A MATTER OF FAT:

Epidemiological studies of dietary and plasma fatty acids in coronary heart disease

Kamalita Pertiwi

Propositions

1. Plasma linoleic acid is an indicator of metabolic health rather than dietary intake in patients with coronary heart disease.
   (this thesis)

2. Dietary intake of fish fatty acids after myocardial infarction offers long-term protection against coronary heart disease mortality.
   (this thesis)

3. Nutritionists must also be trained in food technology to broaden their view on diet and health.

4. Novelty should not be more important in science than rigorousness in methods.

5. Support from a psychologist should be offered to PhD students.

6. Diversity creates better humans and scientists.

Propositions belonging to the thesis entitled:

A matter of fat: Epidemiological studies of dietary and plasma fatty acids in coronary heart disease

Kamalita Pertiwi
Wageningen, 13 January 2021

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This research was conducted under the auspices of the Graduate School VLAG (Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 13 January 2021
at 1.30 p.m. in the Aula.

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A matter of fat: Epidemiological studies of dietary and plasma fatty acids in coronary heart disease
256 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2021)
With references, with summary in English

ISBN: 978-94-6395-611-6
DOI: https://doi.org/10.18174/534842

“The greatest good you can do for another is not just to share your riches, but to reveal to him his own.” (Benjamin Disraeli)

To my teachers

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Chapter 1

General introduction

FATTY ACIDS IN THE HUMAN DIET

Fat is a major part of the human diet. It consists of different fatty acids (FAs) that provide approximately 9 kilocalories of energy (or 37 kilojoules) per gram.[1] Dietary FAs play various roles in human metabolism, and could be involved in cardiometabolic diseases including coronary heart disease (CHD) and type 2 diabetes.

This chapter describes several important dietary FAs, in particular linoleic acid (18:2 n-6, LA), the main polyunsaturated FA (PUFA) in the omega-6 (ω6 or n-6) series, and omega-3 (n-3) PUFAs alpha-linolenic acid (18:3 n-3, ALA), eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA). In addition, odd-chain FAs 15:0 and 17:0 are discussed. Relations of these dietary FAs with cardiometabolic diseases have been increasingly investigated in observational cohort studies, using circulating FAs as biomarkers of intake.[2–4]

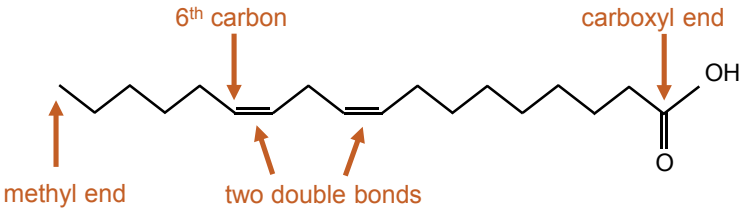

Figure 1 Simplified structure of linoleic acid, the main omega-6 fatty acid

FAs can be characterized based on their structural configuration. In general, a FA consists of a carbon chain with a carboxyl group at one end and a methyl group at the other end, as illustrated in **Figure 1**. FAs can be differentiated by the length of the carbon chain, the presence and number of double bonds in the carbon chain and the type of configuration of the double bond(s). Based on the presence and number of the double bonds, FAs can be categorized into saturated (SFAs; no double bond), monounsaturated (MUFAs; one double bond) or polyunsaturated FAs (PUFAs; >1 double bonds). For the unsaturated FAs (MUFA and PUFA), the location of the nearest double bond from the terminal methyl group determines the omega number.[5] As an example, LA has 18 carbon atoms in the chain and is an omega-6 (n-6) FA with two double bonds (Figure 1); the shorthand nomenclature is therefore 18:2 n-6. Depending on the double bond configuration, a FA is either a *cis* or *trans* FA. Only few FAs exist naturally as *trans* FAs (e.g. *trans* 18:1 n-7 in dairy fat[6]), but a *cis*-FA can be transformed into a *trans* FA via an industrial process known

as partial hydrogenation. Industrial *trans* FAs are considered detrimental to health,[7] and minimizing the consumption of *trans* FAs is therefore recommended.

Several examples of FAs in each class with their shorthand nomenclature, trivial name and dietary sources are given in **Table 1**. In this chapter, the abbreviated trivial names for 18:2 n-6 (LA), 18:3 n-3 (ALA), 20:5 n-3 (EPA) and 22:6 n-3 (DHA) are used and for other FAs, the shorthand nomenclature are used.

Table 1 Examples of FAs and their selected dietary sources

	Shorthand nomenclature	Trivial name	Dietary sources
SFA	15:0	Pentadecyclic acid*	dairy products, meat from ruminants
	17:0	Margaric acid*	dairy products, meat from ruminants
	16:0	Palmitic acid	palm oil
	18:0	Stearic acid	red meat, poultry, cocoa butter
MUFA	16:1 n-7	Palmitoleic acid	red meat, dairy foods, macadamia nuts
	18:1 n-9	Oleic acid	olive oil
PUFA	18:2 n-6	Linoleic acid	sunflower oil, soybean oil
	18:3 n-3	Alpha-linolenic acid	flaxseeds, walnuts
	20:5 n-3	Eicosapentaenoic acid	seafood, mainly oily fish
	22:6 n-3	Docosahexaenoic acid	seafood, mainly oily fish

*Pentadecanoic acid is more commonly used for 15:0 in the literature than the trivial name. For 17:0, heptadecanoic acid is also used.

Food sources may be characterized by their FA composition (**Table 1**). EPA and DHA are found abundantly in seafood, especially oily fish, and in a limited amount in red meat, poultry and eggs. Therefore, EPA and DHA are also termed “seafood n-3” or “marine n-3”. Most FAs in the human diet have a carbon chain with an even-numbered length,[5] although there are exceptions, such as pentadecanoic acid (15:0) and heptadecanoic acid (17:0). These odd-chain FAs are considered to reflect intake of dairy foods because the rumen microbiota in the ruminants can synthesize these odd-chain FAs, while humans and non-ruminants are considered not to produce them.[8,9] In milk fat, these odd-chain FAs are present only in small amounts; 15:0 content in milk fat ranges from 1-2 g/100 g and 17:0 is 0.6-1.5 g/100 g.[10]

Metabolism of dietary FAs

Dietary fats are broken down by digestive enzymes into FAs, which undergo beta-oxidation to yield energy. FAs are also incorporated into various lipid pools for transport as cholesteryl esters, phospholipids and triacylglycerols (or triglycerides), which are components of circulating lipoproteins, and for storage as triacylglycerols

in adipose tissue.[5] The composition of FAs in lipid pools is affected by metabolic processes such as *de novo* lipogenesis, elongation and desaturation. Through *de novo* lipogenesis, the human body is capable of endogenously producing FAs from carbohydrates. This process mainly happens in the liver and adipose tissues, generating SFAs with 16:0 as the main product.[11] FAs can be converted into longer FAs by elongation or be made into a more unsaturated type by desaturation.[5] Stearoyl-coA-desaturase-1 (also known as delta-9-desaturase) catalyzes conversion of a SFA to a MUFA, e.g. 16:0 to 16:1 n-7, whereas delta-5-desaturase and delta-6-desaturase are active in the n-3 and n-6 metabolic pathways. In **Figure 2**, the elongation and desaturation processes in the n-3 and n-6 metabolic pathways are illustrated.

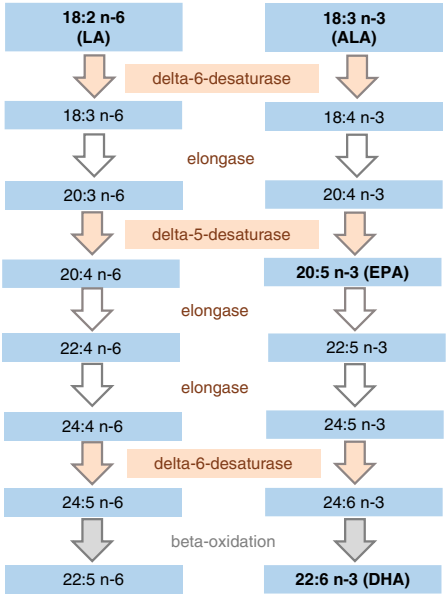

Figure 2 Elongation and desaturation of FAs in the n-3 and n-6 pathways

Text box 1 FAs at the top of the pathways in Figure 2, LA and ALA, are considered essential FAs. Essential FAs cannot be synthesized in the human body and serve as precursors for synthesis of longer chain PUFAs, therefore LA and ALA must be obtained from the diet. Although a conversion from ALA to EPA and DHA is possible, the conversion efficiency is known to be limited to <10% (and even lower from EPA to DHA) in individuals consuming a diverse diet.[12,13] Therefore, EPA and DHA are obtained from the diet.

FATTY ACIDS AND CARDIOMETABOLIC DISEASES

Dietary SFAs raise LDL-cholesterol, as has been repeatedly demonstrated in well-controlled feeding trials.[14] Elevated LDL-cholesterol is a major risk factor for cardiovascular disease (CVD), in particular CHD.[15] Replacement of dietary SFAs by PUFAs or (to a lesser extent) MUFAs is a cornerstone in CVD prevention.[16,17]

Cardiometabolic diseases, with CHD and type 2 diabetes as the main forms, remain the leading causes for death and disability worldwide.[18] In 2017, CHD contributed 54% of the disability-adjusted life years due to CVD, placing a significant burden on the healthcare costs in Europe.[19] In the Netherlands, due to improvement in risk factors and treatment of CHD patients, the number of CHD deaths has declined[20,21] and the number of surviving CHD patients is likely to grow.[20]

Patients with a history of myocardial infarction (MI) may have a more than 30% higher risk of recurrent MI, CVD and all-cause mortality than non-diseased individuals with similar age, as shown in population-based studies in Western societies.[22,23] Regarding FAs, primary and secondary CVD prevention guidelines have recommended replacing dietary SFAs with PUFAs, to eat fish 1-2 times per week, including oily fish, and limiting consumption of *trans* FAs.[16,17] Additionally, for CVD patients, EPA and DHA supplements at high doses (2-4 g/d) may be used to manage dyslipidaemia.[24,25] However, a meta-analysis of supplementation studies has recently shown that providing additional ~0.5 to 5 g/d EPA and DHA did not reduce CVD or all-cause mortality risk in patients with CVD, except for a 10% reduced risk in CHD mortality.[26]

N-3 FAs at habitual levels of dietary intake in Western populations may lower CHD mortality risk via their anti-arrhythmic effects.[27,28] In contrast to what is provided in supplementation studies, habitual dietary n-3 FAs in populations with a Western diet is usually (far) below 500 mg/d,[29,30] corresponding to a fish intake of <2 servings per week. For CHD patients, it is not clear whether habitual dietary n-3 FAs are related to mortality risk, or whether there is any benefit of having higher habitual dietary n-3 FAs on top of advanced cardiovascular drug treatment.

The associations of habitual dietary n-3 FAs with mortality risk are often investigated using observational cohort studies. The few available observational cohort studies in CHD patients (summarized in Rimm et al.[31]) mainly investigated these associations using circulating EPA and/or DHA, but most had small sample sizes and number of events, had relatively short term follow-up time (≤2 y) and are difficult to compare because of differences in the choices of endpoints and confounders.

Type 2 diabetes is often a comorbidity of CVD. The presence of type 2 diabetes is a strong predictor for cardiovascular events[32,33] and mortality in CHD patients.[33,34] Dietary LA has been investigated in relation to type 2 diabetes risk in observational cohorts of largely healthy populations but the results are mixed.[35–44] The lack of consistent results might be explained by the fact that not all studies replaced the same nutrients in theoretical substitution models (SFAs[35,36] or carbohydrates[40,41]). Nevertheless, in recent US cohort studies, substitution of either dietary SFAs or carbohydrates for dietary LA were similarly associated with a lower type 2 diabetes risk.[44] Other cohort studies reporting no associations have also considered measurement errors in the diet estimation due to the use of self-reported questionnaires as the explanation for the null findings.[39–41] A proposed mechanism for the inverse association between LA and type 2 diabetes is that increasing LA intake leads to more incorporation of unsaturated FAs in the membrane lipids. This will increase the membrane fluidity and number of insulin receptors, and decrease the affinity of these receptors, resulting in an improved insulin sensitivity.[45,46] Studies of dietary LA and type 2 diabetes risk in patients with CHD are lacking.

Odd-chain FAs have gained attention because of their beneficial associations with type 2 diabetes and CVD risks. Inverse associations with these cardiometabolic diseases have been reported in several cohorts without prevalent type 2 diabetes or CVD using circulating 15:0[4,47–50] and 17:0[4,50,51] to reflect dairy foods consumption. A more recent randomized controlled trial has shown that circulating proportions of 15:0 and 17:0 also increased after a 7-day supplementation with fermentable fibre.[52] Higher intake of dietary fiber is hypothesized to increase the production of propionate, a three-carbon FA, by the gut microbiome. In the liver, propionyl-CoA (converted from propionate) competes with acetyl-CoA in FA synthesis, leading to increased production of odd-chain FAs, especially 17:0.[52] Further, the investigators proposed that odd-chain FAs, particularly 17:0, reflect dietary fiber rather than dairy intake.[52] Whether circulating 15:0 and 17:0 could be influenced by dietary fiber in MI patients warrants more research.

Limitation of traditional dietary assessment methods

Observational cohort studies use self-reported dietary assessment methods such as the food-frequency questionnaire (FFQ) to estimate habitual diet because of feasibility of administering and data processing of the questionnaire. However, dietary intakes estimated using an FFQ may be prone to recall bias and therefore subjective.[53]

Assessment of FA intakes is especially difficult. Reporting of fat-containing products may be incomplete because fat is not always visible in food, for example when used as ingredient in dressings and sauces, or when used in preparing food (e.g. cooking oils) especially if the person is not involved in the preparation of the food.[54] Reporting of calorie-dense foods, high in fat and sugar, may also be affected by social desirability and weight status of the individuals.[55] Those with a high body mass index (BMI) are more likely to underreport their calorie-dense foods intakes than those with a healthy BMI.[55]

It is also important to recognize that the quality of the underlying food composition tables affects the calculated FAs from reported dietary intakes.[56,57] The FA content and composition of (processed) foods may change over time due to modifications in the food supply, e.g. use of different feed types for cattle, which affects the FA profile in meat and milk, plant breeding to produce certain FA profiles of plant oils, or reformulation of commercial products by the food industry.[56,58] Furthermore, detailed information of specific FAs may not be available for all foods in food composition tables, with data sometimes limited to total fat content.[59] In particular, assessing habitual ALA is difficult. ALA is a constituent of plant oils which are used as ingredients of commercial salad dressings, spreads, margarines and cooking fats, and are difficult to assess accurately using an FFQ because types and amount of plant oil used may be brand-specific,[58,60] while often only average values for these products are available in the food composition tables. Furthermore, the commercial products may be reformulated, e.g. using another type of plant oil, because of costs[58] or other (market-related) reasons,[61] and the values in food tables are not updated, contributing errors in the estimation of ALA intakes. On the other hand, the data of FA composition of fish is likely less affected by these issues.

Text box 2 Dietary assessment based on self-reports may be influenced by recall bias. In addition, estimated fatty acid intakes may be affected by errors or incompleteness of food composition tables. For LA, ALA, EPA and DHA, and odd-chain FAs, their circulating levels are frequently considered as objective measure of dietary intakes, free from the errors that are associated with self-reported dietary assessment.[2–4]

CIRCULATING FATTY ACIDS AS BIOMARKERS OF HABITUAL DIET

To overcome limitations of self-reported dietary assessment methods, observational cohort studies increasingly use the FA composition in blood and its fractions ("circulating FAs") as biomarkers of habitual dietary FA intakes. FA biomarkers measured in lipid pools are considered more objective in reflecting habitual intake of FAs because the levels do not depend on the ability of study participants to recall their food consumption.[62] FA biomarkers are considered as "concentration biomarker". This means that the levels cannot be used to infer absolute intakes but may be useful for ranking individuals according to their habitual intakes, which is the main interest of observational cohort studies.[62]

Factors affecting the utility of circulating FAs

1. Lipid pools

There are various lipid pools in the human body that may be used for the assessment of FA composition, including subcutaneous abdominal adipose tissues, whole blood, blood plasma/serum, red blood cells and various types of white blood cells.[63] Considering the interest of cohorts in long-term habitual intake, adipose tissue is often the preferred pool as a dietary biomarker of FAs because FAs turnover in adipose tissue is slow under energy balance. FA composition of adipose tissue is considered to reflect diet in the past 1 to 2 years.[63,64] However, the use of adipose tissue in cohort studies is rather limited because the tissue sampling is regarded as more invasive for participants, more time-consuming and expensive to measure, therefore it is less feasible in the usually large cohorts.

In cohort studies the most often reported pools for reflecting long-term habitual intakes of FAs are circulating FAs in total plasma or serum, plasma/serum fractions (or compartments) of cholesteryl esters and phospholipids and erythrocytes.[63] Circulating plasma FAs are considered to reflect dietary FAs of past weeks, although circulating cholesteryl esters can be influenced by recent diet.[63] Cholesteryl esters and phospholipids can be found in circulating lipoproteins,[63] as illustrated in **Figure 3**. Total plasma as a lipid pool comprises cholesteryl esters, phospholipids, also triacylglycerols and non-esterified FAs (or free FAs) fractions. Triacylglycerols is found largely in very-low-density lipoprotein. The FA composition of triacylglycerols represents only recent diet, from hours to days, therefore circulating triacylglycerols FAs are less suitable to assess long-term diet.[63] Circulating non-esterified FAs are still rarely used as an indicator of habitual dietary

intakes in cohort studies.[65] Because of the interest in long-term habitual diet, triacylglycerols and non-esterified FAs are not discussed in this Thesis.

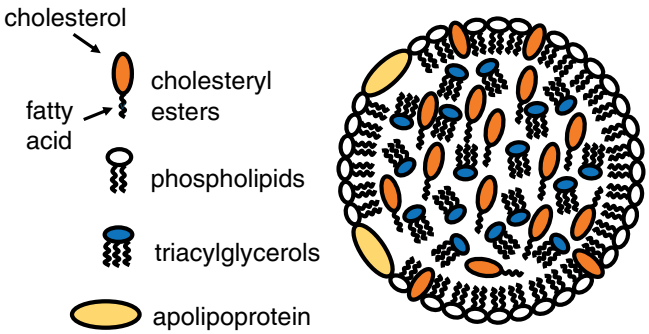

Figure 3 An illustration of the structure of a circulating lipoprotein particle in blood plasma (not to scale). Phospholipids are located in the outer layer while cholesteryl esters can be found in the core.

Studies with a specific interest in EPA and DHA often measure the FA composition of erythrocytes (red blood cells).[66,67] Due to their 120-day turnover rate, erythrocytes are considered to reflect intake for a longer time span than total plasma or plasma fractions, although for LA it has been reported that plasma cholesteryl esters, phospholipids and erythrocytes reflect intakes over almost similar time spans (1-2 weeks).[68,69] However, erythrocyte FAs are more sensitive to degradation during laboratory and field storage than those of plasma, particularly the FAs with a longer (≥20 carbons) and a more unsaturated chain.[70] Additional steps during sample preparation and measurement are needed to minimize oxidation of erythrocyte PUFAs.[67] These reasons may explain why some cohort studies choose plasma over erythrocytes. The main focus in this Thesis is on habitual intake of LA, ALA, EPA, DHA and odd-chain FAs, therefore plasma fractions of cholesteryl esters and phospholipids, and total plasma were investigated.

2. Analytical methods

Analytical methods include measurements techniques of circulating FA composition which can be varied among studies.[62] In general, measurement of circulating FAs in plasma consists of the following steps: 1) extraction of lipids from plasma, 2) separation into different fractions if using a specific fraction, 3) transesterification of FAs into FA methyl esters and 4) measurement of FA methyl esters by a chromatograph equipped with a detector. The resulting identified

individual FAs are presented as proportion to total FAs, in weight or molar percentage. By adding internal standards to the sample, absolute amounts of specific FAs can be provided.[64] Proton-nuclear magnetic resonance can also be used and this technique reports circulating FA data both in absolute concentrations and relative to total FAs.[71] However, the number of FAs measured are limited; EPA, ALA and odd-chain FAs are not available.[71] In observational studies, circulating individual FAs are generally measured and presented as a proportion of total FAs.[2,3,72] Circulating FAs in absolute concentrations seem to be less correlated with diet.[63,73]

3. Dietary factors

For FAs that are primarily obtained from the diet, their circulating levels are generally considered a good marker of their dietary intakes.[64] In cohort studies of generally healthy populations that focus on habitual intakes, correlations between dietary and circulating FAs in various plasma lipid pools are around 0.4 to 0.5 for EPA and DHA, while for LA, correlations range from 0.2 to 0.6.[63] Although ALA can only be obtained from the diet, results from isotope tracer studies have shown that much of ingested ALA is oxidized in the body[74] and only very small amount of dietary ALA is incorporated in plasma fractions.[75] For odd-chain FAs, correlations for 15:0 and dairy intake (correlation of 0.2-0.3) are slightly stronger than for 17:0 (correlation of 0.1-0.2).[72] However, others have also reported associations of odd-chain FAs with fruits and vegetables[51] and ruminant meat intakes.[76] Compared to cohort studies, correlations between dietary and circulating FAs are notably stronger in intervention studies where participants have well-controlled FA intakes, covering a wider range in dietary FAs for generating contrast in biomarker response. These studies are beyond the scope of this Thesis, which focusses on long-term habitual FA intake.

4. Factors related to metabolism

Several factors may affect circulating FA composition through their influence on metabolism. Enzyme activities may be modified by sex hormones,[77,78] smoking, genetic variations and health/disease status.[63,64,79] The utility of circulating FAs as biomarker of dietary FAs have been studied mostly in cohorts of generally healthy individuals.[80–85] In the two Dutch cohorts where approximately half of the participants had CVD or type 2 diabetes, the presence of cardiometabolic diseases was associated with lower circulating LA levels.[73] Circulating LA was also found to be lower in 40 Finnish post-MI patients (67% on statin) when compared to 40 age-matched healthy men, even though their consumption of soft margarines on bread were similar.[86]

When using circulating FAs as biomarkers of diet in CHD patients, it is important to consider how factors related to metabolism affect circulating FA composition, but data in this field are scanty. Patients with prior MI are usually treated with medications such as statin, a lipid-lowering drug. Statins lower the low-density lipoprotein levels by competitive inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is involved in the rate-limiting step of cholesterol synthesis.[87] Changes in circulating lipoproteins may affect the circulating FA composition measured in cholesteryl esters and phospholipids since these fractions are components of circulating lipoproteins.[63] Few trials have demonstrated a decline in circulating LA and ALA and an increase in longer-chain PUFAs such as 20:4 n-6 after statin treatment compared with placebo.[88–90] Whether statin use also modifies the relation of circulating FAs with dietary FAs is not known.

Another factor which affects circulating FA composition is alcohol intake through its chronic effects on metabolism.[91] High alcohol intake is a risk factor for cardiometabolic diseases[92,93] and mortality.[92,94] Chronic high alcohol intake affects lipoprotein metabolism in the liver, notably resulting in reduced very-low-density lipoprotein and increased high-density lipoproteins concentration,[95] and the regulation of several transcription factors involved in *de novo* lipogenesis.[96] Higher alcohol intake has been associated with a lower circulating LA and higher 16:1 n-7 and 18:1 n-9,[97–102] and less consistently with a higher circulating n-3 FAs.[98,101–103]

Circulating FAs may also be affected by underlying insulin resistance. Obesity and dyslipidemia are closely related to insulin resistance and are often present in patients with CHD. Low circulating LA has been associated with a lower type 2 diabetes risk in cohorts of healthy individuals.[3] However, a lower circulating LA together with higher circulating 18:3 n-6, 20:3 n-6 (desaturation products of LA, see Figure 2), and 16:1 n-7 also characterize the circulating FA composition of patients with non-alcoholic fatty liver disease.[104] A recent cross-sectional study showed that circulating LA was also inversely associated with hepatic fat accumulation in healthy older individuals.[105] It is known that hepatic *de novo* lipogenesis is increased in patients with conditions related to insulin resistance.[11] These patterns of circulating FAs may be an early indication of impaired FA metabolism related to the liver, and this may influence relations between diet and circulating FAs.

Text box 3 Correlations between habitual dietary and circulating FAs are relatively strong for EPA and DHA (correlations of 0.4-0.5).[63,106] Meanwhile, associations for LA[63] and the odd-chain FAs[72] are more modest (correlations of ~0.2). Weak or no correlations between dietary and circulating ALA have been reported.[63,106] However, these findings are mainly from cohort studies in generally healthy populations. It is not known to what extent circulating FAs reflect dietary intake in patient populations, especially in those with prevalent CHD. In these patients, however, circulating FA composition may also be affected by the disease, prevalent risk factors and cardiovascular medication such as statins.

OBJECTIVE AND OUTLINE OF THIS THESIS

Polyunsaturated and odd-chain FAs are considered important for cardiometabolic health, but the role of these FAs in the secondary prevention of cardiometabolic disease is not yet clear. In observational studies, circulating FAs as biomarkers of specific FA intakes have repeatedly been studied in relation to type 2 diabetes and CVD. Circulating FAs may be influenced by metabolic processes and other factors, and little is known about their utility as biomarkers of FA intake in patients with CHD.

The **main objective** of this Thesis is to examine the mutual relationship between dietary and circulating FAs, and their associations with cardiometabolic disease outcomes in stable CHD patients. The main focus is on polyunsaturated FAs LA, ALA, EPA and DHA, and the odd-chain FAs 15:0 and 17:0, which are FAs in the human diet that may be important for cardiometabolic disease prevention.

Chapters in this Thesis are largely based on data from the Alpha Omega Cohort, which consists of 4837 Dutch post-MI patients with a clinically diagnosed MI ≤10 y before study enrolment.[107,108] Baseline measurements were conducted in 2002-2006. During the first 40 months of follow-up, patients were randomized to low doses of n-3 FAs (in margarines) or placebo, showing no effect on major cardiovascular events.[108] Because of randomization, the n-3 FA supplementations cannot act as confounders when studying dietary/circulating FA associations with outcomes. Data collection comprised demographic factors, habitual diet (food-frequency questionnaire), lifestyle factors, medical history and medication use, CVD risk factors and biomarkers in blood.[107] The patients have been followed for cause-specific mortality through 31 December 2018.

The Thesis additionally includes baseline data (year 2005) from the cohort of Prospective Urban Rural Epidemiology (PURE) in South Africa. The PURE South Africa study is part of the international PURE study on the role of environment, nutrition and socioeconomic status in the development of chronic non-communicable diseases.[109] In total, 2010 healthy black South African adults aged >30 years from two urban and two rural communities in North West Province participated in the study.[110] Data collection included demographic factors, habitual diet (food frequency questionnaire), lifestyle factors, cardiometabolic risk factors and markers of inflammation, liver function and glycemia in blood.

Figure 4 presents the outline of this Thesis. Grey dashed arrows represent the main relations of interest in this Thesis. Black arrows show the three specific objectives of this Thesis:

a) the relationship of habitual dietary intakes, i.e. FAs or dietary FA sources, with circulating FAs, and metabolic factors that may modify this relation (**Chapter 2 and 3**),
b) the relationship of habitual dietary intakes, i.e. FAs or dietary FA sources, with cardiometabolic outcomes (**Chapter 4, 5 and 6**),
c) the relationship of circulating FAs with cardiometabolic outcomes, and metabolic factors that may modify this relation (**Chapter 4, 5 and 6**).

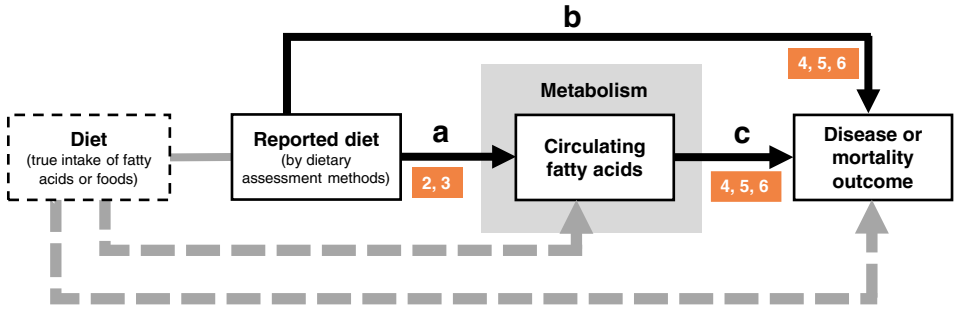

Figure 4 Outline of this Thesis

The Thesis consists of two parts. In the first part, the relationship of dietary and circulating FAs was investigated using cross-sectional analyses for which findings are presented in **Chapters 2 and 3**. In **Chapter 2**, the relations between dietary and circulating LA, ALA, EPA and DHA in three different plasma fractions were examined in the Alpha Omega Cohort. Potential influence of selected cardiometabolic risk factors, statins use and alcohol intake on the relations between dietary and circulating FAs were also examined. In **Chapter 3**, relationships of dairy and fiber intakes with circulating odd-chain FAs measured in cholesteryl esters and phospholipids were studied in the Alpha Omega Cohort. We also investigated whether the relation between dairy intake and circulating odd-chain FAs is modified by fiber intake, and whether the relation of dietary fiber and circulating odd-chain FAs is modified by dairy intake.

In the second part, the relationships of both dietary and circulating FAs with risk of cardiometabolic outcomes were evaluated, namely incident type 2 diabetes and

CHD mortality for which findings are presented in **Chapters 4, 5 and 6**. In **Chapter 4**, association of LA with the risk of type 2 diabetes was investigated in the Alpha Omega Cohort. Both dietary and circulating LA in plasma cholesteryl esters were assessed in relation to incident type 2 diabetes during a median follow up of ~40 months, in which associations for dietary LA were obtained from a theoretical isocaloric substitution model replacing SFAs. Also, the influence of potential confounding or mediating effects of metabolic factors on the associations was examined. In **Chapter 5** we performed a cross-sectional study in the PURE South Africa cohort to examine the associations of dietary and circulating LA in plasma phospholipids in relation to markers of glucose metabolism and liver function. In **Chapter 6** we performed a prospective analysis in the Alpha Omega Cohort to investigate the relations of dietary and circulating EPA and DHA, and ALA with mortality risk during a median follow-up of 12 y. Finally in **Chapter 7** the main findings, interpretations, methodological considerations and implications of this Thesis are discussed, followed by directions for future research.

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Chapter 2

Circulating n-3 fatty acids and linoleic acid as indicators of dietary fatty acid intake in post-myocardial infarction patients

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Nutrition, Metabolism and Cardiovascular Diseases 2019;29(4):343-350

ABSTRACT

Background and Aims: Population-based studies often use plasma fatty acids (FAs) as objective indicators of FA intake, especially for n-3 FA and linoleic acid (LA). The relation between dietary and circulating FA in cardiometabolic patients is largely unknown. We examined whether dietary n-3 FA and LA were reflected in plasma lipid pools in post-myocardial infarction (MI) patients.

Methods and Results: Patients in Alpha Omega Cohort filled out a 203-item food-frequency questionnaire from which eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), alpha-linolenic acid (ALA), and LA intake were calculated. Circulating individual FA (% total FA) were assessed in cholesteryl esters (CE; *n*=4,066), phospholipids (PL; *n*=838), and additionally in total plasma for DHA and LA (*n*=739). Spearman correlation coefficients (r_s) were calculated for dietary vs. circulating FA. Circulating FA were also compared across dietary FA quintiles, overall and in subgroups by sex, obesity, diabetes, statin use, and high alcohol intake.
Patients were on average 69 years old and 79% was male. Moderate correlations between dietary and circulating levels were observed for EPA (r_s ~0.4 in CE and PL) and DHA (r_s ~0.5 in CE and PL, ~0.4 in total plasma), but not for ALA (r_s ~0.0). Weak correlations were observed for LA (r_s 0.1 to 0.2). Plasma LA was significantly lower in statin users and in patients with a high alcohol intake.

Conclusions: In post-MI patients, dietary EPA and DHA were well reflected in circulating levels. This was not the case for LA, which may partly be influenced by alcohol use and statins.

INTRODUCTION

There is continuing interest in the use of circulating fatty acids (FA) as biomarkers of dietary FA intake in epidemiological studies,[1,2] often in relation to cardiometabolic outcomes.[3,4] Dietary FA that are frequently studied include n-3 FA, i.e. eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3), alpha-linolenic acid (ALA, 18:3 n-3), and linoleic acid (LA, 18:2 n-6). LA intakes in Western diet generally range from 4 to 6 energy percent (en%), whereas intake of n-3 FA is much lower (mean intake of <500 mg/d for EPA+DHA and 1.0-1.5 g/d for ALA).[5,6]

In cohorts of mainly healthy participants, correlations between dietary intake and plasma circulating levels are mostly moderate for EPA, DHA (0.4-0.6), and LA (0.2-0.3), and generally weak for ALA (<0.1).[1] However, circulating levels of FA, which are expressed as proportions of total FA, are not only affected by dietary intake but also by fatty acid metabolism. For example, the endogenous synthesis of saturated and monounsaturated fatty acids may affect the proportions of other FA.[1,7] Furthermore, metabolic impairments such as abdominal obesity, type 2 diabetes and/or impaired liver function may influence desaturase activity,[8,9] and hence circulating levels of n-3 and n-6 FA. To what extent lipid-modifying agents (e.g. statins) and lifestyle factors, such as alcohol use, affect circulating FA is not yet clear.

In our cohort of drug-treated post-myocardial infarction (MI) patients, we examined whether dietary LA and n-3 FA intake were reflected in circulating FA, using various plasma lipid pools (plasma cholesteryl esters, CE; plasma phospholipids, PL; total plasma). We also investigated whether these relations were modified by cardiometabolic risk factors, alcohol intake and statin use, which could have implications for using these biomarkers of intake in patient populations.

METHODS

Study design and population

The Alpha Omega Cohort (AOC) is a prospective cohort study and a continuation of the Alpha Omega Trial, which has been described in detail elsewhere.[10,11] Briefly, 4,837 Dutch men and women aged 60-80 y who had an MI up to 10 years before study enrollment participated in a 3-year intervention study of n-3 fatty acid supplementation, which had no effect on recurrent cardiovascular events.[11] The

study was approved by the medical ethics committee at the Haga Hospital (The Hague, The Netherlands) and all patients provided written informed consent before enrollment.

The present study is a cross-sectional analysis of baseline data of the AOC (2002-2006) (**Supplemental Figure S1**). Patients with missing food frequency questionnaire (FFQ) data (*n*=453) and patients with unreliable dietary data (*n*=238) were excluded, as previously described.[12] Patients with missing data on plasma CE (*n*=75) or >5% unknown FA in CE (*n*=5) were also excluded. In total, 4,066 patients with complete data of dietary intake and measurements of plasma FA in CE were analyzed. Circulating FAs were additionally measured in PL in 838 patients (21% of the cohort) and in total plasma in 739 patients (18% of the cohort).

Dietary assessment

Patients filled out a 203-item FFQ, which was an extended and updated version of a previously biomarker-validated FFQ to estimate the intake of different types of fat and cholesterol. The FFQ has shown high reproducibility for assessing daily intake of food groups including edible fats and oils, with Spearman *r* up to 0.9. Intakes of total energy, total fat and saturated fat estimated by FFQ correlated well with estimates from the dietary history method (Pearson *r* ~0.8). Double data entry was performed and returned questionnaires were checked. In case of missing data on relevant parts of the FFQ, patients were called by dietitians for additional data collection. The FFQ included specific questions on types and brands of fats and oils used in food preparation. Food items were linked to the 2006 Dutch food composition table (NEVO)[13] to calculate total energy (MJ/d) and nutrient intakes, including EPA, DHA, ALA and LA. Dietary intakes of EPA and DHA were expressed in mg/d, ALA in g/d and LA as % of total energy intake (en%). Additionally, individual n-3 FA were reported as en% and LA as g/d. Patients used no fish oil supplements, which was an exclusion criterion for the Alpha Omega Trial.[10] The FFQ included detailed questions on alcoholic beverages from which total ethanol intake (g/d) was computed. Alcohol use was categorized as no intake (0 g/d), low intake (>0 to 10 g/d), moderate intake (>10 to 20 g/d for women and >10 to 30 g/d for men), or high intake (>20 g/d for women and >30 g/d for men).

Risk factor assessment

Demographic data and information on lifestyle factors, medical history and medication use were obtained by questionnaires. Physical activity was assessed with the validated Physical Activity Scale for the Elderly (PASE) and classified as low (no activity or only light activity, defined as ≤ 3 metabolic equivalents (METs)), medium (>0 to <5 days per week of moderate or vigorous activity, >3 METs), or high (≥5 days per week of moderate or vigorous activity, >3 METs).[14] Medication was coded according to the Anatomical Therapeutic Chemical (ATC) Classification system, with codes C10AA and C10B for statins.[15] Patients underwent a physical examination at home or in the hospital by trained research nurses. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Obesity was defined as BMI ≥30 kg/m^2. A total of 30 mL of venous blood was drawn from each patient, of which 10 mL was collected in EDTA tubes for FA composition analysis. If patients had their last meal ≥8 hours before blood sampling, they were considered fasted. Samples for plasma FA composition analysis were stored at -80°C. Serum lipids and plasma glucose were measured using standard kits as described previously.[10] Diabetes mellitus was considered present in case of a self-reported physician's diagnosis, use of anti-diabetes medication, or plasma glucose ≥7.0 mmol/L (fasting) or ≥11.1 mmol/L (non-fasting).[16]

Measurement of FA in plasma CE and PL

Circulating FA in CE and PL pools were analyzed at the Division of Human Nutrition and Health, Wageningen University, the Netherlands. Briefly, extracted total lipids from plasma samples were separated into CE and PL by solid phase extraction silica columns. FA were transesterified into fatty acid methyl esters and analyzed by gas chromatography. FA were identified by comparing retention times with FA standards, and expressed as weight percentage relative to total FA (% total FA). Laboratory analyses of FA in CE took place in different years. We observed stable FA over 6-9 years of storage at -80°C, shown by high intraclass correlation coefficients (r>0.90) for EPA, DHA, ALA and LA. Others have also observed stable FA in CE, PL and triglycerides over a time span of 10-12 years when stored at -80°C.[17,18] Detailed laboratory and quality control methods are described in **Supplemental Methods** and **Supplemental Table 1**.

Measurement of FA in total plasma

Stored plasma EDTA samples (300 µl) were sent in 2014 to Nightingale Health, Ltd (Helsinki, Finland; formerly Brainshake, Ltd) for profiling of metabolic markers. A high-throughput proton nuclear magnetic resonance (^{1}H-NMR) metabolomics platform was used to quantify 231 metabolic measures, including selected FA (linoleic acid and DHA) as reported in detail elsewhere.[19] Linoleic acid and DHA were expressed as relative to the total FA content in total plasma (% of total FA) and in absolute concentrations (in mmol/L). To monitor quality of the measurements, two control samples, of which one sample represented human plasma, were placed in each box of 96 samples. The measurement CV, as reported by the Nightingale Health, was calculated for a control sample over 10 boxes and was <5% for both LA and DHA.[20]

Statistical analysis

Normality of distribution of values for all variables was checked visually using histograms and Q-Q plots. Patient characteristics were computed as means ± standard deviation (SD) for normally distributed variables, medians (interquartile range) for non-normally distributed variables, and percentages for categorical variables. Dietary and circulating FA were expressed as medians (interquartile range) because of skewed distributions, and as mean ± SD (linoleic acid only).

Dietary FA were related to circulating levels in different plasma lipid pools by means of Spearman rank correlation coefficients (r_s) and partial r_s, adjusting for age (y), sex, and total energy intake. Fisher's z transformation was used to obtain 95% confidence interval (95% CI) for correlation coefficients. Circulating FA were also reported in quintiles of dietary FA to obtain insight in potential dose-response relationships. Least-squares means and 95% CI of circulating FA in quintiles of dietary FA were obtained by multivariable linear regression, adjusting for age, sex, total energy intake, obesity (absent vs. present), physical activity (low, medium, or high), smoking status (current, former, or never), fasting status at blood collection (yes or no), total serum cholesterol (mmol/L), measurement year (for CE), high alcohol intake, and prevalent diabetes. No adjustment was made for statin use to avoid multicollinearity with serum cholesterol, which was already in the model. Missing data on adjustment variables were 5.9% of total sample. All analyses were done using complete cases. The median values of intake in quintiles of dietary FA were used as explanatory variables to calculate p-for-trend.

For plasma FA in CE (*n*=4,066), analyses in quintiles of dietary FA intake were repeated in strata of sex, obesity (absent vs. present), prevalent diabetes (absent vs. present), statin use (no vs. yes), and high alcohol intake (>30 vs. ≤30 g/d in men; >20 vs. ≤20 g/d in women), while holding all other variables at the observed population mean. Partial Spearman correlations (r_s) between dietary and circulating FA were also obtained in these subgroups. Analyses were carried out with SAS 9.4 (Cary, NC, USA). A two-sided p-value <0.05 was considered statistically significant.

RESULTS

The cohort with CE data was 69±6 years old and mostly male (79%). Most patients were treated with cardiovascular drugs, including statins (86%). Twenty percent had diabetes, 23% had obesity, and 16% had a high alcohol intake (**Table 1**). Patient characteristics in subcohorts of other lipid pools were roughly similar, except for use of statins, use of antihypertensive drugs, serum lipids and fasting status, which were slightly different in patients with PL data.

Relation between intake and circulating n-3 FA

In our study population, median dietary intake of EPA+DHA was 108 mg/d (0.05 energy%) and intake of ALA was 0.45 energy%. Circulating EPA was comparable in CE (median: 1.06% of total FA) and PL (1.13%). DHA was higher in PL (4.49%) than in CE (0.67%) and total plasma (1.24%). Median circulating ALA was 0.49% in CE and 0.14% in PL (**Table 2**). Individual circulating n-3 FA were strongly positively correlated across different plasma lipid pools (all r_s ≥0.80; **Supplemental Table 2**).

Moderate correlations between dietary and circulating EPA and DHA were observed in crude analysis and in analysis with adjustment for age, sex and total energy intake (**Table 2**). Partial correlations were 0.37-0.39 for EPA and 0.43-0.50 for DHA (**Table 2**). Partial correlations between dietary and circulating ALA were weak (r_s -0.02 in CE; **Table 2**), as was the correlation between dietary ALA and circulating EPA (r_s -0.06 in CE; data not in Table). Partial correlations obtained in a subsample that had fasted >12 hours showed similar results (0.35-0.38 for EPA, 0.38-0.42 for DHA, and ~0.01 for ALA in CE; data not in Table). **Figure 1** shows results from multivariable linear regression analysis, with additional adjustment for obesity, physical activity, smoking status, high alcohol intake and prevalent diabetes. A positive dose-response relation between dietary and circulating levels (in CE) was observed for EPA and DHA, whereas for ALA no association was found

(**Figure 1**). Similar associations were observed in PL and total plasma (**Supplemental Figures 2-3**).

Sex, obesity and diabetes did not affect overall circulating levels of n-3 FA (**Supplemental Figures 4-8**). Statin users, however, had higher circulating EPA and DHA and lower circulating ALA compared to non-statin users. Circulating EPA (but not DHA and ALA) was also higher in patients with a high alcohol intake. In all subgroups, there was a dose-response relation between dietary and circulating EPA and DHA (but not for ALA), comparable to the total cohort. Correlation coefficients between dietary and circulating n-3 FA were also comparable to the total cohort in most subgroups (**Supplemental Table 3**). In patients with a high alcohol intake, however, dietary ALA was more strongly correlated with circulating ALA in PL compared to the total cohort, but this may be a chance finding due to small sample size.

Table 1 Characteristics of patients of the Alpha Omega Cohort by availability of FA in different plasma lipid pools[a]

	Cholesteryl esters (*n*=4,066)	Phospholipids (*n*=838)	Total plasma (*n*=739)
Age (y)	69.0 ± 5.6	69.1 ± 5.7	70.0 ± 5.8
Men, *n* (%)	3,226 (79.3)	652 (77.8)	578 (78.2)
BMI (kg/m^2)[b]	27.7 ± 3.8	27.9 ± 4.0	27.6 ± 3.8
Obesity, *n* (%)[b,c]	947 (23.3)	201 (24.0)	166 (22.5)
Total daily energy intake (MJ)[d]	8.0 ± 2.0	8.0 ± 2.0	8.0 ± 2.1
Alcohol intake, *n* (%)[e]			
No	198 (4.9)	55 (6.6)	40 (5.4)
Low	2,157 (53.1)	443 (52.9)	379 (51.3)
Moderate	1,065 (26.2)	215 (25.7)	202 (27.3)
High	646 (15.9)	125 (14.9)	118 (16.0)
Smoking status, *n* (%)[b]			
Never	660 (16.2)	142 (17.0)	117 (15.8)
Former	2,740 (67.4)	571 (68.2)	496 (67.1)
Current	665 (16.4)	124 (14.8)	126 (17.1)
Physical activity, *n* (%)[b,f]			
Low	1,646 (40.7)	310 (37.2)	342 (46.5)
Medium	1,531 (37.8)	345 (41.4)	253 (34.4)
High	868 (21.5)	178 (21.4)	140 (19.1)
Medication use, *n* (%)			
Statins	3,494 (85.9)	750 (89.5)	630 (85.3)
Anti-hypertensive drugs	3,644 (89.6)	789 (94.2)	668 (90.4)
Anti-diabetes drugs	593 (14.6)	126 (15.0)	116 (15.7)
Prevalent diabetes, *n* (%)[g]	811 (20.0)	182 (21.7)	164 (22.2)
Plasma glucose (mmol/L)[b]	5.60 (5.06-6.59)	5.98 (5.38-6.97)	5.64 (5.10-6.67)
Serum lipids (mmol/L)[h]			
Total cholesterol	4.71 ± 0.96	4.45 ± 0.90	4.72 ± 0.98
LDL cholesterol	2.57 ± 0.82	2.26 ± 0.75	2.59 ± 0.83
HDL cholesterol	1.28 ± 0.34	1.37 ± 0.34	1.28 ± 0.35
Triglycerides	1.66 (1.21-2.31)	1.59 (1.18-2.31)	1.63 (1.18-2.30)
Fasting at blood collection, *n* (%)[i]	1,390 (35.5)	226 (28.2)	249 (35.1)

[a] Values are shown as mean ± standard deviation, median (interquartile range) or *n* (%);
[b] Missing values for <1% of patients;
[c] Obesity defined as BMI ≥30 kg/m^2;
[d] To convert MJ to kcal, multiply by 238.8;
[e] Categorized as “no: 0 g/d”, “low: >0 to 10 g/d”, “moderate: >10 to 20 g/d for women and >10 to 30 g/d for men”, and “high: >20 g/d for women and >30 g/d for men”;
[f] Categorized as "low: no activity or only light activity (≤ 3 METs)", "medium: >0 to <5 days per week of moderate or vigorous activity (>3 METs)", and "high: ≥5 days per week of moderate or vigorous activity";
[g] Defined as a self-reported physician’s diagnosis, use of anti-diabetes medication, or plasma glucose ≥7.0 mmol/L (fasting) or ≥11.1 mmol/L (non-fasting);
[h] Missing values for 61 patients for total cholesterol, HDL-C and TG, and for 253 patients for LDL-C;
[i] Defined as consumption of the last meal ≥8 hours before blood sampling; missing values for 155 patients.

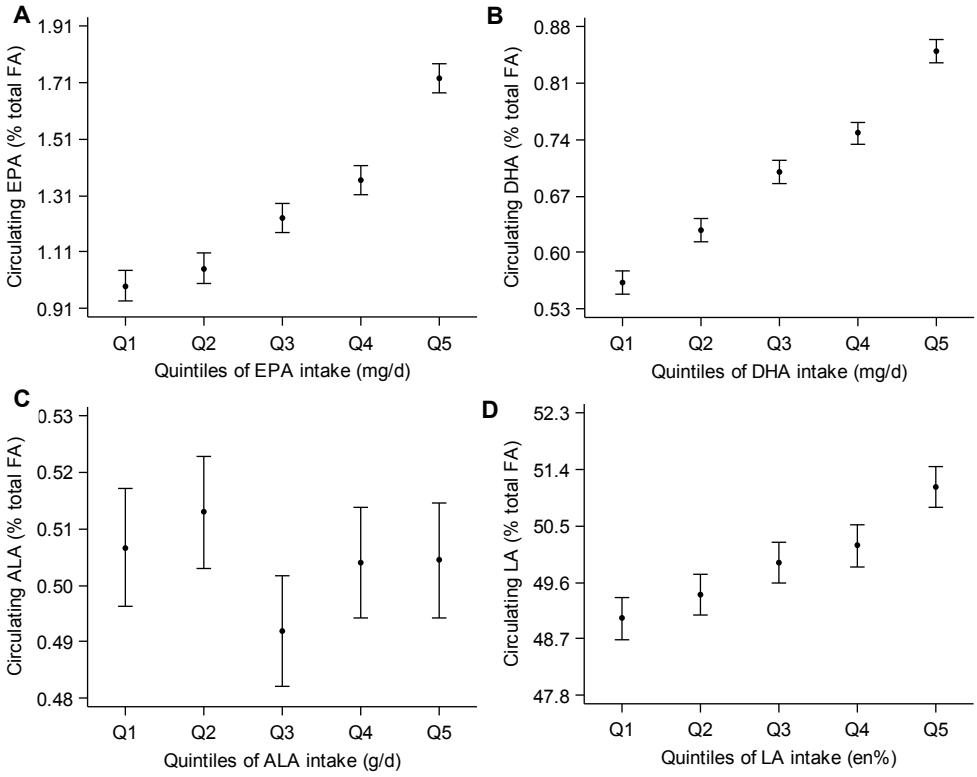

Figure 1 Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA, and (D) LA in plasma cholesteryl esters (*n*=4,066).

Least-squares means and 95%CI were adjusted for age, sex, total energy intake, obesity, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, alcohol intake, and prevalent diabetes. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89); LA (en%; Q1:1.2-3.8, Q3: 4.8-5.9, Q5:7.2-19.3). P-for-trend <0.001 for EPA, DHA, and LA, and 0.80 for ALA. ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LA, linoleic acid.

Table 2 Circulating and dietary n-3 fatty acids and linoleic acid in different plasma lipid pools, and correlations between circulating and dietary fatty acids in the Alpha Omega Cohort

Plasma lipid pool	Circulating levels of fatty acid		Dietary intake of fatty acid		Spearman rank correlation (r_s)[a]			
					Crude	*P*	Partial	*P*
Cholesteryl esters (*n*=4,066)	EPA (%)	1.06 (0.79-1.52)	EPA (mg/d)	41.3 (12.6-72.2)	0.39 (0.36, 0.41)	<0.001	0.39 (0.37, 0.42)	<0.001
	DHA (%)	0.67 (0.53-0.84)	DHA (mg/d)	67.4 (29.7-114.9)	0.44 (0.41, 0.46)	<0.001	0.45 (0.43, 0.48)	<0.001
	ALA (%)	0.49 (0.41-0.59)	ALA (g/d)	0.93 (0.68-1.33)	-0.01 (-0.04, 0.02)	0.56	-0.02 (-0.05, 0.01)	0.25
	LA (%)	49.9±5.0	LA (en%)	5.7±2.2	0.16 (0.13, 0.19)	<0.001	0.15 (0.12, 0.18)	<0.001
Phospholipids (*n*=838)	EPA (%)	1.13 (0.85-1.56)	EPA (mg/d)	45.0 (12.8-74.1)	0.35 (0.29, 0.41)	<0.001	0.37 (0.31, 0.43)	<0.001
	DHA (%)	4.49 (3.68-5.39)	DHA (mg/d)	72.3 (30.9-120.2)	0.48 (0.42, 0.53)	<0.001	0.50 (0.45, 0.55)	<0.001
	ALA (%)	0.14 (0.11-0.17)	ALA (g/d)	0.92 (0.67-1.30)	-0.00 (-0.07, 0.06)	0.89	-0.01 (-0.07, 0.06)	0.85
	LA (%)	18.7±3.0	LA (en%)	5.8±2.2	0.11 (0.05, 0.18)	0.001	0.10 (0.03, 0.17)	0.003
Total plasma[b] (*n*=739)	DHA (%)	1.24 (1.05-1.55)	DHA (mg/d)	63.5 (25.8-109.5)	0.41 (0.35, 0.47)	<0.001	0.43 (0.37, 0.49)	<0.001
	LA (%)	26.3±3.6	LA (en%)	5.7±2.3	0.17 (0.10, 0.24)	<0.001	0.16 (0.08, 0.23)	<0.001
	DHA (mmol/L)	0.14 (0.12-0.18)	DHA (mg/d)	63.5 (25.8-109.5)	0.34 (0.27, 0.40)	<0.001	0.37 (0.31, 0.43)	<0.001
	LA (mmol/L)	3.05±0.72	LA (en%)	5.7±2.3	0.12 (0.04, 0.19)	0.002	0.13 (0.06, 0.20)	<0.001

Values are shown as mean ± standard deviation, median (interquartile range).
[a] r_s (95% CI), with P-values, for association between dietary and circulating fatty acids in corresponding plasma pools. Partial correlations were adjusted for age, sex, and total energy intake.
[b] EPA and ALA were not measured in total plasma.
ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid.

Relation between intake and circulating linoleic acid

Dietary linoleic acid intake was 5.7±2.2 en% (12.1±5.5 g/d) and levels of circulating linoleic acid were 49.9±5.0 % in CE, 18.7±3.0 % in PL, and 26.3 ±3.6 % in total plasma. Correlations for linoleic acid across different plasma lipid pools ranged from 0.71 to 0.85, with the strongest correlation between CE and total plasma (r_s= 0.85). Partial correlations between dietary and circulating linoleic acid (**Table 2)** were <0.2 for all plasma lipid pools, with some evidence for a dose-response relationship (**Figure 1D**). Partial correlations in subsample that had fasted >12 hours showed similar results (r_s=0.06-0.16; data not in Table). When dietary linoleic acid was expressed as % of total fat, the partial correlation with linoleic acid in CE was 0.13, and when expressed in g/d, it was 0.15 (data not shown in Table).

Subgroup analyses for dietary and circulating linoleic acid (in CE) are shown in **Figure 2A-E.** Men had somewhat higher circulating linoleic acid than women. Circulating linoleic acid did not strongly differ between obese and non-obese or diabetic and non-diabetic patients. However, circulating linoleic acid was substantially lower in statin users (**Figure 2D**) and in patients with a high alcohol intake (**Figure 2E**). In all subgroups, there was a dose-response relation between dietary and circulating linoleic acid in CE, comparable to the total cohort (**Figure 2A-E**). The partial correlations between dietary and circulating linoleic acid within subgroups were also comparable to the total cohort, although some fluctuations were seen in PL and total plasma, resulting from smaller sample sizes (**Supplemental Table 4**).

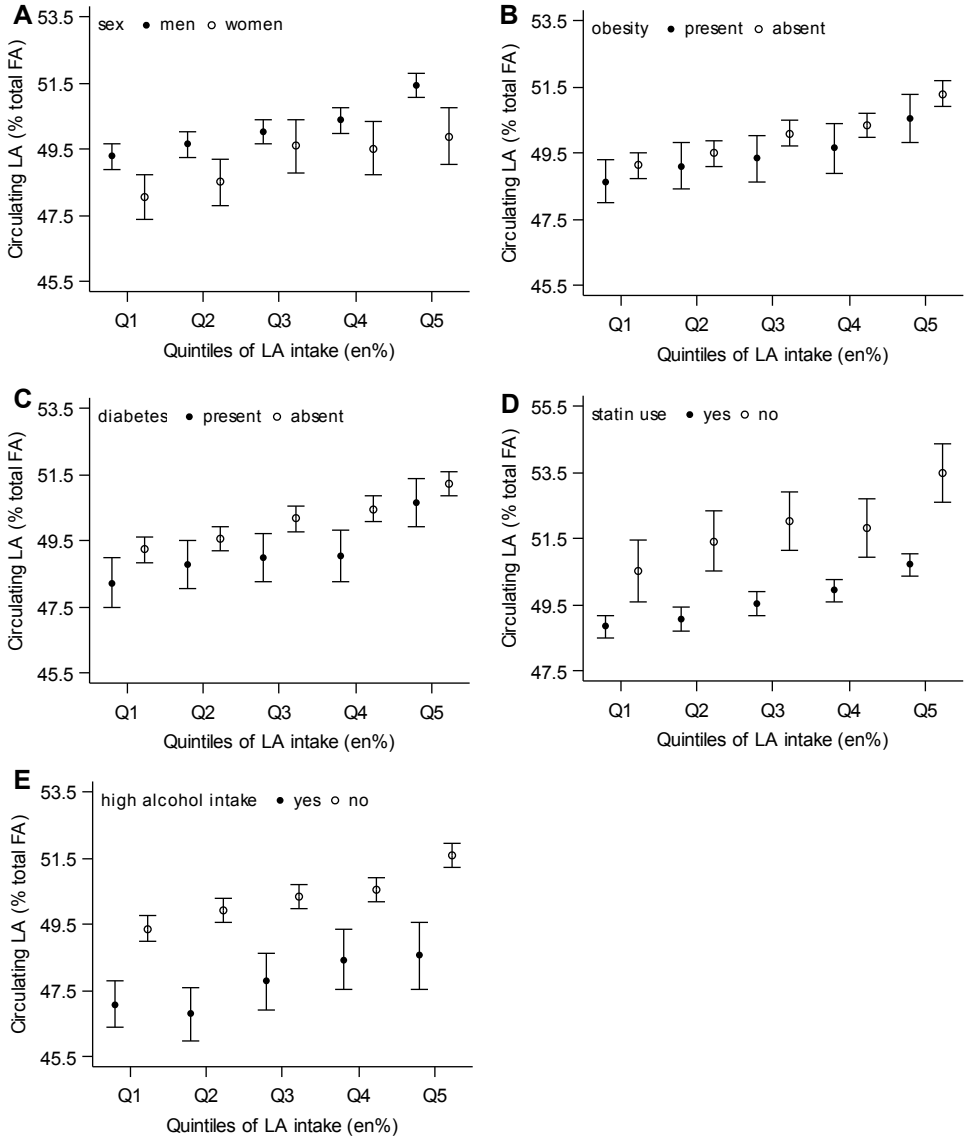

Figure 2 Relation between dietary and circulating linoleic acid (LA) in cholesteryl esters in strata of (A) sex, (B) obesity, (C) diabetes, (D) statin use, and (E) alcohol intake.

Least-squares means and 95%CI were adjusted for age, sex, total energy intake, obesity, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, alcohol intake, and prevalent diabetes. When stratified by statin use, total serum cholesterol was not included as adjustment variable in the model. LA intake ranges (en%): Q1:1.2-3.8, Q3: 4.8-5.9, Q5:7.2-19.3. P-for-trend <0.001 in all subgroups of sex, obesity, statin use, and prevalent diabetes, and in patients without high alcohol intake; P-for-trend=0.003 in patients with high alcohol intake. P-values for interaction terms: sex, p=0.60; obesity, p=0.47; diabetes, p=0.79; statin use, p=0.18; alcohol intake, p=0.85.

DISCUSSION

In our cohort of post-MI patients, we observed moderate correlations between intake and circulating levels of EPA and DHA (0.4-0.5), and no correlations for ALA, which was consistent across various plasma lipid pools. Correlations between dietary and circulating linoleic acid were weak (<0.2). Circulating levels of LA appeared to be lower for statin users and patients with a high alcohol intake.

Circulating FA have repeatedly been used as biomarkers of FA intake in (relatively) healthy populations.[1,4] For individual n-3 FA, the present study in post-MI patients showed similar correlations between dietary and circulating levels to those in population-based studies. In previous reviews[1,21] and in the more recent Multi-Ethnic Study of Atherosclerosis (MESA) cohort,[22] correlations varied between 0.2 and 0.5 for EPA and DHA. For ALA, weak correlations between dietary and circulating levels were also reported by others.[21,22] Rapid oxidation of ingested ALA[23] and dietary measurement error may explain this finding. Conversion of ALA into EPA is not a likely explanation because the correlation between ALA intake and plasma EPA was only weak in our cohort.

We have shown dose-response relationships between dietary and circulating EPA and DHA in CE irrespective of cardiometabolic risk factors and statin use. In four longitudinal UK and Finnish cohorts, statin use was associated with only a small increase in circulating proportion of DHA in total plasma.[24] In Australian adults, associations between dietary and circulating EPA and DHA in PL varied by sex, but were not modified by history of MI (3% of total participants), presence of diabetes (4%), lipid-lowering medication use (3%), or alcohol intake.[25]

Correlations between dietary and circulating linoleic acid in the Alpha Omega Cohort were considerably weaker than in other studies, which were mostly conducted in non-patient populations (r=0.20-0.34).[25–29] The unit for linoleic acid intake may possibly affect the size of the correlation. However, in our study, expressing dietary linoleic acid as % total fat instead of energy% did not result in stronger correlation. Our findings are in line with the MESA study that showed a weak correlation (r=0.13) between dietary linoleic acid, expressed in g/d, and circulating linoleic acid measured in phospholipids.[22] Part of our cohort was not in fasting state during blood sampling. However, this cannot explain the absence of a correlation since we obtained similar results in a subsample of the cohort that had fasted >12 hours. This finding is in line with the study by Hodge et al. in 4439 Australian adults[25] who also found that fasting status did not modify the association between dietary and circulating unsaturated FA. The average level and range of

linoleic acid intake in our cohort was comparable to that of the general older Dutch population[30] and to that of other populations with Western diets.[25–29] Therefore, we consider lack of contrast in linoleic acid intake not a likely explanation for the weak correlation with circulating linoleic acid.

In our study, patients with high alcohol intake had lower mean circulating linoleic acid than patients who consumed less alcohol, despite having the same dietary linoleic acid intake. In several other studies, alcohol intake was inversely associated with circulating linoleic acid and directly with palmitic acid (16:0).[28,31] Palmitic acid and linoleic acid are two major FA in CE, PL and total plasma.[1] Circulating palmitic acid does not reflect only palmitic acid intake but also its endogenous synthesis. Changes in palmitic acid will indirectly affect the measured proportion of linoleic acid, since they are expressed as relative concentrations in lipid pools.[32]

We observed generally lower circulating linoleic acid levels in statin users compared to non-users, despite similar linoleic acid intake. A decrease in circulating linoleic acid levels associated with statin use was also observed in total serum of four population-based cohorts.[24] Hodge et al.,[25] however, did not find that use of lipid-lowering drugs (3% of cohort) modified the association between dietary and circulating FA in 4439 Australian adults. *In vitro* research in human white blood cells suggested that statins may enhance the activity of desaturase enzymes, which converts LA into arachidonic acid (20:4 n-6). However, the underlying mechanisms remain unclear.[33]

Around 20% of our cohort suffered from diabetes. For a given linoleic acid intake, linoleic acid in CE tended to be lower in diabetic patients than in other patients. In a cross-sectional analysis of 70-year-old Swedish men, a lower linoleic acid in CE was associated with reduced insulin sensitivity,[34] and another study observed a lower plasma CE linoleic acid in patients with non-alcoholic fatty liver disease compared to age- and sex-matched healthy individuals.[9] Diabetic patients may be at higher risk of liver dysfunction, which could possibly affect circulating linoleic acid and its correlation with dietary intake. However, we did not assess insulin resistance or liver dysfunction to confirm this hypothesis.

Correlations between dietary and circulating FA partly depend on the dietary assessment method. We used an FFQ that provided very detailed information on sources of fat and types and brands of fats and oils used for food preparation. Using this FFQ, we found a strong inverse association of dietary unsaturated FA (replacing saturated and trans FA) with cardiovascular mortality in the Alpha Omega Cohort.[12]

In the current analysis, we also found expected correlations of dietary with circulating n-3 FA, further supporting the validity of this FFQ.

A limitation needs to be considered while interpreting our results. The proportion of patients in different subgroups was unbalanced (e.g. only 14% non-statin users). This complicated the comparison of correlation coefficients in different subgroups and formal testing of interaction. Our results in subgroups therefore need confirmation in other studies.

To conclude, circulating EPA and DHA in various plasma lipid pools are good indicators of dietary intake in a cohort of post-MI patients, whereas circulating ALA is not. Concerning linoleic acid, circulating levels may not well reflect dietary linoleic acid intake. This may partly be due to alcohol use and/or statins.

List of abbreviations

ALA, alpha-linolenic acid; CE, cholesteryl esters; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; MI, myocardial infarction; PL, phospholipids

Acknowledgments

This research received financial support from the Netherlands Organisation for Scientific Research (NWO) through its Graduate Programme on Food Structure, Digestion and Health. Funding for plasma fatty acid analyses was obtained from Unilever R&D and from the Dutch biobank collaboration BBMRI-NL. Data collection for the Alpha Omega Cohort was funded by the Dutch Heart Foundation (topdown program 200T401) and the National Institutes of Health (NIH/NHLBI grant no. R01HL076200).

We thank Eveline Waterham for management of Alpha Omega Cohort database and biobank and Dr. Paul Hulshof, Robert Hovenier and Marlies Diepeveen-de Bruin for analysis of plasma fatty acids.

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SUPPLEMENTARY MATERIALS

Supplemental Methods

Measurements of fatty acid composition in cholesteryl esters (CE) and phospholipids (PL) in Alpha Omega Cohort

Analysis of fatty acids

A volume of 650 µl plasma was pipetted into a tube. Total lipids were extracted from the plasma samples with an isopropanol/hexane mixture (2:3, v/v) and centrifuged. The upper hexane layer was removed and dried under nitrogen atmosphere. Afterwards, the total lipids were separated into CE and PL fractions using silica column SPE 500 mg (Sopachem Isolute 460-0050-B, Sopachem BV, the Netherlands). Subsequently, fatty acids (FA) in each fraction were transesterified into FA methyl esters (FAME) using boron trifluoride-methanol. FAME samples of 0.5 µl were injected in splitless mode into a model 6890 gas chromatograph equipped with a model 7683 auto sampler (Agilent, Santa Clara, CA) and a flame-ionization detector. The column used for chromatography was a CP WAX 58 CB column (25 m x 0.25 mm i.d. and film thickness of 0.21 µm) (Agilent, Santa Clara, CA). Hydrogen was used as the carrier gas. Injector and detector temperatures were kept at 275°C and 265°C, respectively. Initial temperature of the oven was 60°C and this temperature was held for 1.5 minutes. The temperature was programmed to rise by 30°C/minutes to 180°C, 1.2°C/minutes to 215°C, and 40°C/min to 245°C. The final temperature was held for 10 minutes. Total run time was 45 minutes. FA were identified by comparing elution time of the peak of each FAME with FA standards of known composition (Nu-chek Prep, Elysian, MN). The sum of the all detected area under the peak was considered 100%. In total, 38 FA were identified and quantified, ranging from 12:0 to 24:1. FA were expressed as weight percentage relative to total FA measured in the chromatogram.

FA in CE were measured in different years: 2003-2004 (321 samples), 2006 (770 samples), 2012 (314 samples), and 2016 (2,661 samples), and PL FA were all measured in 2016. A plasma control pool was used to monitor analytical performance of the FA analyses over different batches. In each run of 38 samples, a control sample was analyzed in duplicate. Within-run and between-run CV of the control pool were calculated as measure of repeatability. For CE measurements in 2003-2012, the CV (in %) for within- and between-run were 0.4 and 1.1 for LA, 1.0 and 4.7 for ALA, 1.5 and 4.9 for EPA, 1.4 and 6.7 for DHA, respectively. For

measurements of CE in 2016, within- and between-run CV were 0.6 and 1.2 for linoleic acid, and 3.5 and 7.6 for DHA. For PL, the CV for within- and between-run were 0.9 and 1.0 for linoleic acid, 1.6 and 4.2 for DHA, respectively.

Effect of long-term storage in -80°C to FA composition in CE

Thirty-five blood samples were randomly selected from baseline samples provided by patients in the Alpha Omega Trial (AOT). Sixteen of these samples were collected in 2003 and 19 samples in 2006. These samples were analyzed for FA composition in CE directly after collection. All of the blood samples were again analyzed for FA composition in 2012. Effects of 6-9 years of storage at -80°C were investigated by comparing circulating FA measured in 2003 or 2006 and in 2012. Here, we reported results for selected FA that are within the scope of the current analysis: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), alpha-linoleic acid (ALA) and linoleic acid.

Variance components (between- and within-subject variance) were estimated using PROC VARCOMP with restricted maximum likelihood method, assuming a one-way random effect model ANOVA. Within- and between-subject CV and intraclass correlation coefficients (ICC) as reliability coefficient were calculated using the estimated variance components. ICC represents the proportion of total variation attributed to differences between subjects. All analyses were done using SAS 9.3 (SAS Institute, Cary, NC).

Patients included in the analysis for effect of storage time were on average 70.2 (±SD 5.8) years old and 66% were male. The within-subject CV ranged from 1.3 (linoleic acid) to 8.7 (ALA) while between-subject CV ranged from 9.6 (linoleic acid) to 83.6 (EPA). All FA had high ICC (>0.90) (Supplemental Table 1).

Supplemental Table 1 Stability of individual n-3 fatty acids and linoleic acid in cholesteryl esters after 6-9 years of storage at -80°C in 35 plasma samples from the Alpha Omega Cohort

Fatty acids	Mean values (± SD) of fatty acid (% total fatty acids)		CV (%)		ICC
	Initial analysis	Repeat analysis[a]	Within-subject	Between-subject	
EPA					
2003	1.38 ± 0.81	1.40 ± 0.80	2.1	58.0	0.99
2006	1.22 ± 0.99	1.26 ± 1.08	6.6	83.6	0.99
DHA					
2003	0.76 ± 0.20	0.74 ± 0.22	2.9	27.9	0.99
2006	0.63 ± 0.28	0.65 ± 0.29	3.4	44.5	0.99
ALA					
2003	0.49 ± 0.14	0.52 ± 0.15	5.6	27.8	0.96
2006	0.58 ± 0.17	0.58 ± 0.17	8.7	28.6	0.91
Linoleic acid					
2003	48.6 ± 5.04	49.2 ± 5.25	1.5	10.5	0.98
2006	51.2 ± 5.04	50.9 ± 4.87	1.3	9.6	0.98

[a] Represents measured values (in % of total fatty acids) after long-term (6-9 years) storage at -80°C.
ALA, alpha-linolenic acid; CV, coefficient of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ICC, intraclass correlation coefficient.

Supplemental Table 2 Correlation coefficients for circulating n-3 FA and linoleic acid across different plasma lipid pools

	CE vs PL (n=838)	CE vs total plasma (n=739)	PL vs total plasma (n=151)
EPA	0.93 (0.92, 0.94)	n/a[†]	n/a[†]
DHA	0.93 (0.92, 0.94)	0.88 (0.87, 0.90)[a]	0.92 (0.88, 0.94)[a]
		0.70 (0.66, 0.74)[b]	0.70 (0.61, 0.77)[b]
ALA	0.80 (0.78, 0.83)	n/a[†]	n/a[†]
Linoleic acid	0.84 (0.81, 0.85)	0.85 (0.83, 0.87)[a]	0.71 (0.61, 0.78)[a]
		0.50 (0.43, 0.54)[b]	0.45 (0.31, 0.57)[b]

All values are Spearman rank correlation coefficients and 95% CI. P-value for all the correlations is <0.001.
[†] EPA and ALA were not measured in total plasma and correlation coefficients could not be calculated.
[a] Fatty acid in total plasma is expressed as % of total fatty acids.
[b] Fatty acid in total plasma is expressed as absolute amount (mmol/L).
ALA, alpha-linolenic acid; CE, cholesteryl esters; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PL, phospholipids; n/a, not available.

Supplemental Table 3 Correlations between dietary and circulating EPA, DHA and ALA in plasma lipid pools in subgroups[a]

		Cholesteryl esters (n=4,066)		Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
EPA[b]									
Sex	Men	0.40 (0.37, 0.43) (n=3226)	<0.001	0.37 (0.31, 0.44) (n=652)	<0.001	n/a	n/a	n/a	n/a
	Women	0.36 (0.30, 0.41) (n=840)	<0.001	0.37 (0.23, 0.48) (n=186)	<0.001	n/a	n/a	n/a	n/a
Obesity[c]	Present	0.38 (0.32, 0.43) (n=947)	<0.001	0.35 (0.22, 0.47) (n=201)	<0.001	n/a	n/a	n/a	n/a
	Absent	0.40 (0.37, 0.43) (n=3115)	<0.001	0.38 (0.31, 0.44) (n=637)	<0.001	n/a	n/a	n/a	n/a
Prevalent diabetes	Present	0.33 (0.27, 0.39) (n=881)	<0.001	0.27 (0.13, 0.40) (n=182)	<0.001	n/a	n/a	n/a	n/a
	Absent	0.41 (0.38, 0.44) (n=3255)	<0.001	0.40 (0.33, 0.46) (n=656)	<0.001	n/a	n/a	n/a	n/a
Statin use	Yes	0.40 (0.37, 0.43) (n=3494)	<0.001	0.38 (0.32, 0.44) (n=750)	<0.001	n/a	n/a	n/a	n/a
	No	0.34 (0.27, 0.41) (n=572)	<0.001	0.31 (0.11, 0.49) (n=88)	0.003	n/a	n/a	n/a	n/a

Supplemental Table 3. continued

		Cholesteryl esters (n=4,066)		Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
High alcohol intake[d]	Yes	0.37 (0.30, 0.43) (n=646)	<0.001	0.34 (0.17, 0.48) (n=125)	<0.001	n/a	n/a	n/a	n/a
	No	0.38 (0.35, 0.41) (n=3420)	<0.001	0.36 (0.30, 0.43) (n=673)	<0.001	n/a	n/a	n/a	n/a
DHA									
Sex	Men	0.46 (0.44, 0.49) (n=3226)	<0.001	0.50 (0.44, 0.56) (n=652)	<0.001	0.44 (0.38, 0.51) (n=578)	<0.001	0.39 (0.32, 0.46) (n=578)	<0.001
	Women	0.43 (0.37, 0.48) (n=840)	<0.001	0.51 (0.39, 0.61) (n=186)	<0.001	0.37 (0.23, 0.50) (n=161)	<0.001	0.27 (0.12, 0.41) (n=161)	<0.001
Obesity[c]	Present	0.44 (0.39, 0.49) (n=947)	<0.001	0.51 (0.40, 0.61) (n=201)	<0.001	0.43 (0.30, 0.55) (n=166)	<0.001	0.36 (0.22, 0.48) (n=166)	<0.001
	Absent	0.46 (0.43, 0.48) (n=3115)	<0.001	0.50 (0.44, 0.56) (n=637)	<0.001	0.43 (0.36, 0.49) (n=573)	<0.001	0.37 (0.29, 0.44) (n=573)	<0.001
Prevalent diabetes	Present	0.41 (0.35, 0.47) (n=811)	<0.001	0.44 (0.31, 0.55) (n=182)	<0.001	0.43 (0.29, 0.55) (n=164)	<0.001	0.33 (0.19, 0.46) (n=164)	<0.001
	Absent	0.46 (0.44, 0.49) (n=3255)	<0.001	0.52 (0.46, 0.57) (n=656)	<0.001	0.43 (0.36, 0.50) (n=575)	<0.001	0.38 (0.31, 0.45) (n=575)	<0.001

		Cholesteryl esters (n=4,066)		Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
Statin use	Yes	0.45 (0.43, 0.48) (n=3494)	<0.001	0.51 (0.45, 0.56) (n=750)	<0.001	0.42 (0.35, 0.48) (n=630)	<0.001	0.36 (0.29, 0.43) (n=630)	<0.001
	No	0.46 (0.39, 0.52) (n=572)	<0.001	0.50 (0.32, 0.64) (n=88)	<0.001	0.48 (0.32, 0.61) (n=109)	<0.001	0.39 (0.22, 0.54) (n=109)	<0.001
High alcohol intake[d]	Yes	0.41 (0.34, 0.47) (n=646)	<0.001	0.47 (0.32, 0.60) (n=125)	<0.001	0.50 (0.35, 0.63) (n=118)	<0.001	0.43 (0.26, 0.57) (n=118)	<0.001
	No	0.46 (0.44, 0.49) (n=3420)	<0.001	0.51 (0.45, 0.56) (n=713)	<0.001	0.42 (0.35, 0.48) (n=621)	<0.001	0.35 (0.28, 0.41) (n=621)	<0.001
ALA[b]									
Sex	Men	-0.02 (-0.06, 0.02) (n=3226)	0.18	-0.01 (-0.08, 0.07) (n=652)	0.88	n/a	n/a	n/a	n/a
	Women	0.01 (-0.06, 0.07) (n=840)	0.85	-0.01 (-0.16, 0.13) (n=186)	0.84	n/a	n/a	n/a	n/a
Obesity[c]	Present	-0.02 (-0.08, 0.05) (n=947)	0.62	-0.00 (-0.14, 0.13) (n=201)	0.95	n/a	n/a	n/a	n/a
	Absent	-0.02 (-0.05, 0.02) (n=2948)	0.27	-0.02 (-0.09, 0.06) (n=637)	0.70	n/a	n/a	n/a	n/a

Supplemental Table 3. continued

		Cholesteryl esters (n=4,066)		Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
Prevalent diabetes	Present	-0.00 (-0.07, 0.07) (n=811)	0.97	0.03 (-0.11, 0.18) (n=182)	0.66	n/a	n/a	n/a	n/a
	Absent	-0.02 (-0.06, 0.01) (n=3,255)	0.22	-0.02 (-0.09, 0.06) (n=656)	0.69	n/a	n/a	n/a	n/a
Statin use	Yes	-0.02 (-0.05, 0.02) (n=3,494)	0.33	0.00 (-0.07, 0.08) (n=750)	0.92	n/a	n/a	n/a	n/a
	No	-0.04 (-0.12, 0.04) (n=572)	0.38	-0.04 (-0.25, 0.17) (n=88)	0.71	n/a	n/a	n/a	n/a
High alcohol intake[d]	Yes	0.01 (-0.07, 0.09) (n=646)	0.78	0.30 (0.13, 0.45) (n=125)	<0.001	n/a	n/a	n/a	n/a
	No	-0.02 (-0.05, 0.01) (n=3,420)	0.22	-0.07 (-0.14, 0.01) (n=713)	0.08	n/a	n/a	n/a	n/a

[a] Spearman rank correlation coefficient (r_s) and 95%CI adjusted for age, sex (except when used as stratifying factor), and total energy intake; [b] EPA and ALA were not measured in total plasma and correlation coefficients could not be calculated; [c] Obesity defined as BMI ≥30 kg/m^2; [d] High alcohol intake, yes: >30 g/d in men, >20 g/d in women; no: ≤30 g/d in men, ≤20 g/d in women; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; n/a, not available.

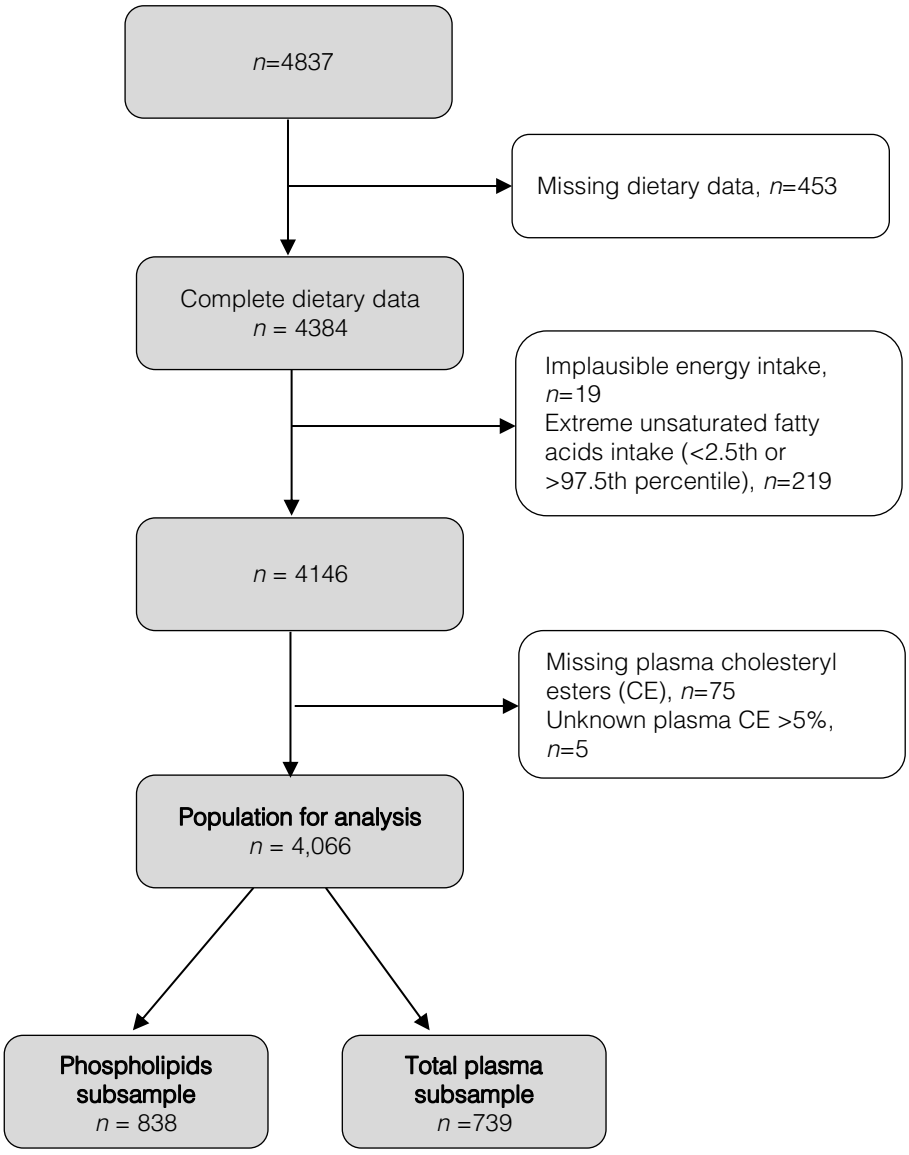
Supplemental Table 4 Correlations between dietary and circulating linoleic acid in plasma lipid pools in subgroups[a]

		Cholesteryl esters (n=4,066)		*P-int*	Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*		r_s	*P*	r_s	*P*	r_s	*P*
Sex	Men	0.16 (0.12, 0.19) (n=3226)	<0.001	0.60	0.11 (0.04, 0.19) (n=652)	<0.001	0.15 (0.06, 0.22) (n=578)	<0.001	0.10 (0.01, 0.18) (n=578)	<0.001
	Women	0.15 (0.09, 0.22) (n=840)	<0.001		0.07 (-0.08, 0.21) (n=186)	0.36	0.19 (0.03, 0.34) (n=161)	0.016	0.24 (0.09, 0.39) (n=161)	0.002
Obesity[b]	Present	0.15 (0.09, 0.21) (n=947)	<0.001	0.47	-0.00 (-0.14, 0.14) (n=201)	0.96	0.27 (0.12, 0.41) (n=166)	<0.001	0.10 (-0.06, 0.25) (n=166)	0.21
	Absent	0.15 (0.12, 0.19) (n=3115)	<0.001		0.13 (0.05, 0.21) (n=637)	<0.001	0.12 (0.04, 0.20) (n=573)	0.004	0.14 (0.06, 0.22) (n=573)	<0.001
Prevalent diabetes	Present	0.14 (0.07, 0.21) (n=881)	<0.001	0.79	0.16 (0.02, 0.30) (n=182)	0.029	0.17 (0.02, 0.32) (n=164)	0.029	0.19 (0.04, 0.34) (n=164)	<0.001
	Absent	0.16 (0.12, 0.19) (n=3255)	<0.001		0.09 (0.01, 0.16) (n=656)	0.028	0.15 (0.07, 0.23) (n=575)	<0.001	0.11 (0.03, 0.19) (n=575)	<0.001
Statin use	Yes	0.15 (0.12, 0.19) (n=3494)	<0.001	0.18	0.11 (0.04, 0.18) (n=750)	0.002	0.18 (0.10, 0.25) (n=630)	<0.001	0.13 (0.06, 0.21) (n=630)	<0.001
	No	0.16 (0.08, 0.24) (n=572)	<0.001		0.03 (-0.19, 0.24) (n=88)	0.81	0.09 (-0.10, 0.27) (n=109)	0.37	0.18 (-0.01, 0.36) (n=109)	0.07

Supplemental Table 4. continued

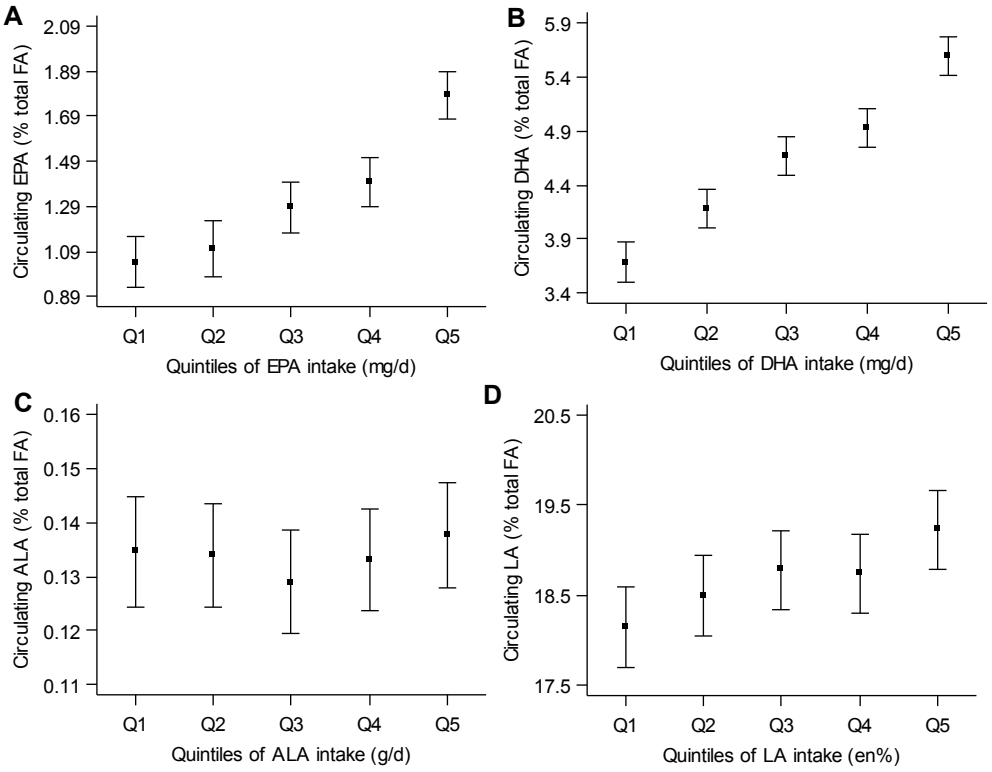
		Cholesteryl esters (n=4,066)		*P-int*	Phospholipids (n=838)		Total plasma (%) (n=739)		Total plasma (mmol/l) (n=739)	
		r_s	*P*		r_s	*P*	r_s	*P*	r_s	*P*
High alcohol intake[c]	Yes	0.09 (0.01, 0.17) (n=646)	0.019	0.85	-0.03 (-0.21, 0.14) (n=125)	0.71	0.09 (-0.10, 0.27) (n=118)	0.35	0.02 (-0.17, 0.20) (n=118)	0.85
	No	0.14 (0.11, 0.17) (n=3420)	<0.001		0.11 (0.03, 0.18) (n=713)	0.005	0.14 (0.07, 0.22) (n=621)	<0.001	0.14 (0.06, 0.22) (n=621)	<0.001

[a] Spearman rank correlation coefficient (r_s) with 95% CI adjusted for age, sex (except when used as stratifying factor), and total energy intake. [b] Obesity defined as BMI ≥30 kg/m^2. [c] High alcohol intake, yes: >30 g/d in men, >20 g/d in women; no: ≤30 g/d in men, ≤20 g/d in women. *P*-int, *P-value* for interaction.



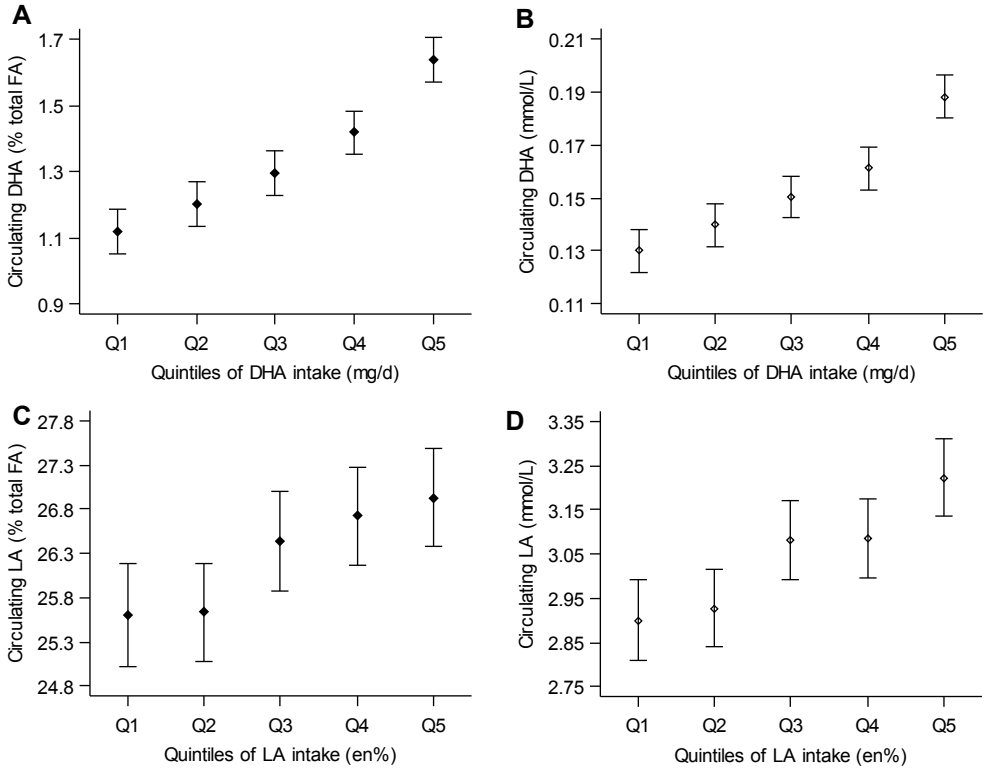
Supplemental Figure 1 Flow diagram for population for analysis[a]

[a] Phospholipids subsample and total plasma subsample overlapped for 151 samples.



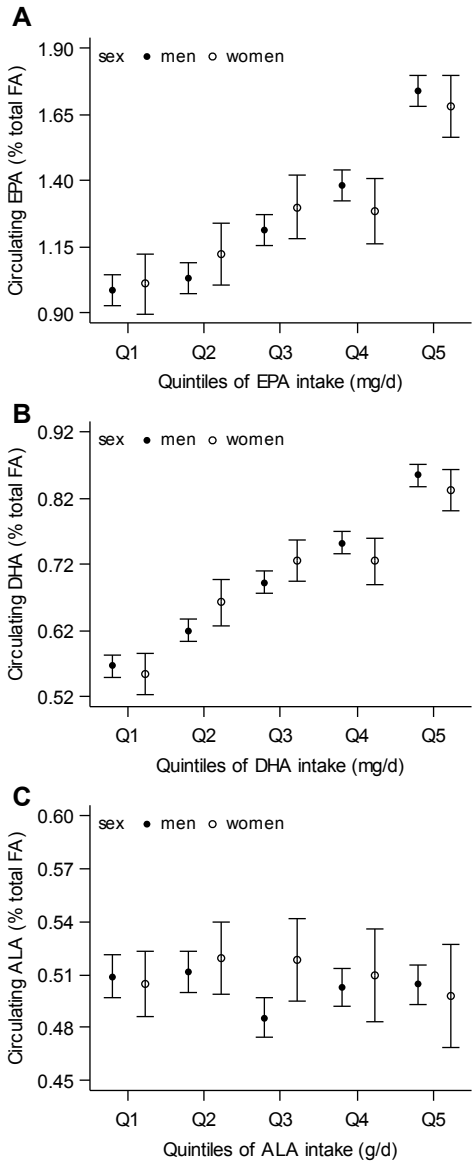
Supplemental Figure 2A-D Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA, and (D) linoleic acid in plasma phospholipids.

Least-squares means and 95%CI were adjusted for age, sex, obesity, physical activity, smoking status, fasting status, total serum cholesterol, total energy intake, alcohol intake, and prevalent diabetes. Intake ranges: EPA (mg/d; Q1:0.0-8.0, Q3:31.5-53.7, Q5:94.3-648.1); DHA (mg/d; Q1:0.5-23.8, Q3:56.7-88.8, Q5:146.3-1061.6); ALA (g/d; Q1:0.24-0.62, Q3:0.80-1.06, Q5:1.44-3.55); LA (en%; Q1:1.5-4.0, Q3: 5.0-5.9, Q5:7.2-16.7). ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LA, linoleic acid.



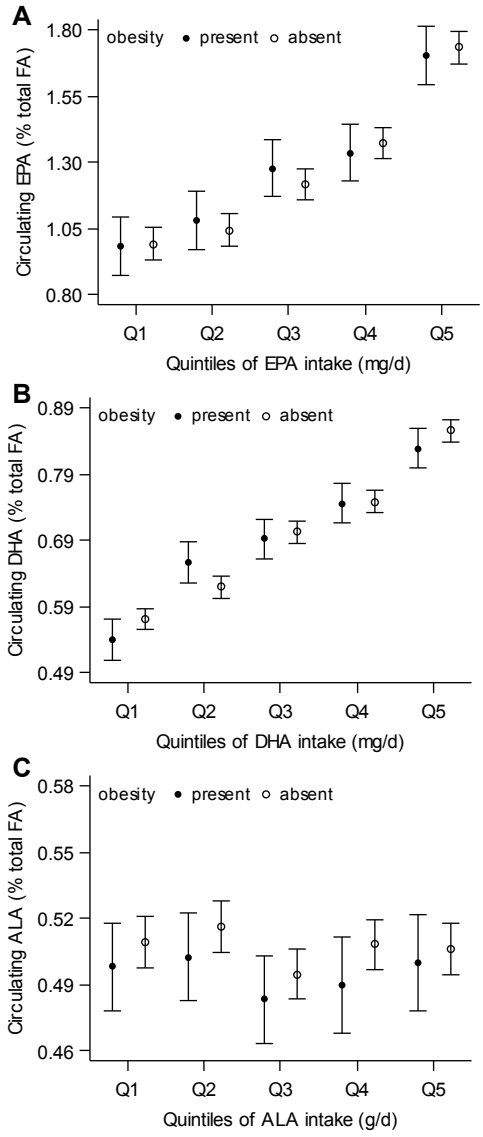
Supplemental Figure 3A-D Relation between dietary and circulating DHA (A, B) and linoleic acid (C, D) in total plasma.

Least-squares means and 95%CI were adjusted for age, sex, obesity, physical activity, smoking status, fasting status, total serum cholesterol, total energy intake, alcohol intake, and prevalent diabetes. Intake ranges: DHA (mg/d; Q1:0.6-20.4, Q3:46.6-80.2, Q5:122.0-962.4); LA (en%; Q1:1.3-3.8, Q3: 4.8-5.8, Q5:7.3-19.3). DHA, docosahexaenoic acid; FA, fatty acids; LA, linoleic acid.



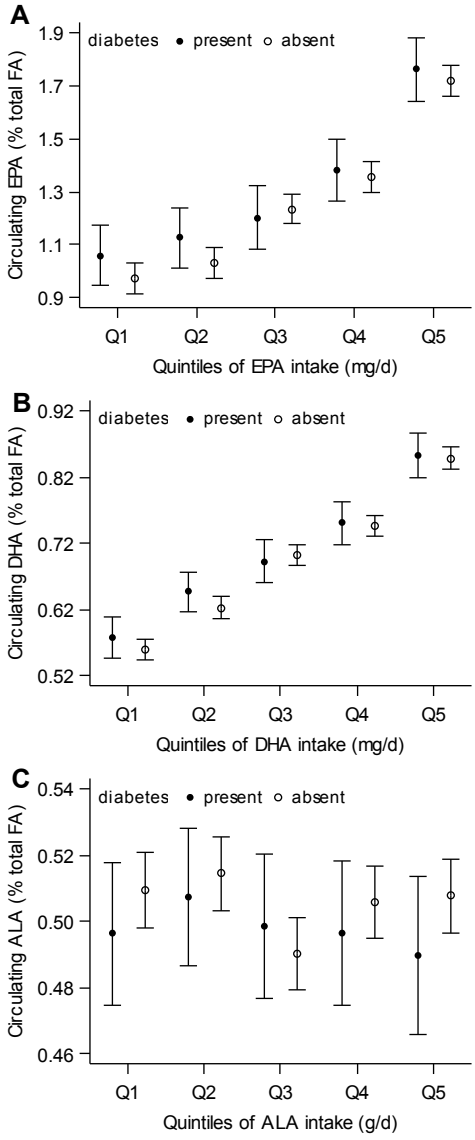
Supplemental Figure 4A-C Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA in cholesteryl esters by sex.

Least squares means and 95%CI were adjusted for age, obesity, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, total energy intake, alcohol intake, and prevalent diabetes. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89). ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids.



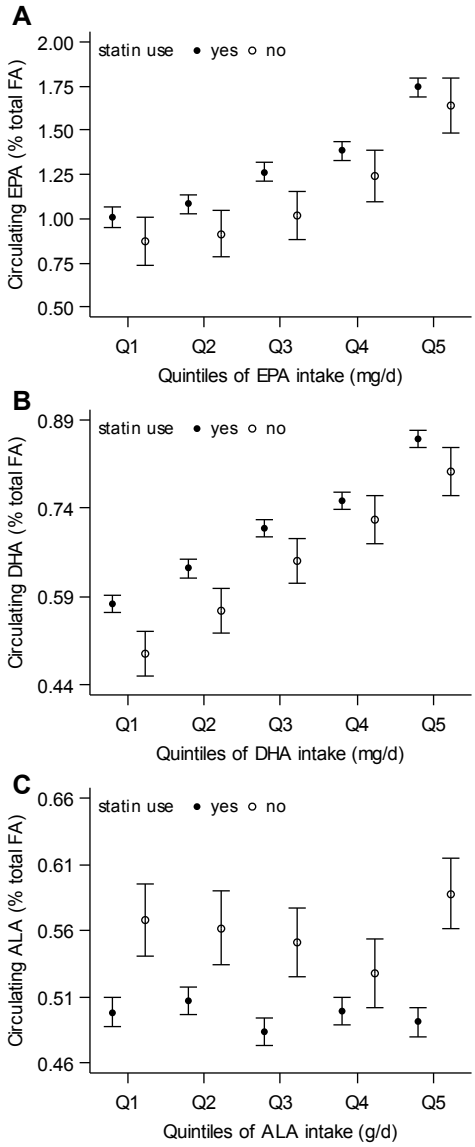
Supplemental Figure 5A-C Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA in cholesteryl esters by presence of obesity.

Least squares means and 95%CI were adjusted for age, sex, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, total energy intake, alcohol intake, and prevalent diabetes. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89). ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid.



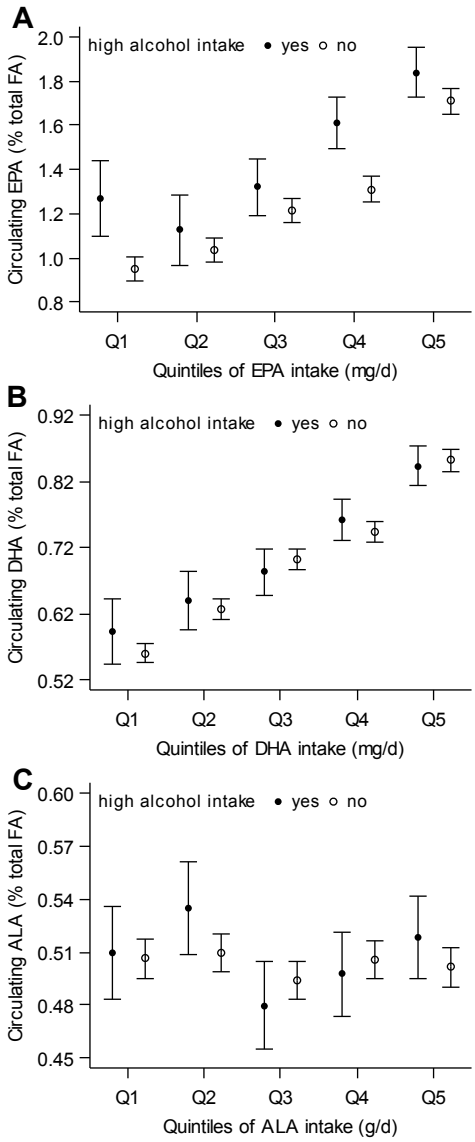
Supplemental Figure 6A-C Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA in cholesteryl esters by prevalent diabetes.

Least squares means and 95%CI were adjusted for age, sex, obesity, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, total energy intake, and alcohol intake. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89). ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids.



Supplemental Figure 7A-C Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA in cholesteryl esters by statin use.

Least squares means and 95%CI were adjusted for age, sex, obesity, physical activity, smoking status, fasting status, measurement year, total energy intake, high alcohol intake, and prevalent diabetes. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89). ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids.



Supplemental Figure 8A-C Relation between dietary and circulating (A) EPA, (B) DHA, (C) ALA in cholesteryl esters by alcohol intake.

Least squares means and 95%CI were adjusted for age, sex, obesity, physical activity, smoking status, fasting status, total serum cholesterol, measurement year, total energy intake, and prevalent diabetes. High alcohol intake, yes: >30 g/d in men, >20 g/d in women; no: ≤30 g/d in men, ≤20 g/d in women. Intake ranges: EPA (mg/d; Q1:0.0-8.3, Q3:28.7-50.9, Q5:85.6-692.0); DHA (mg/d; Q1:0.1-23.4, Q3:51.3-84.3, Q5:134.5-1061.6); ALA (g/d; Q1:0.19-0.63, Q3:0.83-1.07, Q5:1.46-3.89). ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FA, fatty acids.

Chapter 3

Associations of dairy and fiber intake with circulating odd-chain fatty acids in post-myocardial infarction patients

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Nutrition & Metabolism 2019;16(1):78

ABSTRACT

Background: Circulating odd-chain fatty acids pentadecanoic (15:0) and heptadecanoic acid (17:0) are considered to reflect dairy intake. In cohort studies, higher circulating 15:0 and 17:0 were associated with lower type 2 diabetes risk. A recent randomized controlled trial in humans suggested that fiber intake also increased circulating 15:0 and 17:0, potentially resulting from fermentation by gut microbes. We examined the associations of dairy and fiber intake with circulating 15:0 and 17:0 in patients with a history of myocardial infarction (MI).

Methods: We performed cross-sectional analyses in a subsample of 869 Dutch post-MI patients of the Alpha Omega Cohort who had data on dietary intake and circulating fatty acids. Dietary intakes (g/d) were assessed using a 203-item food frequency questionnaire. Circulating 15:0 and 17:0 (as % of total fatty acids) were measured in plasma phospholipids (PL) and cholesteryl esters (CE). Spearman correlations (r_s) were computed between intakes of total dairy, dairy fat, fiber, and circulating 15:0 and 17:0.

Results: Patients were on average 69 years old, 78% was male and 21% had diabetes. Total dairy intake comprised predominantly milk and yogurt (69%). Dairy fat was mainly derived from cheese (47%) and milk (15%), and fiber was mainly from grains (43%). Circulating 15:0 in PL was significantly correlated with total dairy and dairy fat intake (both r_s=0.19, p<0.001), but not with dietary fiber intake (r_s=0.05, p=0.11). Circulating 17:0 in PL was correlated both with dairy intake (r_s=0.14 for total dairy and 0.11 for dairy fat, p<0.001), and fiber intake (r_s=0.19, p<0.001). Results in CE were roughly similar, except for a weaker correlation of CE 17:0 with fiber (r_s=0.11, p=0.001). Circulating 15:0 was highest in those with high dairy intake irrespective of fiber intake, while circulating 17:0 was highest in those with high dairy and fiber intake.

Conclusions: In our cohort of post-MI patients, circulating 15:0 was associated with dairy intake but not fiber intake, whereas circulating 17:0 was associated with both dairy and fiber intake. These data suggest that cardiometabolic health benefits previously attributed to 17:0 as a biomarker of dairy intake may partly be explained by fiber intake.

INTRODUCTION

Circulating odd-chain fatty acids (OCFA) pentadecanoic (15:0) and heptadecanoic acid (17:0) have been used as biomarkers of dairy and dairy fat intake in observational studies.[1,2] The reason is that fatty acids primarily coming from exogenous sources are usually considered good candidates as biomarkers for intake[3] and more objective than self-reported dietary assessment. Since OCFA are considered to be solely produced in rumen of ruminants and cannot be produced by the human body, they have been proposed as good candidate biomarkers for dairy fat and/or total dairy intake.[4–6] A recent meta-analysis of 18 observational studies showed that intake of dairy and dairy fat were more correlated with circulating proportions of 15:0 (with correlation coefficients (*r*) of 0.20 and 0.33, respectively) than 17:0 (*r*=0.10 and 0.19, respectively).[1]

Higher circulating 15:0 and 17:0 have been associated with lower risk of cardiometabolic outcomes, such as type 2 diabetes[7–9] and cardiovascular disease.[10–12] A recent pooled analysis of 16 prospective cohort studies showed that higher circulating 15:0 and 17:0 were associated with 20% and 35% lower risk of type 2 diabetes, respectively, by comparing individuals with circulating 15:0 or 17:0 in the 90th to those in the 10th cohort-specific percentile.[2] In these studies, the beneficial associations between circulating 15:0 and 17:0 and cardiometabolic risk were often attributed to dairy intake only.

In a randomized controlled trial in sixteen healthy participants, supplementation of inulin, a fermentable fiber, increased the level of circulating OCFA in plasma phospholipids (PL).[13] In the European Prospective Investigation into Cancer and Nutrition (EPIC) Inter-Act study, both intake of dairy and fruits and vegetables, which are important sources of fiber, were associated with the sum of circulating 15:0 and 17:0 in PL, with correlations between 0.1-0.2.[7] Another observational study showed associations of circulating PL 15:0 with ruminant meat intake (*r*=0.4) and dairy fat (*r*=0.5).[14] These data suggest that circulating OCFA may not only reflect dairy and/or dairy fat intakes, but also intakes of other foods.

Our primary aim in the present analyses was to examine the associations of dairy, dairy fat and fiber intakes with circulating 15:0 and 17:0 in a cohort of Dutch post-myocardial infarction patients.

METHODS

Study design and population

Cross-sectional analyses were carried out in baseline data of the Alpha Omega Cohort (years 2002-2006) as described previously.[15,16] This cohort consists of 4837 Dutch patients aged 60 through 80 who had a myocardial infarction (MI) up to 10 years before study enrollment. The medical ethics committee at the Haga Hospital (The Hague, The Netherlands) approved the study and all patients provided written informed consent. The present analysis included 869 patients with complete and reliable data on dietary intake and plasma fatty acids both in PL and cholesteryl esters (CE) **(Additional file 1: Figure S1)**.

Dietary assessment

Habitual dietary intake was assessed with a 203-item food frequency questionnaire (FFQ) which was an extended version of a previously validated FFQ to estimate fatty acids and cholesterol intake.[17,18] The FFQ contained 42 items on various dairy products for which questions were grouped by fat contents: whole, semi-skimmed or skimmed. Total dairy included intake of milk, yogurt, cheese, dairy desserts, cream, milk for coffee and creamers, butter and ice-cream. Intakes of different dairy products in grams/day were calculated by multiplying consumption frequencies and portion sizes. Intake of milk and creamers from non-dairy sources such as soy milk and non-dairy creamers were not included. Daily intake of total energy (kcal/d; including alcohol), foods (g/d) and nutrients, including dairy fat (g/d), were calculated after linkage of intake data to the 2006 Dutch food composition table (NEVO).[19]

Dairy products were divided in two groups based on their fat content. Low-fat dairy included semi-skimmed (fat content ≤1.8 g/100 ml) and skimmed (fat content ≤1.5g/100 ml) milk, buttermilk, yogurt drink and other low-fat dairy products. High-fat dairy included full-fat dairy products (fat content >3.0 g/100 g for solids or >1.8 g/100 ml for liquids), all types of cheese, dairy-based coffee creamer, butter, cream and ice-cream **(Additional file 1: Table S1)**.

Total fiber intake was calculated by summing up intakes of fiber from all dietary sources (excluding dietary supplements) (Additional file 1: Table S2). Dietary fiber included plant constituents that cannot be digested by human enzymes in stomach and small intestine, e.g. lignin, cellulose, hemicellulose and pectin, as measured using the recommended Association of Official Analytical Chemists method.[19] Total

meat included beef, pork, chicken, turkey, lamb, mutton and other meats. Ruminant meat included intake of beef, lamb and mutton. Total fish included fatty fish, lean fish and shellfish. Intakes of foods and food groups were expressed as g/d.

Data collection on risk factors

Information about demographic factors, lifestyle characteristics and medication use were obtained by self-administered questionnaires as previously described.[15] The validated Physical Activity Scale for the Elderly was used to assess the physical activity level of the patients.[20] Alcohol intake (ethanol, in g/d) was assessed with the FFQ and categorized as 'no' (0 g/d), 'low' (>0 to 10 g/d), 'moderate' (>10 to 20 g/d in women; >10 to 30 g/d in men) or 'high' (>20 g/d in women; >30 g/d in men). Medications were coded according to the Anatomical Therapeutic Chemical Classification system, with codes C10AA and C10B for statins, C02 for antihypertensive drugs and A10 for antidiabetic drugs.[21] Physical examination took place at home or in the outpatient clinic by trained research nurses. Body mass index was calculated from measured weight divided by height squared (kg/m^2). A fasted or non-fasted venous blood sample (30 mL) was drawn from each patient. Fasting was defined as at least 8 hours since the last meal. Serum lipids and plasma glucose were measured at baseline using standard kits as previously described.[15] Diabetes was considered present if patients reported a physician's diagnosis of diabetes, used anti-diabetes medication, or had plasma glucose ≥7.0 mmol/L (fasting) or ≥11.1 mmol/L (non-fasting).[22] Blood samples were stored at -80°C for future analysis.

Measurement of odd-chain fatty acids

Circulating OCFA 15:0 and 17:0 were measured in plasma PL and CE. Laboratory procedures for measuring fatty acid composition in plasma PL and CE in the Alpha Omega Cohort have been described in detail previously.[23] In short, plasma total lipids from 10 mL EDTA blood samples were extracted and separated into plasma PL and CE fraction of fatty acids by using solid phase extraction method. Subsequently, fatty acids in each fraction were trans-esterified into fatty acid methyl esters. Fatty acid composition in plasma PL and CE were measured using gas chromatography equipped with a flame ionization detector. In total, 38 fatty acids were detected by comparison to pure fatty acid standards. For each plasma fraction, circulating individual fatty acids were expressed as proportion of total fatty acids measured (% total fatty acids).

Statistical analysis

Patient characteristics were expressed as crude means (± standard deviation, SD) or medians (interquartile range, IQR) for continuous variables and numbers (percentage) for categorical variables. Spearman's rank correlation (r_s) was used to measure the associations of total dairy, dairy fat and total fiber intakes with circulating 15:0 and 17:0, for PL and CE separately. Partial correlation coefficients were calculated with adjustment for age, sex and total energy intake and their 95% confidence intervals (CI) were obtained by Fisher's z transformation. Additionally, we assessed the dose-response association of dairy or fiber intake with circulating 15:0 and 17:0 using restricted cubic splines with three knots placed at 5th, 50th and 95th percentile, adjusted for age, sex and total energy intake. To examine whether correlations between dairy and 15:0 and 17:0 were modified by dietary fiber, partial correlations were obtained in strata of low and high total fiber intake (<21.2 g/d versus ≥21.2 g/d, based on median energy-adjusted intake). Likewise, partial correlations between dietary fiber and circulating OCFA were calculated for strata of energy-adjusted total dairy intake (median= 301 g/d) and dairy fat (median= 12.0 g/d). We also assessed circulating 15:0 and 17:0 in combined subgroups of dairy and fiber intake: 'low dairy-low fiber', 'low dairy-high fiber', 'high dairy-low fiber' and 'high dairy-high fiber' by using generalized linear models (normal distribution, identity link function) with robust standard errors, also adjusted by age, sex and total energy intake.

Because ruminant meat and fish can contain 15:0 and 17:0, we also calculated partial correlations of intakes of total meat, ruminant meat and total fish with circulating 15:0 and 17:0 in PL and CE. SAS 9.4 (Cary, NC, USA) was used for all analyses, and a two-sided p-value <0.05 was considered as statistically significant. The %RCS_Reg macro was used to perform restricted cubic splines analyses.[24]

RESULTS

Patients were on average 69.2 (SD= 5.7) years old and enrolled in the study ~3 year after MI (**Table 1**). Of the cohort, 78% were male, 21% had diabetes and 24% was obese. Most patients (>85%) used cardiovascular medication, including statins and anti-hypertensive drugs.

Median total dairy intake was 294 g/d, which was predominantly low-fat dairy (73%) (**Table 2**). Total dairy mainly comprised milk (median= 150 g/d), followed by yogurt (56 g/d), dairy desserts (e.g. quark and custard; 22 g/d), and cheese (20 g/d). Median intake of dairy fat was 11.1 g/d (19% of the total fat intake), of which 1.3 g/d was from milk and 4.5 g/d was from cheese. Median total fiber intake was 21.0 g/d, which was mainly derived from grains (43% of total fiber). Median intakes of total meat, ruminant meat and total fish were 59 g/d, 17 g/d and 14 g/d, respectively.

Circulating proportions of 15:0 were comparable in PL and CE, while for 17:0, proportions in PL were higher than in CE (Table 1). Proportions of 15:0 and 17:0 within each fraction were correlated (crude r_s in PL= 0.56, in CE= 0.47; all $p<0.001$; data not in Table).

Table 1 Characteristics of patients in the subsample of Alpha Omega Cohort for the present analysis[a]

	Value
Age (y)	69.2 ± 5.7
Men, *n* (%)	678 (78.0)
Body mass index (kg/m^2)	27.8 ± 4.0
Obese, *n* (%)[b]	205 (23.6)
Smoking status, *n* (%)[c]	
Never	150 (17.3)
Former	595 (68.6)
Current	123 (14.2)
Physical activity, *n* (%)[c,d]	
Low	327 (37.9)
Medium	356 (41.3)
High	179 (20.8)
Alcohol intake, *n* (%)[e]	
No	58 (6.7)
Low	464 (53.4)
Moderate	223 (25.7)
High	124 (14.3)
Time since last myocardial infarction (y)	3.0 (1.4-5.9)
Prevalent diabetes, *n*(%)[f]	186 (21.4)
Medication use, *n* (%)	
Statins	772 (88.8)
Anti-hypertensive	818 (94.1)
Anti-diabetes	129 (14.8)
Serum lipids (mmol/L)	
Total cholesterol[c]	4.43 ± 0.89
LDL cholesterol[g]	2.25 ± 0.75
HDL cholesterol[c]	1.37 ± 0.35
Triglycerides[c]	1.59 (1.18-2.31)
Odd-chain fatty acids (% total fatty acids)	
Phospholipids 15:0	0.14 (0.12-0.16)
Phospholipids 17:0	0.38 ± 0.08
Cholesteryl esters 15:0	0.16 ± 0.04
Cholesteryl esters 17:0	0.08 (0.07-0.10)

[a] Values are mean ± standard deviation or median (interquartile range) or *n* (%).
[b] Obesity defined as body mass index ≥30 kg/m^2.
[c] Missing values for <1% of patients.
[d] Categorized as "low: no activity or only light activity (≤ 3 METs), "medium: >0 to <5 days per week of moderate or vigorous activity (>3 METs), and "high: ≥5 days per week of moderate or vigorous activity.
[e] Categorized as "no: 0 g/d", "low: >0 to 10 g/d", "moderate: >10 to 20 g/d for women and >10 to 30 g/d for men", and "high: >20 g/d for women and >30 g/d for men".
[f] Defined as a self-reported physician's diagnosis, use of anti-diabetes medication, or plasma glucose ≥7.0 mmol/L (fasting) or ≥11.1 mmol/L (non-fasting).
[g] Missing values for 44 patients for LDL cholesterol.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; MET, metabolic equivalent.

Table 2 Reported intake of dairy products, dairy fat, total fiber and other foods in AOC subsample[a,b]

	Mean ± SD	Median (Q1-Q3)
Total dairy	343 ± 248	294 (191-418)
High-fat dairy	92 ± 98	65 (37-113)
Low fat dairy	251 ± 224	201 (110-321)
Milk	161 ± 183	150 (21-167)
Yogurt	75 ± 85	56 (18-128)
Cheese	26 ± 26	20 (9-32)
Cream	1 ± 2	0 (0-1)
Butter	1 ± 5	0 (0-0)
Dairy desserts	45 ± 66	22 (13-58)
Ice-cream	10 ± 19	9 (0-13)
Milk for coffee and creamers	23 ± 52	5 (0-25)
Dairy fat	13.6 ± 9.8	11.1 (7.3-17.2)
Fat from milk	2.1 ± 3.0	1.3 (0.3-2.5)
Fat from yogurt	0.8 ± 1.2	0.3 (0.1-1.4)
Fat from cheese	6.4 ± 6.4	4.5 (2.3-7.7)
Fat from cream	0.2 ± 0.4	0.0 (0.0-0.3)
Fat from butter	0.9 ± 4.0	0.0 (0.0-0.0)
Fat from dairy desserts	1.6 ± 2.6	0.5 (0.0-2.0)
Fat from ice-cream	0.7 ± 1.4	0.7 (0.0-1.0)
Fat from milk for coffee and creamer	0.9 ± 1.5	0.3 (0.0-1.2)
Total fiber	21.5 ± 6.9	21.0 (16.7-25.1)
Total meat	52 ± 34	59 (30-72)
Ruminant meat	18 ± 15	17 (6-26)
Total fish	17 ± 19	14 (4-17)

[a] Intake (not energy-adjusted) is reported in grams/day;
[b] Individual dairy products were listed in Additional file 1: Table S1.
AOC, Alpha Omega Cohort; Q, quartile.

Associations of circulating 15:0 and 17:0 with dietary intake

Circulating 15:0 in both PL and CE was significantly correlated with intake of total dairy and dairy fat; partial r_s ranged from 0.19 to 0.26 (**Table 3**). Circulating 15:0 in both PL and CE were not significantly associated with total fiber intake (partial r_s ≤0.05). Circulating 17:0 in PL was significantly correlated with intakes of total dairy (partial r_s=0.14), dairy fat (partial r_s = 0.11), and total fiber intake (partial r_s = 0.19). Correlations with 17:0 in CE were similar for total dairy, dairy fat and total fiber. However, the correlation of total fiber with 17:0 in CE (partial r_s = 0.11) was smaller than for PL. Dose-response analysis using restricted cubic splines showed that associations of PL 15:0 and 17:0 with total dairy were non-linearly shaped. For CE, only the association between 15:0 and total dairy intake was non-linear (Additional file 1: Figure S2-S4). No correlations were observed between circulating OCFA and other food groups, except for a weak correlation of 15:0 in CE with total fish intake (partial r_s = 0.09) (Table 3), which persisted when only the fish eaters (n=680) were included in the analysis (partial r_s = 0.10; not shown in Table). Additional adjustments for medications and presence of type 2 diabetes did not appreciably change the results **(Additional file 1: Table S3)**. However, in sensitivity analysis including only patients without type 2 diabetes (n=683), PL 17:0 was weakly correlated with ruminant meat intake (p=0.013) and the correlation of CE 15:0 with fish intake was no longer statistically significant **(Additional file 1: Table S4)**.

In patients with a high fiber intake, partial correlations of dairy intake with circulating OCFA were smaller than in those with a low fiber intake (**Table 4**). In patients with a high total dairy intake, partial correlations of total fiber intake with circulating 17:0 in PL and CE were smaller than in patients with a low total dairy intake, and total fiber intake was not correlated with 15:0 in patients with higher intakes of total dairy or dairy fat (**Table 5**). Assessment of the circulating 15:0 and 17:0 in different strata of dairy and fiber showed that patients with low or high fiber intake had similar mean circulating PL 15:0 when their dairy intake was high (**Figure 1**). The highest mean circulating PL 17:0 was observed for patients with both high dairy and high fiber intake, while patients with either low dairy-high fiber intake or high dairy-low fiber intake had similar mean circulating PL 17:0. Results in CE were similar (data not shown).

Table 3 Correlations between intakes of dairy, fiber and other foods and circulating 15:0 and 17:0[a]

	Fractions	Pentadecanoic acid (15:0)		Heptadecanoic acid (17:0)	
		Crude	Partial	Crude	Partial
Dairy					
Total dairy	Phospholipids	0.19 (0.12, 0.25)***	0.19 (0.12, 0.25)***	0.14 (0.08, 0.21)***	0.14 (0.08, 0.21)***
	Cholesteryl esters	0.22 (0.15, 0.28)***	0.23 (0.17, 0.30)***	0.11 (0.04, 0.17)**	0.14 (0.07, 0.20)***
Dairy fat	Phospholipids	0.17 (0.10, 0.23)***	0.19 (0.13, 0.26)***	0.10 (0.03, 0.16)**	0.11 (0.05, 0.18)***
	Cholesteryl esters	0.22 (0.16, 0.29)***	0.26 (0.20, 0.32)***	0.10 (0.03, 0.16)**	0.12 (0.05, 0.18)***
Total fiber	Phospholipids	0.01 (-0.06, 0.08)	0.05 (-0.01, 0.12)	0.13 (0.07, 0.20)***	0.19 (0.12, 0.25)***
	Cholesteryl esters	0.00 (-0.07, 0.07)	0.03 (-0.04, 0.10)	0.09 (0.02, 0.15)**	0.11 (0.04, 0.18)**
Other foods					
Total meat	Phospholipids	-0.02 (-0.09, 0.04)	-0.01 (-0.08, 0.05)	0.01 (-0.06, 0.07)	0.02 (-0.05, 0.08)
	Cholesteryl esters	-0.05 (-0.11, 0.02)	-0.04 (-0.10, 0.03)	0.01 (-0.05, 0.08)	0.02 (-0.05, 0.08)
Ruminant meat	Phospholipids	0.06 (-0.01, 0.12)	0.06 (-0.01, 0.13)	0.06 (-0.01, 0.12)	0.06 (-0.00, 0.13)
	Cholesteryl esters	0.05 (-0.01, 0.12)	0.06 (-0.01, 0.12)	0.04 (-0.03, 0.10)	0.04 (-0.03, 0.11)
Total fish	Phospholipids	-0.00 (-0.07, 0.06)	0.01 (-0.05, 0.08)	0.06 (-0.01, 0.12)	0.06 (-0.01, 0.13)
	Cholesteryl esters	0.07 (0.00, 0.13)*	0.09 (0.02, 0.15)*	0.02 (-0.04, 0.09)	0.02 (-0.05, 0.09)

*$p<0.05$; **$p<0.01$; ***$p<0.001$.

[a] Crude: Spearman's correlation of intakes (g/d) with circulating 15:0 or 17:0 in phospholipids or cholesteryl esters (% of total fatty acids); partial: Spearman's correlation of energy-adjusted intakes (g/d) with circulating 15:0 or 17:0 in phospholipids or cholesteryl esters (% of total fatty acids), adjusted for age, sex, total energy intake.

Table 4 Partial correlations between dairy intake and circulating 15:0 and 17:0 in strata of fiber intake[a,b]

		Pentadecanoic acid (15:0)		Heptadecanoic acid (17:0)	
		Phospholipids	Cholesteryl esters	Phospholipids	Cholesteryl esters
Total dairy	Low fiber (*n*=438)	0.22 (0.13, 0.30)***	0.27 (0.18, 0.36)***	0.20 (0.11, 0.29)***	0.16 (0.07, 0.25)***
	High fiber (*n*=431)	0.17 (0.07, 0.26)***	0.19 (0.10, 0.28)***	0.10 (0.00, 0.19)*	0.11 (0.02, 0.20)*
Dairy fat	Low fiber (*n*=438)	0.23 (0.14, 0.31)***	0.32 (0.23, 0.40)***	0.20 (0.10, 0.28)***	0.18 (0.08, 0.27)***
	High fiber (*n*=431)	0.18 (0.09, 0.28)***	0.22 (0.12, 0.30)***	0.09 (-0.00, 0.19)	0.09 (-0.01, 0.18)

*$p<0.05$; **$p<0.01$; ***$p<0.001$.

[a] Spearman's correlation of energy-adjusted dietary intakes (g/d) with circulating 15:0 or 17:0 (% of total fatty acids), partially adjusted for age, sex, total energy intake.

[b] "Low fiber" referring to subsample of patients with energy-adjusted total fiber intake below median intake of total sample and "high fiber" to patients with energy-adjusted total fiber intake at median intake or higher. Median energy-adjusted total fiber intake = 21.2 g/d.

Table 5 Partial correlations between fiber intake and circulating 15:0 and 17:0 in strata of dairy intake[a,b,c]

Dietary intake		Pentadecanoic acid (15:0)		Heptadecanoic acid (17:0)	
		Phospholipids	Cholesteryl esters	Phospholipids	Cholesteryl esters
Total fiber	Low total dairy (*n*=434)	0.10 (0.01, 0.19)*	0.10 (0.01, 0.20)*	0.25 (0.16, 0.34)***	0.13 (0.03, 0.22)**
	High total dairy (*n*=435)	0.02 (-0.08, 0.11)	-0.01 (-0.11, 0.08)	0.13 (0.04, 0.22)**	0.10 (0.00, 0.19)*
Total fiber	Low dairy fat (*n*=434)	0.15 (0.06, 0.24)**	0.17 (0.08, 0.26)***	0.26 (0.17, 0.35)***	0.18 (0.08, 0.27)***
	High dairy fat (*n*=435)	0.02 (-0.07, 0.12)	-0.01 (-0.11, 0.08)	0.15 (0.06, 0.24)**	0.09 (-0.00, 0.18)

*$p<0.05$; **$p<0.01$; ***$p<0.001$.

[a] Spearman's correlation of energy-adjusted dietary intakes (g/d) with circulating 15:0 or 17:0 (% of total fatty acids), partially adjusted for age, sex, total energy intake.

[b] "Low total dairy" referring to subsample of patients with energy-adjusted total dairy intake below median intake of total sample and "high total dairy" to patients with energy-adjusted dairy intake at median intake or higher. Median energy-adjusted total dairy intake = 301 g/d;

[c] "Low dairy fat" referring to subsample of patients with energy-adjusted dairy intake below median intake of total sample and "high dairy fat" to patients with energy-adjusted dairy intake at median intake or higher. Median energy-adjusted dairy fat intake = 12.0 g/d.

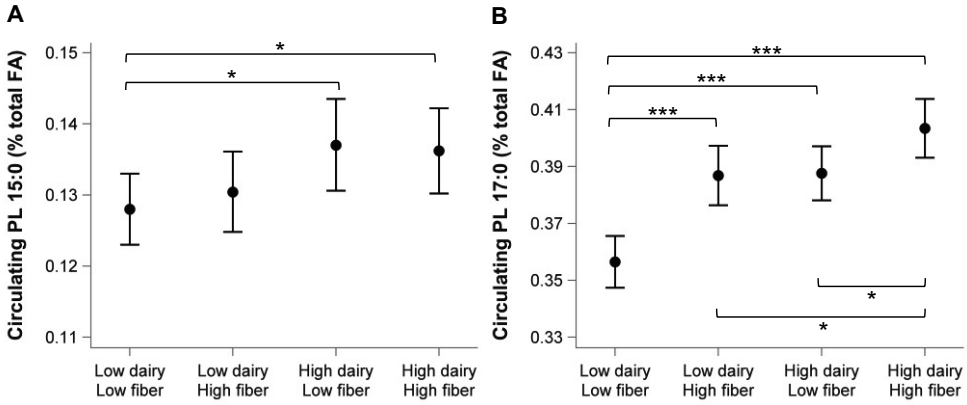

Figure 1 Circulating phospholipids 15:0 (A) and 17:0 (B) in dairy and fiber intake strata[a,b]

[a] Low dairy intake: <301 g/d; high dairy intake: ≥301 g/d;
[b] Low fiber intake: <21.2 g/d; high fiber intake: ≥21.2 g/d;
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

DISCUSSION

In the present analyses of 869 post-MI patients of the Alpha Omega Cohort, total dairy and dairy fat intake were modestly correlated with circulating 15:0, but total fiber intake was not. However, both dairy and fiber intake were correlated with circulating 17:0. Correlations between dairy intake and circulating 15:0 and 17:0 were stronger when fiber intake was low. Likewise, correlations between fiber intake and circulating 15:0 and 17:0 were stronger when dairy intake was low. Circulating 17:0 was highest in patients with both high dairy and high fiber intake, while circulating 15:0 was highest in patients with high dairy intake, regardless of their fiber intake.

The strengths of the correlations of total dairy and dairy fat intake with circulating OCFA were comparable to those reported in a recent meta-analysis of 18 observational studies, mainly in general populations.[1] This meta-analysis reported weaker correlations with total dairy and dairy fat intake for 17:0 as compared to 15:0, which is confirmed by our data. Only few studies have reported correlations between circulating OCFA and intakes of both dairy and fiber-rich foods. Consistent with our findings, a cross-sectional study of 301 healthy Swedish men aged 60-64 years showed significant correlations of 17:0 in PL with intake of dairy fat (r=0.23)

and total fiber (r=0.27).[25] However, in contrast to our observations, PL 15:0 in these Swedish men was also significantly correlated with fiber intake (r=0.20). In an EPIC-InterAct subcohort of almost 16,000 European adults, intakes of both dairy and fruits and vegetables were correlated with the sum of PL 15:0 and 17:0 (r=0.1-0.2).[7] As the proportion of 17:0 in PL tends to be 2-3 times higher than that of 15:0, the observed correlation with fruits and vegetables (key sources of fiber) were likely driven by 17:0, which would be in line with our findings. A cross-sectional analysis in 423 Finnish children aged 6-8 years old[26] found weak but significant correlations of 0.1 for 17:0 in PL and intakes of various high-fat dairy products such as milk (≥1% fat) and cheese (>17% fat) as well as for 17:0 in PL and intake of grain products high in fiber (≥5% fiber). No association was found between 15:0 in PL and intake of high-fiber grain products in these Finnish children, which is also in agreement with our present study.

A randomized controlled trial that studied the effects of fiber intake on circulating PL 15:0 and 17:0 in sixteen healthy participants found an increase of 17% in circulating 15:0 and an increase of 11% in circulating 17:0 after one week daily supplementation of 30 g inulin (a fermentable fiber). Although the average increase was larger for 15:0, it was more consistent for 17:0, with increases in PL 17:0 observed in almost all participants after inulin supplementation.[13] Based on these findings, the authors suggested that fermentation of fiber by gut microbiota could increase formation of propionate, a short chain FA, which would lead to hepatic synthesis of longer chain OCFA, in particular 17:0. The authors hypothesized that circulating OCFA could be a biomarker of fiber intake.[13] Our exploratory analyses showed that associations of dairy intake with circulating OCFA, particularly 17:0 in PL, were weaker in patients with a relatively high fiber intake. These findings suggest that circulating PL 17:0 may not adequately reflect dairy intake in populations with higher fiber intake. We also observed weaker correlations between circulating OCFA and fiber in patients with a relatively high dairy intake, which suggests that circulating OCFA may reflect fiber intake better when dairy intake in the population is low. It is noteworthy that in our exploratory analyses, circulating 17:0 was highest with high intakes of both dairy and fiber, and similar for high fiber or high dairy separately. Together, these findings imply that both dairy and fiber affect circulating proportions of OCFA, especially 17:0. This may also explain, for example, the lower correlation of 17:0 with dairy in high fiber subgroup. Higher fiber intake may contribute to higher circulating 17:0 from fiber, which could weaken the correlation of 17:0 with dairy intake.

Our findings may have implications for biomarker studies of nutrition and cardiometabolic diseases. Consistent inverse associations have been reported

between circulating proportions of OCFA and cardiovascular-related outcomes,[2,11,27] especially for 17:0, which would amount to an estimated 35% lower risk of type 2 diabetes according to a pooled analysis of 16 prospective cohort studies.[2] Observed risk reductions with higher levels of circulating OCFA have mainly been attributed to dairy intake.[2,7] Our findings suggest that these associations, especially for 17:0 in PL, may be partly due to intake of dietary fiber.

We did not observe significant correlations between meat or ruminant meat intake and circulating OCFA, while fish intake was only weakly correlated with 15:0. This is not in line with an analysis from the EPIC-Oxford study, which reported a correlation of 0.4 between intake of ruminant meat and concentrations of 15:0 in PL.[14] This is and also not in line with an analysis from the EPIC study in 16 European regions, which reported ecological correlations with 17:0 in PL of 0.4 for red meat and 0.7 for fish intake.[28] However, the latter analysis used mean values of intake and circulating OCFA in 16 European regions instead of individual data,[28] and such aggregate data analysis is limited in adjustment for potential confounders. In our Alpha-Omega cohort, the intake of ruminant meat (18 g/d) was considerably lower than in the EPIC-Oxford cohort (30 g/d), which may be an alternative explanation why we did not observe an association with circulating OCFA. Furthermore, dairy intake in our cohort was relatively high, which reduces the relative contribution of ruminant meat to circulating OCFA. However, we did find a weak positive correlation between 15:0 in CE and fish, despite that median fish intake was low (14 g/d). Dietary fiber and fish were associated with higher circulating odd-chain fatty acids in our Dutch cohort, despite a relatively low fiber and fish intake. These findings need to be confirmed by other studies, preferably randomized controlled trials in populations with higher fiber and fish intake.

The present analysis has several strengths. We collected information about dairy intake using an FFQ that had been specifically designed for estimating fatty acid intakes.[17,18] Fatty acids intake estimated by the FFQ were highly correlated to estimates by dietary history (Pearson's correlation of ~0.8 for saturated and total fatty acids in g/d). We analyzed circulating OCFA both in PL and CE, which are two commonly used plasma fractions in epidemiological studies. Stability of FA composition over 6-9 years of storage in -80°C was confirmed and inter- and intra-assay variation for measurement of various fatty acids was <8% in CE and <5% in PL.[23] We examined circulating OCFA in relation to dairy intake in subgroups with high and low fiber intake. To the best of our knowledge, this approach has not been explored in other studies of circulating OCFA as biomarkers of dairy intake.

A limitation of our current study is that we could not accurately estimate the intake of specific types of dietary fiber, because the Dutch food composition table (NEVO,[19]) mainly reports on total fiber content of foods. Fiber intake in our cohort (~22 g/d) was in line with a Dutch survey in 2010-2012 among 739 individuals aged 70 years or older, showing mean fiber intakes of 22 g/d in men and 19 g/d in women.[29] These fiber intakes are below the recommended amount of 25 g/d for the Netherlands.[30] Possibly, if fiber intakes had been higher in our cohort, stronger associations with circulating odd-chain fatty acids could have been observed. Despite this limitation, we still observed correlations between circulating 17:0 and fiber, consistent with other studies. Another limitation is the cross-sectional nature of the study, meaning that observed associations cannot be interpreted as causal relationships. Higher fiber intake may be a proxy of better adherence to healthy diet and the possibility of residual confounding from other aspects of healthy diet cannot be fully excluded. Our cohort consisted of post-MI patients and 21% of our cohort had diabetes. The circulating FA composition may be affected by existing chronic conditions, such as insulin resistance, via changes in FA metabolism.[31] Nevertheless, means and ranges of circulating proportion of OCFA in PL and CE in these patients were similar to other studies in generally healthy populations.[1] Analyses on the differences in means of circulating 15:0 and 17:0 between combined groups of dairy and fiber intake were not prespecified and should therefore be considered exploratory.

CONCLUSION

In this population of post-MI patients with a relatively high dairy consumption, circulating proportions of 15:0 were associated with dairy intake, but not fiber intake, whereas circulating 17:0 were associated with both dairy and fiber intake. The utility of 17:0 as a biomarker of dairy intake may depend on the amount of fiber consumed, and likewise, its utility as a marker of fiber intake may depend on the amount of dairy consumed. The data suggest that cardiometabolic health benefits previously attributed to biomarkers of dairy intake, especially 17:0, may partly be attributable to dietary fiber intake.

List of abbreviations

CE: cholesteryl esters
EPIC: European Prospective Investigation into Cancer and Nutrition
FA: fatty acids
FFQ: food frequency questionnaire
MI: myocardial infarction
OCFA: odd-chain fatty acids
PL: phospholipids

Declarations

Ethics approval and consent to participate

The study was approved by the medical ethics committee at the Haga Hospital (The Hague, The Netherlands). All patients provided written informed consent before enrollment.

Acknowledgments

We thank Eveline Waterham for management of the database and biobank of the Alpha Omega Cohort and Dr. Paul Hulshof, Robert Hovenier and Marlies Diepeveen-de Bruin for analysis of plasma fatty acids.

Funding

This research received financial support from the Netherlands Organization for Scientific Research (NWO) through its Graduate Programme on Food Structure, Digestion and Health. Funding for plasma fatty acid analyses was supported by research grants from Unilever R&D before divesting its spreads business, which is since July 2, 2018 operating under the name Upfield™. Data collection for the Alpha Omega Cohort was funded by the Dutch Heart Foundation (topdown program 200T401) and the National Institutes of Health (NIH/NHLBI grant no. R01HL076200).

Availability of data and materials

Datasets generated and/or analysed during this study are available in the DANS repository through the following link: https://doi.org/10.17026/dans-2a3-fg5q. The data catalogue of Alpha Omega Cohort is publicly accessible on www.alphaomegacohort.org.

Competing interests

AJW and PLZ are employed at Unilever R&D, The Netherlands; JMG received financial support from Unilever for epidemiological studies of dietary and circulating fatty acids; KP, LKK, JdG, no competing interest.

Authors' contributions

The authors' contributions were as follows– JMG and JdG designed the research; KP, LKK and AJW conducted the research; KP performed the statistical analyses; KP wrote the draft paper; JMG had overall responsibility for the research. All authors read the paper for important intellectual content and approved the final manuscript.

Consent for publication

Not applicable

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SUPPLEMENTARY MATERIALS

Table S1 Individual dairy products included and grouping into high fat or low fat dairy for the present analysis of Alpha Omega Cohort

Dairy products	Specific dairy food products	High fat or low fat dairy
Milk	Full fat milk	High fat dairy
	Full fat chocolate milk	High fat dairy
	Semi-skimmed and skimmed milk	Low fat dairy
	Semi-skimmed and skimmed chocolate milk	Low fat dairy
	Buttermilk	Low fat dairy
Yogurt	Full fat yogurt	High fat dairy
	Semi-skimmed and skimmed yogurt	Low fat dairy
	Yogurt drink	Low fat dairy
Dairy desserts	Full fat quark	High fat dairy
	Semi-skimmed and skimmed quark	Low fat dairy
	Full fat custard	High fat dairy
	Semi-skimmed and skimmed custard	Low fat dairy
	Other desserts with cream	High fat dairy
	Full fat pudding	High fat dairy
	Low fat pudding	Low fat dairy
	Dessert porridge	Low fat dairy
Cream	Whipped cream	High fat dairy
	Cream with warm meals (crème fraiche, sour cream)	High fat dairy
Cheese	All types	High fat dairy
Milk for coffee and creamers	Full fat milk added to coffee	High fat dairy
	Semi-skimmed milk added to coffee	Low fat dairy
	Full fat powdered milk for coffee	High fat dairy
	Skimmed powdered milk for coffee	Low fat dairy
	Full fat dairy based coffee creamers	High fat dairy
	Skimmed dairy based coffee creamers	High fat dairy
Butter	Salted	High fat dairy
	Unsalted	High fat dairy
Ice-cream		High fat dairy

Table S2 Food sources of dietary fiber for the present analysis of Alpha Omega Cohort

Fiber types	Food sources included	Mean ± SD	Median (IQR)
Grain fiber	Bread (whole wheat, white bread, multigrain, rye), buns, toast Granola, cornflakes, fiber-rich breakfast products Rice, noodles, pasta Biscuits, cookies, pancakes, savory pie	9.3 ± 3.6	9.0 (6.5-11.7)
Fruits fiber	Citrus fruits, apples, pears, bananas, strawberries, blueberries, cherries, grapes, peaches, nectarines, plums, apricot, kiwi, grapefruit, dried fruit, other fruit Apple sauce Orange juice, apple juice, grapefruit juice, other juices	3.9 ± 3.6	2.7 (1.3-5.8)
Vegetable fiber	Endives, spinach, purslane, sprouts, cauliflower, broccoli, other cabbage, carrots, leek, chicory, kohlrabi, beets, mushrooms, bell pepper, onion, tomatoes, lettuce, crudités, other vegetables Tomato juice, vegetable juice Onion soup, tomato soup, other soup with vegetables	2.0 ± 1.1	1.8 (1.3-2.5)
Potato fiber	Boiled potatoes, mashed potatoes, baked potatoes, hotchpot, potato salad, other potatoes, fries, potato chips	3.7 ± 1.8	3.6 (2.4-4.6)
Beans, legumes, nuts fiber	Peas, broad beans, green beans, legumes, legume soup, various nuts	1.6 ± 1.1	1.0 (0.5-1.6)
Other source (unknown)		1.0 ± 0.8	0.8 (0.5-1.3)

Table S3 Spearman correlation coefficients for relation between intake and circulating odd-chain fatty acids additionally adjusted for medications and presence of type 2 diabetes

	Fractions	Pentadecanoic acid (15:0)	Heptadecanoic acid (17:0)
Dairy			
Total dairy	Phospholipids	0.19 (0.13, 0.25)***	0.15 (0.09, 0.22)***
	Cholesteryl esters	0.24 (0.17, 0.30)***	0.13 (0.07, 0.20)***
Dairy fat	Phospholipids	0.20 (0.13, 0.26)***	0.12 (0.05, 0.18)***
	Cholesteryl esters	0.26 (0.20, 0.32)***	0.11 (0.05, 0.18)***
Total fiber	Phospholipids	0.07 (-0.00, 0.13)	0.19 (0.12, 0.25)***
	Cholesteryl esters	0.04 (-0.03, 0.10)	0.11 (0.04, 0.17)**
Other foods			
Total meat	Phospholipids	-0.01 (-0.08, 0.05)	0.01 (-0.05, 0.08)
	Cholesteryl esters	-0.04 (-0.10, 0.03)	0.02 (-0.05, 0.09)
Ruminant meat	Phospholipids	0.06 (-0.01, 0.13)	0.05 (-0.01, 0.12)
	Cholesteryl esters	0.05 (-0.01, 0.12)	0.05 (-0.02, 0.11)
Total fish	Phospholipids	0.02 (-0.05, 0.08)	0.06 (-0.00, 0.13)
	Cholesteryl esters	0.09 (0.02, 0.16)**	0.02 (-0.05, 0.87)

Partial r_s are Spearman's correlation coefficients between dietary intakes and individual circulating odd-chain fatty acids adjusted for age, sex, total energy intake, anti-hypertensive medication use, statins use and presence of type 2 diabetes.

*p<0.05; **p<0.01; ***p<0.001.

Table S4 Spearman correlation coefficients for relation between intake and circulating odd-chain fatty acids including only patients without type 2 diabetes (n=683)

	Fractions	Pentadecanoic acid (15:0)	Heptadecanoic acid (17:0)
Dairy			
Total dairy	Phospholipids	0.17 (0.09, 0.24)***	0.14 (0.06, 0.21)***
	Cholesteryl esters	0.23 (0.15, 0.30)***	0.17 (0.10, 0.24)***
Dairy fat	Phospholipids	0.20 (0.12, 0.27)***	0.10 (0.03, 0.18)**
	Cholesteryl esters	0.27 (0.20, 0.34)***	0.15 (0.07, 0.22)***
Total fiber	Phospholipids	0.03 (-0.04, 0.11)	0.18 (0.11, 0.25)***
	Cholesteryl esters	0.02 (-0.06, 0.09)	0.09 (0.01, 0.16)*
Other foods			
Total meat	Phospholipids	0.02 (-0.06, 0.09)	0.07 (-0.01, 0.14)
	Cholesteryl esters	-0.01 (-0.09, 0.06)	0.03 (-0.04, 0.11)
Ruminant meat	Phospholipids	0.06 (-0.01, 0.14)	0.10 (0.02, 0.17)*
	Cholesteryl esters	0.05 (-0.03, 0.12)	0.03 (-0.04, 0.11)
Total fish	Phospholipids	0.01 (-0.09, 0.06)	0.05 (-0.02, 0.13)
	Cholesteryl esters	0.07 (-0.01, 0.14)	0.01 (-0.06, 0.09)

Partial r_s are Spearman's correlation coefficients between dietary intakes and individual circulating odd-chain fatty acids adjusted for age, sex and total energy intake;

*p<0.05; **p<0.01; ***p<0.001.

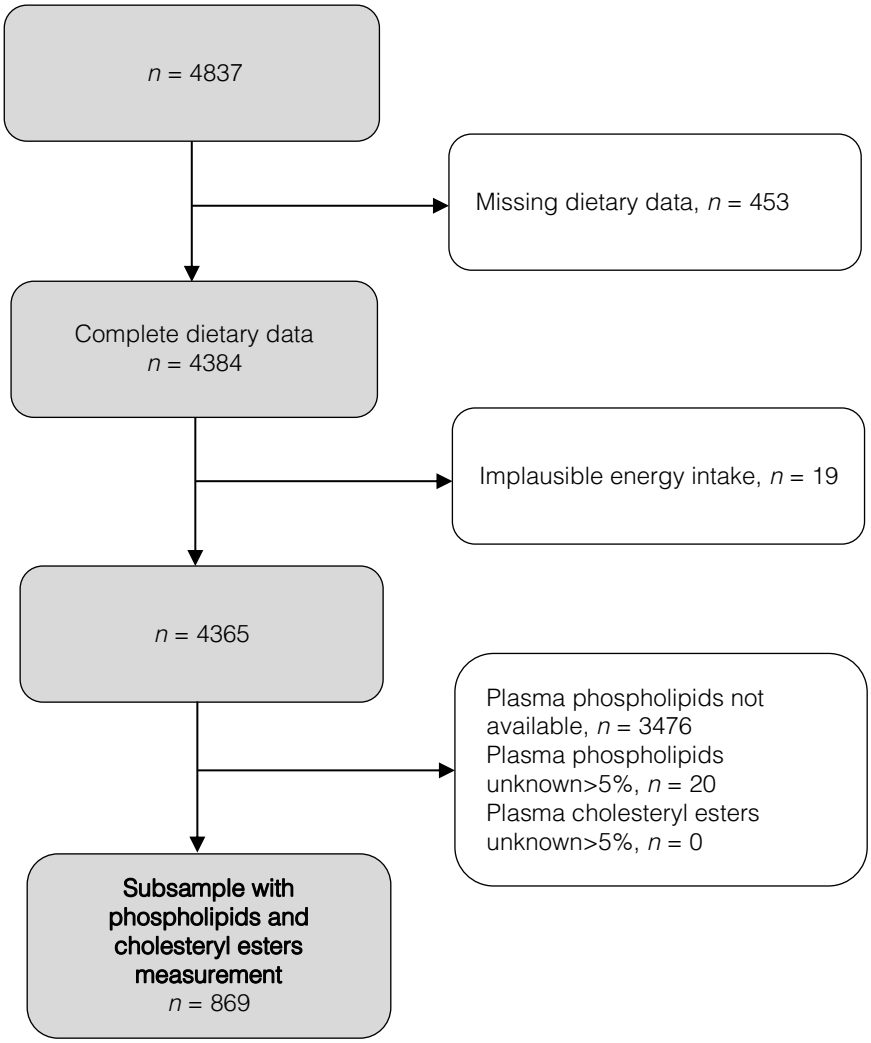

Figure S1 Flow diagram for population for analysis

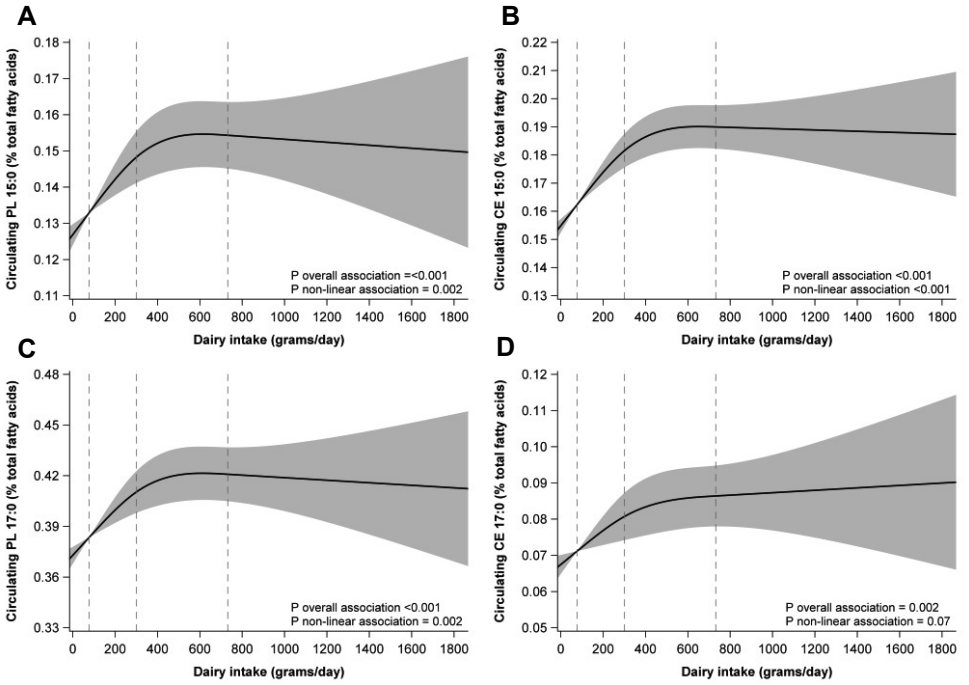

Figure S2 Associations of circulating 15:0 (A, B) and 17:0 (C, D) with dairy intake (energy-adjusted grams/day) evaluated by restricted cubic splines.

Solid lines and grey areas are central estimates of circulating 15:0 or 17:0 and 95% confidence interval with adjustment for age, sex, and total energy intake. Knots were located at 5th, 50th and 95th percentile, represented by dashed vertical lines; Reference value was set at 5th percentile of dairy intake which was 77 g/day. CE, cholesteryl esters; PL, phospholipids.

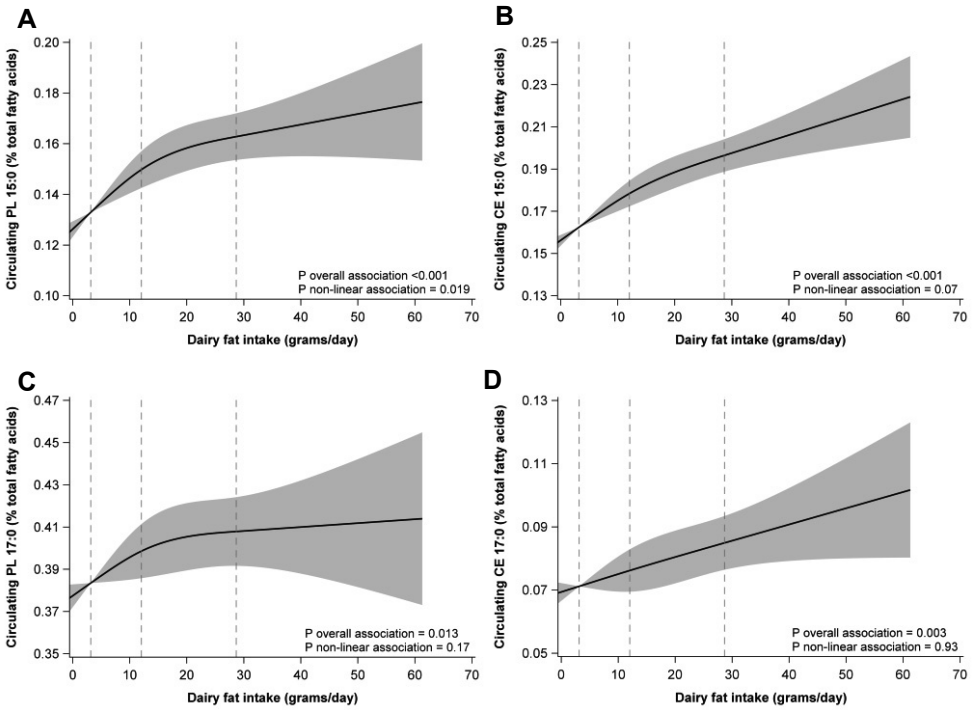

Figure S3 Associations of circulating 15:0 (A, B) and 17:0 (C, D) with dairy fat intake (energy adjusted grams/day) evaluated by restricted cubic splines.

Solid lines and grey areas are central estimates of circulating 15:0 or 17:0 and 95% confidence interval with adjustment for age, sex, and total energy intake. Knots were located at 5th, 50th and 95th percentile, represented by dashed vertical lines. Reference value was set at 5th percentile of dairy fat intake which was 3.2 g/day. CE, cholesteryl esters; PL, phospholipids.

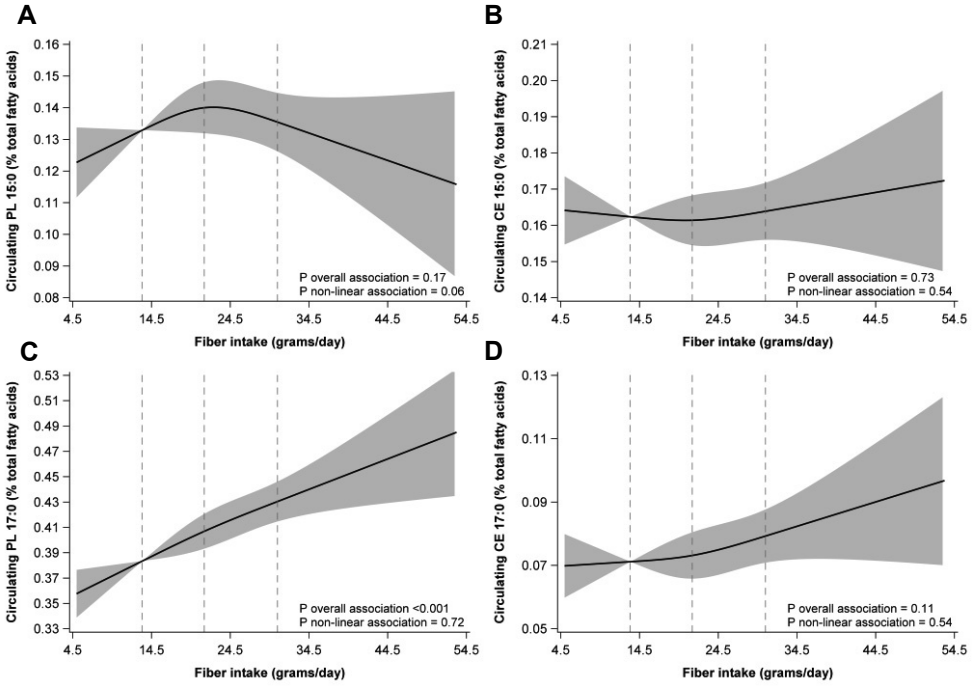

Figure S4 Associations of circulating 15:0 (A, B) and 17:0 (C, D) with fiber intake (energy-adjusted grams/day) evaluated by restricted cubic splines.

Solid lines and grey areas are central estimates of circulating 15:0 or 17:0 and 95% confidence interval with adjustment for age, sex, and total energy intake. Knots were located at 5th, 50th and 95th percentile, represented by dashed vertical lines. Reference value was set at 5th percentile of fiber intake which was 13.3 g/day. CE, cholesteryl esters; PL, phospholipids.

Chapter 4

Plasma and dietary linoleic acid and 3-year risk of type 2 diabetes after myocardial infarction: a prospective analysis in the Alpha Omega Cohort

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Diabetes Care 2020;43(2):358–365

ABSTRACT

Objective: To study plasma and dietary linoleic acid in relation to type 2 diabetes risk in post-myocardial infarction (MI) patients.

Research Design and Methods: We included 3,257 patients aged 60-80 y (80% male) with a median time since MI of 3.5 years from the Alpha Omega Cohort and were initially free of type 2 diabetes. At baseline (2002-2006), plasma LA was measured in cholesteryl esters and dietary LA was estimated with a 203-item food-frequency questionnaire. Incident type 2 diabetes was ascertained through self-reported physician diagnosis and medication use. Hazard ratios (HR, with 95% CI) were calculated by Cox regressions, in which dietary LA isocalorically replaced sum of saturated (SFA) and trans fatty acids (TFA).

Results: Mean (±SD) circulating and dietary LA was 50.1±4.9 % and 5.9±2.1 en%, respectively. Plasma and dietary LA were weakly correlated (Spearman r= 0.13, P <0.001). During a median follow-up of 41 months, 171 patients developed type 2 diabetes. Plasma LA was inversely associated with type 2 diabetes risk (quintile [Q]5 vs. Q1: 0.44 [0.26, 0.75]; per 5%: 0.73 [0.62, 0.86]). Substitution of dietary LA for SFA+TFA showed no association with type 2 diabetes risk (Q5 vs. Q1: 0.78 [0.36, 1.72]; per 5 en%: 1.18 [0.59, 2.35]). Adjustment for markers of de novo lipogenesis attenuated plasma LA associations.

Conclusions: In our cohort of post-MI patients, plasma LA was inversely related to type 2 diabetes risk, whereas dietary LA was not related. Further research is needed to assess whether plasma LA indicates metabolic state rather than dietary LA in these patients.

INTRODUCTION

Dietary guidelines for the prevention of coronary heart disease (CHD) promote the replacement of trans fatty acids (TFA) and saturated fatty acids (SFA) by *cis* unsaturated fat, especially polyunsaturated fatty acids (PUFA).[1] Linoleic acid (18:2n-6, LA) is the predominant type of dietary PUFA with a contribution of 4-6% to total energy intake (en%) in Western diets.[2]

The importance of PUFA for the prevention of type 2 diabetes is not yet clear. Results from population-based studies showed inconsistent associations between type 2 diabetes risk and dietary LA,[3–8] also when studied as total omega-6 FA,[9,10] or as total PUFA.[11,12] Inverse associations were found when SFA was theoretically replaced by PUFA[11,12] and not when PUFA replaced total carbohydrates.[4,9] However, a recent analysis using data from three cohorts in the United States observed that isocaloric replacement by LA for either SFA, TFA or carbohydrates were all associated with lower type 2 diabetes risk.[8] Inconsistencies might also be due to incomplete adjustment for relevant confounders, such as total energy intake[3,5] or dietary fiber.[7,10]

Plasma LA has been investigated as biomarker of dietary LA.[13] Moderate correlations (0.2-0.3) between dietary, assessed by food frequency questionnaire, and plasma LA have been reported in non-patient populations, but correlations up to 0.7 were also reported when dietary history or multiple-day food records were used.[13] Plasma LA has been used to investigate associations of LA with type 2 diabetes risk in mostly general populations.[14,15] A pooling study of 8 European population-based cohorts with ~27,000 individuals showed a relative risk (RR) of 0.54 for incident type 2 diabetes in the upper versus lower quintile of plasma phospholipids LA.[14] This was confirmed in a larger pooling study including 20 prospective cohort studies of ~40,000 individuals, which showed an RR of 0.57 when comparing extreme quintiles of circulating LA in various lipid compartments.[15]

Plasma LA is not only influenced by dietary LA but also endogenous metabolic processes. Liver fat accumulation has been associated with lower plasma LA and higher plasma palmitic acid in an elderly population without diabetes.[16] Higher delta-6-desaturase (D6D) activity, estimated by ratio of 18:3n-6 to LA in blood fractions, predicted worsening of glycaemia.[17]

Patients who have had myocardial infarction (MI) and diabetes had higher risk for recurrent cardiovascular events and mortality as compared to patients who did not have diabetes.[18,19] In the Alpha Omega Cohort, we showed that a better dietary fat

quality, studied by using theoretical replacement of SFA plus TFA by PUFA (predominantly LA), was related to a 34% lower risk of CVD mortality, on top of advanced drug treatment.[20] Whether having better dietary fat quality or plasma fatty acid composition could also lower type 2 diabetes risk in these patients is still unknown. We therefore examined the associations of both plasma LA measured in cholesteryl esters and dietary LA (replacing SFA plus TFA) and the incidence of type 2 diabetes during ~3 years of follow-up in our cohort of post-MI patients.

RESEARCH DESIGN AND METHODS

Patients and study design

The Alpha Omega Cohort is a prospective observational study of 4837 drug-treated Dutch patients aged 60-80 years with a verified clinically diagnosed MI up to 10 y before study enrollment. The Alpha Omega Cohort is registered with ClinicalTrials.gov, NCT03192410. Baseline measurements took place between 2002 and 2006, as described in detail elsewhere[21,22] These patients were free living, mostly men (78%), and some also reported a history of stroke (7%) or type 2 diabetes (17%). Most of the patients were treated with statins (86%) and antihypertensive medications (89%).[22] During the first 40 months of follow-up, patients took part in an intervention study (Alpha Omega Trial) with low doses of omega-3 PUFA (treatment groups: alpha-linolenic acid, eicosapentaenoic acid + docosahexaenoic acid, alpha-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid, and placebo), which had no effect on major cardiovascular events.[21] Patients were monitored for type 2 diabetes incidence (outcome for the current study) for the duration of the intervention study.

The current analysis excluded patients with prevalent type 2 diabetes at baseline (*n*=1014). Prevalent type 2 diabetes at baseline was defined when a patient reported having received the diagnosis from a physician, was taking antidiabetic drugs, or had an elevated plasma glucose level (≥7.0 mmol/L if fasted for ≥4h or ≥11.1 mmol/L if not fasted).[21] We further excluded patients with missing data on plasma cholesteryl esters or patients with >5% of unknown FA (n=61) and missing dietary data or extreme energy intake (<800 or >8,000 kcal/d for men, <600 or >6,000 kcal/d for women) (n=334). Additionally, we excluded 171 patients with extreme unsaturated FA intakes (<2.5th or >97.5th percentile) to obtain reliable risk estimates when analyzing FA intake on a continuous scale, as reported previously.[20] A total of 3257 patients remained for analysis (**Supplementary Figure S1**).

Dietary data collection

Dietary intake data were collected at baseline by a 203-item food frequency questionnaire (FFQ). The FFQ was an extended version of a reproducible, validated 104-item questionnaire that was specifically designed for estimating FAs and cholesterol intake.[23,24] The original FFQ showed good relative validity for LA intake when compared to dietary history interviews (Pearson r = 0.65).[23] Patients were instructed to report their usual food intake over the past month, including the type of food, frequency, amount, and preparation methods if applicable. For example, when patients indicated that they consumed baked potatoes, they were also asked to indicate which type and brand of fat was used for preparation. A trained dietician checked the returned FFQs and obtained additional information by telephone for items that were unclear or missing. Double data entry was performed, and inconsistencies were solved. Total energy and nutrient intakes were calculated from food consumption data using the 2006 Dutch food consumption database (NEVO).[25] Dietary intake of LA and other macronutrients were expressed as percentages of energy (en%), excluding calories from alcohol.

Assessment of plasma FAs

Samples of 30 mL venous blood, either fasting or non-fasting, were drawn at the patients' home or at the hospital. Blood was collected in EDTA containing vacutainers, packed in a sealed envelope and sent over postal mail to a central laboratory.[26] FA were measured in plasma cholesteryl esters. Detailed procedures of FA measurement have been described previously.[27] In short, lipids from EDTA plasma were separated by solid phase extraction silica columns to obtain cholesteryl esters fraction (Chrompack, Middelburg, the Netherlands). Cholesteryl esters FA were identified by gas chromatography through comparison with known standards (Nu-chek prep, Inc. Elysian, MN, USA). Individual FAs were expressed as percentage of total FAs. For LA, the within-run and between-run coefficient of variation was ≤0.6% and ≤1.2%, respectively.

Ascertainment of incident type 2 diabetes

Type 2 diabetes was ascertained based on a self-reported physician's diagnosis and/or the initiation of antidiabetic medication. Type 2 diabetes was assessed after 12 months, 24 months, at midterm examination (20 months) and at the end of the Alpha Omega Trial phase, with a median (interquartile range [IQR]) follow-up of

40.7 (36.8-41.5) months, through telephone calls and questionnaires. The date of diagnosis or start of medication use was reported by the patients. If this information was missing, the midpoint between two interview dates was used (10% of cases). Diagnosis of incident type 2 diabetes was not based on plasma glucose concentrations because blood samples were only collected for part of the cohort during the follow-up.

Other measurements

Body mass index (BMI) was calculated as measured weight (kg) divided by the squared height (m^2). Educational level was assessed in four categories, as primary education, lower secondary education, higher secondary or lower tertiary education, and higher tertiary education. Smoking status was reported in three categories as never, former, or current. Alcohol intake, estimated from the FFQ, was categorized as no (ethanol intake of 0 g/d), low (>0-10 g/d), moderate (>10-20 g/d for women or >10-30 g/d for men), or high (>20 g/d for women or >30 g/d for men). Physical activity was assessed by using the validated Physical Activity Scale for the Elderly[28] and categorized as low (no activity or only light activity, ≤ 3 metabolic equivalents (METs)), intermediate (>0 to <5 days/week of moderate or vigorous activity, >3METs), or high (≥5 days/week of moderate or vigorous activity, >3METs). Medication use was coded according to the Anatomical Therapeutic Chemical Classification System. Codes were A10 for antidiabetic drugs, C02, C03, C07, C08 and C09 for antihypertensive drugs, C10AA and C10B for statins and B01 for antithrombotic drugs.[29] Blood lipids and glucose were analyzed by standard kits using a Hitachi 912 Autoanalyzer (Roche Diagnostics, Basel, Switzerland).[22] Blood was collected in fasting state in 44.5% of the cohort (4 to <12 hours for 414 patients and ≥12 hours for 1035 patients). Prediabetes was defined as plasma glucose between 6.1 and 6.9 mmol/L after ≥4 hours of fasting. Family history of type 2 diabetes was considered present when patients reported at least one parent with type 2 diabetes. To describe overall diet quality of these patients, we computed a healthy nutrient and food score using information from the FFQ, as reported previously.[30]

Statistical analysis

Baseline characteristics are presented as mean and SD for normally distributed variables, median and IQR for skewed variables, or percentage for categorical variables. Spearman correlation was computed between dietary and plasma LA.

Survival time in years was computed from the date of study enrollment until 1) onset of type 2 diabetes, 2) death, or 3) end of follow-up. Associations of dietary and plasma LA with incident type 2 diabetes were analyzed using Cox proportional hazard analysis. Proportional hazards assumption was tested by log-log plots and by including time-dependent covariates in the Cox models; all assumptions were met. Hazard ratios (HRs) and 95% CIs were calculated in quintiles of dietary or plasma LA and the lowest quintile was set as the reference category. The *P* value for trend across quintiles was obtained by assigning each patient median dietary or plasma LA values for the category, and we modeled this value as continuous in the Cox models. Analyses were repeated with LA on a continuous scale (per 5 en% increase in dietary LA; per 5% increase in plasma LA). Per 5% increase for continuous analysis was chosen mainly because it approximated the range between the extreme quintiles of dietary or plasma LA in the present analysis or was also used by other studies.[6,12] Missing values for covariables (n=1 for smoking, n=2 for BMI, n=13 for education level, n=16 for physical activity) were imputed with sex-specific median (continuous variable) or mode (categorical variable) to retain all patients in the analyses.

The following models were used: Model 1 adjusted for age (y), sex and Alpha Omega Trial treatment code (four categories); model 2 additionally adjusted for physical activity (3 categories), smoking status (three categories), educational level (four categories), BMI (kg/m^2), family history of type 2 diabetes (yes/no), total energy intake excluding calories from alcohol (kcal/d), alcohol intake (four categories), dietary fiber (g/d) and dietary cholesterol (mg/d). For dietary LA, we additionally investigated a replacement of sum of SFA and TFA by LA in an isocaloric substitution model (model 3). In model 3, we simultaneously included the percentages of energy from linoleic acid, carbohydrates, protein, and the remaining FA types: n-3 PUFA and *cis*-MUFA in the model, and left SFA and TFA out of the model. Coefficients for dietary LA from this Cox model may be interpreted as estimated effect of replacing a certain percentage of energy contributed by SFA+TFA for equivalent energy from LA, holding constant the total energy intake and other macronutrients. Restricted cubic spline analyses were carried out to evaluate the linearity of the associations in the fully adjusted models. Knots were positioned at the 5th, 50th and 95th percentile, and value at 5th percentile of dietary or plasma LA was considered as the reference value.[31]

Subgroup analyses were carried out by sex, age (<65 or ≥65 y), obesity (BMI <30 or ≥30 kg/m^2) as previously examined in other studies[4,6,12,15] and time since MI (≤5 or >5 y). Additionally, we investigated alcohol intake (no, low, moderate or high) and statin use (yes or no) since in this cohort we observed a lower plasma LA in

statin users and patients with high alcohol intake as compared to patients without these characteristics.[27] Potential effect modification by these variables were statistically tested by including interaction terms with plasma LA in model 2. Sensitivity analyses for dietary and plasma LA were carried out by excluding patients with prediabetes at baseline.

In order to explore additional confounding or mediating effects of metabolic factors on the association between plasma LA and type 2 diabetes incidence, we added the following covariables to the fully adjusted model (one at a time) in post-hoc analyses: triacylglycerols (mmol/L); palmitic acid (16:0, %) and palmitoleic acid to palmitic acid ratio (16:1n-7/16:0) as markers for *de novo* lipogenesis; and oleic acid (18:1n-9, %), as the other major FA present in cholesteryl esters and may be derived from *de novo* lipogenesis.

Finally, to explore associations between FA metabolism and incident type 2 diabetes, we examined associations of selected markers of FA metabolism with incident type 2 diabetes by using these markers as exposure variable in model 2. We selected plasma 16:0, 18:1n-9, the ratio of palmitic acid to LA (16:0/18:2n-6) as markers for liver fat content,[16,32] the ratio of arachidonic acid to dihomo-gamma-linolenic acid (20:4n-6/20:3n-6) as an estimation of delta-5-desaturase (D5D) activity, and the ratio gamma-linolenic acid to LA (18:3n-6/18:2n-6) as estimated D6D activity.

All analyses were performed using SAS 9.4 statistical software (SAS Institute, Cary, NC, USA). Two-sided *P*-values <0.05 were considered statistically significant.

RESULTS

Patients were on average 68.9 ± 5.5 y old, had a BMI of 27.4 ± 3.5 kg/m^2 (75% overweight or obese), and 80.5% were men. Median (IQR) time since MI for these patients was 3.5 (1.6-6.3) years. Most of the patients used antithrombotic drugs (97.7%), antihypertensive drugs (89.0%), or statins (86.4%). Dietary and plasma LA were weakly correlated (r = 0.13, P <0.001). Median dietary LA increased from 3.5 to 8.8 en% across dietary quintiles, with a smaller range (5.6 to 6.3 en%) across plasma quintiles (**Table 1**). Median proportions of plasma LA increased from 49.3 to 51.2% of total FA across quintiles of dietary LA, whereas this range was larger (43.9 to 56.3% of total FA) across quintiles of plasma LA. During a median follow-up of 41 months (10277 person-years), 171 cases of type 2 diabetes occurred. No patients were lost during follow-up.

Dietary LA

Patients with higher dietary LA had a lower BMI and were more likely to be men and to be current smokers. The proportion of patients with high alcohol intake, using statins, and the concentrations of plasma glucose and serum triacylglycerols did not vary across quintiles of dietary LA. Those in the higher quintiles of dietary LA had higher intake of total energy, proportion of energy from total fat, SFA, and *cis* MUFA, and lower protein and carbohydrates. Fiber intake was similar across quintiles (**Table 1**).

Dietary LA was not associated with type 2 diabetes risk after adjusting for demographic, lifestyle characteristics, dietary fiber and dietary cholesterol (model 2) (HR_{Q5vsQ1}: 0.88; 95% CI: 0.54, 1.44; *P*-trend = 0.97) (**Table 2**). When SFA and TFA were theoretically replaced by LA, dietary LA was not associated with type 2 diabetes risk across quintiles (HR_{Q5vsQ1}: 0.78; 95% CI: 0.36, 1.72; *P*-trend = 0.84) and continuously ($HR_{per\ 5\ en\%}$: 1.18; 95% CI: 0.59, 2.35; *P* = 0.64). Exclusion of patients with prediabetes yielded similar results as in the total sample.

Table 1 Baseline characteristics of 3,257 post-MI patients from the Alpha Omega Cohort by quintiles of dietary and plasma LA

	Quintiles of dietary LA (en%)			Quintiles of plasma LA (%)		
	Q1 (n=651)	Q3 (n=651)	Q5 (n=651)	Q1 (n=652)	Q3 (n=651)	Q5 (n=651)
Median (range)	3.5 (1.2-4.1)	5.6 (5.1-6.2)	8.8 (7.6-13.9)	43.9 (28.5-46.1)	50.1 (48.9-51.4)	56.3 (54.3-67.6)
Age, y	69.4±5.7	68.8±5.5	68.6±5.4	68.2±5.7	68.7±5.4	69.8±5.5
Men, *n* (%)	471 (72.4)	536 (82.3)	569 (87.4)	504 (77.3)	511 (78.5)	553 (85.0)
Body mass index, kg/m^2	27.6±3.7	27.4±3.5	27.1±3.2	27.6±3.8	27.4±3.2	26.7±3.3
Time since MI, y	3.6 (1.5-6.3)	3.6 (1.7-6.1)	3.1 (1.4-6.3)	3.1 (1.4-5.9)	3.4 (1.5-6.2)	4.0 (1.8-6.6)
Smoking, *n* (%)						
Never	136 (20.9)	102 (15.7)	86 (13.2)	92 (14.1)	109 (16.7)	112 (17.2)
Former	414 (63.6)	452 (69.4)	432 (66.4)	417 (64.0)	440 (67.6)	435 (66.9)
Current	101 (15.5)	97 (14.9)	133 (20.4)	143 (21.9)	102 (15.7)	103 (15.8)
Physical activity, *n* (%)*						
Low	260 (40.2)	256 (39.4)	259 (40.0)	257 (39.5)	263 (40.4)	269 (41.3)
Middle	228 (35.3)	247 (38.0)	262 (40.5)	266 (40.9)	250 (38.4)	243 (37.3)
High	158 (24.5)	147 (22.6)	126 (19.5)	127 (19.5)	138 (21.2)	139 (21.4)
Highest level of education, *n* (%)						
Primary	124 (19.2)	133 (20.5)	128 (19.8)	120 (18.5)	125 (19.2)	137 (21.1)
Lower secondary	233 (36.0)	228 (35.1)	268 (41.4)	223 (34.4)	249 (38.2)	229 (35.2)
Higher secondary or lower tertiary	209 (32.3)	203 (31.2)	189 (29.2)	208 (32.0)	202 (31.0)	203 (31.2)
Higher tertiary	81 (12.5)	86 (13.2)	62 (9.6)	98 (15.1)	75 (11.5)	81 (12.5)
Alcohol intake, *n* (%)*						
No	32 (4.9)	18 (2.8)	35 (5.4)	27 (4.1)	17 (2.6)	36 (5.5)
Low	337 (51.8)	344 (52.8)	306 (47.0)	262 (40.2)	347 (53.3)	378 (58.1)
Moderate	172 (26.4)	179 (27.5)	202 (31.0)	170 (26.1)	182 (28.0)	183 (28.1)
High	110 (16.9)	110 (16.9)	108 (16.6)	193 (29.6)	105 (16.1)	54 (8.3)
Medication use, *n* (%)						
Statins	560 (86.0)	564 (86.6)	563 (86.5)	602 (92.3)	587 (90.2)	464 (71.3)
Anti-thrombotic drugs	633 (97.2)	640 (98.3)	641 (98.5)	637 (97.7)	631 (96.9)	637 (97.8)
Anti-hypertensive drugs	581 (89.2)	580 (89.1)	590 (90.6)	586 (89.9)	576 (88.5)	572 (87.9)

	Quintiles of dietary LA (en%)			Quintiles of plasma LA (%)		
	Q1 (n=651)	Q3 (n=651)	Q5 (n=651)	Q1 (n=652)	Q3 (n=651)	Q5 (n=651)
Serum lipids, mmol/L[†,‡,§]						
Total cholesterol	4.81±0.95	4.73±0.93	4.70± 0.98	4.71±0.98	4.66±0.88	4.83±0.95
LDL cholesterol	2.67±0.81	2.60±0.76	2.59 ± 0.85	2.49±0.81	2.54±0.75	2.80±0.84
HDL cholesterol	1.34±0.36	1.30±0.32	1.30 ± 0.35	1.32±0.37	1.31±0.35	1.29±0.33
Triacylglycerols	1.58 (1.18-2.11)	1.58 (1.18-2.21)	1.59 (1.16-2.19)	1.76 (1.28-2.48)	1.56 (1.17-2.17)	1.46 (1.11-1.97)
Plasma glucose, mmol/L[‡,§]	5.40 (4.96-5.98)	5.44 (4.96-6.10)	5.38 (4.90-6.00)	5.57 (5.04-6.28)	5.45 (5.00-6.00)	5.33 (4.90-5.84)
Prediabetes, *n* (%)[*]	39 (6.1)	39 (6.1)	36 (5.6)	53 (8.2)	34 (5.3)	36 (5.6)
Family history of type 2 diabetes, *n* (%)	104 (16.0)	104 (16.0)	104 (16.0)	108 (16.6)	101 (15.5)	108 (16.6)
Dietary factors						
Energy, kcal/d	1750±485	1856±502	1938±502	1775±501	1858±516	1938±490
Protein, en%	16.4±3.0	15.6±2.7	14.6±2.5	15.8±2.8	15.5±3.0	15.2±2.6
Total fat, en%	31.5±5.9	32.6±4.6	38.6±4.9	35.0±6.2	35.0±5.9	36.0±6.2
SFA, en%	12.7±3.3	13.1±2.8	13.5±3.2	13.2±3.2	12.9±2.9	13.0±3.0
cis MUFA, en%	8.4±2.0	9.9±1.9	11.4±1.8	10.1±2.4	9.9±2.1	9.9±2.2
PUFA, en%	5.6±1.9	7.1±0.9	10.8±1.6	7.1±2.2	7.6±2.2	8.2±2.3
TFA, en%	0.73±0.22	0.76±0.18	0.78±0.16	0.76±0.20	0.75±0.18	0.75±0.18
Carbohydrates, en%	52.1±6.2	49.7±5.6	44.6±5.4	49.2±6.4	49.4±6.1	48.8±6.4
Fiber, g/d	21.3±7.0	21.7±6.7	21.1±6.2	20.4±6.7	21.6±6.6	23.2±6.9
Cholesterol, mg/d	181±68	189±70	181±68	187±72	185±66	183±68
Healthy nutrient and food score	22.1±6.2	22.7±6.1	22.6±6.1	21.6±6.3	22.6±5.9	23.6±5.8
Plasma FA composition						
SFA, % total FA	13.2±1.2	13.1±1.0	12.9±1.0	14.1±1.1	13.1±0.7	12.1±0.7
MUFA, % total FA	22.9 ±3.2	22.4±3.1	21.6±3.1	26.3±3.0	22.2±1.7	19.1±1.8
PUFA, % total FA	62.4±4.0	63.1±3.9	64.1±3.7	58.0±3.3	63.4±1.8	67.6±2.1
n-3 PUFA, % total FA	2.4 (2.0-3.2)	2.4 (2.0-3.0)	2.3 (1.9-2.8)	2.8 (2.2-3.6)	2.4 (2.0-3.0)	2.0 (1.6-2.4)
n-6 PUFA, % total FA	59.5±4.3	60.3±4.2	61.5±4.1	54.7±3.4	60.6±1.9	65.4±2.2
Arachidonic acid (20:4n-6), % total FA	8.2±2.0	8.3±2.1	8.4±2.1	9.4±2.2	8.6±1.8	6.9±1.5
Palmitic acid (16:0), % total FA	11.4±0.8	11.3±0.8	11.2±0.8	12.1±0.8	11.3±0.6	10.5±0.6

Table 1. continued

	Quintiles of dietary LA (en%)			Quintiles of plasma LA (%)		
	Q1 (n=651)	Q3 (n=651)	Q5 (n=651)	Q1 (n=652)	Q3 (n=651)	Q5 (n=651)
Palmitoleic acid (16:1n-7), % total FA	2.9±1.2	2.6±1.1	2.4±1.0	3.9±1.4	2.5±0.7	1.9±0.6
Oleic acid (18:1n-9), % total FA	18.0±2.2	17.8±2.2	17.3±2.2	20.3±2.0	17.7±1.4	15.6±1.5
Liver fat proxy (16:0/18:2n-6)	0.23±0.04	0.23±0.04	0.22±0.03	0.28±0.03	0.22±0.01	0.18±0.01
Estimated D6D activity (18:3n-6/18:2n-6)	0.021±0.008	0.020±0.008	0.020±0.008	0.029±0.009	0.020±0.006	0.013±0.004

Values are shown as mean ± standard deviation, median (interquartile range) or n (%). en%, percentage of total energy intake, excluding energy from alcohol.
* Classification described in text (Materials and Methods).
† To convert to mg/dL, divide by 0.02586 for total, LDL, HDL cholesterol and by 0.01129 for triacylglycerols.
‡ Fasting <4 hours (n=1,676), fasting 4 to <8 hours (n=314), fasting 8 to <12 hours (n=100), fasting ≥12 hours (n=1,035), fasting status unknown (n=132).
§ Missing values for 61 patients for total cholesterol, HDL-C and TG, for 253 patients for LDL-C, for 31 patients for plasma glucose, and 1 patient for healthy nutrient and food score.

Plasma LA

Patients with higher plasma LA had lower BMI, lower plasma glucose and serum triacylglycerol concentrations. They were less likely to be current smokers, to have high alcohol intake, and to be statin users as compared to those in the lower quintiles. Patients with higher plasma LA also had higher total energy intake, percentage of energy from PUFA, and dietary fiber intake (Table 1).

After multivariable adjustments, there was a strong inverse association between plasma LA across quintiles (HR_{Q5vsQ1}: 0.44; 95% CI: 0.26, 0.75; *P*-trend = 0.001) (**Table 3**). In continuous analyses, each 5% higher plasma LA was associated with 27% lower risk of type 2 diabetes (HR: 0.73; 95% CI: 0.62, 0.86; *P*<0.001). Results from restricted cubic splines analysis indicated a possible linear relation (*P* non-linearity = 0.36; **Figure 1**). Association between plasma LA and incident type 2 diabetes were modified by sex (*P* interaction= 0.009), with inverse association being more pronounced in women (**Supplementary Table S1**). In this cohort, women had a lower median (IQR) alcohol intake (2.7 [0.0-9.6] g/d) than men (9.8 [3.0-22.1] g/d). Age, BMI, alcohol intake and statin use did not modify the associations.

In sensitivity analyses including only patients without prediabetes at baseline (n=3073), the associations were similar to the main analysis, as shown in results across quintiles (HR_{Q5vsQ1}: 0.50; 95% CI: 0.28, 0.88; *P* for trend=0.014) and on a continuous scale ($HR_{per\ 5\%}$: 0.76; 95% CI: 0.63, 0.91; *P*=0.003) (**Supplementary Table S2**).

Table 2 Hazard ratios and 95% CI for dietary LA in quintiles and per 5 en% and incident type 2 diabetes in 3257 post-MI patients from the Alpha Omega Cohort

	Quintiles of dietary LA (en%)					*P*-trend*	Per 5 en%	*P* value
	Q1 (n=651)	Q2 (n=652)	Q3 (n=651)	Q4 (n=652)	Q5 (n=651)			
Median dietary LA, en%	3.5	4.6	5.6	6.8	8.8			
Cases	37	30	34	39	31			
Model 1†	1.00	0.79 (0.49, 1.28)	0.90 (0.56, 1.43)	1.04 (0.66, 1.64)	0.81 (0.50, 1.31)	0.72	1.01 (0.70, 1.44)	0.96
Model 2‡	1.00	0.80 (0.49, 1.29)	0.95 (0.59, 1.51)	1.12 (0.71, 1.76)	0.88 (0.54, 1.44)	0.97	1.09 (0.76, 1.58)	0.64
Model 3§	1.00	0.72 (0.44, 1.19)	0.86 (0.51, 1.45)	1.01 (0.56, 1.81)	0.78 (0.36, 1.72)	0.84	1.18 (0.59, 2.35)	0.64

en%, percentage of total energy intake, excluding energy from alcohol.

* *P* values for trend were obtained by assigning each patient median dietary LA for the category and this value was modeled as continuous. † Model 1 was adjusted for age (continuous; y), sex (men/women), and Alpha Omega Trial treatment code (4 categories).

‡ Model 2: model 1 plus physical activity (3 categories), smoking status (3 categories), educational level (4 categories), BMI (continuous; kg/m^2), family history of type 2 diabetes (yes/no), total energy intake (excluding calories from alcohol, continuous; kcal/d), alcohol consumption (4 categories), dietary fiber (g/d; continuous), and dietary cholesterol (mg/d; continuous).

§ Model 3 (substitution of SFA and TFA for LA): model 2 plus dietary protein (en%; continuous), carbohydrates (en%; continuous), n-3 polyunsaturated FAs (en%; continuous), and *cis*-monounsaturated fatty acids (en%; continuous).

Table 3 Hazard ratios and 95% CI for plasma LA in quintiles and per 5% and incident type 2 diabetes in 3257 post-MI patients from the Alpha Omega Cohort

	Quintiles of plasma LA					*P*-trend*	Per 5%	*P* value
	Q1 (n=652)	Q2 (n=650)	Q3 (n=651)	Q4 (n=653)	Q5 (n=651)			
Median plasma LA, %	43.9	47.6	50.1	52.8	56.3			
Cases	49	38	31	31	22			
Model 1†	1.00	0.76 (0.50, 1.16)	0.63 (0.40, 0.98)	0.61 (0.39, 0.96)	0.42 (0.26, 0.70)	<0.001	0.73 (0.63, 0.85)	<0.001
Model 2‡	1.00	0.74 (0.48, 1.14)	0.64 (0.40, 1.00)	0.60 (0.38, 0.94)	0.44 (0.26, 0.75)	0.001	0.73 (0.62, 0.86)	<0.001

* P-values for trend were obtained by assigning each patient median plasma LA for the category and this value was modeled as continuous.
† Model 1 was adjusted for age (continuous; y), sex (men/women), and Alpha Omega Trial treatment code (4 categories).
‡ Model 2: model 1 plus physical activity (3 categories), smoking status (3 categories), educational level (4 categories), BMI (continuous; kg/m^2), family history of type 2 diabetes (yes/no), total energy intake (excluding calories from alcohol, continuous; kcal/d), alcohol intake (4 categories), dietary fiber (g/d; continuous), and dietary cholesterol (mg/d; continuous).

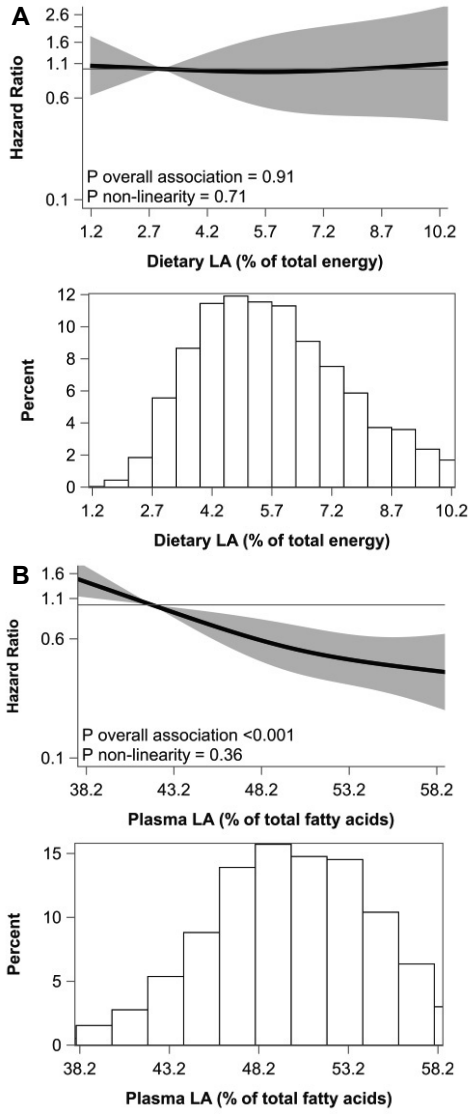

Figure 1 Associations of (A) dietary LA (replacing SFA + TFA) and (B) plasma LA with incident type 2 diabetes in 3257 post-MI patients.

Solid lines are risk estimates evaluated by restricted cubic splines from Cox models, showing the shape of associations on a continuous scale with three knots located at the 5th, 50th and 95th percentiles. The y-axis shows the multivariable hazard ratios for type 2 diabetes incidence for any dietary or plasma LA value, compared to the reference values set at 5th percentile of dietary LA (3.0 en%) or 5th percentile of plasma LA (41.9%). Grey areas indicate 95% confidence intervals. Results are presented for the fully adjusted models for dietary LA (model 3; adjusted for variables in model 2 (age, sex, Alpha Omega Trial treatment code, physical activity, smoking status, educational level, BMI, family history of type 2 diabetes, total energy intake excluding alcohol, alcohol consumption, dietary fiber, dietary cholesterol) plus dietary protein, carbohydrates, n-3 PUFA, *cis*-MUFA) and plasma LA (model 2). Distribution of values of dietary or plasma LA were displayed in a histogram under each spline; en%, percentage of total energy intake, excluding energy from alcohol.

CONCLUSIONS

In the present study, a 5% increase in plasma LA in cholesteryl esters was linearly associated with a 27% lower risk of type 2 diabetes in a high-risk population of post-MI patients, independent of age, sex, BMI, family history and lifestyle characteristics. Dietary LA was not associated with type 2 diabetes risk.

Our findings for dietary LA are not in line with previous observational studies replacing SFA with PUFA in a theoretical substitution which found inverse associations with incident type 2 diabetes in cohorts of women.[11,12] A recent analysis using data from three US cohorts showed that substitution of 5% energy from dietary LA for SFA and 2% energy for TFA was associated with 14% and 17% lower risk of type 2 diabetes, respectively, and that these associations were consistently observed in men and in women.[8] A meta-analysis of 13 randomized controlled feeding trials showed that isocaloric replacement of PUFA for SFA or carbohydrates, where primarily n-6 FA was used as the intervention arm, decreased insulin resistance but did not affect glucose concentrations.[33] Total PUFA also includes n-3 FA, which influence may be limited but cannot be excluded.[34]

Our findings for plasma LA are in line with previous studies in mostly general populations. Two recent large prospective studies, including a comparative meta-analysis[14] and a pooled analysis,[15] consistently showed that increasing proportions of LA in different blood fractions, including cholesteryl esters, are associated with a lower risk of type 2 diabetes. A possible explanation for the association between higher plasma LA and lower type 2 diabetes risk is through the increase of the unsaturation degree of the membrane lipids. Higher degree of unsaturation may increase membrane fluidity and number of insulin receptors and decrease receptor affinity, which could result in higher insulin sensitivity.[35,36] We also observed that the inverse association between plasma LA and type 2 diabetes risk was more pronounced in women. However, this effect measure modification by sex was not observed in other studies.[4,14,15] We suspected that the stronger inverse association in women could be due to that alcohol intake of male patients in this cohort was higher than the intake of the female patients. Furthermore, when the association between plasma LA and type 2 diabetes risk was examined in patients with different alcohol intake categories, patients with no alcohol intake were shown to have the lowest type 2 diabetes risk, although the interaction term did not reach significance. Possible influence of other factors related to metabolic condition in these patients on the association of LA and type 2 diabetes risk will be further discussed below.

In earlier population-based studies that examined both plasma and dietary LA within the same cohort, generally inverse associations with type 2 diabetes were found for plasma LA, while for dietary LA the associations varied.[3–5] To explain the inconsistent observations between plasma and dietary LA, the authors of the earlier studies attributed the null association for dietary LA to the subjective nature of the dietary assessment method, which is suggested to provide a lower ability to detect true associations than objective LA biomarkers.[4,5] Another possible explanation was the existence of metabolic differences between individuals who later develop type 2 diabetes and not.[3,4]

In relation to the dietary assessment method, dietary intake data used in the present analyses were collected by a validated FFQ specifically designed for the assessment of FA intake. We also recently showed that replacing dietary SFA and TFA by unsaturated FA was associated with a lower risk of mortality from CHD in the same cohort.[20] This further supported the qualitative validity of our FFQ to rank patients adequately according to their FA intake. Explanation for the inconsistent observations for plasma and dietary LA in the present study may therefore not solely be related to the dietary assessment method, but also to other factors, such as metabolic conditions influencing plasma composition of FA.

Populations at higher risk for type 2 diabetes may already have altered plasma FA composition, including a decrease in plasma LA proportion. In our population of post-MI patients, those with low plasma LA had a more adverse metabolic risk profile with higher BMI, plasma glucose and serum triacylglycerol concentrations. They also consumed more alcohol and often were statin users. Previously, we reported a generally weak correlation between dietary and circulating LA (Spearman correlation <0.20) and that this weak correlation might be in part due to alcohol and/or statins use.[27] The more adverse metabolic profile observed in patients with low plasma LA was not observed for dietary LA.

It is possible that the patients with a more adverse metabolic risk profile had an impaired liver function, such as in non-alcoholic fatty liver disease, and this may explain why we observed an increased risk of type 2 diabetes in patients with a low plasma LA. Non-alcoholic fatty liver disease has been strongly associated with an increased risk of type 2 diabetes.[37] In non-alcoholic fatty liver disease, de novo lipogenesis is up-regulated and/or FA oxidation is down-regulated.[38] These processes may promote a larger proportion of plasma SFA and MUFA, leading to a smaller plasma LA proportion.[13] In addition, insulin resistance has been associated with high D6D activity and low plasma LA proportion.[4] Indeed, in the post-hoc analyses of the present cohort, we found that higher proportion of the FA in the *de*

novo lipogenesis pathway, oleic acid and palmitic acid, and higher estimated D6D activity were associated with increased type 2 diabetes risk. Furthermore, adjustments for markers of *de novo* lipogenesis (palmitic acid, oleic acid, ratio of palmitoleic acid to palmitic acid) also attenuated associations of plasma LA. These changes in FA composition due to the impaired metabolism may additionally explain the weak correlation between plasma and dietary LA in our population.

This study has some limitations. The Alpha Omega Cohort is a rather homogenous population of predominantly male post-MI patients, therefore range of dietary LA and plasma LA proportion might not be comparable to other studies in healthy populations. Nevertheless, the range of dietary and plasma LA in this patient population were quite similar to studies in generally healthy populations.[3–6,8,15] Other limitations are the relatively short follow up time (median of 41 months) and the self-reported diagnosis of incident type 2 diabetes, that might have led to an underestimation of the number of cases and attenuation of associations.

To conclude, in this cohort of post-MI patients, plasma LA, but not dietary LA, was associated with lower type 2 diabetes risk. Our results suggested that metabolic conditions affecting plasma LA, rather than dietary LA intake, may be responsible for this association. Further research on the factors affecting plasma LA and its utility as a biomarker of LA intake in patient populations is warranted.

Acknowledgements

The authors thank Eveline Waterham (Division of Human Nutrition and Health, Wageningen University) for logistic support and data management and Dr. Paul Hulshof, Robert Hovenier and Marlies Diepeveen-de Bruin (Division of Human Nutrition and Health, Wageningen University) for analysis of plasma FA.

Funding

The Alpha Omega Cohort emerged from the Alpha Omega Trial (ClinicalTrials.gov Identifier NCT00127452), which was funded by the Dutch Heart Foundation (grant 200T401), National Institutes of Health National Heart, Lung, and Blood Institute (grant R01HL076200), and Office of dietary Supplements. This research was supported in part by the Netherlands Organisation for Scientific Research (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) through its Graduate Programme on Food Structure, Digestion and Health.

Duality of interest

This research was supported in part by Upfield. Financial support for plasma FA determinations in the Alpha Omega Cohort was obtained from Unilever R&D Vlaardingen. AJW and PLZ are employed by Unilever, Vlaardingen, the Netherlands. JMG received funding from Unilever R&D for epidemiological studies of dietary and circulating FAs and cardiovascular disease. Unilever is a producer of food consumer products. It divested its spreads business, which is since July 2018 operating under the name Upfield. None of the other authors reported a conflict of interest related to the study.

Author contributions

The authors' responsibilities were as follows: KP, AJW, MCH: wrote the manuscript. KP, AJW, MCH, LKK SSS-M, JdG, PLZ and JMG interpreted the results and critically revised the manuscript for intellectual content. KP and MCH analyzed data. JMG and SSS-M designed research. JMG had primary responsibility for the final content and handled funding and supervision. All of the authors read and approved the final manuscript. JMG is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation

Parts of this study were presented in abstract form at the EPI/Lifestyle 2018 Scientific Sessions of the American Heart Association, New Orleans, LA, 20-23 March 2018.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1 Hazard ratios and 95%CI of plasma linoleic acid and incident type 2 diabetes (per 5%) in subgroups*

Subgroups	HR (per 5 %)	*P* for interaction
Sex		
Men (n=2621)	0.81 (0.67, 0.96)	
Women (n=636)	0.47 (0.33, 0.68)	0.009
Age		
<65 y (n=966)	0.84 (0.62, 1.12)	
≥65 y (n=2291)	0.69 (0.57, 0.83)	0.28
Body mass index		
<30 kg/m^2 (n=2604)	0.72 (0.60, 0.87)	
≥30 kg/m^2 (n=653)	0.72 (0.53, 0.97)	0.96
Alcohol intake†		
No (n=132)	0.49 (0.25, 0.96)	
Low (n=1672)	0.70 (0.56, 0.87)	
Moderate (n=907)	0.88 (0.64, 1.21)	
High (n=546)	0.72 (0.49, 1.06)	0.42
Statin use		
Yes (n=2815)	0.70 (0.58, 0.83)	
No (n=442)	0.94 (0.62, 1.43)	0.19
Time since myocardial infarction		
>5 years (n=1190)	0.78 (0.60, 1.02)	
≤5 years (n=2038)	0.71 (0.58, 0.86)	0.68

* Hazard Ratios with 95% confidence intervals were obtained by Cox regression analysis, using the fully adjusted model (model 2, described in text, Statistical Analysis).
† Categorized as “no: 0 g/d”, “low: >0 to 10 g/d”, “moderate: >10 to 20 g/d for women and >10 to 30 g/d for men”, and “high: >20 g/d for women and >30 g/d for men”.

Supplementary Table S2 Hazard ratios of dietary and plasma LA in quintiles and per 5% with incident type 2 diabetes including only patients without prediabetes at baseline (n=3073)*,†

Exposure	N cases	Q1	Q2	Q3	Q4	Q5	*P*-trend‡	Per 5%	*P* value
Plasma LA (% total FAs)	138	1.00	0.70 (0.43, 1.14)	0.63 (0.38, 1.06)	0.62 (0.37, 1.03)	0.50 (0.28, 0.88)	0.014	0.76 (0.63, 0.91)	0.003
Dietary LA (en%)	138	1.00	0.75 (0.43, 1.29)	0.91 (0.51, 1.62)	1.10 (0.58, 2.10)	0.82 (0.34, 1.99)	0.98	1.16 (0.54, 2.51)	0.70

* Hazard Ratios with 95% confidence intervals were obtained by Cox regression analysis, using the fully adjusted model (model 2 for plasma LA and model 3 for dietary LA, described in text, Statistical Analysis);
† Sensitivity analyses excluding patients with prediabetes at baseline. Prediabetes was defined as plasma glucose between 6.1-6.9 mmol/L after ≥4 h of fasting;
‡ P-values for trend were obtained by assigning each patient median plasma or dietary LA for the category and modeled these values as continuous;
en%, percentage of total energy intake, excluding energy from alcohol; LA, linoleic acid; Q, quintile; SFA, saturated fatty acids; TFA, trans fatty acids.

Supplementary Table S3 Hazard ratios of plasma LA in quintiles and per 5% with incident type 2 diabetes, adjusting for potential confounding or intermediary factors one at a time*

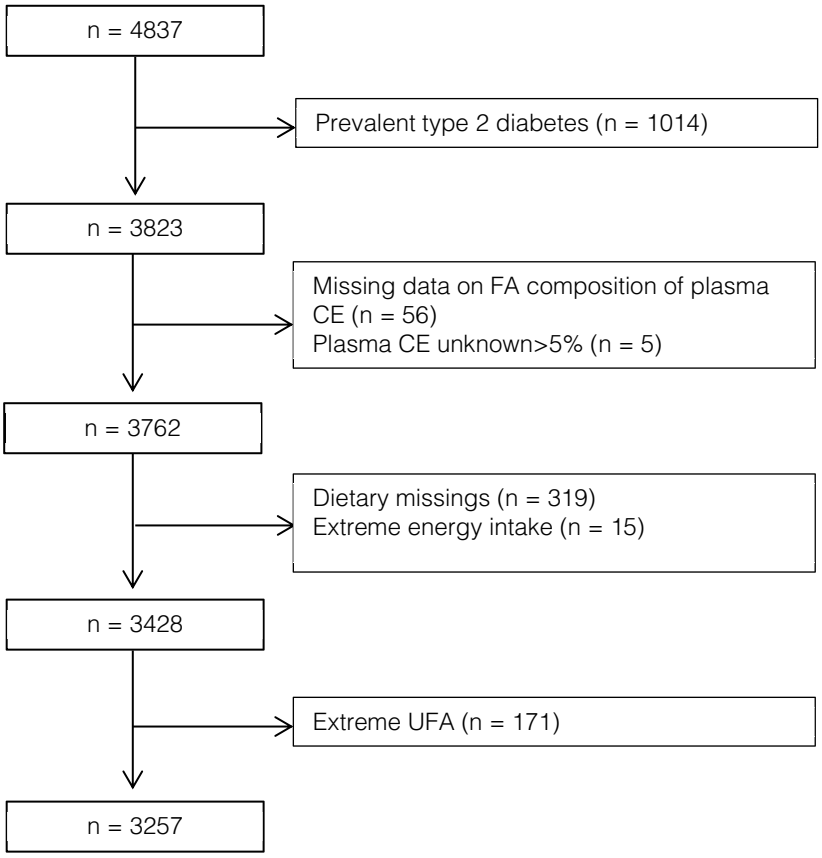
	Quintiles of plasma LA (%)					*P*-trend†	Per 5%	*P* value
	Q1 (n=652)	Q2 (n=650)	Q3 (n=651)	Q4 (n=653)	Q5 (n=651)			
Median, % total FAs	43.9	47.6	50.1	52.8	56.3			
Cases	49	38	31	31	22			
Model 2	1.00	0.74 (0.48, 1.14)	0.64 (0.40, 1.00)	0.60 (0.38, 0.94)	0.44 (0.26, 0.75)	0.001	0.73 (0.62, 0.86)	<0.001
+ Triacylglycerol, mmol/L	1.00	0.77 (0.50, 1.18)	0.65 (0.41, 1.03)	0.64 (0.40, 1.02)	0.47 (0.28, 0.80)	0.003	0.76 (0.64, 0.89)	<0.001
+ Palmitic acid (16:0), % total FAs	1.00	0.80 (0.52, 1.26)	0.72 (0.44, 1.19)	0.71 (0.42, 1.20)	0.58 (0.30, 1.10)	0.09	0.77 (0.62, 0.96)	0.020
+ Palmitoleic to palmitic acid ratio (16:1n-7/16:0)	1.00	0.81 (0.52, 1.26)	0.73 (0.44, 1.18)	0.70 (0.42, 1.16)	0.53 (0.30, 0.95)	0.030	0.77 (0.64, 0.93)	0.008
+ Oleic acid (18:1n-9), % total FAs	1.00	0.85 (0.54, 1.34)	0.78 (0.47, 1.32)	0.80 (0.45, 1.40)	0.66 (0.33, 1.30)	0.25	0.80 (0.63, 1.03)	0.09

* Hazard Ratios with 95% confidence intervals were obtained by Cox regression analysis, using the fully adjusted model (model 2, described in text, Statistical Analysis);
† *P* values for trend were obtained by assigning each patient median plasma LA for the category and we modeled these values as continuous;
FA, fatty acids; LA, linoleic acid.

Supplementary Table S4 Hazard ratios of specific plasma fatty acids and fatty acid ratios with incident type 2 diabetes*

		Quintiles of plasma or plasma ratios of FA					*P*-trend†
		Q1	Q2	Q3	Q4	Q5	
Palmitic acid (16:0), % total FA	N	651	652	649	653	652	
	Median	10.3	10.9	11.2	11.7	12.2	
	Cases	20	33	34	39	45	
	Model 2	1.00	1.69 (0.97, 2.94)	1.76 (1.01, 3.07)	2.02 (1.18, 3.49)	2.23 (1.30, 3.80)	0.003
Oleic acid (18:1n-9), % total FA	N	652	649	653	653	650	
	Median	15.1	16.6	17.7	18.8	20.6	
	Cases	17	31	38	42	43	
	Model 2	1.00	1.83 (1.01, 3.31)	2.27 (1.28, 4.05)	2.43 (1.38, 4.30)	2.65 (1.49, 4.72)	<0.001
Liver fat proxy (16:0/18:2n-6)	N	651	652	651	652	651	
	Median	0.18	0.21	0.22	0.24	0.27	
	Cases	19	32	36	39	45	
	Model 2	1.00	1.70 (0.96, 3.00)	1.90 (1.09, 3.32)	2.07 (1.19, 3.60)	2.38 (1.38, 4.12)	0.002
Estimated D5D activity (20:4n-6/20:3n-6)‡	N	647	648	648	648	647	
	Median	6.8	8.7	10.1	11.9	15.0	
	Cases	38	40	31	35	23	
	Model 2	1.00	1.09 (0.70, 1.70)	0.84 (0.52, 1.36)	1.04 (0.66, 1.66)	0.72 (0.43, 1.22)	0.23
Estimated D6D activity (18:3n-6/18:2n-6)	N	651	652	651	652	651	
	Median	0.011	0.015	0.019	0.023	0.031	
	Cases	25	26	34	42	44	
	Model 2	1.00	1.01 (0.58, 1.75)	1.31 (0.77, 2.21)	1.66 (1.00, 2.77)	1.74 (1.04, 2.91)	0.008

* Hazard Ratios with 95% confidence intervals were obtained by Cox regression analysis, using the fully adjusted model (Model 2 described in text, Statistical Analysis);
† *P* values for trend were obtained by assigning each patient median plasma FA or ratio of plasma FA for the category and we modeled these values as continuous;
‡ Estimated D5D activity was only calculated for 3238 patients with non-zero values for their 20:3n-6 levels;
D5D, delta-5-desaturase; D6D, delta-6-desaturase; FA, fatty acid.



Supplemental Figure S1 Flow diagram for selecting the population of analysis from the Alpha Omega Cohort.

CE, cholesteryl esters; FA, fatty acids; UFA, unsaturated fatty acids.

Chapter 5

Associations of linoleic acid with markers of glucose metabolism and liver function in South African adults

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Lipids in Health and Disease 2020;19:138

ABSTRACT

Background: The relation between dietary and circulating linoleic acid (18:2 n-6, LA), glucose metabolism and liver function is not yet clear. Associations of dietary and circulating LA with glucose metabolism and liver function markers were investigated.

Methods: Cross-sectional analyses in 633 black South Africans (aged >30 years, 62% female, 51% urban) without type 2 diabetes at baseline of the Prospective Urban Rural Epidemiology study. A cultural-sensitive 145-item food-frequency questionnaire was used to collect dietary data, including LA (percentage of energy; en%). Blood samples were collected to measure circulating LA (% total fatty acids (FA); plasma phospholipids), plasma glucose, glycosylated hemoglobin (HbA1c), serum gamma-glutamyl transferase (GGT), alanine (ALT) and aspartate aminotransferase (AST). Associations per 1 standard deviation (SD) and in tertiles were analyzed using multivariable regression.

Results: Mean (±SD) dietary and circulating LA was 6.8 (±3.1) en% and 16.0 (±3.5) % total FA, respectively. Dietary and circulating LA were not associated with plasma glucose or HbA1c (β per 1 SD: -0.005 to 0.010, $P>0.20$). Higher dietary LA was generally associated with lower serum liver enzymes levels. One SD higher circulating LA was associated with 22% lower serum GGT (β (95% confidence interval): -0.25 (-0.31, -0.18), $P<0.001$), but only ≤9% lower for ALT and AST. Circulating LA and serum GGT associations differed by alcohol use and locality.

Conclusion: Dietary and circulating LA were inversely associated with markers of impaired liver function, but not with glucose metabolism. Alcohol use may play a role in the association between LA and liver function.

INTRODUCTION

Circulating linoleic acid (18:2 n-6, LA) has been inversely associated with the risk of type 2 diabetes (T2D) in cohorts of mostly healthy Caucasian populations,[1] whereas the association between dietary linoleic acid and T2D risk remains equivocal.[2–5] This discrepancy has been mainly attributed to the limitation of self-reported dietary LA assessment. However, endogenous fatty acids (FA) metabolism affecting circulating LA proportion may also play a role.

The proportion of circulating LA is not only influenced by dietary LA, but also by endogenous FA metabolism, which may be affected by various factors such as obesity and liver impairment.[6,7] A lower circulating LA was observed in non-alcoholic fatty liver disease patients as compared to healthy individuals.[8] Circulating LA has also been inversely associated with hepatic fat deposition.[9] These findings suggest that low proportions of circulating LA may indicate impairment of liver function.

The influence of dietary LA on glucose metabolism and liver function is not yet clear. Meta-analyses of randomized controlled feeding trials showed that increasing the intake of polyunsaturated fatty acids (PUFA) (mainly LA) potentially affects glucose metabolism.[10,11] On the other hand, a high dietary n-6 FA or n-6/n-3 ratio has been considered to promote the development of fatty liver through inflammation,[12] although evidence from two systematic reviews and meta-analyses of randomized controlled trials in humans showed that increasing dietary n-6 FA did not result in higher concentration of inflammatory markers.[13,14]

Studies on LA and markers related to T2D risk have been performed mainly in Caucasian populations. Populations with African ancestry may have different distributions of genetic variants related to long-chain PUFA metabolism.[15] Therefore, this study aimed to investigate associations of both dietary and circulating LA with markers of glucose metabolism and liver function in the South African leg of the Prospective Urban and Rural Epidemiological (PURE) study.

SUBJECTS AND METHODS

Study population

The PURE North-West Province South Africa (PURE-NWP-SA) is a cohort of black South African participants and part of the larger international PURE study. The design, selection and recruitment of participants for the PURE-NWP-SA study have

been described previously.[16] In brief, apparently healthy black South African adults aged >30 years old were recruited from two urban and two rural communities in North-West Province. Of 3,750 adults who completed the screening questionnaire, 2,010 attended baseline measurements in 2005. All participants gave written consent to participation and the study was approved by the Ethics Committee of the North-West University (04M10 and NW-00016-10-A1).[16]

Baseline PURE-NWP-SA cohort data were used for the present cross-sectional study. Participants without information on dietary LA intake or with implausible energy intake (<3,347 kilojoules (kJ) [<800 kilocalories (kcal)] or >16,736 kJ [>4,000 kcal] for men; <2,092 kJ [<500 kcal] or >14,644 kJ [>3,500 kcal] for women)[17] or missing plasma FA were further excluded. Missing or unreliable plasma glucose (values <2.5 mmol/L) and glycosylated hemoglobin (HbA1c) were excluded. Finally, participants with prevalent diabetes, defined as self-reported diagnosis of type 2 diabetes or use of anti-diabetes drugs, were excluded. A total of 633 participants were included in the present analyses (**Additional file 1: Figure S1**).

Dietary assessment

Information on usual diet was collected with a cultural-sensitive 145-item quantified food-frequency questionnaire (QFFQ). This questionnaire, covering dietary intake in the previous month, was developed and validated in a similar population.[18–20] Dietary FA intakes at baseline (expressed as grams/day (g/d) and a percentage of energy (en%)) were calculated by using the South African food composition database.[21] In addition, information from the QFFQ and food composition database were used to calculate total daily intakes of energy (kJ), macronutrients (en%), dietary fiber (g/d) and cholesterol (mg/d).

Circulating plasma phospholipid FA measurement

Venous blood samples were collected from participants after 8-10 h of fasting by a trained nurse, prepared in conditions required for further processing and stored at -80°C until analysis.[16] Before analysis, frozen EDTA samples were thawed and total lipids were extracted by using a modified Folch method[22] from the plasma samples. Phospholipids FA fraction was isolated by using thin-layer chromatography. FA composition of plasma phospholipids was analyzed using quadrupole gas chromatography electron ionization mass spectrometry at the Centre of Excellence for Nutrition laboratory, Potchefstroom, South Africa as previously described in

detail.[23] A total of 26 FA were quantified as a percentage of total FA identified in the sample (g/100 g total FA; % total FA) including LA and arachidonic acid (20:4n-6). Delta-6-desaturase activity was also estimated by calculating the ratio of circulating dihomo-gamma-linolenic acid to LA (20:3n-6/18:2n-6).

Markers of glucose metabolism and liver function

Plasma glucose (mmol/L) in the fasting state was measured by using Synchron® System (Beckman Coulter Co., Fullerton, CA, USA). HbA1c was assessed in EDTA-treated whole blood by using a D-10 Hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA, USA). Serum liver enzymes (gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST); U/L) were determined using Sequential Multiple Analyzer Computer Konelab20i auto analyzer (Thermo Fischer Scientific, Vantaa, Finland).

Other measurements

Information about demographics, socioeconomic status, lifestyle and physical activity was collected using structured questionnaires that were standardized for the international PURE study.[16] Locality was identified as rural or urban based on the communities where the participants resided. Physical activity index (PAI) was calculated (maximum score of 10.0) and participants were categorized into having 'low'($1<PAI\leq3.33$), 'moderate' ($3.34\leq PAI\leq6.67$), or 'high' ($PAI \geq6.68$) physical activity.[24,25] Participants had to indicate their level of education obtained and their responses were recoded into a dichotomous variable of 'no school or primary' or 'secondary or higher'. Participants' smoking status was recorded as 'non-smoker' or 'past or current smoker'. Alcohol intake was assessed by using the QFFQ and expressed as g/d of pure ethanol.[26] Alcohol intake was also categorized into 'no' (0 g/d), 'light' (>0-10 g/d (women); >0-20 g/d (men)), 'moderate' (>10-20 g/d (women); >20-30 g/d (men)), or 'high'(>20 g/d (women); >30 g/d (men)).

Height, weight and waist circumference were measured using standardized procedure as described previously.[16] Body mass index (BMI) was calculated by dividing weight by height squared (kg/m^2). Obesity was considered present when BMI ≥30 kg/m^2. Other biochemical analyses such as total cholesterol (mmol/L), high-density lipoprotein cholesterol (HDL-C; mmol/L), triglycerides (TG; mmol/L), high-sensitivity C-reactive protein (mg/L) were determined in serum.[27] Interleukin-6 (pg/ml) was measured using ultrasensitive enzyme immunoassays (Elecsys 2010, Roche, Basel, Switzerland).[28] Low-density lipoprotein cholesterol (LDL-C; mmol/L)

was estimated using the equation: LDL-C = total cholesterol – HDL-C – triglycerides/adjustable factor. In this modification of the Friedewald equation specific adjustable factors are used to estimate VLDL-C, depending on the levels of triglycerides and non-HDL-C.[29] Values of serum HDL-C ≤1.0 mmol/L for men or ≤1.3 mmol/L for women were considered low and serum triglycerides >1.7 mmol/L was considered as high. Systolic and diastolic blood pressure (in mmHg) were measured twice on the right upper arm in a seating position with 5 min between measurements as previously reported.[27] Use of medications (anti-diabetes, anti-hypertensive drug) was recorded at study sites by interviewers. Hypertension was considered present if blood pressure was ≥140/90 mmHg or using antihypertensive medication. The human immunodeficiency virus (HIV) status of participants was assessed according to the South African National Department of Health protocol as previously described.[16]

Statistical analysis

Participants' characteristics were examined in the total sample and by categorizing the participants into tertiles according to their dietary or circulating LA values. Linear trends across dietary or circulating LA were calculated by assigning median values of dietary or circulating LA for the tertile and modelling these as a continuous variable in the regression models. The distribution of values for all variables, the residuals and homogeneity of variances were examined before performing the main analyses. The outcome variables (plasma glucose, HbA1c, serum GGT, ALT and AST) were log-transformed before analysis to achieve normality of residuals and homogeneity of variances.

To assess the relationship between dietary and circulating LA, Spearman rank correlation (r) between dietary and circulating LA was calculated and adjusted for age, sex and total energy intake. Least-squares means and 95% confidence interval (CI) of circulating LA (% total FA) across tertiles of dietary LA (en%) were calculated by using linear regression, adjusted for age, sex and total energy intake, also in subgroups by sex (men *vs.* women), age (<65 *vs.* ≥65 y), obesity (present *vs.* absent), glycemic status (plasma glucose <6.1 *vs.* ≥6.1 mmol/L), and alcohol use ('no': intake=0 g/d *vs.* 'yes': intake >0 g/d).

Association of dietary or circulating LA with glycemic and liver function measures were evaluated per 1 SD and in dietary or circulating LA tertiles by using multivariable linear regression. Model 1 included age and sex. Model 2 was additionally adjusted for education level, locality, smoking status, physical activity,

alcohol intake (g/d), total energy intake (kJ/d) and BMI (kg/m^2). Model 3 was further adjusted for available carbohydrates (en%), saturated FA (en%), *trans*-FA (g/d) and dietary fiber (g/d). Dietary factors were considered but not included in the fully adjusted model for circulating LA (model 2) because no indication was found for confounding of circulating LA associations by these factors. Missing values in covariables (education level, n=15; smoking status, n=1; physical activity, n=22) were imputed by using sex-specific modes. Potential effect modification by sex, age, locality, obesity, and alcohol use was evaluated by using stratified analyses and by including product terms in the fully adjusted model (model 3 for dietary LA and model 2 for circulating LA). Stratified analyses by alcohol use were conducted in two categories (yes *vs.* no) due to the relatively small sizes of moderate and high alcohol intake subgroups.

In sensitivity analyses, participants with a high alcohol intake (alcohol >20 g/d for women and >30 g/d for men), with liver enzymes values indicating possible alcohol abuse (serum GGT >80 U/L and AST-to-ALT ratio ≥ 2:1) or having elevated plasma glucose (fasting plasma glucose ≥6.1 mmol/L) were excluded. All analyses were performed in SAS version 9.4 (Cary, NC, USA). A two-sided *P* value <0.05 was considered as statistically significant.

RESULTS

The total sample of 633 subjects was on average 53 years, 63% was women and 51% resided in urban communities (**Table 1**). Fifty-eight percent of the participants were considered hypertensive and 43% had either high serum triglycerides or low HDL-C levels. Mean (±SD) dietary LA intake was 6.8 (±3.1) en% and circulating LA was 16.0 (±3.5) % total FA. Liver function markers values were available for 613 participants (97%), whose characteristics were not different from the total sample (**Additional file 1: Table S1**). The proportion of participants reporting no alcohol intake was lower in urban (48.6%) than in rural communities (66.2%), whereas proportions of participants with high alcohol intake and with obesity were similar in both communities (**Additional file 1: Table S2**).

Dietary LA was weakly associated with circulating LA (r=0.14, *P*<0.001). Margarine and vegetable oils, sources of dietary LA, were also associated with circulating LA with adjusted Spearman's correlations of 0.10 (*P*=0.011) and 0.10 (*P*=0.009), respectively (**Additional file 1: Table S3**). Dose-response associations of circulating with dietary LA intake were evident, despite differences in sex, age or presence of

obesity. Dose-response associations were observed for participants with normal plasma glucose, but not in participants with elevated plasma glucose. Within categories of similar LA intake, individuals with alcohol intake >0 g/d had a lower mean circulating LA than individuals reporting no alcohol intake (**Additional file 1: Figure S2**).

Dietary LA and glucose metabolism and liver function markers

Participants with higher dietary LA had on average a higher BMI, were more likely to reside in urban communities and were less likely to consume alcohol than those with lower dietary LA (**Table 2**). Mean (±SD) circulating LA ranged from 15.5 (±3.5) to 16.4 (±3.5) % total FA across dietary LA tertiles. Participants with higher dietary LA did not have significantly higher high-sensitivity C-reactive protein, interleukin-6 or circulating arachidonic acid than those in the lower dietary LA tertiles (Table 2).

Dietary LA was not associated with plasma glucose or HbA1c after multivariable adjustments of demographic, lifestyle and dietary factors (**Table 3**). Participants in the highest dietary LA tertile had significantly lower geometric mean (95% CI) serum GGT than participants in the lowest tertile (T3 *vs.* T1: 48.8 (42.8, 55.6) *vs.* 65.2 (57.0, 74.5), *P*-trend=0.008). Dietary LA was not significantly associated with serum ALT across tertiles (T3 *vs.* T1: 16.6 (15.2, 18.1) *vs.* 19.1 (17.4, 20.9), *P*-trend=0.06). Geometric mean serum AST was lower with higher dietary LA (T3 *vs.* T1: 25.0 (22.8, 27.5) *vs.* 30.2 (27.4, 33.2), *P*-trend=0.018). In continuous analysis per one SD, however, the associations between dietary LA and log-serum GGT, ALT or AST were not statistically significant (Table 3).

Table 1 Baseline characteristics of participants of PURE-NWP-SA included in the present analyses (n=633)

	Total sample
Age (y)	53.0 ± 10.4
Men	240 (37.9)
Body mass index (kg/m^2)	24.9 ± 6.9
Obesity	143 (22.6)
Locality (urban)	325 (51.3)
Education level[a]	
No school or primary	247 (40.0)
Secondary or higher	371 (60.0)
Smoking status[a]	
Non-smoker	284 (44.9)
Past or current smoker	348 (55.1)
Physical activity[a]	
Low	5 (0.8)
Moderate	265 (43.4)
High	341 (55.8)
Fasting plasma glucose (mmol/L)	4.90 (4.40-5.40)
HbA1c (%)	5.60 (5.30-5.90)
Serum lipids (mmol/L)	
Total cholesterol	5.19 ± 1.31
LDL cholesterol	3.03 ± 1.13
HDL cholesterol	1.58 ± 0.64
Triglycerides	1.13 (0.84-1.69)
Liver enzymes activity (U/L)	
Gamma-glutamyl transferase	45.8 (29.7-88.0)
Alanine transaminase	17.0 (12.6-24.4)
Aspartate aminotransferase	25.0 (18.8-35.0)
HIV-positive[a]	5 (0.8)
Dietary variables	
Daily total energy intake (kJ)	7,356 ± 3,031
Available carbohydrate (en%)	58.8 ± 9.2
Protein (en%)	12.4 ± 2.3
Total fat (en%)	24.2 ± 8.7
Saturated fatty acids (en%)	5.9 ± 3.0
Monounsaturated fatty acids (en%)	6.4 ± 3.3
Polyunsaturated fatty acids (en%)	7.4 ± 3.2
n-3 fatty acids (mg/d)[a]	312 (193-474)
Trans-fatty acids (g/d)	0.18 (0.07-0.49)
Dietary cholesterol (mg/d)	149 (82-251)
Dietary fiber (g/d)	20.4 ± 9.2
Alcohol consumption[c] (g/d)	0 (0-12.3)
No	362 (57.2)
Light	128 (20.2)
Moderate	43 (6.8)
High	100 (15.8)

Values are mean ± SD, median (Q1-Q3), or *n* (%).

[a] Missing values for 15 participants for education level, 1 participant for smoking status, 22 participants for physical activity level, 1 participant for HIV status and 1 participant for n-3 fatty acids.

[b] Available for n=613.

[c] Alcohol consumption categories: 'No': 0 g/d; 'Light': >0 to 10 g/d (women), >0 to 20 g/d men; 'Moderate': >10-20 g/d (women), >20-30 g/d (men); 'High': >20 g/d (women), >30 g/d (men).

Table 2 Baseline characteristics of participants across tertiles of dietary and circulating linoleic acid

	Dietary linoleic acid (en%)				Circulating linoleic acid (% total FA)			
	T1 (n=211)	T2 (n=211)	T3 (n=211)	*P*-trend[a]	T1 (n=211)	T2 (n=211)	T3 (n=211)	*P*-trend[a]
Median (range)	3.9 (0.4-5.3)	6.7 (5.4-8.0)	9.6 (8.0-18.4)		12.5 (6.0-14.4)	16.0 (14.4-17.7)	19.5 (17.7-25.7)	
Age (y)	52.4 ± 9.0	52.8 ± 11.3	53.8 ± 10.6	0.18	53.8 ± 9.4	54.2 ± 11.2	50.9 ± 10.1	0.004
Men	91 (43.1)	80 (37.9)	69 (32.7)	0.09	76 (36.0)	82 (38.9)	82 (38.9)	0.78
Body mass index (kg/m^2)	23.8 ± 6.5	25.0 ± 7.5	25.9 ± 6.7	0.002	24.4 ± 7.1	25.2 ± 7.4	25.0 ± 6.3	0.36
Obesity	40 (19.0)	47 (22.3)	56 (26.5)	0.17	40 (19.0)	52 (24.6)	51 (24.2)	0.30
Waist circumference (cm)	79.6 ± 12.3	80.2 ± 14.3	81.3 ± 12.1	0.20	80.8 ± 13.4	80.6 ± 13.5	79.6 ± 11.9	0.36
Locality (urban)	71 (33.6)	123 (58.3)	131 (62.1)	<0.001	107 (50.7)	113 (53.6)	105 (50.0)	0.72
Education level[b]				0.12				<0.001
No school or primary	94 (45.6)	76 (36.4)	77 (37.9)		108 (51.9)	78 (38.2)	61 (29.6)	
Secondary or higher	112 (54.4)	133 (63.6)	126 (62.1)		100 (48.1)	126 (61.8)	145 (70.4)	
Smoking status[b]				0.008				0.010
Non-smoker	84 (40.0)	87 (41.2)	113 (53.6)		81 (38.4)	92 (43.6)	111 (52.9)	
Past or current smoker	126 (60.0)	124 (58.8)	98 (46.4)		130 (61.6)	119 (56.4)	99 (47.1)	
Physical activity[b]				<0.001				0.006
Low	1 (0.5)	3 (1.5)	1 (0.5)		1 (0.5)	2 (1.0)	2 (1.0)	
Moderate	66 (32.4)	100 (49.0)	99 (48.8)		107 (52.4)	87 (42.4)	71 (35.2)	
High	137 (67.2)	101 (49.5)	103 (50.7)		96 (47.1)	116 (56.6)	129 (63.9)	
Serum lipids (mmol/L)								
Total cholesterol	5.10 ± 1.33	5.18 ± 1.31	5.28 ± 1.30	0.16	5.13 ± 1.31	5.34 ± 1.37	5.10 ± 1.25	0.81
LDL-C	2.92 ± 1.16	3.01 ± 1.14	3.15 ± 1.08	0.047	2.87 ± 1.08	3.15 ± 1.18	3.06 ± 1.11	0.09
HDL-C	1.60 ± 0.71	1.61 ± 0.60	1.55 ± 0.59	0.42	1.65 ± 0.72	1.60 ± 0.61	1.49 ± 0.56	0.012
Triglycerides	1.06 (0.85-1.62)	1.07 (0.82-1.69)	1.20 (0.86-1.78)	0.09	1.17 (0.86-1.85)	1.15 (0.85-1.65)	1.04 (0.80-1.53)	<0.001
High sensitivity C-reactive protein (mg/L)	3.9 (1.3-10.0)	3.2 (1.0-9.5)	3.8 (1.2-11.4)	0.67	4.1 (1.4-14.0)	3.6 (1.5-9.2)	3.5 (0.9-8.8)	0.014
Interleukin-6 (pg/mL)	3.04 (1.58-7.29)	3.00 (0.75-4.91)	2.94 (0.75-5.54)	0.13	4.50 (1.98-8.83)	2.86 (0.76-5.12)	2.09 (0.75-4.16)	0.002
HIV-positive	2 (1.0)	0 (0)	3 (1.4)	0.38	2 (1.0)	0 (0)	3 (1.4)	0.30
Dietary variables[c]								
Total energy intake (kJ/d)	7,413 ± 3,265	7,338 ± 2,979	7,319 ± 2,846	0.75	7,699 ± 3,263	7,282 ± 2,920	7,088 ± 2,875	0.038
Carbohydrate	62.7 ± 10.7	59.3 ± 7.4	54.3 ± 7.2	<0.001	57.8 ± 9.5	59.4 ± 9.2	59.1 ± 9.0	0.15

	Dietary linoleic acid (en%)				Circulating linoleic acid (% total FA)			
	T1 (n=211)	T2 (n=211)	T3 (n=211)	*P*-trend[a]	T1 (n=211)	T2 (n=211)	T3 (n=211)	*P*-trend[a]
Protein	11.4 ± 2.3	12.8 ± 2.1	13.1 ± 2.1	<0.001	12.0 ± 2.4	12.7 ± 2.2	12.7 ± 2.3	0.001
Total fat	16.4 ± 6.1	24.6 ± 5.3	31.7 ± 6.9	<0.001	22.6 ± 9.0	24.0 ± 8.6	26.0 ± 8.3	<0.001
Saturated FA	3.9 ± 2.4	6.2 ± 2.5	7.6 ± 2.9	<0.001	5.4 ± 2.8	5.9 ± 3.0	6.5 ± 3.1	<0.001
Monounsaturated FA	3.8 ± 2.0	6.6 ± 2.4	8.9 ± 3.1	<0.001	5.8 ± 3.1	6.5 ± 3.4	7.0 ± 3.3	<0.001
Polyunsaturated FA	4.3 ± 2.6	7.2 ± 1.1	10.6 ± 2.1	<0.001	7.1 ± 3.7	7.1 ± 3.0	7.9 ± 3.0	0.013
n-3 fatty acids (mg/d)	221.1 (145.5-362.4)	325.3 (209.8-497.2)	391.5 (253.5-543.7)	<0.001	296.0 (182.2-452.5)	319.6 (207.5-487.2)	319.9 (192.6-476.2)	0.37
Trans-FA (g/d)	0.09 (0.03-0.25)	0.20 (0.08-0.48)	0.33 (0.12-0.68)	<0.001	0.18 (0.07-0.43)	0.17 (0.06-0.48)	0.19 (0.07-0.53)	0.71
Total fiber (g/d)	20.4 ± 9.0	21.1 ± 9.3	19.9 ± 9.1	0.55	20.3 ± 9.0	20.4 ± 8.6	20.6 ± 9.9	0.75
Dietary cholesterol (mg/d)	90 (48-159)	159 (99-268)	204 (129-307)	<0.001	151 (78-248)	148 (90-246)	143 (81-254)	0.56
Alcohol use: yes[d]	118 (55.9)	84 (39.8)	69 (32.7)	<0.001	117 (55.4)	88 (41.7)	66 (31.3)	<0.001
(g/d)	4.6 (0-36.2)	0 (0-11.6)	0 (0-4.3)	<0.001	2.9 (0-27.0)	0 (0-8.6)	0 (0-5.8)	<0.001
Fatty acid composition								
Arachidonic acid (20:4n-6), %	13.5 ± 2.7	13.4±2.5	13.7±2.3	0.43	12.9 ± 2.9	14.1 ± 2.4	13.6 ± 2.0	0.011
Estimated D6D activity (20:3n-6/18:2n-6)	0.200 ± 0.061	0.192 ± 0.055	0.187 ± 0.058	0.016	0.234 ± 0.053	0.196 ± 0.047	0.150 ± 0.040	<0.001

Values are mean ± SD, median (Q1-Q3), or *n* (%) unless otherwise stated.

[a] P-values for trend for continuous variables were obtained by assigning median value of dietary or circulating linoleic acid and this value was modelled as continuous; for categorical variables p-values from (exact) Chi-squared tests were displayed.

[b] Missing values for 15 participants for education level, 1 participant for smoking status, 22 participants for physical activity level, 1 participant for HIV status, 1 participant for dietary n-3 fatty acids and 62 participants for interleukin-6.

[c] Dietary variables are expressed as percentage of energy unless otherwise stated.

[d] Alcohol use: 'No': alcohol intake=0 g/d, 'Yes' alcohol intake >0 g/d.

D6D, delta-6-desaturase; FA, fatty acids; HIV, human immunodeficiency virus.

Table 3 Associations of dietary linoleic acid with glucose metabolism and liver function markers[a,b]

	Tertiles of dietary linoleic acid			*P*-trend[c]	β (95% CI) per 1 SD	*P*
	T1	T2	T3			
Glucose metabolism markers						
Plasma glucose, mmol/L						
Model 1	4.88 (4.73, 5.03)	4.84 (4.69, 4.99)	4.92 (4.77, 5.07)	0.71	0.008 (-0.010, 0.026)	0.36
Model 2	4.90 (4.74, 5.06)	4.83 (4.68, 4.98)	4.91 (4.76, 5.06)	0.89	0.007 (-0.013, 0.026)	0.50
Model 3	4.90 (4.72, 5.06)	4.83 (4.68, 4.98)	4.91 (4.76, 5.06)	0.93	0.010 (-0.015, 0.036)	0.43
HbA1c (%)						
Model 1	5.61 (5.51, 5.70)	5.66 (5.56, 5.76)	5.67 (5.57, 5.77)	0.39	0.004 (-0.006, 0.014)	0.40
Model 2	5.64 (5.54, 5.74)	5.65 (5.56, 5.75)	5.65 (5.55, 5.75)	0.89	0.001 (-0.010, 0.011)	0.92
Model 3	5.60 (5.49, 5.71)	5.65 (5.56, 5.75)	5.68 (5.57, 5.80)	0.36	0.008 (-0.007, 0.022)	0.30
Liver function markers (subsample, n=613)						
Serum GGT, U/L						
Model 1	64.8 (57.5, 73.1)	55.6 (49.3, 62.6)	50.2 (44.6, 56.7)	0.004	-0.079 (-0.149, -0.010)	0.025
Model 2	59.3 (52.6, 66.9)	56.4 (50.4, 63.2)	53.9 (48.0, 60.6)	0.28	-0.006 (-0.076, 0.065)	0.88
Model 3	65.2 (57.0, 74.5)	57.0 (50.9, 63.7)	48.8 (42.8, 55.6)	0.008	-0.084 (-0.179, 0.010)	0.08
Serum ALT, U/L						
Model 1	19.0 (17.6, 20.5)	17.2 (16.0, 18.6)	16.8 (15.6, 18.2)	0.027	-0.030 (-0.074, 0.014)	0.18
Model 2	18.5 (17.0, 20.0)	17.4 (16.2, 18.8)	17.1 (15.8, 18.5)	0.21	-0.005 (-0.053, 0.043)	0.84
Model 3	19.1 (17.4, 20.9)	17.4 (16.2, 18.8)	16.6 (15.2, 18.1)	0.06	-0.020 (-0.084, 0.044)	0.54
Serum AST, U/L						
Model 1	29.0 (26.7, 31.5)	26.8 (24.6, 29.1)	26.0 (23.9, 28.3)	0.07	-0.022 (-0.071, 0.026)	0.36
Model 2	28.3 (26.0, 30.9)	26.7 (24.6, 28.9)	26.6 (24.5, 28.9)	0.33	0.004 (-0.047, 0.055)	0.88
Model 3	30.2 (27.4, 33.2)	26.7 (24.6, 28.9)	25.0 (22.8, 27.5)	0.018	-0.041 (-0.109, 0.027)	0.24

Values for outcome variables in dietary linoleic acid tertiles are geometric means and 95% CI. Values for β (95% CI) are natural log-transformed values;
[a] One standard deviation of dietary linoleic acid is 3.1 en%.
[b] Multivariable models: Model 1: adjusted for age, sex; Model 2: model 1 plus education level, locality, smoking status, physical activity, alcohol intake, total energy intake, BMI; Model 3: model 2 plus fiber, available carbohydrates, saturated fatty acids, trans-fatty acids intake.
[c] P-values for trend were obtained by assigning median value of dietary linoleic acid and and this value was modelled as continuous.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

Circulating LA and glucose metabolism and liver function markers

Mean (±SD) dietary LA ranged from 6.5 (±3.2) to 7.4 (±3.0) en% across circulating phospholipids LA tertiles. Participants with higher circulating LA were younger and less likely to consume alcohol. Circulating LA was inversely associated with serum triglycerides and inflammatory markers C-reactive protein and interleukin-6 (Table 2).

Circulating LA was not significantly associated with plasma glucose or HbA1c (**Table 4**). One SD increase in circulating LA (3.5% total FA) was associated with a 0.25 lower (95% CI: -0.31, -0.18; *P*<0.001) log-serum GGT after adjustment for demographic and lifestyle factors, total energy intake and BMI (Table 4). Circulating LA was also inversely associated with log-serum ALT (β= -0.050 (95% CI: -0.096, -0.003), *P*=0.036) and AST (β= -0.090 (95% CI: -0.139, -0.041), *P*<0.001). In tertiles, circulating LA was also significantly inversely associated with serum GGT (T3 *vs.* T1: 42.3 (37.9, 47.2) *vs.* 77.2 (69.1, 86.4), *P*-trend<0.001) and AST (T3 *vs.* T1: 24.8 (22.9, 26.9) *vs.* 30.2 (27.8, 32.8), *P*-trend<0.001).

Associations of circulating LA with serum GGT were modified by alcohol use (*P*<0.001) and locality (*P*<0.001). Stronger inverse associations of circulating LA with GGT were observed for participants who reported any alcohol consumption compared to those who reported no consumption, and for participants residing in the urban compared to those in the rural communities (**Additional file 1: Table S4**). Similar effect modification by locality was observed for associations of circulating LA with serum ALT (*P*=0.006) and AST (*P*=0.001).

Sensitivity analysis

Excluding participants reporting high alcohol intake did not appreciably change the results (**Additional file 1: Table S5**). When participants with an indication of alcohol abuse were excluded, associations of circulating LA with serum GGT, ALT and AST were attenuated (**Additional file 1: Table S6**). Exclusion of participants with elevated plasma glucose levels resulted in a significant inverse association of dietary LA with log-serum GGT; the β (95% CI) per one SD was -0.105 (-0.204, -0.006; *P*=0.038) after exclusion as compared to -0.084 (-0.179, 0.010; *P*=0.08) before exclusion (**Additional file 1: Table S7**).

Table 4 Associations of circulating linoleic acid with glucose metabolism and liver function markers[a,b]

	Tertiles of circulating linoleic acid			*P*-trend[c]	β (95% CI) per 1 SD	*P*
	T1	T2	T3			
Glucose metabolism markers						
Plasma glucose, mmol/L						
Model 1	4.94 (4.79, 5.09)	4.88 (4.73, 5.03)	4.82 (4.67, 4.97)	0.28	-0.009 (-0.027, 0.009)	0.34
Model 2	4.90 (4.75, 5.06)	4.88 (4.73, 5.03)	4.85 (4.70, 5.00)	0.62	-0.005 (-0.024, 0.014)	0.59
HbA1c, %						
Model 1	5.59 (5.50, 5.69)	5.63 (5.54, 5.73)	5.71 (5.61, 5.81)	0.10	0.009 (-0.002, 0.019)	0.10
Model 2	5.61 (5.52, 5.71)	5.63 (5.53, 5.72)	5.69 (5.60, 5.79)	0.28	0.006 (-0.005, 0.016)	0.29
Liver function markers (subsample, n=613)						
Serum GGT, U/L						
Model 1	85.5 (76.4, 95.7)	54.9 (49.1, 61.3)	38.7 (34.6, 43.3)	<0.001	-0.32 (-0.39, -0.26)	<0.001
Model 2	77.2 (69.1, 86.4)	55.4 (49.8, 61.7)	42.3 (37.9, 47.2)	<0.001	-0.25 (-0.31, -0.18)	<0.001
Serum ALT, U/L						
Model 1	19.4 (18.0, 20.9)	17.3 (16.0, 18.6)	16.5 (15.3, 17.8)	0.004	-0.068 (-0.112, -0.024)	0.002
Model 2	18.9 (17.5, 20.4)	17.3 (16.1, 18.7)	16.9 (15.6, 18.2)	0.05	-0.050 (-0.096, -0.003)	0.036
Serum AST, U/L						
Model 1	31.5 (29.0, 34.3)	26.9 (24.7, 29.1)	23.9 (22.0, 25.9)	<0.001	-0.121 (-0.168, -0.073)	<0.001
Model 2	30.2 (27.8, 32.8)	26.9 (24.9, 29.1)	24.8 (22.9, 26.9)	0.001	-0.090 (-0.139, -0.041)	<0.001

Values for outcome variables in circulating linoleic acid tertiles are geometric means and 95% CI. Values for β (95% CI) are natural log-transformed values;

[a] One standard deviation of circulating linoleic acid is 3.5% total fatty acids.

[b] Multivariable models: Model 1: adjusted for age, sex; Model 2: model 1 plus education level, locality, smoking status, physical activity, alcohol intake, total energy intake, BMI.

[c] P-values for trend were obtained by assigning median value of circulating linoleic acid and this value was modelled as continuous.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

DISCUSSION

In this cross-sectional study in 633 black South African men and women, dietary and circulating LA were not significantly associated with plasma glucose or HbA1c. However, both higher circulating and dietary LA were associated with lower levels of GGT. One SD higher circulating LA was associated with a 22% lower serum GGT and participants in the highest tertile of dietary LA had the lowest levels of serum GGT, a marker of liver function that is associated with insulin resistance and hepatic fat accumulation.

Only a few studies have reported on the relation of dietary and circulating LA with markers of glucose metabolism. As LA is the predominant type of dietary PUFA in many populations, including Africans,[30] comparison of the results of the current study was also made with other studies using data on intake of n-6 or PUFA. The findings of no associations between dietary LA and glucose metabolism markers in the present study are in line with findings from previous studies in Caucasians. Dietary PUFA (mainly from plant source) was not associated with fasting glucose or HbA1c in 5,675 Dutch men and women aged 45-65 years without diabetes.[31] Analyses of baseline data of two different trials in healthy middle-aged adults in Finland[32] and adults with metabolic syndrome in Spain[33] also showed no association of PUFA or LA intake with risk of impaired glucose metabolism. Two recent systematic reviews and meta-analyses of randomized controlled trials concluded that dietary n-6 or PUFA had some effect on insulin concentration but little or no effect on fasting glucose,[11,34] also when plant-based PUFA replaced saturated fatty acids intake in populations without diabetes.[11]

As with dietary LA, circulating LA was not associated with glucose metabolism markers in the present study. In contrast, the investigators of the Hoorn study of 667 Dutch Caucasian participants reported a weak inverse association between circulating LA in serum and plasma glucose. However, no association between circulating LA and HbA1c was observed in the Hoorn study,[35] consistent with findings of the present study. In the Hoorn study, about half of the participants had impaired glucose metabolism,[35] while in this current study only 9% had elevated plasma glucose. In normal glucose metabolism plasma glucose is under tight regulation,[36] which may explain null findings in the current study.

For dietary LA, generally either no or weak associations with liver enzymes were observed in the present study. However, circulating LA was inversely associated with serum liver enzymes, most notably serum GGT. Concentrations of liver enzyme GGT have previously been associated with hepatic fat accumulation and insulin

resistance.[37,38] In the Coronary Artery Risk Development in Young Adults study (44% black participants), mean dietary PUFA intake at baseline and year 7 tended also to be inversely related to serum GGT in year 10 of follow-up, although not statistically significant. However, there was no information on whether this association differed in the subgroup of black participants.[39] Results of a 10-week randomized controlled trial in Swedish individuals (15% had diabetes) with abdominal obesity showed that intervention with a diet high in n-6 PUFA (14 en% from LA) resulted in lower liver fat as compared to an isocaloric diet high in saturated fatty acids.[40] In the present study, exclusion of participants with elevated plasma glucose resulted in a somewhat stronger inverse association of dietary LA with serum GGT.

No other study seems to have specifically examined the association of circulating LA and serum GGT, but there is some evidence that circulating LA could be associated with liver fat accumulation.[9,41,42] First, in the European Prospective Investigation into Cancer and Nutrition-Potsdam study, higher delta-6-desaturase activity, resulting in lower circulating LA, was associated with higher serum GGT.[41] The authors suggested that the association of delta-6-desaturase activity with T2D risk might be mediated by liver fat accumulation. Second, higher circulating LA was associated with lower visceral adipose tissue in a study of 287 elderly subjects, and also with lower fat in a subgroup of 73 of these subjects[9]. In another study in 24 overweight men, circulating LA was moderately inversely correlated with visceral fat thickness.[42] A possible mechanism relating LA to liver function is through its influence on *de novo* lipogenesis by attenuating effects of insulin on FA synthase activity and expression.[43] Another possible mechanism may be related to oxidative stress, as elevated serum GGT is a marker of high oxidative stress.[44]

A weak association between dietary and circulating LA in the present study may partly explain the present study finding that dietary LA was weaker associated with GGT than circulating LA. It is possible that alcohol intake plays a role here. Previously, in a cohort of Dutch post-myocardial infarction patients, weak correlations of dietary with circulating LA and lower circulating LA with higher alcohol intakes at similar intakes of LA[45] were reported, which were also observed in the present study. High alcohol intake is an established cause of liver fat accumulation[46] and higher alcohol intake has been associated with lower circulating LA in several cross-sectional studies.[47–49] It is, therefore, possible that the inverse association between circulating LA and GGT in this study may reflect alcohol influence. However, when participants whose liver enzyme values indicated alcohol abuse were excluded, the associations of LA and serum GGT were somewhat

attenuated but remained significant. This suggests that the observed association cannot be entirely explained by alcohol.

The stronger associations between LA and GGT in urban than in rural areas is difficult to explain. A possible explanation might be related to alcohol use in the current study population. The urbanization process in South Africa has been linked to a change in alcohol consumption pattern.[50] A difference in obesity status is not a likely explanation because average BMI and proportion of individuals with obesity in urban and rural communities were similar.

Study strengths and limitations

A novel aspect of the present study is the investigation on the associations of LA with glucose metabolism and liver function in a black South African population, while most other studies investigated white Caucasian populations. The current finding that LA was inversely associated with serum GGT may be relevant because the Atherosclerosis Risk in Communities study[51] showed that GGT is a strong predictor of T2D risk in a black population as compared to ALT or AST.

The present study also contributes to evidence on the relation of dietary fat quality with T2D risk in South Africans. The current recommendation for South Africans is to consume 5-8% energy from n-6 PUFA.[52] Intake in the population seems to be within this range, which was confirmed by the data in the present study. However, most of the evidence underlying the South African dietary guidelines on dietary fat quality is based on studies in Caucasian populations.[52] The present study findings suggest that a higher dietary LA is also associated with better liver function in a black population, which support the current South African recommendation of dietary n-6 PUFA.

An important limitation of the present study is its cross-sectional design, which limits inferences on causal relationships. An additional limitation is that assessment of alcohol intake by QFFQ may not be accurate. The finding that the association of circulating LA with serum GGT was stronger in alcohol consumers than in non-consumers warrants further investigation using a more sophisticated tool to assess alcohol intake. Another limitation of the current study is lack of insulin data. Availability of this data would add more information on insulin resistance in this population.

CONCLUSION

In this population of apparently healthy black South Africans, dietary and circulating LA were weakly correlated. Dietary LA and circulating LA were not significantly related to markers of glucose metabolism. However, dietary LA and in particular circulating LA were inversely related to serum GGT. This suggests that a low LA intake is related to an impaired liver function and may in this way affect long-term insulin resistance, which would explain the inverse relation between circulating LA and T2D risk observed in prospective cohort studies. The role of alcohol in the association between circulating LA and liver function warrants further research.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12944-020-01318-3.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FA, fatty acids; GGT, gamma-glutamyl transferase; HbA1c, glycosylated hemoglobin; HIV, human immunodeficiency virus; LA, linoleic acid; PAI, physical activity index; PUFA, polyunsaturated fatty acids; PURE-NWP-SA, Prospective Urban Rural Epidemiology North-West Province South Africa; QFFQ, quantified food-frequency questionnaire; T2D, type 2 diabetes.

Declarations

Availability of data and materials

The data that support the findings of this study are available from PURE South Africa study investigators but restrictions apply to the availability of these data and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the Health Research Ethics Committee of North-West University.

Acknowledgments

The authors would like to thank all supporting staffs and participants of the PURE study and in particular: 1) PURE-South Africa: Prof. A. Kruger (posthumous), Prof. M. Greeff, Ms. P.G. Molaudzi, Ms. V. Kruger, Ms. J. Brits, Ms. P. Jardim working in the Africa Unit for Transdisciplinary Health Research (AUTHeR), Faculty of Health Sciences, North-West University, Potchefstroom, South Africa, as well as all the field workers over the years; Ms. M. Maritz (Hypertension in Africa Research Team) for her contribution towards finalizing the mortality data. 2) PURE International: Prof. S. Yusuf and the PURE project office staff at the Population Health Research Institute (PHRI), Hamilton Health Sciences and McMaster University, ON, Canada. Any opinion, finding and conclusion or recommendation expressed in this material is that of the authors and the South African National Research Foundation does not accept any liability in this regard.

Funding

This research was supported by the Netherlands Organization for Scientific Research (NWO) through its Graduate Programme on Food Structure, Digestion and Health and Upfield™. Fatty acids measurement was funded by Unilever. PURE South Africa study was supported by South Africa - Netherlands Research Programme on Alternatives in Development (08/15), South African National Research Foundation (NRF GUN numbers 2069139 and FA2006040700010; FA2007042600011), South African Medical Research Council, North-West University, Potchefstroom, South Africa and the Population Health Research Institute, ON, Canada.

Competing interests

This research was supported in part by Upfield. AJW and PLZ are employed by Unilever, the Netherlands. Unilever is a producer of food consumer products. It divested its spreads business, which has operated since July 2018 under the name Upfield. JMG received financial support from Unilever for epidemiological studies of dietary and circulating fatty acids. The funders had no role in the design of the study, collection and analysis of data and decision to publish. No other potential conflicts of interest relevant to this article were reported.

Authors' contributions

HSK, IMK, TVZ and CMS designed the research. KP, LKK, JMG and HSK conducted the research. KP performed the statistical analyses. KP wrote the draft paper. All authors interpreted the results and critically revised the manuscript for intellectual content. CMS had overall responsibility for the research. All authors approved the final manuscript for publication.

Ethics approval and consent to participate

All participants gave written consent to participation and the study was approved by the Ethics Committee of the North-West University.

Consent for publication

Not applicable

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SUPPLEMENTARY MATERIALS

Table S1 Characteristics of patients in the subsample of participants with available liver enzyme values[a,b]

	Values for subsample of 613
Age (y)	53.0 ± 10.4
Men	232 (37.8)
Body mass index (kg/m^2)	24.8 ± 6.9
Obesity	137 (22.4)
Locality (urban)	318 (51.9)
Education level (no school or primary)[a]	238 (39.8)
Smoking status[a]	
Non-smoker	275 (44.9)
Past or current smoker	337 (55.1)
Physical activity[a]	
Low	5 (0.8)
Moderate	256 (43.2)
High	331 (55.9)
Plasma glucose (mmol/L)	4.90 (4.40-5.40)
HbA1c (%)	5.60 (5.30-5.90)
Serum lipids (mmol/L)	
Total cholesterol	5.19 ± 1.31
LDL-C	3.03 ± 1.13
HDL-C	1.59 ± 0.64
Triglycerides	1.13 (0.84-1.67)
Liver enzymes activity (U/L)	
Gamma-glutamyl transferase	45.8 (29.7-88.0)
Aspartate aminotransferase	25.0 (18.8-35.0)
Alanine transaminase	17.0 (12.6-24.4)
HIV-positive	5 (0.8)
Dietary variables	
Daily total energy intake (kJ)	7332 ± 3005
Carbohydrate (en%)	58.7 ± 9.3
Protein (en%)	12.4 ± 2.3
Total fat (en%)	24.2 ± 8.8
Saturated fatty acids (en%)	5.9 ± 3.0
Monounsaturated fatty acids (en%)	6.5 ± 3.3
Polyunsaturated fatty acids (en%)	7.4 ± 3.3
Trans-fatty acids (en%)	0.10 (0.04-0.23)
Dietary cholesterol (mg/d)	148 (81-252)
Total fiber (g/d)	20.3 ± 9.1
Alcohol consumption (g/d)[b]	0 (0-13.2)
No	352 (57.4)
Light	119 (19.4)
Moderate	43 (7.0)
High	99 (16.2)

Values are mean ± SD, median (Q1-Q3), or *n* (%).
[a] Missing values for 15 participants for education level, 1 participant for smoking status, 21 participants for physical activity level and 1 participant for HIV status.
[b] Alcohol consumption categories: 'No': 0 g/d; 'Light': >0 to 10 g/d (women), >0 to 20 g/d men; 'Moderate': >10-20 g/d (women), >20-30 g/d (men); 'High': >20 g/d (women), >30 g/d (men).

Table S2 Baseline characteristics of participants by locality[a,b,c]

	Urban participants (n=325)	Rural participants (n=308)
Age (y)	53.8 ± 10.7	52.1 ± 9.8
Men	136 (41.8)	104 (33.8)
Body mass index (kg/m^2)	25.2 ± 7.1	24.5 ± 6.7
Obesity	74 (22.8)	69 (22.4)
Locality (urban)	325 (100)	0 (0)
Education level[a]		
No school or primary	89 (28.0)	158 (52.7)
Secondary or higher	229 (72.0)	142 (47.3)
Smoking status[a]		
Non-smoker	142 (43.8)	142 (46.1)
Past or current smoker	182 (56.2)	166 (53.9)
Physical activity[a]		
Low	2 (0.6)	3 (1.0)
Moderate	229 (72.0)	36 (12.3)
High	87 (27.4)	254 (86.7)
Plasma glucose (mmol/L)	5.00 (4.30-5.50)	4.70 (4.40-5.20)
HbA1c (%)	5.60 (5.30-5.90)	5.60 (5.30-5.90)
Serum lipids (mmol/L)		
Total cholesterol	5.28 ± 1.33	5.09 ± 1.29
LDL-C	3.04 ± 1.11	3.01 ± 1.16
HDL-C	1.54 (1.18-1.97)	1.39 (1.10-1.82)
Triglycerides	1.16 (0.85-1.78)	1.08 (0.82-1.52)
Liver enzymes activity (U/L)[b]		
Gamma-glutamyl transferase	49.0 (32.4-97.3)	40.7 (27.9-77.0)
Alanine transaminase	17.5 (12.8-25.0)	16.7 (12.1-23.0)
Aspartate aminotransferase	26.0 (20.7-39.0)	23.3 (17.3-32.9)
HIV-positive	1 (0.3)	4 (1.3)
Dietary variables		
Daily total energy intake (kJ)	8172 ± 3117	6496 ± 2684
Available carbohydrate (en%)	54.9 ± 7.2	62.9 ± 9.5
Protein (en%)	13.4 ± 2.1	11.4 ± 2.1
Total fat (en%)	27.8 ± 8.0	20.4 ± 7.8
Saturated fatty acids (en%)	7.2 ± 2.8	4.6 ± 2.6
Monounsaturated fatty acids (en%)	8.0 ± 3.1	4.8 ± 2.6
Polyunsaturated fatty acids (en%)	7.9 ± 2.8	6.8 ± 3.6
Linoleic acid (en%)	7.5 ± 2.8	6.2 ± 3.2
Trans-fatty acids (g/d)	0.43 (0.16-0.72)	0.09 (0.04-0.18)
Dietary cholesterol (mg/d)	212 (136-309)	106 (53-165)
Dietary fiber (g/d)	22.8 ± 10.1	18.0 ± 7.2
Alcohol intake (g/d)[c]	1.9 (0-15.6)	0 (0-6.0)
No	158 (48.6)	204 (66.2)
Light	79 (24.3)	49 (15.9)
Moderate	34 (10.5)	9 (2.9)
High	54 (16.6)	46 (14.9)
Circulating linoleic acid (% total fatty acids)	16.0 ± 3.2	15.9 ± 3.7

Values are mean ± SD, median (Q1-Q3), or *n* (%).

[a] Missing values for 15 participants for education level, 1 participant for smoking status, 22 participants for physical activity level and 1 participant for HIV status.

[b] Only available for n=613.

[c] Alcohol consumption categories: 'No': 0 g/d; 'Light': >0 to 10 g/d (women), >0 to 20 g/d men; 'Moderate': >10-20 g/d (women), >20-30 g/d (men); 'High': >20 g/d (women), >30 g/d (men).

Table S3 Spearman's correlations (r_S) of selected food groups and circulating linoleic acid[a,b]

Dietary components	r_S	*P*
Cereal and cereal products	-0.01	0.86
Vegetables	0.12	0.002
Fruits	0.01	0.73
Meat, meat products and eggs	0.11	0.006
Fish and seafood	-0.03	0.49
Fats and oils	0.14	<0.001
Margarine	0.10	0.011
Vegetable oils	0.10	0.009
Dressings	0.04	0.38
Tallow	0.04	0.32

[a] Spearman correlations adjusted for age, sex and total energy intake.
[b] Food and food group intakes are expressed in grams/day.

Table S4 Results from stratified analyses for associations of circulating LA with markers of liver function[a]

	Serum GGT, U/L			Serum ALT, U/L			Serum AST, U/L		
	β (95% CI) per 1 SD	*P*	*P-int*	β (95% CI) per 1 SD	*P*	*P-int*	β (95% CI) per 1 SD	*P*	*P-int*
Dietary LA									
Sex			0.08			0.53			0.014
Men (n=232)	-0.070 (-0.247, 0.108)	0.44		0.018 (-0.095, 0.130)	0.76		-0.057 (-0.180, 0.066)	0.36	
Women (n=381)	-0.110 (-0.219, -0.0005)	0.049		-0.048 (-0.126, 0.030)	0.23		-0.039 (-0.121, 0.043)	0.35	
Age			0.57			0.11			0.11
<65 y (n=530)	-0.101 (-0.205, 0.003)	0.06		-0.046 (-0.112, 0.020)	0.17		-0.049 (-0.123, 0.025)	0.19	
≥65 y (n=83)	0.162 (-0.069, 0.393)	0.17		0.184 (-0.065, 0.434)	0.14		0.141 (-0.042, 0.325)	0.13	
Locality			0.16			0.15			0.28
Urban (n=318)	0.069 (-0.094, 0.233)	0.40		0.037 (-0.072, 0.146)	0.50		0.012 (-0.090, 0.114)	0.82	
Rural (n=295)	-0.181 (-0.294, -0.068)	0.002		-0.060 (-0.140, 0.020)	0.14		-0.080 (-0.177, 0.018)	0.11	
Obesity			0.58			0.62			0.84
Absent (n=476)	-0.119 (-0.232, -0.006)	0.039		-0.023 (-0.094, 0.049)	0.53		-0.064 (-0.145, 0.017)	0.12	
Present (n=137)	0.082 (-0.077, 0.240)	0.31		-0.012 (-0.169, 0.145)	0.88		0.071 (-0.050, 0.191)	0.25	
Alcohol use			0.12			0.36			0.06
No (n=352)	-0.009 (-0.130, 0.113)	0.89		-0.009 (-0.098, 0.080)	0.84		0.008 (-0.084, 0.100)	0.86	
Yes (n=261)	-0.105 (-0.257, 0.048)	0.18		-0.037 (-0.132, 0.059)	0.45		-0.085 (-0.190, 0.021)	0.11	

Table S4. continued

	Serum GGT, U/L			Serum ALT, U/L			Serum AST, U/L		
	β (95% CI) per 1 SD	*P*	*P-int*	β (95% CI) per 1 SD	*P*	*P-int*	β (95% CI) per 1 SD	*P*	*P-int*
Circulating LA									
Sex			0.08			0.65			0.67
Men (n=232)	-0.37 (-0.49, -0.24)	<0.001		-0.059 (-0.143, 0.024)	0.16		-0.124 (-0.214, -0.034)	0.007	
Women (n=381)	-0.18 (-0.26, -0.11)	<0.001		-0.045 (-0.100, 0.010)	0.11		-0.073 (-0.131, -0.015)	0.014	
Age			0.94			0.79			0.77
<65 y (n=530)	-0.24 (-0.31, -0.16)	<0.001		-0.049 (-0.097, -0.002)	0.042		-0.084 (-0.136, -0.031)	0.002	
≥65 y (n=83)	-0.27 (-0.43, -0.12)	<0.001		-0.045 (-0.230, 0.140)	0.63		-0.154 (-0.284, -0.024)	0.021	
Locality			<0.001			0.006			0.001
Urban (n=318)	-0.44 (-0.54, -0.33)	<0.001		-0.132 (-0.208, -0.056)	<0.001		-0.186 (-0.256, -0.117)	<0.001	
Rural (n=295)	-0.08 (-0.17, 0.002)	0.05		0.014 (-0.045, 0.072)	0.64		-0.009 (-0.080, 0.062)	0.81	
Obesity			0.05			0.29			0.025
Absent (n=476)	-0.28 (-0.36, -0.21)	<0.001		-0.067 (-0.119, -0.015)	0.011		-0.118 (-0.176, -0.060)	<0.001	
Present (n=137)	-0.08 (-0.18, 0.03)	0.17		0.040 (-0.068, 0.148)	0.47		0.044 (-0.040, 0.128)	0.30	
Alcohol use			<0.001			0.38			0.14
No (n=352)	-0.16 (-0.24, -0.09)	<0.001		-0.046 (-0.101, 0.009)	0.10		-0.072 (-0.129, -0.015)	0.013	
Yes (n=261)	-0.41 (-0.54, -0.29)	<0.001		-0.081 (-0.164, 0.003)	0.06		-0.142 (-0.233, -0.051)	0.002	

Values for β (95% CI) are natural log-transformed values.

[a] For dietary LA, results were from model 3 and for circulating LA, results were from model 2 (Subjects and Methods).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; LA, linoleic acid; *P*-int, *P*-value for interaction.

Table S5 Sensitivity analyses for the cross-sectional association of dietary and circulating LA with outcomes, including only participants without high alcohol intake (n=533)[a,b]

	T1	T2	T3	*P*-trend	β (95% CI) per 1 SD	*P*
Dietary LA	*N=148*	*N=184*	*N=201*			
Plasma glucose, mmol/L	4.91 (4.70, 5.14)	4.84 (4.68, 5.01)	4.87 (4.69, 5.06)	0.85	0.012 (-0.019, 0.042)	0.46
HbA1c, %	5.64 (5.50, 5.79)	5.70 (5.59, 5.80)	5.70 (5.58, 5.82)	0.60	0.008 (-0.010, 0.025)	0.39
Serum GGT, U/L	56.4 (48.3, 65.8)	49.8 (44.5, 55.7)	43.4 (38.2, 49.4)	0.025	-0.056 (-0.161, 0.048)	0.29
Serum ALT, U/L	18.0 (16.2, 20.1)	16.7 (15.4, 18.0)	16.2 (14.8, 17.7)	0.20	0.005 (-0.068, 0.077)	0.90
Serum AST, U/L	28.3 (25.3, 31.7)	25.2 (23.2, 27.3)	24.2 (22.1, 26.6)	0.07	-0.010 (-0.085, 0.066)	0.80
Circulating LA	*N=152*	*N=180*	*N=201*			
Plasma glucose, mmol/L	4.91 (4.73, 5.09)	4.88 (4.72, 5.05)	4.84 (4.69, 5.00)	0.61	-0.007 (-0.027, 0.013)	0.49
HbA1c, %	5.66 (5.54, 5.77)	5.63 (5.53, 5.74)	5.75 (5.65, 5.85)	0.22	0.007 (-0.005, 0.018)	0.26
Serum GGT, U/L	66.9 (59.2, 75.7)	49.7 (44.5, 55.4)	38.1 (34.3, 42.3)	<0.001	-0.22 (-0.28, -0.15)	<0.001
Serum ALT, U/L	18.3 (16.8, 20.0)	16.5 (15.2, 17.9)	16.1 (14.9, 17.4)	0.041	-0.053 (-0.101, -0.004)	0.033
Serum AST, U/L	28.8 (26.3, 31.6)	25.5 (23.5, 27.6)	23.6 (21.8, 25.6)	0.002	-0.087 (-0.137, -0.037)	<0.001

Values for outcome variables in tertiles of dietary or circulating LA are geometric means and 95% CI. Values for β (95% CI) are natural log-transformed values.
[a] For dietary LA, results were from model 3 and for circulating LA, results were from model 2 (Subjects and Methods).
[b] High alcohol intake defined as drinking alcohol >20 g/d for women and >30 g/d for men (n=100). For liver enzymes outcomes, results were from a subset of 514 participants (Dietary LA T1=140, T2=180, T3=194; Circulating LA T1=144, T2=176, T3=194).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; LA, linoleic acid.

Table S6 Sensitivity analyses for the cross-sectional association of dietary and circulating LA with outcomes, including only participants without indication of alcohol abuse (n=540)[a,b]

	T1	T2	T3	*P*-trend	β (95% CI) per 1 SD	*P*
Dietary LA	*N=166*	*N=186*	*N=188*			
Plasma glucose, mmol/L	4.99 (4.78, 5.20)	4.85 (4.69, 5.02)	4.96 (4.77, 5.16)	0.93	0.008 (-0.021, 0.038)	0.59
HbA1c, %	5.68 (5.54, 5.81)	5.68 (5.57, 5.78)	5.70 (5.58, 5.83)	0.82	0.007 (-0.010, 0.023)	0.43
Serum GGT, U/L	47.4 (41.7, 54.0)	49.3 (44.6, 54.6)	44.9 (39.8, 50.5)	0.54	0.006 (-0.083, 0.095)	0.90
Serum ALT, U/L	17.6 (15.9, 19.4)	17.2 (15.9, 18.6)	16.5 (15.1, 18.1)	0.43	0.013 (-0.055, 0.081)	0.70
Serum AST, U/L	24.7 (22.4, 27.1)	24.9 (23.1, 26.8)	24.1 (22.1, 26.3)	0.74	0.034 (-0.032, 0.099)	0.32
Circulating LA	*N=160*	*N=183*	*N=197*			
Plasma glucose, mmol/L	4.97 (4.79, 5.16)	4.95 (4.79, 5.12)	4.88 (4.72, 5.04)	0.45	-0.008 (-0.029, 0.013)	0.44
HbA1c, %	5.67 (5.55, 5.79)	5.66 (5.55, 5.76)	5.73 (5.62, 5.83)	0.46	0.005 (-0.006, 0.017)	0.38
Serum GGT, U/L	60.2 (54.0, 67.1)	46.9 (42.5, 51.8)	38.9 (35.3, 42.9)	<0.001	-0.18 (-0.24, -0.11)	<0.001
Serum ALT, U/L	18.1 (16.6, 19.7)	16.7 (15.5, 18.1)	16.7 (15.4, 18.0)	0.19	-0.031 (-0.079, 0.017)	0.21
Serum AST, U/L	25.8 (23.8, 28.1)	24.4 (22.7, 26.3)	23.6 (22.0, 25.4)	0.12	-0.045 (-0.091, 0.001)	0.06

Values for outcome variables in tertiles of dietary or circulating LA are geometric means and 95% CI. Values for β (95% CI) are natural log-transformed values.
[a] For dietary LA, results were from model 3 and for circulating LA, results were from model 2 (Subjects and Methods).
[b] Indication of alcohol abuse by liver enzyme values: serum GGT concentration twice higher than normal range (values >80 U/L), and AST/ALT ratio of at least 2:1 (n =93).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; LA, linoleic acid.

Table S7 Sensitivity analyses for the cross-sectional association of dietary and circulating LA with outcomes, including only participants with normal plasma glucose (n=577)[a,b]

	T1	T2	T3	*P*-trend	β (95% CI) per 1 SD	*P*
Dietary LA	*N=194*	*N=189*	*N=194*			
Plasma glucose, mmol/L	4.73 (4.61, 4.85)	4.56 (4.46, 4.66)	4.69 (4.57, 4.81)	0.76	0.005 (-0.013, 0.023)	0.60
HbA1c, %	5.54 (5.46, 5.62)	5.52 (5.46, 5.59)	5.55 (5.47, 5.63)	0.85	0.005 (-0.005, 0.015)	0.29
Serum GGT, U/L	62.2 (54.0, 71.7)	56.9 (50.6, 64.1)	47.2 (41.2, 54.1)	0.014	-0.105 (-0.204, -0.006)	0.038
Serum ALT, U/L	19.0 (17.2, 20.9)	17.0 (15.7, 18.4)	16.1 (14.7, 17.7)	0.036	-0.035 (-0.102, 0.032)	0.31
Serum AST, U/L	30.3 (27.4, 33.5)	26.9 (24.7, 29.2)	24.7 (22.4, 27.2)	0.012	-0.052 (-0.122, 0.019)	0.15
Circulating LA	*N=190*	*N=191*	*N=196*			
Plasma glucose, mmol/L	4.71 (4.61, 4.81)	4.64 (4.54, 4.74)	4.63 (4.53, 4.73)	0.32	-0.005 (-0.018, 0.008)	0.44
HbA1c, %	5.52 (5.45, 5.58)	5.51 (5.45, 5.58)	5.58 (5.52, 5.65)	0.16	0.005 (-0.002, 0.012)	0.13
Serum GGT, U/L	75.8 (67.3, 85.3)	54.0 (48.2, 60.4)	41.1 (36.7, 46.1)	<0.001	-0.24 (-0.31, -0.17)	<0.001
Serum ALT, U/L	18.3 (16.8, 19.9)	17.0 (15.7, 18.4)	16.6 (15.4, 18.0)	0.12	-0.040 (-0.088, 0.009)	0.11
Serum AST, U/L	30.0 (27.5, 32.7)	26.9 (24.7, 29.2)	25.0 (23.0, 27.1)	0.004	-0.090 (-0.140, -0.040)	<0.001

Values for outcome variables in tertiles of dietary or circulating LA are geometric means and 95% confidence intervals. Values for β (SE) are natural log-transformed values.
[a] For dietary LA, results were from model 3 and for circulating LA, results were from model 2 (Subjects and Methods).
[b] Normal plasma glucose was defined as fasting plasma glucose <6.1 mmol/L (n=56). For liver enzymes outcomes, results were from a subset of 558 participants (Dietary LA T1=186, T2=184, T3=188; Circulating LA T1=182, T2=187, T3=189).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; LA, linoleic acid.

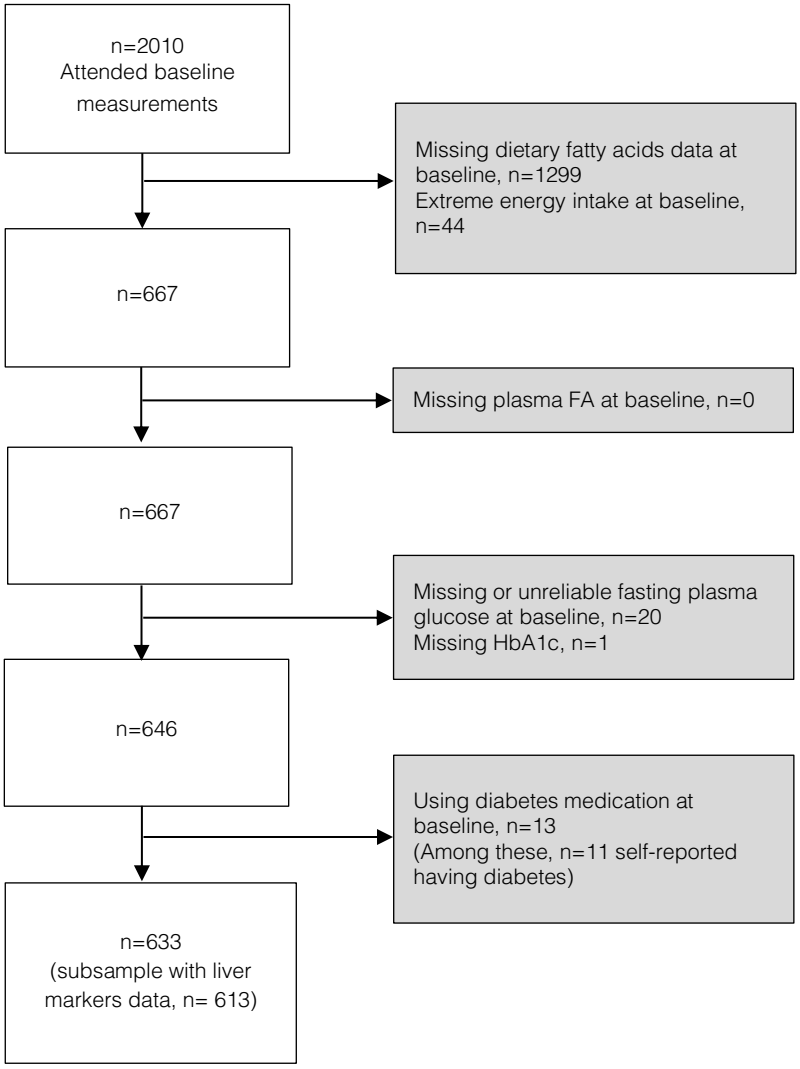

Figure S1 Flow chart of selection of participants

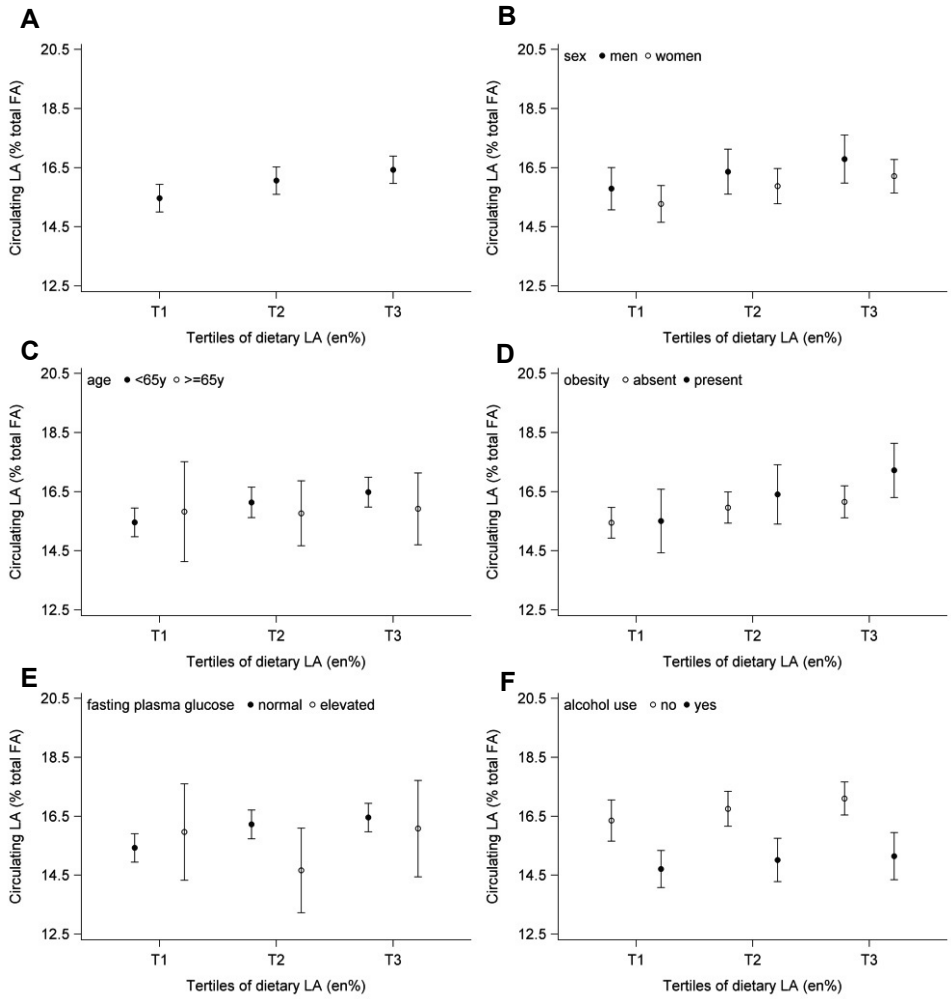

Figure S2 Circulating LA across tertiles of LA intake in total sample (A) and in subgroups of sex (B), age (C), obesity (D), fasting plasma glucose (E) and alcohol use (F)[a,b,c,d]

[a] Values are least-squares means with 95% confidence interval, adjusted for age, sex, total energy intake;
[b] Obesity was defined as 'present' when BMI ≥30.0 kg/m^2 and 'absent' when BMI <30 kg/m^2;
[c] Alcohol use: 'no' included participants with alcohol intake of 0 g/d, 'yes' included alcohol intake >0 g/d;
[d] Fasting plasma glucose categories: 'elevated' was defined as fasting plasma glucose ≥6.1 mmol/L, 'normal' was defined as plasma glucose <6.1 mmol/L;
LA, linoleic acid.

Chapter 6

Dietary and circulating long-chain omega-3 polyunsaturated fatty acids and mortality risk after myocardial infarction: a long-term follow up of the Alpha Omega Cohort

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In preparation

ABSTRACT

Background: Habitual intake of long-chain omega-3 fatty acids (n-3 FAs), in particular eicosapentaenoic and docosahexaenoic acid (EPA+DHA) from fish, has been associated with a reduced risk of fatal coronary heart disease (CHD) in population-based studies. Whether that is also the case for CHD patients is not yet clear. We studied the associations of dietary and circulating EPA+DHA and alpha-linolenic acid (ALA), a plant-derived n-3 FA, with long-term mortality risk after myocardial infarction (MI).

Methods and Results: We analyzed data from 4067 Dutch post-MI patients aged 60-80 y (79% men, 86% on statins) enrolled in the Alpha Omega Cohort from 2002-2006 (baseline) and followed through 2018. Baseline intake of fish and n-3 FAs were assessed through a validated 203-item food-frequency questionnaire, as well as circulating n-3 FAs in plasma cholesteryl esters. Hazard ratios (HRs) with 95% confidence intervals (CIs) were obtained from Cox regression analyses. During a median follow-up period of 12 y, 1877 deaths occurred, of which 515 from CHD and 834 from CVD. Dietary intake of EPA+DHA was significantly inversely associated with CHD mortality (HR of 0.69 (0.52-0.90) for >200 vs. ≤50 mg/d; HR of 0.92 (0.86-0.98) per 100 mg/d), while weak non-significant inverse associations were found for CVD and all-cause mortality. Similar results were obtained for fish intake (HR_{CHD} of 0.74 (0.53-1.03) for >40 vs. ≤5 g/d; P_{trend}: 0.031). Circulating EPA+DHA was inversely associated with CHD mortality (HR of 0.71 (0.53-0.94)) for >2.52 vs. ≤1.29% of total FAs; 0.85 (0.77-0.95) per 1-SD), with significant risk reductions also for CVD and all-cause mortality. Dietary and circulating ALA were not significantly associated with mortality endpoints.

Conclusions: In a cohort of Dutch post-MI patients with long-term follow-up, higher dietary and circulating EPA+DHA from fish were consistently associated with a lower risk of mortality, especially from CHD.

INTRODUCTION

Increasing the intake of long-chain omega-3 fatty acids (n-3 FAs) is often recommended for the prevention of cardiovascular disease (CVD).[1,2] Beneficial effects have mainly been attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are n-3 FAs from oily fish and other seafood.[3] Alpha-linolenic acid (ALA) is the parent n-3 FA derived from plant foods, such as flaxseed, nuts, and vegetable oils. EPA (and DHA) may be formed from ALA, but conversion in the human body is probably less than 10%, as indicated by stable-isotope studies.[4,5]

In the Alpha Omega Trial of 4837 Dutch post-myocardial infarction (MI) patients, we reported that dietary doses of 400 mg/d EPA+DHA or 2 g/d ALA for 40 months did not lower the risk of major CVD events as compared to placebo.[6] Findings were also negative for fatal events including CHD, although the study was underpowered for these endpoints.[6] A recent Cochrane meta-analysis showed a non-significant 10% lower risk of fatal CHD in CVD patients, and smaller non-significant effects on fatal CVD and all-cause mortality, for EPA+DHA supplementation (doses >0.5 to 3 g/d).[7] Effects of ALA supplementation in ≤3 secondary prevention studies, including the Alpha Omega Trial, showed non-significant risk reductions of 5% or less for fatal CHD, fatal CVD or all-cause mortality.[7]

Whether habitual dietary intake of n-3 FAs could improve long-term survival in CHD patients is not yet clear. Dietary intake of EPA+DHA in Western populations is generally below 500 mg/d,[8] and ALA intake is only ~1.5 g/d (<1 energy percent). Concentrations of n-3 FAs in blood may be considered as biomarkers of essential FA intake, especially for EPA+DHA.[1] We examined associations of dietary intakes of EPA+DHA, fish and ALA, as well as circulating levels of n-3 FAs, with fatal CHD (primary outcome), CVD and all-cause mortality during >12 y follow-up in our Alpha Omega Cohort of post-MI patients.

METHODS

Study population

The Alpha Omega Cohort comprises 4837 Dutch men and women aged 60-80 y with a clinically diagnosed MI up to 10 y before entering the study. Patients were examined by trained research nurses at baseline (2002-2006) and have been followed for cause-specific mortality since study enrolment. During the initial 40

months of follow-up, patients received low-dose supplementation of ALA, EPA+DHA, ALA plus EPA+DHA or placebo (Alpha Omega Trial),[9] which did not prevent major recurrent CVD events.[6] Medical-ethical approval was obtained and all patients provided written informed consent.[9] Patients with missing data on circulating n-3 FAs or >5% unknown FAs were excluded (**Figure S1**). Patients with missing dietary data or implausible energy intakes (<800 or >8000 kcal for men; <600 or >6000 kcal for women) and extreme unsaturated FA intakes (<2.5th or >97.5th percentile) were further excluded, as described previously,[10] leaving 4067 patients for analysis.

Dietary assessment

Diet was assessed at baseline using a 203-item semi-quantitative food-frequency questionnaire (FFQ). This was an extended version of a biomarker-validated and reproducible FFQ, which emphasized FAs measurement.[11,12] Food intake, including preparation methods, was assessed over the past month. For foods containing FAs, patients indicated consumed brands on a list. Returned FFQs were checked by trained dietitians who obtained information on missing or unclear answers from patients by telephone, using a predefined protocol. Intakes of total energy and nutrients, including EPA, DHA and ALA, were calculated through linkage with the Dutch Food Composition Table 2006 (NEVO 2006).[13] Correlations between dietary and circulating FAs (in plasma cholesteryl esters) were 0.39 and 0.45 for EPA and DHA, respectively, and -0.02 for ALA.[14] The 2015 Dutch Healthy Diet score was calculated for adherence to dietary guidelines (DHD-15; scale from zero to maximal adherence [0-150]).[15]

Laboratory measurements

Blood samples (fasted ≥8h for 34% of the patients) were collected by trained research nurses to measure serum total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides and plasma glucose using standard laboratory methods.[9] Blood for FAs analysis was collected in EDTA-treated tubes, and stored in -80°C until further analysis. Circulating FAs were measured as described previously.[14] Briefly, plasma cholesteryl esters FAs were separated from total lipids, transesterified into FA methyl esters and measured by gas chromatography equipped with a flame-ionization detector. A total of 38 FAs were quantified and expressed as weight percentage of total FAs (% total FA). For

ALA and EPA, within- and between-run coefficients of variation were <5% and for DHA <8%.[14]

Endpoints

The study focusses on CHD mortality as the primary outcome, and CVD mortality and all-cause mortality as secondary outcomes. The vital status of the patients was monitored through linkage with municipal registries from baseline through 31 December 2018. Follow-up for cause-specific mortality occurred in three phases. From 2002-2009 (Alpha Omega Trial),[6,9] information was obtained from the national mortality registry (Statistics Netherlands [CBS]), treating physicians and close family members. Primary and contributing causes of death were coded by an independent Endpoint Adjudication Committee (EAC), as described previously.[6,9] From 2010-2012, primary and contributing causes of death data were obtained from CBS. From 2013 onwards, CBS provided data on the primary cause of death only and treating physicians were asked to fill out an additional cause-of-death questionnaire (response rate: 67%), which was coded by study physicians who were not involved in the present analysis. Mortality coding was performed according to *International Classification of Diseases*, tenth revision (ICD-10). CHD mortality comprised ICD-10 codes I20-I25 (ischemic heart disease), I46 (cardiac arrest) and R96 (sudden death, undefined). CVD mortality comprised I20-I25, I46, I50 (heart failure), R96, and I60-I69 (stroke). Person-years were calculated from study enrolment to date of death or 31 December 2018, whichever came first. One patient was lost to follow-up and censored after 2.9 y.

Assessment of covariables

Information on demographic, lifestyle, medical history and medication use was collected using questionnaires, as described elsewhere.[9] The highest attained educational level was categorized as primary, lower secondary, higher secondary or lower tertiary, and higher tertiary education. Smoking status was categorized as never, former or current smoker. Physical activity was classified as low (no or only light activity, ≤3 metabolic equivalents (METs)), moderate (>0 to <5 days/week of moderate or vigorous activity, >3 METs) or high (≥5 days/week of moderate or vigorous activity). Medication use was coded according to Anatomical Therapeutic Chemical classification system,[16] with codes C10AA and C10B for statins, B01 for anti-thrombotic drugs and C02, C03, C07, C08 and C09 for antihypertensive drugs. Physical examination was carried out by trained research nurses, in the hospital or

the patient's home.[9] Body weight (kg) and height (m) were assessed, from which BMI was calculated (kg/m^2). Obesity was defined as BMI ≥30 kg/m^2. Systolic and diastolic blood pressures were measured twice in sitting position with an automated device, and values were averaged. Diabetes was considered present if patients reported a diagnosis of diabetes by a physician or the use of antidiabetic drugs, or when they had a fasting plasma glucose ≥7.0 mmol/L or non-fasting level of ≥11.1 mmol/L. Family history of MI was defined as having any parent with MI before the age of 60 y and family history of diabetes was defined as having any parent with diabetes. Alcohol intake (ethanol in g/d) was calculated from the FFQ and categorized into no (0 g/d), low (>0-10 g/d), moderate (>10-30 g/d for men or >10-20 g/d for women) or high (>30 g/d for men or >20 g/d for women).

Statistical analysis

Baseline characteristics of patients are presented as mean (±standard deviation (SD)) or median and interquartile range for continuous variables or percentages for categorical variables. Intakes of FAs were energy-adjusted using the residual method by Willett et al.[17] or expressed as percentage of total energy intake (en%), where appropriate. Dietary EPA+DHA intake was categorized as ≤50 mg/d, >50 to ≤100 mg/d, >100 to ≤200 mg/d and >200 mg/d. Energy-adjusted fish intake was categorized as ≤5 g/d, >5-20 g/d, >20-40 g/d, and >40 g/d). Dietary ALA was categorized into intakes ≤0.5 g/d, >0.5 to ≤1.0 g/d, >1.5 to ≤2.0 g/d and >2.0g/d. Circulating EPA+DHA and ALA were analyzed in quintiles. For circulating EPA+DHA, additional categories were made based on the cut-offs from the Omega-3 index.[18] Because Omega-3 index is based on erythrocytes, converted cut-off values by Stark et al.[19] were used for plasma cholesteryl esters. Linear trends were assessed by entering median values within categories of n-3 FAs as continuous variables into the models. Continuous associations were estimated per 100 mg/d for dietary EPA+DHA or 1 g/d for dietary ALA, and for circulating EPA+DHA and ALA per 1-SD increment.

Age- and sex-adjusted hazard ratios (HRs) with 95% confidence intervals (CIs) for fatal endpoints were obtained from Cox models, in categories of dietary n-3 FAs, circulating n-3 FAs and fish intake, using the lowest categories as the reference. Proportional hazards assumptions were met, based on Schoenfeld residuals. In multivariable Cox models, HRs for dietary intake of n-3 FAs were adjusted for age, sex, education level (4 categories), physical activity (3 categories), smoking status (3 categories), alcohol intake (4 categories), obesity (no/yes), prevalent diabetes (no/yes), cardiovascular drug use (statins, antihypertensive drugs, antithrombotic

drugs, as separate dummies), time since last MI (y), and intake of total energy intake (kcal/d), cholesterol (mg/d), fiber (g/d) and *trans* FAs (g/d).

Multivariable Cox models for circulating n-3 FAs included age, sex, educational level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drug use, time since MI, and serum total cholesterol (mmol/L). In addition, circulating linoleic acid (LA, 18:2n-6; % total FAs) and arachidonic acid (AA, 20:4n-6; % total FAs) were added to the multivariable model because they impact FA composition of plasma cholesteryl esters.[20,21] Circulating EPA and DHA were also analyzed separately per 1-SD.

Covariates with missing values (<5%) were imputed by the sex-specific median (continuous variables) or mode (categorical variables) to retain patients in multivariable models. Potential non-linear associations were explored using restricted cubic spline analysis with 5 knots located at 5th, 27.5th, 50th, 72.5th, and 95th percentiles.[22,23]

Stratified analyses were performed for dietary and circulating n-3 FAs in relation to fatal CHD, fatal CVD, and all-cause mortality, in subgroups of age (<65 vs. ≥65 y), sex, prevalent diabetes (no vs. yes), obesity (no vs. yes), statin use (no vs. yes), diet quality (<median vs. ≥median), time since last MI (<1 vs. ≥1 y), and supplemental intake of EPA+DHA or ALA during the Alpha Omega Trial phase (initial 40 months of follow-up). Analyses for circulating n-3 FAs were also repeated in strata of circulating LA and AA (<median vs. ≥median). *P*-values for interaction were obtained by entering product terms for n-3 FAs and stratification variables into the multivariable models. In sensitivity analyses for dietary and circulating n-3 FAs, we successively excluded 146 deaths during the first 2 y of follow up (subtracting also 2 y of follow-up time for the remaining cohort) and 187 patients who reported the use of fish oil supplements.

SAS software version 9.4 (SAS Institute Inc., Cary, NC) was used for all analyses. A two-sided *P*-value <0.05 was considered statistically significant.

RESULTS

At baseline, patients were on average 69 y old, 79% were men, 17% were current smokers, and 20% had diabetes (**Table 1**). Patients had an MI ~4 y before study enrolment and most patients were treated with cardiovascular medication, such as statins (86%). During a median follow-up period of 12.4 y (total of 45,229 person-years), 1877 patients died, which included 515 CHD deaths (11.4 per 1000 person-years) and 834 CVD deaths (18.4 per 1000 person-years). Patients had a median fish intake of 14 g/d, which included 5 g/d of oily fish. Median EPA+DHA intake was 108 (IQR: 46-187) mg/d, with ~80% having intakes ≤200 mg/d. Mean ALA intake was 1.09 (SD: 0.50) g/d, or 0.5% of energy (en%). EPA+DHA were mainly from fish (89%), while ALA was mainly obtained from cooking oils (26%), grain products (19%) and margarine (14%) (**Table S1**). The median DHD-15 score for adherence to dietary guidelines was 79 (range: 33-125).

Dietary n-3 FAs and fish intake

Patients with higher dietary EPA+DHA intake were more likely to be physically active, had higher alcohol intake, higher HDL cholesterol and slightly lower non-fasting serum triglycerides (**Table S2**). No such associations were observed across quintiles of ALA intake (**Table S3**). Patients with higher EPA+DHA and ALA intakes had higher DHD-15 scores. Patients with EPA+DHA intakes >200 mg/d had a significantly lower risk of CHD mortality (HR: 0.69; 95% CI: 0.52-0.90) than those with intakes ≤50 mg/d (**Table 2**). **Figure 1** shows the continuous association for dietary EPA+DHA with CHD mortality (HR: 0.92; 0.86-0.98; $P_{\text{non-linearity}}$=0.75). Inverse, borderline significant trends were found for CVD mortality (HR per 100 mg/d: 0.96; 0.92-1.01) and all-cause mortality (HR per 100 mg/d: 0.97; 0.94-1.00). Total fish intake was significantly inversely associated with CHD mortality (HR of 0.73 for >20 vs. ≤5 g/d; *P*-trend=0.031) in multivariable analysis, but not with CVD or all-cause mortality (Table 2). Findings were similar for oily fish (HR of 0.72, 95% CI 0.54-0.95, for >11 vs <1 g/d; data not in table). Dietary ALA intake was not associated with CHD mortality (**Figure 2**) or CVD or all-cause mortality (**Figure S2**).

Table 1. Baseline characteristics of population for analysis and across circulating EPA+DHA quintiles

	Total population (n=4067)	Quintiles of circulating EPA+DHA			*P**
		Q1 (*n*=818)	Q3 (*n*=813)	Q5 (*n*=815)	
Age, y	69.0±5.6	69.3±5.6	68.9±5.6	69.1±5.6	0.93
Men	3221 (79.2)	687 (84.0)	612 (75.3)	627 (76.9)	<0.001
Body mass index, kg/m^2†	27.7±3.8	27.3±3.6	27.9±4.0	27.9±3.9	0.014
Obese (≥30 kg/m^2)	953 (23.4)	169 (20.7)	204 (25.1)	196 (24.1)	0.21
Time since MI, y†	3.7 (1.7-6.3)	4.2 (1.9-6.6)	3.3 (1.4-5.8)	3.6 (1.5-6.2)	0.13
Smoking status†					0.22
Never	663 (16.3)	116 (14.2)	145 (17.8)	150 (18.4)	
Former	2730 (67.1)	559 (68.3)	539 (66.3)	534 (65.5)	
Current	673 (16.6)	143 (17.5)	129 (15.9)	131 (16.1)	
Physical activity†					<0.001
Low	1652 (40.8)	359 (44.2)	346 (42.7)	302 (37.3)	
Middle	1528 (37.8)	272 (33.5)	295 (36.4)	335 (41.4)	
High	865 (21.4)	182 (22.4)	170 (21.0)	173 (21.4)	
Highest level of education†					0.002
Primary	803 (19.8)	183 (22.5)	159 (19.7)	139 (17.1)	
Lower secondary	1462 (36.1)	310 (38.1)	297 (36.8)	296 (36.5)	
Higher secondary or lower tertiary	1275 (31.5)	242 (29.8)	257 (31.8)	262 (32.3)	
Higher tertiary	506 (12.5)	78 (9.6)	95 (11.8)	114 (14.1)	
Alcohol intake					<0.001
No	203 (5.0)	50 (6.1)	42 (5.2)	46 (5.6)	
Low	2155 (53.0)	489 (59.8)	417 (51.3)	385 (47.2)	
Moderate	1067 (26.2)	195 (23.8)	242 (29.8)	216 (26.5)	
High	642 (15.8)	84 (10.3)	112 (13.8)	168 (20.6)	
Medication use					
Statins	3494 (85.9)	618 (75.6)	737 (90.7)	728 (89.3)	<0.001
Antithrombotic drugs	3978 (97.8)	795 (97.2)	801 (98.5)	790 (96.9)	0.08
Antihypertensive drugs	3650 (89.8)	719 (87.9)	729 (89.7)	727 (89.2)	0.19
Serum lipids, mmol/L‡,§					
Total cholesterol	4.71±0.95	4.72±0.98	4.63±0.87	4.75±0.96	0.21
LDL cholesterol	2.57±0.81	2.61±0.85	2.49±0.74	2.62±0.84	0.21
HDL cholesterol	1.29±0.34	1.24±0.33	1.27±0.34	1.35±0.35	<0.001
Triglycerides	1.65 (1.21-2.31)	1.68 (1.19-2.37)	1.68 (1.21-2.40)	1.51 (1.18-2.10)	<0.001
Plasma glucose, mmol/L‡	5.61 (5.05-6.59)	5.57 (4.98-6.48)	5.72 (5.08-6.85)	5.62 (5.10-6.45)	0.64

Table 1. continued

	Total population (n=4067)	Quintiles of circulating EPA+DHA			*P*[*]
		Q1 (*n*=818)	Q3 (*n*=813)	Q5 (*n*=815)	
Blood pressure (mmHg)[†]					
Systolic	142±22	142±22	143±21	142±22	0.67
Diastolic	80±11	81±11	80±11	80±11	0.41
Prevalent diabetes	813 (20.0)	154 (18.8)	186 (22.9)	155 (19.0)	0.08
Family history of MI	467 (11.5)	95 (11.6)	84 (10.3)	104 (12.8)	0.55
Family history of diabetes	834 (20.5)	176 (21.5)	180 (22.1)	159 (19.5)	0.07
Dietary factors					
Energy, kcal/d	1921±518	1957±517	1865±496	1761 ± 485	<0.001
Protein, en%	15.0±2.8	14.6±2.9	15.0±2.8	16.4 ± 2.9	<0.001
Total fat, en%	33.8±6.2	34.7±6.1	33.9±6.2	34.6 ± 6.2	<0.001
Saturated FAs, en%	12.5±3.1	12.9±3.0	12.6±3.1	12.6 ± 3.1	<0.001
cis monounsaturated FAs, en%	9.5±2.2	9.6±2.3	9.4±2.2	10.0 ± 2.3	0.20
Polyunsaturated FAs, en%	7.2±2.2	7.5±2.3	7.3±2.3	7.4 ± 2.3	<0.001
Total n-3 FAs, en%	0.71±0.25	0.69±0.26	0.71±0.25	0.75±0.26	<0.001
ALA, g/d	1.09±0.50	1.10±0.51	1.13±0.52	1.06±0.46	0.019
EPA+DHA, mg/day	108 (46-187)	50 (21-104)	104 (52-169)	189 (114-357)	<0.001
Total n-6 FAs, en%	5.5±2.1	6.0 ± 2.2	5.7±2.2	5.2±2.0	<0.001
trans FAs, g/d[‖]	1.6±0.6	1.6±0.6	1.6±0.6	1.4±0.6	<0.001
Carbohydrates, en%	46.7±6.8	47.2±6.8	46.6±6.7	46.3±6.8	0.002
Fiber, g/d[‖]	21.5±6.8	22.2±7.1	21.2±6.4	21.3±6.6	0.026
Cholesterol, mg/d[‖]	184±69	178±68	183±64	187±73	0.016
Total fish (g/d)	14 (5-20)	7 (1-15)	13 (6-19)	18 (13-40)	<0.001
Oily fish (g/d)	5 (1-11)	1 (0-5)	5 (2-10)	11 (6-22)	<0.001
Diet quality score (DHD-15)	79.2±13.6	78.0±13.6	78.2±13.0	81.9±14.1	<0.001
Circulating FAs, % total FAs					
Saturated FAs	13.1±1.1	12.7±1.3	13.2±1.0	13.5±1.0	<0.001
Monounsaturated FAs	22.5±3.2	21.2±3.0	23.0±3.2	23.0±3.2	<0.001
Polyunsaturated FAs	63.0±4.0	64.8±3.9	62.4±4.0	62.1±3.9	<0.001
Total n-3 FAs	2.35 (1.94-2.98)	1.65 (1.51-1.82)	2.32 (2.19-2.46)	3.80 (3.41-4.45)	<0.001
ALA, 18:3n-3	0.51±0.14	0.48±0.15	0.51±0.14	0.52±0.15	<0.001
EPA, 20:5n-3	1.06 (0.79-1.52)	0.61 (0.51-0.70)	1.05 (0.97-1.16)	2.21 (1.88-2.73)	<0.001
DHA, 22:6n-3	0.66 (0.53-0.84)	0.47 (0.40-0.55)	0.67 (0.58-0.75)	0.96 (0.87-1.10)	<0.001
Total n-6 FAs	60.2±4.4	63.0±4.0	59.9±4.0	57.8±4.2	<0.001
Linoleic acid, 18:2n-6	50.0±5.0	53.5±4.5	49.1±4.6	47.8±4.6	<0.001

	Total population (n=4067)	Quintiles of circulating EPA+DHA			*P*[*]
		Q1 (*n*=818)	Q3 (*n*=813)	Q5 (*n*=815)	
Arachidonic acid, 20:4n-6	8.4±2.0	7.8±2.1	8.8±2.0	8.2±1.9	0.07

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DHD-15, 2015 Dutch Healthy Diet score; EPA, eicosapentaenoic acid; FAs, fatty acids; MI, myocardial infarction; Q, quintile. Values are shown as mean ±SD, median (IQR) or *n* (%) unless stated otherwise.

[*] *P* value for linear trend, through median values across categories of intake using a linear regression model, or obtained from chi-square test for categorical variables.

[†] <1% of patients had missing values for body mass index, time since MI, smoking status, physical activity, educational level and blood pressure.

[‡] part of the cohort had missing values for total cholesterol, HDL cholesterol and triglycerides (n=61), LDL cholesterol (n=252) and plasma glucose (n=33).

[§] to convert to mg/dL, divide by 0.02586 for total, LDL, HDL cholesterol and by 0.01129 for triglycerides

[‖] values for dietary TFAs, fiber and cholesterol were non-energy adjusted.

Table 2. Associations of dietary EPA+DHA and total fish intakes with CHD, CVD and all-cause mortality in the Alpha Omega Cohort

	Dietary EPA+DHA intake, adjusted for energy				*P*-trend*
	≤50 mg/d (n=1113)	>50 to 100 mg/d (n=815)	>100 to 200 mg/d (n=1234)	>200 mg/d (n=905)	
Median dietary EPA+DHA, mg/d	26	75	141	339	
Person-years	12065	9069	13826	10269	
CHD mortality					
Cases, *n*	167	103	154	91	
Age- and sex-adjusted HR	1.00 (ref.)	0.82 (0.64-1.04)	0.86 (0.69-1.07)	0.67 (0.52-0.87)	0.005
Multivariable HR†	1.00 (ref.)	0.80 (0.63-1.03)	0.85 (0.68-1.06)	0.69 (0.52-0.90)	0.015
CVD mortality					
Cases, *n*	251	161	262	160	
Age- and sex-adjusted HR	1.00 (ref.)	0.85 (0.70-1.03)	0.98 (0.82-1.16)	0.79 (0.65-0.96)	0.043
Multivariable HR†	1.00 (ref.)	0.85 (0.69-1.03)	0.99 (0.83-1.19)	0.84 (0.68-1.04)	0.22
All-cause mortality					
Cases, *n*	566	368	570	373	
Age- and sex-adjusted HR	1.00 (ref.)	0.86 (0.75-0.98)	0.94 (0.84-1.06)	0.81 (0.71-0.92)	0.007
Multivariable HR†	1.00 (ref.)	0.85 (0.74-0.97)	0.96 (0.85-1.08)	0.86 (0.75-0.99)	0.11
	Total fish intake, adjusted for energy				*P*-trend*
	≤5 g/d (n=1002)	>5 to 20 g/d (n=2069)	>20 to 40 g/d (n=523)	>40 g/d (n=473)	
Median total fish, g/d	1.4	13.6	27.0	44.8	
Median oily fish, g/d	0.6	5.9	15.0	25.1	
Person-years	10917	23091	5914	5307	
CHD mortality					
Cases, *n*	155	253	59	48	
Age- and sex-adjusted HR	1.00 (ref.)	0.81 (0.66-0.99)	0.71 (0.52-0.96)	0.71 (0.51-0.98)	0.013
Multivariable HR‡	1.00 (ref.)	0.85 (0.70-1.04)	0.73 (0.54-0.99)	0.74 (0.53-1.03)	0.031
CVD mortality					
Cases, *n*	221	431	101	81	
Age- and sex-adjusted HR	1.00 (ref.)	0.97 (0.83-1.14)	0.86 (0.68-1.09)	0.84 (0.65-1.09)	0.11
Multivariable HR‡	1.00 (ref.)	1.04 (0.88-1.22)	0.91 (0.72-1.16)	0.91 (0.70-1.18)	0.33
All-cause mortality					
Cases, *n*	494	948	241	194	
Age- and sex-adjusted HR	1.00 (ref.)	0.95 (0.85-1.06)	0.91 (0.78-1.06)	0.90 (0.76-1.06)	0.15
Multivariable HR‡	1.00 (ref.)	1.03 (0.92-1.15)	0.98 (0.84-1.15)	0.97 (0.82-1.15)	0.64

CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ref., reference.
Values in Table represent hazard ratios (HRs) with 95% CIs, estimated from multivariable Cox models.

*P for linear trend, through median values across categories of circulating EPA+DHA, using a linear regression model.
†HRs for EPA+DHA were adjusted for age, sex, educational level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drugs, time since MI, and intake of total energy, cholesterol, fiber and *trans* FAs
‡HRs for fish were adjusted for age, sex, educational level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drugs, time since MI, and energy-adjusted intakes of meat, grains, fruits and vegetables.

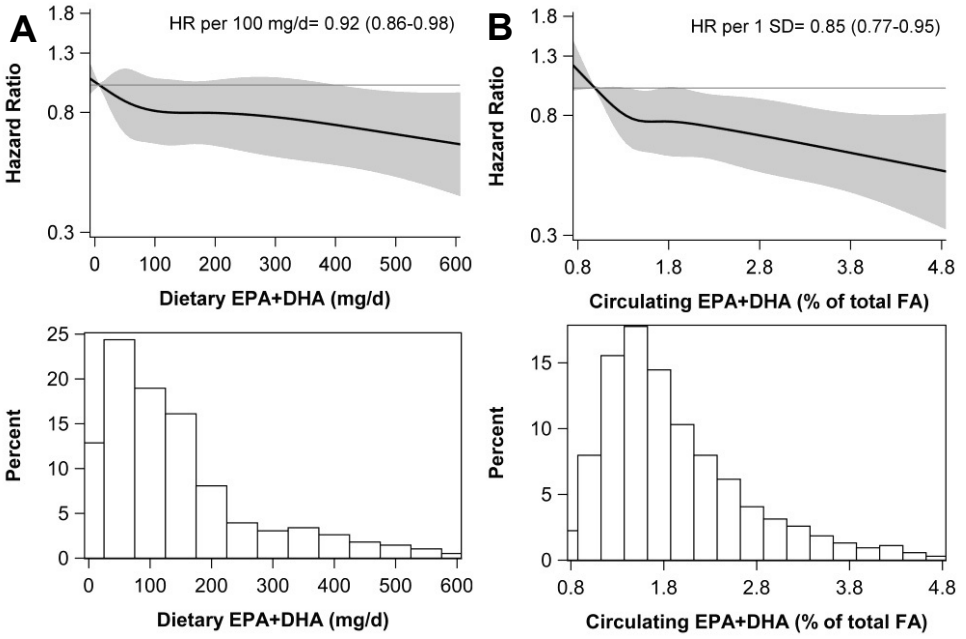

Figure 1. Associations of (A) dietary and (B) circulating EPA+DHA with coronary heart disease (CHD) mortality in 4067 post-MI patients.

Solid lines are risk estimates evaluated by restricted cubic splines from Cox models showing the shape of the associations on a continuous scale with five knots located at 5th, 27.5th, 50th, 72.5th, and 95th percentiles. The y-axis shows the multivariable-adjusted HRs for CHD mortality risk for any dietary or circulating EPA+DHA value, compared to the reference value set at 5th percentile of dietary (7.8 mg/d) or circulating EPA+DHA (0.99% total FA). Gray areas indicated 95% confidence intervals. One SD of circulating EPA+DHA was 0.95% of total FA. Histograms depict the distributions of dietary or circulating EPA+DHA in the Alpha Omega Cohort.

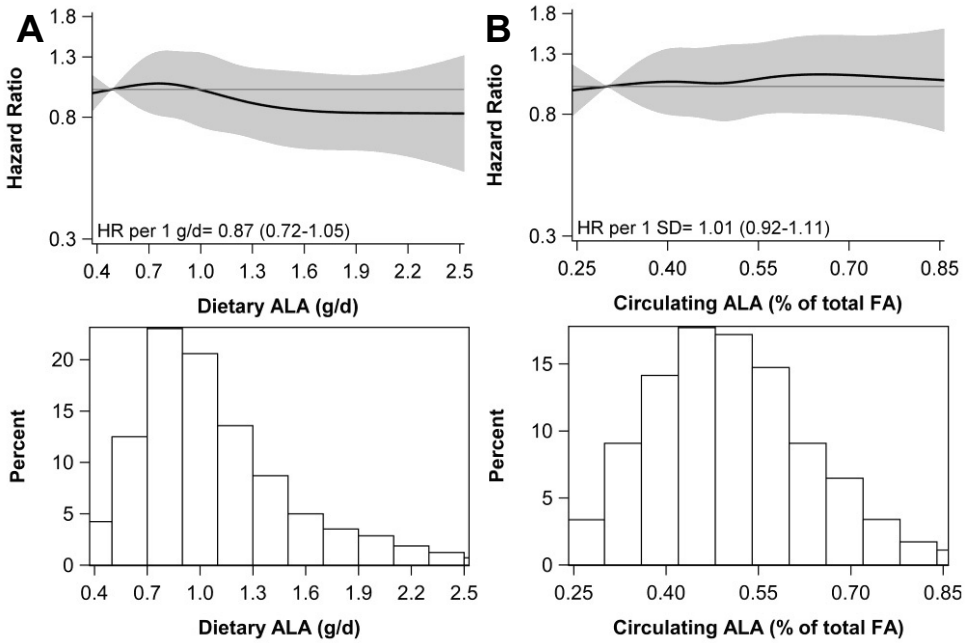

Figure 2. Associations of (A) dietary and (B) circulating ALA with coronary heart disease mortality (CHD) in 4067 post-MI patients.

Solid lines are risk estimates evaluated by restricted cubic splines from Cox models showing the shape of the associations on a continuous scale with five knots located at 5th, 27.5th, 50th, 72.5th, and 95th percentiles. The y-axis shows the multivariable-adjusted HRs for CHD mortality risk for any dietary or circulating ALA value, compared to the reference values set at 5th percentile of dietary (0.49 g/d) or circulating ALA (0.30% of total FAs). Gray areas indicated 95% confidence intervals. One SD of circulating ALA was 0.14% of total FAs. Histograms depict the distributions of dietary or circulating ALA in the Alpha Omega Cohort.

Circulating n-3 FAs

Patients with higher circulating EPA+DHA were more physically active, had higher alcohol intake and a higher DHD-15 score (Table 1). Those in the higher circulating ALA quintiles were less likely to be male and statin users (**Table S4**). Circulating ALA was not associated with DHD-15 score. After adjustment for demographic factors, lifestyle factors, circulating LA and AA, patients in the highest quintile of circulating EPA+DHA had a significantly lower CHD mortality risk (HR: 0.71, 95% CI: 0.53-0.94) than those in the lowest quintile (**Table 3**). Each increment in circulating EPA+DHA (per 1-SD of 0.95% of total FA) was associated with a 15% lower risk of CHD mortality (Figure 1). Associations were similar for CVD and all-cause mortality (Table 3), and for EPA and DHA when analyzed separately (data not shown). In categorical analyses based on cut-offs for Omega-3 index (**Table S5**), reductions in CHD mortality risk were more pronounced (HR: 0.63 for

EPA+DHA >3.1 vs ≤1.3% of total FAs; 95% CI 0.44-0.90). Circulating ALA was not associated with CHD mortality (**Figure 2**), and also not with CVD or all-cause mortality (Figure S2).

Subgroup analyses

HRs for dietary n-3 FAs in subgroups are presented in **Figure S3**. For dietary EPA+DHA, HRs for fatal CHD tended to be lower in patients who did not use statins (HR per 100 mg/d: 0.85, 95% CI: 0.72-1.00) or who had an MI <1 y before study enrolment (HR per 100 mg/d: 0.84, 95% CI: 0.68-1.05), but interaction terms were not statistically significant ($P_{interaction}$ of 0.28 and 0.38, respectively). Associations with fatal CHD were slightly stronger in older patients, non-obese patients, non-diabetic patients, patients with a higher diet quality score, and in patients who did not receive supplemental EPA+DHA during the first 40 months of follow-up. Associations for dietary ALA and fatal CHD in subgroups were all non-significant, but there was a trend towards a lower risk in women (HR of 0.69 (0.40-1.21) per 1 g/d).

HRs for circulating n-3 FAs in subgroups are presented in **Figure S4**. For circulating EPA+DHA, HRs for fatal CHD were lower in patients with circulating arachidonic acid (20:4 n-6) above the median (HR: 0.74 per 1-SD, 95% CI: 0.62-0.90; $P_{interaction}$=0.07) and in patients with MI <1 y before study enrolment (HR: 0.62 (0.41-0.95; $P_{interaction}$=0.13). Slightly stronger associations with fatal CHD were observed in younger and diabetic patients, in patients with circulating linoleic acid (18:2 n-6) below the median, and in patients who received no supplemental EPA+DHA during the first 40 months, Associations for circulating ALA and fatal CHD in subgroups were all non-significant.

Results of sensitivity analyses are presented in **Table S6**. The association between dietary EPA+DHA and fatal CHD was attenuated after excluding the first 2 y of follow-up (HR: 0.95 per 100 mg/d, 95% CI 0.89-1.01), whereas HRs for circulating EPA+DHA remained roughly similar. Exclusion of fish oil supplement users did not change the HRs for EPA+DHA. Results for dietary and circulating ALA did not materially change in sensitivity analyses.

Table 3. Associations of circulating EPA+DHA in quintiles with CHD, CVD and all-cause mortality in the Alpha Omega Cohort

	Circulating EPA+DHA					*P*-trend*
	Q1	Q2	Q3	Q4	Q5	
	≤1.29 (n=818)	>1.29 to 1.56 (n=809)	>1.56 to 1.92 (n=813)	>1.92 to 2.52 (n=812)	>2.52 (n=815)	
Median circulating EPA+DHA, % total FAs	1.12	1.43	1.73	2.17	3.14	
Person-years	8890	8904	8966	9152	9318	
CHD mortality						
Cases, *n*	123	99	107	89	97	
Age- and sex-adjusted HR	1.00 (ref.)	0.85 (0.65-1.11)	0.89 (0.69-1.15)	0.72 (0.55-0.95)	0.75 (0.58-0.98)	0.031
Multivariable HR†	1.00 (ref.)	0.83 (0.63-1.09)	0.88 (0.67-1.16)	0.72 (0.54-0.96)	0.71 (0.53-0.94)	0.020
CVD mortality						
Cases, *n*	183	160	175	159	157	
Age- and sex-adjusted HR	1.00 (ref.)	0.92 (0.74-1.14)	0.96 (0.78-1.18)	0.86 (0.70-1.06)	0.80 (0.65-0.99)	0.032
Multivariable HR†	1.00 (ref.)	0.90 (0.72-1.11)	0.94 (0.75-1.15)	0.85 (0.68-1.07)	0.75 (0.60-0.95)	0.016
All-cause mortality						
Cases, *n*	420	388	370	359	340	
Age- and sex-adjusted HR	1.00 (ref.)	0.97 (0.85-1.12)	0.89 (0.77-1.02)	0.85 (0.74-0.98)	0.76 (0.66-0.88)	<0.001
Multivariable HR†	1.00 (ref.)	0.97 (0.84-1.12)	0.90 (0.77-1.04)	0.86 (0.74-1.00)	0.73 (0.63-0.86)	<0.001

CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; Q, quintile; ref., reference.
Values in Table represent hazard ratios (HRs) with 95% confidence intervals, estimated from multivariable Cox models.
**P* for linear trend, through median values across categories of circulating EPA+DHA, using a linear regression model.
†HRs were adjusted for age, sex, educational level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drugs, serum cholesterol, circulating linoleic acid (18:2n-6), and circulating arachidonic acid (20:4n-6)

DISCUSSION

In this analysis of 4067 Dutch post-MI patients with state-of-the-art drug treatment, higher habitual intakes of EPA+DHA (>200 mg/d) and fish (>20 g/d) were associated with ~30% lower risk of CHD mortality during >12 y of follow-up. A similar association was found for circulating EPA+DHA (in plasma cholesteryl esters), for which also lower risks of CVD and all-cause mortality were found. Dietary and circulating ALA were not significantly associated with mortality. Strengths of the present study include its complete follow-up (only 1 patient censored), extensive data on potential confounders, and the detailed FFQ, which was specifically designed for FAs assessment.[11,12]

Data on habitual dietary intake of EPA+DHA (or fish) and mortality after MI are scarce. In 2412 Norwegian CHD patients aged ~62 y, long-chain n-3 FAs (from diet and supplements) were not significantly associated with fatal CHD or all-cause mortality (137 deaths) during 5 y of follow-up.[24] The Norwegian cohort consumed more fish (64-150 g/d) and marine n-3 FAs (0.6 to 2.6 g/d) than our Dutch cohort.[24] In a 5-y follow-up study of 400 Finnish CHD patients aged ~61 y, fish intakes >57 g/d were associated with a markedly lower risk of all-cause mortality (34 deaths), but not with fatal CHD (16 cases).[25] The Alpha Omega Cohort is characterized by a low fish intake (14 g/d), and 80% of patients consumed <200 mg/d of EPA+DHA. The 30% lower risk of fatal CHD in patients with higher EPA+DHA and fish intakes during long-term follow-up agrees with a meta-analysis of 9 prospective cohort studies in mainly healthy populations, followed for 6-40 y (pooled relative risk [RR] of 0.82, 95% CI 0.69-0.98 for higher EPA+DHA intakes).[26]

EPA+DHA in blood lipid compartments, including plasma cholesteryl esters, is an established biomarker of habitual EPA+DHA and (oily) fish intake,[27] although it may also be influence by other factors and metabolic processes.[28] Correlations between intake and plasma levels were around 0.4 in the Alpha Omega Cohort, comparable to other cohorts.[27] Consistent with findings for dietary EPA+DHA and fish, we found a 30% lower risk of fatal CHD for higher circulating EPA+DHA. In addition, lower risks were observed for fatal CVD and all-cause mortality. Erythrocytes have been proposed as a more suitable biomarker for long-term intake of n-3 FAs.[18,29] When applying criteria for the Omega-3 index,[18] an established indicator of red blood cell EPA+DHA, a 37% lower risk of fatal CHD in the upper category (>3.1%) was found in our Alpha Omega Cohort.

As for dietary EPA+DHA, data on circulating EPA+DHA and mortality risk in CHD patients are limited. In Finnish CHD patients, RRs for all-cause and CHD mortality

were around 0.3-0.5 for high vs low EPA and DHA in serum cholesteryl esters.[25] In 956 CHD patients of the Heart and Soul study, those with erythrocyte EPA+DHA above the median had a 27% lower mortality risk during 6 y of follow-up.[30] Fatal CVD and CHD were not examined in that study. In 3259 German patients referred for coronary angiography (LURIC study),[31] higher circulating EPA+DHA was associated with a 22% lower risk for both fatal CVD and all-cause mortality (975 deaths) during 10 y of follow-up, comparable to our findings. Fatal CHD was not examined in German patients, and observed risk reductions were non-significant for ALA. Our findings of circulating EPA+DHA and fatal CHD agree with a pooling study of population-based cohorts (>45,000 individuals) around 59 y of age, with a median follow-up of 10 y.[21] In multivariable analysis, each 1-SD increase in circulating EPA and/or DHA was associated with a 9-10% lower risk of fatal CHD. ALA was also related to a 9% lower risk of fatal CHD (per 1-SD) in population-based cohorts.[21] This finding differs from the Alpha Omega Cohort where associations with circulating ALA were neutral, for which we have no explanation.

The Alpha Omega Cohort included a trial phase during early follow-up, in which patients were randomized to margarines that provided low doses of EPA+DHA (400 mg/d), ALA (1 g/d), both EPA+DHA and ALA, or placebo (Alpha Omega Trial), for 40 months.[6,9] This intervention did not significantly impact major CVD events or secondary endpoints, including fatal CHD (HR of 0.95 for EPA+DHA vs placebo, 95% CI 0.68-1.32). A Cochrane meta-analysis of RCTs (including the Alpha Omega Cohort) showed a non-significant 10% lower risk of fatal CHD in CVD patients, mostly for higher doses of EPA+DHA.[7] In contrast, the current observational analysis of the Alpha Omega Cohort showed strong cardioprotective associations for dietary and circulating EPA+DHA, which were not modified by supplemental n-3 FAs during early follow-up. The present cohort study differs from the original trial in various aspects, including EPA+DHA doses (dietary intake vs. supplemental intake of 400 mg/d on top of diet), food matrix (fish vs. margarine spreads), duration of follow-up (>12 y vs. 40 months) and number of CHD deaths (515 vs. 138 cases). We cannot exclude the possibility of residual confounding in the present analysis because the intake of fish (and EPA+DHA) was higher in patients who were more educated, and also in alcohol users. We did, however, carefully adjust for these and a large number of other potential confounders.

Interestingly, in 846 female patients of the Alpha Omega Cohort, there was a trend towards a lower risk of fatal CHD with higher ALA intake (HR of 0.69 per 1-SD), although not statistically significant. This observation corroborates results from the earlier Alpha Omega Trial, in which low-dose ALA supplementation (1 g/d) reduced the risk of major CVD events in the same group of women, a finding that approached

significance (HR of 0.73; 95% CI 0.51-1.03).[6] We therefore cannot rule out a beneficial effect of ALA in CVD prevention, specifically in women.

EPA+DHA can reduce blood pressure, plasma triglycerides and blood coagulation,[32] but only for intakes >750 mg/d.[3] For lower intakes of EPA+DHA from fish, as in our cohort, protection against fatal CHD has been attributed to the prevention of cardiac arrhythmias.[3,32–34] Inverse associations of EPA+DHA with fatal CHD in our patients tended to be stronger in those with an MI <1 y before study enrolment. A beneficial role for EPA+DHA in attenuating reperfusion ischemia injury in cardiac tissue has been hypothesized, although the exact mechanism is not yet understood.[35,36]

To conclude, higher levels of dietary and circulating EPA and DHA (mainly from fish) were associated with a lower risk of mortality, in particular from CHD, in Dutch post-MI patients with a low fish intake. This finding could have major implications for dietary advice after MI, and warrants confirmation in other large cohorts of CHD patients.

Acknowledgments

The authors thank Paul Hulshof, Robert Hovenier, and Marlies Diepeveen-de Bruin (Division of Human Nutrition and Health, Wageningen University) for analysis of plasma FAs.

Sources of funding

The Alpha Omega Cohort (ClinicalTrials.gov Identifier NCT03192410), which was funded by the Dutch Heart Foundation (grant 200T401) and NIH/National Heart, Lung, and Blood Institute and Office of Dietary Supplements, grant R01HL076200. Financial support for research on FAs was obtained from Unilever and Upfield, The Netherlands. This research was supported by the Dutch Research Council (NWO) through its Graduate Programme on Food Structure, Digestion and Health.

Disclosures

JMG received funding from Unilever for epidemiological studies of FAs and cardiovascular disease in the Alpha Omega Cohort. PLZ was employed at Unilever until May 2019. Other authors have no disclosures to report.

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SUPPLEMENTAL TABLES AND FIGURES

Table S1. Percentage contribution of selected food groups to total intakes of EPA+DHA and ALA in the Alpha Omega Cohort

	EPA+DHA	ALA
Grain products*	-†	19
Meat and poultry	5	7
Eggs	4	-†
Milk and milk products‡	-†	6
Fish§	89	3
Nuts and seeds‖	-†	4
Vegetables and potatoes	-†	6
Legumes	-†	1
Cooking oils	-†	26
Margarine	-†	14
Dressings#	-†	6
Savory spreads	2	3

*Grain products included bread, pie, pancake, pizza, noodle.
†Contribution <1%.
‡Milk and milk products included butter.
§Fish included oily fish (50%), lean fish and shellfish.
‖Nuts and seeds include peanut butter.
#Dressings included creamy (mayonnaise-based) and oil-vinegar dressings.
Values for ALA do not add up to 100% because snacks and unknown sources are not in the Table.

Table S2. Baseline characteristics of the Alpha Omega Cohort in categories of dietary EPA+DHA intake

	Dietary EPA+DHA intake, adjusted for energy				*P*[*]
	≤50 mg/d (n=1113)	51 to 100 mg/d (n=815)	101 to 200 mg/d (n=1234)	>200 mg/d (n=905)	
Age, y	69.5±5.6	69.3±5.5	68.5±5.6	68.6±5.6	<0.001
Men	882 (79.2)	654 (80.2)	975 (79.0)	710 (78.4)	0.83
Body mass index, kg/m^{2}[†]	27.6±3.8	27.6±3.7	27.9±3.8	27.7±3.9	0.35
Obese (≥30 kg/m^{2})	242 (21.8)	192 (23.6)	304 (24.6)	215 (23.8)	0.42
Time since myocardial infarction, y[†]	3.8 (1.8-6.6)	3.8 (1.7-6.1)	3.8 (1.7-6.4)	3.3 (1.5-6.0)	0.15
Smoking status[†]					0.65
Never	181 (16.3)	119 (14.6)	200 (16.2)	163 (18.0)	
Former	751 (67.5)	559 (68.6)	822 (66.6)	598 (66.1)	
Current	180 (16.2)	137 (16.8)	212 (17.2)	144 (15.9)	
Physical activity[†]					<0.001
Low	522 (47.3)	373 (46.1)	465 (37.9)	292 (32.3)	
Middle	357 (32.4)	288 (35.6)	505 (41.1)	378 (41.8)	
High	224 (20.3)	149 (18.4)	258 (21.0)	234 (25.9)	
Highest level of education[†]					<0.001
Primary	265 (23.9)	187 (23.2)	223 (18.1)	128 (14.2)	
Lower secondary	432 (39.0)	318 (39.5)	418 (34.0)	294 (32.6)	
Higher secondary or lower tertiary	308 (27.8)	229 (28.4)	421 (34.2)	317 (35.2)	
Higher tertiary	104 (9.4)	72 (8.9)	168 (13.7)	162 (18.0)	
Alcohol intake					<0.001
No	67 (6.0)	41 (5.0)	51 (4.1)	44 (4.9)	
Low	651 (58.5)	459 (56.3)	622 (50.4)	423 (46.7)	
Moderate	279 (25.1)	194 (23.8)	342 (27.7)	252 (27.9)	
High	116 (10.4)	121 (14.9)	219 (17.8)	186 (20.6)	
Medication use					
Statins	937 (84.2)	692 (84.9)	1068 (86.6)	797 (88.1)	0.06
Antithrombotic drugs	1084 (97.4)	800 (98.2)	1207 (97.8)	887 (98.0)	0.67
Antihypertensive drugs	995 (89.4)	727 (89.2)	1113 (90.2)	815 (90.1)	0.86
Serum lipids, mmol/L[‡,§]					
Total cholesterol	4.68±0.96	4.74±0.93	4.73±0.94	4.69±0.97	0.79
LDL cholesterol	2.54±0.81	2.61±0.81	2.59±0.80	2.56±0.84	0.92
HDL cholesterol	1.27±0.33	1.25±0.33	1.29±0.33	1.32±0.38	<0.001
Triglycerides	1.68 (1.23-2.32)	1.79 (1.28-2.42)	1.63 (1.20-2.32)	1.57 (1.18-2.21)	0.002

	Dietary EPA+DHA intake, adjusted for energy				P*
	≤50 mg/d (n=1113)	51 to 100 mg/d (n=815)	101 to 200 mg/d (n=1234)	>200 mg/d (n=905)	
Plasma glucose, mmol/L‡	5.63 (5.08-6.59)	5.58 (5.00-6.58)	5.60 (5.08-6.68)	5.59 (5.05-6.46)	0.48
Systolic	141±22	142±21	142±21	143±22	0.16
Diastolic	80±11	80±11	80±11	81±11	0.048
Prevalent diabetes	224 (20.1)	172 (21.1)	248 (20.1)	169 (18.7)	0.65
Family history of myocardial infarction	125 (11.2)	90 (11.1)	139 (11.3)	113 (12.5)	0.76
Family history of diabetes	218 (19.6)	160 (19.6)	250 (20.3)	206 (22.8)	0.28
Dietary factors					
Energy, kcal/d	1959±525	1896±530	1898±505	1931±514	0.65
Protein, en%	14.3±2.7	14.8±2.9	15.1±2.7	15.9±2.8	<0.001
Total fat, en%	34.3±6.4	34.1±6.4	33.7±6.0	33.0±5.9	<0.001
Saturated FAs, en%	13.0±3.2	12.7±3.1	12.4±2.9	11.8±2.9	<0.001
cis monounsaturated FAs, en%	9.4±2.2	9.4±2.2	9.5±2.1	9.7±2.2	<0.001
Polyunsaturated FAs, en%	7.3±2.4	7.3±2.2	7.3±2.2	7.1±2.2	0.11
Total n-3 FAs, en%	0.65±0.25	0.67±0.25	0.71±0.24	0.82±0.25	<0.001
ALA, g/d	1.07±0.53	1.10±0.49	1.11±0.48	1.08±0.47	0.98
EPA+DHA, mg/d	26 (12-37)	75 (63-87)	141 (121-165)	339 (255-454)	<0.001
Total n-6 FAs, en%	5.6±2.2	5.5±2.1	5.5±2.1	5.3±2.0	<0.001
trans-FAs, g/d‖	1.7±0.7	1.6±0.6	1.5±0.6	1.5±0.6	<0.001
Carbohydrates, en%	47.9±6.8	46.7±7.0	46.3±6.6	45.9±6.6	<0.001
Fiber, g/d‖	21.9±7.0	21.0±7.0	21.2±6.5	22.1±6.6	0.13
Cholesterol, mg/d‖	170±67	180±67	187±67	199±71	<0.001
Total fish (g/d)	1.8 (0.1-4.3)	9.6 (6.3-14.8)	15.6 (12.8-17.8)	39.8 (26.7-44.2)	<0.001
Oily fish (g/d)	0.4 (0-1.2)	3.2 (1.8-4.6)	7.5 (5.9-9.6)	20.6 (14.4-28.3)	<0.001
Diet quality score (DHD-15)	75.2±13.1	77.0±13.0	79.8±12.6	85.4±13.5	<0.001
Circulating FAs, % total FAs					
Saturated FAs	13.1±1.1	13.1±1.3	13.2±1.1	13.2±1.1	0.07
Monounsaturated FAs	22.4±3.1	22.4±3.3	22.6±3.2	22.7±3.3	0.027
Polyunsaturated FAs	63.1±4.0	63.1±4.2	62.9±4.0	62.7±4.1	0.027
Total n-3 FAs	2.02 (1.74-2.43)	2.21 (1.87-2.64)	2.46 (2.08-3.03)	2.94 (2.37-3.70)	<0.001
ALA	0.51±0.15	0.51±0.15	0.50±0.14	0.51±0.14	0.47
EPA	0.88 (0.68-1.13)	0.97 (0.73-1.26)	1.14 (0.85-1.58)	1.48 (1.06-2.07)	<0.001
DHA	0.54 (0.44-0.67)	0.62 (0.52-0.75)	0.71 (0.59-0.85)	0.84 (0.66-0.98)	<0.001

Table S2. continued

	Dietary EPA+DHA intake, adjusted for energy				*P**
	≤50 mg/d (n=1113)	51 to 100 mg/d (n=815)	101 to 200 mg/d (n=1234)	>200 mg/d (n=905)	
Total n-6 FAs	60.7±4.2	60.5±4.5	60.1±4.3	59.4±4.4	<0.001
Linoleic acid, 18:2 n-6	50.3±5.0	50.2±5.0	49.8±4.9	49.6±5.0	0.001
Arachidonic acid, 20:4 n-6	8.6±2.1	8.5±2.1	8.4±2.0	8.0±2.0	<0.001

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DHD-15, 2015 Dutch Healthy Diet score; EPA, eicosapentaenoic acid; FA, fatty acids; Q, quintile.
Values are shown as mean ±SD, median (IQR) or *n* (%) unless stated otherwise.
* *P* value for linear trend, through median values across categories of intake using a linear regression model, or obtained from chi-square test for categorical variables.
† <1% of patients had missing values for body mass index, time since MI, smoking status, physical activity, educational level and blood pressure.
‡ part of the cohort had missing values for total cholesterol, HDL cholesterol and triglycerides (n=61), LDL cholesterol (n=252) and plasma glucose (n=33).
§ to convert to mg/dL, divide by 0.02586 for total, LDL, HDL cholesterol and by 0.01129 for triglycerides; ‖ values for dietary TFAs, fiber and cholesterol were non-energy adjusted.

Table S3. Baseline characteristics of the Alpha Omega Cohort in categories of dietary ALA intake

	Dietary ALA intake, adjusted for energy					*P*[*]
	≤0.5 g/d (n=222)	>0.5 to 1.0 g/d (n=1902)	>1.0 to 1.5 g/d (n=1285)	>1.5 to 2.0 g/d (n=412)	>2.0 g/d (n=246)	
Age, y	69.3±5.4	69.2±5.6	68.9±5.6	68.3±5.6	68.6±5.4	0.002
Men	198 (89.2)	1477 (77.7)	976 (76.0)	351 (85.2)	219 (89.0)	<0.001
Body mass index, kg/m^{2}[†]	27.0±3.3	27.8±3.8	27.8±3.9	27.8±3.9	27.7±3.4	0.27
Obese (≥30 kg/m^2)	33 (14.9)	467 (24.6)	311 (24.2)	93 (22.6)	49 (19.9)	0.013
Time since myocardial infarction, y[†]	3.9 (1.5-6.9)	3.7 (1.7-6.3)	3.8 (1.7-6.4)	3.5 (1.6-6.1)	2.9 (1.2-6.2)	0.10
Smoking status[†]						<0.001
Never	20 (9.0)	343 (18.0)	224 (17.4)	50 (12.1)	26 (10.6)	
Former	163 (73.4)	1253 (65.9)	840 (65.4)	306 (74.3)	168 (68.3)	
Current	39 (17.6)	305 (16.0)	221 (17.2)	56 (13.6)	52 (21.1)	
Physical activity[†]						0.30
Low	91 (41.2)	780 (41.3)	538 (42.1)	149 (36.3)	94 (38.4)	
Middle	79 (35.8)	707 (37.4)	468 (36.7)	181 (44.0)	93 (38.0)	
High	51 (23.1)	404 (21.4)	271 (21.2)	81 (19.7)	58 (23.7)	
Highest level of education[†]						0.15
Primary	47 (21.2)	376 (19.9)	251 (19.6)	74 (18.1)	55 (22.6)	
Lower secondary	81 (36.5)	648 (34.3)	473 (37.0)	162 (39.6)	98 (40.3)	
Higher secondary or lower tertiary	63 (28.4)	627 (33.1)	386 (30.2)	126 (30.8)	73 (30.0)	
Higher tertiary	31 (14.0)	241 (12.7)	170 (13.3)	47 (11.5)	17 (7.0)	
Alcohol intake						<0.001
No	5 (2.3)	95 (5.0)	71 (5.5)	23 (5.6)	9 (3.7)	
Low	94 (42.3)	980 (51.5)	730 (56.8)	218 (52.9)	133 (54.1)	
Moderate	58 (26.1)	497 (26.1)	331 (25.8)	108 (26.2)	73 (29.7)	
High	65 (29.3)	330 (17.4)	153 (11.9)	63 (15.3)	31 (12.6)	
Medication use						
Statins	192 (86.5)	1631 (85.8)	1098 (85.5)	355 (86.2)	218 (88.6)	0.76
Antithrombotic drugs	216 (97.3)	1855 (97.5)	1256 (97.7)	408 (99.0)	243 (98.8)	0.29
Antihypertensive drugs	195 (87.8)	1718 (90.3)	1150 (89.5)	373 (90.5)	214 (87.0)	0.41
Serum lipids, mmol/L[‡,§]						
Total cholesterol	4.72±0.92	4.75±0.95	4.71±0.95	4.67±0.93	4.51±0.93	<0.001
LDL cholesterol	2.59±0.85	2.60±0.81	2.56±0.81	2.55±0.78	2.43±0.83	0.007
HDL cholesterol	1.30±0.33	1.30±0.35	1.28±0.34	1.28±0.34	1.23±0.30	0.004

Table S3. continued

	Dietary ALA intake, adjusted for energy					*P*[*]
	≤0.5 g/d (n=222)	>0.5 to 1.0 g/d (n=1902)	>1.0 to 1.5 g/d (n=1285)	>1.5 to 2.0 g/d (n=412)	>2.0 g/d (n=246)	
Triglycerides	1.59 (1.24-2.09)	1.65 (1.19-2.31)	1.66 (1.22-2.35)	1.67 (1.21-2.34)	1.66 (1.21-2.32)	0.98
Plasma glucose, mmol/L[‡]	5.50 (5.08-6.18)	5.62 (5.04-6.68)	5.61 (5.07-6.58)	5.62 (5.08-6.58)	5.61 (5.00-6.40)	0.95
Blood pressure (mmHg)[†]						
Systolic	142±23	142±22	142±21	142±22	141±20	0.59
Diastolic	81±11	80±11	80±11	81±11	80±11	0.76
Prevalent diabetes	26 (11.7)	380 (20.0)	282 (22.0)	84 (20.4)	41 (16.7)	0.006
Family history of myocardial infarction	27 (12.2)	227 (11.9)	142 (11.1)	50 (12.1)	21 (8.5)	0.56
Family history of diabetes	47 (21.2)	369 (19.4)	292 (22.7)	77 (18.7)	49 (19.9)	0.18
Dietary factors						
Energy, kcal/d	2539±466	1879±467	1793±499	2027±524	2186±516	0.17
Protein, en%	13.1±2.6	15.1±2.8	15.4±2.8	14.6±2.6	13.9±2.4	0.028
Total fat, en%	33.6±7.1	32.2±6.3	33.9±5.4	37.1±4.8	39.7±4.9	<0.001
Saturated FAs, en%	13.4±3.5	12.4±3.2	12.3±2.8	12.8±2.9	12.6±3.1	0.93
cis monounsaturated FAs, en%	9.0±2.3	9.1±2.3	9.5±2.1	10.3±1.7	11.0±1.5	<0.001
Polyunsaturated FAs, en%	6.5±2.1	6.2±2.3	7.4±1.6	9.3±1.6	11.4±1.7	<0.001
Total n-3 FAs, en%	0.63±0.28	0.61±0.22	0.72±0.19	0.93±0.19	1.16±0.19	<0.001
ALA, g/d	0.37±0.13	0.79±0.13	1.21±0.14	1.72±0.14	2.40±0.38	<0.001
EPA+DHA, mg/d	72 (17-142)	109 (46-188)	115 (53-194)	107 (47-179)	97 (32-162)	0.98
Total n-6 FAs, en%	4.8±2.0	4.5±1.5	5.6±1.6	7.5±1.6	9.4±1.7	<0.001
trans FAs, g/d[‖]	2.1±0.7	1.5±0.6	1.5±0.6	1.7±0.6	1.8±0.6	<0.001
Carbohydrates, en%	47.5±7.7	47.7±6.9	46.7±6.5	44.1±5.8	42.7±5.2	<0.001
Fiber, g/d[‖]	24.0±8.0	21.4±6.7	21.0±6.4	22.0±7.3	22.5±6.2	0.80
Cholesterol, mg/d[‖]	218±83	181±66	177±69	194±66	192±70	0.94
Total fish (g/d)	9.2 (1.1-15.3)	14.0 (5.2-19.6)	14.5 (6.2-20.8)	13.2 (5.0-19.8)	11.5 (3.5-17.6)	0.80
Oily fish (g/d)	3.6 (0-8.2)	5.5 (1.3-11.0)	5.8 (1.6-11.4)	5.3 (1.1-10.2)	5.0 (0.6-9.4)	0.52
Diet quality score (DHD-15)	74.0±13.4	79.1±13.5	80.5±13.5	78.6±13.6	79.5±13.4	0.014
Circulating FAs, % total FAs						
Saturated FAs	13.4±1.8	13.2±1.1	13.1±1.0	13.0±1.1	13.0±1.0	<0.001
Monounsaturated FAs	23.3±3.3	22.8±3.2	22.2±3.2	22.1±3.4	21.6±2.8	<0.001
Polyunsaturated FAs	61.8±4.6	62.6±4.0	63.4±4.0	63.6±4.1	64.1±3.5	<0.001
Total n-3 FAs	2.40 (1.93-3.09)	2.37 (1.95-3.03)	2.36 (1.93-2.98)	2.30 (1.90-2.82)	2.26 (1.88-2.78)	<0.001

	Dietary ALA intake, adjusted for energy					P*
	≤0.5 g/d (n=222)	>0.5 to 1.0 g/d (n=1902)	>1.0 to 1.5 g/d (n=1285)	>1.5 to 2.0 g/d (n=412)	>2.0 g/d (n=246)	
ALA	0.53±0.15	0.51±0.15	0.50±0.14	0.50±0.14	0.49±0.14	0.035
EPA	1.06 (0.77-1.67)	1.07 (0.80-1.58)	1.08 (0.80-1.51)	1.01 (0.78-1.38)	1.00 (0.78-1.41)	0.001
DHA	0.66 (0.50-0.83)	0.67 (0.53-0.84)	0.67 (0.54-0.84)	0.66 (0.53-0.82)	0.64 (0.50-0.82)	0.13
Total n-6 FAs	58.9±4.9	59.7±4.4	60.6±4.3	60.9±4.4	61.4±3.9	<0.001
Linoleic acid, 18:2 n-6	48.9±5.5	49.6±4.9	50.3±5.0	50.8±5.0	51.0±4.5	<0.001
Arachidonic acid, 20:4 n-6	8.2±1.9	8.3±2.0	8.5±2.1	8.3±2.0	8.6±2.1	0.007

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DHD-15, 2015 Dutch Healthy Diet score; EPA, eicosapentaenoic acid; FA, fatty acids; Q, quintile.
Values are shown as mean ±SD, median (IQR) or *n* (%) unless stated otherwise. **P* value for linear trend, through median values across categories of intake using a linear regression model, or obtained from chi-square test for categorical variables.
† <1% of patients had missing values for body mass index, time since MI, smoking status, physical activity, educational level and blood pressure.
‡ part of the cohort had missing values for total cholesterol, HDL cholesterol and triglycerides (n=61), LDL cholesterol (n=252) and plasma glucose (n=33).
§ to convert to mg/dL, divide by 0.02586 for total, LDL, HDL cholesterol and by 0.01129 for triglycerides.
‖ values for dietary TFAs, fiber and cholesterol were non-energy adjusted.

Table S4. Baseline characteristics of the Alpha Omega Cohort in quintiles of circulating ALA

	Circulating ALA, in quintiles					*P**
	Q1 (n=799)	Q2 (n=806)	Q3 (n=826)	Q4 (n=823)	Q5 (n=813)	
Circulating ALA, % total FAs (range)	0-0.39	0.39-0.46	0.46-0.53	0.53-0.61	0.61-1.66	
Age, y	68.2±5.5	68.9±5.7	68.9±5.5	69.3±5.6	69.5±5.5	<0.001
Men	665 (83.2)	667 (82.8)	654 (79.2)	629 (76.4)	606 (74.5)	<0.001
Body mass index, kg/m^2†	27.6±3.6	28.2±3.8	27.9±3.8	27.8±4.0	27.1±3.6	<0.001
Obese (≥30 kg/m^2)	184 (23.1)	208 (25.8)	204 (24.7)	192 (23.4)	165 (20.3)	0.10
Time since myocardial infarction, y†	3.4 (1.5-6.2)	3.9 (1.8-6.3)	3.7 (1.6-6.4)	3.8 (1.6-6.3)	3.7 (1.7-6.3)	0.37
Smoking status†						0.06
Never	106 (13.3)	124 (15.4)	141 (17.1)	133 (16.2)	159 (19.6)	
Former	551 (69.0)	546 (67.8)	564 (68.3)	548 (66.6)	521 (64.1)	
Current	142 (17.8)	135 (16.8)	121 (14.7)	142 (17.3)	133 (16.4)	
Physical activity†						0.31
Low	341 (43.0)	325 (40.5)	325 (39.6)	321 (39.3)	340 (41.9)	
Middle	268 (33.8)	301 (37.5)	326 (39.8)	323 (39.5)	310 (38.2)	
High	185 (23.3)	176 (22.0)	169 (20.6)	173 (21.2)	162 (20.0)	
Highest level of education†						0.08
Primary	151 (19.0)	152 (19.0)	155 (18.9)	178 (21.7)	167 (20.6)	
Lower secondary	281 (35.4)	266 (33.2)	304 (37.1)	298 (36.3)	313 (38.7)	
Higher secondary or lower tertiary	243 (30.6)	279 (34.8)	252 (30.8)	253 (30.8)	248 (30.7)	
Higher tertiary	120 (15.1)	104 (13.0)	108 (13.2)	93 (11.3)	81 (10.0)	
Alcohol intake						0.32
No	50 (6.3)	34 (4.2)	41 (5.0)	37 (4.5)	41 (5.0)	
Low	421 (52.7)	459 (57.0)	430 (52.1)	425 (51.6)	420 (51.7)	
Moderate	217 (27.2)	189 (23.5)	216 (26.2)	230 (28.0)	215 (26.5)	
High	111 (13.9)	124 (15.4)	139 (16.8)	131 (15.9)	137 (16.9)	
Medication use						
Statins	751 (94.0)	732 (90.8)	732 (88.6)	677 (82.3)	602 (74.1)	<0.001
Antithrombotic drugs	782 (97.9)	791 (98.1)	807 (97.7)	804 (97.7)	794 (97.7)	0.96
Antihypertensive drugs	717 (89.7)	731 (90.7)	722 (87.4)	741 (90.0)	739 (90.9)	0.14
Serum lipids, mmol/L‡,§						
Total cholesterol	4.45±0.84	4.53±0.90	4.72±0.96	4.85±0.95	5.00±0.99	<0.001
LDL cholesterol	2.37±0.75	2.43±0.72	2.58±0.83	2.70±0.84	2.78±0.84	<0.001

	Circulating ALA, in quintiles					P*
	Q1 (n=799)	Q2 (n=806)	Q3 (n=826)	Q4 (n=823)	Q5 (n=813)	
HDL cholesterol	1.29±0.34	1.27± 0.32	1.29±0.35	1.28±0.33	1.30±0.37	0.52
Triglycerides	1.54 (1.11-2.20)	1.59 (1.18-2.20)	1.67 (1.23-2.31)	1.71 (1.28-2.41)	1.79 (1.30-2.52)	<0.001
Plasma glucose, mmol/L‡	5.59 (5.05-6.54)	5.68 (5.07-6.88)	5.60 (5.03-6.50)	5.65 (5.12-6.66)	5.48 (5.00-6.38)	0.29
Blood pressure (mmHg)†						
Systolic	141±21	143±21	143±21	142±23	142±21	0.47
Diastolic	80±11	80±11	81±11	80±12	80±11	0.31
Prevalent diabetes	159 (19.9)	194 (24.1)	140 (17.0)	175 (21.3)	145 (17.8)	0.003
Family history of myocardial infarction	94 (11.8)	92 (11.4)	95 (11.5)	89 (10.8)	97 (11.9)	0.96
Family history of diabetes	177 (22.2)	173 (21.5)	156 (18.9)	180 (21.9)	148 (18.2)	0.15
Dietary factors						
Energy, kcal/d	1908±527	1899±497	1909±492	1919±507	1971±563	0.006
Protein, en%	14.9±2.7	15.2±2.8	14.9±2.8	15.0±2.7	14.9±2.9	0.32
Total fat, en%	33.6±6.3	33.7±5.8	33.6±6.3	34.1±6.3	33.9±6.2	0.26
Saturated FAs, en%	12.2±3.0	12.4±2.8	12.5±3.1	12.7±3.2	12.6±3.2	0.002
cis monounsaturated FAs, en%	9.7±2.2	9.5±2.1	9.4± 2.2	9.5±2.2	9.4±2.2	0.025
Polyunsaturated FAs, en%	7.3±2.3	7.3±2.2	7.1±2.2	7.2±2.2	7.2±2.3	0.59
Total n-3 FAs, en%	0.71±0.26	0.72±0.25	0.70±0.25	0.71±0.25	0.72±0.26	0.34
ALA, g/d‖	1.11±0.52	1.12±0.49	1.06±0.45	1.09±0.51	1.07±0.51	0.06
EPA+DHA, mg/d‖	104 (46-178)	112 (46-189)	111 (48-186)	106 (46-189)	108 (45-189)	0.21
Total n-6 FAs, en%	5.7±2.1	5.6±2.1	5.3±2.0	5.5±2.0	5.4±2.1	0.022
trans-FAs, g/d‖	1.5±0.7	1.5±0.6	1.6±0.6	1.6±0.6	1.6±0.7	0.018
Carbohydrates, en%	47.1±6.8	46.8±6.6	46.8±6.8	46.4±6.8	46.6±6.9	0.12
Fiber, g/d‖	21.3±6.6	21.6±6.6	21.6±6.8	21.5±6.8	21.8±7.0	0.18
Cholesterol, mg/d‖	179±66	183±67	182±69	187±71	188±71	0.004
Total fish (g/d)	13.5 (5.3-19.0)	14.0 (5.5-20.5)	13.7 (5.4-20.3)	13.7 (5.8-19.7)	13.0 (4.6-19.0)	0.84
Oily fish (g/d)	5.3 (1.3-9.8)	5.4 (1.2-10.8)	5.5 (1.2-11.4)	5.7 (1.2-11.1)	5.2 (1.2-10.8)	0.64
DHD-15 score	79.9±14.2	79.5±13.3	79.6±14.0	78.0±12.9	79.2±13.4	0.09
Circulating FAs, % total FAs						
Saturated FAs	13.1±1.2	13.2±1.0	13.2±1.1	13.1±1.0	13.1±1.2	0.39
Monounsaturated FAs	22.1±3.3	22.5±2.9	22.6±3.1	22.6±3.2	22.7±3.4	<0.001
Polyunsaturated FAs	63.5±4.2	63.0±3.7	62.8±3.9	62.8±4.0	62.7±4.2	<0.001
Total n-3 PUFAs	1.98 (1.63-2.55)	2.26 (1.88-2.84)	2.39 (2.01-2.99)	2.44 (2.04-3.05)	2.62 (2.20-3.30)	<0.001

Table S4. continued

	Circulating ALA, in quintiles					*P**
	Q1 (n=799)	Q2 (n=806)	Q3 (n=826)	Q4 (n=823)	Q5 (n=813)	
EPA	0.87 (0.64-1.28)	1.02 (0.78-1.45)	1.10 (0.87-1.56)	1.13 (0.84-1.60)	1.17 (0.86-1.69)	<0.001
DHA	0.68 (0.56-0.84)	0.70 (0.57-0.86)	0.68 (0.55-0.85)	0.64 (0.52-0.82)	0.62 (0.48-0.80)	<0.001
Total n-6 PUFAs	61.1±4.5	60.3±4.0	59.9±4.3	60.0±4.3	59.6±4.6	<0.001
Linoleic acid, 18:2n-6	49.7±5.3	49.5±4.7	49.7±4.9	50.2±4.8	50.7±5.0	<0.001
Arachidonic acid, 20:4n-6	9.6±2.1	9.0±2.0	8.3±1.8	7.9±1.8	7.1±1.6	<0.001

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DHD-15, 2015 Dutch Healthy Diet score; EPA, eicosapentaenoic acid; FA, fatty acids; Q, quintile.
Values are shown as mean ±SD, median (IQR) or *n* (%) unless stated otherwise.
* *P* value for linear trend, through median values across categories of intake using a linear regression model, or obtained from chi-square test for categorical variables.
† <1% of patients had missing values for body mass index, time since MI, smoking status, physical activity, educational level and blood pressure.
‡ part of the cohort had missing values for total cholesterol, HDL cholesterol and triglycerides (n=61), LDL cholesterol (n=252) and plasma glucose (n=33).
§ to convert to mg/dL, divide by 0.02586 for total, LDL, HDL cholesterol and by 0.01129 for triglycerides.
‖ values for dietary TFAs, fiber and cholesterol were non-energy adjusted.

Table S5. Associations of circulating EPA+DHA in categories based on converted Omega-3 index cut-offs with CHD, CVD and all-cause mortality in the Alpha Omega Cohort

	Categories of circulating n-3 fatty acids				*P-trend*[*]
	≤1.3% (n=844)	>1.3 to ≤2.2 (n=2058)	>2.2 to ≤3.1 (n=739)	>3.1 (n=426)	
Median circulating EPA+DHA, % total FA	1.12	1.66	2.54	3.73	
Person-years	9169	22,779	8460	4821	
CHD mortality					
Cases, *n*	127	250	90	48	
Age- and sex-adjusted HR	1.00 (ref.)	0.82 (0.66-1.01)	0.80 (0.61-1.05)	0.71 (0.51-0.98)	0.06
Multivariable HR[†]	1.00 (ref.)	0.80 (0.64-1.01)	0.80 (0.60-1.07)	0.63 (0.44-0.90)	0.026
CVD mortality					
Cases, *n*	189	411	155	79	
Age- and sex-adjusted HR	1.00 (ref.)	0.89 (0.75-1.06)	0.91 (0.73-1.12)	0.77 (0.59-1.00)	0.09
Multivariable HR[†]	1.00 (ref.)	0.87 (0.73-1.05)	0.90 (0.71-1.13)	0.70 (0.53-0.93)	0.038
All-cause mortality					
Cases, *n*	436	945	313	183	
Age- and sex-adjusted HR	1.00 (ref.)	0.89 (0.80-0.99)	0.80 (0.69-0.92)	0.77 (0.65-0.94)	0.001
Multivariable HR[†]	1.00 (ref.)	0.90 (0.80-1.01)	0.81 (0.69-0.94)	0.73 (0.60-0.87)	<0.001

CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; Q, quintile; ref., reference.
Values in Table represent hazard ratios (HRs) with 95% confidence intervals, estimated from multivariable Cox models.
[*]P for linear trend, through median values across categories of circulating EPA+DHA, using a linear regression model.
[†]HRs were adjusted for age, sex, educational level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drugs, serum cholesterol, circulating linoleic acid (18:2 n-6), and circulating arachidonic acid (20:4 n-6).

Table S6. Sensitivity analyses for continuous associations of dietary and circulating n-3 FAs with all-cause, CVD and CHD mortality in the Alpha Omega Cohort

	EPA+DHA			ALA		
	CHD mortality	CVD mortality	All-cause mortality	CHD mortality	CVD mortality	All-cause mortality
Dietary intake						
Main analysis (n=4067)	0.92 (0.86-0.98)	0.96 (0.92-1.01)	0.97 (0.94-1.00)	0.87 (0.72-1.05)	0.91 (0.79-1.05)	0.98 (0.89-1.07)
Excluding ≤2 y follow-up (n=3921)	0.95 (0.89-1.01)	0.98 (0.93-1.03)	0.98 (0.95-1.01)	0.89 (0.73-1.08)	0.93 (0.80-1.08)	1.00 (0.90-1.10)
Excluding fish oil supplement users (n=3880)	0.92 (0.86-0.98)	0.96 (0.92-1.01)	0.97 (0.94-1.00)	0.88 (0.73-1.06)	0.91 (0.79-1.06)	0.97 (0.88-1.07)
Circulating levels						
Main analysis (n=4067)	0.85 (0.77-0.95)	0.88 (0.82-0.96)	0.90 (0.85-0.94)	1.01 (0.92-1.11)	1.02 (0.95-1.10)	1.01 (0.96-1.06)
Excluding ≤2 y follow-up (n=3921)	0.86 (0.77-0.97)	0.89 (0.82-0.97)	0.90 (0.85-0.95)	0.98 (0.89-1.09)	1.00 (0.93-1.08)	1.01 (0.96-1.06)
Excluding fish oil supplement users (n=3880)	0.85 (0.76-0.94)	0.88 (0.81-0.96)	0.90 (0.86-0.95)	1.02 (0.92-1.11)	1.02 (0.95-1.10)	1.02 (0.97-1.07)

ALA, alpha-linoleic acid; CHD, coronary heart disease; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, FAs, fatty acids, MI, myocardial infarction.
Values in Table represent hazard ratios (HRs) with 95% confidence intervals, estimated from multivariable Cox models (see Methods).
HRs are expressed per 100 mg/d of dietary EPA+DHA, per 1 g/d of dietary ALA or per 1-SD of circulating EPA+DHA or ALA.
Numbers (n) refer to patients included in the analysis.

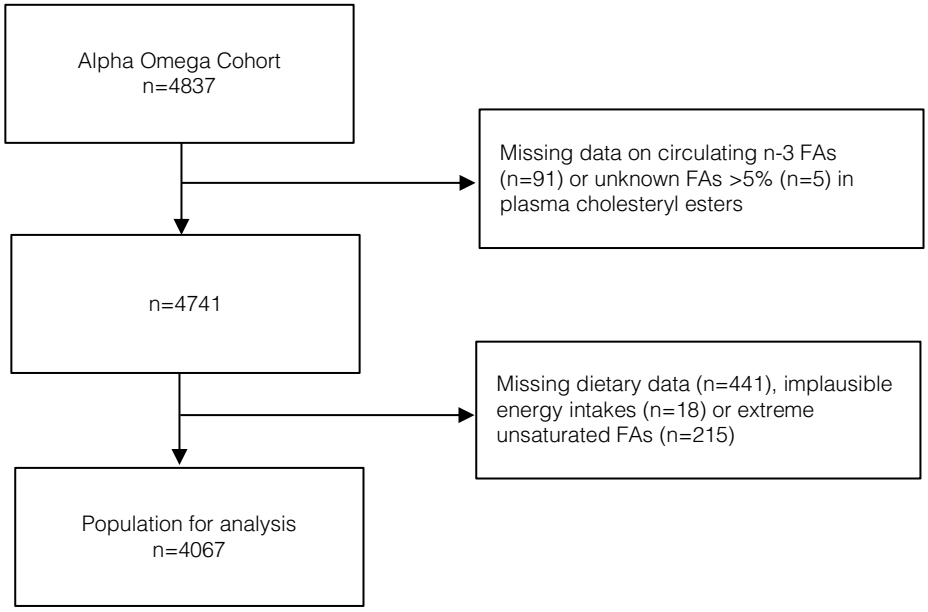

Figure S1. Flow diagram for selecting the population of analysis from the Alpha Omega Cohort. FA, fatty acids.

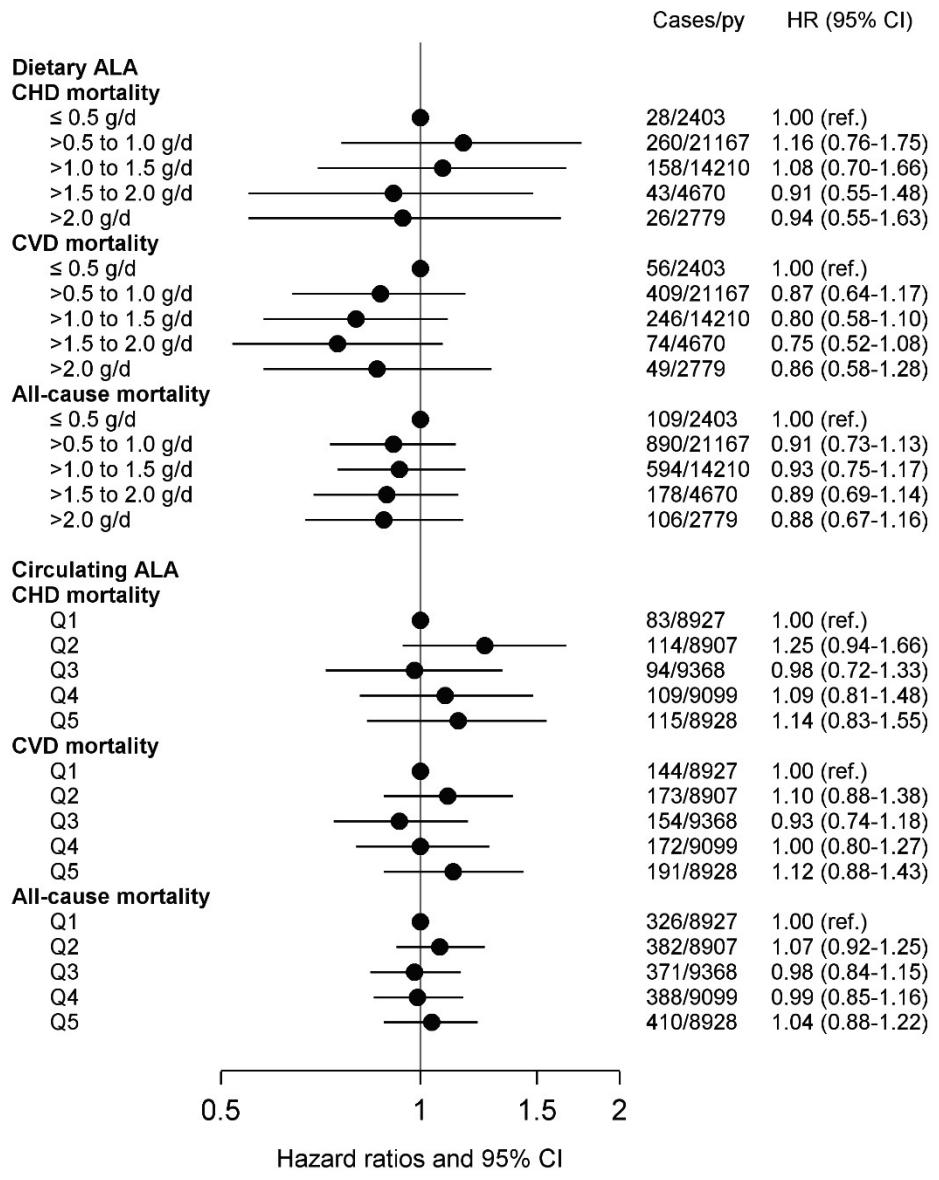

Figure S2. Associations of dietary ALA (in categories) and circulating ALA (in quintiles) with CHD, CVD and all-cause mortality.

ALA, alpha-linoleic acid; CHD, coronary heart disease; CVD, cardiovascular disease; py, person-years; Q, quintile; ref, reference. Hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained from multivariable Cox models, using the lowest category as reference; HRs for dietary ALA were adjusted for age, sex, education level, physical activity, smoking status, alcohol intake, obesity, prevalent diabetes, cardiovascular drugs, time sice MI, and intake of total energy, cholesterol, fiber and *trans* fatty acids; HRs for circulating ALA were adjusted for the same covariates except dietary factors, plus serum total cholesterol, circulating 18:2 n-6 and circulating 20:4 n-6; All *P* values for linear trend were >0.05.

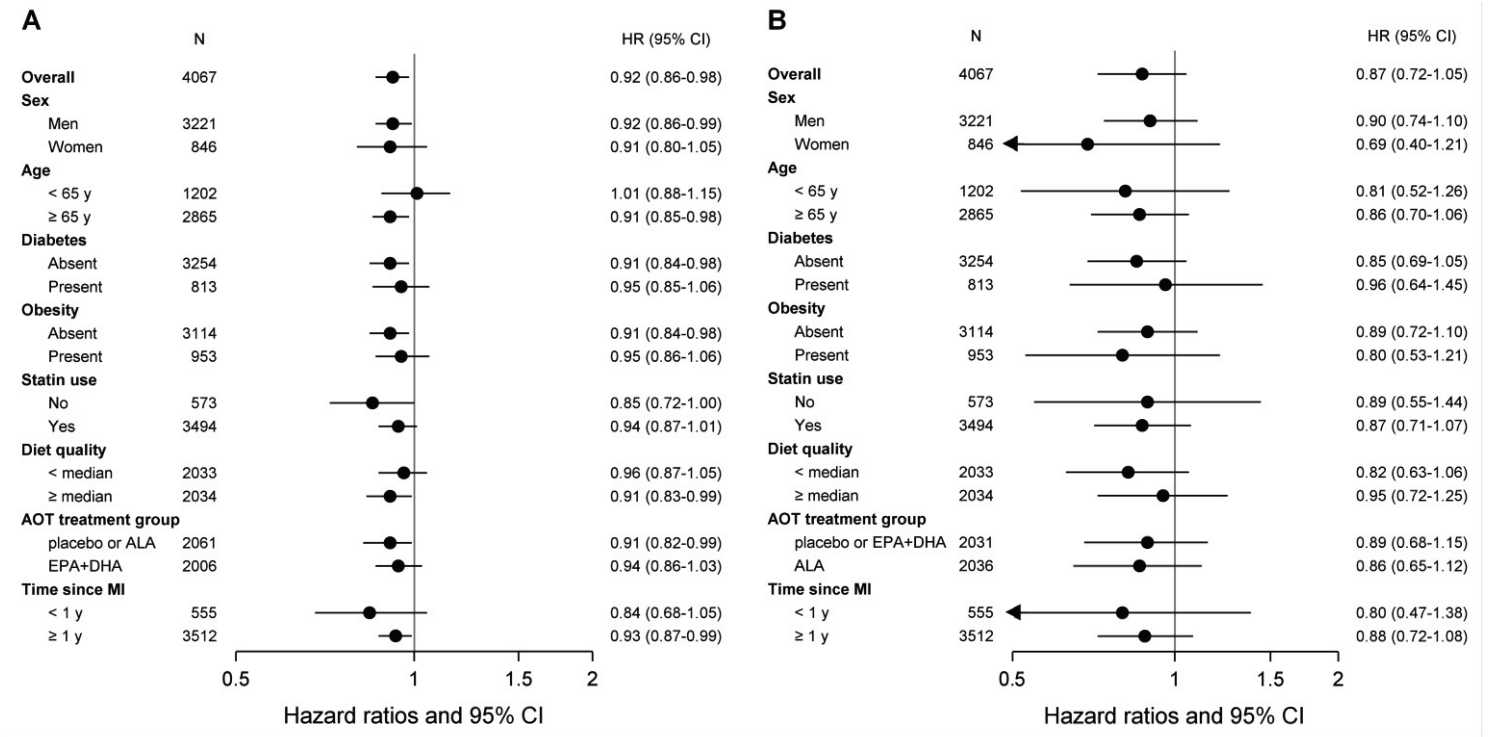

Figure S3. Associations of dietary (A) EPA+DHA (per 100 mg/d) and (B) ALA (per 1 g/d) with CHD mortality in subgroups of the Alpha Omega Cohort.

ALA, alpha-linoleic acid; AOT, Alpha Omega Trial; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained from multivariable Cox models (see Methods); dietary quality was based on the 2015 Dutch Healthy Diet score (DHD-15; median: 79, range: 33-125); all *P* values for interaction were >0.10.

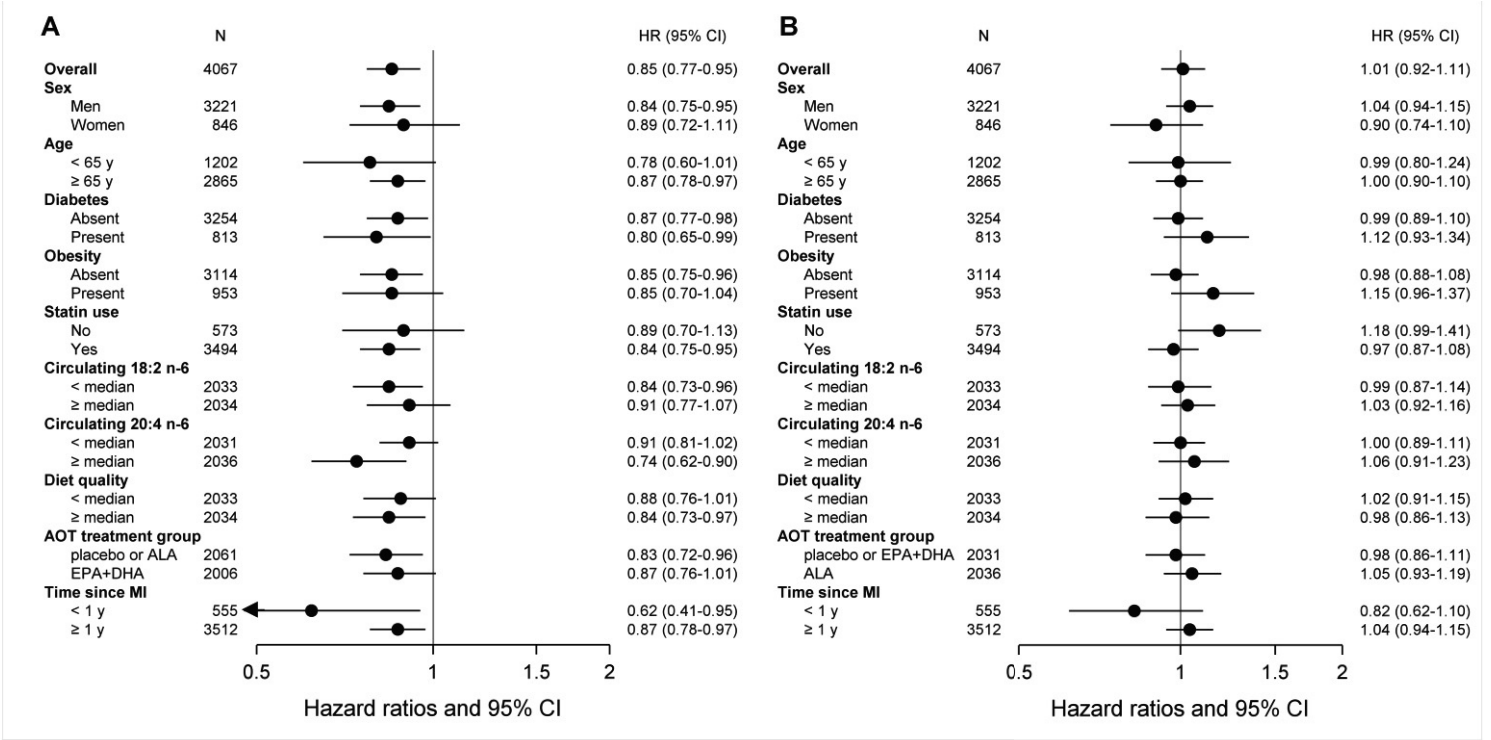

Figure S4. Associations of circulating (A) EPA+DHA (per 1-SD) and (B) ALA (per 1-SD) with CHD mortality in subgroups of the Alpha Omega Cohort.

ALA, alpha-linoleic acid; AOT, Alpha Omega Trial; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained from multivariable Cox models (see Methods); median circulating 18:2 n-6 (linoleic acid) was 49.96 % total FAs; median circulating 20:4 n-6 (arachidonic acid) was 8.26 % total FAs; dietary quality was based on the 2015 Dutch Healthy Diet score (DHD-15; median: 79, range: 33-125); for EPA+DHA, *P* for interaction was 0.07 for circulating 20:4 n-6; for ALA, *P* for interaction was 0.06 for statin use; all other *P* values for interaction were >0.10.

Chapter 7

General discussion

This Thesis set out to investigate the mutual relationship between dietary and circulating fatty acids (FAs) and their associations with cardiometabolic outcomes in prospective cohort studies, mainly in patients with a history of myocardial infarction (MI). This Thesis focuses on FAs that could be important for the prevention of cardiovascular diseases (CVD), of which circulating levels may be influenced by diet. These include polyunsaturated FAs, namely n-3 FAs (EPA, DHA and ALA), linoleic acid (LA, the main n-6 FA), and odd-chain FAs (15:0 and 17:0). Survival of post-MI patients has considerably improved in the past decades and the importance of diet in the prevention of recurrent CVD events and type 2 diabetes in these patients is increasingly acknowledged. It is therefore important to have a good understanding of the role of unsaturated FAs in cardiometabolic health in these patient groups. The concentrations of FAs in blood lipid pools, including plasma cholesteryl esters, are increasingly used as alternative, objective measures for self-reported dietary intakes in cohort studies. The role of dietary and circulating FAs in cardiometabolic health has mainly been studied in general populations, but is largely unknown for patients with coronary heart disease (CHD). Patients with CHD often have an altered metabolism due to multiple risk factors, presence of disease and treatment with cardiovascular medication, which can affect the circulating FAs and their utility as biomarkers of dietary FAs intake.

Results presented in this Thesis were mainly derived from the Alpha Omega Cohort, which consists of 4837 Dutch patients with a history of MI who received state-of-the-art cardiovascular drug treatment (**Chapters 2, 3, 4 and 6**). Additionally, data from the South African PURE cohort were used to study LA in relation to type 2 diabetes risk in more detail, focusing also on the role of the liver (**Chapter 5**). In this last chapter, the main findings of this Thesis are summarized and discussed for each of the FAs. Furthermore, methodological aspects are considered and directions for future studies are given.

MAIN FINDINGS

Table 1 presents the summary of the main findings of this Thesis according to the three specific objectives.

Table 1 Summary of main findings of this Thesis according to the specific objectives

Fatty acids	Objective 1: Association of dietary with circulating fatty acids	Objective 2: Association of dietary fatty acids with cardiometabolic outcomes	Objective 3: Association of circulating fatty acids with cardiometabolic outcomes
Unsaturated fatty acids			
EPA, DHA	Modest correlations (0.4 to 0.5)	Inverse associations with primarily CHD mortality and all-cause mortality	Inverse associations with CHD mortality, CVD and all-cause mortality
ALA	No correlations	Not associated with CHD, CVD and all-cause mortality	Not associated with CHD, CVD and all-cause mortality
LA	• Weak correlations (<0.2) • A lower circulating LA in those with statins use and high alcohol intake for the same intakes.	• No associations with type 2 diabetes risk • No associations with plasma glucose or HbA1c • Weak inverse associations with serum liver enzymes	• Inverse associations with type 2 diabetes risk • No associations with plasma glucose or HbA1c • Inverse associations with serum liver enzymes, especially gamma-glutamyl transferase
Odd-chain fatty acids			
15:0	• Correlated with dairy (~0.2) but not with fiber intakes • Highest circulating levels in those with high dairy intakes, irrespective of fiber intakes.		
17:0	• Correlated with both dairy, and fiber intakes (0.1 to 0.2) • Highest circulating levels in those with high dairy and high fiber intakes		

Polyunsaturated n-3 FAs

In Dutch post-MI patients from the Alpha Omega Cohort, dietary n-3 FAs mainly comprised ALA (72% of total n-3 FAs; median: 0.9 g/d) and EPA+DHA (8%; median: 108 mg/d) (**Chapter 2 and 6**). These patients consumed most EPA+DHA from fish, whereas dietary sources of ALA were cooking oils, grain products (e.g. bread, pasta) and margarine (**Chapter 6**). Circulating levels of EPA+DHA in plasma fractions of cholesteryl esters, phospholipids and total plasma were generally around 1% of total FAs, except for circulating DHA in plasma phospholipids which was 4.5% of total FAs. Circulating ALA was generally low in cholesteryl esters and phospholipids (<0.5%) (**Chapter 2**).

In post-MI patients, dietary EPA+DHA intake was well reflected in circulating EPA+DHA measured in three different plasma lipid fractions, with correlations between 0.4 and 0.5 (**Chapter 2**). Furthermore, dietary and circulating EPA+DHA were both inversely associated with CHD mortality risk (**Chapter 6**). In contrast to the findings for EPA+DHA, dietary and circulating ALA were not correlated (**Chapter 2**), and both dietary and circulating ALA were not associated with CHD mortality risk (**Chapter 6**).

Polyunsaturated n-6 LA

Dietary LA in post-MI patients was on average ~6 % of total energy (en%) (**Chapter 2**), which is within the range previously observed in populations with a Western diet.[1,2] The circulating levels of LA were the highest in plasma fraction of cholesteryl esters (50% of total FAs), followed by total plasma and PL (20 to 30% of total FAs). Correlations between dietary and circulating LA were weak (<0.2) in both post-MI patients (**Chapter 2**) and in South African individuals of whom 40% had hyperlipidemia (**Chapter 5**). Post-MI patients with higher alcohol intake and statin use and black South Africans reporting alcohol use had circulating LA levels that were lower than those without these characteristics, despite having similar dietary LA intake (**Chapter 2 and 5**).

In Dutch post-MI patients, inverse associations with T2D risk were observed for circulating LA in plasma cholesteryl esters but not for dietary LA (**Chapter 4**). The associations of circulating LA with T2D risk were attenuated after adjustments for FAs in *de novo* lipogenesis pathway (16:0 and 18:1 n-9) and 16:1 n-7/16:0. Additionally, the estimated delta-6-desaturase activity (18:3 n-6/18:2 n-6) and FAs in *de novo* lipogenesis (16:0 and 18:1 n-9) showed even stronger direct associations with T2D risk than those of LA. Further, circulating LA was inversely

associated with liver function markers, especially serum gamma-glutamyl transferase levels, whereas a weaker inverse association between dietary LA and serum gamma-glutamyl transferase levels was observed in the South African men and women (**Chapter 5**).

Odd-chain FAs

In Dutch post-MI patients, the average intake of dairy products was in the range of 300-350 g/d and fiber intake was ~22 g/d (**Chapter 3**). Main contributors to total dairy intake were milk (47%) and yogurt (22%), whereas major sources for fiber intake were grain products (43%), followed by fruits and vegetables (27%), potatoes (17%), and small amounts from beans and legumes (<7%). Circulating 15:0 and 17:0 in cholesteryl esters and phospholipids were very low (<0.5% of total FAs), with a higher proportion of 17:0 in phospholipids (as compared to 15:0), whilst in cholesteryl esters the proportion of 15:0 was higher.

In Dutch post-MI patients, circulating 15:0 and 17:0 in cholesteryl esters and phospholipids showed only weak correlations with dairy and dairy fat intakes (0.17-0.26 for 15:0 and 0.10-0.14 for 17:0) (**Chapter 3**). Interestingly, circulating 17:0 was also correlated with fiber intakes (0.09-0.19), whereas circulating 15:0 was not ($r \leq$ 0.05). Patients with high intakes of both dairy and fiber had the highest circulating 17:0. Elevated circulating 15:0 levels were observed in patients with high dairy intake irrespective of their fiber intake.

INTERPRETATION OF FINDINGS

Circulating levels of FAs that cannot be produced endogenously have been considered to be closely related to dietary FAs intake.[3] In observational studies, the identification of biomarkers for habitual FAs intake tends to be based on simple correlations between self-reported intakes and plasma concentrations, using single measurements. Several factors influence these correlations and need to be considered in the interpretation of associations of these FAs with cardiometabolic diseases. An overview of these considerations and how they impact the utility of FA biomarkers in epidemiological studies is given in Table 2, at the end of this section.

n-3 FA: EPA and DHA

Although EPA and DHA can be endogenously produced from ALA, they are mainly derived from the diet because of the limited conversion from ALA to EPA and DHA in the human body.[4] Despite low intake of fish (around 14 g/d) and EPA+DHA intake in the Dutch post-MI patients, the correlations between dietary and circulating EPA and DHA were similar to correlations of 0.4-0.5 previously reported in cohorts of generally healthy populations with varying intakes of EPA and DHA.[5–7] Furthermore, this Thesis shows that correlations between dietary and circulating EPA and DHA in post-MI patients are unlikely to be modified by factors that affect metabolism, including statin use, presence of diabetes, obesity and alcohol use.

In **Chapter 6**, higher dietary and circulating EPA+DHA were associated with ~30% lower long-term CHD mortality risk, respectively. Consistent inverse associations were found in the Alpha Omega Cohort for habitual dietary and circulating EPA and DHA and fish intake, in relation to CHD mortality. These findings suggest that in epidemiological studies in CHD patients, circulating EPA+DHA levels are reliable biomarkers of dietary intake of these FAs. Research on habitual EPA+DHA intake and mortality risk in CHD patients is limited and available studies are difficult to compare because of variations in range of intake, length of follow-up, type of CVD and clinical endpoints.[8,9] A cohort study in Norwegian CHD patients (~90% on statins) did not find an association between intakes of EPA+DHA from diet and/or supplements and CHD mortality.[10] However, the Norwegian patients had an EPA+DHA intake that was much higher (0.6 to 2.6 g/d) than Dutch post-MI patients in **Chapter 6**. An earlier review of cohort studies and randomized controlled trials (RCTs) suggested that dietary EPA+DHA up to 250 mg/d provide most of the reduction in cardiovascular risk in populations varying in CHD risk, with no substantial reduction above this range.[11] Two cohorts of patients with acute

coronary syndrome in Norway[12] and Argentina[13] reported no associations of circulating EPA and/or DHA with cardiac or sudden death. These studies had shorter follow-up (<5 y) and less events (<200 cases) than the Alpha Omega Cohort described in **Chapter 6**. Two cohort studies of 400-1000 patients with stable CHD with at least 5 y of follow-up showed a reduction of all-cause mortality risk ranging from 25% to 70%, comparing extreme categories of circulating EPA+DHA,[14,15] similar to our observations in a larger sample of post-MI patients. Evidence from the most recent meta-analysis of RCTs in those at high risk of CVD including CHD patients has shown that increasing EPA+DHA intake reduced CHD mortality risk by 10%, while not affecting CVD or all-cause mortality,[16] in agreement with our findings described in **Chapter 6**.

The inverse association of dietary EPA+DHA with CHD mortality in the Alpha Omega Cohort differs from findings of the Alpha Omega Trial which showed no effect of EPA+DHA supplementation on cardiovascular events for post-MI patients.[17] Because the associations in the Alpha Omega Cohort concern the habitual EPA+DHA intakes, which reflect a lifetime exposure to dietary EPA+DHA, a possible interpretation for the null findings in the Alpha Omega Trial is that there was no additional benefit of n-3 FAs supplementation above the habitual levels of EPA+DHA intake in these patients. The length of follow-up period might also be an explanation since Alpha Omega Cohort had a longer follow-up period than the Alpha Omega Trial (40 months). Other potential explanations are related to fish intake because most EPA+DHA intakes in post-MI patients were derived from fish. The inverse associations of EPA+DHA with CHD mortality might have reflected a higher consumption of other beneficial nutrients associated with fish consumption or a lower intake of other foods such as meat, and not necessarily EPA+DHA. The inverse associations in the Alpha Omega Cohort might also be due to the residual confounding associated with EPA+DHA or fish intake. EPA+DHA intake was higher in alcohol users and correlated with socioeconomic status in the Alpha Omega Cohort, as well as other studies.[18,19]

Dietary EPA and DHA assessed as self-reported intake and circulating levels are consistently correlated (0.4-0.5) in CHD patients, irrespective of blood lipid pool (plasma cholesteryl esters, phospholipids or total plasma). Thus, in epidemiological studies of CHD patients circulating EPA+DHA may be considered as a good biomarker of dietary EPA+DHA. Furthermore, in CHD patients of the Alpha Omega Cohort, both higher habitual dietary and circulating EPA+DHA (as well as fish intake) showed similar associations with a lower CHD mortality risk.

n-3 FA: ALA

The finding of no correlation between dietary and circulating ALA (**Chapter 2**) agrees with most observational studies, largely conducted in apparently healthy populations.[6,7] There are several possible explanations for the absence of correlation between dietary and circulating ALA. Results from isotope tracer studies have shown that a large proportion of dietary ALA (>75%) is directed towards oxidation for energy[4,20] and that only a small amount is incorporated in plasma phospholipids, which also explains its low conversion to longer-chain EPA and DHA.[20–22] In this Thesis, dietary ALA at habitual level was also not related to circulating EPA in the Dutch post-MI patients (**Chapter 2 and 6**). Additionally, incomplete or imprecise data of ALA content in the Dutch food composition table (NEVO)[23] may affect the validity of dietary ALA estimation.[24] The main food sources of ALA in these post-MI patients were (plant-derived) cooking oils (26%), margarines (14%), and grain products (19%) such as baked goods, which might include plant oils or seeds and walnuts as ingredients (**Chapter 2**). Any change in types and amount of plant oils used by the food industry affects ALA content in these foods of which data may not be quickly adopted into the food composition tables, thereby contributing to errors in dietary ALA intake estimation. Cohort studies of ALA intakes should pay attention to completeness and accuracy of ALA data in food composition tables, and enrich these tables with recent data from the literature and/or perform additional measurements of ALA content in foods, which was not done in this Thesis. Poor data on ALA intake will lead to misclassification of subjects, which impacts the associations with cardiometabolic disease endpoints (if present), likely resulting in an attenuation of relative risks (i.e. bias towards the null).

Generally, there is a lack of cohort studies on the associations of dietary and circulating ALA with mortality, and even less in CHD patients. Considering that

dietary ALA was not correlated with circulating ALA in this Thesis, the associations with mortality are discussed separately because the interpretation for dietary ALA associations may be different to those of circulating ALA. Dietary ALA was not significantly associated with risk of CHD mortality in Dutch post-MI patients (**Chapter 6**), in line with a recent meta-analysis of RCTs in patients at high risk of CVD.[16] However, in the small group of women (n=846) of the Alpha Omega Cohort, a HR of 0.69 (0.40-1.21) per 1 g/d higher ALA intake was observed. Previously, in the Alpha Omega Trial that took place during the first 40 months of follow-up, low-dose ALA supplementation (1 g/d) was related to a lower risk of CVD events specifically in women, a finding that approached significance (HR of 0.73; 95% CI 0.51-1.03).[17] Based on these findings, we cannot rule out the possibility that dietary ALA plays a role in the prevention of recurrent CVD in women. A recent meta-analysis of nine healthy cohorts indicated dietary ALA to be inversely associated with CHD mortality in younger populations (<60 y), in studies with ≥50% women and in Asian but not in European or North American cohorts.[25] However, for those with history of CVD in the Asian cohort, no association was observed between dietary ALA and CVD mortality which included deaths from CHD and stroke.[26] A study in 667 healthy older Dutch population showed that a higher intake of food sources of ALA that also contained *trans* FAs, such as margarine, cooking fat and baked goods, was associated with a higher risk of CHD, which disappeared after adjustment of *trans* FA intake.[27] This result suggests that ALA intake might be related to *trans* FAs. ALA intake was also associated with *trans* FAs in the post-MI patients (**Chapter 6**) and adjusted for in the analyses. Overall, the limited evidence including findings presented in this Thesis indicates that dietary ALA is not associated with mortality risk in CHD patients.

No significant associations with CHD, CVD and all-cause mortality were observed for circulating ALA in the Alpha Omega Cohort, although there was some evidence for a lower risk of fatal CHD specifically in women (HR of 0.90 per 1-SD; 95% CI 0.74-1.10), for which the study was underpowered (**Chapter 6**). To the best of our knowledge, there is one other study on the relation between circulating ALA and mortality risk in CHD patients. Kleber et al. studied 3259 German men and women referred for coronary angiography (78% with CHD) and showed that a higher circulating ALA in erythrocytes was associated with a lower all-cause mortality risk in women but not in men.[28] In the study of Kleber et al., the women were younger, had less CHD and used less lipid-lowering drugs compared to the men,[29] which may explain the different associations in women than in men in that study. In the Alpha Omega Trial (described above), supplemental ALA intake of 1 g/d was well reflected in plasma ALA levels, resulting in a borderline significantly lower risk of

recurrent CVD in women.[17] Based on the totality of evidence, it may be possible that cardiovascular benefits of ALA in women are only achieved at higher levels of ALA intake (e.g. >2 g/d). Whether higher circulating ALA through dietary or supplemental intake could play a role in secondary prevention of CVD warrants confirmation in statin-treated CHD patient cohorts with a larger number of women.

ALA intake in the range commonly consumed in Western populations (around 1 g/d) was not correlated with circulating ALA in Dutch post-MI patients. This hampers the use of circulating ALA as a biomarker of intake in cohort studies of CHD patients. In this Thesis, dietary and circulating ALA were not significantly associated with mortality risk, which supports the overall (limited) evidence that habitual ALA intake plays no major role in secondary CVD prevention. However, cardioprotective effects of ALA in female CHD patients, for which the study was underpowered, cannot be ruled out. Neutral results for dietary ALA may also be due to misclassification of exposure, resulting from inaccurate and incomplete dietary assessment and food composition tables.

n-6 FA: LA

Only weak correlations (<0.2) between dietary and circulating LA were observed in CHD patients (**Chapter 2**), despite the fact that LA is an essential FA that is exclusively obtained from the diet. The main contributors of dietary LA were similar to ALA in the Dutch post-MI patients, namely margarines (32%) and cooking oils (21%) (unpublished data). As discussed for ALA, errors may have occurred in dietary LA assessment, but the Dutch food composition table (NEVO 2006) used in **Chapters 2 and 4** in this Thesis showed more accuracy and completeness for LA than for ALA. Dietary LA is the major PUFA in the Western diet (4-6 en%),[2] and common cooking oils, e.g. sunflower and corn oil, contain much more LA (~60% of total fat) than ALA (~1.0%),[30] making estimations of LA intake less prone to error. Although not very likely, inaccuracies in LA intake assessment may have contributed to the weak correlation between dietary and circulating LA in post-MI patients (**Chapter 2**). The intake of LA of 5.7 en% estimated by FFQ in the Alpha Omega Cohort (**Chapters 2 and 4)** matches that of a general older population of Dutch men (5.6 en%) and women (5.5 en%), who completed two non-consecutive 24h dietary recalls in the Dutch Food Consumption Survey.[31] Furthermore, LA intake

of the post-MI patients was comparable to other large cohorts in the generally younger and healthy Western populations (4.4-5.5 en%) which reported stronger correlations (0.20 to 0.34) between dietary and circulating LA.[32–36]

For a given dietary LA intake, post-MI patients using statins or reporting high alcohol (>30 g/d for men or >20 g/d for women) had lower circulating LA than those without these characteristics (**Chapter 2**). Statin use and high alcohol intake in post-MI patients might affect circulating LA through influencing desaturase enzymes, in various ways.

First, an increased activity of the desaturase enzymes in the n-6 FA pathway results in a lower circulating LA and higher circulating levels of its desaturation products. Statin was shown to increase desaturation of LA in an *in vitro* study using human THP-1 cell line.[37] Additionally, some trials in hyperlipidemic individuals have shown a lower circulating LA[38–40] and higher 18:3 n-6,[39] 20:3 n-6[39] and 20:4 n-6[38,40] after statin treatment, suggesting that statin causes an overall increase in the conversion of LA to 20:4 n-6. A lower circulating LA in statin users compared to non-users were also shown in a more recent analysis of two British and two Finnish cohorts,[41] which further confirmed their findings in Mendelian randomization analyses of eight cohorts using the rs12916 in *HMGCR* gene, used as a proxy of life-long influence of statin.[41] The lower circulating LA in those using statins compared to non-users in post-MI patients might therefore be due to an increased delta-6-desaturase activity. Furthermore, statin use might also explain the weak correlation for LA in post-MI patients of whom the majority (~90%) were on statins (**Chapter 2**), while other cohorts reporting stronger correlations were performed in mainly healthy populations without users of lipid-lowering drugs[32,33,35,36] or with only small proportion (2-3%) using lipid-lowering drugs.[34]

Second, alcohol may induce changes in other major circulating FAs in the lipid pool, which in turn also affect circulating LA since circulating FAs in cohort studies are often measured relative to total FAs. A review on alcohol and PUFA has postulated a direct effect of ethanol, the main component of alcoholic drinks, on circulating PUFAs.[42] Ethanol metabolism in the liver causes an increase in lipid peroxidation, with chronic high intake of ethanol resulting in lower circulating PUFAs.[42] However results from later studies in different cohorts[43,44] indicated that the stearoyl-coA-desaturase-1 may also be involved. Similar to the post-MI patients in **Chapter 2**, cross-sectional and longitudinal analyses of three Finnish cohorts which comprised ~9800 adults aged 24-45 y showed that a higher ethanol intake was associated with not only a lower circulating LA but also a higher circulating total MUFAs, both relative to total FAs in serum.[45] Interestingly, ethanol intake was also directly

associated with the absolute concentration of total serum MUFAs, but not with absolute concentration of circulating LA.[45] A mechanism that could explain the observations in this Finnish study,[45] in the post-MI patients (**Chapter 2**) and South Africans reporting alcohol use (**Chapter 5**) is related to the effect of ethanol on the modulation of *de novo* lipogenesis by increasing activity of sterol regulatory element binding protein-1c (SREBP-1c) which regulates stearoyl-coA-desaturase-1, the enzyme converting SFAs to MUFAs.[46] It is therefore possible that the low circulating LA associated with (high) alcohol intake observed in this Thesis is a consequence of increased plasma circulating MUFAs.

Results presented in **Chapter 4** showed that dietary LA, theoretically modelled to replace similar energy from SFAs, was not associated with T2D risk in Dutch post-MI patients. This was found also in some,[47–49] but not all[50–52] population-based cohorts. For circulating LA and T2D risk, findings in this Thesis were in line with the pooled analysis of 20 cohorts in mostly healthy populations.[53] Apart from possible errors in dietary assessment of LA, the discrepancy between dietary and circulating LA associations with T2D risk in post-MI patients may be explained by an impaired liver metabolism affecting circulating LA. In post-MI patients circulating LA associations with T2D risk were attenuated after adjustments for 16:0, 16:1 n-7/16:0 and 18:1 n-9 (**Chapter 4**). These FAs and estimated delta-6-desaturase activity (18:3 n-6/LA) were also strongly associated with T2D risk in post-MI patients (**Chapter 4**). A lower circulating LA accompanied by higher LA desaturation products (18:3 n-6, 20:3 n-6), 16:1 n-7 and 18:1 n-9 characterizes the circulating FAs composition in individuals with non-alcoholic fatty liver disease.[54] In another cohort of slightly overweight but healthy elderly, circulating LA was inversely associated with liver fat while 16:0 was directly associated with liver fat.[55] Excess liver fat may also be caused by high alcohol intake,[46] which relationship with low circulating LA has been discussed in the previous paragraph. To verify the possible relation with an impaired liver function, the associations between circulating LA and liver enzymes were further examined (**Chapter 5**). A lower circulating LA was associated with higher levels of liver enzymes, particularly gamma-glutamyl transferase indicating worse liver function, but not with fasting glucose or glycated hemoglobin (HbA1c) in black South Africans. As liver fat accumulation is related to insulin resistance,[56] the associations of a low circulating LA with an impaired liver function may reflect insulin resistance in those with low circulating LA, which would explain the relation to a higher risk of T2D. However, this could not be confirmed in this Thesis due to lack of insulin data.

RCTs can provide more insight in whether dietary LA affects T2D risk. A meta-analysis of 13 RCTs (duration ≤16 weeks) in individuals without diabetes showed

that increasing dietary PUFAs (mainly LA) to replace SFAs and carbohydrates was associated with a decrease in fasting insulin and insulin resistance, but not with fasting glucose.[57] However, another meta-analysis including 11 RCTs of longer duration (≥24 weeks) concluded that the effect of n-6 FAs on plasma glucose, insulin resistance and the incidence of T2D is not yet clear because there was only a limited number of RCTs and these RCTs were considered to have a high risk of bias.[58] Experimental evidence in CHD patients is lacking. RCTs with a sufficiently large study sample and of longer duration (e.g. >24 weeks) of isocaloric replacement of dietary SFAs by LA or PUFAs on T2D risk are needed, specifically in statin-treated post-MI patients.

In post-MI patients, circulating LA was inversely associated with T2D risk whereas dietary LA was not. The correlation between dietary and circulating LA in these patients was weak (r <0.2). This weak correlation might partly be due to an increased delta-6-desaturase activity in statin users and/or increased circulating MUFAs due to high alcohol intake in these patients. Furthermore, impaired liver metabolism might also be associated with lower circulating LA. This suggests that the higher T2D risk in post-MI patients with low levels of circulating LA could reflect the (preclinical) metabolic disease process, rather than low dietary LA intake.

Odd-chain FAs

Odd-chain FAs (15:0 and 17:0) have been proposed as biomarkers of dairy intake because these FAs are only produced by gut microbiota in ruminants,[59,60] which therefore meets the assumption that these FAs originate from exogenous sources. Indeed, in this Thesis, circulating odd-chain FAs 15:0 and 17:0 were correlated with dairy intakes in post-MI patients (**Chapter 3**), similar to previous cohorts of healthy individuals.[61] Interestingly, circulating 17:0 was also correlated with fiber intakes in post-MI patients, and those with both high dairy and high fiber intakes had the highest circulating 17:0. This supported an earlier finding of an RCT in healthy individuals which reported increased circulating odd-chain FAs particularly 17:0 after a 7-day supplementation of inulin, a fermentable fiber,[62] and a limited number of cohort studies in healthy populations reporting correlations of 17:0 with dietary fiber or fiber-rich foods.[63–65]

Increased production of short-chain FAs by the gut microbiota from fiber fermentation, especially the three-carbon propionate that can be used as substrate for FA synthesis, may explain the relationship between dietary fiber and circulating odd-chain FAs.[62] However, this is not the only proposed mechanism for explaining variation in circulating odd-chain FAs levels in the human body. Another possible explanation is the biosynthesis of 17:0 through alpha-oxidation of 18:0,[66,67] although a direct relationship between fat from ruminant sources (including dairy) and circulating 15:0 was also confirmed in the trials.[66] Possible biosynthesis of odd-chain FAs from valine, a branched-chain amino acid which can be derived from dairy intake, has also been proposed.[68] In experiments involving *in vitro* and mouse (*in vivo*) models, valine has been shown to enhance production of 15:0 and 17:0 by activating PPARα-dependent alpha-oxidation and increasing availability of propionyl-coA as the precursor for *de novo* FA synthesis.[68] Controlled dose-response feeding studies in humans are warranted to assess and compare the effects of different fibers and dairy foods on circulating odd-chain FAs.

Although it remains to be confirmed, the relation between circulating 17:0 and fiber intake could have major implications for the interpretation of results from observational cohort studies. Higher levels of circulating odd-chain FAs were associated with a reduced risk of T2D in an analysis involving 16 prospective cohorts of non-diabetic individuals,[69] and mortality from CVD in a cohort of older adults.[70] In most studies, inverse associations with circulating odd-chain FAs were linked to dairy intake because of previously known associations of dairy intake and circulating odd-chain FAs.[59,60] However, the associations between 17:0 and fiber intake observed in our study and others[62–65] suggest that fiber intake (partly) accounts for the inverse associations of circulating 17:0 with cardiometabolic diseases. Potential pathways that could link circulating odd-chain FAs to a lower cardiometabolic disease risk may be elucidated through fundamental, (animal) experimental research, which was beyond the scope of the present Thesis.

Circulating odd-chain FAs 15:0 and 17:0 were related to dairy intake in post-MI patients, but circulating 17:0 was also related to fiber intake. Therefore, circulating odd-chain FAs may not be exclusive biomarkers of dairy intake. This has major implications for research in the field of nutritional epidemiology, when studying dietary intakes and cardiometabolic risk. Inverse associations of circulating odd-chain FAs, especially 17:0, with T2D risk in recent cohort studies may (partly) be attributable to dietary fiber, rather than dairy.

Table 2 An overview of assessment methods for selected fatty acids and their utility in cohort studies of CHD patients

Fatty acid	Assessment methods	Utility*	Considerations†	Interpretation of associations with cardiometabolic outcomes
EPA and DHA	Reported diet, with FFQ	+++	• Specific and limited major dietary sources (fish and seafood) allowing good estimation (+) • Intake is low, but major dietary sources less affected by changes in the food industry (+)	Reflecting intake of EPA and DHA and (oily) fish
	Circulating CE, with GC	+++	• Limited conversion from precursor (ALA) to EPA and DHA (+) • Levels are less affected by obesity, statin use and high alcohol intake (+)	Likely reflecting intake of EPA and DHA and (oily) fish
	Circulating PL, with GC	+++	• Same as for CE	Likely reflecting intake of EPA and DHA and (oily) fish
	Circulating total plasma, with ^{1}H-NMR	++	• Same as for CE • Not all individual n-3 FAs can be measured (EPA not available) (-)	Likely reflecting intake of DHA (and possibly also EPA and fish) when expressed relative to total FAs
ALA‡	Reported diet, with FFQ	+/-	• Relatively low intake (~1 g/d), and errors from food composition tables likely affect reliability of estimated intake (-)	May reflect diet, but improvements and updates of food composition tables may be necessary for accurate estimation
	Circulating CE, with GC	-	• Circulating levels likely not reflecting intake at habitual levels in Western diet due to rapid oxidation of most ingested dietary ALA (-)	Likely not reflecting diet at lower levels (~1 g/d) of intake
	Circulating PL, with GC	-	• Same as for CE (-)	Likely not reflecting diet at lower levels (~1 g/d) of intake
LA	Reported diet, with FFQ	++	• Relatively high intake (~12 g/d), from commonly consumed foods and more complete data in food composition tables due to LA being a major PUFA (+)	Likely reflecting diet
	Circulating CE, with GC	+	• Major FA in the fraction, better reliability coefficient than for other FAs with smaller proportions (+) • Levels may be influenced by an impaired metabolism, e.g. from metabolic syndrome, obesity, diabetes, (high) alcohol intake or statin use (-)	Likely reflecting metabolic (disease) processes, rather than diet
	Circulating PL, with GC	+	• Same as for CE	Likely reflecting metabolic (disease) processes, rather than diet

Table 2. continued

Fatty acid	Assessment methods	Utility*	Considerations†	Interpretation of associations with cardiometabolic outcomes
LA (*continued*)	Circulating total plasma, with ^{1}H-NMR	+	• Same as for CE	Likely reflecting metabolic (disease) processes, rather than diet
Odd-chain 15:0‡	Reported diet, with FFQ	++ (dairy)	• Ranking based on fat content of dairy products can be well estimated (+) • Content of odd-chain FAs in dairy foods may be affected by various factors, e.g. the feed types of the cattle (-)	Likely to reflect intake of dairy and dairy fat
	Circulating CE, with GC	++ (dairy)	• Ratio of 15:0 to 17:0 is similar to ratio found in dairy foods (+) • Correlation with dairy food is generally better than 17:0 (+) • Very low amount in plasma; reliability unknown (-)	Likely to reflect intake of dairy and dairy fat
	Circulating PL, with GC	+ (dairy)	• Very low amount in plasma; reliability unknown (-)	Likely to reflect intake of dairy and dairy fat
Odd-chain 17:0‡	Reported diet, with FFQ	++ (dairy)	• Same as for 15:0	Likely to reflect intake of dairy and dairy fat
	Circulating CE, with GC	+ (dairy)	• Very low amount in plasma; reliability unknown (-)	Likely to reflect intake of dairy, dairy fat and fiber
	Circulating PL, with GC	+/- (dairy) +/- (fiber)	• Not only reflecting dairy but also fiber intakes (-) • Very low amount in plasma; reliability unknown (-) • Proportion of 17:0 is higher than in CE (+)	Likely to reflect intake of dairy, dairy fat and fiber

CE, cholesteryl esters; FFQ, food frequency questionnaire; GC, gas chromatography; ^{1}H-NMR, proton-nuclear magnetic resonance; PL, phospholipids.
*Utility of the assessment method for studying associations between habitual dietary fatty acids and cardiometabolic diseases in CHD patients with Western diets: - not useful, + limited usefulness, ++ useful, +++ very useful, +/- not clear.
†Symbol "+" indicates an advantage and "-" indicates a disadvantage of the method.
‡ALA, odd-chain 15:0 and 17:0 cannot be measured using ^{1}H-NMR, therefore the utility of the circulating levels measured with this method is not evaluated in this Thesis.

METHODOLOGICAL CONSIDERATIONS

Methodological choices related to exposures and endpoints, study design, and analytic models could impact the validity of findings in this Thesis. In this section, several methodological aspects related to internal and external validity of the findings are discussed.

Measurement of dietary FAs

Most large cohort studies, including Alpha Omega Cohort and the South African PURE cohort, use the FFQ for estimating habitual intake, a dietary assessment method that is relatively easy to administer and process. The 203-item FFQ used in the Alpha Omega Cohort was an extended version from an original 104-item FFQ developed to specifically estimate intakes of FAs and cholesterol.[71,72] Separate questions were asked for foods with different fat content and a list of brand names for products was provided, which support its validity to accurately discriminate intake of foods with different fat content. For each food item, patients were asked to indicate the usual portion sizes and the frequency of consumption. When patients indicated consumption of a prepared food, such as cooked fish, additional questions were asked on the preparation method (e.g. fried, oven-baked, boiled, or steamed), and if during preparation fats/oils were used, the type of fats/oils was also asked, for which a list of brand names of fats and oils was provided to the patients.

The FFQ has been previously validated against dietary history and had high reproducibility for assessing the intake of most food groups, including edible fats and oils, with Spearman correlations up to 0.9.[72] The reported intakes were less likely affected by recall bias because the FFQ covered the intake of past month and not a longer time period, and patients in the Alpha Omega Cohort were less likely to have cognitive impairment, which was an exclusion criteria of the study.[73] The reported intakes of FAs (**Chapter 2**), dairy and fiber (**Chapter 3**) by post-MI patients in this Thesis were comparable with results from detailed food records obtained from the general elderly Dutch population.[31]

In PURE South Africa study, assessment of dietary intake was performed using a 145-item quantitative FFQ. This quantitative FFQ was a culture-sensitive FFQ covering the past month,[74] and was developed and validated in a similar population.[75,76] To facilitate better estimation of portion sizes, a food-portion photograph book that was specifically developed for this population was also

provided.[77] Preparation methods were asked in detail in the FFQ, which for example could include 13 additional questions related to preparation methods of carrots in different communities.[78] Recipes for traditional dishes used in the local communities were also collected during the study and used to complete information from the South African food composition table.[79]

In the cohort studies described in this Thesis, single measurements of dietary FAs obtained at baseline were examined in relation to cardiometabolic outcomes. Dietary FAs were assumed to be relatively stable over time. The Alpha Omega Cohort comprised post-MI patients aged 60-80 y consuming a traditional Dutch diet, and modification of dietary habits is not likely to occur at older age.[80] However, even when patients maintained their habitual diets during follow-up, the nutritional content of foods, particularly the commercial products, could have changed over time due to reformulation by the food industry. This may have caused misclassification of exposure and attenuated the associations of dietary FAs with type 2 diabetes and long-term risk of mortality, although this is less likely for associations of EPA+DHA that which almost exclusively obtained from fish (**Chapter 6**). Baseline measurements of dietary FAs were also used from PURE South Africa data, however, cross-sectional analyses were performed for associations of LA with serum liver enzymes and glucose metabolism markers (**Chapter 5**). Therefore, the misclassification of exposure due to changes in nutritional content of foods is not likely to affect the associations, because exposures and outcomes were measured at the same time point.

In studies of relation between dietary and circulating FAs, variations exist in units of dietary FAs, which may be expressed as percentage of total fat, percentage of energy intake or absolute intake. Several investigators argued that dietary FAs should be expressed as percentage of total fat to facilitate comparison with circulating FAs, often expressed as percentage of total FAs in lipid pools.[33,34] In this Thesis, dietary LA was expressed as percentage of energy, while EPA, DHA and ALA were presented as absolute intake (in grams or milligrams per day). LA substantially contributes to total daily energy intake, and therefore intake is expressed relative to energy intake. EPA+DHA, however, hardly contribute to energy intake and show no correlation with total energy intake. The use of these units facilitates comparison of this Thesis with those of other studies and with dietary recommendations. Furthermore, weaker correlations were observed when expressing the dietary FAs as percent of total fat (**Chapter 2**), which further support the choice of units used in this Thesis.

Measurement of circulating FAs

Circulating FAs are frequently measured as FA composition of a lipid pool and expressed in relative concentrations (% of total FAs) in cohort studies. **Figure 1** depicts circulating levels of selected FAs in cholesteryl esters and phospholipids in the Alpha Omega Cohort. The use of relative rather than absolute concentrations is preferred because of better correlations between dietary and circulating FAs,[81,82] as confirmed in **Chapter 2** for LA and DHA. However, expressing circulating FAs relative to total FAs also implies that the circulating levels of different FAs in a plasma fraction are interrelated. Therefore, circulating FA cannot be studied in isolation, because as the levels of one FA increase, the levels of one or more FAs decrease by default. It is therefore difficult to conclude whether associations between circulating FAs and cardiometabolic diseases are causal, and fully attributable to that specific FA. The interrelation of circulating FAs was recently illustrated in a Mendelian randomization study, showing that the variations in the circulating levels of most FAs related to type 2 diabetes risk were explained by variants in *FADS1/2* gene cluster encoding enzymes in n-3 and n-6 pathways[83] even though some of those FAs (e.g. 18:1 n-9 and 18:0) are not in the n-3 and n-6 pathways and therefore are not directly influenced by the enzymes.

In epidemiological studies of circulating FAs, it is recommended to also consider correlations with other major FA(s) in the studied lipid pool, and to consider associations of the different FAs with cardiometabolic diseases in a broader context. Different lipid pools have specific FAs composition, as shown in Figure 1. The relative influence of other FAs in lipid pools on risk estimates for LA in relation to type 2 diabetes have been examined in this Thesis, for example by examining the effect of additionally adjusting for 18:1 n-9 (oleic acid) on the risk estimate in the association of circulating LA in cholesteryl esters and type 2 diabetes, and by analyzing the association of 18:1 n-9 with type 2 diabetes (**Chapter 4**).

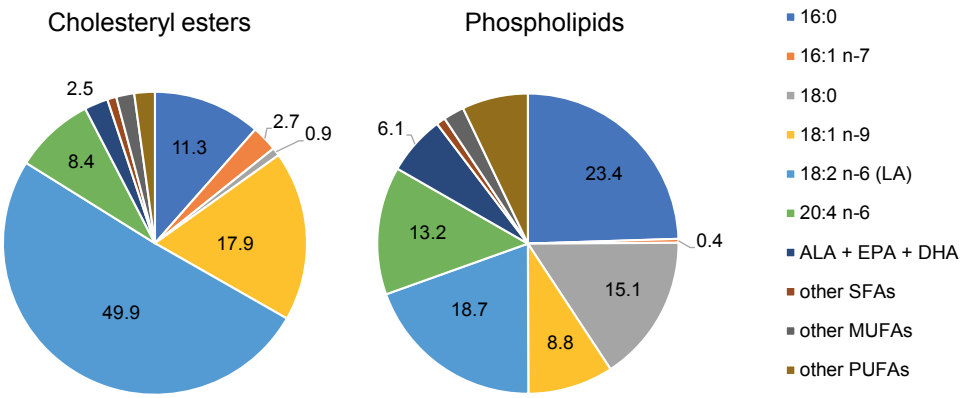

Figure 1 Fatty acid composition in plasma cholesteryl esters and phospholipids.*

*All values are expressed as percentage of total FAs. 16:0 indicates palmitic acid, 16:1 n-7, palmitoleic acid; 18:0, stearic acid; 18:1 n-9, oleic acid; 20:4 n-6, arachidonic acid.

In this Thesis only single measurements for circulating FAs at baseline were used, similar to most other cohort studies. It has been shown in a study of Ma et al.[84] using repeated measurements of circulating FAs in post-menopausal women that circulating levels of major FAs in cholesteryl esters and phospholipids, which are LA, 16:0 and 20:4 n-6, had a high reliability coefficient (R>0.65, with R being the proportion of total variance from between-person component). The short (2-weeks) and long-term (3-year) reliability for these FAs were comparable, with slightly better reliability for cholesteryl esters than phospholipids. As FA composition of plasma fractions are generally considered to reflect intake of past weeks, results of study by Ma et al. provided an evidence that circulating levels of FA in these fractions are relatively stable and may be used to reflect intake over a longer period, which also support the use of single baseline measurement of circulating FAs in cholesteryl esters and phospholipids in Alpha Omega Cohort and PURE South Africa study.

Bias and confounding

In prospective cohort studies, selection bias may occur, especially in case of differential loss to follow up. In the Alpha Omega Cohort, follow up was almost complete because only one patient was lost to follow up, making selection bias unlikely. In South African PURE study, of 3750 adults completing the screening questionnaire, only 2010 (54%) attended baseline measurement, of whom 633 participants with available circulating FAs data were analyzed. Compared to the

total cohort, more healthy participants may have been selected for the analytic sample, since the prevalence of HIV-infected individuals was very low (<1%) (**Chapter 4**) compared to the prevalent HIV in total cohort of PURE South Africa (16%).[85] Compared to the participants without HIV, those with HIV had a lower high-density lipoprotein cholesterol and a higher ratio of triglycerides to high-density lipoprotein cholesterol, although their serum concentration of liver enzymes and glucose (the outcomes of interest in **Chapter 5**), were similar.[85]

Observational studies may also suffer from information bias, related to differential misclassification of dietary and lifestyle exposures. To illustrate, in the present Thesis, alcohol consumption was an important confounder and effect modifier in associations of FAs with cardiometabolic endpoints. In the Alpha Omega Cohort, patients who were classified as non-drinkers may have deliberately underreported their alcohol intakes, or stopped drinking for health reasons ("sick quitters"). Alcohol use was inversely associated with circulating LA, and positively with dietary EPA+DHA in the Alpha Omega Cohort. Misclassification of patients for alcohol use, which is known to have a J-shaped relationship with fatal CHD, may have impacted our risk estimates in either direction. Underreporting and errors in alcohol intake assessment may also have occurred in the PURE South Africa cohort.[86] This could potentially have weakened the inverse association between dietary LA and serum gamma-glutamyl transferase in South-African individuals (**Chapter 5**). Likewise, misclassification may have occurred for other dietary and lifestyle factors. Patients wanting to give socially desirable answers may have underreported foods that are rich in calories, added sugars and salt, and overreported healthy foods, such whole-grains, fruit and vegetables. This could potentially have affected associations between dietary FAs and cardiometabolic endpoints, but is unlikely to have affected those for circulating FAs.

Misclassification of outcomes might have happened for type 2 diabetes (**Chapter 4**) and cause-specific mortality (**Chapter 6**), which could have caused imprecision in risk estimates for FAs with wider confidence intervals. Type 2 diabetes incidence was based on a physician diagnosis and/or start of anti-diabetes medication, which were both self-reported in the Alpha Omega Cohort. Missing data on plasma glucose during follow-up for the Alpha Omega Cohort will have led to underdiagnosis of incident T2D cases. In relation to mortality outcomes, since patients had prevalent CHD, it is possible that their disease status influenced the ascertainment of death causes. However, for both incidence of type 2 diabetes and mortality, the outcome misclassification is likely not affected by exposure status (non-differential) since the exposure (dietary or circulating FA) is not known by the physicians who ascertained the diagnosis of T2D or mortality causes.

Finally, residual confounding cannot be ruled out in observational studies. Fish intake, for example, tends to be part of a more healthy lifestyle in the Netherlands, as also shown by its positive association with education level, which may be a proxy for socioeconomic status (**Chapter 6**). Incomplete adjustment for factors related to fish intake may have led to overestimation for dietary EPA+DHA in relation to fatal CHD. In both cohorts presented in this Thesis, an FFQ was used for dietary assessment. The FFQ may not be optimal for some dietary exposures, for example salt consumption. High salt (sodium) intake is a risk factor for CVD, and salt intake may be associated with dietary FAs. Salt added to fish, for example, may have confounded associations of EPA+DHA with fatal CVD. Because salt increases CVD risk through an adverse effect on blood pressure, lack of adjustment may have masked some of cardioprotective associations for EPA+DHA. Risk estimates presented in this Thesis were adjusted for a wide variety of dietary and lifestyle factors, socioeconomic status, cardiovascular medication, and cardiovascular risk factors, where appropriate. Adjustment of overall dietary quality was additionally performed, by adding the 2015 Dutch Health Diet score (DHD15) to the multivariable models (**Chapter 6**).

External validity

Findings in this Thesis are mainly from the Alpha Omega Cohort in post-MI patients whose characteristics are rather different from general populations. Although they were stable, free-living patients who had similar dietary intakes as the general elderly Dutch population,[31] ~90% of the patients were treated with cardiovascular medication. As shown in this Thesis, associations between FAs and cardiometabolic endpoints may be influenced by the presence of disease and medical treatment. Therefore, the findings may not be generalizable to healthy individuals. However, our findings may be applicable to populations at high risk of developing CVD, for example those with metabolic syndrome. In line with results from the Alpha Omega Cohort, weak correlations between dietary and circulating LA were found in the South Africa PURE study, where approximately half of the cohort had dyslipidemia and/or hypertension. The Alpha Omega Cohort includes mainly patients of Dutch origin (94%) and findings may not be generalizable to non-Caucasian populations. Also, around 80% was male, and subgroup analyses in female patients lacked statistical power. Therefore, data on FAs and cardiometabolic endpoints in women and extrapolation of findings to female CHD patients in general should be done with caution.

FUTURE RESEARCH DIRECTIONS AND IMPLICATIONS

The Thesis indicates and highlights various research needs, which include studies that provide more insight in the role of particular FAs and the underlying biological pathways, as well as methodological studies in this field. Several suggestions for further research are given below.

1. *Elucidating the role of dietary FAs and modulating factors in the pathogenesis of cardiometabolic diseases.*

This Thesis showed that circulating LA was inversely associated with type 2 diabetes risk. It is important to investigate the mechanistic pathways for the relation of LA with type 2 diabetes risk, particularly the role of liver for LA. Whether statins and alcohol intake also affect type 2 diabetes risk through modification of circulating LA levels should be further investigated.

Odd-chain FAs have also been shown to be inversely related to type 2 diabetes in several cohort studies,[69] and in this Thesis it was shown that circulating 17:0 is also associated with fiber intake, which may be related to fiber fermentation by the gut microbiota. Future studies should investigate the mechanistic pathway for the relation of odd-chain FAs, including the role of gut microbiota.

2. *Improving the quality of FA data in food composition tables, particularly for ALA.*

Food composition tables may be incomplete and/or lack accurate data on ALA content of foods, especially for industrially processed products (e.g. margarines and cooking fats) for which product formulations tend to change over time. This Thesis showed a poor correlation between circulating ALA and dietary ALA intake, which may partly be due to inaccurate dietary assessment.

3. *Further study into the potential cardioprotective effects of ALA, specifically in female CHD patients.*

Although the studies in this Thesis lacked power for subgroup analyses in women, data suggested that higher intake of ALA could play a role in the prevention of recurrent events in female CHD patients. This finding needs to be confirmed in other secondary prevention studies, and asks for exploration of possible gender-related mechanisms related to ALA and CHD.

4. *Understanding differences in the relation of EPA+DHA with CHD mortality between Alpha Omega Cohort and Trial*

In the Alpha Omega Trial, post-MI patients who received supplemental EPA+DHA (400 mg/d) through margarines for 40 months experienced no reduction in risk of recurrent CVD events, including fatal CHD. In the Alpha Omega Cohort that included the same patients, baseline dietary and circulating EPA+DHA and fish consumption were associated with a ~30% lower risk of fatal CHD during >12 y of follow-up. Further research into these discrepant results could provide insight in the role of n-3 FAs and fish in CVD prevention and treatment.

5. *Exploring new methodologies to account for the intercorrelation of various circulating FAs in the lipid pool.*

Due to interrelationship of various circulating FAs, it is difficult to establish independent causal associations of circulating FAs with cardiometabolic disease, even when Mendelian randomization analysis was employed.[83] To take into account the correlations among circulating FAs, some studies[87,88] have used data-driven techniques such as principal component analysis and cluster analysis to derive FA patterns. However, the current practice of these data-driven techniques has been criticized, for example due to subjective or arbitrary methodological choices during the analysis that may affect the resulting pattern.[89] As these techniques can also be useful to discover new pathways, a guideline or good practice in performing these data-driven techniques needs to be established.

6. *Clarifying the role of dietary and non-dietary factors influencing circulating FAs in CHD patients.*

This Thesis showed that statin and (high) alcohol intake was also associated with circulating LA, and circulating 17:0 was associated with both dairy and fiber intake. However, the relative influence of statin use or alcohol intake compared to dietary LA for circulating LA, or influence of fiber compared to dairy intake for circulating 17:0 were not studied in this Thesis. To clarify the relative influences of various factors affecting circulating FAs, a controlled feeding study using a subsample of cohort study could be performed, such as done by Song et al. in the Women's Health Initiative cohort.[82] Unlike the approach used in most cohort studies which only collect information about participants' habitual diet, measure their circulating FAs and calculate the correlations between the two measures, the investigators of the study designed a two-week dietary intervention which maintained the variation in the habitual intakes of their participants.[82] This was done to mimic the dietary variation in cohort studies while reducing measurement error due to reporting of

intakes by the participants. The utility of circulating FAs was assessed by how much variation explained by dietary intake, which can be compared with the explained variation by other factors.[82] The information from such a study will also aid interpretation of the associations of circulating FAs when used as biomarker of dietary FAs in relation to cardiometabolic health.

7. *Exploring the use of combined measures of dietary and circulating FAs in relation to cardiometabolic outcomes.*

A combined measure of reported intake and biomarkers potentially increases the statistical power to detect diet-disease association,[90] but only few studies have used this, including an investigation on the relation of EPA, DHA and fish intake with incident atrial fibrillation.[91] Several important challenges for this approach are in determining the study design in which the use of combined measure is most cost-effective, in selecting which confounders to adjust for in the association of combined measure with disease, and the interpretation of results for public health implication.

8. *Examining of genetic variations in circulating FAs in relation to cardiometabolic outcomes.*

Possible influence of genetic variations is not yet accounted for and not studied in this Thesis although it may be relevant in post-MI patients. Circulating levels of n-3 and n-6 FAs are modified by the desaturase enzymes which are affected by the genetic variations in the *FADS* gene cluster.[92–95] Genetic variations may also influence the associations of dietary and circulating FAs[94] or associations of dietary or circulating FAs with cardiometabolic outcomes in cohort studies.[92,96] Statin use was not associated with higher circulating erythrocytes 20:3 n-6 (dihomo-gamma-linolenic acid) and 20:4 n-6 (arachidonic acid) in persons with TT genotype of rs174546 in the EPIC-Potsdam study.[93] In healthy populations, associations of circulating n-3 FAs with CHD mortality were not modified by variations in the *FADS* genotypes.[96] More investigations in populations with a different genetic background and distinct dietary patterns such as Africans and Asians are needed. Insight in genetic modifiers of FA metabolism could be important for personalized dietary advice to CHD patients.

Implications

This Thesis yields several new insights, which may have implications for the field of nutritional epidemiology, in particular research of FAs and cardiometabolic disease. These implications have been discussed in detail in the various Chapters and in the General Discussion, and are briefly summarized below.

- Plasma cholesteryl esters and phospholipids measured by gas chromatography are equally useful as lipid pools to measure circulating FAs in a cohort study, but measurement by ^{1}H-NMR may be less useful because of limited number of FAs that can be measured, although laboratory assessment is more cost-effective and relatively fast. Specifically for LA and DHA, total plasma measurements using ^{1}H-NMR seem to be as useful and reliable as other plasma fractions measured by gas chromatography.
- Not all circulating FAs are useful as biomarkers of habitual diet, which may even be true for FAs that are exclusively obtained from the diet. Circulating EPA and DHA were the most useful biomarkers among the FAs studied in this Thesis, while circulating ALA was not, and results for odd-chain FAs were mixed. Carefully interpreting the results from epidemiological studies using circulating ALA, and odd-chain FAs seems prudent.
- Results on circulating LA in cohorts of CHD patients or individuals with features of the metabolic syndrome warrant careful interpretation, since circulating LA may not reflect dietary LA intake in these populations.
- A low circulating LA may indicate metabolic impairment that involves the liver, which appears to be related to T2D. Therefore, low levels of circulating LA may be useful in clinical practice to identify CHD patients at risk of T2D. Its predictive value compared to other biomarkers of cardiometabolic health should be further studied.
- Higher intake of EPA and DHA from fish, reflected in circulating EPA+DHA levels, is associated with a markedly lower long-term risk of CHD mortality after MI. This information is important for the construction of dietary guidelines for CHD patients.

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7

English Summary

Fatty acids (FAs) are considered important dietary components for the prevention of cardiometabolic diseases. Epidemiological studies have been performed on dietary FAs, as well as circulating FAs in blood lipid pools, such as plasma cholesteryl esters. The role of omega-3 (n-3) FAs, which include eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) from fish and alpha-linolenic acid (ALA, 18:3 n-3) from plant sources, is extensively studied in relation to cardiovascular disease (CVD). More recently, omega-6 (n-6) linoleic acid (LA, 18:2 n-6) has received increased attention in relation to type 2 diabetes (T2D). In addition, circulating odd-chain FAs pentadecanoic acid (15:0) and heptadecanoic acid (17:0) in blood are increasingly used as biomarkers of dairy intake in cardiometabolic research.

Self-reported dietary FAs intake, often based on food frequency questionnaires, is a subjective measure and may be prone to measurement errors. Circulating FAs have been proposed as objective indicators of dietary intake of essential FAs, which need to be obtained from the human diet ("biomarkers of dietary FAs"). However, circulating FAs are also subject to metabolic processes. Patients with coronary heart disease (CHD), such as myocardial infarction (MI), and T2D patients likely have an altered metabolism due to the presence of disease and drug treatment, which may affect circulating FAs.

The main objective of this Thesis was to investigate the mutual relationships of dietary and circulating FAs and their associations with cardiometabolic outcomes. We first examined correlations between dietary and circulating FAs in post-MI patients (**Chapter 2 and 3**). Then, we obtained associations of both dietary and circulating FAs with risk of cardiometabolic outcomes, specifically T2D and mortality from CHD, CVD and all causes in these patients (**Chapter 4, 5 and 6**). Findings are largely based on the Alpha Omega Cohort of >4000 Dutch patients aged of 60-80 y (~80% men) with an MI up to 10 y before study enrolment, who received state-of-the-art cardiovascular drug treatment. Additionally, data from the Prospective Urban Rural Epidemiology (PURE) South Africa study among 633 black men and women over 30 y of age were analyzed.

In **Chapter 2,** circulating FAs were measured in plasma cholesteryl esters and phospholipids using gas chromatography, and in total plasma by proton-nuclear magnetic resonance (^{1}H-NMR). Dietary and circulating levels of EPA and DHA in the Alpha Omega Cohort were significantly correlated (r ~0.4), indicating that circulating EPA and DHA can be considered as biomarkers of intake in post-MI patients. For ALA, dietary and circulating levels were not correlated. For LA, correlations between dietary and circulating were weak (r <0.2). Patients using

statins and those reporting high alcohol intake (>20 g/d for women and >30 g/d for men) had consistently lower circulating LA levels, despite having similar dietary LA intake. This suggests that statins and alcohol impact the utility of plasma LA as a biomarker of dietary intake in post-MI patients.

Circulating odd-chain FAs (15:0 and 17:0), which comprise <1% of total FAs in blood, are increasingly used as biomarkers of dairy intake in epidemiological studies. Recent data, however, suggests that dietary fiber may also affect plasma odd-chain FAs, especially 17:0. **Chapter 3** presents correlations of dairy and dairy fat intakes with circulating 15:0 and 17:0 both in plasma cholesteryl esters and phospholipids in 869 post-MI patients. Correlations with dairy intake were 0.2-0.3 for 15:0 and ~0.1 for 17:0, similar to those found in healthy populations. Correlations of circulating 15:0 with dairy intake were not modified by fiber intake. Circulating 17:0, however, was associated with dietary fiber to the same extent as dairy. Highest 17:0 levels were found in patients who were in the upper categories of both fiber and dairy intake.

In **Chapter 4** we performed a prospective analysis of dietary and circulating LA in plasma CE (50% of total FAs) with incident T2D during 40 months of follow-up in 3257 post-MI patients without T2D at baseline. Dietary LA was not associated with T2D when replacing saturated FAs in a theoretical substitution model. However, higher circulating LA was significantly associated with a 27% lower T2D risk (per 5% increase). This discrepancy in findings for dietary and circulating LA may be related to their weak correlations, reported in Chapter 2, possibly due to impaired metabolism. Weaker associations were observed after adjusting for circulating 16:0 and 18:1 n-9 (FAs in *de novo* lipogenesis pathway), of which higher levels were also positively associated with T2D risk. This pattern of FAs (lower LA along with higher 16:0, 18:1 n-9, and downstream products of LA metabolism) is known to be associated with liver fat accumulation. These findings suggest that impaired liver function may partly explain the associations of circulating LA with T2D in post-MI patients, as further described in Chapter 5.

Chapter 5 describes the cross-sectional associations of dietary and circulating LA with markers of glucose metabolism and liver function in 633 black South Africans who took part in the PURE study. In South Africans men and women (~50% with dyslipidemia and/or hypertension), dietary and circulating LA were not associated with plasma glucose or glycated hemoglobin (HbA1c). Those with higher dietary LA had a non-significant better liver function, indicated by lower serum gamma-glutamyl transferase (GGT), although this association was only weak (8% lower serum GGT per 1-standard deviation increase (SD)). Higher circulating LA was

markedly associated with lower serum liver enzyme levels, in particular GGT (22% lower per 1-SD), which is associated with insulin resistance and liver fat accumulation. This association seemed to be stronger in alcohol users vs. non-users. These results further indicate the involvement of liver in circulating LA associations, which may partly be related to alcohol use.

Chapter 6 presents results for baseline dietary and circulating EPA+DHA and ALA, as well as fish intake, in relation to long-term mortality from CHD, CVD or any cause in the Alpha Omega Cohort. Patients consumed around 14 g/d of fish, and ~80% consumed less than 200 mg/d of EPA and DHA. During a median follow-up period of 12 y, 1877 patients died of whom 515 from CHD. Higher dietary EPA+DHA (>200 mg/d) was associated with a ~30% lower risk of CHD mortality, while weaker non-significant associations were observed for CVD and all-cause mortality. Circulating EPA+DHA showed a similar inverse association with CHD mortality, and also with CVD and all-cause mortality. These findings were corroborated by inverse associations with habitual fish intake, the main source of EPA and DHA. In contrast, dietary and circulating ALA were not significantly related to mortality endpoints, although there was some evidence for a lower risk of fatal CHD with dietary ALA specifically in women.

Chapter 7 provides an overall discussion of findings presented in this Thesis, with an overview of FAs biomarkers and their usefulness in epidemiological studies. This is followed by methodological considerations and suggestions for future research. As described in the different chapters, plasma cholesteryl esters and phospholipids can be considered as useful lipid pools for the assessment of circulating FAs in CHD patients. Total plasma measurement using ^{1}H-NMR may be a useful alternative for LA, but this technique also has limitations, including lack of information on EPA. Circulating LA, ALA and odd-chain FAs (particularly 17:0) may not reflect dietary intakes of these FAs or their dietary sources in CHD patients. With regard to cardiometabolic outcomes, lower plasma LA may be an indicator of a higher T2D risk. More research is needed to understand the role of LA in the pathogenesis of cardiometabolic diseases, possibly involving the liver. Circulating EPA and DHA are valid biomarkers of n-3 FAs from fish. This Thesis suggests that a higher intake of EPA and DHA may lower the long-term risk of CHD mortality after MI. The findings presented in this Thesis have implications for research in the field of nutritional epidemiology, and for the prevention and treatment of cardiometabolic diseases.

Acknowledgments

Finally I am at the end of my PhD journey! This is something I have always wanted to do, and although it took a while to get to this point, I am really proud of myself that I have worked hard, did not give up and stayed true to myself. This would not have been possible without help and support I received from many people, and I would like to take this opportunity to express my appreciation to them.

First, I would especially like to thank my promotor, **Marianne Geleijnse**. Marianne, thank you for your guidance, trust and support. I really appreciated your insightful questions during our discussions, all the opportunities and appreciation you have given me, and your advices on career and life. My daily supervisor, **Leanne Küpers**, thank you for your huge support and guidance. Leanne, I very much appreciated our meetings and your flexibility, especially during the thesis writing. It is really nice that I could also share my thoughts with you about academia and life in general, and that you also shared your thoughts and experience. Many thanks, **Dieuwertje Kok**, for your supervision at the start of my thesis. I really liked our weekly meetings, Dieuwertje. You always give very nice presentations in the MENU-D! I am so lucky to have worked with all of you, and for sure I learned a lot from you!

I would like to thank my thesis committee **Prof. Sander Kersten, Prof. Ingeborg Brouwer, Prof. Joline Beulens and Prof. Ronald Mensink** for your willingness to read and evaluate my thesis and for being present at the defense as opponents.

Many thanks to my collaborators: **Janette de Goede, Sabita Soedamah-Muthu, Marjolein Harbers, Anne Wanders, Peter Zock and Daan Kromhout**. Janette, many thanks for your help and guidance during the proposal writing and also for sharing about AOT. Thank you, Anne and Peter, for your support and collaboration during my PhD, and of course, your fatty acids knowledge! Sabita, Marjolein, Daan, thank you for your critical inputs! I also would like to thank the PURE study investigators for a good collaboration: **Salome Kruger, Tertia van Zyl, Lanthé Kruger, Marius Smuts**. Salome, thank you for your patience and for your quick replies for my many questions. Tertia and Lanthé, for your quick help to provide additional data during the peer-review round, and Marius for your critical inputs. I hope to be able to visit you all someday!

Eveline, thanks so much for your first introduction to the AOC folder, and for your patience for the many times I bothered you with my questions about AOC data. Thank you **Marlies** and **Paul** for your time and knowledge on fatty acid measurement. **Els** and **Jeanne**, thanks for your expertise on FFQ/diet data.

WUR, HNH and NAD group: **Ellen**, thanks so much for your trust, support and encouragement. **Cora** and **Agnes**, thanks for the opportunities and advices in

teaching, I learned a lot from both of you! **Nicole** and **Franzel**, thanks for your friendliness and curiosity! **Anneleen**, thanks for sharing your experiences and help during Rothman lunches. **Lydia**, thanks for connecting me to Marianne during the research master track. **NECo members**, thanks for letting me join and learn from you all. **Jasmijn, Gea, Lyda, Riekie, Rianne, Karin, Sjanneke,** thank you for your huge help! Thanks also to team **Vital@work De Bongerd** who kept me active during these COVID-19 times! SG program team and supervisory board: **Angela, Adri, Wiebe, Daphne, Brechtje, and Cor and other board members**: Thanks for having me! I had really good times during Open Mind Labs, was happy to contribute to SG programming, and I learned so much from all the discussions!

The MSc period was a defining moment for me to start a PhD. Thank you **Diewertje** and **Jeanne** for your supervision during my master thesis. I appreciated the freedom and all discussions we had. It was a very nice experience! **Linda**, thanks for your supervision during my internship. It was a tough period and things were not going well with the visa, but you were willing to think with me how to make the period as fruitful as possible. Thank you for all your help and flexibility.

Paulina and Esther, my super paranymphs, I thank you both for your support and good chats during my PhD. **Paulina**, you have been there from the start of my days in NL. It is so weird that we even ended up in the same office again (well, I guess I was the weird one in that office), and although we have totally different personalities, I am very thankful for this friendship. Thank you for your patience, different perspective of things, and for just being there, always! I hope to be there also at the end of your PhD, but if not, be sure that I am always supporting you wherever I am! **Esther**, it is not that long time ago that we know each other but it is always nice to have some chats with you! I also learned a lot from you during this short time. I wish you a good luck for your PhD! I am sure you will rock it. And of course, so nice that I can talk about football with both of you!

To all PhD colleagues, it was nice knowing you. I enjoyed the lunch walks and PhD tour with you. Thank you for being my dinner buddies during the tour, **Ruoxuan, Tsitsi, Xiaolin, and Anouk**. Thanks to fellow Newtrition editorial board members (**Janet, Merel, Jesca, Pol, Tesfaye, Matjaz**) for the collaboration, and **Marielle**, thanks for remembering me for NSD rides and your occasional help for my 'farm'! **Maria Salazar**, it's so sweet of you to remember my birthday, thanks very much! To friends who were formerly in WUR: **Ellen Smeets**, buddy! Although we were so busy in that period, it was also nice to do it together! Thanks for being my RMT buddy! **Famke Mölenberg**, you're the very first colleague of AOC when I started my PhD! It was so nice having Epi discussions with you. Thanks for the adventure in Portland

and for inviting me for BBQ at your house! Good luck for your PhD! **Stefania**, thanks for all the support, discussions and adventures! **Apple**, thanks for all the chit-chats, I will surely visit you when I'm in SG!

To (former) office roommates in 1055 (**Guido, Irene, Suzanne, Yfke, Ilse, Paulina, Rachelle, Erna, Maria Salazar, Carlijn, Berber**) and 2059 (**Iris, Miranda, Xiaolin, Annick, Susanne**). Thanks for your understanding and comradeship! Special thanks for **Dr. Guido Camps**, to whom I am deeply indebted for many things. Thanks for your advices, and also for letting me taking care of your shrimps and your friend! :)

I also would like to thank PhD colleagues in NAD group: **Moniek, Jesca, Harm, Merel, Anne, Liangzi, Vera, Iris, Carlijn, Laura, Ilse, Judith, and Koen**, and especially AOC colleagues: **Elly, Esther, Anniek, Maria Gorety**. Thank you for your feedback for my manuscripts and for learning together during the paperclub/MENU-D. To my students, **Sylvia, Ellen, Katinka, Emma, Luc**: I learned a lot during your master thesis, too! I wish you all the best for your future endeavors!

It was a pleasure to be part of **ICC international Choir**, **Hoev 4B**, and **LA5A**. You were part of my wonderful time in Wageningen! Let's keep in touch! Indonesian friends in NL: **Jovian & Cheri, Sisi & Willy, Sari & Royce, Sammy & Tammy, Geby & Rieza**, thanks for the stay over/dinner/lunch invitations and the fun board game times, and of course the eating adventures!

Thank you **ci Astri, ci Lina and Bu Susan**, for the trust, support, and opportunities during my time in Nutrifood, also for the Wageningen inspiration!

I am grateful for my long-time friends: **Hesti, Cath, Emilia**, I can't thank you enough for your friendship all these years! The wise oenjoe-ers**: Ivana, Rieza, Theo and Liem**, thanks for sharing your wisdom, friendship, and fun (karaoke) times!

The songs of **Epik High** have helped me through many difficult times. Tablo, Mithra Jin, DJ Tukutz, 고맙습니다! I've (also) done something wonderful :)

Mama, papa, Alin and Abe, terima kasih untuk semuanya. Let's be healthy and happy. Alin and Abe, sometimes I feel that I am the younger one. I am so lucky to have you as my brothers!

I am truly grateful for the wonderful times spent with the **Bieger and Sierink family**: **Annette, Mark, Theo & Claudia, Valentijn, Matthijs & Suz, oom John & tante Monique, Thierry, tante Carla, tante Ingrid, oom Henk Jan & tante Netty, oom Menno, and the Laheij family**. Bedankt voor alle warmte, aandacht en liefde die jullie mij altijd geven!

Oom John & tante Monique, so nice that you share my love for Indonesian foods, and your spare ribs BBQ are the best! Annette, thanks for your caring attention! It always felt like home whenever I visited your house. I will never forget your kindness.

Last but not least, dear **Jordi**, this thesis is also for you. Thank you for your patience, love and support during my PhD, and all the exciting football matches we watched together. The world is brighter with you! WZAWZDB!

Kamalita

About the author

CURRICULUM VITAE

Kamalita Pertiwi was born on the 21st of August 1987 in Jakarta, Indonesia, and raised in Bekasi. She went to Institut Pertanian Bogor (IPB) in 2005 where she majored in Food Technology and minored in Functional Management. She obtained her BSc degree in 2009 after investigating the safety of irradiated *rendang*. In 2009, her team representing IPB and Indonesia won the 3rd prize in the international Developing Solutions for Developing Countries competition organized by the Institute of Food Technologists Student Association in Anaheim (USA). She worked for five years at Nutrifood Indonesia, a food company, where she provided scientific support and helped organize two Mister International events. In 2014, she was awarded a scholarship and went to Wageningen University and Research (WUR), the Netherlands, to study Nutrition and Health with a specialization in Epidemiology and Public Health. She worked with Dr. Linda Oude Griep at Imperial College London on her minor thesis about potatoes and blood pressure, and obtained her MSc degree in 2016 with a master's thesis on the relation of glycemic index and load with blood lipids, supervised by Dr. Diewertje Sluik and Dr. Jeanne de Vries (WUR). Her PhD research proposal – written during the VLAG Research Master Track – was one of the four that was awarded funding through the Graduate Programme on Food Structure, Digestion and Health of the Dutch Research Council (NWO). In September 2016, Kamalita started her PhD trajectory in the Nutrition and Disease group. During her PhD, she was involved in the Fatty Acids and Outcomes Research Consortium (FORCE). She attended various (international) conferences and courses, won the 2nd prize for the best poster during NuGOweek 2017 and was a runner-up for the Foppe ten Hoor prize 2020. She enjoys teaching and was involved as supervisor of five MSc theses and in several BSc/MSc courses (on-campus and distance learning), and was also a member of the Education Committee of the Division of Human Nutrition and Health. She was an editorial board member of Newtrition (2016-2019) and was the Wageningen representative for the Indonesian Student Association in the Netherlands (PPI Belanda) (2018). She was also appointed the PhD member of the supervisory board of Studium Generale WUR (2019-2020). She holds a brown belt in karate, likes to sing, read (comic) books, watch football matches and Korean variety shows. She aspires to become a university teacher and researcher. Kamalita can be contacted by e-mail: iwitrepk@live.com.

LIST OF PUBLICATIONS

Peer-reviewed publications

Pertiwi K, Kok DE, Wanders AJ, de Goede J, Zock PL, Geleijnse JM. Circulating n-3 fatty acids and linoleic acid as indicators of dietary fatty acid intake in post-myocardial infarction patients. *Nutrition, Metabolism and Cardiovascular Disease*. 2019;29(4):343-350.

Pertiwi K, Küpers LK, Wanders AJ, de Goede J, Zock PL, Geleijnse JM. Associations of dairy and fiber intake with circulating odd-chain fatty acids in post-myocardial infarction patients. *Nutrition & Metabolism.* 2019;16:78.

Pertiwi K, Wanders AJ, Harbers MC, Küpers LK, Soedamah-Muthu SS, de Goede J, Zock PL, Geleijnse JM. Plasma and dietary linoleic acid and 3-year risk of type 2 diabetes after myocardial infarction: A prospective analysis in the Alpha Omega Cohort. *Diabetes Care*. 2020;43(2):358-365.

Pertiwi K, Küpers LK, Geleijnse JM, Zock PL, Wanders AJ, Kruger HS, van Zyl T, Kruger IM, Smuts CM. Associations of linoleic acid with markers of glucose metabolism and liver function in South African adults. *Lipids in Health and Disease.* 2020;19:138.

Aljuraiban GS, **Pertiwi K**, Stamler J, Chan Q, Geleijnse JM, Van Horn L, Daviglus ML, Elliott P, Oude Griep LM. Potato consumption, by preparation method and meal quality, with blood pressure and body mass index: The INTERMAP study. *Clinical Nutrition*. 2020;39(10): 3042-3048.

Expected publication

Pertiwi K, Küpers LK, de Goede J, Zock PL, Kromhout D, Geleijnse JM. Dietary and circulating long-chain omega-3 fatty acids and mortality after myocardial infarction: a 12-year follow up of the Alpha Omega Cohort. *In preparation.*

Van Westing A, Eckl MR, Küpers LK, **Pertiwi K**, Hoogeveen EK, Geleijnse JM. Plasma fatty acids and kidney function decline: a prospective analysis of the Alpha Omega Cohort. *Under review.*

Published abstracts

Pertiwi K, Kurniati A, Antono L, Sie S, Wu M. Glycemic index values may be correlated with body composition: preliminary findings. *Obesity Reviews*. 2014;15(Suppl 2):92.

Angela A, Antono L, Kurniati A, **Pertiwi K**, Sie S. Wu M. The effect of whey-to-casein protein ratio in chocolate-vanilla milk beverage on satiety and energy intake. *Obesity Reviews*. 2014;15(Suppl 2):189.

Pertiwi K, Lizuardi AB, Kurniati A, Sie S. Being first-degree relatives of type 2 diabetes: On body composition, insulin resistance and physical activity. *Diabetes Research and Clinical Practice.* 2014;106(Suppl 1):S218.

Pertiwi K, Lizuardi AB, Kurniati A, Antono L, Xie S, Yolanda V. The relationship between body composition, glycemic index, and glycemic response of foods in healthy Indonesian adults. *Diabetes Research and Clinical Practice.* 2016;120(Suppl 1):S68.

Pertiwi K, Oude Griep LM, Stamler J, Chan Q, Geleijnse JM, Steffen LM, Rodriguez B, Daviglus ML, Van Horn L, Elliott P for the INTERMAP Research Group. Relationship of potato consumption, total and by preparation method with blood pressure and body mass index: The International Population Study on Macronutrients and Blood Pressure (INTERMAP) US Study. *Circulation*. 2017;135:AP272.

Pertiwi K, Kok D, Geleijnse JM. Circulating biomarkers of linoleic acid intake. *Scripta Scientifica Pharmaceutica*. 2017;4:1

Pertiwi K, Wanders AJ, Zock PL, Harbers MC, Geleijnse JM. Circulating and dietary linoleic acid and prevalent diabetes in post-myocardial infarction patients. *Circulation.* 2018;137:AP227.

Harbers MC, **Pertiwi K**, Soedamah-Muthu SS, de Goede J, Molenberg FJ, Wanders AJ, Zock PL, Kromhout D, Geleijnse JM. Plasma and dietary linoleic acid and diabetes incidence after myocardial infarction. *Circulation.* 2018;137:AP215.

Pertiwi K, Küpers LK, Wanders AJ, Zock PL, Geleijnse JM. Circulating odd-chain fatty acids in relation to intake of dairy and fiber in post-myocardial infarction patients. *Circulation*. 2019;139:AP034.

Other publications

Irawati Z, **Pertiwi K**, and Zakaria FR. Toxicity test on malondialdehyde content and antioxidant capacity of irradiation sterilization rendang: in vitro (*Uji toksisitas terhadap kadar malondialdehida dan kapasitas antioksidan pada rendang steril iradiasi: in vitro)*. *Jurnal Ilmiah Aplikasi Isotop dan Radiasi*. 2010;6(1):31-45.

Angela A, Antono L, Kurniati A, **Pertiwi K**, Wu M. Effect of whey-to-casein protein ratio in chocolate-vanilla milk beverage on satiation and acute energy intake. *Kasetsart J. (Nat. Sci.).* 2015;49:738–746.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

	Organizer and location	Year
Metabolic Profiling in Disease Diagnosis and Personalised Healthcare	Imperial College London, London (UK)	2016
American Heart Association (AHA) EPI/Lifestyle Scientific Sessions	AHA, Portland (US)	2017
Nutriscience	VLAG/Human Nutrition and Health, Wageningen (NL)	2017
NuGOweek "*Molecular nutrition – understanding how food influences health"*	NuGO and Medical University of Varna, Varna (BG)	2017
AHA EPI/Lifestyle Scientific Sessions	AHA, New Orleans (US)	2018
FOODBALL final meeting	JPI FOODBALL, Wageningen (NL)	2018
Exposure Assessment in Nutrition Research	VLAG, Wageningen (NL)	2018
Dutch Nutritional Science Days	Nederlandse Academie van Voedingswetenschappen (NAV), Heeze (NL)	2018
AHA EPI/Lifestyle Scientific Sessions	AHA, Houston (US)	2019
Wageningen Indonesia Scientific Exposure	Wageningen Indonesia Platform, Wageningen (NL)	2019
Dutch Epidemiological Conference (WEON)	Vereniging voor Epidemiologie (VvE) and Universitair Medisch Centrum Groningen (UMCG), Groningen (NL)	2019
NuGOweek *"From Foodomics to Nutrigenomics: Translating food composition data into healthy diets"*	NuGO and Agroscope, Bern (CH)	2019
Dutch Nutritional Science Days	NAV, Heeze (NL)	2019
Symposium Pioneering Nutrition	Human Nutrition and Health, Wageningen (NL)	2019
TIFN Food Summit: Personalized nutrition – High hopes from the Low Lands	Top Institute Food and Nutrition, Wageningen (NL)	2019

General courses

	Organizer and location	Year
VLAG PhD week	VLAG, Baarlo (NL)	2017
4th Wageningen PhD symposium	Wageningen PhD council, Wageningen (NL)	2017
Introduction to R	VLAG, Wageningen (NL)	2017
Chemometrics	VLAG, Wageningen (NL)	2017
Scientific Writing	Wageningen Graduate School (WGS), Wageningen (NL)	2018
Teaching and Supervising Thesis Students	WGS, Wageningen (NL)	2019
Last Stretch of the PhD programme	WGS, Wageningen (NL)	2019
Career Orientation	WGS, Wageningen (NL)	2020
Philosophy and Ethics of Food Science and Technology	VLAG, Wageningen (NL)	2020

Optionals

	Organizer and location	Year
Preparation of research proposal	WU, Wageningen (NL)	2016
PhD study tour to East Canada	Division of Human Nutrition and Health, East Canada (CA)	2019
Staff seminars, group meetings, paper club	Nutrition and Disease, Wageningen (NL)	2016-2020

COLOPHON

The research described in this thesis was financially supported by the Dutch Research Council (NWO) through the VLAG Graduate Programme on Food Structure, Digestion and Health (number 022.006.009); and Unilever R&D Vlaardingen (until 1 January 2019) and Upfield™ (after 1 January 2019).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design and layout by Kamalita Pertiwi

Printed by ProefschriftMaken