patients are ε4 carriers (Van Cauwenberghe *et al.*, 2016). Large-scale collaborative genome-wide association studies (GWAS), like the International Genomics of Alzheimer's Project (IGAP), have significantly advanced scientific knowledge regarding the genetic underpinnings of LOAD by identifying at least 20 additional genetic risk loci. These newly-identified genes point toward pathways implicated in the immune system and inflammatory responses, cholesterol and lipid metabolism, and endosomal-vesicle recycling (Harold *et al.*, 2009; Seshadri, 2010; Hollingworth *et al.*, 2011; Lambert *et al.*, 2013; Kunkle *et al.*, 2019). A recent GWAS has identified nine additional novel loci using the unconventional approach of including a proxy phenotype for AD to further increase the sample size (Jansen *et al.*, 2019). *In silico* functional follow-up analysis has emphasized the crucial causal role of the immune system, rather than an immune response as a consequence of AD pathology. Together with the AD-associated genetic effects on lipid metabolism in that

study and the previous GWAS, these biological implications strengthen the hypothesis that neurodegenerative pathogenesis involves an interplay between inflammation and lipids, as lipid changes might harm immune responses of microglia and astrocytes, and vascular health of the brain (Jansen *et al.*, 2019).

Individuals who have a first-degree relative, such as a parent or sibling, diagnosed with AD are predisposed to develop the disease with a 4-10 times increased risk, compared to individuals who do not (Eid *et al.*, 2019). It is important to note that family history represents a combination of both genetic factors and shared environments among family members that contributes to the risk of dementia/AD. The same may be true for ethnicities and population, which share the same environment and similar ways of diets (Babulal *et al.*, 2019). Over the age of 65, AD and other dementias have been diagnosed in 10.3 % of Caucasians, 12.2 % of Hispanics and 13.8 % of black/African-Americans (Alzheimer Association, 2019). Initially it was thought that genetic factors do not account for large prevalent differences among racial/ethnic groups, however, more recent evidence suggests that the genetic architecture of an individual plays a role in developing both EOAD and LOAD (Eid *et al.*, 2019). It therefore cannot be ruled out that the genetic make-up of a population group has an influence on the risk of AD, or more specifically has a higher susceptibility to AD under certain lifestyle and environmental conditions (Grant, 1999; Chilton *et al.*, 2014; Mathias, 2015; Babulal *et al.*, 2019) in which diet may play a major role.

### 1.2 Modifiable factors and their contribution to the risk of dementia

Understanding the disease's multi-causal pathogenesis and interactions presents an incredible challenge particularly at the individual level (Eid *et al.*, 2019). Furthermore, the multi-causal factors that play a role in aetiology and progression of the disease make it more likely that dementia/AD may be prevented rather than cured (Scheltens *et al.*, 2016).

In 2017, the Lancet commission on Dementia prevention, intervention, and care published a report outlining nine modifiable risk factors that if prevented reduce the risk for dementia; here, it was estimated that the life-course contribution of modifiable risk factors to dementia is 35 % (Livingston *et al.*, 2017). The population-based Rotterdam Study reported a comparable reduction in dementia incidence of 30 % by eliminating six modifiable risk factors like being overweight, hypertension, diabetes mellitus, high

cholesterol, smoking, and lower education (de Bruijn *et al.*, 2015). Three years after their first publication the Lancet commission added three additional factors based on new convincing evidence, namely excessive alcohol consumption, head injury, and air pollution. Combined with the nine previously described factors (i.e. lower education, hypertension, hearing impairment, smoking, obesity, depression, physical inactivity, diabetes, and infrequent social contact) they now modelled 12 risk factors that potentially prevent or delay 40 % of dementias (Livingston *et al.*, 2020). By adding these three new factors, the attributable fraction of modifiable risk factors for dementia increased by 5 %. It is challenging to estimate the total amount of modifiable risk conferred solely by diet, but diet is nonetheless one of the fundamental risk factors for health and disease in the world. Diet has been associated with many non-communicable diseases and it influences many of the aforementioned modifiable risk factors, including obesity, diabetes and CVD (Micha *et al.*, 2014; Scarmeas *et al.*, 2018).

Diet is by far the dominant factor that determines the FA composition in the human body. This aspect should be given more consideration when non-modifiable risk factors, such as family history and ethnicity, are examined and discussed in terms of the risk of dementia. As described above, it is important to consider that family and ethnicity are a combination of genetic factors as well as shared environments among family members and populations.

### 1.3 Fatty acid profiles as biomarker for relative dietary fatty acid intake

The great number of literature on FAs in all fields of molecular biology indicates that the choice of dietary fats is a key modifiable factor determining numerous aspects of health and disease throughout the course of a person’s life (Morris, 2012; Brenna *et al.*, 2018). Collecting dietary intake data is associated with many challenges, primarily related to the subjective nature of data collection tools such as food frequency questionnaires (FFQ), multiple-day food records and 24-hour dietary recalls (Hedrick *et al.*, 2012). Common problems arise with the aforementioned self-administered questionnaires, in that individuals are not always able to recall all foods consumed or the specific components of the food, and have difficulty determining accurate portion sizes and typically underreport dietary intake. Furthermore, dietary recalls are typically costly (resource-intensive), time consuming, place a high burden on respondents and are therefore not always feasible in

large-scale investigations. In this regard, FFQ, food records and dietary recalls may provide a more or less precise estimate of a population's habitual dietary intake but are not representative of typical intake over time (Hedrick *et al.*, 2012).

In contrast, biomarkers of food or nutrient intake objectively assess dietary intake and status without the bias of self-reported dietary intake errors, and more accurately associate dietary intake with disease risk and nutritional status. Depending on the type of sample used (e.g. blood, hair, or adipose tissue) biomarkers can be categorized into short-term (reflecting intake over past hours/days), medium-term (reflecting intake over weeks/months) and long-term markers (reflecting intake over months/years) (Hedrick *et al.*, 2012). For example, a change to the proportions of FAs in the diet is reflected in the serum triglycerides within the first hours, while the FA composition of the serum cholesterol esters and phospholipids is related to the average dietary FA composition during the previous three to six weeks. The FA profile of the erythrocyte membrane phospholipids reflects the dietary fat composition during the preceding months, and the FAs in the adipose tissue triglycerides reflect it over many months or years (Vessby *et al.*, 2002; Calder, 2018). Although dietary biomarkers generally provide a more proximal measure of dietary intake, factors which may not appear in traditional dietary assessment methods could skew biomarker measures of dietary intake. Such factors could include genetic variability, lifestyle (e.g., smoking) and physiologic factors, dietary factors (e.g., nutrient-nutrient interaction), biological sample and analytical methodology (Hedrick *et al.*, 2012) which will in-part be discussed later in this work.

With regards to using blood FA analysis as a biomarker for dietary intake, studies have shown that that FAs measured in blood phospholipids correlate with relative intake of FAs in the diet. This is true for saturated fatty acids (SFA), the essential fatty acids (EFA) alpha-linolenic (ALA) and linoleic acid (LA), as well as for omega-6 (ω-6) and omega-3 (ω-3) polyunsaturated fatty acids (PUFA) (Hedrick *et al.*, 2012; Brenna *et al.*, 2018), Therefore, the FA profiling in blood serum phospholipids as it has been done in the current study is a reliable, easy method to obtain a minimally invasive biomarker for dietary intake of FAs. Additionally, FA profiles can easily be monitored over time to determine whether dietary recommendations were adhered to and are independent of self-reported dietary intake errors. Moreover, direct measurement of important ω-3 FAs in the serum overcomes the

relatively imprecise assessment of most questionnaires that employ general questions on fish consumption and thus do not capture well the different levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contained in different types of fish (Morris, 2012).

As health sciences trend toward individualized nutrition, developing biomarkers that measure intake of specific foods, rather than nutrients, may become a primary focus (Hedrick *et al.*, 2012). Therefore, it is important to understand how certain diets differ in their FA composition, as this knowledge provides a more comprehensive evaluation of how major changes in dietary patterns over a relatively short geological period affect human health. Moreover, this knowledge provides an understanding of how dietary patterns that result in different FA profiles unknowingly contribute to the primary prevention of chronic, vascular, and neurodegenerative diseases.

### 1.4 Evolution of the human diet and current dietary recommendations

Throughout evolution, the foods consumed by humans have changed dramatically and nowadays differ greatly across the various regions of the world. The current diet consumed in North America and parts of Europe is generally referred to as the Modern Western Diet (MWD). Before the development of agriculture and animal husbandry, hominin dietary choices were limited to minimally processed, wild plant and animal foods (Cordain *et al.*, 2005). Major changes to the type and amount of fat and micronutrients consumed have taken place over the past 10,000 years since the beginning of the Agricultural Revolution, and again during the past 150 years, while a human's genetic profile today is very similar to that of their ancestor's during the Palaeolithic period 40,000 years ago (Simopoulos, 2003, 2006). Fatty acids are categorised into three major groups: SFAs, monounsaturated FAs (MUFAs), and PUFAs. With increasing industrial food preparation, the presence of trans-fatty acids (TFAs) in the diet has substantially increased. Additionally, essential PUFAs separate into two biologically important families, the omega-6 (ω-6) PUFAs and the omega-3 (ω-3) PUFAs, namely linoleic acid (LA, ω-6) and alpha-linolenic acid (ALA, ω-3). With the MWD, individuals now consume more SFAs and TFAs and less MUFAs and PUFAs, with low vegetable intake and low intake of fish and seafood. Two specific ω-3 PUFAs, EPA and DHA, are particularly low in the MWD. This change in diet has culminated in a total daily energy uptake of 72.1 % based on foods

such as dairy products, cereals, refined sugars, refined vegetable oils, and alcohol in the USA that would have contributed to little or none of the energy in the typical preagricultural hominin diet (Cordain *et al.*, 2005).

Each of the four mentioned types of fats, TFAs, SFAs, MUFAs and PUFAs, has unique physicochemical properties and their influence on human health has been studied particularly in relation to chronic and widespread diseases such as CVD, diabetes, cancer, obesity, inflammatory and autoimmune diseases (Simopoulos, 2006). Here, evidence shows that in order to prevent health risks, the type of fat is more important than the absolute amount of dietary fat (Cordain *et al.*, 2005; Vannice and Rasmussen, 2014). Beneficial fats are MUFAs and some PUFAs, whereas most SFAs and TFAs are detrimental when consumed in high quantities and increase the risk of CVD by elevating blood concentrations of total and LDL cholesterol. The Agricultural Revolution 10,000 years ago induced decisive changes to the Western world's diet through the cultivation of cereals and the domestication of livestock. Cereal grains are high in carbohydrates and ω-6 FAs, mainly the essential ω-6 FA LA, but low in ω-3 FAs, particularly in comparison to leafy green vegetables (Simopoulos, 2006). The industrial processing of oil-seed at the beginning of the 20th century significantly raised the total intake of vegetable fat, which further directly increased the dietary level of ω-6 PUFAs at the expense of a lowered level of ω-3 PUFAs because of the inherently higher concentrations of ω-6 PUFAs and lower concentrations of ω-3 PUFAs in most vegetable oils (Blasbalg *et al.*, 2011). The trend toward a higher ratio of ω-6 to ω-3 PUFAs was exacerbated as meat from grain fed cattle and livestock became the norm over the past 150-200 years. With the advent of animal husbandry, it became more feasible to prevent or attenuate the seasonal decline in body fat that mainly consists of SFAs by feeding domesticated animals stored plant foods. This meat from modern grain-fed cattle and livestock has a high absolute SFA content, high ω-6 FA content and low ω-3 FA content (Cordain *et al.*, 2005). These significant changes to the MWD have led to the introduction of foods that had not been components of hominin diets before the advent of animal husbandry or the Industrial Revolution, with major sources of SFAs and TFAs included in the diet in the form of fatty meats, baked goods, cheese, milk, margarine, and butter, and a marked increase of plant based ω-6 FA in the form of LA (Cordain *et al.*, 2005; Blasbalg *et al.*, 2011; Stehle, 2014). As a result, the ratio of ω-6 to ω-3 PUFAs has risen to 10:1 and higher (Simopoulos, 2003), whereas the ratio

in hunter-gatherer diets predominant in wild animal foods (Simopoulos, 2003) has been estimated to be between 1:1 and 3:1 (Simopoulos, 2003; Cordain *et al.*, 2005; Sheppard and Cheatham, 2018).

It is recommended that individuals should increase their intake of MUFAs and PUFAs, while decreasing their intake of SFAs (Vannice and Rasmussen, 2014). Overall, dietary guidelines in Central Europe and North America recommend several fruit and vegetable servings a day, at least two servings of fish per week, processed meats and unprocessed red meats limited to one or 1.5 servings per week respectively, an increase of whole grains and a limit on refined grains intake, along with a limit on the intake of sugar-containing beverages (Lichtenstein *et al.*, 2006; Hercberg *et al.*, 2008; Heuer *et al.*, 2015; Mozaffarian, 2016; Voortman *et al.*, 2017). Furthermore, SFAs are recommended to be consumed at less than 7 % of the daily total energy (en%), and TFAs are recommended to be consumed as little as possible, or less than 1 en% (Micha *et al.*, 2014; Vannice and Rasmussen, 2014). Currently, SFAs make up the highest percentage of fat intake in Germany, with a median 16 en% in men and 15 en% in women (Stehle, 2014), which is comparable to the intake in North America, although country-specific intakes can vary dramatically from 2.3 to 27.5 en% (Micha *et al.*, 2014). Furthermore, huge differences for ω-6 and ω-3 PUFA intake are observed across different regions around the world (Micha *et al.*, 2014). Different dietary amounts of ω-6 and ω-3 PUFA based on diverse food habits result in specific FA balances in the body tissue of different populations. The proportion of ω-3 for example varies between 72 % for Greenland Eskimos down to 30 % and lower in parts of Europe and North America (Lands and Lamoreaux, 2012). Following the dietary guidelines of two servings of fatty fish per week would correspond to an intake of approximately 300-500 mg EPA and DHA per day (Lichtenstein *et al.*, 2006; Stehle, 2014; Heuer *et al.*, 2015; Calder, 2018). In combination with the recommended limited intake of processed meats and unprocessed red meats the proportion of ω-3 in the body tissue would result in a preferred value around 50 % which has been associated with beneficial health outcomes (Lands and Lamoreaux, 2012). It is important to note when making dietary recommendations that FAs compete for the same enzymes and incorporation into the plasma membranes, and play opposing roles in inflammatory processes in the human body (Schaeffer *et al.*, 2006; Zárate *et al.*, 2017; Calder, 2018). Particularly ω-6 and ω-3 FAs compete for the same FA desaturase enzyme as described in a following chapter.

For example, plasma levels of the ω-6 FA arachidonic acid (ARA) increase as dietary LA (ω-6) is increased from 0 to ~3.5 en%. Increasing dietary LA above 3.5 % no longer impacts levels of plasma ARA, but reduces plasma levels of ω-3 PUFAs such as EPA (Chilton *et al.*, 2014).

### 1.5 Diet, human health and the prevention of dementia

Long recognized differences in heart attack mortality rates for people in Greenland, Japan and Mediterranean countries, all of which were lower than those for Americans and Northern Europeans (Keys and Grande, 1957) inspired numerous scientific studies that identified reasons for this observation. Native populations in Greenland, Northern Canada and Alaska consuming their traditional diet were found to have much lower rates of death from CVD than predicted, despite their high dietary fat intake (Calder, 2018). The Greenland diet and the traditional diet in Japan are characterized by a high intake of ω-3 FAs and a low intake of ω-6 FAs, resulting in blood proportion of ω-6 highly unsaturated fatty acid (HUFA, with 20 or more carbon atoms and 3 or more double bonds) levels of 28 % to 45 % (Lands and Lamoreaux, 2012). In 1995, a 25-year follow-up study of the Seven Country Study reported an association of elevated serum cholesterol with risk of death from coronary heart disease (CHD) in the USA and Northern Europe, but to a lesser extent in Southern Europe, and no clear association for Serbia and Japan (Toshima *et al.*, 1995; Verschuren, 1995). In fact, it has been shown that proportions of ω-6 in tissue HUFA correlates strongly with CHD mortality (Lands, 2003).

Numerous studies assessed the effect of ω-3 FAs (DHA and EPA) on the risk of dementia/AD, with inconclusive results. Epidemiological studies showed a positive relation between fish intake and reduced risk of dementia/AD incidence, global cognition and an inverse relation to cognitive decline, while others show no effect (Cederholm *et al.*, 2013; Thomas *et al.*, 2020). Interventional studies also show inconclusive results. A Cochrane review of randomised controlled trials (RCT) studying the role of ω-3 FAs in preventing cognitive decline in healthy older people showed no benefits (Calder, 2018). A more recent meta-analysis of six RCT identified a slower rate of cognitive decline in those receiving ω-3 FAs (Calder, 2018). In contrast, a meta-analysis of three RCT involving participants with mild to moderate AD found no evidence of a benefit from ω-3 FAs on any outcome that was assessed (Calder, 2018; Power *et al.*, 2019).

Although the prevalence of dementia worldwide is increasing, the current distribution of dementia around the world seems to vary according to cultural and socioeconomic differences among nations, with a higher prevalence of dementia and AD in developed countries compared to developing countries (Rizzi *et al.*, 2014). Currently, the dementia prevalence is highest in North America, Western Europe, and Latin America. The prevalence is lower in North Africa, Middle Eastern, and countries such as Indonesia, Thailand, and Sri Lanka (Prince *et al.*, 2013; Rizzi *et al.*, 2014). The lack of methodological uniformity among studies, including diagnostic criteria and different mean population ages, partially accounts for different dementia rates. Nonetheless after considering these potential sources of bias, differences in age-adjusted prevalence of dementia still exist in different regions of the world. In China, India, and Latin America, dementia is rapidly becoming the major public health problem due to aging demographic transition. The prevalence of dementia in Latin America is already higher than expected for its level of population aging, which might be explained by the combination of low average education and high vascular risk profile (Rizzi *et al.*, 2014). In general, low education and other socioeconomic factors have been associated with high risk of obesity, diabetes, hypertension, dyslipidemia, and metabolic syndrome, and these entire factors raise the risk for dementia (Morris *et al.*, 2014). Furthermore, rapid dietary changes are leading to obesity and cardiometabolic disease in China. Specifically, the increased percentage of calories from fat (from 22 % to 32 %) and a higher percentage of energy intake from protein (adjusted for the percentage of calories from fat) from 1991 to 2011 is positively associated with an increased likelihood of having high LDL, high TAG and high CRP, and an increased risk of being overweight (Adair *et al.*, 2014). Furthermore, a causal link between recent increases in obesity among middle-aged Chinese and dementia prevalence has been discussed (Loef and Walach, 2013a). This is well in line with early observation that the prevalence of AD is associated with fat consumption per day, as well as total calories per day (Grant, 1997).

Another revealing observation is that vascular dementia (VaD) used to be more common than AD in China and Japan, but the ratio of VaD/AD prevalence decreased from 2:1 to 1:1 in Japan. Reduced salt intake and treatment of hypertension seem to have reduced the incidence of both stroke and VaD, while increased life expectancy and westernization of lifestyle, including diet, may have contributed toward the increased prevalence of AD

(Rizzi *et al.*, 2014). Among developed countries, Japan has the lowest prevalence of both dementia in general and AD in particular. As mentioned before, the traditional food habits from Japan achieve high tissue proportions of ω-3 HUFAs. The westernization of dietary habits has led to a decrease in the proportions of ω-3 in the tissue to 55 % (Lands and Lamoreaux, 2012), which is still high compared to Europe and North America, but up to 20 % lower compared to traditional Japanese food habits.

Strong evidence for the hypothesis that a change in lifestyle and environment affects the risk of AD can be seen from cross-cultural studies that compare the prevalence of dementia among immigrants with that observed in their countries of origin. Studies show that the heart disease rates (Sekikawa *et al.*, 2008; Lands, 2014) and AD rates among Japanese men living in the United States are significantly higher than those of Japanese men living in Japan (White *et al.*, 1996). Another study showed that the dietary pattern of Japanese immigrants from Okinawa living in Brazil, characterized by low fish consumption and large meat intake, increased the prevalence of dementia when compared to those who remained in Okinawa (Yamada *et al.*, 2002). Another interesting dementia project compared the incidence rates of AD in two elderly Yoruba community-dwelling populations, one in Ibadan, Nigeria, and the other in Indianapolis, USA. The Yoruba people of the two communities are culturally diverse but genetically related, and results showed that age-standardized annual incidence rates were significantly lower among native Yoruba than among African Americans for both overall dementia (2.3 % and 8.3 %, respectively) and AD (1.4 % and 6.3 %, respectively) (Ogunniyi *et al.*, 2000; Hendrie *et al.*, 2001). These results emphasize the role of the environment and diet as a major factor on the risk of dementia. Interestingly, in the Nigeria-USA study, the prevalence of dementia was higher in the USA despite the better education system. These results do not necessarily argue against the education-related “cognitive reserve” hypothesis in dementia but may imply that other environmental factors, such as diet and physical activity, might be even more important risk factors for dementia than low educational background (White, 1996; Rizzi *et al.*, 2014). All this evidence suggests that efforts to prevent, detect and control obesity, hypertension, diabetes, dyslipidaemia, and to promote a balanced diet are likely to have maximum positive impact upon brain health and dementia risk in later life (Prince *et al.*, 2016).

### 1.6 Dietary patterns and the balance between ω-6 and ω-3 fatty acids

Beside studies that focus solely on ω-3 FAs, a growing body of evidence suggests that certain diets are associated with a lower incidence of dementia and that combinations of foods and nutrients into certain patterns may act synergistically to provide stronger health effects than those conferred by their individual dietary components (Solfrizzi *et al.*, 2017). Prominent dietary patterns that were studied throughout recent years on the prevention of cognitive decline are the Mediterranean diet (MeDi), the Dietary Approaches to Stop Hypertension (DASH) and the Mediterranean-DASH diet Intervention for Neurodegenerative Delay (MIND) diets (Scarmeas *et al.*, 2018). While the MeDi is a typical dietary pattern of Mediterranean countries, characterized by high consumption of fruits, vegetables, legumes and cereals, olive oil as the main added lipid, moderate consumption of alcohol (mainly wine and during meals), low consumption of red meat and dairy products, and low intake of SFAs, the DASH diet is characterized by low consumption of SFAs, TFAs, and commercial pastries and sweets, and higher intake of dairy than in the MeDi. The MIND diet is based on the dietary components of the MeDi and DASH diets with modifications that highlight the foods and nutrients shown to be associated with dementia prevention: leafy green vegetables, other vegetables, nuts, berries, beans, whole grains, seafood, poultry, olive oil, and wine, but it also includes five unhealthy food groups (red meats, butter and stick margarine, cheese, pastries and sweets, and fried/fast food) (Solfrizzi *et al.*, 2017; Scarmeas *et al.*, 2018). These three dietary patterns all contain low amounts of unhealthy FAs, such as SFA and TFA, as well as a more even balance between ω-6 and ω-3 FAs. Solfrizzi *et al*. and Scarmeas *et al*. reviewed the evidence from observational and interventional studies showing that higher adherence to the Mediterranean diet is associated with a slower decline in performance on various cognitive test batteries and a reduced risk of dementia, mild cognitive impairment, or progression from mild cognitive impairment to dementia/AD. Furthermore, this dietary pattern approach has proven to be the most fruitful in terms of association with cognitive outcomes. Moreover, healthy dietary patterns, such as the DASH and the MIND diets, were also associated with slower rates of cognitive decline and significant reduction in AD rate. Even though dietary recommendations focus on dietary fat and single vascular risk factors (e.g., hypertension, blood cholesterol, etc.), total calories and obesity, the full health impact of each diet extends far beyond these pathways (Solfrizzi *et al.*, 2017).

Important nutrients that are part of the described healthy diets are folate, vitamin E, vitamin D, carotenoids, flavonoids, polyphenols and other antioxidants, dietary fibre, coffee and caffeine, each of which has been associated separately with cognitive outcomes (Solfrizzi *et al.*, 2017; Scarmeas *et al.*, 2018).

Interventional studies on ω-3 FAs and dietary patterns are challenged by certain limitations. Negative results to risk reduction for AD and cognitive decline/improvements warrant longer durations of studies or higher dosages of EPA and DHA (Andrieu *et al.*, 2015; Cederholm, 2017). Challenges of dietary patterns are the mentioned diversity of nutrients taken up with certain dietary patterns and the multiple causal biological pathways that are affected by each nutritional element. Furthermore, there are non-dietary factors such as physical activity and aspects of cognitive reserve (e.g., education, profession, socioeconomic status, intellect, and social activities) that influence the outcome of these studies (Scarmeas *et al.*, 2018). To account for underlying confounders and to make use of additive or synergistic effects, multidomain interventional studies have been conducted. Some of these multidomain studies showed promising results, of which the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) is a prominent example (Ngandu *et al.*, 2015). In the FINGER trial, 1,260 individuals with an increased dementia risk aged 60-77 years were randomly assigned to receive multidomain lifestyle counselling (nutritional guidance, group and individual physical activity, cognitive training, and intensive monitoring of vascular and metabolic risk factors). Individuals who were assigned to the multidomain intervention showed slower cognitive decline, particularly in executive functioning and processing speed. These results suggest that a multidomain intervention could improve or maintain cognitive functioning in at-risk elderly people from the general population (Ngandu *et al.*, 2015). Given the multidomain nature of these interventions, it is not clear whether the nutritional aspect or other components are responsible for the noted effects (Scarmeas *et al.*, 2018). Accordingly, another analysis from the FINGER study investigated the role of diet and particularly dietary changes. Adherence to a healthy diet at baseline predicted improvement in global cognition, regardless of intervention group, whereas dietary improvement was associated with beneficial changes in executive function, especially in the intervention group. This clear protective effect of a healthy baseline diet for the subsequent changes in cognition underlines the importance of a healthy diet throughout life (Lehtisalo *et al.*, 2019). Future

studies therefore must better identify the dietary factors to focus on in elderly and specific subgroups (e.g., those with more vascular risk factors, higher future dementia risk scores, or brain amyloid positivity, or carriers of *APOE* ε4), and those that would require intervention already at midlife (Scarmeas *et al.*, 2018; Lehtisalo *et al.*, 2019). These findings underpin again that long interventional periods are vital to successfully prevent late-life dementia given that pathological processes begin several decades prior to the stage of clinically detectable symptoms (Jack *et al.*, 2010; Sperling *et al.*, 2014). In this context, it may well be that traditional dietary habits from Japan and the Mediterranean achieve healthy balanced tissue proportions of ω-6 and ω-3 and thereby unknowingly maintain a form of long-term food-based primary prevention of CVD and dementia (Lands, 2014), implying that dietary and lifestyle modifications are viable options for the prevention and treatment of lifestyle-related diseases.

Finally, a recent study has shown that increased maternal consumption of ω-6 PUFAs, in association with a high LA/ALA ratio, might favour the continuous development of adipose tissue during the pregnancy and lactation period and during infancy leading to childhood overweight and obesity (Ailhaud *et al.*, 2007). No significant changes to fat intake in the general diet were reported, but rather a shift in the overall FA composition from saturated fats towards fats enriched with ω-6 PUFAs was observed, predominantly LA and ARA, that led to high LA/ALA and ARA/DHA ratios in breast milk of US and European women (Ailhaud *et al.*, 2007). These findings are in line with other studies that showed a link between a Western-like diet (high ω-6/ω-3 ratio) and a gradual enhancement in fat mass over generations (Simopoulos, 2016). Overall, it seems important that pregnant and breastfeeding women have adequate intakes of long-chain ω-3 FAs while avoiding excessive ω-6 FA intake in the form of ω-6 PUFAs, underlining the importance of a balanced ω-6/ω-3 ratio from the very beginning of life.

### 1.7 Genetic modulation of fatty acid levels

The field of nutrigenetics investigates how the genetic make-up of a population or individual influences the body's response to diets or specific food components in relation to various diseases and is anticipated to lay the future foundation for personalized diet recommendations (Simopoulos, 2010; Roke *et al.*, 2017; Barrea *et al.*, 2020). The *FADS* gene cluster is a highly interesting object to study in the context of gene-environment

interaction and the way modern humans eat in the Western world, as variants within these genes show a marked contribution to the variability of blood ω-6 and ω-3 PUFA levels (Schaeffer *et al.*, 2006; Tanaka *et al.*, 2009; Lemaitre *et al.*, 2011; Guan *et al.*, 2014; Hu *et al.*, 2016). In the report by Schaefer *et al*., variants in the *FADS1/2* gene explained up to ~28 % of variations in ARA levels whereas the overall association with ω-3 FA levels was less pronounced. Here ~7 % of the variability of EPA levels in blood FAs was explained by these variants (Schaeffer *et al.*, 2006). Importantly, the relationship between variants in the *FADS1/2* gene cluster and blood FA levels has been confirmed in various populations (Martinelli *et al.*, 2008; Bokor *et al.*, 2010; Merino *et al.*, 2011).

The *FADS1* and *FADS2* genes lie head-to-head (5' to 5') on chromosome 11 and code for the delta-5 desaturase (D5D) and delta-6 desaturase (D6D) enzymes, respectively. These desaturases in connection with elongases catalyse the conversion of PUFAs in humans (Figure 1, Schaeffer *et al.*, 2006). The D5D and D6D are known to be the key enzymes of the ω-FA pathways and are expressed in a majority of human tissues, with the highest levels in the liver but also with major amounts in the brain, heart and lungs (Schaeffer *et al.*, 2006). In the ω-6 pathway, the conversion of the EFA LA to ARA involves both desaturase steps and an elongase step. The same desaturase and elongase enzymes are responsible for the conversion of the EFA ALA to EPA, and further to DHA (Figure 1). The preferred substrate for the D6D is ω-3 > ω-6 > ω-9, but due to competition between the substrates and product inhibition, the efficiency of the desaturation of ω-3 is dependent on competing ω-6 FAs in the diet and in tissues (Vessby *et al.*, 2002). For example, it has been shown that a high LA/ALA ratio intake as occurs in the MWD, decreases EPA blood concentration (Greupner *et al.*, 2018b).

Those studies that analysed the *FADS* pathway activity consistently associated minor allele carriers for single nucleotide polymorphisms (SNPs) in *FADS1/2* with lower estimated pathway activity (Bokor *et al.*, 2010; Merino *et al.*, 2011; Al-Hilal *et al.*, 2013; Gillingham *et al.*, 2013; Cormier *et al.*, 2014). Considering the evolution of the *FADS* gene cluster, these results become increasingly interesting. Two studies independently published data on the evolution of the human *FADS1/2* gene region that showed a positive selection of a haplotype associated with an enhanced ability to produce ARA and DHA from their precursor FAs in the lineage leading to modern humans (Ameur *et al.*, 2012;

Mathias *et al.*, 2012). The advantageous mutations within the *FADS* gene region swept to fixation within African but not in European or Asian populations, suggesting this enhanced metabolism is the driving force behind positive selection at the *FADS* gene cluster (Mathias *et al.*, 2012). Furthermore, it has been shown that SNPs associated with increased expression of *FADS1* and increased production of ARA and EPA have been favoured in Europeans since the Bronze Age (Buckley *et al.*, 2017).

The findings on MWD and FA metabolism from the last two decades provide an immediate insight into how a formerly positive selection of the *FADS* haplotype leads to an increased risk of disease through diet and lifestyle in the Western world. Scientific evidence shows that the *FADS* haplotype in combination with a particular diet (environment) shifts the balance between ω-6 and ω-3, which increases health risks (Chilton *et al.*, 2014; Hester *et al.*, 2014). A diet high in ALA and low in LA, in combination with the high efficiency haplotype shifts the balance towards EPA (ω-3) in relation to ARA. A high LA/ALA diet, on the other hand, in combination with the high efficiency haplotype shifts the balance towards ARA, which is unfavourable, and probably shifts conditions from normal physiology to pathophysiology when ARA is the dominant HUFA available (Lands, 2015).

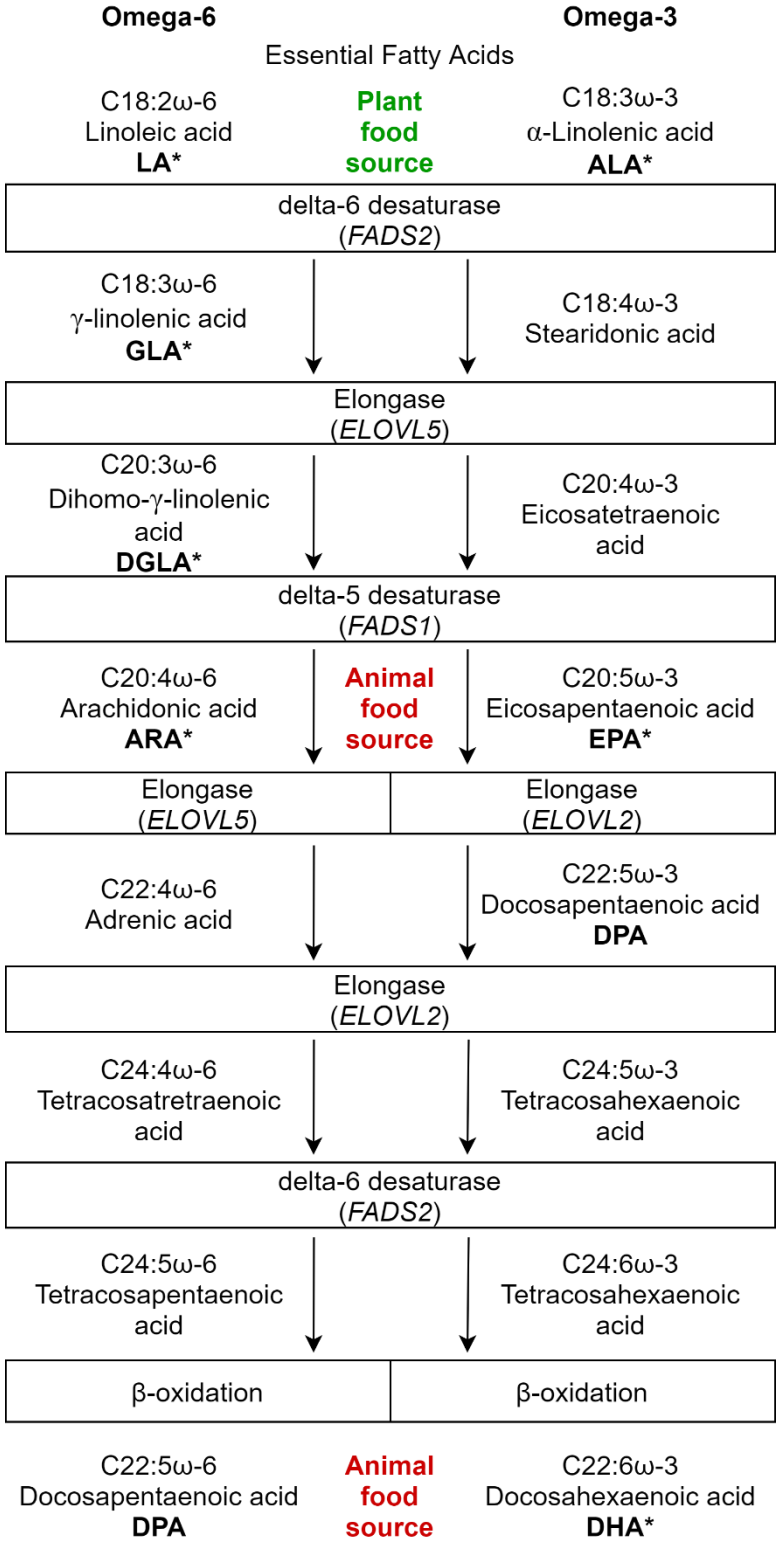


**Figure 1:** Biosynthetic pathway for the convertion of ω-6 and ω-3 EFAs to long chain PUFAs. Desaturases and elongases are both involved in the FA metabolism. Genes from the *FADS* gene cluster and the *ELOVL* gene family encode these enzymes. *FAs that were available for the analysis in the AgeCoDe cohort.

Many diseases such as hypertension, diabetes, stroke, CVD, and AD that have been associated with lipids and therefore lipid metabolism may play a role in the aetiology (Simopoulos, 2006; Saini and Keum, 2018). Consequently, diet-gene interactions may help decipher human health disparities, both among different populations and on an individual level. Understanding the effect of genetic variation and gene-nutrient interaction on physiological processes helps to determine nutritional requirements for human health. Adding genetic information might be a useful tool to discriminate between responders and non-responders in nutritional studies, which would transform dietary recommendations and help guide the development of individualized diets informed by genomics. Furthermore, knowledge of personal genetic variants and individualized risk profiles based on FA levels could potentially promote behavioural changes in individuals, resulting in health improvements and the prevention of chronic diseases (Simopoulos, 2010).

### 1.8 Aims of the present study

The intensive study of FAs and their metabolism has shown how FAs influence each other as they compete for enzymes and incorporation into various tissues. Through this, the balance of FAs in the diet determines physiological properties of tissues, which in turn may play a role in health and healthy aging.

The main aim of the study is to investigate whether the balance between competing ω-6 and ω-3 FAs measured in blood serum phospholipids contributes to the risk of dementia. These FAs, more specifically EFAs and particular ω-3 and ω-6 PUFAs, are representative of dietary FA intake derived from plants and different animal products. Within this work, these dietary FAs are combined into a PUFA index that could potentially be used as a health risk assessment for the risk of developing dementia. The availability of biomarkers that provide estimates of the intake of specific foods and dietary components could greatly enhance nutritional research targeting the compliance to national recommendations.

*APOE* ε4 is still the strongest genetic risk factor found for late-life dementia. Since it is a lipid transporter, it is investigated whether the *APOE* ε4 status shows a biological interaction with FAs and the *FADS* genotype that contributes to the risk of dementia.

Motivated by the strong influence of SNPs in the *FADS* gene cluster on FA levels and altered *FADS* pathway activity in the body, it is further investigated as to whether this

influence of the *FADS* genotype on FA levels is mediated by methylation. Including the *FADS* genotype, other FAs beside ω-3, and lifestyle factors may reveal stronger findings and should emphasize health-related risks due to ω-6 FAs, which are overabundant in the MWD.

# 2 Material and Methods

## 2.1 Data sets and study design

The study samples consist of participants from the German Study on Ageing, Cognition, and Dementia (AgeCoDe). For the current study two data sets from the AgeCoDe cohort were used. The main FA analysis was carried out on the entire data set using available FA values, and a further analysis was performed on a smaller subsample including additional DNA methylation data (Figure 2). The AgeCoDe study is a multicenter prospective general practice-based cohort study ongoing since 2001 including community dwelling elderly. Participants included in this study were age 75 years or older, had at least one contact with their general practitioner (GP) in the past 12 months, and were dementia free according to their GP. These participants were recruited at six study sites in Germany (Bonn, Düsseldorf, Hamburg, Leipzig, Mannheim, and Munich) (Luck *et al.*, 2007; Jessen *et al.*, 2011). Written informed consent was collected from all subjects prior to participation in the study. As a follow-up, participants underwent at-home interviews every 18 months by trained physicians and psychologists. For the following study, follow-up 3 (FU-3) was set as a baseline, since DNA, blood serum, and blood plasma were all available from the participants with roughly 1,600 individual's serum available at FU-3. In 1,472 individuals the composition of 25 FAs was successfully measured from serum phospholipids. 35 individuals were excluded because they did not meet the initial AgeCoDe study inclusion criteria of being 75 years or older and free of dementia. Further, only those subjects were selected for this study that had no clinical diagnosis of dementia at FU-3, which led to the exclusion of an additional 105 individuals. At the time of analysis, neuropsychological assessment data was available from a total of eight follow ups. Thus, all subjects who received clinical diagnosis of AD type or AD/mixed during the subsequent five follow ups ($n = 196$) were included as cases. Of the remaining undiagnosed individuals participating in the study, 70 individuals were excluded due to other dementias

and ten had no follow up information on diagnosis, leaving 1,056 individuals remaining as controls for the analysis (Figure 2).

### 2.2 The methylation subset

This subset was initially filtered from the AgeCoDe study participants with neuropsychological assessment data available until FU-5. Therefore, all subjects with a clinical diagnosis of dementia at FU-4 and FU-5 (*n* = 102) after the set study baseline at FU-3 were included as cases. For each of these cases two subjects (*n* = 204) who remain cognitively healthy over the time of two FUs were matched for age, sex, and education. The matching was conducted using the FUZZY plug-in for SPSS. In brief the FUZZY extension command uses the case group as a *demander* dataset and potential controls as a *supplier* dataset. The user then has the possibility to define *BY* variables which are used for the matching (IBM, 2021). For the categorical variables sex and education, an exact matching was specified with a tolerance of ± 3 years specified for the age variable for the first matching round in order to find a matching pair for each case. For the second match, the tolerance on age needed to be set to ± 5 years in order to achieve complete matching for each case. Over the course of the study new FU data became available in which eleven controls converted to AD and four subjects were excluded due to vascular dementia or other dementias. Finally, three subjects were excluded because their sex from the clinical files was incongruent with the genetically determined sex. Overall, this led to a study sample of 113 cases and 186 controls that were followed up over the time period of 7.5 years. Despite all changes from the initial dataset every case had at least one matched control.

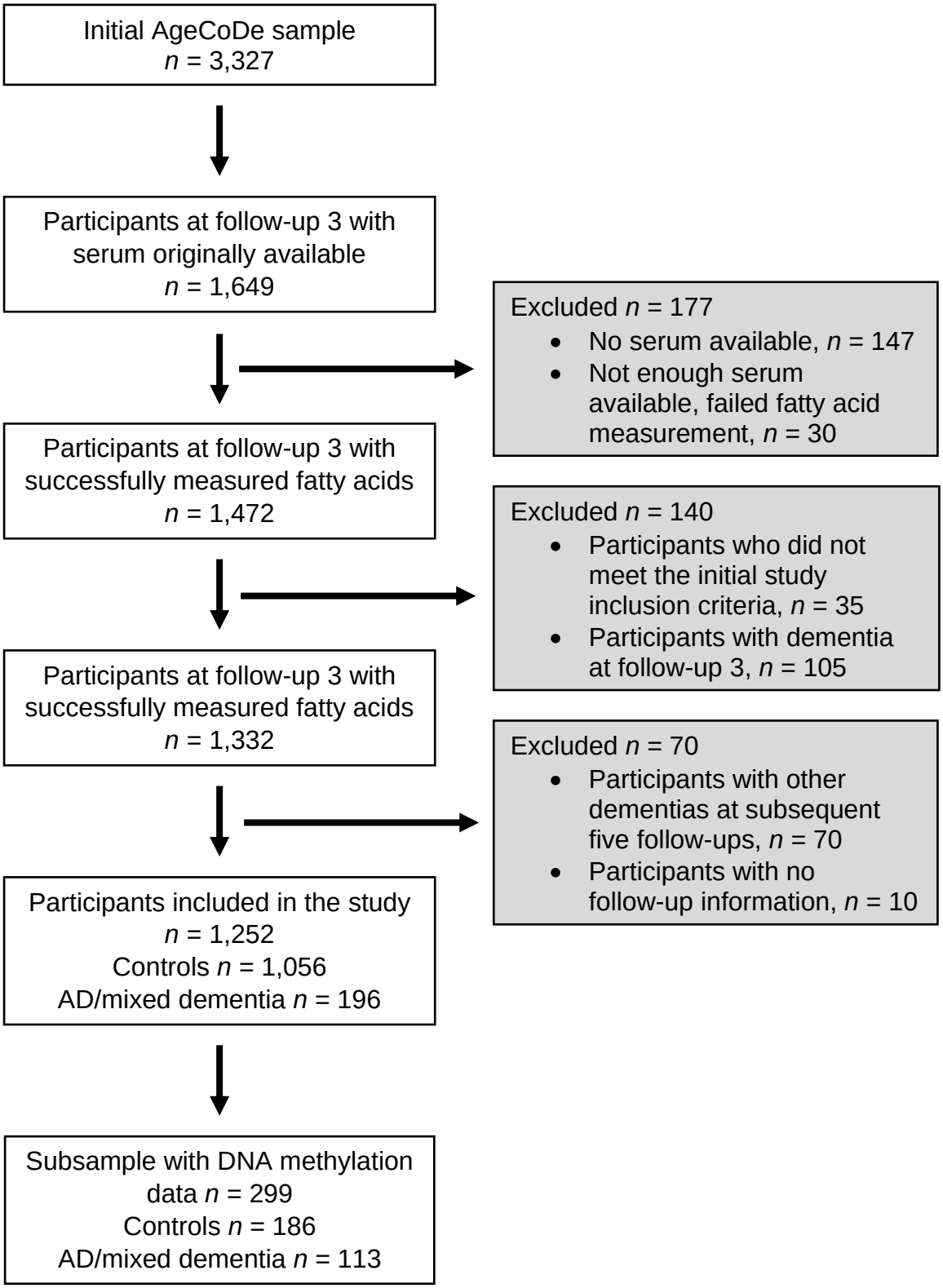


**Figure 2:** Flow chart of the participants included in the study.

## 2.3 Cognitive assessment in the AgeCoDe cohort

Cognitive function and dementia were assessed by consensus of the interviewing investigator and an experienced geriatrician or geriatric psychiatrist according to the Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV) and International Classification of Diseases (ICD-10) criteria that are implemented as a standardized diagnostic algorithm in the Structured Interview for Diagnosis of Dementia of Alzheimer type, Multi-infarct Dementia and Dementia of other Aetiology according to DSM-IV and ICD-10 (SIDAM) (Zaudig *et al.*, 1991; Zaudig and Hiller, 1996). The SIDAM is specifically designed to diagnose dementia, comprising of cognitive impairment, defined by the total SIDAM cognitive score (SIDAM cognitive (SISCO) score), a 55-item neuropsychological test battery including the Mini-Mental State Examination (MMSE) (Folstein *et al.*, 1975) score (0-30) and 25 additional items, and impairment of activities of daily living (ADL) as assessed by a 14-item scale (SIDAM-ADL-scale). The diagnosis of dementia in AD was established according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD dementia (McKhann *et al.*, 1984). For diagnoses of vascular dementia (i.e., evidence for cerebrovascular events (Hachinski-Rosen scale, medical history) and a temporal relationship between the cerebrovascular event and the occurrence of cognitive decline), the National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherché et l'Enseignement en Neurosciences (NINDS-AIREN) criteria were used (Roman *et al.*, 1993). Mixed dementia was diagnosed in cases of cerebrovascular events without temporal association to cognitive decline. For statistical analyses, AD dementia and mixed dementia were combined into one AD dementia group. Dementia diagnosis in participants who were not personally interviewed was based on the Global Deterioration Scale (GDS) (Reisberg *et al.*, 1982) and the Blessed Dementia Rating (BDR) scale (Blessed *et al.*, 1968). A score of at least four on the GDS was used as the diagnostic criteria for dementia. In these cases, an etiological diagnosis was established if the information provided was sufficient to judge aetiology according to the aforementioned criteria.

## 2.4 *APOE* and genome-wide genotyping

Leucocyte DNA was isolated with the Qiagen blood isolation kit according to the manufacturer's instructions (Qiagen, Germany). Standard procedures were performed for *APOE* ε4 genotyping (Hixson and Vernier, 1990).

Genomic DNA, serum, and plasma were isolated from whole blood samples. DNA concentration and purity were determined using the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific).

The AgeCoDe cohort DNA samples were genotyped with the Illumina Infinium Global Screening Array (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Samples were removed with genotype call rates < 97 %, excess heterozygosity, duplicates, samples genetically related to other individuals in the cohort, or sample mix-up (PIH AT > 0.1875). If a discrepancy was detected between the clinical file and the genetically determined sex, the sample was removed unless the discrepancy was safely resolved. To detect population outliers of non-European ancestry (>6 SD from European population mean), principal component analysis was conducted using SMARTPCA from EIGENSOFT 6.1.4. Variants with a call rate < 95 % or that grossly deviated from Hardy-Weinberg equilibrium in controls ($p$-value ≥ $1 \times 10^{-6}$) were excluded; we also excluded markers with a different missing rate between case and control ($p$-value, $5 \times 10^{-4}$ for the difference) or a minor allele frequency (MAF) < 0.01. Imputation was carried out using the Haplotype Reference Consortium panel on the Michigan Imputation Server. Only common markers (MAF > 0.01) with a high imputation quality ($R^2$ > 0.30) were selected for downstream analyses.

The methylation subset of the AgeCoDe cohort was genotyped prior to the entire sample, and a total of 200 ng of DNA was analysed using the Illumina Infinium OmniExpressExome (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Illumina iScan was used for imaging of the array and GenomeStudio version 2011.1 (Illumina, San Diego, CA, USA) was used to extract the signal intensities of the images. Raw data was exported into the PLINK format. Variant and sample quality control was performed before imputation. Samples were excluded if their ancestry was non-European, if samples were related, if they had an outlying heterozygosity rate, or if they had a sample call rate < 98 %. SNPs with a Hardy-Weinberg equilibrium $p$-value > $10^{-6}$,

a MAF > 0.01, and a call rate > 95 % were included. Genotype imputation was performed using IMPUTE2 software (Howie *et al.*, 2009) and 1000 Genomes phase 3 release (Oct14) as reference.

### 2.5 Genome-wide methylomic profiling

Bisulfite conversion of 500 ng genomic DNA was applied using Qiagen EpiTect 96 Bisulfite Kit (Cat No./ID: 59110; Qiagen, Hilden, Germany) according to the manufacturer's protocol. A total of 200 ng of bisulfite converted DNA was analysed using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Illumina iScan was used for imaging of the array and signal intensities of the images were extracted using GenomeStudio version 2011.1 (Illumina, San Diego, CA, USA). The methylumi package (Davis *et al.*, 2014) was used to import signal intensities into R as a methylumi object. Quality filtering of the 485,513 CpG probes was carried out based on the following exclusion criteria: (i) site selection to exclude sites with multimaps, indels, SNP-at-CpG and repeats, which led to the exclusion of 72,001 sites and (ii) the pfilter quality control function in wateRmelon which discards probes that have a detection $p > 0.01$ in at least 5 % of samples and less than three beat counts per probe in at least 5 % of the samples (607 sites excluded). The dasen function in the R package wateRmelon was used for normalization (Pidsley *et al.*, 2013). After quality filtering, 412,905 CpGs were included in the analysis. Beta values were next logit transformed into M values (Du *et al.*, 2010), adjusted for technical covariates (plate and Sentrix ID) using ComBat (Leek *et al.*, 2012) and finally adjusted for the first six principal component analysis axes.

### 2.6 Fatty acid profiling

Blood was collected by participants' GPs in tubes with EDTA and without anticoagulant, and stored at -80 °C. FA composition of serum phospholipids was determined in duplicate by gas chromatography (Shimadzu GmbH, model GC 2010 plus, Duisburg, Germany, flame ionization detector [FID]) as described elsewhere (Burak *et al.*, 2017; Egert *et al.*, 2018). Briefly, after Folch extraction was performed on serum samples (Folch *et al.*, 1957), the phospholipid fraction was separated using a silica thin-layer chromatography plate in a solvent mixture of petroleum ether and acetic acid (Christophe and Matthijs, 1967). After scraping off the phospholipid band under ultraviolet light, the phospholipid fraction was

methylated by transesterification with methanol/HCl and incubated at 95 °C for 4 h. The FA methyl esters were extracted with petroleum ether, dissolved in heptane and injected into the gas chromatograph. Peaks of interest were identified by comparing with authentic FA methyl ester standards (37 Component FAME Mix certified reference material, C14-C24, Sigma-Aldrich, St. Louis, MI, USA). Selected FAs were expressed as a percentage of the total area by dividing the integrated area under the peak by the total area of all FAs and as absolute concentration. FAs were also determined quantitatively from the internal standard and expressed as µmol/L serum (Melo van Lent *et al.*, 2021).

## 2.7 Statistical analyses

### 2.7.1 Fatty acid analysis

Calculations were performed with SPSS software (version 25.0; SPSS Inc, Chicago, IL). The main analysis was carried out with concentration values, whereas the percentage values were used for comparative purposes. The sum of either all available SFAs, MUFAs, and PUFAs were calculated for the control and the case group. From the available measured FAs, the individual ω-6 FAs LA, DGLA, and ARA were analysed. From the available ω-3 FAs ALA, EPA, and DHA were analysed, as well as the ratios LA/ALA, ARA/LA, ARA/EPA, and EPA/ALA. Moreover, palmitic acid (PA, 16:0) and stearic acid (SA, 18:0) were analysed as individual FAs. EPA+DHA were used as an ω-3 index variable, and PA+SA as an SFA index variable. Furthermore, the ratio SFA/ω-3 was calculated.

The proportion of ω-6 FA in PUFAs (%ω-6) was calculated with concentration values of FAs with four different ω-6 PUFAs (LA, 18:2ω-6 = A; GLA, 18:3ω-6 = B; DGLA, 20:3ω-6 = C; ARA, 20:4ω-6 = D), and three ω-3 PUFAs (ALA, 18:3ω-3 = E; EPA, 20:5ω-3 = F; DHA, 22:6ω-3 = G) that are part of the ω-6 and ω-3 synthesis pathway.

$$\%\omega\text{-}6 \text{ in PUFA} = 100 \times (A + B + C + D) / (A + B + C + D + E + F + G) \quad (1)$$

The %ω-6 was also calculated in HUFA, meaning only 20-carbon FAs.

$$\%\omega\text{-}6 \text{ in HUFA} = 100 \times (C + D) / (C + D + F + G) \quad (2)$$

The proportion of an individual FA was calculated accordingly to formula (3).

$$\%\text{single FA in PUFA} = 100 \times \text{single FA}_{A\text{-}G} / (A + B + C + D + E + F + G) \quad (3)$$

For comparison purposes, the %ω-6 in PUFA was also calculated with percentages of total FAs according to formulas (1) and (3).

Product-to-substrate ratios were used as surrogate estimates for desaturase activity. D5D activity was estimated from the ARA, 20:4ω-6/DGLA, 20:3ω-6 ratio and D6D activity was estimated from the DGLA, 20:3ω-6/LA, 18:2ω-6 ratio (Warensjö *et al.*, 2009).

Before performing statistical analysis of the FA levels and the desaturase activity, the Kolmogorov-Smirnov as well as the Shapiro-Wilk tests were used to identify the presence of normal distributions in the data, with further visual examination of the distributions being conducted. Potential outliers were identified using the outlier labelling rule, using the difference between the lower and upper quartile multiplied by a factor to determine the lower and upper demarcation points for determining an outlier as described by Hoaglin *et al*. (Hoaglin *et al.*, 1986) and revised in Hoaglin *et al*. (Hoaglin and Iglewicz, 1987). Group differences between controls and cases in FA levels were analysed with continuous variables using a student's *t*-test. Continuous variables were also used in linear regression analysis and to determine the correlation between FAs. Further analyses of individual FAs and ratios between FAs were performed with tertiles. Tertiles were used for two reasons: first, tertiles are robust against outliers; and second, it is possible to identify a U-shaped relationship, meaning that one tertile could have an association in the opposite direction to the others, which is harder to detect with continuous variables. The Pearson chi-square ($\chi^2$) test was used to compare between the tertile groups.

### 2.7.2 Survival analysis

Multivariable Cox proportional hazards regressions were used to assess individual associations of FA tertiles (at FU-3) with dementia (at FU-4-8) as the dichotomous dependent outcome variable. The following demographic and genetic variables were used as covariates: age, sex, education, *APOE* ε4 status, and a *FADS1* SNP (rs174546). The lifestyle covariates body mass index (BMI), smoking, physical activity, total cholesterol, triglycerides, and lipid lowering medication were added. In addition, cognitive decline was used as a covariate. All covariates in the regression models are based on their potential relationship with risk for AD or their influence on FA levels. For each variable included in the regression models, hazard ratios (HRs) and Wald 95 % confidence intervals (CIs) were calculated.

Cognitive decline was calculated using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological assessment battery (the validated German version (Thalmann *et al.*, 2000) 10-item Word List Delayed Recall subtest (scoring 0-10; higher scores indicating a better memory performance) was applied). The cognitive test data of FU-3 was subtracted from the baseline test score to estimate the cognitive decline over time. Education was classified into three levels (low, middle and high) according to the revised version of the Comparative Analysis of Social Mobility in Industrial Nations (CASMIN) classification system (Brauns and Steinmann, 1999). Lipid lowering medication was categorized into users and non-users. Smoking was divided into three categories: never smoker, former smoker, and current smoker. Assessment of physical activity was conducted at FU-3. A modified physical activity score based on Verghese *et al*. was constructed (Verghese *et al.*, 2003). Participants reported the frequency of usual engagement in each of the six physical activities: bicycling, walking, swimming, gymnastics, chores/gardening, and a category of other physical leisure activities (e.g., bowling, jogging, or golfing) using five possible options to answer: (1) “each day”; (2) “several times per week”; (3) “once a week”; (4) “less than once a week”; and (5) “never”. For the present study, the five frequency categories were collapsed into two categories whether the participant usually engaged in one of the six activities (each day-once a week= 1) or not (less than once a week, never= 0). For each participant, these values (0 or 1) were summed up across the six activities to a total physical activity score (scoring 0-6). Participant were then assigned to lower (0-1), middle (2), and high (3-6) physical activity groups based on the total physical activity score.

The Cox regression model was used in two runs (Run A, Run B) and covariates were used in blocks as shown in Table 1. Run A determines the risk of dementia only for the FA variables (Block 1, Run A). Run B shows the change in $\chi^2$ *p*-value when the FA variables are added as a last block to the model, describing if there is an added value to the model conferred by FA variables (Block 4, Run B). Benjamini-Hochberg false discovery rate (FDR) correction was used to control for multiple testing.

For the proposed harmful FA variables, the lowest tertile was set as the reference group, which includes the %ω-6 index, the SFA index, LA and ARA as single FAs, and the ratios LA/ALA, ARA/LA, ARA/EPA, SFA index/ω-3 index. For the beneficial FA variable, the

highest tertile was set as the reference group, including EPA, ALA, the ω-3 index and the ratio EPA/ALA.

**Table 1:** Covariates used in the Cox regression analysis

| Covariates | Run A | Run B |
|---|---|---|
| Block 1 | FA/ratio [terz] | age, gender, education and *APOE* ε4 status, *FADS1* SNP |
| Block 2 | Block 1, age, gender, education and *APOE* ε4 status, *FADS1* SNP | Block 1, for the lifestyle factors BMI, smoking status and physical activity, total cholesterol, triglycerides, and lipid lowering medication |
| Block 3 | Block 1+2, for the lifestyle factors BMI, smoking status and physical activity, total cholesterol, triglycerides, and lipid lowering medication | Block 1+2, cognitive decline |
| Block 4 | Block 1+2+3, cognitive decline | Block 1+2+3, FAs/Ratios [terz] |

Abbreviations: *APOE* ε4, apolipoprotein epsilon 4; BMI, Body Mass Index; FA, fatty acid; *FADS1*, Fatty acid desaturase 1; SNP, single nucleotide polymorphism.

### 2.7.3 Gene-environment interaction analysis

The presence of gene-environment interaction (*APOE*-FA levels) and the risk of dementia was tested by two approaches: the Cox regression model and the relative excess risk due to interaction (RERI) measure of biological interaction. An indication for interaction using Cox regression analysis would be if the OR of the interaction term (variable 1 × variable 2) clearly differs from the sum of the individual ORs. The Logistic regression model and the Cox regression model are exponential and are inherently multiplicative, thus, an interaction implies departure from multiplicativity, rather than from additivity, with the latter being a measure of biological interaction between risk factors. In order to determine if the departure from multiplicativity from the Cox regression analysis indicates an interaction between variables, the RERI measure was used as an index on an additive scale.

#### *2.7.3.1 Cox regression model analysis for interaction*

Groups were formed for the three tertiles of FA variables and the *APOE* genotype as a dichotomous variable resulting in six possible combinations. For the proposed harmful FAs, or ratios, i.e., %ω-6, LA/ALA, ARA/EPA, and SFA/ω-3, the group of the lowest tertile and no *APOE* ε4 allele was set as the reference group for the Cox regression model. For

the beneficial FAs, such as EPA, DHA, ω-3 index, and the ratio of EPA/ALA, the highest tertile and no *APOE* ε4 allele was set as the reference group.

*2.7.3.2 RERI measure of biological interaction*

The presence of gene-environment interaction (*APOE*-FA levels), or gene-gene interaction (*APOE-FADS1*) and the risk of dementia was tested by calculating the RERI index on an additive scale (Andersson *et al.*, 2005). This index indicates whether the combined effect of two exposures is larger or smaller than the sum of the individual effects of the exposures, compared to indices on a multiplicative scale, e.g. interaction terms in logistic regression or Cox regression models (Ahlbom and Alfredsson, 2005; Knol *et al.*, 2011).

The Cox regression model, can be defined in order to produce the output that is needed for assessment of biological interaction. Testing additive interaction between risk factors requires binary coding of the risk factors; therefore, the median was used to describe low levels or high levels of FA variables. The *APOE* ε4 status was divided into either no ε4 alleles or at least one ε4 allele. The *FADS1* SNP rs174546 was coded the same way as the *APOE* ε4 status, with no minor alleles, or at least one minor allele. With two dichotomous risk factors, there are four possible combinations and, thus, four exposure categories. The model is set up to include terms for three of the four possible combinations of exposure, coded as indicator variables, while the fourth category serves as a reference category (Table 2). In the model, $RR_{ij}$ is the relative risk in exposure category *i*,*j*. Thus, $RR_{11}$, $RR_{10}$, $RR_{01}$, and $RR_{00}$ are the relative risks for each of the four categories. Those subjects who are unexposed to both the first and the second risk factor are defined as the reference category, i.e., $RR_{00} = 1$. $RR_{11}$ is the relative risk of a disease if both risk factors are present, $RR_{10}$ is the relative risk of the disease if only the first risk factor is present, and $RR_{01}$ is the relative risk of the disease if only the second risk factor is present. Thus, there are three relative risks to be estimated and all three estimates will be required for assessment of biological interaction. Based on these assumptions and definitions, the three relative risk estimates can be obtained from a Cox regression model, with the corresponding covariance matrix needed for calculation of CIs.

**Table 2:** RERI dummy coding for different exposure combinations

| Exposure level | Ind01 | Ind10 | Ind11 |
|---|---|---|---|
| *i* = 0, *j* = 0 | 0 | 0 | 0 |
| *i* = 0, *j* = 1 | 1 | 0 | 0 |
| *i* = 1, *j* = 0 | 0 | 1 | 0 |
| *i* = 1, *j* = 1 | 0 | 0 | 1 |

Abbreviations: *i*, exposure category 1; *j* exposure category 2; Ind, indicator variables.

Andersson *et al*. provide an Excel sheet which can be used to calculate the measures of interaction and their CIs based on the results from a Cox regression model, which can be found at www.epinet.se (Andersson *et al.*, 2005). The Excel sheet requires the regression coefficients for each of the three exposure categories as input, and the covariance matrix in order to be able to carry out the CI calculations. The same covariates are used in this Cox regression as for the calculation of the aforementioned risk of AD.

The output of the RERI measure for interaction can be interpreted in the following manner:

RERI = 0: no interaction or exactly equal to additivity of the individual effects of the two risk factors

RERI > 0: positive interaction or more than additivity of the individual effects of the two risk factors

RERI < 0: negative interaction or less than additivity of the individual effects of the two risk factors.

SPSS does not provide the covariance matrix that is needed for the RERI analysis from the Cox regression model. Therefore, the covariance matrix needed in the provided Excel sheet had to be calculated from the correlation matrix from the Cox regression model and the variances of the indicator variables by using a syntax in Rstudio.

### 2.7.4 Linear regression analysis for association of *FADS1/2* SNPs with fatty acid levels

A linear regression model was used to assess the association and the direction between genotype status (rs174546) and FA levels, and the enzyme activity estimates with the

same covariates as in the Cox regression analysis (see chapter 2.7.2, Table 1). The SNP genotype was used as an additive model, homozygous for the major allele, heterozygous, and homozygous for the minor allele. The standardised beta (β) is a measure for the strength of the effect of each independent variable as well as for the effect direction. A positive β indicates that the minor allele is associated with higher FA levels, whereas a negative β means the minor allele is associated with lower FA levels.

Three SNPs were chosen to investigate the association of the *FADS* genotype with FA levels and enzyme activity estimates in the subset analysis. These three SNPs were also used in the mediation analysis (chapter 2.7.5.2). The first SNP rs174546 is located in the *FADS1* gene and the first LD block of the *FADS1/2* gene cluster, rs968567 is located in the *FADS2* promotor region that is included in the first LD block that has been shown to be functional (Bokor *et al.*, 2010), and rs174611 is located in the *FADS2* gene in the second LD block of the *FADS1/2* gene cluster (Ameur *et al*., 2012, Figure 6). These three SNPs are also used in the mediation analyses within the subset.

### 2.7.5 Mediation analysis

#### *2.7.5.1 EstiMeth package*

The EstiMeth package can be used to estimate genetically driven DNA methylation. The model is based on a multiple regression approach to obtain weights between SNPs and corresponding DNA methylation. The package was developed using a reference dataset for which both methylation and genotypic data are available. The performance of the model was then verified in independent validation datasets. Subsequently, the genetically driven DNA methylation signal can be estimated in independent individuals as the linear combinations of the inferred weights and observed genotypes (Freytag *et al.*, 2018).

In case genotypic data are not accessible, the association between methylation and a trait can be approximated using the model's weight, a trait GWAS summary statistics (SNP to trait association), and the covariance structure of the model's SNPs inferred from reference sample (this function is called MetaMeth within the EstiMeth package). The MetaMeth function was used with the summary statistics from the genome-wide association study (GWAS) of the CHARGE consortium on ω-3 FAs (Lemaitre *et al.*, 2011) to obtain CpG sites that are significantly associated with EPA levels, based on the GWAS results. The methylation values for these resulting CpG sites were used to build a

summary score that was used in the mediation analysis in the present study (Freytag *et al.*, 2018).

*2.7.5.2 Mediation analysis using the SPSS macro PROCESS*

The reason for testing mediation is to better understand the mechanism through which the causal variable affects the outcome, in this case, how SNPs in the *FADS1/2* gene affect FA levels. Mediation is a key part of the process analysis. Mediation models can be estimated by multiple regression and are based on four steps: (i) the causal variable (X, SNP) is correlated with the outcome (Y, FA), (ii) the causal variable (SNP) is correlated with the mediator (M, DNA methylation), (iii) the mediator (M, DNA methylation) affects the outcome variable (Y, FA), (iv) the mediator completely mediates the causal variable - outcome relationship, so that the effect of the causal variable on the outcome should disappear when controlling for a mediator.

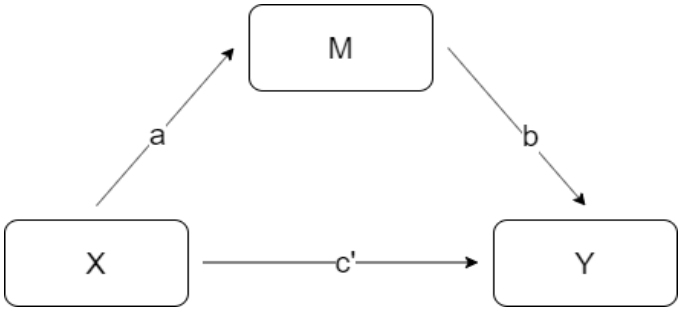


**Figure 3:** Mediation schematic.

By convention, the association between the causal variable (X, independent variable) and the outcome (Y, dependent variable) without a mediator is called the path c, or total effect. Within the mediation analysis, the association between the causal variable (X) and the mediator (M) is called path a, the association between the mediator (M) and the outcome (Y) is called path b, and the path between X and Y is then called direct effect, or path c'. The path of a and b combined is called the indirect effect (ab), and thus the total effect equals the direct effect plus the indirect effect, or c = c' + ab (Figure 3).

The SPSS macro PROCESS by Hayes *et al*. was used to perform the mediation analyses. The PROCESS macro uses ordinary least squares regression, yielding unstandardized path coefficients for total, direct, and indirect effects (Hayes, 2018). Bootstrapping with

5,000 samples was employed to compute the CIs and inferential statistics, and effects were deemed significant when the CI did not include zero.

The CpG sites that associated significantly with EPA levels based on the MetaMeth function were used to construct a methylation summary score. This summary score was then used as the mediator in the mediation analysis to test if the association between genetic variants in the *FADS1/2* gene cluster and FA levels is mediated by the methylation of the *FADS1/2* genes. Because the genetic variant has more than two states, homozygous for the common allele, heterozygous with the common and rare allele and homozygous for the rare allele, the predictor variable is multicategorical. By indicating X as a multicategorical variable, the coding system in PROCESS allows different modes of comparison between the categorical groups and does the dummy coding automatically. For the present analysis, the category “homozygous for the common allele” was defined as the reference group, so the output compares the other two groups with the reference group. In this case, the analysis produces two results for each path a, path c, path c’, and the indirect effect (ab) (Figure 4).

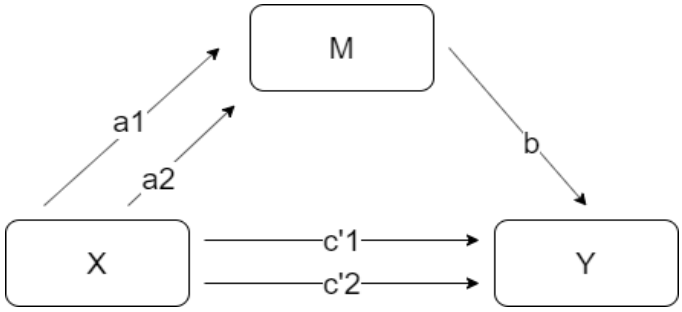


**Figure 4:** Mediation schematic categorical X variable.

G*Power version 3.1 was used to calculate the sample size to predict Y with 80 percent power (Faul *et al.*, 2007). For this, the *F*-test multiple regression - omnibus (deviation of $R^2$ from zero) fixed model was used. The type of power analysis was set *a priori* to calculate the sample size. The α error probability was set to 0.05, and the effect size $f^2$ was set to 0.054, which is a conventional small value for the effect size based on Cohen *et al*. (Cohen, 1988). The number of predictors was set to three: two genetic predictors based on the dummy coding (against the reference group) by PROCESS and the mediator variable. Age, sex, education, BMI and smoking were used as covariates.

# 3 Results

## 3.1 Characteristics of the AgeCoDe study sample and fatty acid levels

After the exclusion of all individuals as it is summarised in Figure 2 the final sample for the analysis consists of 1,252 individuals, 196 of whom receive a diagnosis of the AD dementia type and 1,056 who remain without a diagnosis over the follow up period of 7.5 years. The dementia group is slightly older at baseline with 84.6 years of age against 83.6 in the dementia-free group ($t(1248) = -4.0$, $p < 0.001$, $d = -0.23$), and there are more females in the dementia group ($p = 0.003$, $\chi^2$ test, Table 3) compared to the control group. The age of onset in the dementia group and the age at last follow up in the dementia-free group do not differ. There is also no difference on education between groups, whereas the dementia-free group shows higher physical leisure activity ($p < 0.001$, $\chi^2$ test). Individuals with the *APOE* ε4 genotype appear significantly more frequently in the dementia group ($p < 0.001$, $\chi^2$ test). The genotypes for rs174546 as well as the use of lipid lowering medication are equally distributed between the two groups. The inflammatory marker high-sensitivity C-reactive protein (hs-CRP) is on a trend level lower in the dementia group ($t(266.3) = 1.932$, $p = 0.054$, $d = 0.24$).

**Table 3:** Characteristics of the AgeCoDe study sample

| Characteristics | Dementia-free (*n*) | Dementia (*n*) | *d*, *p*-value [##] |
|---|---|---|---|
| Age [years] | 83.6 ± 3.2 (1054) | 84.6 ± 3.2 (196) | -0.23, < 0.001 |
| Age at last follow-up / AAO [years] | 88.4 ± 3.4 (1056) | 88.0 ± 3.8 (196) | NS |
| Female [%] | 62.2 (1056) | 73.5 (196) | 0.003 |
| Education [Group %] | | | NS |
| Lower | 58.7 | 59.7 | |
| Middle | 28.4 | 30.1 | |
| High | 12.9 | 10.2 | |
| Physical activity [Group %] | | | < 0.001 |
| Lower | 27.7 | 44.0 | |
| Middle | 34.6 | 30.1 | |
| High | 37.6 | 25.9 | |
| *APOE* ε4 allele [Group %] | 16.2 | 26.5 | < 0.001 |
| rs174546, MaA = C [Group %] | | | NS |
| C/C | 43.1 | 40.9 | |
| C/T | 44.4 | 45.5 | |

| Characteristics | Dementia-free (*n*) | Dementia (*n*) | *d*, *p*-value [##] |
|---|---|---|---|
| T/T | 12.5 | 13.6 | |
| hs-CRP [mg/L] | 2.97 ± 2.64 (956) | 2.60 ± 2.28 (175) | 0.24, NS (0.054) |
| Lipid lowering medication [Group %] | Yes 20.2 | Yes 20.4 | NS |
| SFAs | 2236.73 ± 546.07 (1045) | 2270.64 ± 505.25 (193) | NS |
| MUFAs | 459.08 ± 129.56 (1039) | 465.14 ± 122.25 (193) | NS |
| PUFAs | 2050.88 ± 564.18 (1048) | 2060.62 ± 504.97 (193) | NS |
| %ω-6 weight percentage (wt%) | 85.84 ± 3.98 (1045) | 86.00 ± 3.86 (194) | NS |
| %ω-6 concentration | 85.14 ± 5.42 (1050) | 86.45 ± 5.49 (196) | -0.18, 0.002 |
| 16:0 PA [µmol/L] | 1517.37 ± 372.02 (1047) | 1535.89 ± 340.05 (193) | NS |
| wt% | 30.40 ± 1.49 (1035) | 30.33 ± 1.46 (193) | NS |
| 18:0 SA [µmol/L] | 577.78 ± 152.43 (1043) | 593.71 ± 150.71 (194) | NS |
| wt% | 13.25 ± 1.15 (1043) | 13.07 ± 1.34 (196) | 0.22, NS (p=0.076) |
| SFA index (PA+SA) [µmol/L] | 2096.05 ± 513.66 (1046) | 2126.91 ± 471.96 (193) | NS |
| 18:2ω-6 LA [µmol/L] | 962.96 ± 315.33 (1047) | 1005.99 ± 317.49 (194) | -0.10, NS (p=0.081) |
| wt% | 18.02 ± 2.82 (1054) | 18.33 ± 2.72 (196) | NS |
| 20:3ω-6 DGLA [µmol/L] | 148.71 ± 53.64 (1049) | 154.90 ± 53.36 (195) | NS |
| wt% | 2.71 ± 0.65 (1052) | 2.81 ± 0.63 (195) | -0.11, 0.049 |
| 20:4ω-6 ARA [µmol/L] | 604.05 ± 204.94 (1045) | 611.70 ± 193.48 (194) | NS |
| wt% | 9.11 ± 2.02 (1051) | 9.20 ± 1.81 (196) | NS |
| 20:4ω-6 / 18:2ω-6 ratio (ARA/LA) [µmol/L] | 0.65 ± 0.21 (1043) | 0.63 ± 0.19 (195) | NS |
| 18:3ω-3 ALA [µmol/L] | 9.40 ± 4.57 (1028) | 9.13 ± 4.47 (193) | NS |
| wt% | 0.16 ± 0.07 (1041) | 0.15 ± 0.06 (194) | NS |
| 20:5ω-3 EPA [µmol/L] | 68.17 ± 35.72 (1028) | 60.72 ± 32.75 (193) | 0.34, 0.005 |
| wt% | 1.02 ± 0.46 (1023) | 0.92 ± 0.42 (189) | 0.15, 0.007 |
| 20:5ω-3 / 18:3ω-3 ratio (EPA/ALA) [µmol/L] | 7.81 ± 4.12 (1028) | 6.97 ± 3.59 (188) | 0.34, 0.004 |

| Characteristics | Dementia-free (*n*) | Dementia (*n*) | *d*, *p*-value [##] |
|---|---|---|---|
| 22:6ω-3 DHA [µmol/L] | 213.27 ± 93.66 (1043) | 194.52 ± 81.62 (193) | 0.33, 0.005 |
| wt% | 3.74 ± 1.09 (1054) | 3.82 ± 1.04 (195) | NS |
| ω-3 index (EPA+DHA) [µmol/L] | 284.28 ± 119.02 (1042) | 254.17 ± 99.26 (192) | 0.43, < 0.001 |
| 18:2ω-6 / 18:3ω-3 ratio (LA/ALA) [µmol/L] | 112.79 ± 45.22 (1041) | 121.44 ± 46.15 (190) | -0.14, 0.016 |
| 20:4ω-6 / 20:5ω-3 ratio (ARA/EPA) [µmol/L] | 10.43 ± 5.25 (1039) | 11.89 ± 5.74 (192) | -0.20, 0.001 |
| SFA index / ω-3 index (PA+SA/EPA+DHA) [µmol/L] | 8.22 ± 2.99 (1049) | 9.20 ± 3.18 (195) | -0.24, < 0.001 |

Abbreviations: µmol/L, micromole per liter; ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; AAO, age at onset; ALA, alpha-linolenic acid; *APOE* ε4, apolipoprotein epsilon 4; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; hs-CRP, high-sensitivity C-reactive protein; LA, linoleic acid; MaA, major allele; MUFA, monounsaturated fatty acid; NS, not significant; PA, palmitic acid; PUFA, polyunsaturated fatty acid; SA, stearic acid; SFA, saturated fatty acid; wt%, weight percent; [##]student's t-test for continuous variables, Pearson $\chi^2$ test for categorical variables; *d*, Cohen's *d*.

The means of all available SFAs, MUFAs and PUFAs calculated with concentration values are depicted in Table 3. The sum of all FAs of these species shows no differences between the dementia group and the dementia-free group. Single FAs shown in Table 3 are presented as concentration values and as percentage values. There is a higher proportion of ω-6 FAs in the dementia group compared to the dementia-free group, but only for the %ω-6 index build with concentration values of the FAs, and not for the %ω-6 index from FA percentage values (%ω-6 percentage, *NS*, %ω-6 concentration, $t(1244) = -3.1$, $p = 0.002$, $d = -0.18$, Table 3). PA and SA are not different between the two groups. For the wt% the t-test of SA is on a trend level lower in the dementia group ($t(252.1) = 1.782$, $p = 0.076$, $d = 0.22$). EPA and DHA levels are significantly reduced in the dementia group ($t(284.6) = 2.9$, $p = 0.005$, $d = 0.34$, $t(293.8) = 2.9.1$, $p = 0.005$, $d = 0.33$). EPA levels are reduced expressed as concentration values as well as wt% levels, whereas the wt% level for DHA shows no significant difference. The ratio of EPA and ALA is lower in the dementia group, showing that there is less EPA in relation to its precursor FA ALA ($t(284.8) = 2.9$, $p = 0.004$, $d = 0.34$). No ω-6 FA is significantly different between the groups, only LA is elevated in the dementia group on a trend level measured

with concentration levels ($t(1239) = -1.744$, $p = 0.081$, $d = -0.10$). DGLA levels are elevated in the dementia group when compared with wt% ($t(1245) = -2.0$, $p = 0.049$, $d = -0.11$). To be highlighted, ratios between ω-6 and ω-3 FAs show a difference between the groups, specifically ratios between ARA and EPA ($t(1229) = -3.5$, $p = 0.001$, $d = -0.20$), and LA and ALA ($t(1229) = -2.4$, $p = 0.016$, $d = -0.14$). For both ratios the values are higher in the dementia group. The ratio SFA index/ω-3 index is significantly higher in the dementia group ($t(1242) = -4.2$, $p < 0.001$, $d = -0.24$, Table 3).

### 3.2 Interdependencies of fatty acids and lifestyle impact on fatty acids

All available ω-6 FAs that are part of the ω-6 PUFA synthesis pathway, namely LA, GLA, DGLA, and ARA have a moderate to strong positive correlation with each other with a Pearson correlation coefficient of around 0.4 to 0.6 (Table 4). The relation of ALA and EPA is a moderate positive one with a Pearson correlation coefficient of 0.36. ALA and DHA show a weak relation with a positive Pearson correlation coefficient of 0.16. EPA and DHA are showing a moderate to strong positive relation with each other with a Pearson correlation coefficient of 0.47.

**Table 4:** Pearson correlation between ω-6 fatty acids and between ω-3 fatty acids

| ω-6 | GLA | DGLA | ARA |
|---|---|---|---|
| LA | $r = 0.50$** | $r = 0.56$** | $r = 0.50$** |
| GLA | | $r = 0.44$** | $r = 0.51$** |
| DGLA | | | $r = 0.60$** |
| ARA | | | |

| ω-3 | EPA | DHA |
|---|---|---|
| ALA | $r = 0.36$** | $r = 0.16$** |
| EPA | | $r = 0.47$** |
| DHA | | |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GLA, gamma-linolenic acid; LA, linoleic acid, **$p < 0.01$.

A higher ratio of LA/ALA is associated with a higher level of ARA and a lower level of EPA ($p = 0.001$, and $p < 0.001$ respectively, $\chi^2$ test, Table 5). This can clearly be seen in the association of the ratio LA/ALA and the ratio ARA/EPA. Higher levels of LA in relation to

ALA are associated with a higher ratio of ARA/EPA ($p < 0.001$, $\chi^2$ test). This ratio LA/ALA is less affecting to the level of DHA. The association shows the same direction as with EPA, but is not significant ($p = 0.182$, $\chi^2$ test). A higher ratio of LA/ALA is also associated with the proportion of ω-6 in HUFA (PUFA with 20 or more carbon atoms and 3 or more double bonds) including ARA, DGLA, EPA and DHA ($p < 0.001$, $\chi^2$ test).

**Table 5:** The influence of the LA/ALA ratio on fatty acid levels

| Fatty acid | *n* (%) with high EPA levels | *n* (%) with high DHA levels | *n* (%) with high ARA levels | *n* (%) with high ARA/EPA levels | *n* (%) with high %ω-6 HUFA levels |
|---|---|---|---|---|---|
| LA/ALA lowest tertile | 204 (48.9) | 151 (36.2) | 123 (29.5) | 66 (15.8) | 105 (25.2) |
| LA/ALA middle tertile | 131 (31.4) | 146 (35.0) | 126 (30.2) | 132 (31.7) | 123 (29.5) |
| LA/ALA highest tertile | 82 (19.7) | 120 (28.8) | 168 (40.3) | 219 (52.5) | 189 (45.3) |
| $\chi^2$ test *p*-value | < 0.001 | 0.182 | 0.001 | < 0.001 | < 0.001 |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HUFA, highly unsaturated fatty acid; LA, linoleic acid.

Table 6 shows the proportion of ω-6 in PUFA and the proportion of individual FAs in PUFA calculated as concentration values and wt% values. The %ω-6 calculated with concentration values ranges from 62.29 % to 95.16 % with a mean of 85.24 %. The %ω-6 calculated with wt% values ranges from 62.52 % to 95.43 % with a mean of 85.69 %. LA and ARA contribute the highest portion to the %ω-6 in PUFA with a mean for LA of 47.67 % and for ARA of 29.93 %. Both FAs are positively associated with the %ω-6 index with a Pearson correlation coefficient for LA of 0.49 and for ARA of 0.27 as shown in Table 7. The proportion of DGLA measured with concentration values has a range from 2.56 % to 17.04 % and a mean of 7.40 % (Table 6). The correlation with %ω-6 has a positive Pearson correlation coefficient of 0.13 (Table 7). ALA is the least abundant FA shown here with a range between 3.43 % and 0.10 % and a mean of 0.49 % (Table 6). The correlation with %ω-6 is the lowest with a negative Pearson correlation coefficient of -0.090 (Table 7). EPA and DHA are also anti-correlated with %ω-6, with Pearson

correlation coefficients of -0.68 and -0.94, respectively (Table 7). The proportion of EPA ranges from 17.79 % to 0.55 % (mean 3.51 %), while DHA is the most abundant ω-3 FA with a range between 29.21 % and 2.64 % and a mean of 10.76 %. EPA and DHA expressed as ω-3 index range from 37.31 % to 4.53 % with a mean of 14.27 % (Table 6). The ω-3 index is anti-correlated with the proportion of ω-6 in PUFA with a Pearson correlation coefficient of -0.99 (Table 7). Every ω-6 FA is correlated with the %ω-6 in PUFA whereas EPA, DHA and the ω-3 index are anti-correlated with %ω-6 in PUFA, with overall higher variance seen in the ω-6 FAs (Table 7), as shown in Figure 5.

**Table 6**: Range comparison of fatty acid proportions for concentration values and percentage values

| % of FA in PUFAs | Concentration (mean) | Percentage wt% (mean) |
|---|---|---|
| %ω-6 | 62.29 – 95.16 (85.24) | 62.52 – 95.43 (85.69) |
| %LA | 0.09 – 74.25 (47.67) | 0.08 – 73.36 (51.49) |
| %DGLA | 2.56 – 17.04 (7.40) | 2.58 – 18.16 (7.84) |
| %ARA | 12.61 – 69.36 (29.93) | 8.91 – 65.91 (26.11) |
| %ALA | 3.43 – 0.10 (0.49) | 3.29 – 0.10 (0.48) |
| %EPA | 17.79 - 0.55 (3.51) | 15.33 – 0.46 (3.10) |
| %DHA | 29.21 – 2.64 (10.76) | 25.84 – 2.73 (10.74) |
| %ω-3 (EPA+DHA) | 37.31 – 4.53 (14.27) | 37.29 – 3.95 (13.83) |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid; wt%, weight percent.

For comparison purposes between concentration and wt% values, and to be further concordant with the current literature the ranges and mean values were also calculated with wt% values (Table 6, column 3). The proportions of ω-6 to the individual FAs do not vary substantially when either concentration or wt% are considered (Table 6). The upper or lower ends of the ranges vary by a few percentage points (i.e., %ARA, %EPA, %DHA), but the mean values are similar (Table 6).

**Table 7:** Correlation of fatty acid proportions with the %ω-6 index

| | %LA | %DGLA | %ARA | %ALA | %EPA | %DHA | %ω-3 index |
|---|---|---|---|---|---|---|---|
| %ω-6 | $r$ = 0.49 | $r$ = 0.13 | $r$ = 0.27 | $r$ = -0.090 | $r$ = -0.68 | $r$ = -0.94 | $r$ = -0.99 |
| $p$-value | < 0.001 | < 0.001 | < 0.001 | 0.002 | < 0.001 | < 0.001 | < 0.001 |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

Beside the associations of FAs with each other, physical activity also has an influence on FA levels. As shown in Table 8, more physical activity is associated with higher levels of EPA ($p$ = 0.015, $\chi^2$ test) and DHA; in the latter case the association is not significant ($p$ = 0.106, $\chi^2$ test). In the group with the highest physical activity, fewer subjects have a high ratio of ARA/EPA ($p$ = 0.027, $\chi^2$ test). There is no relationship between physical activity and the ratio of LA/ALA ($p$ = 0.529, $\chi^2$ test).

**Table 8:** Association of physical activity with fatty acid levels

| Fatty acid | $n$ (%) with high EPA levels | $n$ (%) with high DHA levels | $n$ (%) with high ARA/EPA levels | $n$ (%) with high LA/ALA levels |
|---|---|---|---|---|
| Physical activity low | 107 (26.4) | 103 (25.4) | 139 (34.2) | 131 (32.3) |
| Physical activity mid | 139 (34.3) | 146 (36.0) | 143 (35.2) | 139 (34.2) |
| Physical activity high | 159 (39.3) | 157 (38.7) | 124 (30.5) | 136 (33.5) |
| $\chi^2$ test $p$-value | 0.015 | 0.106 | 0.027 | 0.529 |

Abbreviations: ARA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

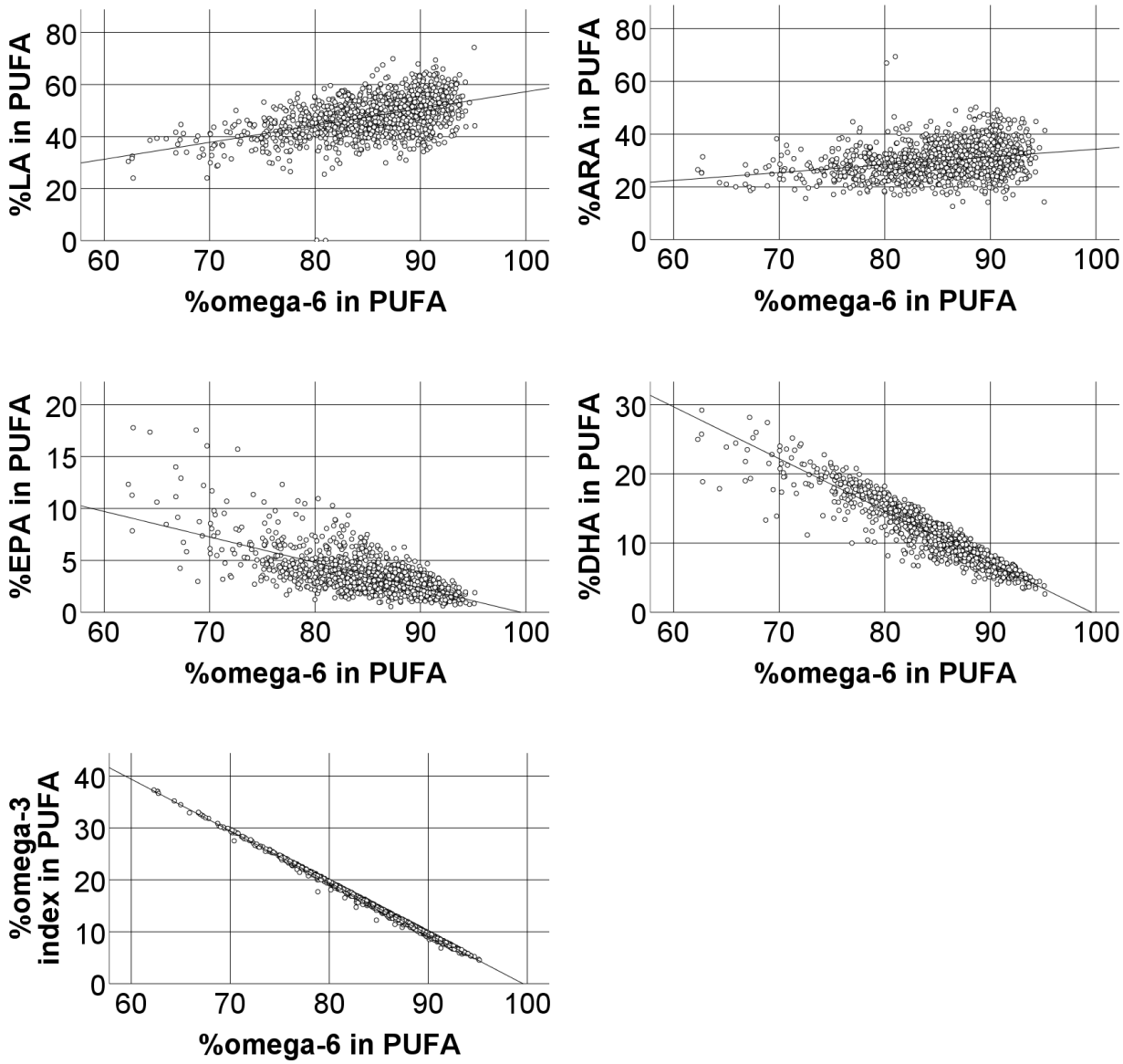


**Figure 5:** Fatty acid competition for accumulation in serum phospholipids. Analysis of individual fatty acid proportions in total %omega-6 in PUFA show lower proportions of EPA and DHA when the proportions of LA and ARA are higher.

### 3.3 Fatty acids and the risk of dementia

The association of FAs with the risk for dementia/AD was analysed by Cox proportional hazards model. The time-to-event variable (dementia) has been calculated to tenths of years. Table 9 shows the results for the Cox regression analysis of the different FA variables. The first column of *p*-values describes the risk of only the respective FA variable. The second *p*-value column shows the change in $\chi^2$ *p*-value when the FA variable

is added as a last block to the model, describing if there is an added value to the model by adding the FAs to it (for covariates used, see chapter 2.7.2, Table 1).

**Table 9:** Cox regression analysis to evaluate the risk of dementia conferred by fatty acid levels and fatty acid ratios

| Fatty acid | *p*-value | Exp(β) / OR | 95 % CI [low, high] | *p*-value single FA/ratio | *p*-value change in $\chi^2$ | FDR corr. |
|---|---|---|---|---|---|---|
| %ω-6 | | | | 0.001 | 0.007 | sig. |
| lowest tertile | reference | | | | | |
| middle tertile | 0.51 | 1.17 | 0.74, 1.83 | | | |
| highest tertile | 0.004 | 1.86 | 1.22, 2.85 | | | |
| LA/ALA | | | | NS (0.054) | 0.012 | sig. |
| lowest tertile | reference | | | | | |
| middle tertile | 0.042 | 1.58 | 1.02, 2.47 | | | |
| highest tertile | 0.004 | 1.82 | 1.21, 2.75 | | | |
| LA | | | | NS (0.573) | NS (0.583) | |
| lowest tertile | reference | | | | | |
| middle tertile | 0.34 | 1.24 | 0.80, 1.93 | | | |
| highest tertile | 0.38 | 1.23 | 0.78, 1.96 | | | |
| ALA | | | | NS (0.737) | NS (0.359) | |
| highest tertile | reference | | | | | |
| middle tertile | 0.23 | 1.29 | 0.85, 1.95 | | | |
| lowest tertile | 0.20 | 1.32 | 0.86, 2.02 | | | |
| ARA/EPA | | | | 0.007 | 0.031 | |
| lowest tertile | reference | | | | | |
| middle tertile | 0.061 | 1.52 | 0.98, 2.35 | | | |
| highest tertile | 0.012 | 1.75 | 1.13, 2.69 | | | |
| ARA | | | | NS (0.810) | NS (0.708) | |
| lowest tertile | reference | | | | | |
| middle tertile | 0.45 | 0.85 | 0.57, 1.29 | | | |
| highest tertile | 0.48 | 0.86 | 0.56, 1.31 | | | |
| EPA | | | | 0.002 | 0.002 | sig. |
| highest tertile | reference | | | | | |
| middle tertile | 0.027 | 1.64 | 1.06, 2.55 | | | |
| lowest tertile | 0.001 | 2.13 | 1.38, 3.28 | | | |
| DHA | | | | 0.051 | NS (0.135) | |
| highest tertile | reference | | | | | |
| middle tertile | 0.048 | 1.53 | 1.00, 2.32 | | | |
| lowest tertile | 0.230 | 1.32 | 0.84, 2.07 | | | |

| Fatty acid | *p*-value | Exp(β) / OR | 95 % CI [low, high] | *p*-value single FA/ratio | *p*-value change in $\chi^2$ | FDR corr. |
|---|---|---|---|---|---|---|
| EPA/ALA | | | | 0.004 | 0.007 | sig. |
| highest tertile | reference | | | | | |
| middle tertile | 0.80 | 1.06 | 0.69, 1.64 | | | |
| lowest tertile | 0.005 | 1.83 | 1.20, 2.78 | | | |
| ARA/LA | | | | NS | NS | |
| lowest tertile | reference | | | (0.335) | (0.238) | |
| middle tertile | 0.85 | 1.04 | 0.70, 1.54 | | | |
| highest tertile | 0.20 | 0.72 | 0.44, 1.18 | | | |
| SFA/ω-3 | | | | < 0.001 | 0.002 | sig. |
| lowest tertile | reference | | | | | |
| middle tertile | 0.23 | 1.32 | 0.84, 2.10 | | | |
| highest tertile | 0.001 | 2.08 | 1.34, 3.23 | | | |
| SFA index | | | | NS | NS | |
| lowest tertile | reference | | | (0.657) | (0.754) | |
| middle tertile | 0.67 | 1.10 | 0.71, 1.71 | | | |
| highest tertile | 0.45 | 1.20 | 0.75, 1.91 | | | |
| ω-3 index | | | | 0.019 | NS | |
| highest tertile | reference | | | | (0.056) | |
| middle tertile | 0.072 | 1.49 | 0.97, 2.30 | | | |
| lowest tertile | 0.021 | 1.70 | 1.08, 2.66 | | | |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; CI, confidence interval; corr., correction; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Exp(B), exponentiation of the B coefficient; FA, fatty acid; FDR, false discovery rate; LA, linoleic acid; NS, not significant; OR, odds ratio; SFA, saturated fatty acid; sig., significant.

The proportion of ω-6 (%ω-6) variable shows a significant risk for dementia (Table 9). The *p*-value is 0.001 with no other covariates, and 0.007 for the change in $\chi^2$ when added to the model after all other blocks of covariates. The highest tertile is significantly different to the lowest tertile with an observed odds ratio (OR) of 1.86 ($p = 0.004$, 95 % CI 1.22, 2.85) for the highest tertile. This means a higher proportion of ω-6 FAs is associated with a higher risk of dementia. The ratio of LA/ALA is borderline significant when analysed with no covariates ($p = 0.054$). With all covariates the *p*-value for the change in $\chi^2$ is 0.012. Therefore, a higher ratio of LA/ALA is associated with a higher risk of dementia. The middle tertile and the highest tertile are significantly different to the lowest tertile, showing an increase of the OR from 1.58 ($p = 0.042$, 95 % CI 1.02, 2.47) for the middle tertile to

1.82 ($p$ = 0.004, 95 % CI 1.21, 2.75) for the highest tertile. Neither of the two FAs is individually associated with a higher risk of dementia. ARA is individually also not associated with the risk of dementia, but the ratio with EPA shows that a higher level of ARA in relation to EPA is associated with a higher risk of dementia. The middle tertile is borderline significantly different from the lowest tertile and increases the risk for dementia 1.52-fold compared to the lowest tertile ($p$ = 0.061, 95 % CI 0.98, 2.35). The OR for the highest tertile of this ratio is 1.75, which is significantly different from the reference tertile with a $p$-value of 0.012 (95 % CI 1.13, 2.69). This ratio is significantly associated with the risk of dementia with a $p$-value of 0.007 without covariates and a $p$-value of 0.031 for the change in $\chi^2$when added to the model after all other blocks of covariates.

EPA has protective properties against the risk of dementia. The middle tertile has a 1.64 ($p$ = 0.027, 95 % CI 1.06, 2.55) higher risk compared the highest tertile (reference tertile). The lowest tertile has a twofold higher risk compared to the highest tertile with an OR of 2.13 ($p$ = 0.001, 95 % CI 1.38, 3.28). Both tertiles are significantly different from the reference tertile. Both the $p$-values with no covariates and the change in $\chi^2$ when added to the model after all other blocks of covariates are 0.002. The ratio of EPA and ALA also shows a protective effect of higher levels of EPA in relation to ALA. The ratio without covariates has a $p$-value of 0.004, with all other covariates the change in $\chi^2$ has a $p$-value of 0.007 when added to the model after all other blocks of covariates. The OR of the lowest tertile is 1.83 ($p$ = 0.005, 95 % CI 1.20, 2.78). The middle tertile is not significantly different to the highest tertile. The ratio ARA/LA poses no further risk to dementia. The index of the SFAs PA and SA (SFA index) also shows no risk for dementia, but in relation to the ω-3 index highest tertile increases the risk for dementia with an OR of 2.08 ($p$ = 0.001, 95 % CI 1.34, 3.23). The SFA/ω-3 ratio is significantly associated with the risk of dementia with a $p$-value of < 0.001 without covariates and a $p$-value of 0.002 for the change in $\chi^2$ when added to the model after all other blocks of covariates. The ω-3 index is not significantly associated with the risk of dementia after the inclusion of all covariates ($p$ = 0.056). This may be due to the low risk of DHA itself on dementia in this analysis. With all covariates in the Cox's regression model DHA is not associated with the risk of dementia ($p$ = 0.135). After FDR correction %ω-6, the ratio of LA and ALA, EPA, the ratio of EPA and ALA, and the ratio of SFA and ω-3 remained significant (Table 9).

## 3.4 Interaction of *APOE* ε4 status with fatty acids and the risk of dementia

### 3.4.1 Cox regression model

Only those FA variables are analysed for interaction with *APOE* ε4 that were nominally (based on the change in $\chi^2$ *p*-value) significantly associated with the risk of dementia in the Cox proportional hazard regression in the present study (see Table 9).

Within the *APOE* ε4 negative group there is no difference between the three tertiles of the %ω-6 variable. The middle tertile and the highest tertile in the *APOE* ε4 positives group are significantly different to the reference group, whereas the lowest tertile shows no difference to the reference group. The middle tertile has an OR of 3.02 ($p < 0.001$, 95 % CI 1.63, 6.60) and the highest tertile has an OR 5.04 ($p < 0.001$, 95 % CI 2.72, 9.34, Table 10). The change in $\chi^2$ for the %ω-6 variable has a *p*-value of 7.87E-7 when all other covariates were included in the model, and a *p*-value of 2.0E-6 without covariates.

**Table 10:** Cox regression analysis to evaluate the interaction of the *APOE* ε4 status with fatty acid levels for dementia risk

| Combination ε4 status / fatty acid | *p*-value | Exp (β) / OR | 95 % CI [low, high] | *p*-value single FA/ratio | *p*-value change in $\chi^2$ | FDR corr. |
|---|---|---|---|---|---|---|
| no ε4 / %ω-6 | | | | 2.0E-6 | 7.87E-7 | sig. |
| low | ref. | | | | | |
| mid | 0.66 | 0.89 | 0.52, 1.52 | | | |
| high | 0.14 | 1.44 | 0.89, 2.33 | | | |
| ε4 / %ω-6 | | | | | | |
| low | 0.41 | 1.41 | 0.63, 3.13 | | | |
| mid | < 0.001 | 3.02 | 1.63, 6.60 | | | |
| high | < 0.001 | 5.04 | 2.72, 9.34 | | | |
| no ε4 / LA/ALA | | | | 4.27E-4 | 7.0E-6 | sig. |
| low | ref. | | | | | |
| mid | 0.039 | 1.77 | 1.03, 3.05 | | | |
| high | 0.007 | 2.00 | 1.21, 3.31 | | | |
| ε4 / LA/ALA | | | | | | |
| low | < 0.001 | 3.40 | 1.76, 6.57 | | | |
| mid | < 0.001 | 4.28 | 2.02, 9.05 | | | |
| high | 7.92E-7 | 5.27 | 2.72, 10.19 | | | |

| Combination ε4 status / fatty acid | *p*-value | Exp (β) / OR | 95 % CI [low, high] | *p*-value single FA/ratio | *p*-value change in $\chi^2$ | FDR corr. |
|---|---|---|---|---|---|---|
| no ε4 / ARA/EPA | | | | 2.7E-5 | 9.0E-6 | sig. |
| low | ref. | | | | | |
| mid | 0.035 | 1.74 | 1.04, 2.89 | | | |
| high | 0.043 | 1.69 | 1.02, 2.80 | | | |
| ε4 / ARA/EPA | | | | | | |
| low | 0.006 | 2.84 | 1.36, 5.94 | | | |
| mid | 0.001 | 3.15 | 1.58, 6.28 | | | |
| high | 6.49E-8 | 5.50 | 2.97, 10.21 | | | |
| no ε4 / EPA | | | | 4.0E-6 | 9.16E-8 | sig. |
| high | ref. | | | | | |
| mid | 0.002 | 2.34 | 1.38, 3.99 | | | |
| low | < 0.001 | 2.65 | 1.56, 4.50 | | | |
| ε4 + EPA | | | | | | |
| high | <0.001 | 5.11 | 2.55, 10.23 | | | |
| mid | 0.002 | 3.46 | 1.60, 7.48 | | | |
| low | <0.001 | 6.72 | 3.50, 12.91 | | | |
| no ε4 / EPA/ALA | | | | 9.3E-5 | 5.0E-6 | sig. |
| high | ref. | | | | | |
| mid | 0.89 | 1.04 | 0.62, 1.73 | | | |
| low | 0.021 | 1.77 | 1.09, 2.87 | | | |
| ε4 / EPA/ALA | | | | | | |
| high | 0.013 | 2.44 | 1.21, 4.93 | | | |
| mid | 0.005 | 2.72 | 1.36, 5.43 | | | |
| low | <0.001 | 4.85 | 2.66, 8.83 | | | |
| no ε4 / SFA/ω-3 | | | | 3.57E-7 | 4.56E-7 | sig. |
| low | ref. | | | | | |
| mid | 0.54 | 1.18 | 0.69, 2.02 | | | |
| high | 0.050 | 1.65 | 1.00, 2.73 | | | |
| ε4 / SFA/ω-3 | | | | | | |
| low | 0.29 | 1.59 | 0.68, 3.72 | | | |
| mid | 0.001 | 2.91 | 1.51, 5.61 | | | |
| high | <0.001 | 6.24 | 3.39, 11.48 | | | |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; CI, confidence interval; corr., correction; EPA, eicosapentaenoic acid; Exp(B), exponentiation of the B coefficient; ε4, apolipoprotein epsilon 4 allele; FA, fatty acid; FDR, false discovery rate; LA, linoleic acid; NS, not significant; OR, odds ratio; ref, reference; SFA, saturated fatty acid; sig., significant.

All combinations of the *APOE* ε4 allele and the ratio of LA/ALA are significantly different to the reference group, with a gradually increasing OR. The middle tertile in the *APOE* ε4 negative group has an OR of 1.77 ($p$ = 0.039, 95 % CI 1.03, 3.05) and the highest tertile

has a twofold increased risk with an OR of 2.0 ($p$ = 0.007, 95 % CI 1.21, 3.31). The OR ranges from 3.40 to 5.27 for the combinations of this FA ratio and the *APOE* ε4 positives, starting from the lowest to the highest tertile. The change in $\chi^2$ has a $p$-value of 7.0E-6 with all other covariates when added to the model, and a $p$-value of 4.27E-4 without covariates (Table 10).

For the ratio between ARA and EPA all groups are significantly different from the reference group, which is the lowest tertile of the FA ratio with no *APOE* ε4 allele. In the groups with no *APOE* ε4 allele the middle tertile has an OR of 1.74 ($p$ = 0.035, 95 % CI 1.04, 2.89) and the highest tertile has an OR of 1.69 ($p$ = 0.043, 95 % CI 1.02, 2.80). Within the group of *APOE* ε4 allele carrier the OR rises from 2.84 ($p$ = 0.006, 95 % CI 1.36, 5.94) for the lowest tertile to 3.15 ($p$ = 0.001, 95 % CI 1.58, 6.28) for the middle tertile and to 5.50 ($p$ = 0.001, 95 % CI 2.97, 10.21) in the highest tertile. The ratio without covariates has a $p$-value of 2.7E-5, with all other covariates the change in $\chi^2$ has a $p$-value of 9.0E-6 when added to the model after all other blocks of covariates.

Except for the middle tertile and no *APOE* ε4 allele, all combinations of the ratio of EPA and ALA and the *APOE* ε4 status were significantly different from the reference group. Note that the highest tertile with no *APOE* ε4 allele is the reference group. All other groups have an OR bigger than one, starting from 1.77 ($p$ = 0.021, 95 % CI 1.09, 2.87) for the group of *APOE* ε4 negatives and a low ratio. *APOE* ε4 positives with a high ratio show an OR of 2.44 ($p$ = 0.013, 95 % CI 1.21, 4.93), *APOE* ε4 positives with the middle tertile have an OR of 2.72 ($p$ = 0.005, 95 % CI 1.36, 5.43), and *APOE* ε4 positives with the lowest tertile have an OR of 4.85 ($p$ < 0.001, 95 % CI 2.60, 8.83). This shows that *APOE* ε4 carriers with a low level of EPA in relation to ALA have the highest risk for dementia. The change in $\chi^2$ has a $p$-value of 5.0E-6 with all other covariates when added to the model, and a $p$-value of 9.3E-5 without covariates. Looking at EPA individually shows a similar picture. Here all groups show a significant difference to the reference group (no *APOE* ε4 and high level of EPA). In the group of *APOE* ε4 non-carrier the OR increases with lower EPA level, from 2.34 ($p$ = 0.002, 95 % CI 1.38, 3.99) to 2.65 ($p$ < 0.001, 95 % CI 1.56, 4.50) for the lowest tertile. Interestingly, the highest level of EPA in combination with the *APOE* ε4 allele has a higher OR than the middle tertile, with values of 5.11 ($p$ < 0.001, 95 % CI 2.55, 10.23) and 3.46 ($p$ = 0.002, 95 % CI 1.60, 7.48), respectively. The highest

OR of 6.72 ($p < 0.001$, 95 % CI 3.50, 12.91) is observed for the combination of *APOE* ε4 allele carrier and the lowest levels of EPA. The variable has a *p*-value of 4.0E-6 without covariates, with all other covariates the change in $\chi^2$ has a *p*-value of 9.16E-8 when added to the model.

Within the *APOE* ε4 negative group and the SFA/ω-3 ratio, only the highest tertile is significantly different to the reference group with an OR of 1.65 ($p = 0.050$, 95 % CI 1.00, 2.73). In the *APOE* ε4 positive group the middle tertile and the highest tertile are significantly different to the reference group and ORs of 2.91 ($p = 0.001$, 95 % CI 1.51, 5.61) and 6.24 ($p < 0.001$, 95 % CI 3.39, 11.48), respectively. The ratio without covariates has a *p*-value of 3.57E-7, with all other covariates the change in $\chi^2$ has a *p*-value of 4.56E-7 when added to the model after all other blocks of covariates. After FDR correction all *p*-values remained significant.

### 3.4.2 RERI analysis for biological interaction

The OR of the *APOE* ε4 genotype in the Cox regression models varies between 2.6 and 2.8 (taken from the SPSS outputs), depending on the FAs that are in the Cox regression model. Adding the OR of individual FA variables (Table 9) to the OR of *APOE* totals an OR that is less than the ORs of the groups of *APOE* added up with the respective FA variables (Table 10). It is hard to assess if there is a synergistic effect between *APOE* ε4 allele and the FAs by comparing the ORs alone. Therefore, to investigate if there is a biological interaction between the FAs and the *APOE* ε4 status the RERI index was calculated.

Table 11 shows the RERI estimates and the CIs for the different exposure categories. A positive RERI estimate indicates a positive interaction or more than additivity of the individual effects of the two risk factors. All calculated RERI estimates are bigger than 0, with the biggest values for the exposure categories ARA and *APOE* ε4, LA/ALA and *APOE* ε4, and %ω-6 and *APOE* ε4, with the estimates 1.58, 1.18, 2.16, and 2.20, respectively. Based on the CIs, no estimate reached a significant level except for the exposure category SFA/ω-3 and *APOE* ε4 (95 % CI 0.028, 4.30). The exposure category ARA and *APOE* ε4 is close to being significant with the lower boundary of the CI being slightly below zero with a value of -0.077. With a lower bound of -0.24, the exposure category %ω-6 and *APOE* ε4 is also potentially significant.

**Table 11:** Estimates of additive interaction of the *APOE* ε4 status with fatty acids for dementia risk

| Exposure | RERI estimate | 95% CI [low, high] |
|---|---|---|
| EPA and *APOE* ε4 | 0.45 | -2.66, 3.57 |
| ARA and *APOE* ε4 | 1.58 | -0.077, 3.23 |
| ARA/EPA and *APOE* ε4 | 0.42 | -2.00, 2.83 |
| LA and *APOE* ε4 | 0.60 | -1.52, 2.71 |
| ALA and *APOE* ε4 | 0.54 | -1.51, 2.59 |
| LA/ALA and *APOE* ε4 | 1.18 | -1.28, 3.64 |
| %ω-6 and *APOE* ε4 | 2.20 | -0.24, 4.64 |
| *FADS1* and *APOE* ε4 | 0.36 | -1.76, 2.49 |
| SFA/ω-3 and *APOE* ε4 | 2.16 | 0.028, 4.30 |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; *APOE* ε4, apolipoprotein epsilon 4 allele; ARA, arachidonic acid; CI, confidence interval; EPA, eicosapentaenoic acid; *FADS1*, fatty acid desaturase 1; LA, linoleic acid; RERI, relative excess risk due to interaction; SFA, saturated fatty acid.

## 3.5 The effect of *FADS1/2* variants on fatty acid profiles

### 3.5.1 AgeCoDe cohort

The single nucleotide polymorphism (SNP) rs174546 was analysed as a proxy for the effect of *FADS* genotypes on FA levels and estimated desaturase activity (Table 12). The strongest association can be seen for the ratio of ARA and LA, and for the ratio ARA and DGLA, which is used as an estimate for the D5D enzyme activity, with a β of -0.48 and -0.42, respectively. For ARA and the ratio between EPA and ALA the β is -0.25 and -0.30, respectively. EPA on its own has a β of -0.16, whereas no association can be observed for DHA. The ratio of DGLA and LA, which is used as an estimate for the D6D enzyme activity has a β of -0.068. Furthermore, the ratio of GLA and LA is another estimate used for the D6D enzyme activity, that does not include the elongase step from GLA to DGLA. For this second D6D (GLA/LA, D6D2) estimate the β is -0.17 ($p < 0.001$). Here it is to be emphasized that DGLA is positively associated with the minor allele with a β of 0.13 ($p < 0.001$) that accounts for the β difference between the two D6D estimates. The same direction applies for LA and ALA with a β of 0.21 and 0.15, respectively. The %ω-6 increases with the minor allele SNP status with a β value of 0.080 ($p = 0.013$). The ratio SFA/ω-3 has a β of 0.11 ($p = 0.001$), but it can be suggested that this association is driven by the effect of the SNP on PA and EPA. As it can be seen in Table 12, there is no

association for either DHA or SA and the SNP. In contrast, the SNP rs174546 shows a positive association with PA on a trend level ($p$ = 0.088).

**Table 12:** Associations of SNP rs174546 with fatty acid levels and desaturase estimates

| Fatty acid / ratio | | β | *p*-value | CI 95% [low, high] |
|---|---|---|---|---|
| LA | *n* = 950 | 0.21 | < 0.001 | 73.12, 127.03 |
| ln ALA | *n* = 958 | 0.15 | < 0.001 | 0.071, 0.17 |
| LA/ALA | *n* = 941 | -0.015 | 0.65 | -5.23, 3.28 |
| ln EPA | *n* = 958 | -0.16 | < 0.001 | -0.19, -.083 |
| ln EPA/ALA | *n* = 958 | -0.30 | < 0.001 | -0.30, -0.20 |
| ARA | *n* = 948 | -0.25 | < 0.001 | -91.67, -57.05 |
| ARA/LA | *n* = 946 | -0.48 | < 0.001 | -0.15, -0.12 |
| ln ARA/EPA | *n* = 958 | 0.002 | 0.95 | -0.049, 0.052 |
| %ω-6 | *n* = 956 | 0.080 | 0.013 | 0.14, 1.15 |
| D5D ω-6 | *n* = 949 | -0.42 | < 0.001 | -0.89, -0.68 |
| D6D ω-6 | *n* = 949 | -0.068 | 0.024 | -0.009, - 0.001 |
| D6D2 ω-6 | *n* = 955 | -0.17 | < 0.001 | -0.001, <0.001 |
| ln DHA | *n* = 958 | -0.028 | 0.36 | -0.058, 0.021 |
| GLA | *n* = 954 | -0.050 | 0.118 | -0.55, 0.062 |
| DGLA | *n* = 953 | 0.13 | < 0.001 | 5.88, 14.62 |
| SFA/ω-3 | *n* = 958 | 0.11 | 0.001 | 0.025, 0.093 |
| PA | *n* = 958 | 0.049 | 0.088 | -0.003, 0.042 |
| SA | *n* = 958 | 0.005 | 0.86 | -0.022, 0.026 |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; CI, confidence interval; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; D5D, delta-5 desaturase; D6D, delta-6 desaturase; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; PA, palmitic acid; SA, stearic acid; SFA, saturated fatty acid; SNP, single nucleotide polymorphism.

### 3.5.2 Methylation subsample analysis

Overall, all associations and directions for rs174546 could be replicated as they are found for this SNP in the entire dataset of the AgeCoDe cohort (Table 12 / 13). All precursor FAs, namely LA, ALA, DGLA, and PA have elevated levels, and all product FAs, namely GLA, ARA, and EPA have reduced levels when the minor alleles of the analysed SNPs are present, and thusly the product to precursor ratios (i.e., ARA/LA, EPA/ALA) are reduced as indicated by a negative β. Furthermore, the enzyme activity estimates D6D2 and D5D are reduced when the minor alleles are present (Table 13). Table 14 shows the frequencies and the genomic positions of the analysed SNPs in the subsample data set as well as the genotyping success rate for the analysed SNPs, which are 96 % for SNP

rs968567 and 97 % for the SNPs rs174546 and rs174611. Additionally, frequencies for rs174546 for the entire AgeCoDe dataset are included in Table 14; here the genotyping success rate is 85 %.

The highest associations for rs174546 can be observed for ratios, i.e., ARA/LA (β ~ -0.5), EPA/ALA (β ~ -0.4), and for the D5D (β ~ -0.5), and D6D2 (β ~ -0.37) estimates. The associations with individual FAs are weaker; here ARA shows the strongest associations with the analysed SNPs with a β of ~ -0.3. The other FAs are also significantly associated, but with lower β values. Interestingly, DHA and D6D levels, as well as ratios between ω-6 and ω-3 FAs (i.e., LA/ALA, ARA/EPA), are not associated with the analysed SNPs (Table 13).

**Table 13:** Associations of *FADS1/2* SNPs with fatty acid levels and desaturase estimates in the subsample

| SNP | rs174546 | | rs968567 | | rs174611 | |
|---|---|---|---|---|---|---|
| | β | *p*-value | β | *p*-value | β | *p*-value |
| LA | 0.26 | < 0.001 | 0.09 | 0.17 | 0.17 | 0.005 |
| GLA | -0.19 | 0.004 | | NS | | NS |
| ln DGLA | 0.17 | 0.005 | 0.26 | < 0.001 | 0.19 | 0.002 |
| ARA | -0.31 | < 0.001 | -0.17 | 0.007 | -0.21 | 0.001 |
| ln ALA | 0.23 | < 0.001 | | NS | 0.18 | 0.005 |
| ln EPA | -0.23 | < 0.001 | -0.18 | 0.007 | -0.15 | 0.02 |
| DHA | | NS | | NS | | NS |
| ln D6D | | NS | 0.24 | < 0.001 | | NS |
| ln D6D2 | -0.38 | < 0.001 | -0.12 | 0.088 | -0.19 | 0.004 |
| ln D5D | -0.52 | < 0.001 | -0.45 | < 0.001 | -0.45 | < 0.001 |
| ln ARA/LA | -0.52 | < 0.001 | -0.23 | < 0.001 | -0.35 | < 0.001 |
| ln EPA/ALA | -0.40 | < 0.001 | -0.23 | 0.001 | -0.29 | < 0.001 |
| ln LA/ALA | -0.13 | 0.064 | | NS | -0.13 | 0.055 |
| ln ARA/EPA | | NS | | NS | | NS |
| %ω6 | | NS | | NS | | NS |
| ln SFA/ω3 | 0.12 | 0.069 | | NS | | NS |
| ln PA | 0.10 | 0.092 | | NS | | NS |

Abbreviations: ω-3, omega-3 fatty acid; ω-6, omega-6 fatty acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; D5D, delta-5 desaturase; D6D, delta-6 desaturase; D6D2, delta-6 desaturase 2; EPA, eicosapentaenoic acid; *FADS1/2*, fatty acid desaturase 1/2; GLA, gamma-linolenic acid; LA, linoleic acid; NS, not significant; PA, palmitic acid; SFA, saturated fatty acid; SNP, single nucleotide polymorphism.

Overall, the SNP rs174611 (*FADS1/2* LD block 2, Figure 6) shows weaker associations with the analysed FAs and their respective ratios in comparison to the SNP rs174546 from the *FADS1/2* LD block 1 (Figure 6). Furthermore, SNP rs174611 and rs968567 show no association with FA GLA. The SNP rs174611 shows a trend association with the ratio LA/ALA, like rs174546, with *p*-values of 0.064, and 0.055, and similar β values of -0.13 for rs174546 and -0.13 for rs174611 (Table 13).

**Table 14:** Characteristics of SNPs within the *FADS1/2* genes

| SNP | Position | MaA / MiA | Number (%) of subjects with genotype | | | Genotyping success rate (%) |
|---|---|---|---|---|---|---|
| | Chr11 | Hg19 | 1 1 | 1 2 | 2 2 | |
| AgeCoDe | | | | | | |
| rs174546 | 61569830 | C / T | 455 (42.8) | 474 (44.5) | 135 (12.7) | 85.0 |
| Subsample | | | | | | |
| rs174546 | 61569830 | C / T | 123 (42.4) | 119 (41.0) | 48 (16.6) | 97.0 |
| rs968567 | 61595564 | C / T | 192 (66.9) | 81 (28.2) | 14 (4.9) | 96.0 |
| rs174611 | 61627881 | T / C | 130 (44.8) | 128 (44.1) | 32 (11.0) | 97.0 |

Abbreviations: 1, major allele; 2, minor allele; Chr, chromosome; del, deletion; *FADS1/2*, fatty acid desaturase 1/2; MaA, major allele; MiA, minor allele; SNP, single nucleotide polymorphism.

The SNP rs968567 (located in the *FADS2* promotor region, Figure 6) generally shows weaker associations to the FAs and their ratios compared to rs174546. Interestingly rs968567 shows no association with single FAs, except for ARA and DGLA. In fact, rs968567 has the strongest association with DGLA from all analysed SNPs in this study. Even more interesting is the only association for the D6D enzyme activity measured with the ratio between DGLA and LA (D6D) with rs968567. The association is highly significant and has a β of 0.24 (Table 13), meaning the minor allele of rs968567 is associated with a higher *FADS2* enzyme activity estimate. In comparison, rs174546 shows an effect in the opposite direction with a β of -0.068 in the entire AgeCoDe dataset (Table 12). The association of SNPs and the ratio SFA/ω-3 is not significant in the subsample. The direction is similar to the result with rs174546 in the AgeCoDe sample but the *p*-value is above the threshold of 0.05 ($p = 0.069$). The same applies for PA analysed as single FA and the SNPs rs174546 and rs174553 ($p = 0.092$, Table 13).

### 3.6 MetaMeth results and mediation analysis

Methylation was measured in a smaller subsample that consists of 299 subjects (Figure 2) divided into 113 dementia cases and 186 matched controls, as described in the study sample characterisation (see chapter 2.2). For the mediation analysis, 288 subjects were available with all data on SNPs, DNA methylation, and FA levels for EPA. Power calculation with a small effect size ($f2 = 0.052$) of the predictor X (multicategorical, number of predictors = 3) resulted in a needed sample size of 203 individuals to predict Y with a power of 80 %. Therefore, all analysed mediation models have a sufficient number of individuals to predict mediation with more than 80 % power (Table 16).

Based on the GWAS data from the CHARGE consortium, the largest GWAS of phospholipid ω-3 PUFAs with 8,866 participants of European ancestry, and the MetaMeth function (see chapter 2.7.5), 17 CpG sites were identified that were reasonably associated with EPA levels. After false discovery rate (FDR) correction, six out of 17 CpG sites were significantly associated with EPA (Table 15). The calculated methylation summary score based on these six CpG sites was used as mediator, and EPA levels are used as an outcome variable in the mediation analysis.

**Table 15:** MetaMeth results

| CpG site | Position Chr11 | Gene | Functional region | Spearman *p*-value | Spearman *r* | FDR |
|---|---|---|---|---|---|---|
| cg19610905 | 61596334 | *FADS2* | transcription | <0.001 | 0.222 | sig. |
| cg11250194 | 61601938 | *FADS2* | highly regulatory | 0.011 | 0.155 | sig. |
| cg15598662 | 61582890 | *FADS1* | transcription | 0.013 | 0.152 | sig. |
| cg06781209 | 61594998 | *FADS2* | promotor | 0.014 | 0.150 | sig. |
| cg00603274 | 61596627 | *FADS2* | transcription | 0.016 | 0.147 | sig. |
| cg01400685 | 61598026 | *FADS2* | transcription | 0.017 | 0.146 | sig. |

Abbreviations: Chr, chromosome; *FADS1*, fatty acid desaturase 1; *FADS2*, fatty acid desaturase 2; FDR, false discovery rate; sig., significant.

As described above the dummy coding of the categorical predictor variable results in two outputs at a time for the paths a, c, c' and ab. The first number (a1, c1, c1', ab1) represents the comparison of the homozygous major allele group with the heterozygous group, and the second value (a2, c2, c2', ab2) indicates the comparison of the homozygous major allele group with the homozygous group for the minor allele. By convention the path c is

called the total effect, the path c' is called the direct effect and the path of a and b combined is called the indirect effect. Path a is the relationship between the SNP and the methylation value and path b is the relationship of the methylation value and the level of EPA (Figures 3 and 4).

Based on the PROCESS results for rs174546 only the minor allele in a homozygous state shows a significantly different EPA level from the reference group (homozygous major allele) for the total effect (c2: β = -13.96, $p$ = 0.020). The negative regression coefficient β indicates that carriers of the minor allele have lower levels of EPA, which is well in line with the analysis depicted in Table 12 and Table 13. After entering the mediator into the model, rs174546 predicted the methylation level (path a), which can be seen for both genotypes heterozygous (a1: β = -0.15, $p$ < 0.001) and homozygous (a2: β = -0.33, $p$ < 0.001) for the minor allele. In both cases the minor allele reduces the amount of methylation, and the effect is stronger in the homozygous state compared to the heterozygous state. The methylation level in turn predicted EPA levels (path b) significantly (b: β = 52.07, $p$ = 0.015). The positive regression coefficient β indicates that a higher level of methylation is associated with higher levels of EPA. The relationship between rs174546 and EPA level is fully mediated by the level of methylation for both minor allele states, indirect effect ab1 (95 % CI -14.53, -2.11) and ab2 (95 % CI -31.81, -4.49), as zero is not included between the lower and upper limit of the bootstrapping CI (Table 16).

The same directions and effects can be observed with rs968567. The EPA level for the minor allele in a homozygous state is significantly different from the reference group with a β of -21.38, $p$ = 0.023 (path c2). After entering the mediator into the model, rs968567 predicted the methylation level in both genotypes heterozygous (a1: β = -0.21, $p$ < 0.001) and homozygous (a2: β = -0.47, $p$ < 0.001) for the minor allele. The β values for path a are slightly higher compared to rs174546 or rs174611 indicating that rs968567 might have a stronger effect on the level of methylation of the used CpG sites than the other two SNPs. Within the model with rs968567, the methylation level predicted EPA levels significantly, b: β = 72.43, $p$ = 0.006. The effect of rs968567 on EPA level is fully mediated by the level of methylation for both minor allele states, indirect effect ab1 (95 % CI -24.94, -5.74) and ab2 (95 % CI -55.79, -12.81, Table 16).

In this analysis the EPA level for the minor allele of rs174611 in a homozygous state is on a trend level different from the reference group with a β of -12.54, $p = 0.079$ (path c2), indicating that the effect on EPA levels might not be as pronounced as it is for the other analysed SNPs. As can be seen for the other two SNPs the methylation level predicted EPA levels significantly, b: β = 50.31, $p = 0.006$. The effect of rs174611 on EPA levels, even though not significant, is fully mediated by the level of methylation for both minor allele states, indirect effect: ab1 (95 % CI -11.42, -1.88) and ab2 (95 % CI -28.01, -4.85, Table 16).

Similar to the analysis done for EPA, the analysis was also carried out for ARA levels but without any mediation by methylation occurring (results not shown).

**Table 16:** Mediation effects of DNA methylation levels on the association between *FADS1/2* SNPs and EPA levels

| Geno-type | Path a a1 (X1), a2 (X2) | | Path b | | Total effect c1 (X1), c2 (X2) | | Direct effect c1' (X1), c2' (X2) | | Bootstrap analysis: Indirect effect ab1 (X1), ab2 (X2) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β, *t*() | *p* | β, *t*() | *p* | β, *t*() | *p*1 | β, *t*() | *p*2 | Boot LLCI | Boot ULCI |
| rs174546, *n* = 242 | | | | | | | | | | |
| X1 | -0.15, *t*(235) = -10.72 | < 0.001 | 52.07, *t*(234) = 2.44 | 0.015 | -0.91, *t*(235) = -0.20 | NS | 6.92, *t*(234) = 1.24 | NS | -14.53 | -2.11 |
| X2 | -0.33, *t*(235) = -18.29 | < 0.001 | | | -13.96, *t*(235) = -2.342 | 0.020 | 3.21, *t*(234) = 0.35 | NS | -31.81 | -4.49 |
| rs968567, *n* = 243 | | | | | | | | | | |
| X1 | -0.21, *t*(235) = -18.12 | < 0.001 | 72.43, *t*(234) = 2.78 | 0.006 | -3.35, *t*(235) = -0.72 | NS | 11.82, *t*(234) = 1.65 | NS | -24.94 | -5.74 |
| X2 | -0.47, *t*(235) = -20.39 | < 0.001 | | | -21.38, *t*(235) = -2.30 | 0.023 | 12.51, *t*(234) = 0.82 | NS | -55.79 | -12.81 |
| rs174611, *n* = 243 | | | | | | | | | | |
| X1 | -0.13, *t*(235) = -7.85 | < 0.001 | 50.31, *t*(234) = 2.80 | 0.006 | -2.06, *t*(235) = -0.46 | NS | 4.33, *t*(234) = 0.86 | NS | -11.42 | -1.88 |
| X2 | -0.32, *t*(235) = -12.66 | < 0.001 | | | -12.54, *t*(235) = -1.76 | 0.079 | 3.63, *t*(234) = 0.40 | NS | -28.01 | -4.85 |

Abbreviations: EPA, eicosapentaenoic acid; *FADS1/2*, fatty acid desaturase 1/2; LLCI, Lower Limit Confidence Interval; NS, not significant; SNP, single nucleotide polymorphism; ULCI, Upper Limit Confidence Interval.

# 4 Discussion

The current study examines the FA composition in blood serum and its associated risk for developing dementia/AD in elderly people above 80 years of age. Given the research design of the original AgeCoDe study, the analysed sample can be considered representative of community-dwelling older subjects (Jessen, 2010). Hence, the FAs measured are based on the participants' dietary habits and not on administered food. Late-life dementia is caused by multiple factors that can be divided into non-modifiable factors like age, sex and genetic predisposition, and modifiable factors that have been described in detail in the introduction. In the current study sample the mean age of the dementia group is one year higher at baseline compared to dementia-free controls ($t(1248) = -4.0$, $p < 0.001$, $d = -0.23$), and there are more females in the dementia group ($p = 0.003$, $\chi^2$ test, Table 3). The age of onset in the dementia group and the age at last follow up in the dementia-free group do not differ. Overall, the sample characteristics are as expected for a study on dementia at this age group. It has long been observed that more women than men have dementia/AD. The overall lifetime risk for AD irrespective of *APOE* genotype is 11 % for men and 14 % for women by the age of 85 (Genin *et al.*, 2011). Briefly, it is discussed that on average women live longer than men and older age is still the greatest risk factor for dementia/AD, while conversely many studies found no significant difference between men and women in the actual risk of developing AD at the same age. Some studies suggest differences in other related health factors that attribute to the different risks of developing dementia/AD. For example, men have a higher rate of death from CVD in middle age, causing a survival bias because older men included in studies tend to be healthier and may have a lower risk of developing AD, as described in the introduction (Alzheimer Association, 2019). Furthermore, it is possible that lower education levels in women than in men born in the first half of the 20th century could account for a higher risk of AD and other dementias in women, as low education is a risk factor for dementia (Rocca *et al.*, 2014).

It is important to note that individual factors do not add up to an overall risk for disease; instead, there is often a complex interplay between genetic, environmental, and lifestyle factors that makes it challenging to develop or provide disease-modifying therapies. An extensive body of evidence has recently emerged on the beneficial properties of ω-3 FAs

on non-communicable diseases connected to dementia, such as diabetes and CVD, and on cognitive decline and dementia itself (Scarmeas *et al.*, 2018). In general, FA levels are modulated by genetic and epigenetic factors that affect the metabolism or the lipid transport, but the major modulator is diet itself. There have been abundant observational studies, interventional studies, and experimental studies on ω-3 FAs and their effect on cognitive functioning and clinical endpoints such as dementia and AD, but studies in elderly above the age of 80, like in the AgeCoDe cohort, are limited (Cederholm *et al.*, 2013; Loef and Walach, 2013b; Cederholm, 2017; Calder, 2018). Furthermore, the majority of the studies performed limit the scope to ω-3 FAs whilst ignoring the background diet, even though ω-6 FAs have been shown to have a strong influence on ω-3 FA metabolism (Rahbar *et al.*, 2017; Greupner *et al.*, 2018b). Moreover, ω-3 and ω-6 compete for the incorporation into the plasma membranes, where the FA composition modulates the physical properties of membranes, and ω-3 and ω-6 play opposing roles in inflammatory processes in the human body (Simopoulos, 2010; Dyall, 2015; Zárate *et al.*, 2017; Desale and Chinnathambi, 2020). To consider these relationships, the FAs in the present work were used in ratios to each other, and a PUFA-based index that includes both ω-3 and ω-6 of functional relevance was used as health risk index for dementia. This index can be seen in part as a dietary proxy that involves more than just fish consumption and is therefore gaining importance in the discussion of dietary patterns and their health-promoting effects. Furthermore, the present study is, to the best of our knowledge, the first to investigate a possible mediating role of methylation on PUFA levels in relation to dementia/AD in elderly people.

### 4.1 Fatty acids as dietary biomarkers and modifiable risk factors for dementia

Beside the 12 risk factors described in the introduction that potentially prevent or delay 40 % of dementias, diet is a fundamental factor for health and disease that influences many modifiable risk factors, including obesity, diabetes and CVD. A clear but small number of studies investigated the balance between ω-6 and ω-3 FAs, or dietary patterns that inherently achieve balanced tissue proportions of ω-6 and ω-3 FAs, and their influence on health conditions (Loef and Walach, 2013b; Lands, 2014; Micha *et al.*, 2014; Solfrizzi *et al.*, 2017; Scarmeas *et al.*, 2018). Research has shown that chronic diseases such as CHD, hypertension, cancer, arthritis, allergies and other autoimmune diseases are associated with PUFA composition of phospholipids. Furthermore, PUFAs play an

important role in normal growth and brain development (Simopoulos, 2010). Moreover, ω-3 FAs are inversely associated with cognition and the risk of dementia (Cederholm *et al.*, 2013; Dyall, 2015; Cederholm, 2017; Zárate *et al.*, 2017; Calder, 2018), but only a limited number of studies have investigated the balance between ω-6 and ω-3 FAs and the conferred risk for cognitive decline and neurodegenerative diseases as reviewed by Loef *et al*. (Loef and Walach, 2013b). This association is supported by the increasing recognition of the pleiotropic effects of ARA in the brain (Thomas *et al.*, 2015, 2016; Olivier *et al.*, 2016).

In the current study, the relative risks conferred by ω-6 and ω-3 FAs and their ratios (balance) were estimated by Cox regression analysis. The results indicate that the balance between ω-6 and ω-3 is a key to cognitive health measured by the risk of dementia (Table 9). This can be seen in the proportional amount of ω-6 (%ω-6) in blood serum phospholipids and particularly in the ratio LA/ALA as a marker for the uptake of EFAs that can only be obtained from the diet. Therefore, the ratio of these two EFAs is a pure indicator for dietary habits of a subject or a population of a geographical region (country). The endogenous synthesis of PUFA and HUFAs starts with the EFAs LA (ω-6) and the ALA (ω-3) (Figure 1). EFAs and their long-chain derivatives are important components of membranes, and often have important opposing physiological functions, which demands a balance of EFA for good health and normal development (Simopoulos, 2003). The results of the present study further support the importance of a balanced intake of these two EFAs. Both EFAs are not associated with the risk of dementia when analysed independently from each other, but the ratio LA/ALA is and yields an OR of 1.82, which is almost as high as for EPA, which has the highest OR of 2.13 in this study (Table 9).

The ratio ARA/EPA has long been recognized as a surrogate marker of balance between ω-6 and ω-3 FAs and as a risk factor for CVD and vascular cognitive impairment and dementia (Simonetto *et al.*, 2019). In the present study, the ratio of ARA/EPA is nominally significantly associated with the risk of dementia. The highest tertile increases the risk 1.75-fold compared to the lowest tertile (Table 9). Interestingly, none of the individual ω-6 FAs are associated with the risk of dementia, but in relation to ω-3 they seem to play a role for the risk of developing dementia. The Cox's regression analysis of the %ω-6 value shows a significant higher risk of converting to dementia with an OR of 1.86 for individuals

in the highest tertile of the %ω-6 in PUFA (Table 9). This study finds that unfavourable FA levels or ratios increase the risk of dementia by about two-fold, independent of the association analysed (individual FA, ratios, %ω-6 index). These results are well in line with the aforementioned studies on ω-3 FAs and general health, and emphasizes the importance of the balance between ω-3 and ω-6 FAs.

There are no food questionnaires available for the AgeCoDe cohort, which makes it difficult to assess if the measured serum phospholipid FAs are coherent with the diet of the participants. Apart from this “weakness”, current research is moving away from the relatively imprecise assessment of PUFA intake through food questionnaires and instead advises for the monitoring of the diet by directly measuring PUFAs from blood samples, as questionnaires do not capture well the varying levels of EPA and DHA contained in different types of fish (Hedrick *et al.*, 2012; Morris, 2012). Dietary changes are detectable in various tissues after a certain amount of time. As described in the introduction the FA composition of the serum cholesterol esters and phospholipids is related to the average dietary FA composition during the last 3 to 6 weeks (Vessby *et al.*, 2002; Calder, 2018). Thus, the measured FA profiles in the present study reflect the diet of each participant during the preceding weeks. Studies showed that FAs measured in blood phospholipids correlate with relative intake. Therefore, the FA profiling in blood serum phospholipids as it has been done in the current study is a reliable, easy to obtain and minimally invasive biomarker for dietary intake of FAs that is independent of self-reported dietary intake errors (Cunnane *et al.*, 2012; Hedrick *et al.*, 2012; Brenna *et al.*, 2018).

### 4.2 Modern Western Diet and dietary patterns determine fatty acid levels

In the AgeCoDe cohort, the measured relative amounts (wt%) of LA and ARA were the highest ω-6 FAs with ~18 % and ~9 %, respectively. The SFAs PA and SA are also observed with high amounts of ~30 % and ~13 %, respectively. The ω-3 FAs ALA, EPA and DHA, in contrast, are meaningfully lower with relative amounts of ~0.15 %, ~1 % and ~3.5 %, respectively (Table 3). These observations in the AgeCoDe cohort of excess ω-6 FAs, primarily LA and ARA, and lower amounts of ω-3 FAs in form of ALA, EPA and DHA are well in line with studies showing that the intake of LA and ALA that used to be balanced with a ratio of 1:1, changed drastically in modern Western societies, where this ratio exceeds 10:1 due to high intake of vegetable oil products which are high in LA (Hibbeln

*et al.*, 2006; Simopoulos, 2010; Blasbalg *et al.*, 2011; Sheppard and Cheatham, 2018). Furthermore, only three cereals (wheat, maize, and rice) together account for more than 75 % of the world's grain production and are high in ω-6 FAs, but low in ω-3 FAs and in antioxidants, particularly in comparison to leafy green vegetables (Simopoulos, 2003). Beside seed oils, eggs, poultry, and meat contain high amounts of the ω-6 FAs LA and ARA (Cordain *et al.*, 2005; Hibbeln *et al.*, 2006). Therefore, unfavourable FA profiles most likely originate from the present-day MWD.

In the German National Nutrition Survey II, a total of 19,329 men and women aged between 14 and 80 years were interviewed throughout Germany between November 2005 and January 2007, providing substantial information about the nutritional situation in Germany (Stehle, 2014). In general, meat consumption exceeds the food-related benchmarks of the German Nutrition Society (DGE), thereby increasing ω-6 intake. For men, the consumption of meat, meat products and cold cuts clearly exceeds the benchmark (300 g to 600 g per week) and lies near the upper limit with women. Young males (aged 15 to 19 years) and senior citizens (65 to 80 years) have a lower meat intake compared to the other male age groups. Correspondingly, ARA and LA intake follows exactly this pattern. In women, ARA intake is the lowest in the youngest age group, and from there rises until it peaks in the oldest age group. LA shows the same pattern as in men, but overall one quarter less in total amount (Stehle, 2014). Both men and women do not reach the quantities recommended for fish intake on average. The DGE food-related benchmark is 80 g to 150 g of low-fat saltwater fish per week and 70 g high-fat saltwater fish per week. Men eat on average slightly more fish and fish products, crustaceans and shellfish than women. Most fish and fish products, crustaceans and shellfish are eaten by men and women in the age group 51 to 80 years, and accordingly, the highest values of EPA and DHA can be seen in this age group in both men, and women (Stehle, 2014). This fish intake results in considerably lower values than the references from various global organisations that recommend between 250 mg/day and 1,000 mg/day of EPA and DHA (Calder, 2018).

A study from the USA that quantified the changes in the apparent consumption of EFAs during the 20th century from 1909 to 1999 showed major increases in the consumption of poultry, margarine, shortening, and beef tallow; here the estimated per capita

consumption increased by 454 %, 1038 %, 170 %, and 371 %, respectively. The general consumption of oil increased, with canola and soybean oil being the primary driver of the overall increase of oil consumption. Canola was introduced to the market in 1986 and within the 13 years until 1999 the per capita consumption increased 167-fold. Remarkably, the per capita consumption of soybean oil increased 1163-fold during the 20th century. These foods have high amounts of LA and together with the high consumption of grains the ratios of total ω-6/ω-3 and LA/ALA increased 77 % and 55 %, respectively. In addition, the meat from modern grain-fed cattle and livestock has a high absolute SFA content, low ω-3 FA content and high ω-6 FA content (Cordain *et al.*, 2005; Blasbalg *et al.*, 2011). In terms of ω-3 and ω-6 FAs, the MWD can thus be summarized as follows. Due to the low consumption of fish, few ω-3 FAs are absorbed. At the same time, a high intake of ω-6 FAs occurs through the consumption of cereals, vegetable oils, poultry, pork and beef.

Table 3 shows two %ω-6 indices that each can be used as a food-based health risk assessment (see chapter 4.3), one based on percentage values of total FA (wt%), and the other based on concentration values of FAs. Expressing FAs as concentration values differs from the technical report of FA composition, which describes relative amounts of FAs as a percent by weight (wt%, percentage) of all FAs analysed in a sample. The wt% value has a simple “housekeeping” rationale, but it has no clear metabolic or biological significance (Bibus and Lands, 2015; Brenna *et al.*, 2018). Therefore, although percentage values of total FAs is by far the most common way of describing FA profiles, the evaluation of FA concentrations can be used as a more detailed type of assessment (Marangoni *et al.*, 2007; Brenna *et al.*, 2018). Hence, all statistical analyses in the present study were performed with concentration values. In order to compare the FA levels with other studies, percentage values (wt%) were also given for the FAs shown in Table 3. The %ω-6 index is based on FAs which are part of the synthesis pathway of ω-3 and ω-6 PUFAs in humans (Figure 1). Focusing on the synthesis pathway has several rationales. The exclusion of saturated, monoenoic and dienoic acids avoids noise from FAs that have little to do with the aetiology of dementia/AD. This is not to say that none of the excluded FAs is associated with dementia/AD, but that using them all in a sum score obscures the meaningful FAs behind noise as might be seen with the sum scores of SFAs, MUFAs and PUFAs in Table 3, where there is no difference between the two groups. In order to determine a simple marker of both ω-3 PUFA intake and status, the %ω-6 index has been

proposed (Marangoni *et al*., 2007, see chapter 2.7.1), differing from the previously established ω-3 index (EPA + DHA as wt% values) that serves as a CHD risk factor (Harris and Von Schacky, 2004; Harris *et al.*, 2018), and is nowadays also used for dementia/AD (Tan *et al.*, 2012; Hooper *et al.*, 2017; Thomas *et al.*, 2020). Both %ω-6 indices in Table 3 are slightly elevated in the dementia group, but only the %ω-6 index based on concentration values is significantly higher in the dementia group. Cox proportional hazard regression analysis showed that the %ω-6 index (concentration) is significantly associated with the risk of dementia/AD (Table 9), indicating that the balance between ω-6 and ω-3 FAs is critical for the risk of dementia.

The MWD is contrasted with diets that naturally contain a more balanced ratio of ω-6 and ω-3 FAs due to their dietary components and quantities. Consuming certain dietary patterns determines the tissue composition as shown for the blood PUFA proportions (Lands and Lamoreaux, 2012). The lowest proportion of ω-6 is found for example in the Greenland diet and the traditional diet in Japan with levels ranging from 28 % to 45 %. Through westernization of eating habits the modern Japanese diet results in a higher proportion of ω-6 between 40 % to 55 % (Drewnowski and Popkin, 1997; Lands and Lamoreaux, 2012). This is followed by the Mediterranean diet resulting in proportions of ω-6 between 50 % to 70 %. The MWD consumed in Northern Europe and the USA results in higher tissue levels, rising up to over 80 % in the proportion of ω-6 (Lands and Lamoreaux, 2012). With increasing levels of ω-6, the risk for diseases of the cardiovascular system and brain increases, supporting the findings that traditional dietary habits from Japan and the Mediterranean Sea maintain a form of food-based primary prevention of CVD and dementia (Lands, 2003, 2014).

### 4.3 The balance of competing ω-6 and ω-3 as a useful health risk assessment score

Even though ALA is present in high amounts in some plant oils, particularly linseed, chia, perilla and walnut oil, the conversion process to EPA and particularly to DHA is generally low (Burdge, 2004; Simopoulos, 2011; Gillingham *et al.*, 2013), and therefore the main source of EPA and DHA is fatty sea fish. This may explain the moderate correlation of ALA and EPA and the low correlation between ALA and DHA in the current study sample (Table 4), whereas EPA and DHA are taken up together with fish dishes, resulting in a

higher correlation between the two FAs. The same is true for ω-6 FAs: these are all taken up in higher amounts with the typical MWD resulting in higher correlations as seen in Table 4 (Cordain *et al.*, 2005; Blasbalg *et al.*, 2011; Tallima and El Ridi, 2018). In addition, the excess of LA leads to a greater net conversion of LA compared with ALA, even though ALA is the preferred substrate for the desaturase enzyme (Burdge, 2004). Table 5 shows that the number of people with high EPA levels is declining with higher ratios between LA and ALA, with the same decreasing trend for DHA from low to high levels of LA/ALA, but not significant, whereas a high ratio of LA/ALA is associated with high levels of ARA, high levels of the ratio ARA/EPA and a high level of the proportion of ω-6 HUFA. Note that this proportion of ω-6 variable is different to the otherwise used %ω-6 variable for statistical analysis. Here, the proportion of ω-6 HUFA includes only 20-carbon and 22-carbon FAs, namely DGLA, ARA, EPA, DHA, to look specifically at the effect of the ratio LA/ALA on higher carbon FAs.

A recent interventional study investigated the effect of low and high dietary LA/ALA ratio on long-chain PUFA concentrations in healthy men (Greupner *et al.*, 2018b). In order to control for FA intake, the participants were requested to abstain from fish, seafood and ALA-rich plant oils. They used a cross-over design, so each subject acted as its own control, which minimizes interindividual variability of blood FA levels. Two distinct diets were administered as daily lunches: the first diet corresponded to an LA/ALA ratio of 0.56 ± 0.27:1, which resembles the widely discussed palaeolithic ratio for LA/ALA of 1:1, and the second diet consisted followed a ratio of 25.6 ± 2.41:1, which more closely follows a typical MWD where this ratio exceeds 10:1 (Simopoulos, 1999; Cordain *et al.*, 2005). The low-LA/high-ALA diet was effective in increasing ALA and EPA concentrations, but the DHA concentration remained unchanged. In contrast, when subjects were given the high-LA/low-ALA diet, EPA levels decreased, while the level of DHA did not change. ALA levels decreased within the first seven days but recovered to baseline values after 14 days. Additionally, they looked at levels of ARA, which in their study slightly decreased with the low-LA/high-ALA diet and increased with the high-LA/low-ALA diet, but in both diets the change was not significant (Greupner *et al.*, 2018b). Previously, the same group did an interventional study with a high-ALA diet without LA restriction (Greupner *et al.*, 2018a). In comparison, the intervention study with LA restriction resulted in higher increases of ALA and EPA. Both studies were conducted with the same amount of ALA

and the study sample was similar. They concluded that the incorporation and metabolism of ALA is dependent on LA levels, as they are competing for incorporation into cell membranes and for the D6D enzyme (Greupner *et al.*, 2018b). As a side note, TFAs also interfere with the desaturation and elongation of both LA and ALA, and since the amount of trans fats in the Western world's diet is also high, this leads to an additional decrease in ω-3 metabolism (Simopoulos, 2016). In the study of Greupner *et al*., the authors used red blood cells (RBCs) to measure the FA levels, which has an inherent downside in that the turnover time of RBCs is 120 days, while the intervention phase was two weeks, and thus PUFA changes did not fully reach the RBCs, as the authors themselves concluded (Greupner *et al.*, 2018b). The kinetics of incorporating dietary FAs into tissues varies significantly, and so perhaps changes to ARA and DHA would have been more pronounced with a longer intervention phase. Another explanation for the minor changes in the concentrations of LA, ALA, ARA and DHA compared to baseline levels when observing the high-LA/low-ALA diet is that the interventional diet possibly resembles the participants' regular MWD of 10-20:1 FA ratio, making the almost constant FA pattern plausible (Brenna *et al.*, 2009; Greupner *et al.*, 2018b).

Overall, the discussed results are well in line with the observations of the systematic review that examined the effect of modified dietary LA and ALA intakes on ω-3 PUFA status in human adults. This review suggests that it is possible to increase EPA, but only to some extent DHA, if at all. The authors concluded that the most effective strategy for improving ω-3 PUFA status appears to be a combination of increased ALA and reduced LA intakes (Wood *et al.*, 2015). According to the mutual influence of FAs and their competition for metabolism and physiological effects, a study was performed to estimate healthy dietary allowances for ω-3 PUFAs that would meet the nutrient requirements of 97-98 % of the population (worldwide) and prevent mortality outcomes, seeing that the required increase of ω-3 PUFAs can likely be reduced to one-tenth by consuming fewer ω-6 fats (Hibbeln *et al.*, 2006). Dietary balances of ω-3 and ω-6 result in different proportions of competing PUFA in tissue lipids. In the present study, the proportion of LA and ARA shows a higher variance in relation to the proportion of ω-6 in PUFA (%ω-6), whereas the ω-3 FAs EPA and DHA show a strong dependence on the %ω-6 as can be seen in Figure 5. In particular, DHA and the ω-3 index show a strong dependence on the %ω-6 (Table 7). The MWD is overall high in ω-6 FAs, explaining the high variance of LA

and ARA. Dietary sources for ARA are abundant in the MWD, therefore both dietary uptake and biosynthesis contribute to the levels of ARA explaining the smaller correlation to the %ω-6 seen in Table 7. The same applies for EPA, even though the conversion rate from ALA is lowered due to competition for enzymes in the MWD. With fatty sea fish as the primary source, EPA is less abundant in the diet and tissue concentrations are more dependent on competing ω-6 FAs shown by the higher correlation to the %ω-6 seen in Table 7 compared to ARA. The conversion rate from ALA to DHA is particularly low, which likely explains the high dependence of DHA tissue concentrations on the %ω-6. The amount of measured ALA is very low in the AgeCoDe sample, and therefore the high correlation of -0.99 for the ω-3 index (ALA+EPA+DHA) must result from the formula used (see chapter 2.7.1). These results are in line with a study of 1,015 individuals that showed the strongest associations for ARA, EPA, and DHA with proportion of ω-6 in HUFA (20- and 22-carbon ω-3 and ω-6 FAs). Furthermore, they showed lower proportions of ARA when proportions of EPA and DHA are higher, indicating that ω-3 FAs compete with ARA for accumulation in tissue lipids and thereby for subsequent downstream action (Bibus and Lands, 2015). The authors of that study used percent by weight (wt%, or percentage) values of total FA to calculate the proportion of FA in PUFA. As shown in Table 6 there is no substantial difference between the use of concentration and percentage values when calculating proportions of FAs in the present study, making the results from the current study comparable to the literature. Importantly, the proportion of ω-6 in PUFA and the proportion of FA in PUFA variables are created differently to the literature due to different FAs that were available in the present study. In order to determine a simple marker of both ω-3 PUFA intake and status, a mathematical index considering exclusively the HUFA (20-carbon FAs) has been proposed (Marangoni *et al.*, 2007; Stark, 2008; Lands, 2009). The proposed HUFA-based index is defined as the proportion of total ω-3 HUFA (EPA+DPA+DHA) / total HUFA (total ω-3 HUFA + total ω-6 HUFA [DGLA+ARA+22:4ω-6+22:5ω-6]). In essence, the proposed HUFA-based index appears to be a possible predictor of the ω-3 functional role since the HUFAs (20-carbon and higher FAs) of both the ω-6 and ω-3 series are endowed with greater functional relevance at the cellular level (Marangoni *et al.*, 2007). For the current study, docosapentaenoic acid (DPA) is only available as percentage value (wt%), not as concentration value. Furthermore, the ω-6 FAs 22:4ω-6 and 22:5ω-6 are not available (Figure 1); therefore, it

was not possible to create the same HUFA-based index for the AgeCoDe sample. Another publication describes an Omega 3-6 Balance Food Score that is a single score allowing a quantitative estimate of the impact of each food item on the proportions of ω-3 and ω-6 that accumulate in blood HUFA (Lands and Lamoreaux, 2012). This score is based on the balance among eleven ω-3 and ω-6 FAs in food, including both 18-carbon EFAs LA and ALA as well as the FAs used in the HUFA-based index. By combining the 18-carbon FAs and the available 20-carbon and 22-carbon FAs into the %ω-6 value used in the work presented in this thesis (Figure 1), this value represents a combination between the food-based Omega 3-6 Balance Score and the HUFA-based index that is used as a health risk assessment.

Examples are given in the publication from Lands *et al*. for two different FA profiles and their corresponding sum scores and abundancy values. These FA profiles show no difference in the SFA, MUFA and HUFA sum scores, but the proportion of ω-6 in HUFA (%ω-6) shows a completely different picture. One FA profile represents 79 % of ω-6 in HUFA, whereas the second FA profile represents 49 % of ω-6 in HUFA (Lands, 2014). Even though the total HUFA profiles look the same, the health risk assessment based on the proportion of ω-6 in HUFA is very different between the two example FA profiles. Lands and Lamoreaux showed how daily food choices cause different PUFA proportions. Based on that study, a proportion of ω-6 in HUFA of 79 % corresponds to the food habits of the USA, while a proportion of ω-6 in HUFA of 49 % corresponds to the food habits of the Mediterranean Sea or modern Japanese society. The traditional Japanese diet corresponds to an even lower proportion of ω-6 in HUFA (Lands and Lamoreaux, 2012). The FA profiles of the two examples from Lands *et al*. were used with the %ω-6 index as calculated in the present study; the FA profile of 79 % of ω-6 in PUFA from the publication corresponds to a value of 93 % in the present study, and that of 49 % from the publication corresponds to a value of 77 %. These numbers correspond to the lower and upper range of the %ω-6 values created with wt% values of FAs in Table 6, third column. Regarding the FA profile of participants from the AgeCoDe, it can be concluded that the upper range of the %ω-6 corresponds to the typical MWD, whereas the lower range of the %ω-6 corresponds to the Mediterranean or modern Japanese diet, with a more balanced ω-3 to ω-6 relation. In general, the proportion values in Table 6 created with concentration and wt% values are very comparable. Furthermore, as mentioned above, even though

percentage values of total FA is by far the most common way of describing FA profiles, the evaluation of FA concentrations can be used as a more detailed type of assessment (Marangoni *et al.*, 2007; Brenna *et al.*, 2018). The Cox's regression analysis of the diet-related %ω-6 value shows a significant higher risk of converting to dementia with an OR of 1.86 for individuals in the highest tertile of the proportion of ω-6 in PUFA (Table 9). Thus, the balance of ω-3 and ω-6 acids in blood HUFA, or as measured as serum PUFA in the present study, is a useful dementia health risk assessment biomarker in addition to being a useful measure of daily average intakes of competing ω-3 and ω-6 nutrients in foods (Bibus and Lands, 2015). Furthermore, it is not recommended to use summary variables of sets of FAs like SFA, MUFA, or PUFA as a risk assessment, as they probably do not show any difference in total, despite large differences in terms of individual FAs, or their ratios and proportion variables with greater functional relevance (Table 3).

### 4.3.1 Evolution of the *FADS* gene cluster and its disadvantages when consuming the Modern Western Diet

Ever since Schaeffer *et al*. published their results on the effect of common variants within the *FADS1/2* gene cluster on ω-6 and ω-3 FA levels, several subsequent studies have contributed toward unravelling the functional consequences of genetic variants within this region of desaturase activities. The *FADS* region consists of two linkage disequilibrium (LD) blocks: one LD block includes the entire *FADS1* gene and spans well into the gene *FADS2*, and the second LD block includes the other part of *FADS2* and stretches until the intergenic region between the 3' end of *FADS2* and the gene *FADS3* (Figure 6, Ameur *et al.*, 2012). By utilizing three (two desaturation and one elongation) enzymatic steps, humans convert LA to ARA (Figure 1). The enzymes of the two desaturation steps are encoded by two genes (*FADS2* and *FADS1*) and have long been recognized as the rate-limiting steps in ω-3 and ω-6 PUFA biosynthesis (Hester *et al.*, 2014). The desaturases prefer substrate ω-3 FAs families, but due to competition between the substrates and due to product inhibition, the efficiency of the desaturation is dependent on the competing FAs in the diet and in the tissues (Vessby *et al.*, 2002). All previous studies on *FADS* variants and FA levels showed the same results as the present study, i.e., all precursor FAs, namely LA, ALA and DGLA show elevated levels, and all product FAs, namely GLA, ARA, and EPA show reduced levels when the minor allele of the variants is present (Schaeffer *et al.*, 2006; Merino *et al.*, 2011; Al-Hilal *et al.*, 2013; Cormier *et al.*, 2014). Furthermore,

the surrogate estimates for desaturase activity, based on product-to-substrate ratios, decrease for the minor alleles (Table 12, Table 13), which is well in line with previous studies that showed lower desaturase activity estimates associated with the minor alleles of *FADS* variants (Al-Hilal *et al.*, 2013; Cormier *et al.*, 2014). Additionally, PA is on a trend level elevated when the minor allele is present in the AgeCoDe cohort. Clearly, the SFA/ω-3 ratio is higher in minor allele carriers, which is congruent with the findings that PA is also metabolised by the D6D (*FADS2*) enzyme and thereby affecting the ω-3 metabolism (Park *et al.*, 2016). This shows that the variant rs174546 serves as a plausible proxy for the SNPs in the *FADS1/2* LD block 1 including the *FADS1* gene, and the possible actual functional variant(s) within that LD block 1 (Figure 6).

In 2012, two independent studies published data on the evolution of the human *FADS1/2* gene region. Both studies showed a positive selection of a haplotype associated with an enhanced ability to produce ARA and DHA from their precursor FAs in the lineage leading to modern humans (Ameur *et al.*, 2012; Mathias *et al.*, 2012). Based on five European population cohorts, Ameur *et al*. identified two distinct haplotypes in present-day humans called haplotype A and haplotype D. The latter haplotype consists of derived alleles that are specific for modern humans and separate from the ancestral haplotype A that is present in archaic hominins (Denisovan), African apes, and Neanderthal sequences (Ameur *et al.*, 2012). The second study showed similar results and estimated the time to the most recent common ancestor between chimpanzee and human haplotypes to be 1.49 million years based on the *FADS1* block (Mathias *et al.*, 2012). From Ameur *et al*., the haplotype D of the modern human appeared after the split from Neanderthals (around 500,000 years ago) but prior to the exodus of modern humans from Africa (50,000-100,000 years ago). Estimates of the geographic distribution of haplotypes A and D differ dramatically between continents. Using the human genome diversity panel (HGDP-CEPH) Ameur *et al*. showed that haplotype A is essentially absent in African HGDP populations (1 % of chromosomes), whereas in Europe, West, South, and East Asia, and Oceania, it occurs at a frequency of 25-50 %. Among 126 Native Americans included in HGDP, haplotype A accounts for 97 % of the chromosomes. These results have been confirmed in a complementary analysis in population samples from HapMap and the 1000 Genomes Project (Ameur *et al.*, 2012). In the AgeCoDe sample, the frequency of the high efficiency variant of rs174546 is 42.8 % in the homozygous state (Table 14), which is well

in line with the mentioned studies. Both cited research groups speculated that one or more mutations in the *FADS* cluster and the ability to more efficiently convert plant-based EFAs (LA, ALA) to HUFAs helped to maintain the very rapid increase in brain size of hominoids that probably involved selection of several loci to support the greater need of HUFAs. Furthermore, this was an important advantage when expanding and settling in a variety of ecological locations (Ameur *et al.*, 2012; Mathias *et al.*, 2012). The present-day geographic distribution of these haplotypes indicates that both haplotype A and haplotype D were present in Africa at the time of the exodus of modern humans, therefore, both haplotypes are present in European, Asian, and Oceanian populations. The low frequency of haplotype D in the Native American populations included in HGDP indicates that this haplotype might have been lost because of a bottle-neck effect in the colonization of the American continent, and possibly in combination with reduction of the selective pressure as a result of a diet higher in important HUFAs (Ameur *et al.*, 2012; Mathias *et al.*, 2012). The advantageous mutations within the *FADS* gene region swept to fixation within African but not in European or Asian populations, suggesting this enhanced metabolism is the driving force behind positive selection at the *FADS* gene cluster (Mathias *et al.*, 2012). Importantly, SNP rs174546, which has been used in the present study, tags the derived haplotype D in Africans (Ameur *et al.*, 2012) and is in high LD with the derived allele of rs174537, a SNP located in the middle of the selection peak in the study of Mathias *et al*. (Mathias *et al.*, 2012). Both studies speculated as to why the evidence of selective pressure is restricted within Africa and not visible outside of it, but in 2017 a study showed that SNPs associated with increased expression of *FADS1* and increased production of ARA and EPA have been favoured in Europeans since the Bronze Age (Buckley *et al.*, 2017). Furthermore, the authors showed that selective pressures in Europe, Greenland, Africa, and South Asia have driven allele frequency changes in different *FADS* SNPs that are in weak LD with each other, indicating that there are many SNPs in the region affecting FA desaturase activity that can serve as substrates for selection under varying environmental and dietary conditions. The selection in Europe for alleles potentially associated with an improved capacity to increase EPA may be an adaptation to the introduction and spread of agriculture in Europe that likely produced a radical dietary shift away from fish or mammals to plant sources (Buckley *et al.*, 2017). The strong selection on common alleles segregating in Europeans in the *FADS* region

suggests that these alleles may be important in determining the relative nutritional benefits of diets differing in their FA composition, and that these variants might help guide the development of individualized diets informed by genomics (Buckley *et al.*, 2017). Ameur *et al*. also pointed out that the acquisition of a *FADS* haplotype, which is beneficial when food sources rich in the important HUFAs were limited, would now, with regard to the present MWD, be a disadvantage and represent a risk factor for lifestyle-related diseases (Ameur *et al.*, 2012). In fact, one year before the results of *FADS* haplotype analysis for different populations around the globe were published, Mathias *et al*. published a study already discussing the high allele frequency in African Americans of the G allele for rs174537, which is associated with enhanced conversion of DGLA to ARA. The study included African Americans ($n$ = 174) and European Americans ($n$ = 155), wherein, 79-82 % of African Americans carried two copies of the G allele compared to only 42-45 % of European Americans. African Americans had significantly higher circulating levels of plasma ARA and lower DGLA levels than European Americans, and the *FADS* locus revealed significant association with ARA, DGLA and the ARA/DGLA ratio (Mathias *et al.*, 2011). Importantly, the enzymatic efficiency (measured with ARA/DGLA ratio) was similar in both groups and the SNP association with FA levels was highly significant in both groups. Therefore, the authors concluded that the impact of *FADS* genetic variants on PUFA metabolism is likely more pronounced in African Americans due to the larger proportion of individuals carrying the genotype associated with increased *FADS1* enzymatic activity (Mathias *et al.*, 2011). The *FADS1/2* genes are of particular interest because variants within that region have a huge impact on FA levels, especially ARA. Schaeffer *et al*. showed that the variability of ARA levels is explained by the genetic variants of *FADS1/2* by almost 30 % (Schaeffer *et al.*, 2006). As a tagging SNP, rs174546 explained 7.4 % and 10.3 % of the variability of ARA in the entire AgeCoDe dataset and the subsample, respectively. Therefore, as can be seen in the present data (Table 14) and the cited articles, almost half of the German (Eurasian) population carry the high efficiency variants (haplotype D), indicating that for almost half of the German population the derived *FADS* genotype may be disadvantageous in combination with the MWD.

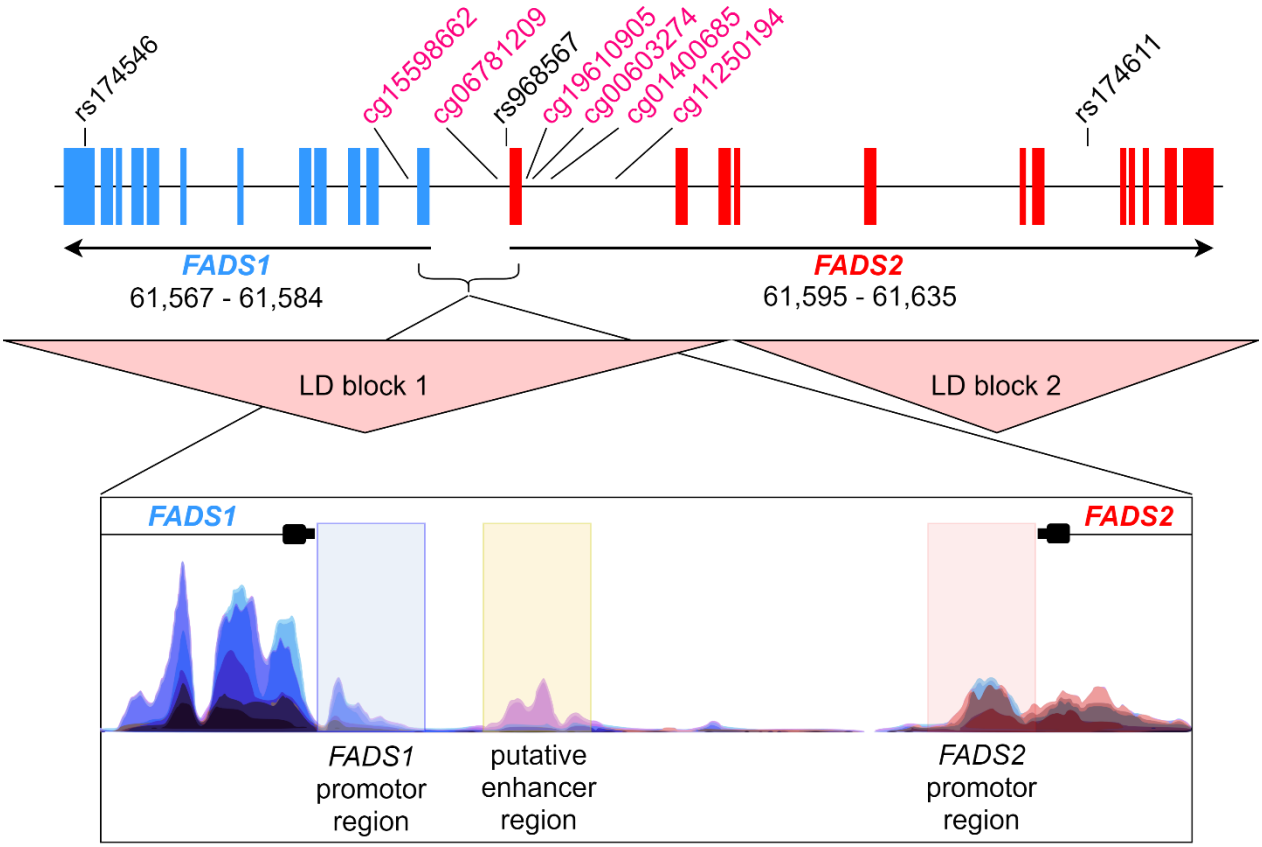


**Figure 6:** Illustration of the *FADS1/2* gene cluster. The three SNPs used for association analysis with FA levels are marked in the upper illustration, as well as the CpG sites of the MetaMeth/mediation analysis. The haploblocks are indicated by LD block 1 and 2. The lower illustration highlights the key regulatory regions depicted by the H3K27Ac cell line from the ENCODE project between the *FADS1/2* genes with the *FADS1* promotor region (blue), *FADS2* promotor region (red) and the putative enhancer region (yellow).

### 4.3.2 Biological interactions of *APOE* with PUFAs are not observed

The *APOE* gene is the strongest common genetic risk factor for AD, of which the *APOE* ε4 allele confers the risk (van der Lee *et al.*, 2018b). In the current study, the frequency of the *APOE* ε4 allele in individuals that are dementia-free is 16.2 % and in the dementia group the frequency is 26.5 % (Table 3). In the general population only 20-25 % carry one or more *APOE* ε4 alleles, whereas 40-65 % of AD patients are *APOE* ε4 carriers (Van Cauwenberghe *et al.*, 2016). The frequencies measured in the current study (16.2 %, 26.5 %) are both lower. The *APOE* ε4 allele confers an increased risk of AD development with an earlier age of onset, and additionally the *APOE* ε4 genotype combines synergistically with atherosclerosis (ATH), peripheral vascular disease, or diabetes in contributing to an increased risk of AD and possible compounding effects of the *APOE* ε4 risk allele and CVD on cognitive decline in AD (Liu *et al.*, 2013). It can be assumed that

these effects combined lead to an earlier disease onset or death of the individuals leading to a reduced frequency of the *APOE* ε4 allele at higher age that is observed in the current study. Furthermore, physiological mechanisms and lifestyle factors presumably help people to surpass the age span with the highest OR for the *APOE* ε4 allele that has been observed for the ages of 60 to 70 years and declines after the age of 70 (Farrer *et al.*, 1997; Genin *et al.*, 2011).

*APOE* plays an important role in the intercellular transport of lipids, lipid metabolism, and lipid homeostasis in various body tissues including the brain, and it has been suggested that the pathological effects of *APOE* ε4 are driven by a lipid-related mechanisms (Barberger-Gateau *et al.*, 2011; Grimm *et al.*, 2017). Epidemiological studies are inconsistent on the effect of sea food consumption and the risk of cognitive decline/AD, and some report stronger effects in *APOE* ε4 non-carriers (Morris, 2012; Samieri *et al.*, 2013). Therefore, it has been investigated if the *APOE* genotype interacts with PUFA levels, leading to an altered risk of dementia/AD. The first approach used six group combinations between *APOE* as dichotomous genotypes and tertiles of FA levels and ratios in a Cox regression analysis. An indication for interaction would be identified if the OR of the most extreme group (*APOE* ε4 + highest or lowest tertile) clearly differs from the sum of the individual ORs of the *APOE* ε4 allele and the respective FA (ratio). The analysis of EPA and the ratio SFA/ω-3 were the only two analyses that resulted in a higher OR in the group with the *APOE* ε4 allele compared to the sum of the individual ORs. In the analysis of EPA, the OR for the lowest tertile of EPA is 2.1(Table 9), the OR for *APOE* is 2.6 (not shown), whereas the combination of the *APOE* ε4 allele and the lowest tertile of EPA has an OR of 6.7 (Table 10), which is higher than the calculated OR of 4.7 combined with the individual ORs. The highest tertile of the SFA/ω-3 ratio has an OR of 2.1 (Table 9) and the OR for *APOE* is 2.7 (not shown) in that analysis, whereas the combination of the *APOE* ε4 allele and the lowest tertile of EPA has an OR of 6.2 (Table 10). These two results may suggest an interaction, but it is hard to tell if the increase of the OR is different enough to deduce an interaction. However, the logistic regression model and the Cox regression model are exponential and are inherently multiplicative, thus, an interaction implies departure from multiplicativity rather than from additivity, with the latter being a measure of biological interaction between risk factors (Andersson *et al.*, 2005). Therefore, the Cox regression analysis was conducted and

defined similar to Andersson *et al*., in order to calculate the RERI index and thereby assess biological interaction. The ratio SFA/ω-3 is the only analysed FA (ratio) to show a significant interaction with the *APOE* ε4 allele, with ARA and the %ω-6 being close to significant (Table 11). Several studies suggest that *APOE* confers its risk on vascular and cognitive health through its influence on SFAs and serum lipoproteins rather than through its influence on PUFAs (Tso *et al.*, 1998; Shatwan *et al.*, 2017; Satizabal *et al.*, 2018). Epidemiological studies showed the strongest, most consistent evidence for the association between cognitive aging and dementia with the dietary intakes of vitamin E, fish and ω-3 FAs, the B-vitamins, particularly folate and vitamin B12, and a high ratio of polyunsaturated to saturated fats (Morris, 2012). This ratio of polyunsaturated to saturated fats is represented by the ratio SFA/ω-3 in the current study, making it likely that the previously observed modifying effects of the *APOE* genotype on the association between ω-3 FAs and dementia were influenced by the background diet of ω-6 FAs and SFA. The influence of *APOE* on SFAs rather than ω-3 PUFAs is well in line with two recent studies that showed no influence of the *APOE* ε4 allele on DHA, or other circulating metabolites, and the association with general cognitive ability, all-cause dementia and AD (van der Lee *et al.*, 2018a), or on the association of fish intake with cognitive decline, global cognition and memory (Samieri *et al.*, 2017).

Even more encouraging are two studies published in 2019 showing that a favourable lifestyle reduces the risk of dementia independently from the genetic risk. One study was conducted with data from participants of the Rotterdam Study, a prospective population-based cohort study in Rotterdam (Licher *et al.*, 2019), and the other was conducted with data from the UK Biobank, a population-based cohort with participants from assessment centers across the United Kingdom (Lourida *et al.*, 2019). Both studies established three categories of genetic risk: low, intermediate, and high based on GWAS studies on AD. Furthermore, both studies generated healthy lifestyle categories from modifiable dementia risk factors including smoking status, physical activity, diet, and alcohol consumption (UK Biobank) and furthermore depression, diabetes, and social isolation (Rotterdam study) to analyse if a healthy lifestyle may offset genetic risk for dementia. In both studies, the polygenic risk score (PRS) and a healthy lifestyle were independently associated with the risk of incident dementia. Both studies added diet to the list of modifiable risk factors

underscoring the results from Livingston *et al*. that a healthy lifestyle reduces the risk of dementia (Livingston *et al.*, 2020) even independently from the genetic risk.

### 4.3.3 *FADS* methylation mediates EPA levels

Throughout the evolution of modern humans, the distance between *FADS1* and *FADS2* has been reduced through deletion to only 11 kb in humans from over 75 kb in rhesus macaques and chimpanzees. This deletion brought the promoters of *FADS1* and *FADS2* closer to each other, and may have resulted in a coordinated regulation of *FADS* expression (Ameur *et al.*, 2012) and potentially increased the efficiency of FA metabolism in modern humans. Genetic variants within the *FADS* gene cluster are important regulators of long-chain PUFA biosynthesis in the liver and consequently have been associated with circulating PUFA levels (Schaeffer *et al.*, 2006; Tanaka *et al.*, 2009; Lemaitre *et al.*, 2011; Hu *et al.*, 2016). Interventional studies showed that ω-3 supplementation changes the desaturase activity for both key enzymes D6D and D5D (Al-Hilal *et al.*, 2013; Cormier *et al.*, 2014). In the AgeCoDe sample used in the current study ω-3 FA levels are also associated with the desaturase activity estimates (data not shown). These observations raise the question of how both genetic variants and FAs can influence enzyme expression/activity. Epigenetic modifications such as DNA methylation or histone modifications can influence gene expression levels (Portela and Esteller, 2010). Therefore, mediation analysis was used in the AgeCoDe sample to investigate if the strong association between variants in the *FADS1/2* genes and EPA levels are mediated by DNA methylation. At the time of analysis DNA methylation data was available for a subsample of 299 subjects from the AgeCoDe cohort (Figure 2). Here, the use of the MetaMeth function (Freytag *et al.*, 2018) identified six CpG sites that were significantly associated with EPA levels (Table 15). A sum score of these six CpG sites with methylation data from the present study was used as the mediator variable in the mediation analyses.

The results from the mediation analyses show that the minor allele of all tested SNPs reduces the level of methylation based on the used methylation score indicated by the negative β of path a (Table 16). It also shows that the effect is stronger for the homozygous genotype of the minor allele (X2, path a2) than for the heterozygous genotype (X1, path a1), and thus methylation is lowest for the minor allele in the

homozygous genotype. The methylation level was positively associated with EPA levels for all analysed SNPs, as indicated by a positive β for path b (Table 16). In the mediation model, the homozygous minor allele genotype showed significantly lower EPA levels compared to the homozygous major allele genotype which was set as the reference group. For the variant rs174611, this association had a *p*-value of 0.079, which may arise from a lower association of this particular SNP with EPA levels and therefore probably due to lack of power for this variant. This path between the SNP and EPA levels is called the total effect, or path c. When the mediator is added to the model, the total effect is called the direct effect, or path c'. In the current analysis, the direct effect is not significant, which shows that the relationships between the genetic variants and EPA levels are fully mediated by methylation. Furthermore, bootstrapping with 5,000 resamples was employed to compute the CIs. The CIs do not include zero for all analysed variants, and as such mediation is considered significant (Table 16).

Ameur *et al*. showed significantly lower *FADS1* expression levels in individuals homozygous for haplotype A (ancestral) compared to individuals homozygous for haplotype D, based on the results from gene expression microarray measurements in human liver samples (Schadt *et al.*, 2008; Ameur *et al.*, 2012). Further studies have uncovered genetic and epigenetic mechanisms in this key regulatory region in the *FADS* gene cluster. Two studies and a follow-up study showed an important putative enhancer region located ~3.5 kb from the *FADS1* and ~7.8 kb from the *FADS2* transcription initiation sites (Howard *et al.*, 2014; Rahbar *et al.*, 2017, 2018). The six CpG sites identified by the MetaMeth function in the current study lie in either the *FADS1* (cg15598662) or *FADS2* transcription initiation site (Table 15, Figure 6). Here, the analysis showed a significant mediation of the effect of the analysed SNPs on EPA levels by the methylation, indicated by a non-significant direct effect (path c') and bootstrapped CI that does not include zero (Table 16). However, the model has a causality problem, as path b shows a positive association (positive β) between methylation and EPA, which does not fit with the assumption that higher methylation is associated with lower EPA levels based on the results from the current mediation analysis (Table 16). Provided that increased DNA methylation at promoter regions is generally associated with lower gene expression levels, the positive association between methylation and EPA would not suggest that decreased promoter methylation is responsible for higher levels of EPA. In fact, Hoile *et al*. showed

that ω-3 supplementation increased the methylation of specific CpG sites in the *FADS2* promotor region. This increased methylation significantly reduced *FADS2* expression, all measured in peripheral blood mononuclear cells (Hoile *et al.*, 2014). Furthermore, studies have shown that ω-3 supplementation decreases the conversion of 18-carbon EFAs to their longer chain metabolites (Haban *et al.*, 2004). Paired with the results from Hoile *et al*. it can be suggested that increased intakes of ω-3 long chain PUFA may reduce PUFA biosynthesis via changes in the epigenetic regulation of *FADS2* that affect gene transcription, possibly in addition to any effect of product inhibition (Hoile *et al.*, 2014). The three studies that described strong allele-specific methylation associations in the putative enhancer region between the *FADS1* and *FADS2* genes showed that the major allele (derived allele) is associated with reduced methylation in the putative enhancer region (Howard *et al.*, 2014; Rahbar *et al.*, 2017, 2018). The same direction of this association is found in the present AgeCoDe study between the major allele of the analysed variants and the CpG site cg27386326, which is located in the putative enhancer region and is the only available CpG site within this region on the Illumina 450 methylation chip. Therefore, the finding in the methylation subsample in the current study is consistent with these previous studies. This is further encouraging, since the studies from Howard *et al*. and Rahbar *et al*. from 2017 measured the methylation in liver tissue, whereas the current AgeCoDe results as well as the findings from Rahbar *et al*. from 2018 were measured in blood, which is an easily accessible tissue from which to conduct these kinds of studies.

Given that the mediation results in the current study were promising but difficult to interpret, a linear regression analysis was conducted to determine whether the methylation sum score used in the mediation analysis would be associated with D5D and D6D enzyme activity approximated with the ω-6 product-to-substrate ratios. Here, a higher methylation was significantly associated with lower D6D activity but with higher D6D2 and D5D activity (data not shown). As discussed above, the D6D2 (GLA/LA) might be a more accurate D6D activity approximation, as it does not include the elongase step from GLA to DGLA. Furthermore, the evolutionary events in the *FADS* cluster suggest a coordinated expression of both genes, which makes a positive association with both desaturases likely. The association between a higher methylation and a higher D5D activity is well in line with the results of Rahbar *et al*. (Rahbar *et al.*, 2018). In their study, they also found a positive association between methylation within the *FADS2* promotor region and the

ARA/DGLA (D5D) ratio. He *et al*. analysed *FADS1/2* genetic polymorphisms and their association with DNA methylation and actual *FADS* mRNA expression in adipose tissue. In their study, they found that the minor allele of the analysed SNP (rs174570), which is in high LD with rs174546 in East Asian populations, was associated with lower *FADS1* and *FADS2* expression levels. Furthermore, correlations between DNA methylation levels and *FADS1* and *FADS2* gene expressions showed that the *FADS1* and *FADS2* expression levels increased with the increasing methylation levels of the two CpG sites in the *FADS2* promotor region, while the *FADS1* and *FADS2* expression levels decreased with the increasing methylation levels of the three CpG sites in the *FADS1* promotor region (He *et al.*, 2018). The positive association of increased methylation in the *FADS2* promotor region and increased *FADS2* gene expression is well in line with the results of the current study of the AgeCoDe sample and Rahbar *et al*. 2018, but opposing to the aforementioned study of Hoile *et al*. (Hoile *et al.*, 2014). In contrast, the results from He *et al*. showed that methylation in the *FADS1* promotor region was negatively associated with *FADS1* and *FADS2* expression mRNA levels. In a mediation analysis of DNA methylation levels on the association between rs174570 and gene expression as outcome, the authors showed that several but not all CpG sites that were significantly associated with both rs174570 and gene expression mediated the genetic effect on expression. These results suggest that DNA methylation is not the only pathway explaining the association between rs174570 and gene expression (He *et al.*, 2018). In the current study, methylation level of cg27386326 in the putative enhancer region is strongly associated with variants in the *FADS* cluster and associated with D6D and D5D enzyme activity, but the association points in the opposite direction to the results from the methylation sum score (data not shown). Howard *et al*. also used the GLA/LA ratio as a *FADS2* efficiency marker. Their results measured in liver tissue showed the same results as the current AgeCoDe analysis, in that less methylation of cg27386326 is associated with higher D5D and D6D (GLA/LA) efficiency (Howard *et al.*, 2014).

Taken together, these results demonstrate the potential of analysing methylation level and desaturase activity within the blood, but indicate that there is still more research needed to arrive at a better understanding of the role methylation plays on gene expression in interaction with genetic variants, nutrition and dietary changes that affect methylation in this gene region. In this regard the study of Hoile *et al*. showed that supplementation with

ω-9 monounsaturated FAs also altered DNA methylation in specific sites in the *FADS1/2* promotor regions (Hoile *et al.*, 2014). Furthermore, Howard *et al*. identified four other sites, namely cg16213375 (*FADS1* promotor), cg10515671 (*FADS1* promotor), cg03805684, and cg19610905 (*FADS2* transcription initiation site) that reached a Bonferroni-adjusted level of significance. The CpG site with the strongest association with EPA levels from the MetaMeth analysis is cg19610905 (Table 15). Unfortunately, Howard *et al*. focused their analysis only on cg27386326 in the putative enhancer region and did not investigate the direction of the association for cg19610905 (Howard *et al.*, 2014). It would have been interesting to see if the direction in the liver samples would also be opposite to the effect of cg27386326 as it was observed in the current study. Overall, studies on the *FADS* gene cluster showed that this cluster is dynamic and a region that quickly reacts to dietary changes.

### 4.4 Unbalanced fatty acid diets increase human (brain) health risks

There is a long history of recommendation to reduce dietary intake of SFAs, specifically PA and SA, to reduce cholesterol and thereby reduce the risk of CVDs and CHDs (Page *et al.*, 1961). High consumption of SFAs generates inflammation in body tissues as well as in the brain, by affecting the NF-κB pathway, TLR-4 receptors, interferon-γ (IFN-γ), and tumour necrosis factor-α (TNF-α) (Desale and Chinnathambi, 2020). The hypothesis is that replacing SFAs mainly with vegetable oil rich in PUFAs (LA) will reduce cardiovascular deaths by lowering serum cholesterol, ever since it was observed that higher levels of cholesterol are linked to events of CHD and death (Page *et al.*, 1961; Ramsden *et al.*, 2016). Ramsden and colleagues re-evaluated unpublished data from the Minnesota Coronary Experiment, a double blind randomized controlled trial on replacement of saturated fat in the diet with LA. The study diets were provided for a year or longer to more than 9,400 participants aged 20-97. The replacement of SFA in the diet with LA lowered serum cholesterol but did not result in a lower risk of death from CHD or all causes (Ramsden *et al.*, 2016). A 25 year follow-up report of the Seven Country Study, a long-term cross-cultural study that aimed at identifying associations of lifestyles and food choices of different ethnic groups with CVD, described an association of elevated serum cholesterol with risk of death from CHD in the USA and Northern Europe, but to a lesser extent in Southern Europe, and no clear association for Serbia and Japan (Keys *et al.*, 1986; Toshima *et al.*, 1995; Verschuren, 1995). The authors concluded that the reduction

in serum cholesterol levels is not likely to bring cultures with a high CHD risk, such as the USA and Northern Europe, down to a CHD mortality level characteristic for the Mediterranean and Japanese cultures unless additional factors are also changed. They suggested dietary factors that affect LDL oxidation and thereby inflammatory processes and thrombosis were of great importance (Verschuren, 1995; Lands, 2014). In fact, as Lands further discussed, a recent meta-analysis including more than 170,000 Japanese men and women showed lower all-cause mortality with higher blood cholesterol levels (Ogushi *et al.*, 2009; Lands, 2014). This is an interesting finding since the traditional Japanese and Greenland diets are characterized by high intake of ω-3 FAs and low intake of ω-6 FAs, resulting in blood %ω-6 HUFA levels of 28 % to 45 % (Lands and Lamoreaux, 2012), causing less inflammatory and thrombotic mediators derived from ω-6 FAs.

Studies showed that disease rates vary within the same ethnic group when compared between their homeland and other countries they migrated too. This observation was previously described for African/American or Hispanics in the USA (Alzheimer Association, 2019). Moreover, the heart disease rates (Sekikawa *et al.*, 2008) and the prevalence of AD is greater for Japanese-American men compared to men and women in Japan (White *et al.*, 1996). Here, increased westernisation of traditional diets seems to strongly affect the risk of dementia and AD, while in Japan, the prevalence of AD markedly increased over the past few decades and correlates closely with the intake of animal products which are high in ω-6, especially when grown on industrial food (Drewnowski and Popkin, 1997; Cordain *et al.*, 2005; Grant, 2014). A similar trend linking lifestyle, including diet, and the increase of metabolic risk factors with the burden of dementia was found for China (Chan *et al.*, 2013; Loef and Walach, 2013b; Rizzi *et al.*, 2014; Winblad *et al.*, 2016). This increased westernisation leads to an increase of the proportions of ω-6 in tissue HUFAs, which showed a strong correlation ($r^2 = 0.986$) with CHD mortality in several countries (Lands, 2003). The studies discussed here raise questions about the hypothesis that blood cholesterol levels cause CHD and that SFAs should be replaced with vegetable oil rich in LA to lower cholesterol levels (Lands, 2014). While there is evidence that substituting SFAs with dietary LA does reduce serum levels of cholesterol, it increases the rates of death from all causes, CHD, and CVD (Ramsden *et al.*, 2013, 2016). In the analysed AgeCoDe sample, there is a strong correlation between SFAs (PA, SA) and cholesterol ($p < 0.001$, $\chi^2$ test, data not shown). In addition, a reduction of

cholesterol by unsaturated FAs (i.e., %ω-6, which consists to a large extent of LA and ARA) was observed ($p$ = 0.015, $\chi^2$ test, data not shown). However, in the current study both the %ω-6 index and a higher ratio between LA and ALA increase the risk of dementia/AD almost 2-fold in the highest tertile (Table 9).

A recent study on the effect of circulating metabolites on cognitive function reported an inverse relation between DHA blood level and the risk of all-cause dementia and AD, by using blood measures of DHA in up to 22,887 individuals (van der Lee *et al.*, 2018a). This study further showed that eating fish raises blood levels of DHA and concluded that high levels of DHA could be beneficial for cognitive function, potentially also reducing the risk of dementia and AD (van der Lee *et al.*, 2018a). Interventional studies and RCT however showed inconclusive results on the benefit from ω-3 supplementation on cognitive aging and the risk of dementia and AD as reviewed elsewhere (Dyall, 2015; Cederholm, 2017; Calder, 2018; Canhada *et al.*, 2018; Power *et al.*, 2019). Interestingly, some studies show higher plasma EPA, but not DHA, to be associated with slower cognitive decline (Samieri *et al.*, 2011), dementia risk (Samieri *et al.*, 2008) and lower gray matter atrophy of the hippocampal / parahippocampal area and amygdala (Samieri *et al.*, 2012; Dyall, 2015). Furthermore, a meta-analysis found that only EPA was significantly lower in patients with pre-dementia syndrome, leading the authors to suggest that EPA may be a risk factor and biomarker for age-related cognitive impairment (Lin *et al.*, 2012; Dyall, 2015). In the present study both EPA and DHA levels are significantly lower in the dementia group (Table 3), but only EPA confers a significant risk to the development of dementia/AD as examined by the Cox proportional hazard regression analysis (Table 9). This discussion raises the following question: which mechanisms are subject to the action of ω-3 and ω-6 FAs.

In the introduction, it was pointed out that a large proportion of dementia is caused by mixed AD and vascular pathology, especially in older individuals. With the focus on FAs, the EPA/ARA ratio has been shown to be a robust marker of future cardiovascular events in a number of clinical settings for many types of CVD (Nelson and Raskin, 2019). Furthermore, EPA has been shown to lower triglycerides and to reduce the levels of atherogenic lipoproteins, including remnant lipoprotein cholesterol, inflammatory markers (including hs-CRP), and inflammatory cytokines. Moreover, EPA has beneficial effects on

endothelial function, oxidative stress, atherosclerotic plaque formation/progression and rupture, platelet aggregation, and thrombus formation (Zárate *et al.*, 2017; Shabir *et al.*, 2018; Nelson and Raskin, 2019). In the current study, EPA showed no effect on the level of hs-CRP (data not shown), but low levels of EPA showed the highest risk of developing dementia/AD (Table 9). Interestingly, the DHA/ARA ratio has little prognostic value for cardiovascular risk (Nelson and Raskin, 2019) and showed no reduction in the risk of dementia in the current study, which strengthens the role of EPA in the protection of the vascular system through mechanisms that may contribute to the functioning of the neurovascular unit, which facilitates the structural and functional connection between neurons and blood vessels in the brain (Shabir *et al.*, 2018). This might be an overlooked mechanism in contributing substantially to the risk of dementia and thereby make EPA as important as DHA, which has long been considered an important component of cognitive health. In this context, studies have identified intracranial ATH (not coronary or aortic ATH), oxidative stress, vascular inflammation, endothelial dysfunction and increased arterial stiffness, alongside with other endothelial alterations that are signs of vascular aging as risk factors for cognitive decline, either through disruption of the communication between neurons and blood vessels, or through disruption of the blood brain barrier (Ungvari *et al.*, 2010; Dolan *et al.*, 2011; Chakraborty *et al.*, 2017).

Both ATH and AD rise in numbers and can partly be related to the Western lifestyle with a diet high in SFAs, ω-6 FAs and sugars. Not only the absolute amounts of ω-6 and ω-3 FA intake, but increased ω-6/ω-3 ratios set a highly pro-thrombotic and pro-inflammatory milieu, and contribute to the prevalence of ATH, obesity, diabetes, inflammatory-autoimmune diseases, and specifically dementia/AD (Loef and Walach, 2013b; Simopoulos, 2016), which is also the result of the current study (Table 9). Overall, the evidence suggests that AD and ATH represent a spectrum of related conditions, with vascular involvement as a common predisposing factors like midlife diabetes, hypertension and obesity (Zlokovic, 2011), and shared risk factors like hyperlipidemia and polymorphisms in genes that are associated with disordered lipid metabolism (Liu *et al.*, 2011; Leeper *et al.*, 2012; Lambert *et al.*, 2013). Hence, an explanation might be, that large vessel intracranial ATH could be a marker for dysfunction of small cerebral vessels and their endothelium that might be the proximate cause of cognitive decline (Dolan *et al.*, 2011).

Structural changes occur in the brain in aging populations that potentially make the aging brain more susceptible to develop ATH, which can lead to vascular cognitive impairment and dementia and to the development of neurodegenerative diseases (Svennerholm *et al.*, 1997; Tarantini *et al.*, 2017; Simonetto *et al.*, 2019). Therefore, the prevention of vascular diseases and the protection of the vascular system should play a central role in the prevention of dementia/AD. A number of therapeutic interventions ranging from dietary and lifestyle interventions to anti-inflammatory and antioxidant treatments have been identified with endothelial protective effects. Furthermore, regular physical activity strongly benefits health by improving vascular endothelial function and by anti-inflammatory effects (Ungvari *et al.*, 2010). Results from the AgeCoDe cohort used in the current study have shown that physical activity even in late life may be effective in reducing conversion to dementia and AD or in delaying the onset of clinical manifestations (Luck *et al.*, 2014). This result is well in line with other publications on the association between higher levels of physical activity and a reduced risk of cognitive decline and dementia (Sumic *et al.*, 2007; Blondell *et al.*, 2014). It could be argued that the association between physical activity and dementia is due to the fact that physically active individuals generally eat healthier diets, which is true to certain extent. Nevertheless, it has been shown that both a healthy diet and participating in physical activity may independently lower the risk for dementia (Scarmeas *et al.*, 2009). In the current study, physical activity is associated with higher levels of EPA and a lower ARA to EPA ratio (table 8), therefore, it cannot be ruled out that the association between physical activity and FA levels is due to higher health awareness; thus, physical activity is used as a confounding factor in the Cox regression analysis (Table 9).

DHA, EPA and ARA are crucial for brain development and functioning of the brain (Akbar *et al.*, 2005; Corsinovi *et al.*, 2011; Bazinet and Layé, 2014; Dyall, 2015; Thomas *et al.*, 2015, 2016; Olivier *et al.*, 2016; Weiser *et al.*, 2016; Power *et al.*, 2019), and with diet being the main source for these FAs, their sufficient supply and balanced ratio are an important basis for brain function throughout life. It has been shown that ω-3 FAs supplementation can lead to an increase of EPA and DHA and a decrease of ARA and thereby to a lower ω-6/ω-3 ratio in plasma, and importantly also in cerebrospinal fluid (CSF) (Freund Levi *et al.*, 2014). A higher content of EPA and DHA modifies the structure of cell membranes and the function of membrane proteins, and through the effects on

intracellular cell signalling, EPA and DHA modulate transcription factor activation and downstream gene expression in beneficial health related ways (Calder, 2018). Once released from the plasma membrane through hydrolase action from the cytosolic phospholipase A2 (cPLA2), which does not discriminate considerably between the ω-6 and ω-3 PUFA (Bibus and Lands, 2015), PUFAs can regulate a wide set of homeostatic and inflammatory processes either directly or via transformation into locally acting bioactive metabolites (eicosanoids). Eicosanoids are key signalling molecules in the central nervous system (CNS) and are reported to be involved in numerous processes including memory and learning, cerebral blood flow, as well as regulation of neuroinflammation (Biringer, 2019). Here again, ω-6 and ω-3 FAs are processed by the same set of enzymes and particularly ARA, EPA and DHA are substrates for bioactive eicosanoids (Bannenberg and Serhan, 2010; Dyall, 2015; Zárate *et al.*, 2017; Calder, 2018).

The ARA-derived prostaglandins, thromboxane, and leukotrienes have been associated with AD pathology and neuronal cell death, activation of microglia and astrocytes, the pathogenesis of CVD, vasoconstriction and platelet activation (Shabir *et al.*, 2018; Biringer, 2019) and the development and maintenance of inflammation that plays a major role in the progression of inflammatory diseases, including CVD and ATH (Haeggström, 2000; Arita *et al.*, 2007; Simopoulos, 2010; Zárate *et al.*, 2017). In contrast, ARA derived lipoxins were the first specialized pro-resolving mediators (SPMs) identified that mediate resolution of inflammatory events (Wang *et al.*, 2015) and operate contrarily to the other mostly pro-inflammatory prostanoids and thromboxanes derived from ARA. In addition to lipoxins, ω-3-derived lipid mediators (SPMs) are potent anti-inflammatory, tissue-protective, resolution-stimulating mediators that counter-regulate ARA-derived pro-inflammatory actions and control the magnitude and duration of inflammation (Arita *et al.*, 2007; Bannenberg and Serhan, 2010; Simopoulos, 2010; Serhan *et al.*, 2015). Reduced levels of anti-inflammatory eicosanoids in CSF and the hippocampus were found to correlate with MMSE in AD patients, which indicates that failed resolution mechanisms may contribute to cognitive function (Wang *et al.*, 2015) and the conservation of homeostasis and health (Bannenberg and Serhan, 2010). Beside their potent pro-resolving and tissue regenerative actions, ω-3-derived lipid mediators stimulate pro-inflammatory to anti-inflammatory phenotype-switch in microglia (Serhan, 2015).

Abundant dietary SFAs such as PA and SA, which in addition to LA and ARA, can lead to an activation of the microglia and thus represent potent components of neuroinflammation. PA is the most potent candidate of the SFAs to cause increased expression of pro-inflammatory cytokines, which might be a mechanism by which the SFA/ω-3 ratio in the current study conveys its risk for the development of late-life dementia (Table 9). Furthermore, SFAs change lipid rafts and thereby showed effect on downstream inflammatory reaction (Desale and Chinnathambi, 2020). Studies have shown that the pro-inflammatory/activated phenotype of microglia dominates in AD condition, which impairs healing mechanisms, and results in ongoing low-grade inflammation that contribute directly to the progression of neurodegeneration (Desale and Chinnathambi, 2020; Webers *et al.*, 2020).

A proposed model suggests that cumulative effects of many inflammatory events throughout life (immunosenescence), where the resolution of inflammation becomes dysfunctional and leaves residual pro-inflammatory effects, builds up to chronic inflammation and chronic activation of astrocytes and microglia cells (Newcombe *et al.*, 2018). This would fit with the discussed observation of inflammation in midlife with neurodegeneration and cognitive aging (Walker *et al.*, 2017a; Walker *et al.*, 2017b). It is further discussed that it is possible to reduce immunosenescence by modifying lifestyle, including low-fat diet with a balanced ω-6/ω-3 ratio, and physical exercise, leading to a reduced cumulative inflammation, which may subsequently decrease the risk of developing dementia and AD in later life (Newcombe *et al.*, 2018). A balanced diet of ω-6 and ω-3, with a reduced intake of SFAs can therefore help to mitigate these harmful mechanisms and reinforce the need for primary prevention of connected pathologies of inflammation, the vascular system and brain functioning in old age.

Beside the assessment of active and beneficial FAs, the duration of interventional studies and RCTs is often discussed. Most intervention studies are brief in duration, making the results from the long-term Multidomain Alzheimer Preventive Trial (MAPT) highly anticipated. MAPT was a 3-year, multicentre, randomised, placebo-controlled superiority trial on community-dwelling, non-demented participants aged 70 years or older. More than 1,500 participants were randomly assigned to four parallel groups consisting of either the multidomain intervention plus ω-3 PUFA, the multidomain intervention plus placebo, ω-3

PUFA alone, or placebo alone. The multidomain intervention and ω-3 PUFAs, either alone or in combination, had no significant effects on cognitive decline over three years in elderly people with memory complaints (Andrieu *et al.*, 2017). The multidomain intervention consisted of cognitive training, advice about and demonstrations of physical activity and nutritional advice that was based on The French National Nutrition and Health Program, a nutritional advice program that aimed to reduce total fat intake and SFA intake (PNNS, Hercberg, Chat-Yung, & Chauliac, 2008). Within the PNNS program, the advice for eggs and meat, as a main source of ω-6 FAs (LA, ARA), was to reduce portion sizes at each meal when eaten twice daily and to limit fried and breaded preparations. Even though the advice was also to eat fish at least twice a week, this was simply advice given in group sessions and not followed up strictly with food frequency questionnaires. Participants were deemed adherent if they attended at least 75 % of the multidomain group sessions (Andrieu *et al.*, 2017). However, the main sources of ω-6 PUFA were thereby not considerably reduced (Hibbeln *et al.*, 2006; Blasbalg *et al.*, 2011; Lands, 2014). Thus, with only loose advice to reduce portion sizes and no strict follow-up, it can be assumed that the diet was still “Western” and would not reach a desired ω-6/ω-3 balance of 2:1. In a systematic review and meta-analysis on the effect of long-term ω-3 FA supplementation on cognition and AD pathology in animal models of AD, the authors found clear and consistent results of reduced Aβ deposition, improved cognition, and reduced hippocampal neuron loss upon EPA and DHA supplementation, which was administered for 10-50 % of the animals’ expected lifetime (Hooijmans *et al.*, 2012; Cederholm *et al.*, 2013). Consequentially, even though the three years from the MAPT study is a long period for an interventional study, it is still roughly half of the minimum interventional time described by Hooijmans *et al*., keeping in mind that this time period is derived from animal studies. These observations support the notion of a preclinical stage of neurodegenerative disease and demonstrate the challenging prospect that primary prevention trials might require more than two decades to prevent disease pathology (Sperling *et al.*, 2014).

Long and very long primary prevention phases, meaning several decades, are supported by two publications from the Atherosclerosis Risk in Communities Study (ARIC), which recently reported the association of systemic inflammation as early as in midlife with neurodegeneration and cognitive aging (Walker *et al.*, 2017a). The second study reported the effect of midlife systemic inflammation on the development of white matter

abnormalities and small vessel disease in the elderly (Walker *et al.*, 2017b). The latter study used hs-CRP to measure the state of systemic inflammation. In the present study, only the ratio between ARA and LA is the only FA marker associated with the inflammation indicator hs-CRP (data not shown). No ω-3 FA or ratio where a ω-3 FA is included could be associated with hs-CRP. These results are well in line with the work of Martinelli *et al*. on *FADS* genotypes and FAs and the association with inflammation and coronary artery disease (CAD). Here, the authors also only found an association with hs-CRP and the ratio ARA/LA and not for the ratio EPA/ALA, and were also the first study that associated CAD and hs-CRP with the ω-6 ratio ARA/LA (Martinelli *et al.*, 2008). The connection between the ratio ARA/LA and inflammation, as described in the present study and by Martinelli *et al*., is particularly interesting, since it has been shown that peripheral chronic low-grade inflammation, measured with CRP, coupled with the *APOE* ε4 allele is associated with an increased risk of AD, especially in the absence of CVDs. This suggests that chronic low-grade inflammation, either sustained or frequently episodic, may be a risk factor for AD (Tao *et al.*, 2018).

Considering the discussed health risk on the vascular system that emerges from ω-6 PUFA and their relation to ω-3, and the apparent involvement of the vascular system in dementia, it is a bit surprising how little the balance between ω-6/ω-3 has been investigated in the context of dementia/AD. Most studies on AD limit their focus on ω-3 levels only. In 2013, Loef and Wallach published their systematic review on the ω-6/ω-3 ratio and dementia or cognitive decline. They concluded, that the ω-6/ω-3 ratio might be a pivotal element in a multicausal chain leading to dementia and AD, and is thus also decisive as a means for potentially preventing it (Loef and Walach, 2013b). The studies discussed in the review support the idea that the ratio between ω-6 and ω-3 FAs is associated with the risks of dementia and cognitive decline in both blood and on dietary ratios. Furthermore, the authors pointed out that a low ω-6/ω-3 ratio is achievable by high intake of ω-3 PUFA in comparison to ω-6 PUFA, or by a reduction of ω-6 PUFA consumption, in order to sustain cognitive function and thus for the prevention of dementia and AD. The cited effect sizes of the association between an increased ω-6/ω-3 ratio and cognitive decline or dementia ranged between ORs of 1.25 and 1.8 or 1.1 and 1.6, respectively, which are comparable to the OR of FAs, or their ratios within the present study (Table 9). It has already been described above that none of the individual ω-6 FAs

on its own is associated with the risk of AD, which is well in line with the literature, even though there are a few studies that found an association of individual ω-6 FAs with cognitive impairment measured by MMSE, like in the Zutphen Elderly Study (Kalmijn *et al.*, 1997). Conversely, the study failed to show an association of LA with the decline of cognitive performance over time, which might be because a balanced dietary ω-6/ω-3 ratio is more important for cognitive decline than a single FA. There are further nutrients that contribute to maintaining cognitive performance that are part of particular dietary patterns (traditional Japanese diet, Mediterranean diet), which are not further discussed in the present work, like B vitamins, antioxidants, vitamin D, vegetables and fruits, but that should not be ignored in dementia and AD studies (Scarmeas *et al.*, 2018). Beyond that, the Mediterranean and traditional Japanese diets have lower levels of added sugar and a lower glycaemic index. Still, the overall key ingredient is a higher ω-3 intake in relation to ω-6 FAs (Cole *et al.*, 2010; Grant, 2014).

### 4.5 Resulting dietary recommendations and precision nutrition

The results from the current study and the discussed publications clearly suggest that a reduction of abundant ω-6 FAs typically found in the Western diet in combination with an increase of ω-3 FAs would show the strongest beneficial effects on health and cognition. Accordingly, recommendations on SFAs and TFAs are well acknowledged in that SFAs are to be consumed at less than 7 en% and TFAs to be consumed as little as possible, whereas caution is warranted with the discussed PUFAs throughout this current work. It has recently been shown that a healthy lifestyle/diet reduces the risk of dementia independently of the genetic risk (Licher *et al.*, 2019; Lourida *et al.*, 2019) (see chapter 4.3.2). For the study on participants from the UK Biobank a healthy diet was based on consumption of at least four of seven commonly eaten food groups following dietary recommendations (Lourida *et al.*, 2019). To be assigned to a healthy diet, a participant for the Rotterdam Study needed to adhere to at least half of the Dutch dietary guidelines (Licher *et al.*, 2019). A study on adherence to the 2015 Dutch dietary guidelines in the Rotterdam Study showed that only 12.8 % followed the guidelines on red and processed meat, meaning that almost 90 % eat more meat than recommended, and only 32.9 % eat fish servings according to the guidelines, whereas 73.7 % used unsaturated fats and oils instead of saturated fats (Voortman *et al.*, 2017). As discussed in a previous section, the unsaturated fats and oils are 90 % LA. Therefore, combined with the amount of meat, fish,

and vegetable oils intake by the participants of the Rotterdam Study, it cannot be assumed that a balanced ratio of ω-6 and ω-3 FAs has been achieved even in individuals on a healthy diet. The same can be assumed for participants of the UK Biobank, as they most presumably eat a MWD typical for Europe and North America. These results offer further room for improving the discussed balance between dietary ω-6 and ω-3 FAs and long-term positive health benefits that are associated with a balanced ratio as discussed throughout the current work. Particularly beneficial effects on cognition are to be expected, as the protective effect of a healthy baseline diet for the subsequent changes in cognition from the FINGER study has shown (Lehtisalo *et al.*, 2019). Even though research is far from understanding the impact of the MWD on human health and disease (Chilton *et al.*, 2014; Christ *et al.*, 2019), all three aforementioned studies emphasize the potential of associations between a balanced ratio of ω-6 and ω-3 FA and dementia to be measured in population studies.

Recent discovery on the evolution of the *FADS* gene cluster help to better understand possible health-related mechanisms based on genetic background and dietary patterns. In this context, a study showed an association of variant rs174537 that is in high LD with the previously discussed variant rs174546 and the synthesis of eicosanoids in the form of for example leukotriene B4 (LTB4), in addition to the known association with ARA and the ARA/LA ratio (see chapter 4.3.1, Table 12/13). Furthermore, they showed a strong correlation between the ARA/LA ratio and LTB4 raising the question whether African ancestry populations (derived haplotype D carrier) have greater capacity to produce eicosanoids (Hester *et al.*, 2014). This question and research should not be limited to populations of African descent as up to half of the European population is carrier of the derived haplotype D. In the AgeCoDe sample used in the current analysis the frequency is 42.8 % with both derived alleles (Table 14). The results from the present study also revealed a positive association of the ARA/LA ratio with low-grade inflammation measured with the inflammatory marker hs-CRP (data not shown). Given that African Americans have higher rates of hypertension, diabetes, stroke, CVD and certain types of cancer and often experience a more severe course of these diseases than Caucasians, diet-gene interactions may help decipher human health disparities among different populations (Chilton *et al.*, 2014). Enzyme activity and diet-gene interactions are often overlooked and should be considered not only in different populations, but also within a population. The

derived haplotype D in combination with a particular diet (environment) probably shifts the balance between ω-6 and ω-3, in that a high ALA/LA diet in combination with the high efficiency haplotype D shifts the balance towards EPA in relation to ARA. Contrarily, a high LA/ALA diet as found in the MWD in combination with the high efficiency haplotype D shifts the balance towards ARA, which is unfavourable and probably shifts conditions from normal physiology to pathophysiology when ARA is the dominant HUFA available (Gillingham *et al.*, 2013; Lands, 2015), as discussed above (see chapter 4.3).

The *FADS* cluster is one gene locus that is slowly being understood for its effect on the interaction with the MWD and the risk of diseases. It is to be expected that further gene variants in subsequent enzymatic steps may also influence biosynthetic efficiencies that result in different lipid mediator levels among different populations or within populations. The concept here is that variation in the DNA does not on its own lead to a disease but instead alters molecular traits that go on to affect disease risk (Chilton *et al.*, 2014). This is of particular importance when major dietary changes occur, as it has been described for Central Europe, North America and the south east Asian region, and when general dietary recommendations are given for a heterogeneous population like from the American Heart Association recommending 5-10 % energy intake of ω-6 PUFA, of which 90 % typically are LA. This leads to a large shift in the ratio of dietary LA/ALA (> 10:1), which results in a marked increase of ARA levels and a dramatic decrease of EPA levels (Chilton *et al.*, 2014). Especially high efficiency variant (derived haplotype D) carriers are at potential risk, which means a possible risk for heterogeneous populations. The substantial shift in MWD towards ω-6 PUFA is clearly visible in the data from the current study and corresponds to the context just described, in that a high dietary LA/ALA ratio increases ARA and decreases EPA (Table 5). Gene-diet interactions in the context of different effects of lifestyle factors on disease risk in individuals with different genotypes become exceptionally relevant but are yet understudied in a disease as complex as late-life dementia/AD, in which a multitude of different aetiologies are present (Eid *et al.*, 2019). Eid *et al*. discussed non-modifiable and modifiable risk factors of dementia/AD, and how they can be used to provide a more personalized therapeutic intervention for patients. The assessment should include demographics, family history of disease, genetic screening, environmental exposure assessment, and a lifestyle questionnaire. After identification of risk factors, patients would be tested for disease biomarkers via neuroimaging or

serum/CSF markers, with an aim to remove or reduce modifiable risk factors and unfavourable lifestyle habits and then provide a therapeutic option with a mode of action tailored to the mechanism most relevant to their individual risk profile (Eid *et al.*, 2019). The genetic screening proposed by Eid *et al*. includes the *APOE* genotype, and more genes from the IGAP consortium GWAS results (Lambert *et al.*, 2013). The proposed biomarkers comprise typical AD markers such as Aβ, tau or p-tau. Based on the literature and the results of an unfavourable FA profile on the risk of dementia from the present study, the *FADS* genotype and relevant FAs should be included in such an assessment. Numerous studies have shown that the composition of PUFAs in phospholipids has been associated with wide range of diseases and have a major impact on human health (Lattka *et al.*, 2010). A health risk assessment based on tissue PUFA status, as proposed by the %ω-6 index in the current study, together with the *FADS* genotype included in the genetic screening would presumably add valuable information on general health and many potential health risks. Both can easily be obtained from a blood sample and are therefore minimally invasive. Furthermore, blood PUFAs can easily be monitored over time and allow for a close follow up as to whether or not dietary recommendations were adhered to, removing the need for food questionnaires and relatively imprecise assessment of ω-3 intake (Hedrick *et al.*, 2012; Morris, 2012). Determining the *FADS* genotype will furthermore help to establish accurate dietary recommendations, for example informing individuals that carry the derived haplotype D and regularly eat fish to balance their ω-6/ω-3 ratio by reducing the ω-6 intake. For individuals carrying the minor allele that do not eat fish regularly it may not be sufficient to reduce the ω-6 intake, due to the reduced desaturase activity and the limited metabolism from ALA to EPA and further to DHA. The analysis of the *FADS* genotype on desaturase activity in the present work showed the same direction (Table 12/13) and is well in line with a study that found the minor alleles in *FADS* genes showing the lowest estimated desaturase activity for both enzymes D5D (*FADS1*) and D6D (*FADS2*), before as well as after the 6-week ω-3 FA supplementation (Cormier *et al.*, 2014).

Lattka *et al*. discussed another significant interaction between a variant in the *FADS2* gene and low ALA intake on myocardial infarction. Here, the protective effect of ALA is attenuated among carriers of the minor allele in individuals with low ALA intake. The authors therefore suggest tightly balanced regulatory mechanisms between dietary and

endogenous FAs as well as desaturase activity, which shows that appropriate dietary intake of FAs can overcome genetic defects (Lattka *et al.*, 2010). Speaking of “genetic defect” is particularly interesting since it is now known that the minor alleles in the evolution of the *FADS* gene cluster represent the ancestral genotype, demonstrating how positive selection led to the high frequency of the derived genotype that is now the major allele. This again strengthens the need to carefully evaluate the modern style of diet against the background of the genetic architecture of modern humans. Therefore, large gene-nutrition-interaction studies on complex phenotypes like late-life dementia are needed to determine the influence of *FADS* polymorphisms on the onset of such complex diseases in the context of individual dietary FA intake. In this regard, Lattka and colleagues strongly recommend the inclusion of *FADS* genotypes as well as diet and lifestyle factors in future randomized control trials addressing biological effects of PUFAs, because this will lead to enhanced sensitivity and precision of such studies and will reveal stronger findings (Lattka *et al.*, 2010). Furthermore, FAs can easily be integrated into an individualized risk profile, as previously discussed in the context of the proposed personalized therapeutic approach to AD (Eid *et al.*, 2019), to better understand the mode of action of dietary components and to decipher the impact of dietary patterns on human health.

### 4.6 Strengths and Limitations

A major strength of this study is its prospective longitudinal design, which allowed for the investigation into the predictive value of ω-3 and ω-6 PUFAs in participants above 75 years of age. The comprehensive AgeCoDe sample data furthermore allowed for the inclusion of a wide range of important confounders. To specifically account for reverse causation, the assessment of memory decline prior to the baseline measurement of PUFAs was used. Furthermore, this study utilised the measurement of absolute concentrations of ω-3 and ω-6 FAs rather than percentage values, which provided a more detailed type of assessment.

This study was an observational study focused on an elderly German population, limiting the generalizability of results to other ethnicities and to persons younger than 75 years of age. Residual confounding may still be present, including survival bias, despite adjusting for multiple confounders. Finally, interventional studies are required to establish a causal relationship.

# 5 Abstract

Dementia health care costs are rapidly rising due to the increasing number of people with dementia worldwide that is expected to increase to 66 million by 2030 and 115 million by 2050 (Prince *et al.*, 2013). Intensive research has clearly shown that dementia is caused by multiple factors, making it challenging to provide therapy. Understanding the complex interactions between genetic, lifestyle and environmental factors is crucial to developing efficient preventive measures and recommendations that help to significantly reduce the risk of dementia. The disease's multi-causal pathogenesis and interactions presents an incredible challenge particularly at the level of an individual person (Eid *et al.*, 2019). Furthermore, the multi-causal factors that play a role in aetiology and progression of the disease make it more likely that dementia/AD may be prevented rather than cured (Scheltens *et al.*, 2016). Diet has been associated with many non-communicable diseases that are shown to increase the risk of dementia (Simopoulos, 2006; Micha *et al.*, 2014; de Bruijn *et al.*, 2015; Scarmeas *et al.*, 2018; Livingston *et al.*, 2020), and thus plays an important role in the prevention of lifestyle and chronic diseases. Scientific evidence points to an important role of nutrition and ω-3 FAs in particular on human health and cognition, while interventional studies also show inconclusive results (Cederholm, 2017; Calder, 2018; Power *et al.*, 2019).

The current diet in North America and parts of Europe has changed dramatically in the type and amount of fat and micronutrients consumed during the past 150-200 years. As a result, the ratio of ω-6 to ω-3 PUFAs has risen to 10:1 and higher, compared to a hunter-gatherer diet ratio that has been estimated to be between 1:1 and 3:1 (Simopoulos, 2003; Cordain *et al.*, 2005). With the MWD, individuals now consume more SFAs and TFAs and less MUFAs and PUFAs, and this change in diet has culminated in a total daily energy uptake of 72.1 % based on foods that would have contributed to little or none of the energy in the typical preagricultural hominin diet (Cordain *et al.*, 2005). The PUFA composition of phospholipids has been shown to be associated with normal growth and development, as well as chronic diseases such as coronary heart disease, hypertension, obesity, inflammatory and autoimmune diseases (Simopoulos, 2006). Since these are also risk factors for dementia, it is therefore of interest whether the ω-6/ω-3 ratio is associated with the risk of dementia. This has been addressed in the current work, as dietary FAs are not

solely analysed individually but also in ratios between competing ω-6 and ω-3 FAs, as well as in a proportion of ω-6 in PUFA (%ω-6) index, in regard to their contribution to the risk of dementia. The analyses in this current work show that the ratios LA/ALA, ARA/EPA, and the %ω-6 are associated with an increased risk of dementia. Of the individual FAs analysed, only EPA showed a protective effect on the risk of dementia. No interaction between the investigated FAs, the *FADS* genotype and the *APOE* ε4 status was found in the investigated sample, except for the ratio SFA/ω-3 that possibly indicates an interaction of the *APOE* ε4 status and SFAs.

Additionally, recent findings on the evolution of the human *FADS1/2* gene region that code for the desaturase enzymes in the metabolic pathways of ω-6 to ω-3 PUFAs are discussed in this current work, demonstrating how a formerly positive selection of advantageous mutations in the *FADS1/2* gene region can lead to an increased risk of disease through diet and lifestyle in the modern Western world.

Finally, an outline is given for integrating FAs into an individualized risk profile and details how genetic information might be a useful tool to discriminate between responders and non-responders in nutritional studies. This additional genetic information would transform dietary recommendations and help guide the development of individualized diets. Furthermore, knowledge on personal genetic variants and individualized risk profiles based on FA levels could potentially promote behavioural changes in individuals, resulting in health improvements and prevention of chronic diseases (Simopoulos, 2010) and neurodegenerative diseases.

# 6 List of figures



# 7 List of tables

# 8 References

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