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Fatty acids as biomarkers for health status and nutritional intake

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Fatty acids as biomarkers for health status and nutritional intake

Focus on dairy and fish

Ilse G. Pranger

Pranger, I.G.

Fatty acids as biomarkers for health status and nutritional intake. Focus on dairy and fish.

Thesis, University of Groningen, Groningen, The Netherlands

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Focus on dairy and fish

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CHAPTER 1

Introduction and aims of thesis

GENERAL INTRODUCTION

In the general population, cardiovascular disease (CVD) is a common cause of death[1,2]. Across Europe, CVD causes more than 4 million deaths each year, which accounts for almost 45% of all deaths[2]. Dietary and nutritional components are relevant players in the development and prevention of cardiovascular disease[3,4]. Fatty acids have been proposed as one of these components, which can be found in dairy (e.g. odd-chain fatty acids), fish (e.g. omega-3 fatty acids) and vegetable oils (e.g. Linolenic acid), common nutritional products of the Dutch diet[5].

Especially dairy products are an interesting source of investigation. First of all, the intake in the Netherlands is high. Additionally, it contains many different types of fatty acids, including both saturated and unsaturated, as well as both cis and trans fatty acids (*see framework*). Some of these fatty acids, such as the saturated odd-chain fatty acids as well as the trans fatty acids Vaccenic acid and Conjugated Linoleic acid, are thought to be rather unique for dairy products. Interestingly, recent research has suggested that the odd-chain fatty acids may reduce the risk to develop CVD[6-8]. Dairy fat is therefore an interesting target for further research to reduce cardiovascular risk in the general population.

Determining the precise effect of fatty acids from dairy products on health remains difficult. Current investigations rely mostly on dietary assessment tools, in which people record their daily consumption of products. These dietary assessment tools have their limitations. The data are not fully objective, and prone to bias and mistakes. Furthermore, dietary assessment tools only measure dairy fat intake, but do not take into account the uptake efficiency of the dairy fat into the human body and metabolism. To improve the reliability of the association between (dairy) fatty acids and cardiovascular health, new quantification methods are required, of which a direct measurement of fatty acids in blood samples represents an interesting example. Unique (combinations of) fatty acids present in blood can give insight in the consumption and uptake rate of the dairy products. In this thesis, we will explore the potential of different (combinations of) dairy fatty acids as potential biomarkers of dairy fat intake in the general population. In addition, we want to explore whether the potential biomarkers can be linked to (cardiovascular) health status.

An introduction to fatty acids

Biochemistry

Fatty acids are rarely available as free fatty acid in nature. Most of the time they are the basic structural components of triglycerides, but they are also found as part of phospholipids or linked to cholesterol[9,10]. Naturally occurring fatty acids are typically composed of carbon chains with a methyl group at one end of the molecule, and a carboxyl group at the other end[11,12]. Fatty acids can be categorized according to chain length, number of double bonds, position of the double bonds (positional isomers) and configuration of the double bonds (geometric isomers)[13]. The most common fatty acids have chain lengths between 10 and 22 carbon atoms and are in large quantities present in plant oils and animal fats[11,12]. Fatty acids can be divided into saturated fatty acids (SFA), which are usually straight fatty acids with no double bonds, and unsaturated fatty acids, fatty acids with one (MUFA) or more double bonds (PUFA)[14]. Saturated fatty acids typically have an even number of carbon atoms (C12:0 – C18:0), but odd chain fatty acids can be found in nature as well (C15:0 and C17:0). These odd-chain fatty acids may either be de novo synthesized by some bacterial species using propionic acid or some other odd-short chain fatty acid as starter unit (instead of acetic acid), or by alpha-oxidation of even-chain fatty acids such as C16:0 (to C15:0) or C18:0 (to C17:0)[15]. Unsaturated fatty acids are either in cis- or trans configuration[14]. Well known cis fatty acids are the omega-3 fatty acids present in fish and some vegetables oils. Trans fatty acids are often produced during industrial hydrogenation (e.g. trans-C18:1(n-9)), but can also be found in natural sources such as dairy (e.g. trans-C18:1(n-7))[16]. The trans fatty acids in milk derive from synthesis by the bacterial flora in the rumen of the animal.

Metabolism

The liver and adipose tissue are capable of synthesizing fatty acids de novo, particularly in the case of a carbohydrate rich diet. De novo lipogenesis (DNL) mainly produces palmitic acid (16:0), which can subsequently become elongated to stearic acid (18:0)[17]. 16:0 and 18:0 can then be desaturated into palmitoleic (16:1(n-7)) and oleic (18:1(n-9)) acids, respectively by $\Delta 9$ -desaturase (also called stearoyl-CoA dehydrogenase (SCD))[18,19]. Two other fatty acid metabolic pathways that can be found in the human body are the omega-6 and omega-3 pathway. The omega-6 fatty acid Linoleic acid (LA) and the omega-3 fatty acid alpha-linolenic acid (ALA) are two parent essential fatty acids that cannot be synthesized in the human body, and have therefore to be taken up from the diet (notably from vegetable oils, nuts and seeds). Subsequently, LA and ALA can undergo elongation and desaturation by $\Delta 5$ and $\Delta 6$ -desaturase[20-22]. LA can be converted into arachidonic acid (AA), which is a precursor of prostaglandins, while ALA can be converted to eicosapentaenoic acid (EPA) and eventually to docosahexaenoic acid (DHA)[20-22]. The last step from EPA to DHA includes β -oxidation as well[22].

Fatty acids from dairy products

Worldwide intake of dairy products is high, particularly in developed countries such as North America and the European Union, including the Netherlands[23]. According to the Dutch Food Consumption Survey, intake of the Dutch adult population (age 31-50 years) is approximately 283 and 253 g/d for respectively males and females[24]. Dairy contains more than 400 different fatty acids[25]. Seventy percent of the fatty acids present in milk are SFA, with palmitic acid (C16:0) (22-35wt%), stearic acid (C18:0) (9-14wt%) and myristic acid (C14:0) (8-14wt%) as the most prominent ones[25,26]. The odd-chain fatty acids pentadecanoic acid (C15:0 (1-2wt%)) and heptadecanoic acid (C17:0 (0.5-1.5 wt%)) can also be found in dairy products[26]. These are mainly produced from C16:0 and C18:0, under influence of microbial fermentation. This process is called α -oxidation and is considered rather unique for the bacterial flora of the rumen[15,27]. C15:0 and C17:0 are therefore thought to mainly originate from dairy fat[15,25,26]. Approximately 25% of the fatty acids in milk are MUFA, of which oleic acid (C18:1) is the main one (20-30wt%). Poly-unsaturated fatty acids, such as linoleic acid (C18:2) and α -linolenic acid (C18:3), account for around 0.5-3wt% of the total fatty acids in milk[25,26,28]. Approximately 2.7% of the milk fats are trans fatty acids[25,29,30]. Dairy fat mainly contains the trans fats Vaccenic acid (trans-C18:1(n-7))[31-33] and Trans-palmitoleic acid (trans-C16:1(n-7))[34], which are formed by microbial fermentation in the rumen of the animal. Dairy products also contain conjugated linoleic acids (CLA) of which rumenic acid (cis-9, trans-11 CLA) comprises more than 90% of the total CLA[30,32,35-37].

Intake of dairy fat and cardiovascular health

Total dairy intake and dairy fat intake have been proposed as nutritional components that may have a beneficial effect on cardiovascular health. For example, dairy intake may be associated with a reduced risk of coronary heart disease and stroke[38]. There is also evidence to suggest that higher intake of dairy is linked to a lower risk of type 2 diabetes[39,40]. In addition to total dairy intake, fat from dairy products may have beneficial effects on cardiovascular health and risk factors of cardiovascular disease[41-48]. These risk factors for cardiovascular disease include (1) traditional risk factors such as BMI, blood pressure and total triglycerides; (2) diabetes related risk factors such as fasting glucose and HbA1c%; and (3) non-traditional risk factors such as inflammation[49-52] (**Figure 1**). However, due to the presence of saturated and trans fatty acids in dairy, there is also skepticism about the potential protective effects and it has even been suggested that dairy fat is unhealthy[53,54]. Therefore, more research is warranted to unravel the association between dairy fat and cardiovascular health.

Figure 1: The potential effects of fat from fish and dairy on cardiovascular risk factors.

Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin; HDL, high density lipoprotein; Hs-CRP, High-sensitive C-reactive protein; LDL, low density lipoprotein

Up till now, the most common method to measure the association between dairy (fat) intake and cardiovascular disease risk has been done by measuring dairy (fat) intake by dietary assessment tools such as food frequency questionnaires (FFQ), food diaries or 24h recalls. Of these dietary assessment tools, the FFQ is the most convenient one. This latter questionnaire is able to capture usual, individual, and long-term dietary intakes[55,56]. Although questionnaires and diaries are widely used, data are subjective and often biased. The methods are observational and based on self-report, which can lead to misclassification, underreporting and/or overreporting of intake[57]. A definite conclusion about the causality of the relationship between dairy fat intake and cardiovascular disease risks is therefore impossible to find. Researchers have therefore shown an increasing interest in biomarkers of dairy fat intake. Biological markers are considered to be more objective parameters of dairy intake.

Dairy fat biomarkers

The fatty acid compositions of plasma/serum (cholesterol esters (CE), triglycerides (TG), phospholipids (PL), free fatty acids (FFA), or a combination of the four), erythrocytes and adipose tissue have been shown to partially reflect the fatty acid composition of the diet[9,41,42,58]. Adipose tissue can be investigated to determine long-term dairy fat intake, while plasma and

serum are used to estimate short-term intake, in which triglycerides reflect intake of the past few hours, and cholesterol esters and phospholipids of the past few days[9,59].

Several fatty acids are to a large extent unique for dairy fat, and are therefore an interesting target for further research on dairy fat biomarkers. In short, C14:0, C15:0, C17:0 and Trans-C16:1(n-7)) are commonly used dairy fat biomarkers[8,41-45,60,61]. And although these biomarker are widely accepted and used, interest in other potential dairy fat biomarkers is growing. For instance, trans-C18:1(n-7) and CLA have been suggested as potential dairy fat biomarkers, since these are also thought to mainly originate from dairy derived products[31,32,62,63]. In **Chapter 2**, we aim to summarize the commonly used dairy fat biomarkers and investigate which fatty acids can serve as potential dairy fat biomarkers as well (**Table 1**).

Table 1: Fatty acids as biomarkers for nutritional intake in the general population

	Animal Fat		Industrial Fat
	Dairy	Fish	
Previously investigated biomarkers	C14:0	C20:5(n-3)	Trans-C18:1(n-9)
	C15:0	C22:6(n-3)	
	C17:0		
	Trans-C16:1(n-7)		
Potentially new biomarkers	Trans-C18:1(n-7)	C15:0	
	CLA	C17:0	

Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; C20:5(n-3), Eicosapentaenoic acid, C22:6(n-3), Docosahexaenoic acid; CLA, Conjugated Linoleic acid; Trans-C16:1(n-7), Trans-Palmitoleic acid; Trans-C18:1(n-7), Vaccenic acid; Trans-C18:1(n-9), Elaidic acid

The less commonly investigated biomarkers trans-C18:1(n-7) and CLA have hardly been explored. Potentially, this might be because by difficulties to separate trans-C18:1(n-7) and other trans fatty acids in commonly applied measurement techniques. One of the trans fatty acid that is hard to separate from trans-C18:1(n-7) is trans-C18:1(n-9), or otherwise called Elaidic acid, an industrially produced fatty acid. To be able to investigate trans-C18:1(n-7) as a new potential dairy fat biomarkers, we aim to investigate a new method to separate trans-C18:1(n-7) from trans-C18:1(n-9) (**Chapter 3**). Additionally, we aim to investigate the commonly and less commonly used dairy fat biomarkers in **Chapter 4**.

Dairy fat biomarkers and cardiovascular health

The commonly investigated biomarkers C15:0, C17:0 and to a lesser extent C14:0 and trans-C16:1(n-7) may potentially have a positive influence on cardiovascular health[41-48]. Although this earlier research showed promising results, as of today some researchers question whether the association between the biomarkers C15:0 and C17:0 with cardiovascular health can only be

ascribed to dairy fat intake. Recently, C15:0 and C17:0 have also been suggested as biomarkers for fish fat intake[64-66]. In addition, both fish intake and fish fat intake are inversely related to cardiovascular diseases[67-69]. Consequently, associations of fatty acids such as C15:0 and C17:0 with cardiovascular health may as well be attributable to fish fat intake rather than to dairy intake. Therefore, in **Chapter 5**, we aim to investigate whether the commonly and less commonly investigated dairy fat biomarkers are solid biomarkers to predict dairy fat intake in the general population. Furthermore, we aim to investigate whether these fatty acids are related to good cardiovascular health, and so whether the potential cardiovascular health effects can be ascribed to dairy fat intake in the general population.

Intake of fish, omega-3 fatty acids and (cardiovascular) health

In observational studies both fish intake and fish fat intake have been found to be inversely related to the development of cardiovascular diseases and mortality[67,68,70,71]. Although these effects might be significantly associated with the fatty acids C15:0 and C17:0, the actual beneficial effects have predominantly been ascribed to the omega-3 fatty acids EPA and DHA[72-74]. Several papers have suggested that EPA and DHA may cause a reduction in cardiovascular events[69,73,75,76]. Evidence suggest that this may be by influencing fatty acid ratios in the body (e.g. AA/EPA ratio)[77,78]. However, evidence from Randomized Controlled Trials are still confusing. In 2018 two studies were published on the association between omega-3 fatty acid intake and cardiovascular diseases[78]. One study (VITAL-Study) found that the intake of omega-3 fatty acids was not associated with cardiovascular health benefits[79], while the other study (REDUCE-IT Trials) showed significant cardiovascular benefits[80]. Potentially, this might be due to the dose used in the RCTs (0.84 grams vs 3.8 grams of omega-3 fatty acids in respectively the VITAL-Trial and the REDUCE-IT Trials). From clinical trials and observational studies it has been demonstrated that omega-3 fatty acid consumption may have a favorable effect on traditional risk factors, such as serum triglycerides, systolic and diastolic blood pressure, but also on other risk factors such as inflammation[81-89] (**Figure 1**). Furthermore, in experimental animal studies EPA and DHA prevent the development of impaired glucose tolerance[90], while in humans it has been suggested to have a clinical significance in the prevention and reversal of insulin resistance[91,92].

Omega-3 fatty acids and (cardiovascular) health in renal transplant recipients

Renal disease patients are an important group that may particularly benefit from omega-3 fatty acid intake. Renal transplantation is the standard treatment for most renal disease patients[93,94], which improves survival. However, the survival of renal transplant recipients (RTR) continues to be significantly lower compared to the general population[95]. Importantly, one of the excessive contributors to mortality in RTR is cardiovascular disease[96,97]. Since omega-3 fatty acids may

protect against cardiovascular risk in the general population, supplements of EPA and DHA have been suggested to reduce all-cause and cardiovascular mortality in RTR[98]. Whether this is also true for the less investigated omega-3 fatty acid ALA is unknown. We therefore aim to explore the (cardiovascular) health associations of the omega-3 fatty acids EPA, DHA and ALA intake in RTR in **Chapter 6 and 7**.

While the association between the omega-3 fatty acid status and (cardiovascular) health can potentially be improved by the intake of the omega-3 fatty acids EPA and DHA, the association might be worsened by the use of drugs such as glucocorticoids (e.g. prednisolone) [20,21]. Glucocorticoids are anti-inflammatory drugs commonly used to treat both acute and chronic inflammatory diseases[88,99], and are also commonly used in RTR to protect against graft failure[100-102]. From animal studies, it is suggested that glucocorticoids may negatively influence long chain omega-3 polyunsaturated fatty acid status, by inhibiting their formation through diminishment of delta-6 desaturase (D6D) activity[21,103]. Whether this is also the case in humans is unknown. Therefore, in **chapter 8**, we aim to investigate the dose-dependent influence of prednisolone on the EPA and DHA status, D6D activity, and the influence on cardiovascular risk factors in healthy young males.

Aims and outline of the thesis

In this thesis, we will explore commonly and less commonly investigated biomarkers for dairy and dairy fat intake in the general population. To explore the less commonly investigated biomarkers, we aim to develop a new method to separate the dairy-derived trans-C18:1 from other trans fats. Furthermore, to investigate whether the dairy fat biomarkers are solid markers for dairy fat intake in the general population, we aim to investigate whether the association between dairy fat biomarkers and dairy fat intake is independent of fish fat intake. A second aim of the thesis is to investigate the relationship of the commonly and less commonly investigated dairy fat biomarkers with (cardiovascular) health outcomes. Since the intake of omega-3 fatty acids also plays an important role in cardiovascular health, we aim to examine those as well.

First of all, we will explore fatty acids as biomarkers for dairy and dairy fat intake. In **chapter 2**, a systematic review and meta-analysis is carried out to identify and validate the current biomarkers of dairy and dairy fat intake in the general population. The pros and cons of the current fatty acid biomarkers for dairy and dairy fat intake are summarized. Subsequently, new potential biomarkers are presented which will be further explored in our own dataset, the LifeLines Biobank and Cohort study.

Current evidence suggests that dairy fat biomarkers have positive effects on cardiovascular health. It has, however, been doubted whether associations of dairy fat biomarkers with

cardiovascular health are genuine. Firstly, most studies still use a suboptimal method to separate the trans-C18:1 fatty acids from each other, causing overestimation of the trans-C18:1(n-7) dairy fat biomarker. Therefore, in **chapter 3** we describe a new method to separate the dairy-derived trans-C18:1(n-7) from the industrially-derived trans-C18:1(n-9). Subsequently, in **chapter 4**, we will try to confirm the commonly and less commonly used dairy fat biomarkers (i.e. trans-C18:1(n-7) and CLA) in the LifeLines Biobank and Cohort study. Furthermore, a combination of biomarkers will be explored to investigate whether this is a better predictor for dairy fat intake compared to single markers.

Secondly, some researchers are concerned that the current biomarkers are not sufficiently explicit for the prediction of dairy fat intake, since some of these fatty acids, such as C15:0 and C17:0, have also been identified as fish fat biomarkers. Consequently, associations of fatty acids such as C15:0 and C17:0 with cardiovascular health may as well be attributable to fish fat intake. Therefore, in **chapter 5**, we will investigate whether the dairy fat biomarkers are solid markers to predict dairy fat intake in the LifeLines Biobank and Cohort study. In addition, we will zoom in on the association between the identified dairy biomarkers and risk factors for cardiovascular disease.

In the general population, a reduction in cardiovascular risk has mainly been ascribed to fish fat intake and the omega-3 fatty acid supplements EPA and DHA. Cardiovascular disease is one of the excessive contributors to mortality in RTR. It has therefore been suggested that these omega-3 fatty acid supplements might as well reduce all-cause and cardiovascular mortality in RTR. Whether this is also true for the less investigated omega-3 fatty acid ALA is unknown. Therefore, in **chapter 6 and 7**, we aim to investigate the association of the intake of omega-3 fatty acids EPA, DHA and ALA with (cardiovascular) mortality in RTR.

In contrast to the omega-3 supplements, drugs (i.e. glucocorticoids) have been thought to increase cardiovascular risk in RTR. Glucocorticoids are widely used in RTR to reduce inflammation. However, the drugs are believed to modify the plasma EPA and DHA status what may results in a worsened cardiovascular health status. We therefore aim to examine the impact of glucocorticoids on plasma EPA and DHA status, and its consequent effect on cardiovascular health outcomes in healthy young males in **chapter 8**.

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CHAPTER 2

Fatty acids as biomarkers of total dairy and dairy fat intakes: A systematic review and meta-analysis

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ABSTRACT

Context: Dairy intake is commonly assessed using questionnaires, however, such data are often biased. Therefore, interest in dairy fat biomarkers is growing. An overview of fatty acids that can be used as biomarker for dairy fat intake is lacking. **Objective:** We performed a systematic review and meta-analysis of observational studies to identify circulating fatty acids as biomarkers total dairy and dairy fat intakes in the general population. **Data Sources:** We searched MEDLINE, EMBASE and Web of Knowledge for eligible studies until June 2017. **Study selection:** Articles were included when researchers assessed the correlation between circulating dairy fatty acids and total dairy (fat) intake as measured by dietary assessment tools. **Data Extraction:** Two independent reviewers extracted data and assessed the risk of bias. **Data synthesis:** Data were pooled with the random-effects model. Meta-analysis revealed that the fatty acids in plasma/serum were significantly correlated with dairy (C14:0 (r [95%CI] = 0.15 [0.11 – 0.18]), C15:0 (0.20 [0.13 – 0.27]) and C17:0 (0.10 [0.03 – 0.16]) and dairy fat intake (C14:0 (0.16 [0.10 – 0.22], C15:0 (0.33 [0.27 – 0.39]), C17:0 (0.19 [0.14 – 0.25]) and trans-C16:1(n-7) (0.21 [0.14 – 0.29])). **Conclusions:** This paper identified C14:0, C15:0, C17:0 and trans-C16:1(n-7) as biomarkers of dairy and dairy fat intake in the general population. Trans-C18:1(n-7) and CLA need more investigation because of suboptimal measurement techniques.

INTRODUCTION

Dairy products contain numerous essential nutrients and can therefore be beneficial for almost all humans, including infants, adults and elderly people[1,2]. Bovine milk contains around 87% water, 4.6% lactose, 3.4% protein, 4.2% fat, 0.8% minerals and 0.1% vitamins[3,4]. The composition and content of milk fat depends on several factors including bovine race, time of lactation, physiological status of the animal and type of diet[5,6]. Milk fat has been documented to contain more than 400 different fatty acids[4]. The fatty acids can be directly derived from the bovine diet, and indirectly from rumen microbial *de novo* lipogenesis, which can both be subject to rumen microbial fermentation[7]. Usually, feed of bovines consists of grass during summer and conserved forage (maize or grass silage and hay) during winter[8]. The type of feed influences the bacterial flora in the rumen, and thereby microbial *de novo* lipogenesis and ruminal fermentation[9,10]. The produced fatty acids are taken up from the rumen and can be extracted in milk[10].

By weight, seventy percent of the fatty acids present in milk are saturated fatty acids, with palmitic acid (C16:0) (22-35wt%), stearic acid (C18:0) (9-14wt%) and myristic acid (C14:0) (8-14wt%) as the most prominent ones[4,6]. Microbial *de novo* lipogenesis and microbial fermentation add to the fatty acids present in milk[9,10]. During microbial *de novo* lipogenesis, C16:0 and C18:0 are produced, with C16:0 as the dominant end product [9,10]. Ruminal microbial fermentation can in turn convert C16:0 and C18:0 into respectively the odd-chain fatty acids pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). This process is called α -oxidation and is considered unique for the bacterial flora of the rumen[5,10]. C15:0 (1-2wt%) and C17:0 (0.5-1.5 wt%) are therefore thought to mainly originate from dairy fat[5,6,10]. This is in contrast to C14:0, C16:0 and C18:0, which also occur in high amounts in meat, including beef, pork and poultry, as well as fish and grain products[11,12]. Medium- and long-chain fatty acids produced by the cow's mammary gland may be desaturated to their mono-unsaturated counterparts[4]. Approximately 25% of the fatty acids in milk are mono-unsaturated fatty acids, of which oleic acid (C18:1) is the most prominent one (20-30wt%). Poly-unsaturated fatty acids, such as linoleic acid (C18:2) and α -linolenic acid (C18:3), account for around 0.5-3wt% of the total fatty acids in milk[4,6]. Approximately 2.7% of the milk fats are trans fatty acids[4,13,14], which are synthesized via microbial fermentation of C18:0 unsaturated fatty acids[15,16]. Vaccenic acid (trans-C18:1(n-7)) is the most prominent trans fatty acid[15-17]. Trans-palmitoleic acid (trans-C16:1(n-7)) is another trans fatty acid which can be found in dairy products[18]. Ruminantly derived products also contain conjugated linoleic acids (CLA) of which more than 90% is rumenic acid (cis-9, trans-11 CLA) [14,17,19-21]. In the rumen, CLA can be formed as an intermediate during the biohydrogenation of C18:2 into C18:0[22,23]. Additionally, Vaccenic acid, another intermediate of rumen biohydrogenation of C18:2 and C18:3, can be desaturated into rumenic acid (cis-9, trans-11 CLA)[23,24].

Commonly used methods to measure dairy fat intake include validated food frequency questionnaire (FFQ), food diary and 24h recall; they are frequently used in many published studies before. The use of an FFQ remains popular to date, as is evidenced by two recent large prospective cohort studies from 2014 and 2016, both reporting on prospective associations of dairy consumption with the risk of developing type 2 diabetes[25,26]. Although these methods provide specific information about dairy products, data are subjective and often biased. The methods are observational and based on self-reporting, which can lead to misclassification, underreporting and/or overreporting of intake[27]. Alternative or complementary methods for measuring dairy intake are therefore needed.

Researchers have shown an increased interest in biomarkers of dairy intake, since biological markers are considered a more objective parameter of this intake. As previously mentioned, dairy fat consists of several fatty acids, of which some are (to a large extent) unique for dairy fat. These fatty acids are present in quantitatively measurable amounts, even in the human circulation and body tissues making them interesting biomarkers of dairy fat intake. Biomarkers for dairy fat intake are most commonly measured in plasma/serum (cholesterol esters (CE), triglycerides (TG), phospholipids (PL), free fatty acids (FFA), or a combination of the four), erythrocytes and adipose tissue (AT)[28-31]. Adipose tissue can be investigated to determine long-term dairy fat intake, while plasma and serum are used to estimate short-term intake; for instance, triglycerides reflect intake over the past few hours, and cholesterol esters and phospholipids reflect intake of the past few days[31,32]. A convenient and efficient tool to measure the fatty acids is a gas chromatograph (GC) equipped with a flame ionization detector (FID) and a capillary polar column[33]. Polar columns are typically used for the analyses of complex fatty acids (i.e. trans fatty acids), since the polar columns can reach a higher selectivity and separation compared to apolar columns[34]. After analyzing, fatty acid samples remain stable for at least four years when stored at -80°C[35].

A common belief is that dairy fats are potentially unhealthy due to the presence of saturated and trans fatty acids. For trans fatty acids, a distinction can be made between industrially and ruminantly produced trans fatty acids[16]. Trans fats from industrial origin (e.g. Elaidic acid (trans-C18:1(n-9))) have been shown to be associated with increased risk of coronary heart disease, insulin resistance, inflammation, breast cancer, prostate cancer and colorectal cancer[36,37]. There is growing body of evidence indicating that adverse health effects associated with industrially produced trans fatty acids intakes may not necessarily apply for trans fatty acids found in dairy products (e.g. trans-C18:1(n-7))[38,39]. In contrast to what has been reported for industrialized trans fatty acids, there does not seem to be an association between ruminant trans fatty acid intake and risk for coronary heart disease[16,19,40]. In addition, positive effects on immune function, cardiovascular health and cancer risk have been ascribed to dairy fatty acids such as rumenic acid (cis-9, trans-11 CLA)[39].

Recently, studies indicate that the fatty acids C14:0, C15:0, C17:0, trans-C16:1, trans-C18:1(n-7) and CLA might be useful biomarkers of dairy fat intake[29,41,42]. However, a clear overview of fatty acids that can be used as biological markers of dairy and dairy fat intake is lacking. To examine which circulating fatty acids are suitable as biomarkers of dairy and dairy fat intake in the general population, we reviewed observational studies that assessed the correlation between circulating dairy fatty acids and total dairy (fat) intake as measured by dietary questionnaires. The research question also includes the current laboratory techniques that are used to measure the biomarkers of interest (fraction, column and equipment).

METHODS

For the main objective, we followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[43] (**Appendix 1**). Inclusion criteria and methods of analysis were specified in advance in a review protocol (**Appendix 2**).

Data sources and searches

A systematic search for relevant literature has been carried out to identify fatty acids as biomarkers of dairy and dairy fat intake in the general population. We searched MEDLINE, EMBASE and Web of Knowledge till June 2017. We developed a search string for each database consisting of the search terms biomarker, dairy fat intake, fatty acids and synonyms. We excluded animal studies. Searches were limited to papers that were published in English. Duplicates were removed before analyses. All included studies were screened for potential references that were not included in the main search. An overview of the search strategies can be found in **Supplemental Table 1**.

Study selection and data extraction

The PICOS criteria used to perform the systematic review are listed in **Table 1**. All observational studies were considered for inclusion. Intervention studies, letters to the editor and reviews were not included. Articles were included when researchers were analyzing circulating fatty acids as biomarkers of dairy (fat) intake. Fatty acids had to be measured in plasma/serum, erythrocytes or adipose tissue, while dairy (fat) intake had to be measured by FFQ, food records and/or 24-hour recalls. We required potentially relevant articles to report on the correlation of biomarkers with total dairy intake or intake of total dairy fat. We limited our search to studies with an epidemiological perspective, focusing on investigation of correlations of biomarkers with dairy (fat) intake. Studies in which dairy fat intake was regulated by supplements or a controlled diet were not included in the review. Title and abstract were screened by two independent reviewers (I.G.P and M.L.J). Studies which were in agreement with the eligibility criteria were retrieved as full text. Disagreements were resolved by discussion between the two researchers. Reasons

for exclusion and percentage of agreement, as Cohen's kappa, between the assessors were documented. In addition, the two independent reviewers extracted data and assessed the risk of bias for each study. From the included studies, the following data were extracted: name first author, publication year, potential dairy fatty acids measured in plasma/serum, erythrocytes and adipose tissue, dairy intake by dietary questionnaires, correlation between dairy intake and circulating fatty acids. Selected papers were also screened for used laboratory techniques with focus on fraction, column and equipment.

Table 1. The PICOS criteria which were used to perform the systematic review

PICOS	
Population	The general population. This includes all age, sex , race and ethnicity groups.
Intervention (exposure)	Dairy (fat) intake measured by dietary assessment tools (Food Frequency Questionnaires, food record, 24-h recalls
Comparator (control group)	There is no comparison against a control group.
Outcome	Potential dairy fatty acid biomarkers (circulating fatty acids) measured in plasma/serum (cholesterol esters, triglycerides, phospholipids, free fatty acids or a combination of all four), erythrocytes or adipose tissue.
Study Design	All observational studies (cross-sectional and prospective studies) were considered for inclusion. Intervention studies, letter to the editor and reviews were not included. Studies in which intake of dairy fat was regulated by supplements or a controlled diet were not included in the review. Relevant articles had to report on the correlation of circulating dairy fatty acids with total dairy intake or total dairy fat intake (as measured by questionnaires).

Quality assessment

Only observational studies were included in this review. Previous studies have emphasized the need for a uniform methodological assessment tool for observational studies[44,45]. The STROBE recommendations are thereby frequently used in an inappropriate way, i.e. as a methodological guideline (to assess the methodological quality of observational epidemiological studies, or to conduct or design observational studies), rather than as a reporting guideline to ensure a clear and complete report of study design, conduct and findings[46]. The Cochrane Non-Randomized Studies Methods Working Group recommends the Newcastle-Ottawa Scale (NOS) for assessing the quality of non-randomized studies in meta-analyses[47]. We therefore used the NOS and adapted it so it is in line with the current review (Appendix S3). The adapted NOS makes it possible to judge studies that focus specifically on the correlation between dairy (fat) intake and potential circulating dairy fat biomarkers. The 'start' system has developed in which a study is judged on three domains: (1) the selection (representativeness of the sample population,

appropriate sample size, ascertainment of the exposure, i.e. validated tool to measure dairy fat intake); (2) the comparability (appropriate control of confounding factors); and (3) the outcome (appropriate assessment of the outcome (validated tool to measure circulating dairy fat biomarkers), appropriate description of statistical test). The item 'respondents comparability and response rate' was excluded from the original quality tool since it is not applicable to the current review. Instead, we added the item 'Sample Size' whereby a sample size of $n \geq 100$ was justified and satisfactory [48-50]. The maximum attainable quality score was 6. A minimum of two-thirds of the quality score was chosen as cut-off to represent reasonable quality studies (> 4).

Data synthesis and analysis

We first conducted an overview of available biomarker studies. Characteristics of the included studies were systematically listed to generate a clear overview of the current literature on potential dairy fat biomarkers in the general population. Papers were classified by type of fraction. For those biomarkers with $\geq$ two studies available, we did quantitative syntheses on aggregated data. When articles investigated dairy intake both by FFQ and food record, only the result of the FFQ was included in the analysis since the FFQ is the most common type of dietary questionnaire and seen as the most reliable method to measure long term intake. When dairy intake was investigated by both food record and 24h-recall, only the result of the food record was included in the analysis. Dairy intake and dairy fat intake were analyzed separately. In addition, separate meta-analyses were carried out for different lipid fractions. In the separate analyses for the different fractions, plasma/serum PL and CE were preferred over plasma TG [51,52]. In addition, fatty acids were measured in plasma FFA only once and therefore not incorporated in the meta-analysis. For the syntheses, data was pooled with the random effects model of meta-analysis, using MedCalc for Windows, version 17.6 (MedCalc Software, Ostend, Belgium). To allow pooling across studies, we used the Fisher Z transformation of the correlation coefficients. Subsequently, the Fisher Z for each study was weighted by their inverse variance and the corresponding 95%CI were calculated. The variance of the Fisher Z transformation was calculated with the formula $z' = 0.5 \times [\ln(1+r) - \ln(1-r)]$. Then, the summary values were converted back to correlations for presentation. The existence of heterogeneity among studies was assessed by Q-tests, and the degree of the heterogeneity was quantified by calculating the I-squared (I^2) value. Publication bias was inspected visually by a funnel plot. Sensitivity analyses were performed including studies with more than two-thirds of the quality score (> 4 quality points), if more than two studies with a sufficient quality score were available. Findings were considered statistically significant if $P < 0.05$.

RESULTS

Results of the systematic review and meta-analysis are presented in a flow diagram (**Figure 1**). Cohen's kappa for the abstract selection was 0.97, indicating high agreement between the two researchers. Out of the 19 studies included for full text review, 18 were used for the qualitative synthesis and meta-analysis.

Figure 1: Flow Diagram of the literature search process

Study inclusion

Characteristics of the included studies can be found in **Table 2**[28-30,42,53-66]. The summary shows that C14:0, C15:0, C17:0 and trans-C16:1(n-7) were investigated as potential biomarkers of dairy (fat) intake. trans-C18:1(n-7) and CLA were also mentioned as potential biomarkers, but the correlation of trans-C18:1(n-7) and CLA with total dairy intake has only been investigated once and for reasons of conciseness we did not incorporate reporting on these fatty acids in **Table 2**.

The circulating fatty acids were derived from plasma/serum (CE, TG, PL, FFA or a combination of the four), erythrocytes or adipose tissue. Circulating fatty acids were detected with a gas (liquid) chromatography (G(L)C)-(Flame ionization detector (FID)) using polar columns with a length ranging from 25 till 100m long.

As described in the method section, we only included studies that measured dairy intake as total dairy fat or total dairy intake. Total dairy (fat) intake was measured with an FFQ, 24h-recall or food record of which the FFQ was the most common methodology.

Table 2: Characteristics of included studies in the systematic review

Fraction	Plasma/ Serum	Paper	Method Biomarker	Column	Dairy intake		Biomarker status (% of total fatty acids)				
					Method	Type of intake	Amount	C14:0	C15:0	C17:0	Trans-C16:1(n-7)
CE+ TG + PL+ FFA	Plasma	Giovannelli et al. (2014)[53]	HPLC	CP-SII 88	FFQ	Dairy intake	3.42 servings/d		0.33	0.38	
					Food record (3-days)	Dairy intake	259 g/d				
		Sun et al. (2007)[29]	GLC-FID	SP2560 (100m x 0.25mm x 0.20µm)	FFQ	Dairy fat intake	12.5 g/d 19.6 % of total fat intake	0.58	0.16	0.30	0.15
		Yakoob et al. (2014)[54]	GLC	SP2560 (100m x 0.25mm x 0.20µm)	FFQ	Dairy intake	2.1 servings/d (cases) ¹ 2.2 servings/d (controls)	0.70	0.17	0.33	0.22
	Serum							0.66	0.17	0.33	0.22
	Yakoob et al. (2016)[55]	GLC	SP2560 (100m x 250mm x 0.20µm)	FFQ	Dairy intake	2.1 servings/d (females) 2.1 servings/d (males)	0.55	0.16	0.32	0.19	
							0.51	0.14	0.31	0.15	
	Brevik et al. (2005)[56]	GLC	SP2560 (100m x 0.2mm x 0.2µm)	FFQ	Dairy fat intake	22.6 % of total fat intake		0.22	0.37		
				Weighted food record	Dairy fat intake	21.2 % of total fat intake					
	Gollev et al. (2014)[57]	GC	BPX-70 (30m x 0.53mm)	24-hour recall (3x)	Dairy intake	439 g/d	0.78	0.30	0.34		
					Dairy fat intake	24 g/d					
		Santaren et al. (2014)[28]	GC-FID	HP-88 30m	FFQ	Dairy intake	5.53 servings/wk		0.25		0.30

Table 2: Continued

Fraction	Plasma/ Serum	Paper	Method Biomarker	Column	Dairy intake		Biomarker status (% of total fatty acids)			
					Method	Type of intake	Amount	C14:0	C15:0	C17:0 Trans-C16:1(n-7)
CE	Serum	Biong et al. (2006)[30]	GC-FID	SP-2560 (100m x 0.25mm)	FFQ	Dairy fat intake	24.6 g/d	0.81	0.23	
		Snedman et al. (1999)[58]	GLC	NS-351	Food record (7 days)	Dairy fat intake	20.3 g/d		0.22	
					Food record	Dairy fat intake	29.6 g/100g total fat intake	1.04	0.22	0.10
TG	Serum	Biong et al. (2006)[30]	GC-FID	SP-2560 (100m x 0.25mm)	24-hour recall	Dairy fat intake	29.9 g/100g total fat intake			
					FFQ	Dairy fat intake	24.6 g/d	1.87	0.34	

Table 2: Continued

Fraction	Plasma/ Serum	Paper	Method Biomarker	Column	Dairy intake		Biomarker status (% of total fatty acids)				
					Method	Type of intake	Amount	C14:0	C15:0	C17:0	Trans-C16:1(n-7)
PL	Plasma	Lund-Blix et al. (2016)[60]	GC-FID	SP-2380 (30mx 0.22mm, 0.25µm)	FFQ	Dairy intake	1.97 servings/d	0.24			
						Dairy fat intake	1.30 servings/d				
		Oliveira et al. (2013)[42]	GC-FID	Unknown	FFQ	Dairy intake	1.5 servings/d	0.26	0.17		0.05
	Serum	Warensjö et al. (2015)[61]	GC-FID	Unknown	Food record (4 days)	Dairy intake	319 g/10MJ	0.41	0.26	0.43	
						Dairy fat intake	6.7 g/10MJ				
		Biong et al. (2006)[30]	GC-FID	SP-2560 (100m x 0.25mm)	FFQ	Dairy fat intake	24.6 g/d	0.48	0.84		
FFA	Plasma	Rosell et al. (2005)[62]	GLC	OV-351 (25m)	Food record (7 days)	Dairy fat intake	30 g/100g fat	NR	NR	NR	
		Smedman et al. (1999)[58]	GLC	NS-351	Food Record (7 days)	Dairy fat intake	20.3 g/d		0.25		
		Thiebaut et al. (2009)[63]	GC-FID	BPX-70 (30m x 0.32mm x 0.25 µm)	FFQ	Dairy intake	286.2 g/d		0.20	0.44	0.17
		Wolk et al. (2001)[59]	GLC	CP-sil 88	Food record	Dairy fat intake	29.6 g/100g total fat intake	0.47	0.22	0.44	
					24-hour recall	Dairy fat intake	29.9 g/100g total fat intake				
		Biong et al. (2006)[30]	GC-FID	SP-2560 (100m x 0.25mm)	FFQ	Dairy fat intake	24.6 g/d	2.10	0.37		

Table 2: Continued

Fraction	Plasma/ Serum	Paper	Method Biomarker	Column	Dairy intake		Biomarker status (% of total fatty acids)				
					Method	Type of intake	Amount	C14:0	C15:0	C17:0	Trans-C16:1(n-7)
Erythrocytes	-	Sun et al. (2007)[29]	GLC-FID	SP-2560 (100m x 0.25mm)	FFQ	Dairy intake	12.5 g/d 19.6 % of total fat intake	0.19	0.10	0.34	0.14
		Yakoob et al. (2014)[54]	GLC	SP-2560 (100m x 0.25mm)	FFQ	Dairy intake	2.1 servings/d (cases) ¹ 2.2 servings/d (controls)	0.45	0.14	0.50	0.17
		Yakoob et al. (2016)[55]	GLC	SP2560 (100m x 0.25mm x 0.20µm)	FFQ	Dairy intake	2.1 servings/d (females) 2.1 servings/d (males)	0.27	0.12	0.39	0.16

Table 2: Continued

Fraction	Plasma/ Serum	Paper	Method Biomarker	Column	Method	Dairy intake		Biomarker status (% of total fatty acids)			
						Type of intake	Amount	C14:0	C15:0	C17:0	Trans-C16:1(n-7)
AT	-	Aslibekyan et al. (2012)[64]	GLC	SP-2560	FFQ	Dairy intake	244 g/d (cases) 237 g/d (controls)		0.21	0.20	
		Baylin et al. (2002)[65]	GLC	SP-2560 (100m x 0.25mm)	FFQ	Dairy intake	NR		0.19	0.21	
		Brevik et al. (2005)[56]	GLC	SP-2560 (100m x 0.25mm)	FFQ	Dairy fat intake	22.6 % of total fat intake		0.32	0.34	
		Biong et al. (2006)[30]	GC-FID	SP-2560 (100m x 0.25mm)	Weighted record	Dairy fat intake	21.2 % of total fat intake				
					FFQ	Dairy fat intake	24.6 g/d	3.60	0.39	0.27	
					Food record (7 days)	Dairy fat intake	30 g/100g fat intake	NR	NR	NR	
		Rosell et al. (2005)[62]	GLC	OV-351 (25m)	FFQ	Dairy fat intake	NR		0.35	0.24	
		Wolk et al. (1998)[66]	GLC	OV-351 (25m)	Food record	Dairy fat intake	NR				
		Wolk et al. (2001)[59]	GLC	CP-sil 88 (50m)	Food record	Dairy fat intake	29.6 g/100g total fat intake	3.60	0.36	0.25	
					24-hour recall	Dairy fat intake	29.9 g/100g total fat intake				

Abbreviations: AT; Adipose tissue; C14:0; Myristic acid; C15:0; Pentadecanoic acid; C17:0; Heptadecanoic acid; CE, Cholesterol esters; FFQ, Food Frequency Questionnaire; FFA, Free fatty acids; GC-Cl= Gas chromatography – chemical ionization; GC-FID = Gas chromatography-Flame ionization detector; GLC= Gas liquid chromatography; GLC-FID = Gas liquid chromatography-Flame ionization detector; PL, Phospholipids; NR, Not Reported; TG, Triglycerides; Trans-C16:1(n-7), Trans-Palmitoleic acid.

¹The paper used data from two cohorts. Only data from the NHS cohort is shown, data of the HPSF cohort shows comparable intakes and status.

Quality assessment

The results of the quality assessment (NOS) can be found in **Table 3**[28-30,42,53-66]. The NOS revealed good study quality in 11 out of 18 studies ($n > 4$ quality points). Representativeness of the general population was not always found in the studies ($n = 7/18$). The majority of the studies had an appropriate sample size ($n \geq 100$) ($n = 16/18$) and validated measurement tools to measure the exposure (Dairy intake) ($n = 18/18$). Around half of the studies adjusted their analyses for any potential confounder ($n = 10/18$). All 18 studies reported a correlation, including the probability level (p-value). One study failed to report on how they measured the outcome (circulating fatty acids) exactly. The study was included in the main analyses, but excluded in the sensitivity analyses.

Table 3: Results of the quality assessment of observational studies

Paper	Selection score (max 3 stars)	Comparability (max 1 star)	Outcome (max 2 stars)	Total Score (max 6 stars)
Aslibekyan et al. (2012)[64]	**	*	**	5
Baylin et al. (2002)[65]	***	*	**	6
Biong et al. (2006)[30]	**	*	**	5
Brevik et al.(2005)[56]	***		**	5
Giovannelli et al. (2014)[53]	***		*	4
Golley et al. (2014)[57]	**		**	4
Lund-Blix et al. (2016)[60]	**		**	4
Oliveira et al. (2013)[42]	***	*	**	6
Rosell et al. (2005)[62]	**		**	4
Santaren et al. (2014)[28]	***		**	5
Smedman et al. (1999)[58]	*	*	**	4
Sun et al. (2007)[29]	***	*	**	6
Thiebaut et al. (2009)[63]	**	*	**	5
Warensjo et al. (2015)[61]	***	*	**	6
Wolk et al. (1998)[66]	**		**	4
Wolk et al. (2001)[59]	**		**	4
Yakoob et al. (2014)[54]	**	*	**	5
Yakoob et al. (2016)[55]	**	*	**	5

According to the adapted Newcastle-Ottawa Scale (NOS) for assessing the quality of non-randomized studies in meta-analyses (**Appendix B**). The maximum attainable quality score is 6. A minimum of two-thirds of the quality score represent a reasonable quality study (> 4).

Systematic review

From the papers included in the qualitative analyses, the majority of the studies measured the fatty acids in plasma/serum ($n = 15/18$), of which C15:0 was the most commonly measured

dairy fat biomarker, followed by C17:0 and C14:0. Eight papers reported on measurement of dairy fatty acid biomarkers in more than one fraction (plasma/serum (CE, TG, PL, FFA, or a combination of the four), erythrocytes and adipose tissue), which allow for evaluation of differences in correlations of the biomarkers assessed in different fractions with dairy (fat) intake (**Supplemental Table 2**) [29,30,54-56,58,59,62].

Results of the studies investigating C14:0, C15:0, C17:0 and trans-C16:1(n-7) which were not included in the meta-analyses can be found in **Table 4** [29,30,53,56,59,66]. One study investigated the correlation of dairy fat intake measured by FFQ with circulating dairy fatty acids in serum TG and FFA [30]. In both serum TG and FFA, C14:0 had a correlation of 0.18 with dairy fat intake. C15:0 measured in serum TG had a correlation of 0.45 with dairy fat intake, while C15:0 measured in FFA was not correlated with dairy fat intake. The single study reporting on trans-C18:1(n-7) in plasma and erythrocytes was not correlated with dairy fat intake [29]. The single study reporting on CLA in adipose tissue and total dairy fat intake found a correlation of 0.24 ($p < 0.001$) [64]. There were no papers that reported on a correlation of trans-C16:1(n-7) in adipose tissue with dairy (fat) intake.

Meta-analysis

C14:0, C15:0, C17:0 and trans-C16:1(n-7) in plasma/serum and erythrocytes, and C14:0, C15:0 and C17:0 in adipose tissue were reported in more than two studies and were therefore investigated using meta-analysis. Overall, fatty acids in plasma/serum were positively correlated with dairy (C14:0 (r [95%CI] = 0.15 [0.11 – 0.18]), C15:0 (0.20 [0.13 – 0.27]), C17:0 (0.10 [0.03 – 0.16]) and trans-C16:1(n-7) (0.08 [-0.01 – 0.16])) and dairy fat intake (C14:0 (0.16 [0.10 – 0.22], C15:0 (0.33 [0.27 – 0.39]), C17:0 (0.19 [0.14 – 0.25]) and trans-C16:1(n-7) (0.21 [0.14 – 0.29])) (**Figure 2**) [28-30,42,53-66]. Separate analyses for fatty acids in plasma/serum showed that the total correlations of fatty acids measured in plasma/serum CE with dairy fat intake were higher compared to the total correlations of fatty acids measured in total plasma/serum and plasma/serum PL with dairy fat intake. The dairy fatty acid biomarkers assessed in erythrocytes were also correlated with dairy fat intake (C14:0 (0.08 [-0.002 – 0.16]), C15:0 (0.16 [0.03 – 0.28]), C17:0 (0.07 [-0.04 – 0.18]) and trans-C16:1(n-7) (0.20 [0.09 – 0.30])). A correlation with dairy intake was not investigated. The dairy fatty acid biomarkers assessed in adipose tissue were positively correlated with dairy (C15:0 (0.34 [0.31 – 0.36]) and C17:0 (0.23 [0.08 – 0.37])) and dairy fat intake (C14:0 (0.53 [0.43 – 0.62]), C15:0 (0.51 [0.37 – 0.63]) and C17:0 (0.22 [0.12 – 0.32])).

Analysis revealed substantial to considerable heterogeneity for plasma/serum C14:0 ($I^2 = 66.9\%$, $P = 0.004$ for the analysis with dairy fat intake), C15:0 ($I^2 = 80.9\%$ and 71.2% , $P \leq 0.001$, respectively for the analysis with dairy intake and dairy fat intake), C17:0 ($I^2 = 49.7\%$ and 55.8% , $P \leq 0.03$) and trans-C16:1(n-7) ($I^2 = 82.5\%$ and 71.7% , $P \leq 0.02$). Substantial heterogeneity was also found for the erythrocytes and adipose tissue fatty acids. Funnel plots of the meta-analysis were created to inspect publication bias (**Supplemental Figure 1**) [28-30,42,53-66].

Table 4: Correlations between Total dairy (fat) intake and circulating dairy fatty acid biomarkers which are not included in the meta-analyses

Fraction	Paper	n	Method	Dairy intake	Biomarker ¹		
					C14:0	C15:0	C17:0 Trans-C16:1(n-7)
CE+TG+PL+FFA	Plasma	Giovannelli et al. (2014)[53]	420/429 (C15:0/C17:0)	Food record (3-days)	Dairy intake	0.03	0.09
	Serum	Brevik et al. (2005)[56]	110	Food record	Dairy fat intake	0.43	0.05
CE	Serum	Wolk et al. (2001)[59]	104	Food record	Dairy fat intake	0.40	0.47
				24-hour recall	Dairy fat intake	0.34	0.35
TG	Serum	Biong et al. (2006)[30]	205	FFQ	Dairy fat intake	0.18	0.45
				24-hour recall	Dairy fat intake	0.30	0.39
PL	Serum	Wolk et al. (2001)[59]	104	FFQ	Dairy fat intake	0.18	0.06
				FFQ	Dairy intake	0.11	0.23
FFA	Plasma	Biong et al. (2006)[30]	205	FFQ	Dairy fat intake	0.18	0.06
				FFQ	Dairy intake	0.11	0.18
Erythrocytes	-	Sun et al. (2007)[29]	313	FFQ	Dairy intake	0.11	0.18
AT	-	Brevik et al. (2005)[56]	107	Food record	Dairy fat intake	0.52	0.07
				Food record	Dairy fat intake	0.63	0.42
		Wolk et al. (1998)[66]	81	Food record	Dairy fat intake	0.63	0.42
		Wolk et al. (2001)[59]	114	24-hour recall	Dairy fat intake	0.64	0.74
					Dairy fat intake	0.74	0.23

¹Correlation (r) between potential circulating dairy fatty acid biomarkers and Dairy (fat) intake
Abbreviations: AT, Adipose tissue; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; FFQ, Food Frequency Questionnaire; n, number; Trans-C16:1(n-7), Trans-Palmitoleic acid.

Some of the funnel plots are asymmetric, which is suggestive of publication bias for these analyses.

Sensitivity analyses were performed including studies with more than half the maximum study quality score (> 4 quality points), if more than two studies with a sufficient quality score were available (**Supplemental Figure 2 and 3**) [28-30,42,54-56,61,63-65]. Meta-analyses were not repeated in the sensitivity analyses when the included studies for the sensitivity analyses were the same as for the main analyses. Therefore, these analyses are not incorporated in **Supplemental Figure 2 and 3**. Correlations between the circulating fatty acids and dairy (fat) intake did not materially differ compared to the regular analyses. However, a decreased heterogeneity for C14:0 and C15:0 in plasma/serum was observed. Interestingly, the heterogeneity for the analyses of C17:0 and dairy intake in plasma/serum decreased from an I^2 of 49.7% with $P = 0.01$ to an I^2 of 0.0% with $P = 0.82$, while the heterogeneity for the analyses of C17:0 and dairy fat intake in plasma/serum increased from an I^2 of 55.8% with $P = 0.03$ to an I^2 of 65.2% with $P = 0.02$. The heterogeneity for fatty acids in adipose tissue did not materially differ from the main analyses, except for the analyses in which the association between C17:0 and dairy fat intake was investigated. The heterogeneity increased from an I^2 of 47.5% with $P = 0.11$ to an I^2 of 81.1% with $P = 0.02$.

Figure 2: Forest plots of studies investigating potential circulating dairy fatty acid biomarkers. A: Meta-analysis C14:0 and dairy intake, B: Meta-analysis C14:0 and dairy fat intake, C: Meta-analysis C15:0 and dairy intake, D: Meta-analysis C15:0 and dairy fat intake, E: Meta-analysis C17:0 and dairy intake, F: Meta-analysis C17:0 and dairy fat intake, G: Meta-analysis trans-C16:1(n-7) and dairy intake, H: Meta-analysis trans-C16:1(n-7) and dairy fat intake

B**Meta-analysis C14:0 and dairy fat intake** **Figure 2: Continued**

C

Figure 2: Continued

Figure 2: Continued

E**Meta-analysis C17:0 and dairy intake** **Figure 2: Continued**

F

Figure 2: Continued

Figure 2: Continued

DISCUSSION

To date, there is no clear overview of biomarkers that can be used as marker of dairy fat intake. This paper systematically reviewed current literature to identify and summarize the fatty acids that can potentially be used as biomarkers of dairy and dairy fat intake in the general population. Meta-analysis revealed that C14:0, C15:0, C17:0 and trans-C16:1(n-7) in plasma/serum can be used as biomarkers for dairy and dairy fat intake. Correlations were even higher in adipose tissue.

The current study has several strengths. Although the subject of biomarkers for dairy and dairy fat intake has been reviewed before[67], this is to our knowledge the first systematic review and meta-analysis investigating the correlation between dairy (fat) intake and circulating dairy fatty acids in the general population. Another strong point of the study is that the vast majority of the studies were of good quality. Results from the overall analyses and sensitivity analyses (studies > 4 quality points) did not materially differ from each other.

We must acknowledge that this study also has its limitations. Studies included in this systematic review and meta-analysis are all observational studies. In general, observational studies have a lower validity than randomized controlled trials and they are more susceptible to bias (e.g. selection and information bias) and confounding factors. Analyses were adjusted for confounding factors in about half of the studies. The results of the current study may be affected by the methodological weaknesses of observational studies. Other study types (e.g. randomized controlled trials, intervention studies) may show a different strength of the correlation. Another limitation is the fact that we found indicators for the presence of substantial to considerable heterogeneity and publication bias. Although we used random effect models to account for the observed heterogeneity, it cannot be excluded that the presence of heterogeneity adversely influenced the validity of the effect estimates of the meta-analysis that we performed. Publication bias might affect the validity of meta-analysis also. Publication bias was inspected visually by funnel plots. Asymmetric funnel plots, as visible for some of the analyses, is suggestive of selective reporting. For instance, for the correlation between C14:0 and dairy intake (**Supplemental Figure 1A**), studies reporting relatively low correlation coefficients at a high standard error may be missing. Lack of such studies may have led to overestimation of the meta-analysis derived correlation between C14:0 and dairy intake. A third limitation is the lack of data on fatty acids measured in liver tissue. The liver is the main metabolizing organ after dietary intake and it would therefore have been interesting if it would have been possible to incorporate data on the liver as well.

The review showed that the correlations of dairy fatty acid biomarkers in plasma/serum with total dairy (fat) intake did not materially differ from the correlations found in erythrocytes. Correlations of dairy fatty acid biomarkers in adipose tissue with dairy (fat) intake were higher compared to plasma/serum and erythrocytes. Although the strength of the correlation of dairy fatty acid biomarkers with dairy (fat) intake varies between the papers, all show a similar trend.

Biong *et al* (2006) investigated the correlation of potential dairy fatty acid biomarkers in plasma/serum, erythrocytes and adipose tissue with dairy (fat) intake. The strongest correlations for the dairy fatty acid biomarkers with dairy (fat) intake were found when the biomarkers were measured in adipose tissue[30]. Additionally, a study from 1999 showed that the correlation of the dairy fatty acid biomarker CLA in adipose tissue with milk fat was stronger than the correlation of the dairy fatty acid biomarker CLA in serum with milk fat[41]. The paper also showed that the concentration of the biomarker in adipose tissue was twice as high as the concentration of the biomarker in serum [41]. That adipose tissue dairy fatty acid biomarkers relate better with dairy and dairy fat intake than circulating fatty acid biomarkers, might be a consequence of the fact circulating fatty acids are more influenced by short-term changes in diet, while data of food frequency questionnaires, to which they are compared usually reflect long-term intake. Because turnover of adipose tissue is much slower, fatty acid biomarkers measured in adipose tissue might more strongly relate to data of food frequency questionnaires, which also reflect long-term intake[31]. Furthermore, fatty acids from dairy are incorporated into serum lipid fractions differently than in adipose tissue[30]. This could be due to different turnover rates of the fatty acids in various fractions, or due to selective mobilization and incorporation[30]. These differences may also influence the strength of correlations.

The meta-analysis revealed the strongest correlations of circulating dairy fatty acid biomarkers with total dairy (fat) intake for C15:0 and C17:0. The correlations of the dairy fatty acid biomarkers C15:0 and C17:0 with dairy (fat) intake show the same overall trend, however the correlations of C15:0 with dairy (fat) intake are slightly higher than the correlations of C17:0 with dairy (fat) intake. Recently, a review about the odd-chain fatty acids C15:0 and C17:0 was published which described that the ratio of C15:0 and C17:0 is approximately 2:1 within ruminant milk fat, while it is approximately 1:2 across all lipid classes in humans[10]. It was suggested that the 2:1 ratio in ruminants can be explained in part by the fact that C15:0 and C17:0 can be produced from respectively C16:0 and C18:0 via an alternative route, the α -oxidation[10]. During the *de novo* lipogenesis more C16:0 will be produced compared to C18:0. Thus, the process of α -oxidation is expected to result in a higher conversion of C16:0 to C15:0 compared to conversion of C18:0 to C17:0. Furthermore, it is known that the apparent oxidation of individual fatty acids decreases with increase in carbon chain length. This will further favor the availability of C16:0 for conversion to C15:0. These effects would together result in the approximate 2:1 ratio[68]. The contrasting C15:0 to C17:0 ratio across all lipid classes in humans may point to a higher endogenous synthesis of C17:0, a more efficient oxidation of C15:0, or both[10]. The fact that C15:0 has a stronger correlation with total dairy and total dairy fat intake compared to C17:0 suggests that C15:0 is more specific to ingestion of dairy products.

The dairy fatty acid biomarker C14:0 was positively correlated with dairy intake. An important finding of our meta-analyses was that the correlation of C14:0 in plasma/serum and

erythrocytes with total dairy intake was weaker compared to the correlation of C15:0 and C17:0 in plasma/serum and erythrocytes with total dairy intake. The weaker correlation of C14:0 in plasma/serum and erythrocytes with dairy intake may be caused by the presence of C14:0 in other products such as meat, coconut oil and palm kernel oil[69,70]. Therefore, C14:0 is not considered to be a good specific marker for dairy intake.

Total dairy (fat) intake has predominantly been measured with validated FFQs, 24-h recalls and food records. As expected, the FFQ was the most common type of methodology, but did not show better results when compared to the 24-h recall and food record[56,59,66]. In the current review, only those studies measuring total dairy (fat) intake were taken into account to overcome the problem of having a widespread variation in reporting dairy intake. Despite this narrowing down of included studies, we still saw variation between studies regarding the strength of the correlation of circulating fatty acids with dairy intake. This variation could have been due to differences in defining total dairy (fat) intake. A study of Santaren *et al* (2014) defined dairy intake as sum of whole milk, 2% milk, skim milk, 1% buttermilk, buttermilk, cottage and ricotta cheese, cheese, flavored yoghurt, low-fat flavored yoghurt, ice cream, frozen yoghurt, ice milk, milk in coffee or tea, and cream or half-and-half in coffee or tea[28]. Another study defined the dairy intake as a sum of milk, sour milk, yoghurt, cheese, ice cream and butter[30]. Unfortunately, several studies did not define dairy (fat) intake at all. Discrepancy between results also occurred due to differences in reporting dairy intake. Dairy intake was described in terms of ‘% of total fat’, ‘servings per week’, or ‘grams per day’. Such differences in reporting may cause variability between study outcomes making comparison between studies difficult, and may also confound conclusions. Furthermore, questionnaires are observational and based on self-report; this may reduce the accuracy and precision of measuring dietary intake, and also lead to systematic errors[27,71,72].

We included studies on the ruminant trans fatty acids trans-C18:1(n-7) and CLA as dairy fatty acid biomarkers in our review. So far, studies in which these ruminant trans fatty acids have been used as dairy fatty acid biomarkers are limited. Trans-C18:1(n-7) and cis-9, trans-11 CLA are naturally occurring fatty acids which are synthesized via bacterial metabolism of unsaturated fatty acids in ruminant animals and found in ruminant-derived foods[16]. Trans-C18:1(n-7) is the most common trans fatty acid found in dairy fat, and is therefore an interesting target for further research[73]. However, the current equipment and column possibilities may be inadequate to measure the specific dairy fatty acids such as trans-C18:1(n-7). Most studies investigating these two fatty acids use polar columns up till 100 m long, however such a column may not have the separation efficiency to prevent coeluting of trans-C18:1(n-7) acid with other trans fatty acids, making quantification difficult. Furthermore, both trans-C18:1(n-7) and CLA have a low measurement intensity when FID is used, potentially leading to the introduction of larger error margin. It is therefore more common to identify a cluster of trans-C18:1 isomers instead of trans-

C18:1(n-7) only. A disadvantage of this method is that a cluster of trans-C18:1 isomers contains both trans fatty acid isomers of ruminant as well as of industrial origin, and is therefore not a pure measurement of dairy fat intake. Several studies investigated the correlation between total trans-C18:1 and dairy intake[41,74,75] and/ or the association of total trans-C18:1 as a marker of dairy intake with health outcomes[74-80].

Overall, this review showed that several dairy fatty acid biomarkers correlate with dairy (fat) intake. Nevertheless, the association between dairy (fat) intake and circulating C15:0, C17:0 and trans-C16:1(n-7) has been called into question in some studies. For instance, trans-C16:1(n-7) raised questions in some studies, since it is a fatty acids that cannot only be found in the diet, but has recently been found to be produced by β -oxidation of trans-C18:1(n-7) as well[81]. In addition, a study from 2017 found that the intake of dietary fibers may influence the odd-chain fatty acids C15:0 and C17:0 [82]. The researchers showed that supplementation with inulin (fermentable dietary fiber) and propionate (major product of intestinal bacteria fermentation of dietary fibers) for a week increased both plasma C15:0 and C17:0, while this was not seen after supplementation with cellulose (control). Furthermore, a study from 2015 demonstrated that adipocytes can convert the branched chain amino acids, valine and isoleucine into propionyl-CoA, which can subsequently act as precursor for the synthesis of odd-chain fatty acid[83]. Thus, catabolism of branched chain amino acids derived from dietary proteins, including dairy proteins, can theoretically also contribute to circulating concentrations of C15:0 and C17:0. Moreover, besides dairy products, C15:0 and C17:0 can also be found in marine water fish and have recently found to be related to DHA, a common fatty acid found in fatty fish[84,85]. C15:0 and C17:0 might therefore be an invalid biomarker of dairy intake in a population with a high fish consumption. However, in countries such as the Netherlands, where consumption of dairy is high (a median of 321 g/day according to the Dutch Food Consumption Survey)[86] and fish intake low (70 g/week)[87], use of C15:0 and C17:0 as dairy fat biomarkers should give no concerns. For example, in salmon, the most common eaten fatty fish in the Netherlands, the amount of C15:0 and C17:0 is around 40mg respectively 20mg per 100g[88], while the intake of C15:0 and C17:0 via dairy products is around 1.10 and 0.56 g per 100g[9,10].

CONCLUSION

In the systematic review, C14:0, C15:0, C17:0 trans-C16:1(n-7), trans-C18 :1(n-7) and CLA in plasma/serum (cholesterol esters, triglycerides, phospholipids, free fatty acids or a combination of the four), erythrocytes, and adipose tissue were validated as potential dairy fatty acid biomarkers. The meta-analysis confirmed C14:0, C15:0, C17:0 and trans-C16:1(n-7) as biomarkers of dairy and dairy fat intake, with weaker correlations for dairy intake than for dairy fat intake. For example, for C17:0 the correlation coefficient with dairy intake was 0.10 (95%CI 0.03-0.16), which suggests that only about 1% of the variance of serum/plasma can be explained by self-

reported dairy intake, while the correlation coefficient with dairy fat intake was 0.19 (95%CI 0.14-0.25), which suggest that a higher, but still limited amount of about 3.6% of the variance of serum/plasma can be explained by self-reported dairy fat intake. Unfortunately, until now, research has mainly focused on use of single markers, and little is known about the performance of prediction models for self-reported dairy and dairy fat intake based on inclusion of two or more biomarkers at the same time. Given the low explained variance of single biomarkers by self-reported dairy and dairy fat intake, it would be interesting if future cross-sectional and prospective studies in the field would provide such information. , Among different fractions, we found fatty acid biomarkers measured in adipose tissue to be most strongly associated with dairy and dairy fat intake. Adipose tissue is, however, not always available in studies. In most fractions, including adipose tissue, C15:0 was found to be the strongest fatty acid biomarker among the set of biomarkers investigated in this review. Thus, future prospective studies in the field would preferably include C15:0 as a biomarker, either alone or in combination with other fatty acid biomarkers. Caution has to be taken with C14:0 as dairy fat biomarker, since this marker is also present in dietary products other than dairy. Trans-C18:1(n-7) and CLA still need further investigation, because of problems with separation between trans-C18:1(n-7) and other trans fatty acids in commonly applied measurement techniques and a small number of studies.

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Supplemental Table 1: Systematic search strategy for MEDLINE, EMBASE and Web of Knowledge

Type of database	Search strategy	Items (June 2017)	Potential relevant or extra papers
MEDLINE	("Biomarkers"[Mesh] OR biomarker*[tiab] OR marker*[tiab])	290	18
	AND		
	("Dairy Products"[Mesh] OR dairy[tiab] OR dairy fat[tiab])		
	AND		
	("Fatty Acids"[Mesh] OR fatty acid*[tiab] OR dairy fat[tiab])		
EMBASE	NOT		
	((("Animals"[Mesh] NOT "Humans"[Mesh]) OR cow[ti] OR cows[ti])		
	('biological marker'/exp OR (biomarker* OR marker*);ab,ti)	347 (new = 187)	0
	AND		
	('dairy product'/exp OR (dairy OR 'dairy fat*');ab,ti)		
	AND		
	('fatty acid'/exp OR ('fatty acid*' OR 'dairy fat*');ab,ti)		
	NOT		
	((('animal'/exp OR 'nonhuman'/exp) NOT 'human'/exp) OR (cow OR cows);ti)		
	TS=(biomarker* OR marker*)	369 (new = 225)	1
Web of Knowledge	AND		
	TS=dairy		
	AND		
	TS=("fatty acid" OR "fatty acids" OR "dairy fat")		
	NOT		
	TI=("cow" OR "cows" OR "animal" OR "cattle")		

Supplemental Table 2: Papers measuring the correlation between potential circulating dairy fatty acid biomarkers and total dairy (fat) intake in multiple fractions

	Fraction								
	Plasma	Serum	Plasma/ Serum CE	Plasma/ Serum TG	Plasma PL	Serum PL	FFA	Erythrocytes	AT
Biong et al. (2006)			x	x		x	x		x
Brevik et al. (2005)		x							x
Rosell et al. (2005)						x			x
Smedman et al. (1999)			x			x			
Sun et al. (2007)	x							x	
Wolk et al. (2001)			x			x			x
Yakoob et al. (2014)	x							x	
Yakoob et al. (2016)	x							x	

Abbreviations: CE = Cholesterol esters, TG= triglycerides, PL= Phospholipids, FFA= free fatty acids, AT = Adipose tissue

Supplemental Figure 1: Funnel plots. From left to right: A1. C14:0 and dairy intake in plasma/serum, B1. C14:0 and dairy fat intake in plasma/serum, B2. C14:0 and dairy fat intake in plasma/serum cholesterol esters, B3. C14:0 and dairy fat intake in erythrocytes, B4. C14:0 and dairy fat intake in adipose tissue, C1. C15:0 and dairy intake in plasma/serum, C2. C15:0 and dairy intake in adipose tissue, D1. C15:0 and dairy fat intake in plasma/serum, D2. C15:0 and dairy fat intake in plasma/serum cholesterol esters, D3. C15:0 and dairy fat intake in erythrocytes, D4. C15:0 and dairy fat intake in adipose tissue, E1. C17:0 and dairy intake in plasma/serum, E2. C17:0 and dairy intake in adipose tissue, F1. C17:0 and dairy fat intake in plasma/serum, F2. C17:0 and dairy fat intake in erythrocytes, F3. C17:0 and dairy fat intake in adipose tissue, G1. Trans-C16:1(n-7) and dairy intake in plasma/serum, H1. Trans-C16:1(n-7) and dairy fat intake in plasma/serum, H2. Trans-C16:1(n-7) and dairy fat intake in erythrocytes.

Supplemental Figure 1: Continued

Supplemental Figure 1: Continued

Supplemental Figure 1: Continued

Supplemental Figure 2: Forest plots of studies investigating dairy fat biomarkers (Subgroup analyses, only studies with > 4 quality points are included). A. Meta-analysis C14:0 with dairy and dairy fat intake. B. Meta-analysis C15:0 with dairy and dairy fat intake. C. Meta-analysis C17:0 with dairy and dairy fat intake.

B**Meta-analysis C15:0**

Supplemental Figure 2: Continued

C

Meta-analysis C17:0

Supplemental Figure 2: Continued

Supplemental Figure 3: *Continued*

Appendix S1: PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-6
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	7, Table 1
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	7, Appendix A
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	7,8
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	7
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Table S1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	7-10
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7,8
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	7,8
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	8,9, Appendix B
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	9, 10

Appendix S1: Continued

Section/topic	#	Checklist item	Reported on page #
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	9, 10
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	10
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	10
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	10, Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2, 11
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table 3, 11, 12
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Figure 2 A-H, Table 4, 12-14
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Figure 2 A-H, 13,14
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Figure S1 A-H, 14
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	Figure S2 A-C, Figure S3 A-C, 14
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	14-22
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	15,16
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	21, 22
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	23

Appendix S2: Research protocol

Title

Fatty acids as Biomarkers of Dairy and Dairy fat intake: A systematic review and Meta-analysis.

Authors

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Review Question

Which circulating fatty acids can be used as potential biomarkers of dairy fat intake in the general population?

Sub question:

What is the association between circulating fatty acids and dairy fat intake measured by dietary questionnaires in the general population?

Searches

The databases MEDLINE, EMBASE and Web of Knowledge will be systematically searched for eligible studies. Duplicates will be removed and animal studies will be excluded. Searches will be limited to papers which are written in English.

Types of study to be included

All observational studies will be considered for inclusion. Intervention studies whereby intake of dairy fat was regulated by supplements or a controlled diet, (systematic) reviews, letters to the editor, and other study designs will be excluded.

Condition or domain being studied

A frequently used method to measure dairy fat intake is a validated food frequency questionnaire (FFQ)[S1-S3]. Although these methods provide specific information about dairy products, data are subjective and often biased [S4]. Alternative or complementary methods for measuring dairy intake are therefore needed. Researchers have shown an increased interest in biomarkers of dairy intake, since biological markers are considered to be a more objective parameter of this intake. Dairy fat consist of several fatty acids, of which some are (to a large extend) unique for dairy fat [S5]. These fatty acids are present in quantitatively measurable amounts, which makes

them interesting as biomarkers for dairy fat intake. Studies recently showed that the fatty acids C14:0, C15:0, C17:0, Trans-C16:1, Trans-C18:1(n-7) and CLA might be useful biomarkers of dairy fat intake [S6-S8]. However, a clear overview of fatty acids that can be used as biological markers of dairy fat intake is lacking.

Participants/population

The population that will be studied is the general population, with no restriction for age and sex. Animal studies will be excluded.

Exposure

Dairy fat intake should be assessed by a dietary questionnaire (FFQ, Food record or 24h recall) and reported in the article. Studies whereby dairy intake was regulated by supplements or a controlled diet will be excluded. Measurements of dairy intake will be restricted to 'Total dairy intake' or 'Total dairy fat intake' to overcome the problem of having a widespread variation in reporting on dairy intake.

Outcome

Circulating fatty acids are preferably measured by a GC (Gas Chromatograph). Fatty acids should be measured in plasma/serum, erythrocytes or adipose tissue. Furthermore, articles should report on the correlation between exposure (dairy fat intake) and outcome (circulating fatty acids), including the probability level (p-value).

Data selection and extraction

Title and abstract will be screened by two independent reviewers (I.G.P and M.L.J). Articles will be rated separately. Disagreements between the two researchers will be resolved by consensus. Studies which are in agreement with the eligibility criteria will be retrieved as full text. Reasons for exclusion and percentage of agreement, as Cohen's kappa, between the assessors will be documented. When data of interest are unclear, authors of the corresponding article will be contacted for more information.

Two independent reviewers will be extracting data and assess the risk of bias for each study. From the included studies, the following data will be extracted: name first author, publication year, study design, mean status of potential dairy fatty acids measured in plasma/serum, erythrocytes and adipose tissue, mean dairy intake by dietary questionnaires, correlation between dairy intake and circulating fatty acids. Selected papers will be screened for used laboratory techniques as well (fatty acid analysis, fraction, column and equipment).

Risk of bias (quality assessment)

Only observational studies will be included in this review. Previous studies have emphasized the need for a uniform methodological assessment tool for observational studies [S9,S10]. The STROBE recommendations are thereby frequently used in an inappropriate way to assess the methodological quality of observational epidemiological studies [S11]. The Cochrane Non-Randomized Studies Methods Working Group recommends the Newcastle-Ottawa Scale (NOS) for assessing the quality of non-randomized studies in meta-analyses [S12]. We therefore designed a methodological study quality tool that assesses the quality of studies that focuses specifically on the correlation between dairy (fat) intake and potential circulating dairy fat

biomarkers. A study will be judged on three domains: the selection (representativeness of the sample population, appropriate sample size, ascertainment of the exposure (validated tool to measure dairy fat intake)), the comparability (appropriate control of confounding factors), and the outcome (appropriate assessment of the outcome (validated tool to measure circulating dairy fat biomarkers), appropriate description of statistical test). The item 'respondents comparability and response rate' was excluded from the original quality tool since it is not applicable in the current review. Instead, we added the item 'Sample Size' whereby a sample size of $n \geq 100$ is justified and satisfactory. The maximum attainable quality score is 6.

Strategy for data synthesis

We will first perform a narrative overview of available biomarker studies. Articles will be classified by type of fraction (plasma/serum, erythrocytes, adipose tissue). For those biomarkers with more than four studies available, we plan quantitative synthesis on aggregate data.

For the quantitative synthesis, data will be pooled with the random effects model of meta-analysis using MedCalc. The existence of heterogeneity among studies will be assessed by Q-tests, and the degree of the heterogeneity will be quantified by calculating the I-squared value.

Analysis of subgroups or subsets

Subgroup analyses will be performed including studies with more than two-thirds of the quality score (> 4 quality points) if more than three studies are available.

Language

English

Funding/Sponsors

Ilse G. Pranger and Stephan J.L. Bakker obtained funding from FrieslandCampina.

References

- S1 Diaz-Lopez, A.; Bullo, M.; Martinez-Gonzalez, M.A.; Corella, D.; Estruch, R.; Fito, M.; Gomez-Gracia, E.; Fiol, M.; Garcia de la Corte, F.J.; Ros, E. *et al.* Dairy Product Consumption and Risk of Type 2 Diabetes in an Elderly Spanish Mediterranean Population at High Cardiovascular Risk. *Eur. J. Nutr.* **2016**, *55*, 349-360.
- S2 Chen, M.; Sun, Q.; Giovannucci, E.; Mozaffarian, D.; Manson, J.E.; Willett, W.C.; Hu, F.B. Dairy Consumption and Risk of Type 2 Diabetes: 3 Cohorts of US Adults and an Updated Meta-Analysis. *BMC Med.* **2014**, *12*, 215-014-0215-1.
- S3 Willett, W.C.; Stampfer, M.J.; Manson, J.E.; Colditz, G.A.; Speizer, F.E.; Rosner, B.A.; Sampson, L.A.; Hennekens, C.H. Intake of Trans Fatty Acids and Risk of Coronary Heart Disease among Women. *Lancet* **1993**, *341*, 581-585.
- S4 Kristal, A.R.; Peters, U.; Potter, J.D. Is it Time to Abandon the Food Frequency Questionnaire? *Cancer Epidemiol. Biomarkers Prev.* **2005**, *14*, 2826-2828.
- S5 Mansson, H.L. Fatty Acids in Bovine Milk Fat. *Food Nutr. Res.* **2008**, *52*, 10.3402/fnr.v52i0.1821. Epub 2008 Jun 11.
- S6 de Oliveira Otto, M.C.; Nettleton, J.A.; Lemaitre, R.N.; Steffen, L.M.; Kromhout, D.; Rich, S.S.; Tsai, M.Y.; Jacobs, D.R.; Mozaffarian, D. Biomarkers of Dairy Fatty Acids and Risk of Cardiovascular Disease in the Multi-Ethnic Study of Atherosclerosis. *J. Am. Heart Assoc.* **2013**, *2*, e000092.

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- S9 Sanderson, S.; Tatt, I.D.; Higgins, J. Tools for Assessing Quality and Susceptibility to Bias in Observational Studies in Epidemiology: A Systematic Review and Annotated Bibliography. *Int. J. Epidemiol.* **2007**, *36*, 666-676.
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- S12 Wells, G.; Shea, B.; O'Connell, D.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P. The Newcastle-Ottawa Scale (NOS) for Assessing the Quality if Nonrandomized Studies in Meta-Analyses. <http://www.ohri.ca/programs/clinicaLepidemiology/oxford.asp>. Accessed June 2017

Appendix S3**NEWCASTLE - OTTAWA QUALITY ASSESSMENT SCALE****(adapted for cross-sectional studies)****Selection:** (Maximum 3 stars)

1) Representativeness of the sample:

- a) Truly representative of the average in the general population. * (all subjects or random sampling)
- b) Somewhat representative of the average in the general population. * (non-random sampling)
- c) Selected group of users.
- d) No description of the sampling strategy.

2) Sample size:

- a) Justified and satisfactory (namely $n \geq 100$). *
- b) Not justified.

3) Ascertainment of the exposure (risk factor; dairy (fat) intake):

- a) Validated measurement tool (FFQ, food record, 24h recall). *
- b) Non-validated measurement tool, but the tool is available or described.
- c) No description of the measurement tool.

Comparability: (Maximum 1 stars)

1) The subjects in different outcome groups are comparable, based on the study design or analysis. Confounding factors are controlled.

- a) The study controls for the most important factor (age, sex, BMI). *
- b) The study controls for any additional factor. *
- c) The study did not control for any additional factors.

Outcome: (Maximum 2 stars)

1) Assessment of the outcome (circulating fatty acids):

- a) Validated measurement tool (Quantitative determination of fatty acids, G(I)C-FID, capillary (a)polar column). *
- b) Non-validated measurement tool, but the tool is available or described *
- c) No description of the measurement tool

2) Statistical test:

- a) The statistical test used to analyze the data is clearly described and appropriate, and the measurement of the correlation is presented, including the probability level (p value). *
- b) The statistical test is not appropriate, not described or incomplete.

This scale has been adapted from the Newcastle-Ottawa Quality Assessment Scale for cohort and case-control studies to perform a quality assessment of cross-sectional studies for the systematic review, "Fatty acids as Biomarkers of Total Dairy and Dairy Fat Intake: A Systematic Review and Meta-analysis". This scale was a modified version of the NOS scale, as also used by several other studies that have felt the need to adapt the NOS scale so as to appropriately assess the quality of cross-sectional studies.

A maximum obtainable quality score of the modified version of the NOS is 6. A minimum of two-thirds of the quality score was chosen as cut-off to represent reasonable quality studies (> 4).

CHAPTER 3

Industrial versus ruminant trans fatty acids and their association with dairy and cardiovascular risk factors

Ilse G. Pranger
Frits A.J. Muskiet
Ido P. Kema
Cécile Singh-Povel
Stephan J.L. Bakker

ABSTRACT

Background: Trans-C18:1 fatty acids in the diet derive from two distinct sources: industrially produced trans-C18:1 (i-TFA) and ruminant trans-C18:1, which is mainly from dairy (r-TFA). I-TFA has shown to be associated with increased cardiovascular risk, whereas r-TFA has been suggested to have a protective effect. We investigated whether the association between r-TFA and cardiovascular risk factors is different compared to the association between i-TFA and cardiovascular risk factors.

Materials and Methods: Data were obtained from the Lifelines Cohort (n=769). Intakes of dairy were determined by food frequency questionnaire. R-TFA and i-TFA were analyzed in fasting plasma triglycerides (TG) and phospholipids (PL) and measured with an GC-FID equipped with an 200 m polar column.

Results: Mean $\pm$ SD age of included subjects was 53 $\pm$ 16 years. Median(25th-75th) intakes of dairy and dairy fat were 322(209–447) and 12.3(8.4–17.4) g/d respectively. Dairy fat was associated with r-TFA (TG std. β =0.249; PL std. β =0.264), and remained associated after adjustment for i-TFA. Plasma TG r-TFA was positively associated with total TG (std. β =0.127) and cholesterol (std. β =0.128). Plasma PL r-TFA was inversely associated with cardiovascular risk factors such as weight (std. β =-0.114), waist circumference (std. β =-0.102), BMI (std. β =-0.107), hs-CRP (std. β = -0.147) and uric acid (std. β =-0.243). R-TFA remained associated with cardiovascular risk factors after adjustments for age, sex, total TG and cholesterol. These associations were not found for i-TFA.

Conclusion: r-TFA, which can be seen as a predictor for the intake of dairy, was inversely associated with cardiovascular risk factors, this in contrast to i-TFA.

INTRODUCTION

In the general population, cardiovascular disease (CVD) has become the number one cause of death worldwide[1]. The total number of CVD deaths has increased globally from 14.1 million in 1990 till 17.7 million in 2015[1,2]. In Europe, more than 85 million people had CVD in 2015, and around 11.3 million new cases were detected[3]. Recognized risk factors for the development of CVD are an increased BMI, elevated levels of total cholesterol and triglycerides, hypertension, insulin resistance and diabetes[4-10]. Other reported predictors of CVD are the inflammatory marker high-sensitive C-reactive protein (Hs-CRP), and the kidney marker uric acid[11-15]. The diet plays an important role in the prevention of CVD and its risk factors[16,17]. However, the optimal dietary composition for CVD prevention is still debated. One of the dietary factors on which more research is needed is dairy fat. Fat from dairy products may have beneficial effects on cardiovascular risk factors such as BMI, fasting glucose and insulin sensitivity[18-21]. However, due to presence of saturated and trans fatty acids such as trans-C18:1, there is skepticism regarding the potential protective effects and even the notion that dairy fat is unhealthy[22,23].

Evidence on the effects of trans-C18:1 fatty acids in cardiovascular disease risk is contradictory. Data from the Cardiovascular Health Study, a prospective study on cardiovascular disease risk factors among older men and women from the USA, showed that high levels of plasma phospholipid trans-C18:1 are inversely associated with overall cardiovascular risk and sudden cardiac death[24]. In another study it was found that high levels of trans-C18:1 fatty acids in adipose tissue were related to an increased risk of myocardial infarction[25]. Importantly, these studies investigated the trans-C18:1 fatty acid cluster as a whole rather than its individual components. It should be realized that the individual components derive from two distinct sources, i.e. industrially produced trans-C18:1 (i-TFA) and ruminant trans-C18:1, mainly from dairy origin (r-TFA)[26]. I-TFA (e.g. elaidic acid or trans-C18:1(n-9)) has been associated with increased cardiovascular risk, while it has been suggested that this is not the case for r-TFA (e.g. vaccenic acid or trans-C18:1(n-7))[27,28]. It was therefore suggested that the effects of i-TFA and r-TFA should be investigated separately. Few studies have indeed done so[29-31], however these studies found the same result for i-TFA and r-TFA or found no significant result. Therefore, most studies still report the TFA as a cluster of trans-C18:1. Furthermore, in the studies analyzing separate effects, the employed method for separating plasma TFA may have been suboptimal[32,33]. The commonly used method to measure trans fatty acids is a GC-FID[34], equipped with a polar column up till 100 m long (e.g. CP-Sil 88)[32,35-37]. However, this method may potentially overestimate the status of i-TFA and r-TFA due to poor separation. Since 2014, polar columns with lengths up till 200 m (i.e. CP Select for FAME) became available that are specifically developed to allow better separation between trans-C18:1 fatty acids[38].

The first aim of the current study was to investigate whether r-TFA was associated with dairy and dairy fat intake, and whether this was independent of i-TFA. For this purpose, we

measured the plasma trans fatty acids on a 200 m polar column. Secondly, we examined the associations of i-TFA and r-TFA with cardiovascular risk factors. A subset of the data from the population based Lifelines Biobank and Cohort study was used to explore these questions.

MATERIALS AND METHODS

Study design and population

The LifeLines Cohort Study is a large observational population-based cohort study and Biobank that examines the health and health-related behaviors of more than 167,000 persons[39]. The participants were recruited from the three Northern provinces of the Netherlands between 2006 and 2013. A more detailed description of the Lifelines Cohort study can be found elsewhere[40,41]. In short, the first group of 25-50 years old participants was recruited via general practitioners. Participants could indicate whether family members were interested as well. In addition, individuals who were interested in the study had the possibility to register via an online self-registration. Individuals with insufficient knowledge of the Dutch language, with severe psychiatric or physical illness, and those with limited life expectancy (< 5 years) were excluded from the study. Participants (> 18 years old) completed several questionnaires, including topics such as occurrence of diseases, general health, medication use, diet, physical activity and personality. Participants were invited to the Lifelines Research sites for a comprehensive health assessment. All participants provided written consent. The current study used a randomly selected subset from the cross-sectional data set (n = 864). The Lifelines Cohort Study was conducted according to the principles of the Declaration of Helsinki and approved by the Medical ethical committee of the University Medical Center Groningen, The Netherlands.

Dietary intake

To assess dietary intake in the Lifelines Cohort, a 110-item semi-quantitative baseline food frequency questionnaire (FFQ) assessing food items over the previous month was developed and validated by the Wageningen University using the Dutch FFQTOOL™, in which food items were selected based on the Dutch national Food Consumption Survey of 1997/1998[42]. Seven answer categories were used to assess consumption frequency, ranging from 'not this month' to '6-7 days a week'. Portion size was estimated by fixed portion sizes (e.g. slices of bread, pieces of fruit) and commonly used household measures (e.g. cups, spoons). Energy and macronutrient intake, including dairy consumption, was estimated from the FFQ data by using the Dutch food composition database of 2011 (NEVO)[43]. For this study, 'total dairy intake' and 'total dairy fat intake' (consisting of cheese, milk, buttermilk, yoghurt, sweetened yoghurt drinks, custard, curd cheese, ice cream, whipped cream and porridge) were calculated.

Cardiovascular risk factors

A standardized protocol was used to obtain blood pressure, height, weight and waist circumference. Systolic and diastolic blood pressures were measured 10 times during a period of 10 min, using an automated Dinamap Monitor (GE Healthcare, Freiburg, Germany). The average of the final three readings was used for each blood pressure parameter. Anthropometric measurements were done without shoes, for body weight to the nearest 0.1 kg and height and waist circumference to the nearest 0.5 cm. Height was measured with a stadiometer and waist circumference was measured in standing position with a tape measure all around the body, at the level midway between the lower rib margin and the iliac crest. BMI was calculated by dividing weight in kilograms by the squared height in meters (kg/m^2).

For analysis of lipids, glucose, uric acid, creatinine and the inflammation marker high sensitivity C-reactive protein (hs-CRP), blood samples were drawn in the morning between 8:00 and 10:00 am after a period of overnight fasting. Serum levels of total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with an enzymatic colorimetric method, low-density lipoprotein (LDL) cholesterol with an enzymatic method and total triglycerides with a colorimetric UV method, all on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). Fasting blood glucose was measured using a hexokinase method. HbA1c was determined in whole blood (EDTA-anticoagulated) by means of turbid metric inhibition immunoassay on a Cobas Integra 800 CTS analyzer (Roche Diagnostics Netherland BV, Almere, The Netherlands). Insulin resistance was estimated by calculating the ratio total triglycerides/HDL cholesterol[44]. Serum uric acid and creatinine were measured on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). The Hs-CRP was determined by nephelometry (BN II system Siemens, Marburg, Germany).

Data on education and smoking habits

Information about education, smoking and general health was collected from the self-administered questionnaire. Educational level was categorized in four groups (1. never been to school or elementary school only, 2. lower vocational or secondary schooling, 3. intermediate vocational schooling or intermediate/higher secondary schooling or 4. higher vocational schooling or university). Subjects were also classified as non-smokers, former smokers or current smokers.

Sample preparation and analyses

Fatty acid analyses

EDTA-plasma samples were collected at baseline and stored at -80°C until use for assessment of fatty acid profiles. Analyses of fatty acids were performed at the Department of Laboratory Medicine of the University Medical Center Groningen, The Netherlands using the methodology

as described by Hoving *et al* [45]. In short, total lipids were extracted by the method of Folch *et al.*, using 6 ml of chloroform-methanol (2:1) and 200 μ l EDTA-plasma sample[46]. In addition, a shortened version of the method of Kaluzny *et al.* was used to isolate plasma cholesterol esters (CE), triglycerides (TG) and phospholipids (PL), using aminopropyl SPE columns for the separation (Isolute, Biotage)[47]. Fatty acids were transmethyated with methanolic-HCL into fatty acid methyl esters (FAME). The samples were extracted with hexane and eventually redissolved into 100 μ l hexane. 100 μ l of an internal standard for the quantification of fatty acids in CE (17:0) (50.1 mg/100 ml chloroform-methanol, 2:1 v/v), and 100 μ l of an internal standard for the quantification of fatty acids in TG (19:0) (19.9 mg/100 ml chloroform-methanol, 2:1 v/v), both obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), were added before isolation of lipid classes. For the quantification of fatty acids in PL, 100 μ l of free fatty acid 19:0 (50.0 mg/100 ml methanol), obtained from Larodan (Solna, Sweden), was added after isolation of lipid classes. 100 μ l Butylated Hydroxytoluene (1 g/100 ml methanol) from Sigma-Aldrich (Zwijndrecht, The Netherlands) was added to prevent fatty acid oxidation. An overview of the sample preparation and analyses can be found in **Figure 1**.

Conditions of the Gas Chromatography and Flame ionization Detector

Aliquots of 2 μ l were injected into an Agilent model 6890 gas chromatography (GC) equipped with a 200 m x 0.25 mm polar column (CP Select for FAME) and detected with an Agilent 7683 series flame ionization detector (FID). The carrier gas was helium, there was a constant pressure of 550 kPa and a split ratio of 1:20. The injector temperature was 260°C and the detector temperature was 300°C. The temperature program started with a temperature of 150°C and increased with a rate of 1°C/min till a temperature of 250°C. The temperature was subsequently increased with 20°C/min till a final temperature of 270°C for 15 min. The conditions of the GC-FID can be found in **Table A** of the supporting information 'Methods and Validation'. Chromatograms of the trans-C18:1 fatty acids in plasma TG and PL are shown in **Supplemental Figures A and B**.

Identification, quantification and quality control

FAME were identified by comparing retention times with those of known standards (Supelco 37 component FAME mix (Sigma-Aldrich)). Peak areas were calculated using Atlas 8.2.3. Chromatography Data System (Thermo Fisher Scientific Inc.) and Atlas Report Manager (Blaise software Series Inc.). Data on fatty acids were calculated into mol%. Variation coefficients (CV) of the fatty acids were determined using quality-control samples (pooled plasma samples). CVs were obtained from 10 replicates measured in a single series. CV% of I-TFA and r-TFA in both plasma TG and PL were $\leq 10\%$ (Supporting information 'Methods and Validation' **Table B**). All heights of the identified peaks in plasma TG and PL exceeded 100 times the average background noise.

Figure 1: Sample preparation and analyses of fatty acids in plasma cholesterol esters (CE), triglycerides (TG) and Phospholipids (PL)

Pilot study

Prior to the current analyses, fasting EDTA plasma samples of the selected Lifelines participants had been frozen at -80°C. They were transported to the Central Laboratory of the University Medical Center Groningen. We first performed a pilot study for which we measured trans-C18:1 fatty acids in plasma CE, TG and PL of 96 participants. In this pilot, we found that trans-C18:1 fatty acids were not detectable in plasma CE, while they were well above the detection limit in TG and PL. Subsequently, we decided to move forward with measurements only in plasma TG and PL. In total, 864 samples were analyzed.

Statistical analyses

All analyses were performed using IBM SPSS Statistics, version 22.0 for Windows software (IBM Armonk, NY, USA) and GraphPad Prism, version 5.03 for Windows software (Graphpad Software, La Jolla, CA, USA). In total, circulating fatty acids were measured in 864 participants, whereof 776 had completed the FFQ. Cases with missing data on either dietary intake or circulating fatty acids were removed before analyses leaving 769 participants in the lifelines cohort with complete data. Characteristics of the Lifelines population are reported as mean $\pm$ SD for continuous data with a normal distribution and as median (25th-75th percentile) for continuous data with a skewed distribution. Categorical data are presented as number (%). Dairy and dairy fat intake were measured as absolute and relative intakes, of which relative intakes were stratified for sex and adjusted for total energy intake using the residual method[48]. Multivariate linear regression analyses were used to investigate the association of absolute and relative dairy and dairy fat intake with I-TFA, R-TFA and I-TFA+R-TFA in plasma TG and PL. Non-normal data were transformed (square root) before analyses. Models were adjusted for I-TFA or R-TFA, age, education and smoking habits, and total carbohydrates and total fat intake. Multivariate linear regression analyses were also used to examine the cross-sectional association of trans-C18:1 fatty acids measured in plasma TG and PL with cardiovascular risk factors. Associations were adjusted for age and sex, and education and smoking habits. Associations with plasma TG were further adjusted for total triglycerides, while associations with plasma PL were further adjusted for total triglycerides and total cholesterol. Associations between dairy fat intake (relative intake) and cardiovascular risk factors were investigated as an extra analyses. The analyses were adjusted for age, education, smoking habits, total carbohydrate and total fat intake. All reported probability values are two-tailed, and $P \leq 0.05$ was considered statistically significant.

RESULTS

Study population

In the present study, 769 subjects with complete data on dietary intake and circulating trans fatty acids were included. Characteristics of the study population are shown in **Table 1**. Age of the included subjects was 53.0 ± 15.5 years and 404 subjects were male (52.5%). The subjects were slightly overweight with a BMI of 26.0 ± 4.0 kg/m² and blood pressure was within normal boundaries (127/74 mmHg). Total TG/HDL-c ratio (mol/mol) was 0.72 (0.45 - 1.17), whereas glucose and HbA1c were 5.1 ± 0.7 and $5.6 \pm 0.4\%$ mmol/L, respectively. The study population had a serum Hs-Crp of 1.0 (0.5 – 2.2) mg/L and a serum uric acid level of 0.31 ± 0.1 mmol/L. Energy intake of the population was 1975 ± 624 kcal/d (8274 ± 2613 KJ/d). Of the total intake, 46 En% was derived from carbohydrates, 15 En% from proteins, and 35 En% from fats. Absolute intakes of dairy and dairy fat intake were 322 (209 – 447) g/d and 12.3 (8.4 – 17.4) g/d respectively. Total dairy intake consisted for 12.8% of cheese, for 35.1% of milk and for 10.0% of yoghurt. Of the products, 25% were skimmed dairy products, 23% semi-skimmed dairy products and 24% full-fat dairy products. Status of i-TFA was 0.12 (0.09 – 0.15) mol% in plasma TG and 0.05 (0.04 – 0.06) mol% in plasma PL. Status of r-TFA was 0.10 (0.07 – 0.13) mol% in plasma TG and 0.09 (0.07 – 0.11) mol% in plasma PL.

Table 1: Characteristics of the Lifelines Population (n = 769)

Baseline Characteristics	Mean/Median/Amount	SD/25 th -75 th percentile/%
General		
Age visit 1, years	53.0	15.5
Male sex (%)	404	52.5%
Weight, kg	79.3	14.2
BMI, kg/m ²	26.0	4.0
Smoking, yes (%)	115	15.0%
SBP, mmHg	126.5	17.0
DBP, mmHg	73.7	9.7
Laboratory measurements		
Total Cholesterol, mmol/L	5.2	1.0
HDL-c, mmol/L	1.5	0.4
LDL-c, mmol/L	3.3	0.9
Total TG, mmol/L	1.0	0.8 - 1.5
TG/HDL-c ratio, mol/mol	0.72	0.45 – 1.17
Glucose, mmol/L	5.1	0.7
HbA1c, %	5.6	0.4

Table 1: Continued

Baseline Characteristics	Mean/Median/Amount	SD/25 th -75 th percentile/%
Creatinine, umol/L	76.0	13.6
C-reactive protein (hs-CRP), mg/L ¹	1.0	0.5 – 2.2
Uric acid, mmol/L ¹	0.31	0.1
Dietary intake (FFQ)		
Energy intake, Kcal/d	1971	624
Total carbohydrates, g/d	228.8	76.7
Total protein, g/d	74.8	20.9
Total fat, g/d	77.2	30.0
Dairy intake (FFQ)		
Total Dairy intake (absolute intake), g/d	322	209 – 447
Total Dairy fat intake (absolute intake), g/d	12.3	8.4 – 17.4
Total Dairy intake (relative intake), g/d	331	217 – 443
Total Dairy fat intake (relative intake), g/d	12.7	9.2 – 16.4
Trans-fatty acid status in Plasma TG		
I-TFA, mol%	0.12	0.09 – 0.15
R-TFA, mol%	0.10	0.07 – 0.13
I-TFA + R-TFA, mol%	0.22	0.17 – 0.29
Trans-fatty acid status in Plasma PL		
I-TFA, mol%	0.05	0.04 – 0.06
R-TFA, mol%	0.09	0.07 – 0.11
I-TFA + R-TFA, mol%	0.14	0.11 – 0.17

Data are presented as mean $\pm$ SD, median (25th-75th percentile) or number (%). Abbreviations: SD, standard deviation; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, Triglycerides; TG/HDL-c ratio, Total Triglycerides/HDL cholesterol ratio; HbA1C, Hemoglobin A1C; FFQ, Food Frequency Questionnaire; Kcal, Kilocalories; PL, Phospholipids; I-TFA, industrial trans fatty acid (Elaidic acid; trans-C18:1(n-9)); r-TFA, ruminant trans fatty acid (Vaccenic acid; trans-C18:a(n-7)). ¹ Hs-CRP was available in n = 412, uric acid was available in n = 348

Dairy intake and trans-C18:1 fatty acids

The associations of i-TFA and r-TFA with dairy and dairy fat intakes (absolute and relative) are shown in **Table 2**. In plasma TG, i-TFA and r-TFA were associated with absolute intakes of dairy (std. β = 0.078 and std. β = 0.101, $p \leq 0.03$) and with dairy fat (std. β = 0.181 and std. β = 0.261, both $P < 0.001$) (Model 1). I-TFA and r-TFA were also associated with relative intakes of dairy (std. β = 0.087, $p = 0.01$ and std. β = 0.105, $p = 0.003$) and dairy fat (std. β = 0.217, std. β = 0.292, both $p < 0.001$). The associations were also found for i-TFA and r-TFA measured in plasma PL, and the combination of i-TFA and r-TFA in plasma TG and PL. The associations of i-TFA, measured in both plasma TG and PL, with absolute and relative dairy and dairy fat intakes did

not remain after adjustment for r-TFA (Model 2). In contrast, the associations of r-TFA with absolute and relative dairy and dairy fat intakes remained after adjustment for i-TFA, for which the associations between r-TFA and relative dairy fat intakes were strongest in both plasma TG and PL (std.β = 0.249 and std.β = 0.264, $p < 0.001$, respectively) (Model 2). The association of trans-C18:1 with dairy and dairy fat intakes was further explored by adjusting the model for age, education and smoking habits (**Supplemental Table 1, Model S1**), and total carbohydrates and total fat intakes (**Supplemental Table 1, Model S2**). R-TFA and i-TFA + r-TFA remained related to absolute and relative dairy and dairy fat intakes.

Trans-C18:1 in plasma TG and cardiovascular risk factors

The associations of industrial and ruminant TFA measured in plasma TG with cardiovascular risk factors are shown in **Table 3**. I-TFA, r-TFA and i-TFA + r-TFA were positively associated with age (std.β = 0.120, std.β = 0.088 and std.β = 0.122, respectively, all $P \leq 0.01$). Additionally, r-TFA and i-TFA + r-TFA were positively associated with total cholesterol (std.β = 0.128 for r-TFA and std.β = 0.131 for i-TFA + r-TFA, $P < 0.001$), LDL cholesterol (std.β = 0.119 for r-TFA and std.β = 0.106 for i-TFA + r-TFA, $P \leq 0.003$) and total TG (std.β = 0.127 for r-TFA and std.β = 0.133 for i-TFA + r-TFA, $P < 0.001$). The associations remained after adjustment for age and sex (**Figure 2**). I-TFA was associated with glucose (std.β = 0.119, $p = 0.001$) and HbA1c% (std.β = 0.114, $p = 0.001$), however only the association with glucose remained after adjustment for age and sex (std.β = 0.085, $p = 0.01$) and there was no association with the insulin sensitivity marker TG/HDL-c ratio. The association of i-TFA + r-TFA with glucose and HbA1c% showed the same trends. The inflammatory marker Hs-CRP was not associated with the trans fatty acids measured in plasma TG. Uric acid was inversely associated with r-TFA after adjustment for age and sex (std.β = -0.108, $p = 0.03$).

In further analyses, the associations were also adjusted for education, smoking and total triglycerides (**Supplemental Table 2**). The association between r-TFA and BMI became inverse (std.β = -0.067, $P = 0.05$). The association of r-TFA with total cholesterol and LDL cholesterol became weaker and disappeared respectively, while the inverse association with uric acid became stronger (std.β = -0.140, $p = 0.001$).

Table 2: Association of dairy and dairy fat intake with the industrial and ruminant trans-fatty acids

Absolute intake										Relative intake									
Dairy intake (g/d)					Dairy fat intake (g/d)					Dairy intake (g/d)					Dairy fat intake (g/d)				
Model 1		Model 2			Model 1		Model 2			Model 1		Model 2			Model 1		Model 2		
Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value	Stand β	P-value
<i>Plasma TG</i>																			
I-TFA	0.078	0.03	0.032	0.46	0.181	<0.001	0.051	0.087	0.01	0.041	0.34	0.217	<0.001	0.076	0.06				
R-TFA	0.101	0.01	0.083	0.05	0.261	<0.001	0.232	0.105	0.003	0.082	0.06	0.292	<0.001	0.249	<0.001				
I-TFA + R-TFA	0.103	0.004			0.261	<0.001		0.109	0.02			0.297	<0.001						
<i>Plasma PL</i>																			
I-TFA	0.081	0.03	0.045	0.26	0.096	0.01	0.009	0.082	0.03	0.046	0.25	0.114	0.002	0.013	0.73				
R-TFA	0.111	0.002	0.095	0.02	0.232	<0.001	0.228	0.111	0.002	0.095	0.01	0.269	<0.001	0.264	<0.001				
I-TFA + R-TFA	0.118	0.001			0.218	<0.001		0.120	0.001			0.256	<0.001						

Model 1: Crude model **Model 2:** Model 1 + additional adjustment for R-TFA or i-TFA.

Dependent: dairy (fat) intake, independent: trans fatty acid. Abbreviations: TG, Triglycerides; I-TFA, Industrial trans-fatty acid; R-TFA, Ruminant trans fatty acid; PL, phospholipids

Table 3: Association between trans-fatty acids in plasma triglycerides (TG) and cardiovascular risk factors

	Plasma TG					
	I-TFA			R-TFA		
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Age (years)	Stand β 0.120	P-value <0.001	Stand β 0.088	P-value 0.01	Stand β 0.122	P-value <0.001
Sex	0.054	0.14	-0.058	0.11	-0.013	0.73
Weight (kg)	0.019	0.59	0.041	0.21	0.032	0.38
Waist circumference	0.082	0.02	0.062	0.05	0.077	0.03
BMI (kg/m ²)	0.063	0.08	0.041	0.25	0.032	0.38
SBP (mmHg)	0.059	0.09	0.015	0.63	0.045	0.20
DBP (mmHg)	0.065	0.07	0.041	0.21	0.072	0.04
Total Cholesterol (mmol/L)	0.098	0.01	0.059	0.09	0.131	<0.001
HDL-c (mmol/L)	0.037	0.31	0.003	0.94	-0.019	0.60
LDL-c (mmol/L)	0.062	0.09	0.033	0.36	0.106	0.003
Total TG (mmol/L)	0.093	0.01	0.088	0.01	0.133	<0.001
TG/HDL-c ratio (mol/mol)	0.056	0.12	0.065	0.06	0.109	0.003
Glucose (mmol/L)	0.119	0.001	0.085	0.01	0.106	0.003
HbA1c (%)	0.114	0.001	0.049	0.11	0.087	0.01
Hs-CRP (mg/L) ¹	0.085	0.09	0.076	0.12	0.004	0.93
Creatinine (umol/L)	0.011	0.77	0.026	0.37	0.039	0.28
Uric acid (mmol/L) ¹	-0.032	0.56	-0.008	0.88	-0.058	0.28
						0.22

Model 1: Crude model; **Model 2:** Model 1 + additional adjustments for age and sex.Dependent: cardiovascular risk factors, independent: trans fatty acid status. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1c; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-Crp data (n = 412), Uric acid data (n = 348).

Figure 2: Age and sex adjusted associations between ruminant TFA measured in plasma triglycerides and cardiovascular risk factors. * = $P < 0.05$

Abbreviations: WC, waist circumference; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C, Total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Total TG, Total triglycerides; HbA1c%, hemoglobin A1c; Hs-CRP, High-sensitive C-reactive protein.

Trans-C18:1 fatty acids in plasma PL and cardiovascular risk factors

The associations of industrial and ruminant TFA measured in plasma PL with cardiovascular risk factors are shown in **Table 4**. I-TFA, r-TFA and i-TFA + r-TFA were positively associated with age (std. β = 0.104, std. β = 0.071 and std. β = 0.093, all $p \leq 0.03$, respectively) and sex (std. β = 0.124, std. β = 0.102 and std. β = 0.130, all $p \leq 0.01$, respectively). R-TFA was inversely associated with weight (std. β = -0.114, $P = 0.002$), waist circumference (std. β = -0.102, $p = 0.01$), BMI (std. β = -0.107, $p = 0.003$), and systolic (std. β = -0.081, $p = 0.02$) and diastolic blood pressure (std. β = -0.072, $P = 0.05$), while this was not the case for i-TFA. R-TFA was also inversely associated with total TG (std. β = -0.098, $p = 0.01$), the TG/HDL-c ratio (std. β = -0.108, $p = 0.003$) and glucose (std. β = -0.077, $p = 0.03$), while this was again not the case for i-TFA. Both r-TFA and i-TFA + r-TFA were inversely associated with the inflammatory marker Hs-CRP (std. β = -0.147 and std. β = -0.131, respectively, all $p \leq 0.01$), and with the kidney marker uric acid (std. β = -0.243 and std. β = -0.222, respectively, all $p < 0.001$). All associations remained significant after adjustment for age and sex (**Figure 3**).

In additional analyses, associations were additionally adjusted for education, smoking, total triglycerides and total cholesterol (**Supplemental Table 3**). Although weaker compared to model 1 and 2, significant inverse associations of r-TFA with waist circumference, BMI, systolic

blood pressure, glucose, Hs-CRP and uric acid remained after adjustment for age, sex, total triglycerides and total cholesterol.

Dairy (fat) intake and cardiovascular risk factors

As an extra analysis, relative intakes of dairy and dairy fat intake were associated with cardiovascular risk factors (**Supplemental Table 4**). Dairy and dairy fat intake were associated with several cardiovascular risk factors such as waist circumference, blood pressure, cholesterol, glucose, HbA1c% and uric acid. However, these associations disappeared after adjustment for age, education, smoking, and total carbohydrates and total fat intake.

Figure 3: Age and sex adjusted associations between ruminant TFA measured in plasma phospholipids and cardiovascular risk factors. * = $P < 0.05$

Abbreviations: WC, waist circumference; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C, Total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Total TG, Total triglycerides; HbA1c, hemoglobin A1c; Hs-CRP, High-sensitive C-reactive protein

Table 4: Association between trans-fatty acids in plasma phospholipids (PL) and cardiovascular risk factors

	Plasma PL									
	I-TFA					R-TFA				
	Model 1		Model 2		Stand β	Model 1		Model 2		Stand β
	Stand β	P-value	Stand β	P-value		Stand β	P-value	Stand β	P-value	
Age (years)	0.104	0.003			0.071	0.03		0.093	0.01	
Sex	0.124	0.001			0.102	0.01		0.130	< 0.001	
Weight (kg)	-0.050	0.18	0.004	0.90	-0.114	0.002	-0.069	-0.113	< 0.001	-0.056
Waist circumference	0.007	0.84	0.016	0.63	-0.102	0.01	-0.092	-0.082	0.03	-0.070
BMI (kg/m ²)	0.018	0.64	0.003	0.94	-0.107	0.003	-0.119	-0.083	0.03	-0.098
SBP (mmHg)	0.013	0.72	-0.011	0.74	-0.081	0.02	-0.095	-0.066	0.07	-0.086
DBP (mmHg)	-0.013	0.74	-0.010	0.76	-0.072	0.05	-0.066	-0.067	0.07	-0.060
Total Cholesterol (mmol/L)	0.104	0.01	0.064	0.08	0.047	0.20	0.017	0.076	0.04	0.038
HDL-c (mmol/L)	0.103	0.01	0.039	0.25	0.093	0.01	0.042	0.115	0.002	0.050
LDL-c (mmol/L)	0.065	0.08	0.043	0.24	0.033	0.37	0.018	0.049	0.19	0.030
Total TG (mmol/L)	0.020	0.59	0.033	0.37	-0.098	0.01	-0.088	-0.072	0.05	-0.058
TG/HDL-c ratio (mol/mol)	-0.027	0.47	0.007	0.85	-0.108	0.003	-0.080	-0.098	0.01	-0.063
Glucose (mmol/L)	0.010	0.78	-0.011	0.76	-0.077	0.03	-0.095	-0.061	0.10	-0.083
HbA1c (%)	0.002	0.96	-0.057	0.08	-0.057	0.11	-0.099	-0.052	0.15	-0.107
Hs-CRP (mg/L) ¹	-0.037	0.46	-0.057	0.26	-0.160	0.001	-0.179	-0.139	0.01	-0.163
Creatinine (umol/L)	-0.060	0.11	-0.001	0.97	-0.093	0.01	-0.044	-0.102	0.01	-0.039
Uric acid (mmol/L) ¹	-0.084	0.13	-0.040	0.44	-0.243	< 0.001	-0.208	-0.222	< 0.001	-0.178

Model 1: Crude model; **Model 2:** Model 1 + additional adjustments for age and sex.
Dependent: cardiovascular risk factors, independent: trans fatty acid status. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1c; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-CRP data (n = 412), Uric acid data (n = 348)

DISCUSSION AND CONCLUSION

In a population-based study of 769 participants, we demonstrated that r-TFA was significantly associated with absolute and relative intakes of dairy and dairy fat. Furthermore, we showed that r-TFA, but not i-TFA were inversely associated with cardiovascular risk factors. To the best of our knowledge, we are the first to have performed such an analysis with r-TFA and i-TFA measured by a 200 m polar column, a technique which is superior in separating r-TFA's from i-TFA's.

Previous studies on the association between TFA and dairy (fat) intake have mainly used a total cluster of trans-C18:1 fatty acids, not separating the industrial and ruminant variant. Earlier cross-sectional studies on the trans-C18:1 cluster showed contradicting results. Two studies from 2014 showed that a cluster of trans-C18:1 fatty acids measured in erythrocytes was positively correlated with dairy intake[49,50]. In contrast, a cluster of trans-C18:1 fatty acids measured in adipose tissue was not associated with dairy intake[51]. In our cohort, in line with the studies of 2014, the combinations of industrial and ruminant trans fatty acids, in both plasma TG and PL, were significantly correlated with dairy intake in the general population.

In our cohort, the associations between dairy (fat) intake and trans-C18:1 fatty acids were stronger for the relative intakes compared to absolute intakes. This is in line with the current literature. Results from the Nurses' Health Study gathered between 1989-1990 showed that the correlation between trans fatty acids measured in plasma and trans fatty acid intake measured by an FFQ changed from an β of 0.17 for the unadjusted correlation (absolute intake) to an β of 0.30 when adjusted for energy intake (relative intake). The same was seen when the trans fatty acids were measured in erythrocytes (unadjusted: $\beta = 0.25$ and adjusted: $\beta = 0.37$)[52]. The authors explained the differences between absolute and relative intakes by the fact that energy intake can operate as a confounding variable and that failure to adjust for total energy intake can lead to attenuation of the associations[48,52]. In the current study, there was a high inter-correlation between energy intake and dairy (fat) intake measured with the FFQ (std. $\beta = 0.34$; std. $\beta = 0.48$, both $P < 0.001$, respectively dairy intake and dairy fat intake), which indicates that energy intake might be a confounding factor in our dataset as well. It was therefore to be expected that the energy-adjusted models (relative intakes) had a higher significant correlation with dairy and dairy fat intake compared to the unadjusted models (absolute intakes).

In the current study both the industrial and ruminant trans fatty acids correlated with absolute and relative intakes of dairy (fat), with a slightly stronger association between dairy and r-TFA. The correlation between r-TFA and dairy (fat) intake remained after adjustment for the i-TFA, while the correlation between i-TFA and dairy (fat) intake disappeared after adjustment for the r-TFA. A combination of i-TFA and r-TFA was associated with dairy as well. That i-TFA and r-TFA were both associated with dairy (fat) intake can be explained by the fact that both trans fatty acids can be found in ruminant derived products, such as dairy. Ruminant and industrial food items contain elaidic acid as well as vaccenic acid, however, vaccenic is the major trans-C18:1

in ruminant food items (> 40 wt% of total trans-C18:1 in ruminant products vs <15 wt% of total trans-C18:1 in industrial products), while elaidic acid is the major trans-C18:1 in industrial food items (< 10 wt% of total trans-C18:1 in ruminant products vs >20wt% of total C18:1 in industrial products)[53,54]. In theory, r-TFA and i-TFA+r-TFA may both act as biomarker for dairy and dairy fat intake. It is, however, important to note that the content of i-TFA in apparently similar foods may vary considerably in the same country and from time to time[26]. Concentration of r-TFA in ruminant fat may vary as well, particularly via the feed of the animal and therefore with season[55]. The variation is however much lower compared to the variation observed for i-TFA[26]. It is therefore preferable to use r-TFA as marker of dairy (fat) intake only.

Dairy intake and fats from dairy products may have a favorable effect on cardiovascular risk factors such as body weight, blood pressure, abnormal glucose homeostasis, insulin resistance and dyslipidemia [18,29,56-59]. Since r-TFA is associated with dairy (fat) intake, an inverse association between r-TFA and cardiovascular risk factors was therefore expected in this cohort. R-TFA measured in plasma PL was inversely associated with several cardiovascular risk factors, such as weight, waist circumference, BMI, systolic and diastolic blood pressure, total TG, TG/HDL-c ratio. All associations remained after adjustment for age and sex. In line with our results, a Canadian study with 100 obese and 100 non-obese participants showed that the ones with a higher percentage of r-TFA in plasma PL had a lower systolic (124.4 vs 118.4 mmHg, $p < 0.01$) and diastolic blood pressure (80.3 vs 75.6 mmHg, $p = 0.04$) compared to the ones with a lower percentage of r-TFA in plasma PL[60]. Furthermore, a study in which the intake of r-TFA was measured by FFQs also suggested a favorable effect on cardiovascular health as results from this cohort showed an inverse association between intake of r-TFA and changes in weight[61].

In our cohort, plasma PL r-TFA showed to be inversely associated with glucose and HbA1c%. Literature reports on the associations of r-TFA with the markers glucose and HbA1c% are contradictory to those results. In a randomized controlled trial with 42 healthy young men, glucose levels did not change after taken an r-TFA enriched butter for 5 weeks[62]. Additionally, fasting plasma glucose levels were also not affected by dietary r-TFA in obese and insulin resistant rats[63]. In contrast, literature does report on inverse associations between the dairy fat biomarkers C15:0, C17:0 and trans-C16:1(n-7) and diabetes related outcomes. In a cross-sectional study with 17 men and women without nonalcoholic fatty liver disease, it was found that serum PL C15:0, C17:0 and trans-C16:1(n-7) were inversely associated with fasting glucose[21]. Furthermore, a higher PL trans-C16:1(n-7) status was found to be associated with lower insulin resistance and lower incidence of diabetes[19]. In the Lifelines cohort, the dairy fat biomarkers C15:0, C17:0 and trans-C16:1(n-7) correlated well with r-TFA. PL r-TFA exhibited correlation coefficients of 0.38 with PL C15:0, of 0.53 with PL C17:0 and of 0.56 with PL trans-C16:1(n-7). The inverse association of r-TFA with glucose and HbA1c% in our cohort may therefore not be surprising.

An inverse association was found with the inflammatory marker Hs-CRP in plasma PL. Yet, the association between plasma r-TFA and Hs-CRP in humans has not been investigated in detail. There is an intervention study that investigated the effect of a butter enriched r-TFA on Hs-CRP[62]. However, the authors of the paper did not find a significant decrease in the inflammatory marker. On the other hand, an experimental study on the effect of r-TFA and dairy proteins in human endothelial cells found a reduction in inflammatory responses[64]. Although the researchers focused on different outcomes, such as prostaglandin excretion and inflammatory gene expression, it showed that r-TFA may have an impact on human inflammation.

The strongest association in our study was found between uric acid and r-TFA in both plasma TG and PL. Additionally, an inverse association between uric acid and dairy fat intake was found. This inverse association between uric acid levels and dairy intake has been observed before[65]. An increased uric acid level may increase the risk for the development of cardiovascular diseases[13,66]. It can be hypothesized that the opposite may actually reduce the risk for the development of cardiovascular diseases. It therefore seems like r-TFA, in this cohort, may have a beneficial effect on cardiovascular health by reducing uric acid levels.

Until now we discussed the results of the phospholipids fraction. In the TG fraction, i-TFA+r-TFA was positively associated with total cholesterol, LDL cholesterol, total triglycerides and TG/HDL cholesterol ratio. The associations with cholesterol were driven by the ruminant variant. The industrial variant in triglycerides adversely associated with glucose and waist circumference. This study is the first to find those associations with r-TFA or i-TFA measured in TG. Interestingly, the beneficial associations in the PL fraction between trans fatty acids and cardiovascular risk factors were neutral or even adverse in the TG fraction and some neutral association in the PL were adverse in the TG. This might be due to the fact that the association between plasma TG trans fatty acids and cardiovascular risk factors can be influenced by recent dietary intakes and *de novo* lipogenesis, whereas this is less important for plasma PL[67]. The latter is confirmed by the fact that fatty acids from *de novo* lipogenesis (i.e. C14:0, C16:0, C16:1(n-7)) measured in plasma TG) were significantly associated with total triglycerides (data not shown), which might explain the discrepant findings.

Some strengths and limitations of the study have to be addressed. A main strength of the study was the separation between the industrial and ruminant trans fatty acids in plasma TG and PL, what made it possible to investigate the association of the rumen derived C18:1 trans fat and cardiovascular risk factors, independently of the industrial derived C18:1 trans fat. This separation was made by using a 200 m polar column, which is a superior method for the separation of industrial and ruminant derived fatty acids. A second strong point is that we measured the fatty acids in two different compartments, i.e. plasma TG and PL, providing the opportunity to investigate whether the association of i-TFA and r-TFA with cardiovascular risk factors were consistent in plasma lipid classes with widely differing functions and half-

lives. Another strong point is the relatively large number of participants included in this study. Furthermore, Lifelines is building a biobank and database. For this purpose, Lifelines has collected and is still collecting information via questionnaires, physical examinations and biological samples, including information on several cardiovascular risk factors. This huge collection of data and samples allowed us to investigate the association of trans fatty acids with multiple cardiovascular risk factors.

This study also has its limitations. Firstly, we utilized cross-sectional data from which we cannot infer causality. It is therefore not possible to rule out reverse causality between the trans fatty acids and cardiovascular risk factors. Secondly, the Lifelines cohort study uses an FFQ to measure dietary intake of the participants which is a method based on self-report, and therefore subject to recall bias. It is possible that the intakes of absolute and relative dairy and dairy fat are over- or underreported. On the other side, the current FFQ is specifically developed for the Dutch population and consists therefore of a relatively large number of dairy items, which makes it a reliable measurement for this paper. Furthermore, since there is no gold standard for measuring dietary intakes so far, FFQs are still seen as one of the best methods since they are able to capture usual, individual, long-term dietary intakes[68,69].

In summary, we found that the ruminant trans-C18:1 fatty acid, that was found to be a predictor for the intake of dairy and dairy fat intake in our population, was inversely associated with cardiovascular risk factors, including Hs-CRP and uric acid. This in contrast to the industrial trans-C18:1 fatty acid, which were not associated with cardiovascular risk factors. Research in intervention and longitudinal studies is warranted to further unravel the association between r-TFA and cardiovascular risk factors.

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The manuscript is based on data from the LifeLines Cohort Study. LifeLines adheres to standards for open data availability. The data catalogue of LifeLines is publicly accessible on www.LifeLines.net. All international researchers can apply for data at the LifeLines research office (LLscience@umcg.nl). The LifeLines system allows access for reproducibility of the study results.

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SUPPORTING INFORMATION: METHODS AND VALIDATION**Supplemental Table A:** Conditions of the GC-FID

Overall	
Technique	GC model 6890
Injection technique	Split, 1:20
Analytical column	Agilent CP select CB for FAME
Detection technique	FID 7683 series
Parameters	
Column	0.25 mm x 200 m
Carrier gas	Helium
Pressure	Constant pressure, 550 kPa
Flow	33.3
Injection temperature	260°C
Detection temperature	300°C
Injection volume	2 µl
H ₂	35
Air flow	300
Make up gas N ₂	Constant make up, 30
Temperature program	
Start	150°C
Rate 1	1°C/min
Final temperature and time	250°C for 0 min
Rate 2	20°C/min
Final temperature and time	270°C for 15 min

Abbreviations: GC, Gas chromatography; FID, Flame Ionization Detector; FAME, fatty acid methyl esters; kPa, kilo Pascal

Supplemental Table B: Variation coefficients of quality-control samples (10 pooled samples)

Fatty acid	Plasma TG		Plasma PL	
	Mean $\pm$ SD	CV %	Mean $\pm$ SD	CV %
SFA				
C14:0	2.62 $\pm$ 0.05	2.0	0.56 $\pm$ 0.03	5.5
C15:0	0.28 $\pm$ 0.01	4.1	0.24 $\pm$ 0.02	8.6
C16:0	30.0 $\pm$ 0.25	0.8	33.52 $\pm$ 0.23	0.7
C17:0	0.43 $\pm$ 0.01	2.2	0.39 $\pm$ 0.01	2.4
C18:0	5.58 $\pm$ 0.11	2.0	13.66 $\pm$ 0.06	0.4
MUFA (cis)				
C16:1(n-7)	3.41 $\pm$ 0.11	3.2	0.46 $\pm$ 0.02	5.0
C18:1(n-9)	31.94 $\pm$ 0.30	1.0	9.65 $\pm$ 0.08	0.8
C18:1(n-7)	1.80 $\pm$ 0.07	3.7	1.15 $\pm$ 0.02	1.3
TFA				
Trans-C18:1(n-9)	0.15 $\pm$ 0.01	7.6	0.05 $\pm$ 0.001	7.8
Trans-C18:1(n-7)	0.11 $\pm$ 0.01	8.5	0.06 $\pm$ 0.01	8.7

Abbreviations: TG, Triglycerides; PL, Phospholipids; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; TFA, Trans-fatty acids

Supplemental Figure A: Chromatogram of the cluster of C18:1 in plasma Triglycerides measured on a 200m polar column (Agilent CP select CB for FAME)

Supplemental Figure B: Chromatogram of the cluster of C18:1 in plasma Phospholipids measured on a 200m polar column (Agilent CP Select CB for FAME)

Supplementary Table 1: Association of dairy and dairy fat intake with the industrial and ruminant trans-fatty acids, extra adjusted for age, total carbohydrates and fat

	Absolute intake						Relative intake					
	Dairy intake (g/d)			Dairy fat intake (g/d)			Dairy intake (g/d)			Dairy fat intake (g/d)		
	Model S1	Model S2	Stand β	Model S1	Model S2	Stand β	Model S1	Model S2	Stand β	Model S1	Model S2	Stand β
	P-value	P-value	P-value	P-value	P-value	P-value	P-value	P-value	P-value	P-value	P-value	P-value
Plasma TG												
I-TFA	0.017	0.69	0.031	0.43	0.036	0.39	0.072	0.04	0.019	0.64	0.015	0.72
R-TFA	0.089	0.04	0.068	0.09	0.239	<0.001	0.174	<0.001	0.076	0.07	0.086	0.04
I-TFA + R-TFA ¹	0.095	0.01	0.087	0.01	0.252	<0.001	0.223	<0.001	0.084	0.02	0.089	0.01
Plasma PL												
I-TFA	0.023	0.57	0.033	0.37	0.006	0.88	0.004	0.90	0.018	0.65	0.025	0.53
R-TFA	0.102	0.01	0.105	0.004	0.236	<0.001	0.197	<0.001	0.089	0.02	0.105	0.01
I-TFA + R-TFA ¹	0.109	0.003	0.121	<0.001	0.223	<0.001	0.187	<0.001	0.096	0.01	0.116	0.001

Model S1: Adjusted for R-TFA or I-TFA and age, education and smoking; **Model S2:**Model S1+ additionally adjusted for total carbohydrates and total fat intake.
Abbreviations: TG, Triglycerides; I-TFA, Industrial trans-fatty acid; R-TFA, Ruminant trans fatty acid; PL, phospholipids.
Dairy (fat) intake and trans fatty acids were transformed before analyses. ¹. not adjusted for i-tfa or r-tfa

Supplementary Table 2: Association between trans-fatty acids in plasma TG and cardiovascular risk factors.

	Plasma TG									
	I-TFA					R-TFA				
	Model S1	P-value	Stand β	Model S2	P-value	Stand β	Model S1	P-value	Stand β	Model S2
Weight (kg)	0.045	0.16	0.019	0.54	0.011	0.73	0.011	0.73	-0.026	0.40
Waist circumference	0.070	0.03	0.011	0.75	0.023	0.48	0.023	0.48	-0.033	0.26
BMI (kg/m ²)	0.049	0.16	0.034	0.25	-0.010	0.79	-0.010	0.79	-0.067	0.05
SBP (mmHg)	0.023	0.45	0.000	0.99	-0.013	0.68	-0.013	0.68	-0.050	0.11
DBP (mmHg)	0.044	0.18	0.025	0.43	0.014	0.68	0.014	0.68	-0.013	0.68
Total Cholesterol (mmol/L)	0.055	0.11	0.026	0.43	0.104	0.003	0.104	0.003	0.070	0.03
HDL-c (mmol/L)	-0.005	0.87	0.042	0.14	-0.063	0.06	-0.063	0.06	0.005	0.87
LDL-c (mmol/L)	0.031	0.38	0.004	0.91	0.093	0.01	0.093	0.01	0.062	0.06
Total TG (mmol/L)	0.096	0.01			0.126	<0.001		<0.001		0.131
TG/HDL-c ratio	0.073	0.03	-0.016	0.15	0.121	<0.001	0.121	<0.001	0.000	0.99
Glucose (mmol/L)	0.085	0.01	0.063	0.06	0.035	0.31	0.035	0.31	0.000	0.99
HbA1c (%)	0.052	0.10	0.036	0.24	0.011	0.72	0.011	0.72	-0.022	0.47
Hs-CRP (mg/L) ¹	0.082	0.10	0.061	0.21	-0.047	0.36	-0.047	0.36	-0.085	0.09
Creatinine (umol/L)	0.027	0.35	0.015	0.51	0.001	0.98	0.001	0.98	-0.008	0.78
Uric acid (mmol/L) ¹	0.000	0.99	-0.054	0.22	-0.091	0.07	-0.091	0.07	-0.140	0.001

Model S1: Adjusted for age, sex, education and smoking; **Model S2:** Adjusted for age, sex and total triglycerides.Dependent: cardiovascular risk factors, independent: trans fatty acid status. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1c; Hs-CRP, High sensitive C-reactive protein. Hs-crp and total TG were ln transformed before analyses. ¹ Hs-CRP data (n = 412), Uric acid data (n = 348)

Supplementary Table 3: Association between trans-fatty acids in plasma PL and cardiovascular risk factors.

	Plasma PL									
	I-TFA					R-TFA				
	Model S1	P-value	Stand β	Model S2	P-value	Model S1	P-value	Stand β	Model S2	P-value
Stand β	Model S1	P-value	Stand β	Model S2	P-value	Model S1	P-value	Stand β	Model S2	P-value
Weight (kg)	0.007	0.83	-0.003	0.93	-0.056	0.10	-0.047	-0.044	0.19	-0.041
Waist circumference	0.016	0.63	0.007	0.83	-0.070	0.03	-0.063	-0.052	0.12	-0.050
BMI (kg/m ²)	0.000	0.99	-0.007	0.84	-0.099	0.01	-0.087	-0.083	0.03	-0.076
SBP (mmHg)	-0.023	0.48	-0.018	0.57	-0.087	0.01	-0.083	-0.084	0.01	-0.078
DBP (mmHg)	-0.013	0.72	-0.022	0.51	-0.063	0.06	-0.057	-0.058	0.09	-0.057
Total Cholesterol (mmol/L)	0.065	0.08			0.006	0.87		0.030	0.42	
HDL-c (mmol/L)	0.043	0.22	0.036	0.19	0.022	0.52	-0.015	0.035	0.31	0.002
LDL-c (mmol/L)	0.048	0.20	-0.017	0.23	0.011	0.77	-0.001	0.026	0.49	-0.008
Total TG (mmol/L)	0.027	0.47			-0.073	0.04		-0.049	0.19	
TG/HDL-c ratio	0.001	0.98	-0.016	0.12	-0.061	0.08	0.008	-0.050	0.17	0.000
Glucose (mmol/L)	0.000	0.99	-0.015	0.67	-0.084	0.01	-0.069	-0.069	0.05	-0.063
HbA1c (%)	-0.053	0.10	-0.058	0.07	-0.084	0.02	-0.081	-0.093	0.004	-0.093
Hs-CRP (mg/L) ¹	-0.057	0.25	-0.061	0.21	-0.172	0.001	-0.168	-0.155	0.003	-0.155
Creatinine (umol/L)	-0.013	0.67	-0.004	0.88	-0.055	0.07	-0.032	-0.053	0.08	-0.031
Uric acid (mmol/L) ¹	-0.053	0.32	-0.043	0.34	-0.197	<0.001	-0.128	-0.176	0.001	-0.118

Model S1: Adjusted for age, sex, education and smoking; **Model S2:** Adjusted for age, sex and total triglycerides and total cholesterol.

Dependent: cardiovascular risk factors, independent: trans fatty acid status. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. Hs-crp and total TG were ln transformed before analyses. ¹ Hs-CRP data (n = 348)

Supplementary Table 4: Association between dairy (fat) intake (relative intake) and cardiovascular risk factors

	Dairy intake						Dairy fat intake					
	Model 1			Model 2			Model 3			Model 1		
	Stand β	P-value		Stand β	P-value		Stand β	P-value		Stand β	P-value	
Age (years)	0.282	< 0.001								0.304	< 0.001	
Weight (kg)	0.037	0.31	0.014	0.71	0.022	0.57	0.022	0.57	0.032	0.37	0.005	0.89
Waist circumference	0.090	0.01	-0.016	0.65	-0.006	0.88	-0.006	0.88	0.104	0.004	-0.008	0.82
BMI (kg/m ²)	0.057	0.12	-0.024	0.51	-0.010	0.78	-0.010	0.78	0.046	0.21	-0.027	0.46
SBP (mmHg)	0.154	< 0.001	0.010	0.76	0.009	0.80	0.009	0.80	0.106	0.003	-0.040	0.24
DBP (mmHg)	0.099	0.01	0.003	0.93	0.005	0.90	0.005	0.90	0.121	0.001	0.012	0.73
Total Cholesterol (mmol/L)	0.000	0.99	-0.084	0.02	-0.084	0.02	-0.084	0.02	0.100	0.01	0.005	0.89
HDL-c (mmol/L)	-0.027	0.45	-0.046	0.22	-0.020	0.59	-0.020	0.59	0.068	0.06	0.056	0.14
LDL-c (mmol/L)	0.018	0.06	-0.054	0.14	-0.063	0.09	-0.063	0.09	0.081	0.02	-0.006	0.87
Total TG (mmol/L)	-0.026	0.47	-0.077	0.04	-0.088	0.02	-0.088	0.02	0.020	0.58	-0.030	0.42
TG/HDL-c ratio	-0.006	0.87	-0.036	0.34	-0.054	0.16	-0.054	0.16	-0.011	0.76	-0.045	0.24
Glucose (mmol/L)	0.061	0.09	-0.029	0.41	-0.017	0.64	-0.017	0.64	0.084	0.02	-0.028	0.44
HbA1c (%)	0.154	< 0.001	0.014	0.66	0.015	0.64	0.015	0.64	0.181	< 0.001	0.018	0.58
Hs-CRP (mg/L) ¹	0.014	0.77	0.020	0.68	0.028	0.58	0.028	0.58	0.002	0.96	0.011	0.83
Creatinine (umol/L)	0.034	0.35	-0.016	0.67	-0.023	0.55	-0.023	0.55	0.018	0.61	-0.031	0.40
Uric acid (mmol/L) ¹	-0.089	0.10	-0.086	0.11	-0.087	0.12	-0.087	0.12	-0.110	0.004	-0.102	0.06

Model 1: Crude model; **Model 2:** Model 1 + additional adjustments for age, education and smoking; **Model 3:** Model 2 + total carbohydrates and total fat intake
Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. Dairy intake and dairy fat intake were sqrt transformed before analyses. Hs-crp and total TG were ln transformed before analyses. ¹ Hs-Crp data (n = 412), Uric acid data (n = 348)

CHAPTER 4

Circulating fatty acids as biomarkers of dairy fat intake: Data from the Lifelines biobank and cohort study

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ABSTRACT

Background: C14:0, C15:0, C17:0 and trans-C16:1(n-7) are often used as biomarkers for dairy fat intake. Trans-C18:1(n-7) and CLA, two fatty acids which are also present in dairy, have hardly been explored. We investigated whether trans-C18:1(n-7) and CLA can enrich the existing biomarker portfolio.

Methods: Data were obtained from Lifelines (n=769). Dairy fat intake was determined by FFQ. Fatty acids were measured in fasting plasma triglycerides (TG), phospholipids (PL) and cholesterol esters (CE).

Results: Median (25th-75th percentile) intakes of dairy and dairy fat were 322(209-447) and 12.3(8.4-17.4) g/d respectively. A pilot study showed that trans-C18:1(n-7) and CLA were only detectable in TG and PL. Of the established markers, TG C15:0 was most strongly associated with dairy fat intake (standardized β (std. β)=0.286, R^2 =0.111). Of the less established markers, TG trans-C18:1(n-7) was most strongly associated with dairy fat intake (Std. β =0.292, R^2 =0.115), followed by PL CLA (Std. β =0.272, R^2 =0.103) and PL trans-C18:1(n-7) (Std. β =0.269, R^2 =0.099). In TG, a combination of C15:0 and trans-C18:1(n-7) performed best (R^2 =0.128). In PL, a combination of C14:0, C15:0, trans-C18:1(n-7) and CLA performed best (R^2 =0.143).

Conclusion: Trans-C18:1(n-7) and CLA can be used as biomarkers of dairy fat intake. Additionally, combining established with less established markers allowed even stronger predictions for dairy fat intake.

INTRODUCTION

Dairy consumption is part of the food culture in many countries. Worldwide intake of dairy products is high, particularly in developed countries such as Canada, North America and parts of the European Union. For instance, in the European Union and in the USA, the average per capita milk consumption is 1.01 and 0.91 servings per day respectively [1]. While dietary recommendations tend to set an upper limit to certain types of dairy products as a result of the high saturated fat or sodium content, dairy intake is generally considered healthy, particularly because of the numerous essential nutrients it contains, including calcium, proteins, phosphorus, magnesium, and several vitamins and minerals [2]. It has been observed that high dairy intake is associated with a reduced risk for hypertension [3]. Furthermore, dairy has been suggested to have a positive influence on bone health by increasing bone mineral density [4,5]. There is also increasing evidence that fat from dairy products (as reflected by circulating dairy fat biomarkers) has a positive effect on various cardiovascular risk factors such as BMI, fasting glucose and insulin sensitivity [6-8]. Moreover, prospective studies have reported lower risk of stroke and coronary heart disease with higher levels of circulating dairy fat biomarkers [9,10]. Additionally, high circulating concentrations of dairy fat biomarkers were found to be associated with reduced risk of development of type 2 diabetes as was evidenced in several longitudinal observational studies [11-14].

Dairy fat consists of more than 400 different fatty acids [15]. The fatty acids in dairy fat derive either directly from the animal's diet, or indirectly from ruminal fermentation. The fatty acid composition of plasma, serum, erythrocytes and adipose tissue are known to partially reflect the fatty acid composition of the diet. Several fatty acids are to a large extent unique for dairy fat intake. They are present in measurable amounts in the circulation and tissue of humans [6]. Myristic acid (C14:0), pentadecanoic acid (C15:0), heptadecanoic acid (C17:0) and trans-palmitoleic acid (Trans-C16:1(n-7)) are commonly used dairy fat biomarkers [13,16-22]. Particularly the odd-chain fatty acids C15:0 and C17:0 [13,16,17,20-23], and the natural ruminant trans fat trans-C16:1(n-7) are often considered as fatty acids that mainly originate from dairy fat [13,20,21], because these fatty acids are synthesized by the bacterial flora in the rumen of the animal and cannot be synthesized in the human body [24]. Correlations of C15:0, C17:0 and trans-C16:1(n-7) with dairy fat intake are variable. Papers that reported on the correlation between circulating dairy fat biomarkers in plasma and dairy fat intake showed correlations of 0.10 - 0.53 with C15:0 [6,13,16,18-22], of 0.16 - 0.36 with C17:0 [13,16,18-21], and of 0.13 - 0.30 with Trans-C16:1(n-7) [13,20,21]. Despite the specific origin of these fatty acids, the correlations are relatively low and interest in other potential dairy fat biomarkers is therefore growing.

Trans fatty acids which are also thought to mainly originate from rumen derived products are vaccenic acid (trans-C18:1(n-7)) and rumenic acid, a conjugated linoleic acid with the lipid name cis-9, trans-11 conjugated linoleic acid (CLA)). The trans fatty acids are synthesized via

microbial fermentation of C18 unsaturated fatty acid in the rumen of the animal [25,26]. They can be found in several ruminant derived foods [9,13,20,21,27-30]. Despite their occurrence in dairy products, vaccenic acid and CLA are hardly explored as potential dairy fat biomarkers. Current studies on the association of trans fats with dairy intake use a cluster of trans-C18:1, which is actually a combination of mainly rumen-derived trans-C18:1(n-7) and industrially (trans-C18:1(n-9) produced trans fats, possibly leading to biased associations [31,32]. It is therefore of interest to determine the association of dairy fat intake and the genuine rumen derived Trans-C18:1(n-7).

The main objective of this study was to investigate the fatty acids trans-C18:1(n-7) and CLA as potential biomarkers of dairy fat intake and to compare them with more commonly used dairy fat biomarkers. Secondly, we investigated whether a combination of biomarkers provides a better predictor for dairy fat intake compared to single biomarkers. For this we developed and validated a model for the prediction of dairy fat intake in the general population. We used data from the LifeLines Biobank and Cohort Study, a large observational cohort study of initially healthy participants living in the Northern part of the Netherlands.

Clinical significance

- Despite their occurrence in dairy products, trans-C18:1(n-7) and CLA are hardly explored as potential dairy fat biomarkers in the general population.
- The present study confirmed the commonly used biomarkers C14:0, C15:0, C17:0 and trans-C16:1(n-7). and identified the biomarkers trans-C18:1(n-7) and CLA as additional dairy fat biomarkers. A combination of the commonly used biomarkers with less established biomarkers allowed even stronger predictions for dairy fat intake.

MATERIALS AND METHODS

Study Design and Subjects

The LifeLines Cohort Study is a large observational population-based cohort study and Biobank that examines the health and health-related behaviors of more than 167,000 persons [33]. The participants were recruited from the three Northern provinces of the Netherlands between 2006 and 2013. The first group of 25-50 years old participants was recruited via general practitioners. Participants could indicate whether family members were interested as well. In addition, individuals who were interested in the study had the possibility to register via an online self-registration. Individuals with insufficient knowledge of the Dutch language, with severe psychiatric or physical illness, and those with limited life expectancy (< 5 years) were excluded from the study. Participants (> 18 years old) completed several questionnaires, including topics such as occurrence of diseases, general health, medication use, diet, physical activity

and personality. Participants were invited to the Lifelines Research sites for a comprehensive health assessment. A more detailed description of the Lifelines Cohort study can be found elsewhere [34,35]. For the present study, we used a subset of the cross-sectional data. 864 participants were randomly selected from the Lifelines biobank. Additionally, 776 of the 864 participants had filled in a food frequency questionnaire (FFQ). Cases with missing data on either FFQ or circulating fatty acids were removed before analysis leaving 769 participants in the lifelines cohort with complete data (**Figure 1**). All participants provided written consent. The Lifelines Cohort Study was conducted according to the principles of the Declaration of Helsinki and approved by the Medical ethical committee of the University Medical Center Groningen, The Netherlands.

Dietary assessment

To assess dietary intake in the Lifelines Cohort, a 110-item semi-quantitative baseline food frequency questionnaire (FFQ) assessing food items over the previous month was developed and validated by the Wageningen University using the Dutch FFQTOOL™, in which food items were selected based on the Dutch national Food Consumption Survey of 1997/1998 [36]. Seven answers categories were used to assess consumption frequency, ranging from 'not this month' to '6-7 days a week'. Portion size was estimated by fixed portion sizes (e.g. slices of bread, pieces of fruit) and commonly used household measures (e.g. cups, spoons). Energy and macronutrient intake, including dairy consumption, was estimated from the FFQ data by using the Dutch food composition database of 2011 (NEVO) [37]. For this study, 'total dairy intake' and 'total dairy fat intake' (cheese, milk, buttermilk, yoghurt, sweetened yoghurt drinks, custard, curd cheese, ice cream, whipped cream and porridge) were calculated. Butter was not included in the 'total dairy intake' and 'total dairy fat intake' calculation.

Clinical measurements

Anthropometric measurements (weight, height, and waist circumference) and blood pressure were measured by well-trained staff. The anthropometric measurements were measured without shoes. Body weight was measured to the nearest 0.1 kg. Height was measured to the nearest 0.5 cm with a stadiometer placing their heels against the rod and the head in Frankfort Plane position. Waist circumference was measured to the nearest 0.5 cm in standing position with a tape measure all around the body, at the level midway between the lower rib margin and the iliac crest. BMI was calculated as weight (kg) divided by height squared (m²). Systolic and diastolic blood pressures were measured 10 times during a period of 10 minutes, using an automated Dinamap Monitor (GE Healthcare, Freiburg, Germany). The average of the final three readings was used for each blood pressure parameter.

Figure 1: Flow diagram of the participants in the study

Data on smoking and general health

Information about smoking and general health was collected from the self-administered questionnaire. Subjects were classified as non-smokers, former smokers or current smokers. Information on history and prevalence of hypertension, hypercholesterolemia and diabetes type 2 were collected in the same questionnaire.

Biochemical measurements

Blood samples were collected in the fasting state, between 8.00 and 10.00 a.m. and transported on ice to the Central Lifelines Laboratory in the University Medical Center Groningen. Serum levels of total and HDL cholesterol were measured using an enzymatic colorimetric method, triglycerides using a colorimetric UV method, and LDL cholesterol using an enzymatic method, all on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). Fasting blood glucose was measured using a hexokinase method. HbA1c was determined in whole blood (EDTA-anticoagulated) by means of turbid metric inhibition immunoassay on a Cobas Integra 800 CTS analyzer (Roche Diagnostics Netherland BV, Almere, The Netherlands). The hs-CRP was determined by nephelometry (BN II system Siemens, Marburg, Germany). Serum creatinine was measured on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland).

Fatty acid analyses

EDTA-plasma samples were collected at baseline and stored frozen at -80°C until use for assessment of fatty acid profiles. Analyses of fatty acids were performed in the Department of Laboratory Medicine of the University Medical Center Groningen, The Netherlands using the methodology as described by Hoving *et al.* [38]. In short, total lipids were extracted by the method of Folch *et al.*, using 6 ml of chloroform-methanol (2:1) and a 200 µl EDTA-plasma sample [39]. After that, a shortened version of the method of Kaluzny *et al.* was used to isolate plasma cholesterol esters (CE), triglycerides (TG) and phospholipids (PL), using aminopropyl SPE columns (Isolute, Biotage) [40]. Fatty acids were transmethyalted with methanolic-HCL into fatty acid methyl esters (FAME). The samples were extracted with hexane and eventually redissolved into 100 µl hexane. Internal standards for the quantification of fatty acids in CE (100 µl of a solution of 50.1 mg C17:0/100 ml chloroform-methanol, 2:1 v/v) and TG (100 µl of a solution of 19.9 mg of C19:0/100 ml chloroform-methanol, 2:1 v/v), both obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), were added before isolation of lipid classes. An internal standard for the quantification of fatty acids in PL (100 µl of a solution of 50.0 mg free fatty acid 19:0/100 ml methanol), obtained from Larodan (Solna, Sweden), was added after isolation of lipid classes. 100 µl Butylated Hydroxytoluene (1 g/100 ml methanol) from Sigma-Aldrich (Zwijndrecht, The Netherlands) was added to prevent fatty acid oxidation.

Aliquots of 2 µl were injected into an Agilent model 6890 gas chromatography equipped with a 200 m x 0.25 mm polar column (CP Select for FAME) and detected with an Agilent 7683 series

flame ionization detector. The 200 m polar column is specifically developed to allow for better separation between trans C18:1 fatty acids [41]. FAME were identified by comparing retention times with those of known standards (Supelco 37 component FAME mix (Sigma-Aldrich)). Fatty acid compositions were expressed into mol%. Firstly, circulating fatty acid compositions were analyzed in 96 samples to investigate whether the potential dairy fat biomarkers, and especially trans-C18:1(n-7) and CLA, could be detected in the three fractions (plasma CE, TG and PL). Subsequently, we proceeded with analyses in those fractions in which all potential dairy fat biomarkers could be detected. The precision of the potential dairy fat biomarkers was calculated by measuring 10 quality-control samples. Potential dairy fat biomarkers had an average variation coefficient of $\leq 10\%$. In TG, the CVs for the potential new biomarkers Trans-C18:1(n-7) and CLA were 8.5% and 7.9% respectively, and in PL, the CVs for Trans-C18:1(n-7) and CLA were 9.3% and 9.6% respectively.

Statistical analyses

All analyses were performed using IBM SPSS Statistics, version 22.0 for Windows software (IBM, Armonk, NY, USA) and STATA/MP version 13.1 for Windows software (Statacorp, College Station, Texas, USA). In total, circulating fatty acids in plasma TG and PL were measured in 864 participants, whereof 776 participants had also completed an FFQ. Cases with missing data on either FFQ or circulating fatty acids were removed before analysis leaving 769 participants in the lifelines cohort with complete data. Baseline data are presented for the total population and separately for males and females to check for potential differences between males and females. Data are presented as N (%), mean $\pm$ SD or as in case of non-normally distributed data as median (25th-75th percentile). Differences in baseline data between males and females were tested with an independent t-test (continuous data), Mann-Whitney U test (non-normally distributed data) or Chi-square test (categorical data). For the main analyses, we used energy-adjusted total dairy fat intake. Energy-adjusted intakes were generated by adjustment for total energy intake using the residual method [42]. Because men usually have a higher energy and macronutrient intake compared to women, adjustment for energy intake was performed for men and women separately. Energy-adjusted intakes are expressed in grams/day. Linear regression models were used to examine the association between circulating fatty acids and total dairy fat intake. We also examined the association of circulating fatty acids with (semi-) skimmed dairy products, full fat dairy products, fermented dairy products, milk and cheese. As sensitivity analyses, we tested whether removal of outliers for energy intake (participants with energy intakes lower or higher than 3 SDs from the mean value of energy intake) influenced the associations between dairy fat biomarkers and energy-adjusted dairy fat intake. We also tested for potential effect modification of the associations of circulating fatty acid biomarkers with total dairy fat intake by sex. Furthermore, in subsidiary analyses, the associations were adjusted for age, sex, BMI, total

meat intake, and the industrialized trans fatty acid trans-C18:1(n-9) (Elaidic acid). Skewed data were transformed before analyses (square root or log-transformed). Multivariate backwards regression analyses were carried out with all potential dairy fat biomarkers in TG, PL and a combination of both to identify the best combination of markers to predict energy-adjusted dairy fat intake in our population (cutoff for removal $p < 0.10$). We compared the performance of the models against each other by comparing the explained variance (R^2). Bland-Altman plots were created to indicate the agreement of observed (FFQ-derived data) compared to the estimated models (based on circulating fatty acids). The mean of the observed and estimated models were plotted against the difference between the observed and estimated models. To be able to compare both models with each other the observed measurement as well as the estimated measurement were standardized before analyses. Validity of the models was evaluated by ranking ability (cross-classification between the observed (FFQ-derived) intake data and the estimated intake data (based on circulating fatty acids)) [43]. For the ranking ability, both observed (FFQ-derived) intake data and estimated intake data (based on circulating fatty acids) were ranked into quintiles. To validate the models, we repeated the multivariate backwards regression analyses in STATA, by implementing the *swboot* command which created a table with the number of times each variable is selected after 1000 bootstrap samples [44]. Variables were included in the bootstrap models when these were present in the majority of the bootstrap attempts (>50%). In secondary analyses, variables were included in the bootstrap models when these were present in >70% of the bootstrap attempts. As additional analyses, we repeated the multivariate backwards regression analyses for unadjusted dairy fat intake (rather than energy-adjusted dairy fat intake which was used for the primary analyses), and also assessed predictive capacity for energy-adjusted dairy intake. All reported probability values are two-tailed, and $P \leq 0.05$ was considered statistically significant.

RESULTS

The baseline characteristics of the 769 participants overall and for males and females separately are shown in **Table 1**. The total population had a mean age of 53.0 ± 15.5 years and a BMI of 26.0 ± 4.0 kg/m². Blood pressure was slightly higher in males (130/77 mmHg) compared to females (123/71 mmHg). Total and HDL cholesterol were higher in females, whereas total triglycerides was higher in males. Energy intake of the population was 1975 ± 624 kcal/d (8274 ± 2613 KJ/d). Of the total intake, 46 En% was derived from carbohydrates, 15 En% from proteins, and 35 En% from fats. The Lifelines dataset confirms that males overall have a higher dietary intake compared to females. Total meat intake of the population was 73.5 g/d, which contributed for 13% to the daily fat intake. Median dairy intake of the population was 322 (209 – 447) g/d and consisted for 12.8% of cheese, for 35.1% of milk, for 10.0% of yoghurt, and for 42.1% of other milk (based) products. Of the total dairy intake, 34% was from fermented dairy products. Median dairy fat

intake of the population was 12.3 (8.4 – 17.4) g/d. Skimmed dairy products contributed for 2% to total dairy fat intake, while semi-skimmed and full-fat products contributed for 20% and 78% respectively. Median dairy fat intake, rather than median dairy intake overall, was significantly higher in males compared to females (median intakes males: 13.5 (9.0 – 18.3) g/d vs females: 11.3 (8.0 – 15.6) g/d; $p < 0.001$). Males consumed more semi-skimmed and full-fat dairy products, while females consumed more skimmed dairy products.

Table 1: Baseline Characteristics of the Lifelines Population

Baseline Characteristics	N = 769	Males N = 404	Females N = 365	P-value
General				
Age visit 1, years	53.0 ± 15.5	53.3 ± 15.2	52.7 ± 16.0	0.60
BMI, kg/m ²	26.0 ± 4.0	26.3 ± 3.4	25.8 ± 4.6	0.08
Smoking, yes (%)	115 (15.0%)	68 (16.8%)	47 (12.9%)	0.13
SBP, mmHg	126.5 ± 17.0	129.5 ± 15.1	123.1 ± 18.2	< 0.001
DBP, mmHg	73.7 ± 9.7	76.6 ± 9.8	70.5 ± 8.4	< 0.001
Laboratory measurements				
Total Cholesterol, mmol/L	5.2 ± 1.0	5.1 ± 1.0	5.3 ± 1.1	0.03
HDL cholesterol, mmol/L	1.5 ± 0.4	1.3 ± 0.3	1.7 ± 0.4	< 0.001
LDL cholesterol, mmol/L	3.3 ± 0.9	3.3 ± 0.9	3.3 ± 1.0	0.27
Total Triglycerides, mmol/L	1.0 (0.8 - 1.5)	1.2 (0.9 - 1.6)	1.0 (0.7 - 1.3)	< 0.001
Glucose, mmol/L	5.1 ± 0.7	5.2 ± 0.6	5.0 ± 0.7	0.003
HbA1c, %	5.6 ± 0.4	5.6 ± 0.4	5.6 ± 0.4	0.99
Creatinin, umol/L	76.0 ± 13.6	83.6 ± 12.3	67.6 ± 9.3	< 0.001
C-reactive protein (hs-CRP), mg/L	1.0 (0.5 - 2.2)	0.9 (0.4 - 1.9)	1.1 (0.5 - 2.9)	0.05
General health				
Diabetes Type 2, yes (%)	22 (2.9%)	12 (3.0%)	10 (2.8%)	0.86
Hypertension, yes (%)	200 (26.0%)	96 (23.8%)	104 (28.6%)	0.21
High cholesterol, yes (%)	165 (21.5%)	95 (23.5%)	70 (19.3%)	0.35
Dietary intake (FFQ)				
Energy intake, Kcal/day	1971 ± 624	2206 ± 665	1711 ± 449	< 0.001
Total carbohydrates, g/d (En%)	228.8 ± 76.7 (46%)	254.4 ± 80.7 (46%)	200.4 ± 60.6 (47%)	< 0.001
Total protein, g/d (En%)	74.8 ± 20.9 (15%)	81.5 ± 22.6 (15%)	67.3 ± 15.8 (16%)	< 0.001
Total fat, g/d (En%)	77.2 ± 30.0 (35%)	87.1 ± 32.7 (36%)	66.2 ± 22.0 (35%)	< 0.001
Total Meat, g/d	73.5 ± 37.5	83.8 ± 40.1	62.0 ± 30.7	< 0.001
Total Meat fat, g/d	10.2 ± 5.8	11.9 ± 6.3	8.3 ± 4.5	< 0.001
Dairy intake (FFQ)				
Total Dairy intake, g/d	322 (209 - 447)	315 (202 - 444)	324 (216 - 447)	0.99
Total Dairy fat intake, g/d	12.3 (8.4 - 17.4)	13.5 (9.0 - 18.3)	11.3 (8.0 - 15.6)	< 0.001

Table 1: Continued

Baseline Characteristics	N = 769	Males N = 404	Females N = 365	P-value
Circulating fatty acids in plasma TG				
C14:0, mol%	1.87 (1.40 – 2.48)	1.98 (1.49 – 2.68)	1.74 (1.35 – 2.35)	< 0.001
C15:0, mol%	0.30 (0.25 – 0.35)	0.29 (0.24 – 0.35)	0.30 (0.25 – 0.35)	0.27
C17:0, mol%	0.49 (0.41 – 0.59)	0.48 (0.39 – 0.58)	0.52 (0.44 – 0.62)	< 0.001
T-C16:1(n-7), mol%	0.026 (0.018 – 0.036)	0.025 (0.017 – 0.036)	0.026 (0.019 – 0.037)	0.05
T-C18:1(n-7), mol%	0.098 (0.064 – 0.141)	0.099 (0.067 – 0.147)	0.096 (0.070 – 0.138)	0.30
CLA, mol%	0.077 (0.050 – 0.095)	0.068 (0.049 – 0.094)	0.071 (0.050 – 0.097)	0.24
Circulating fatty acids in plasma PL				
C14:0, mol%	0.49 (0.41 – 0.57)	0.47 (0.40 – 0.55)	0.50 (0.43 – 0.58)	< 0.001
C15:0, mol%	0.28 (0.24 – 0.33)	0.28 (0.24 – 0.33)	0.29 (0.25 – 0.33)	0.09
C17:0, mol%	0.40 (0.36 – 0.44)	0.39 (0.35 – 0.44)	0.40 (0.36 – 0.45)	0.08
T-C16:1(n-7), mol%	0.020 (0.014 – 0.025)	0.019 (0.013 – 0.024)	0.020 (0.015 – 0.025)	0.002
T-C18:1(n-7), mol%	0.091 (0.068 – 0.115)	0.086 (0.063 – 0.111)	0.094 (0.072 – 0.119)	0.002
CLA, mol%	0.033 (0.022 – 0.043)	0.030 (0.020 – 0.041)	0.032 (0.017 – 0.044)	0.01

Data are presented as mean $\pm$ SD, median (25th – 75th percentile) or number (%). Differences between sex were tested by independent t-test, Mann-Whitney U test or Chi-square test.

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1C, Hemoglobin A1C; FFQ, Food Frequency Questionnaire; Kcal, Kilocalories; TG, Triglycerides; PL, Phospholipids; C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), T-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid.

Circulating fatty acids were measured in the first 96 samples (**Supplemental Table 1**). Trans-C18:1(n-7) appeared not detectable in plasma CE, since it was below the detection limit. All potential dairy fat biomarkers, including trans-C18:1(n-7) and CLA, were detectable in plasma TG and PL. Since we were especially interested in the potentially less established biomarkers, we decided to further only analyze plasma TG and PL. Data on potential circulating dairy fat biomarkers in plasma TG and PL of all 769 participants are shown in **Table 1**. Dairy fatty acids in plasma TG were higher compared to plasma PL. The quantitatively most abundant dairy fat marker was C14:0 (Total TG: 1.87 mol%, PL: 0.49 mol%), followed by C17:0 and C15:0 in both fractions. Trans-C18:1(n-7) was the highest trans fat in both fractions (TG: 0.10 mol%, PL: 0.09 mol%).

Energy-adjusted dairy fat intake

In the univariate main analyses, all potential dairy fat biomarkers in plasma TG, except C17:0, were associated with energy-adjusted dairy fat intake (**Table 2, Model 1**). The highest associations were found for the commonly used biomarker C15:0 (standardized β (std β) = 0.286, $P < 0.001$) and the less established biomarker trans-C18:1(n-7) (std β = 0.292, $P < 0.001$). All potential dairy

fat biomarkers in plasma PL were also moderately associated with energy-adjusted dairy fat intake (**Table 2, Model 1**). Slightly higher, but still moderate associations were found with the less established biomarkers trans-C18:1(n-7) (std β = 0.269, $P < 0.001$) and CLA (std β = 0.272, $P < 0.001$) compared to the commonly used biomarkers. The highest associations with more commonly used biomarkers were found for C14:0 (std β = 0.214, $P < 0.001$) and C15:0 (std β = 0.215, $P < 0.001$), but these were still moderate. In sensitivity analyses, removal of outliers for energy intake did not materially change the association between dairy fat biomarkers and energy-adjusted dairy fat intake. In multivariate regression analyses, associations between fatty acids in plasma TG and PL with energy-adjusted dairy fat intake remained after adjustments for age, sex, BMI, total meat intake, and the industrial trans fatty acid Trans-C18:1(n-9) (**Supplemental Table 2A**). The association between the biomarkers and energy-adjusted dairy fat intake did not differ between males and females, except for PL C14:0 ($P=0.03$ for interaction, association in males: std β = 0.264, $P < 0.001$, association in females: std β = 0.173, $P < 0.001$) (**Supplemental Table 2B, Model 1**).

In plasma TG, multivariate backwards regression analyses identified a combination of C15:0 and trans-C18:1(n-7) as the best combination to predict energy-adjusted dairy fat intake (**Table 2, Model 3**). The explained variance of this combination was 0.128, which was higher than the explained variance of the single markers (TG model: R^2 = 0.128 vs C15:0: R^2 = 0.111 and trans-C18:1(n-7): R^2 = 0.115). In plasma PL, the fatty acids C14:0, C15:0, trans-C18:1(n-7) and CLA were identified as the best combination to predict energy-adjusted dairy fat intake in the general population. This model had an explained variance of 0.143, which is again higher than the explained variance of the single markers (PL model: R^2 = 0.143 vs C14:0: R^2 = 0.074, C15:0: R^2 = 0.075, trans-C18:1(n-7): R^2 = 0.099 and CLA: R^2 = 0.103). A combination of fatty acids from TG (C15:0, trans-C18:1(n-7), CLA) and PL (C15:0, trans-C16:1(n-7), trans-C18:1(n-7), CLA) explained energy-adjusted dairy fat intake in the general population with an R^2 of 0.154. Based on these data, a combination of biomarkers can be seen as a better predictor for energy-adjusted dairy fat intake compared to single biomarkers.

Total dairy intake, total dairy fat intake and dairy product groups

For all, but one, circulating potential dairy fatty acid biomarkers in TG and PL, associations with total dairy fat intake were present and strongest compared to associations with total dairy intake and dairy product groups (**Supplemental Table 3**). C17:0 is a single exception, with absence of an association with total dairy fat intake for TG and a stronger association with total dairy intake than with total dairy fat intake for PL. All fatty acid biomarkers, except C17:0 in TG and PL and Trans-C16:1(n-7) in PL were relatively strongly associated with intake of cheese and full fat dairy.

Table 2: Univariate regression analyses and multivariate models to predict dairy fat with circulating fatty acids in plasma TG, plasma PL and a combination of the two in the Lifelines Cohort

	Model 1			Model 2		Model 3	
Plasma TG	Std β	R ²	P-value	Std β	P-value	Std β	P-value
TG C14:0	0.204	0.071	< 0.001	0.049	0.27		
TG C15:0	0.286	0.111	< 0.001	0.133	0.01	0.162	0.001
TG C17:0	0.049	0.032	0.19	0.016	0.67		
TG T-C16:1(n-7)	0.233	0.072	< 0.001	0.031	0.54		
TG T-C18:1(n-7)	0.292	0.115	< 0.001	0.162	0.002	0.181	< 0.001
TG CLA	0.215	0.076	< 0.001	-0.004	0.94		
				R ² =	0.130	R ² =	0.128
Plasma PL	Std β	R ²	P-value	Std β	P-value	Std β	P-value
PL C14:0	0.214	0.074	< 0.001	0.073	0.07	0.074	0.06
PL C15:0	0.215	0.075	< 0.001	0.104	0.02	0.085	0.03
PL C17:0	0.132	0.047	< 0.001	-0.017	0.70		
PL T-C16:1(n-7)	0.130	0.046	< 0.001	-0.062	0.13		
PL T-C18:1(n-7)	0.269	0.099	< 0.001	0.181	< 0.001	0.148	< 0.001
PL CLA	0.272	0.103	< 0.001	0.154	< 0.001	0.151	< 0.001
				R ² =	0.146	R ² =	0.143
Plasma TG+PL				Std β	P-value	Std β	P-value
TG C14:0				0.033	0.57		
TG C15:0				0.088	0.15	0.111	0.04
TG C17:0				0.005	0.90		
TG T-C16:1(n-7)				0.023	0.66		
TG T-C18:1(n-7)				0.095	0.10	0.113	0.04
TG CLA				-0.092	0.07	-0.088	0.08
PL C14:0				0.023	0.64		
PL C15:0				0.087	0.06	0.081	0.05
PL C17:0				-0.024	0.62		
PL T-C16:1(n-7)				-0.071	0.11	-0.077	0.06
PL T-C18:1(n-7)				0.131	0.01	0.113	0.02
PL CLA				0.151	0.001	0.169	< 0.001
				R ² =	0.160	R ² =	0.158

Model 1: Univariate linear regression analysis of the fatty acid with dairy fat intake; **Model 2:**

Multivariate model with all fatty acids from our population in one model; **Model 3:** Multivariate model with the best combination of fatty acids in our population (backwards regression).

C14:0, C17:0, Trans-C16.1(n-7), Trans-C18.1(n-7) and CLA in plasma TG and Trans-C16.1(n-7), Trans-C18.1(n-7) and CLA in plasma PL were transformed before analyses. Dairy fat intake was transformed and adjusted for energy intake according to the residual method. β 's are standardized beta's.

Abbreviations: TG, Triglycerides; PL, Phospholipids, C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), T-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid

Agreement and ranking ability

To indicate the agreement between the observed intake (FFQ-derived) and estimated intake (based on circulating fatty acids) Bland-Altman plots were made. Plots were made for the best single markers (from a set of markers) which were found by backward regression analyses (**Figures 2 and 3**). In addition, validity of the models was evaluated by ranking ability (cross-classification between the observed intake data (FFQ-derived) and the estimated intake data (TG, PL and TG+PL model based on circulating fatty acids)). Both the observed and estimated intake data were ranked into quintiles and compared. For all three estimated models, $\approx 67\%$ of the participants were classified in the same or adjacent quintile of the observed model, whereas 2-3% of the participants were classified in extreme quintiles. Overall, both methods showed acceptable agreement between the observed and estimated models.

Figure 2: Bland-Altman plots for the agreement between the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma TG) for the measurement of dairy fat intake in the general population. Plots of the differences between the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma TG) vs the mean of the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma TG) method, with (a) representing C15:0 and (b) Trans-C18:1(n-7) as the estimated intake. The red line displays the mean of the difference. The black dotted lines represents the limits of agreement (lower limit: mean - SD*1.96; upper limit: mean + SD*1.96). *Abbreviation: C15:0, Pentadecanoic acid; FFQ, food frequency questionnaire; SD, standard deviation; TG, triglycerides; Trans-C18:1(n-7), Trans-Vaccenic acid*

Figure 3: Bland-Altman plots for the agreement between the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma PL) for the measurement of dairy fat intake in the general population. Plots of the differences between the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma PL) vs the mean of the observed intake (FFQ-derived) and the estimated intake (based on the best circulating fatty acids in plasma PL), with (a) representing C14:0, (b) C15:0, (c) Trans-C18:1(n-7) and (d) CLA as the estimated intake. The red line displays the mean of the difference. The black dotted lines represents the limits of agreement (lower limit: mean - SD*1.96; upper limit: mean + SD*1.96). *Abbreviation: C14:0, Myristic acid; C15:0, Pentadecanoic acid; CLA; Conjugated Linolenic acid; FFQ, food frequency questionnaire; PL; phospholipids; SD, standard deviation; Trans-C18:1(n-7), Trans-Vaccenic acid*

Internal consistency of the models

To judge the internal consistency of the models, we performed backwards multivariate analyses using a bootstrap selection method (**Supplemental Table 4**). In the TG model, C15:0 and trans-C18:1(n-7) were present in more than half of the bootstrap attempts, whereas in the PL model this was the case for C14:0, C15:0, trans-C18:1(n-7) and CLA. When fatty acids from TG and PL were combined, C15:0, trans-C18:1(n-7) and CLA from both plasma TG and PL were found in the majority of the bootstrap attempts. Trans-C16:1(n-7) in plasma PL did not remain in the

prediction model. Complete bootstrap models of TG, PL and a combination of the two can be found in **Table 3**. Overall, the internal consistency for the fatty acids in plasma TG and PL was good, with exception for trans-C16:1(n-7) measured in plasma PL.

Table 3: Multivariate models to predict dairy fat with circulating fatty acids in plasma TG, plasma PL and a combination of the two in the Lifelines Cohort based on 1000 bootstraps

Variable	Beta $\pm$ SE	Std (beta)	P-value	95%CI
Plasma TG				
TG C15:0	1.752 $\pm$ 0.55	0.162	0.001	0.74 ; 2.86
TG T-C18:1(n-7)	1.798 $\pm$ 0.48	0.181	0.001	0.85 ; 2.71
Plasma PL				
PL C14:0	0.538 $\pm$ 0.27	0.074	0.04	0.02 ; 1.07
PL C15:0	1.102 $\pm$ 0.47	0.085	0.02	0.19 ; 2.02
PL T-C18:1(n-7)	2.032 $\pm$ 0.52	0.148	0.001	0.94 ; 3.05
PL CLA	2.844 $\pm$ 0.74	0.151	0.001	1.41 ; 4.31
Plasma TG+PL				
TG C15:0	1.083 $\pm$ 0.63	0.100	0.08	-0.20 ; 2.31
TG T-C18:1(n-7)	1.144 $\pm$ 0.59	0.115	0.05	-0.10 ; 2.56
TG CLA	-1.131 $\pm$ 0.67	-0.091	0.09	-2.53 ; 0.15
PL C15:0	0.912 $\pm$ 0.52	0.070	0.08	-0.11 ; 1.96
PL T-C18:1(n-7)	1.155 $\pm$ 0.59	0.084	0.04	0.04 ; 2.28
PL CLA	3.112 $\pm$ 0.78	0.166	0.001	1.56 ; 4.63

Fatty acids are included in the multivariate model when the circulating fatty acids were present in more than 50% of the models during the bootstrap process based on the backwards stepwise algorithm. C14:0, C17:0, Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma TG and Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma PL were transformed before analyses. Dairy fat intake was transformed and adjusted for energy intake according to the residual method. β 's are standardized beta's. Abbreviations: TG, Triglycerides; PL, Phospholipids, C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1(n-7), T-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; Trans-C18:1(n-9), Eilaidic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid

In secondary analyses, the bootstrap selection method was repeated with selection of fatty acids that were present in more than 70% of the 1000 bootstrap attempts. In the TG model, C15:0 and trans-C18:1(n-7) were again present, whereas the PL model included C15:0, trans-C18:1(n-7) and CLA, but not C14:0. The stricter selection criteria slightly worsened the internal consistency of the PL model. Fatty acids from TG and PL were also combined for this analyses. Interestingly, only the fatty acids trans-C18:1(n-7) and CLA from plasma PL remained in the model.

Additional analyses

As additional analyses, we repeated the multivariate backwards regression analyses for unadjusted dairy fat intake (rather than energy-adjusted dairy fat intake), and also assessed predictive capacity for energy-adjusted total dairy intake (**Supplemental Table 5 and 6**). The associations of the fatty acids with unadjusted dairy fat intake were weaker compared to the associations of the fatty acids with energy-adjusted dairy fat intake. However, C15:0 and trans-C18:1(n-7) remained the best predictive fatty acids in plasma TG, whereas trans-C18:1(n-7) and CLA remained the best predictive fatty acids in plasma PL.

DISCUSSION

The main objective of the current study was to investigate whether the circulating fatty acids trans-C18:1(n-7) and CLA are an addition to the already existing dairy fat biomarkers in the general population. First of all, we found that the less established biomarkers for dairy fat intake were best quantifiable in plasma TG and PL. Additionally, we showed that trans-C18:1(n-7) and CLA, in both plasma TG and PL, were moderately associated with dairy fat intake. In plasma PL, these biomarkers were slightly higher compared to the commonly used biomarkers. Lastly, we developed and validated a model to predict dairy fat intake in the general population. Compared to the single markers, we found that a combination of less established and commonly used biomarkers, including the C15:0, trans-C18:1(n-7) and CLA, showed a slightly higher association with dairy fat intake in the general population.

Significant associations of C15:0 and C17:0 with dairy fat intake were reported in the literature before [13,16,17,19-23,45-48]. In the Lifelines Cohort, C15:0 was also associated with dairy fat intake, while there was no consistent independent association of C17:0 with dairy fat intake. Plasma TG C17:0 was not associated with total dairy fat intake. Plasma PL C17:0 was associated with total dairy fat intake, however in backwards regression analyses taking all potential dairy fat biomarkers from plasma PL into account, plasma PL C17:0 did not remain in the PL model. Additionally, the bootstrapping with stepwise backwards algorithm showed that C17:0 remained in the minority of the 1000 bootstrap attempts (TG: 91x, PL: 149x) (**Supplemental Table 4**). A Norwegian study with 110 healthy men (age 21-55 years), published in 2005, also found that C15:0 from serum and adipose tissue were significantly correlated with dairy fat intake (both $r = 0.28$), while this was not the case for C17:0 (serum: $r = -0.10$, adipose tissue: $r = 0.03$) [23]. Overall, C17:0 might not be such a useful marker for dairy fat intake. Instead, in the Lifelines cohort other biomarkers were found to be better predictors of dairy fat intake.

C15:0 and trans-C18:1(n-7) in plasma TG and PL, emerged as the best biomarkers from the set of investigated biomarkers for the prediction of dairy fat intake. Every prediction model in the lifelines cohort included C15:0 as an important marker of dairy fat intake with standardized beta's for the association with dairy fat intake of 0.29 in plasma TG and 0.22 in plasma PL. These

results are in line with the current literature. For instance, in a study with more than 3000 subjects, published in 2016, the correlation for total plasma C15:0 with dairy fat intake was 0.29 [13]. In addition, in a study with 279 subjects, published in 2015, the correlation of plasma PL C15:0 with dairy fat intake was 0.32 [16].

This is one of the first studies investigating the association between trans-C18:1(n-7), measured in plasma TG and PL, and dairy fat intake. A single study measured trans-C18:1(n-7) in total plasma and erythrocytes, but did not find a significant correlation ($r = 0.06$ and $r = -0.01$ respectively) [21]. Most other studies investigated a cluster of trans-C18:1 fatty acids, which is a combination of the rumen (trans-C18:1(n-7)) and industrial derived (trans-C18:1(n-9)) trans fatty acids, rather than trans-C18:1(n-7) alone [31,32]. One of these studies is a Chinese study of 2091 adult participants aged 50-70 years, published in 2014, which found an association of 0.37 between the cluster of trans-C18:1 fatty acids assessed in erythrocytes and total dairy consumption [31]. The current study showed a lower, but significant association between the ruminant derived trans fatty acid trans-C18:1(n-7) and dairy fat intake with a standardized beta of 0.29 for TG and of 0.27 for PL.

CLA in plasma PL was also found to be associated with dairy fat intake in the Lifelines population, both as a single marker and in combination with C14:0, C15:0 and trans-C18:1(n-7), while CLA in plasma TG was less strongly associated with dairy fat intake. CLA in plasma TG as a single marker was significantly associated with dairy fat intake ($\text{std.}\beta = 0.22$), however the association of CLA with dairy fat intake did not remain in the backwards regression model when only plasma TG fatty acids were included. Current papers report mainly on the correlation between dairy fat intake and CLA in adipose tissue. For example, in a study of 3630 Costa-Rican adults, published in 2012, the correlation of CLA with dairy fat intake was 0.24 [29]. The correlation in adipose tissue is in line with the correlation found with CLA in plasma PL.

In the current cohort, we found that a combination of biomarkers is a slightly better predictor for dairy fat intake in the general population when compared to single markers. C15:0 and trans-C18:1(n-7) were found in all three models and in the bootstrap models. Therefore, a combination of dairy fat biomarkers, particularly models including these two, may be the best combination to predict the dairy fat intake in the general population.

The combined fatty acids assessed in PL provided a slightly better prediction of dairy fat intake than the combined fatty acids in TG (R^2 PL: 0.143 vs R^2 TG: 0.128). Consistent with this, when the selection criterium for inclusion in the model was set at present in > 70% of the models, it was found that only plasma PL trans-C18:1(n-7) and plasma PL CLA remained in the model, if both TG and PL fatty acids were combined. This suggests that fatty acids in PL are stronger predictors than fatty acids in TG. This might be due to the fact that plasma TG is influenced by recent dietary intake (intake in the last few hours), whereas this is less so for plasma PL [49]. Recent meals, including large amounts of carbohydrates, can for instance turn on *de novo*

lipogenesis which changes the fatty acid balance in plasma triglycerides and may therefore change the association with dairy fat intake. Researchers of the FORCE consortium therefore recommend to measure biomarkers of dietary intake in plasma CE and PL over plasma TG [50-52]. Indeed, most of the biomarker studies are carried out in plasma PL [6,16,17,19,22,45], while only one study reported on biomarkers in serum TG [17]. Importantly, however, one of the most promising markers, i.e. ruminant trans-C18:1(n-7), was only detectable in PL and TG, and not in CE. Furthermore, by measuring the fatty acids in both plasma TG and PL, it was possible to identify differences between both fractions.

Prediction of energy-adjusted dairy fat intake was the primary outcome for this paper. In additional analyses, we repeated the analyses for unadjusted dairy fat intake, and also assessed predictive capacity for energy-adjusted dairy intake. Both C15:0 and trans-C18:1(n-7) in plasma TG and both trans-C18:1(n-7) and CLA in plasma PL were associated with unadjusted dairy fat intake, albeit with slightly lower effect sizes than with energy-adjusted dairy fat intake. The associations between dairy biomarkers and energy-adjusted dairy intake were weaker than for energy-adjusted dairy fat intake. Obviously, dairy fatty acid biomarkers serve best to predict dairy fat intake.

This study has several strong points. Firstly, we are one of the first studies to identify trans-C18:1(n-7) and CLA in both plasma TG and PL as biomarkers of dairy fat intake. Another strength of the present study is its large population size. In total, complete data of 769 participants were used for analyses. Additionally, we were able to measure a large amount of dairy fat biomarkers in both plasma TG and PL in one cohort.

We must acknowledge that the current study has some limitations. First of all, the R-squares of the models for prediction of dairy fat intake were relatively low. This means that the dairy fatty acid biomarkers are not strongly related to the dairy fat intake from the FFQ. However, it cannot be discerned whether the weak associations are intrinsic to the dairy fatty acid biomarkers, or that the FFQ as a reference is not optimal. Concerning the dairy fatty acid biomarkers, it has been shown that some of the fatty acids in plasma are not only influenced by dairy fat intake, but can also be affected by metabolic processes (e.g. trans-C16:1(n-7) can be influenced by β -oxidation of trans-C18:1(n-7)), or by the intake of dietary fibers (e.g. C17:0 can be influenced by inulin), meat and coconut oil (e.g. C14:0) [53-56]. A limitation of this study is that we have no specific data on types of oils and types of meat, which precluded adjustment of these potential confounders. However, we had data on total meat intake. In our cohort adjustment for total meat intake did not alter the association between fatty acid biomarkers and energy-adjusted dairy fat intake (**Supplemental Table 2A**). Another limitation is that circulating fatty acids as biomarkers for dietary intake were expressed as proportions rather than as absolute concentrations, which makes them relative and therefore dependent on the presence of other major circulating fatty acids. The reason that we report on proportions of dairy fatty acids is to align with existing

literature and make results comparable. It should also be noted that the fatty acid composition of dairy fat can vary depending on factors such as cow genetics, forage content, production level, time of lactation, physiological status of the animal and seasonal variation (German *et al.* 2006; Jensen 2002, Mansson 2008, Hanus *et al.* 2018). Nevertheless, fatty acid biomarkers can provide an objective estimation of dairy fat intake, while this is intrinsically not the case with FFQ, which may be subject to under- and over reporting by participants [43,57]. Overall, there is no gold standard for measuring dietary intakes and the FFQ can therefore be seen as the best method so far.

This study is the first observational study investigating commonly used biomarkers in combination with less investigated trans fatty acid biomarkers. A first step towards validation would be performance of similar studies in other populations. Furthermore, as the applied assessment method is labor intensive, the results of this study should first be replicated in other studies to show that incorporation is worthwhile. Concluding, this paper showed associations between circulating dairy fatty acids and dairy fat intake in the general population, which can be seen as a first step towards better measurement techniques for dairy fat intake.

CONCLUSION

In this study, models were developed and validated for the prediction of dairy fat intake in the general population. The study confirmed the utility of commonly used biomarkers and identified the biomarkers trans-C18:1(n-7) and CLA as potential dairy fat biomarkers, which are one of the best biomarkers to predict dairy fat intake in the Lifelines population. Moreover, a combination of the biomarkers may be even a stronger predictor for dairy fat intake. A first step towards validation would be performance of similar studies in other populations.

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The manuscript is based on data from the LifeLines Cohort Study. LifeLines adheres to standards for open data availability. The data catalogue of LifeLines is publicly accessible on www.LifeLines.net. All international researchers can apply for data at the LifeLines research office (LLscience@umcg.nl). The LifeLines system allows access for reproducibility of the study results.

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Supplementary Table 1: Potential dairy fat biomarkers in plasma cholesterol esters (CE), triglycerides (TG) and phospholipids (PL) measured in the pilot study of the Lifelines cohort (n = 96)

Fatty Acid	Plasma CE (mol%)	Plasma TG (mol%)	Plasma PL (mol%)
C14:0	0.78 (0.66 – 0.95)	1.62 (1.25 – 2.24)	0.46 (0.40 – 0.56)
C15:0	0.20 (0.17 – 0.23)	0.30 (0.26 – 0.37)	0.29 (0.25 – 0.33)
C17:0	IS	0.49 (0.42 – 0.58)	0.39 (0.35 – 0.42)
T-C16:1(n-7)	0.025 (0.017 – 0.036)	0.026 (0.022 – 0.036)	0.023 (0.017 – 0.027)
T-C18:1(n-7)	BDL	0.10 (0.07 – 0.15)	0.11 (0.08 – 0.13)
CLA	0.052 (0.037 – 0.065)	0.069 (0.042 – 0.091)	0.037 (0.028 – 0.046)

Data are raw data (mol%) presented as Median (25th – 75th percentile).

Abbreviations: CE, cholesterol esters; TG, Triglycerides; PL, Phospholipids, C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1(n-7), Trans-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid; IS, Internal standard; BDL, below detection limit.

Supplementary Table 2A: Univariate regression analyses and multivariate models for the association of circulating potential dairy fat biomarkers and energy-adjusted dairy fat intake (g/d) in the Lifelines cohort study (n = 769)

Potential Circulating Dairy fat Biomarkers												
C14:0			C15:0		C17:0		T-C16:1(n-7)		T-C18:1(n-7)		CLA	
Std β	p-value		Std β	p-value	Std β	p-value	Std β	p-value	Std β	p-value	Std β	p-value
Plasma TG												
Model 1	0.204	<0.001	0.286	<0.001	0.049	0.19	0.233	<0.001	0.292	<0.001	0.215	<0.001
Model 2	0.191	<0.001	0.267	<0.001	0.057	0.12	0.228	<0.001	0.271	<0.001	0.203	<0.001
Model 3	0.191	<0.001	0.268	<0.001	0.052	0.15	0.226	<0.001	0.269	<0.001	0.202	<0.001
Model 4	0.194	<0.001	0.267	<0.001	0.054	0.15	0.225	<0.001	0.270	<0.001	0.201	<0.001
Model 5	0.133	0.001	0.231	<0.001	0.047	0.19	0.185	<0.001	0.240	<0.001	0.160	<0.001
Plasma PL												
Model 1	0.214	<0.001	0.215	<0.001	0.132	<0.001	0.130	<0.001	0.269	<0.001	0.272	<0.001
Model 2	0.199	<0.001	0.197	<0.001	0.111	0.001	0.129	<0.001	0.253	<0.001	0.258	<0.001
Model 3	0.195	<0.001	0.195	<0.001	0.109	0.002	0.126	<0.001	0.250	<0.001	0.255	<0.001
Model 4	0.197	<0.001	0.196	<0.001	0.108	0.002	0.126	<0.001	0.252	<0.001	0.257	<0.001
Model 5	0.186	<0.001	0.189	<0.001	0.099	0.01	0.118	0.001	0.254	<0.001	0.249	<0.001

Model 1: Crude analyses; **Model 2:** Adjusted for Model 1 + age and sex; **Model 3:** Adjusted for Model 2 + BMI; **Model 4:** Adjusted for Model 3 + Total Meat Intake; **Model 5:** Adjusted for Model 3 + Trans-C18.1(n-9), C14:0, C17:0, Trans-C16.1(n-7), Trans-C18.1(n-7) and CLA in plasma TG, and Trans-C16.1(n-7), Trans-C18.1(n-7), CLA, Trans-C18.1(n-9) in plasma PL were transformed before analyses. Dairy fat intake was transformed and adjusted for energy intake according to the residual method. β 's are standardized beta's.

Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1(n-7), Trans-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Conjugated Linolenic acid; TG, Triglycerides; PL, Phospholipids; BMI, Body Mass Index; Trans-C18:1(n-9), Elaidic acid.

Supplementary Table 2B: Univariate regression analyses and multivariate models for the association between PL C14:0 and energy-adjusted dairy fat intake (g/d) stratified for males (n = 403) and females (n = 364)

	Males		Females	
	Std β	p-value	Std β	p-value
Plasma PL				
Model 1	0.264	<0.001	0.173	<0.001
Model 2	0.248	<0.001	0.147	0.003
Model 3	0.250	<0.001	0.148	0.003
Model 4	0.250	<0.001	0.141	0.004
Model 5	0.232	<0.001	0.147	0.003

Model 1: Crude analyses; **Model 2:** Adjusted for Model 1 + age; **Model 3:** Adjusted for Model 2 + BMI; **Model 4:** Adjusted for Model 3 + Total Meat Intake; **Model 5:** Adjusted for Model 3 + Trans-C18:1(n-9). Trans-C18:1(n-9) in plasma PL was transformed before analyses. Dairy fat intake was transformed and adjusted for energy intake according to the residual method. β 's are standardized beta's. Abbreviations: C14:0, Myristic acid; PL, Phospholipids; BMI, Body Mass Index; Trans-C18:1(n-9), Elaidic acid.

Supplementary Table 3: Univariate associations of circulating potential dairy fat biomarkers with unadjusted total dairy intake, total dairy fat intake and intake of dairy product groups (g/d) in the Lifelines cohort study (n = 769)

	Potential circulating dairy fat biomarkers											
	C14:0			C15:0			C17:0			T-C16:1(n-7)		
	Std β	p-value	Std β	p-value	Std β	p-value	Std β	p-value	Std β	p-value	Std β	p-value
Plasma TG												
Total dairy intake	0.092	0.01	0.140	<0.001	0.065	0.08	0.059	0.14	0.101	0.01	0.040	0.26
Total dairy fat intake	0.222	<0.001	0.257	<0.001	0.030	0.43	0.188	<0.001	0.261	<0.001	0.171	<0.001
Skimmed dairy	-0.044	0.22	0.12	0.74	0.036	0.33	-0.001	0.99	-0.041	0.26	-0.048	0.19
Semi-Skimmed dairy	0.082	0.02	0.027	0.45	-0.004	0.92	-0.023	0.57	0.016	0.66	-0.004	0.90
Full Fat Dairy	0.164	<0.001	0.155	<0.001	0.025	0.51	0.122	0.003	0.195	<0.001	0.120	<0.001
Fermented Dairy	-0.018	0.63	0.059	0.10	0.069	0.07	0.026	0.52	0.032	0.38	-0.012	0.75
Milk	0.118	0.001	0.080	0.03	-0.008	0.84	0.051	0.22	0.092	0.01	0.047	0.19
Cheese	0.164	<0.001	0.203	<0.001	-0.002	0.96	0.173	<0.001	0.198	<0.001	0.144	<0.001
Plasma PL												
Total dairy intake	0.092	0.01	0.143	<0.001	0.174	<0.001	0.054	0.14	0.111	0.002	0.079	0.03
Total dairy fat intake	0.198	<0.001	0.192	<0.001	0.127	<0.001	0.085	0.02	0.232	<0.001	0.228	<0.001
Skimmed dairy	-0.02	0.74	0.009	0.81	0.099	0.01	0.008	0.82	0.001	0.98	-0.008	0.83
Semi-Skimmed dairy	0.010	0.78	0.043	0.23	0.025	0.49	-0.020	0.59	-0.021	0.58	-0.021	0.56
Full Fat Dairy	0.142	<0.001	0.136	<0.001	0.071	0.05	0.060	0.10	0.170	<0.001	0.157	<0.001
Fermented Dairy	0.055	0.13	0.071	0.05	0.125	0.001	0.058	0.11	0.072	0.05	0.053	0.14
Milk	0.044	0.22	0.105	0.004	0.081	0.03	0.026	0.47	0.063	0.08	0.055	0.13
Cheese	0.166	<0.001	0.137	<0.001	0.066	0.07	0.061	0.10	0.163	<0.001	0.206	<0.001

C14:0, C17:0, Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma TG, and Trans-C16:1(n-7), Trans-C18:1(n-7), CLA, Trans-C18:1(n-9) in plasma PL were transformed before analyses. Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1(n-7), Trans-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Conjugated Linolenic acid; TG, Triglycerides; PL, Phospholipids.

Supplementary Table 4: Number of times each variable is selected after 1000 bootstrap attempts in the lifelines cohort
(n = 769)

Variable	Number of times selected
TG model	
TG C14:0	292
TG C15:0	871
TG C17:0	91
TG T-C16:1(n-7)	173
TG T-18:1(n-7)	943
TG CLA	148
PL model	
PL C14:0	611
PL C15:0	786
PL C17:0	149
PL T-C16:1(n-7)	454
PL T-C18:1(n-7)	994
PL CLA	987
TG+PL model	
TG C14:0	337
TG C15:0	540
TG C17:0	90
TG T-C16:1(n-7)	146
TG T-18:1(n-7)	523
TG CLA	511
PL C14:0	232
PL C15:0	651
PL C17:0	182
PL T-C16:1(n-7)	454
PL T-18:1(n-7)	857
PL CLA	954

C14:0, C17:0, Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma TG and Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma PL were transformed (sqrt) before analyses. Dairy fat intake was transformed and adjusted for energy intake according to the residual method.

Abbreviations: TG, Triglycerides; PL, Phospholipids; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1(n-7), Trans-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Conjugated Linolenic acid

Supplementary Table 5: Univariate regression analyses and multivariate models to predict total dairy fat intake (g/d) with circulating fatty acids in plasma TG, plasma PL and a combination of the two in the Lifelines cohort (n = 769)

	Model 1			Model 2		Model 3	
Plasma TG	Std β	R²	P-value	Std β	P-value	Std β	P-value
TG C14:0	0.222	0.066	< 0.001	0.096	0.02	0.091	0.03
TG C15:0	0.257	0.084	< 0.001	0.125	0.02	0.117	0.02
TG C17:0	0.030	0.019	0.43	-	-		
TG T-C16:1(n-7)	0.188	0.045	< 0.001	0.013	0.80		
TG T-C18:1(n-7)	0.261	0.086	< 0.001	0.151	0.01	0.135	0.01
TG CLA	0.171	0.047	< 0.001	-0.047	0.32		
				R²=	0.104	R²=	0.103
Plasma PL	Std β	R²	P-value	Std β	P-value	Std β	P-value
PL C14:0	0.198	0.057	< 0.001	0.083	0.04	0.083	0.04
PL C15:0	0.192	0.054	< 0.001	0.090	0.04	0.095	0.02
PL C17:0	0.127	0.034	0.001	0.010	0.82		
PL T-C16:1(n-7)	0.085	0.025	0.02	-0.096	0.02	-0.096	0.02
PL T-C18:1(n-7)	0.232	0.070	< 0.001	0.167	0.001	0.167	< 0.001
PL CLA	0.228	0.070	< 0.001	0.123	0.003	0.123	0.003
				R²=	0.110	R²=	0.110
Plasma TG+PL				Std β	P-value	Std β	P-value
TG C14:0				0.120	0.04	0.129	0.003
TG C15:0				0.059	0.35		
TG C17:0				-	-		
TG T-C16:1(n-7)				0.013	0.80		
TG T-C18:1(n-7)				0.083	0.16	0.102	0.07
TG CLA				-0.122	0.02	-0.111	0.03
PL C14:0				-0.008	0.87		
PL C15:0				0.086	0.06	0.111	0.004
PL C17:0				0.024	0.62		
PL T-C16:1(n-7)				-0.087	0.05	-0.074	0.08
PL T-C18:1(n-7)				0.125	0.02	0.140	0.01
PL CLA				0.132	0.01	0.124	0.01
				R²=	0.130	R²=	0.128

Model 1: Univariate linear regression analysis of the fatty acid with total dairy fat intake; **Model 2:** Multivariate model with all fatty acids from our population in one model; **Model 3:** Multivariate model with the best combination of fatty acids in our population (backwards regression). C14:0, C17:0, Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma TG and Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma PL were transformed before analyses. Dairy fat intake was transformed. β 's are standardized beta's.

Abbreviations: TG, Triglycerides; PL, Phospholipids, C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), T-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid

Supplementary Table 6: Univariate regression analyses and multivariate models to predict energy-adjusted dairy intake (g/d) with circulating fatty acids in plasma TG, plasma PL and a combination of the two in the Lifelines Cohort (n = 769)

	Model 1			Model 2		Model 3	
Plasma TG	Std β	R²	P-value	Std β	P-value	Std β	P-value
TG C14:0	0.073	0.045	0.04	0.013	0.77		
TG C15:0	0.141	0.059	< 0.001	0.144	0.01	0.141	< 0.001
TG C17:0	0.066	0.043	0.08	0.051	0.21		
TG T-C16:1(n-7)	0.069	0.043	0.09	-0.039	0.45		
TG T-C18:1(n-7)	0.105	0.050	0.003	0.052	0.35		
TG CLA	0.053	0.042	0.14	-0.058	0.23		
				R²=	0.064	R²=	0.059
Plasma PL	Std β	R²	P-value	Std β	P-value	Std β	P-value
PL C14:0	0.080	0.046	0.02	0.020	0.62		
PL C15:0	0.143	0.060	< 0.001	0.057	0.20	0.075	0.07
PL C17:0	0.170	0.068	< 0.001	0.140	0.002	0.131	0.002
PL T-C16:1(n-7)	0.071	0.044	0.05	-0.033	0.45		
PL T-C18:1(n-7)	0.111	0.051	0.002	0.008	0.86		
PL CLA	0.082	0.046	0.02	0.047	0.27		
				R²=	0.075	R²=	0.072
Plasma TG+PL				Std β	P-value	Std β	P-value
TG C14:0				0.085	0.17	0.087	0.01
TG C15:0				0.039	0.54		
TG C17:0				0.011	0.79		
TG T-C16:1(n-7)				-0.038	0.48		
TG T-C18:1(n-7)				0.024	0.69		
TG CLA				-0.097	0.07		
PL C14:0				-0.034	0.51		
PL C15:0				0.062	0.19		
PL C17:0				0.147	0.004	0.177	< 0.001
PL T-C16:1(n-7)				-0.012	0.80		
PL T-C18:1(n-7)				0.006	0.91		
PL CLA				0.075	0.13		
				R²=	0.083	R²=	0.075

Model 1: Univariate linear regression analysis of the fatty acid with energy-adjusted dairy intake; **Model 2:** Multivariate model with all fatty acids from our population in one model; **Model 3:** Multivariate model with the best combination of fatty acids in our population (backwards regression). C14:0, C17:0, Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma TG and Trans-C16:1(n-7), Trans-C18:1(n-7) and CLA in plasma PL were transformed before analyses. Dairy intake was transformed and adjusted for energy intake according to the residual method. β 's are standardized beta's.

Abbreviations: TG, Triglycerides; PL, Phospholipids; C14:0, Margaric acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), T-Palmitoleic acid; T-C18:1(n-7), Vaccenic acid; CLA, Cis-9, Trans-11 Conjugated linoleic acid

CHAPTER 5

Potential biomarkers for fat from dairy and fish and their association with cardiovascular risk factors: Cross-sectional data from the Lifelines biobank and cohort study

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ABSTRACT

Dairy fat intake, reflected by the biomarkers C14:0, C15:0, C17:0, trans-C16:1 (*n*-7), trans-C18:1 (*n*-7) and CLA, may have beneficial effects on cardiovascular health. It has, however, been questioned whether this association is genuine, since C15:0 and C17:0 are also biomarkers from fish. We investigated whether the above biomarkers are reliable markers for dairy fat intake in 864 healthy subjects. Subsequently, we explored the association between these biomarkers and cardiovascular risk factors. Intakes of dairy and fish were determined by Food Frequency Questionnaires (FFQs). Fatty acids were analyzed in plasma triglycerides (TG) and phospholipids (PL). Median intakes of dairy and fish fat were 12.3 (8.4–17.4) g/day and 1.14 (0.53–1.75) g/day. All fatty acids, except TG C17:0, were associated with dairy fat (std.β range TG: 0.12 for C14:0 till 0.25 for C15:0 and Trans-C18:1 (*n*-7); and std.β range PL: 0.12 for C17:0 and Trans-C16:1 (*n*-7) till 0.24 for Trans-C18:1 (*n*-7) and CLA; $P < 0.001$). TG C17:0 was associated with fish fat (std.β = 0.08; $P = 0.03$), whereas PL C17:0 was not. Associations remained after adjustment for fish/dairy fat intake. Strongest inverse associations with biological variables were found with PL C17:0 and Trans-C18:1 (*n*-7) (Std.βs: waist circumference: -0.18 , $P < 0.001$ and -0.10 , $P < 0.05$; BMI: -0.17 , $P < 0.001$, -0.11 , $P < 0.01$; glucose: -0.10 , $P < 0.01$ and -0.08 , $P < 0.05$; high sensitive C-reactive protein (hs-CRP): -0.22 , $P < 0.001$ and -0.16 , $P < 0.01$; uric acid: -0.27 , $P < 0.001$ and -0.24 , $P < 0.001$). In conclusion, fatty acid biomarkers, except plasma TG C17:0, were associated with dairy fat intake, independent of fish fat intake. PL C17:0 and trans-C18:1 (*n*-7) were inversely associated with adiposity, diabetes, inflammation and uric acid.

INTRODUCTION

Cardiovascular diseases have become the number one cause of death worldwide [1]. Dietary and nutritional components are relevant players in the development and progression of cardiovascular diseases. Fatty acids are one of the nutritional components that may have influence on cardiovascular diseases. Whether this impact is positive or negative can for instance depend on the dietary source of the fatty acid. Earlier research has already shown that this was the case for the associations between saturated fatty acids and incident cardiovascular diseases [2,3]. For example, saturated fatty acids from meat were found to increase cardiovascular risk, while saturated fatty acids from dairy were inversely associated with cardiovascular risk [3–7]. Additionally, researchers have reported that trans fatty acids from processed foods, margarines, frying fast foods and commercial baked goods may increase risk for cardiovascular disease [8–11]. In contrast, trans fatty acids from dairy may actually be inversely related to cardiovascular risk factors [5]. Saturated and trans fatty acids from dairy products therefore seem to have a positive effect on cardiovascular health.

The saturated fatty acids pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and to a lesser extent myristic acid (C14:0), and the trans fatty acids trans-palmitoleic acid (trans-C16:1(*n*-7)), vaccenic acid (trans-C18:1(*n*-7)) and the major conjugated linoleic acid (CLA) (cis-9, trans-11 CLA) were identified as dairy fat biomarkers. Additionally, dairy fat intake, measured with the dairy fat biomarkers, was found to be inversely associated with cardiovascular risk factors [4–6,12–16]. Despite the evidence, researchers raised concerns on the association between dairy fat intake, measured by dairy fat biomarkers, and cardiovascular health, since dairy fat biomarkers may not be explicit enough for the prediction of dairy fat intake [17]. For instance, C15:0 and C17:0 were also identified as possible biomarkers for fish fat intake [18,19]. Both fish intake and fish fat intake have already been found to be inversely related to the development of cardiovascular diseases [20]. In a meta-analysis of 19 observational studies, it was found that fish consumption was associated with a risk reduction of 17% for fatal coronary heart disease [21]. Additionally, a meta-analysis with 14 randomized controlled trials demonstrated that an intervention group who consumed omega-3 fatty acid supplements (eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA), two fatty acids that are well-known biomarkers of fish intake) had an 8% reduction for cardiac death compared to the control group [22]. Because of the potential association of C15:0 and C17:0 with fish intake, researchers are concerned that the current circulating fatty acids that have been identified as dairy fat biomarkers, are not explicit enough for the prediction of dairy fat intake [19]. Therefore, inverse associations of the fatty acids with cardiovascular health may potentially not be ascribed to dairy fat intake, but fish fat intake instead.

One of the main purposes of this paper was therefore to find out whether the dairy fat biomarkers are solid biomarkers for dairy fat intake in the general population. We therefore

investigated the association of the saturated fatty acids C15:0 and C17:0 with dairy fat intake, and additionally for fish fat intake. We repeated these measurements for the other dairy fat biomarkers (C14:0, trans-C16:1 (*n*-7), trans-C18:1 (*n*-7) and CLA). Secondly, we wanted to explore whether these circulating fatty acids were associated with cardiovascular risk factors, and so whether the association with cardiovascular risk factors could be ascribed to dairy fat intake, fish fat intake or both. To answer these questions, a subset from the Lifelines Biobank and Cohort study was used, a large observational cross-sectional study with initially healthy participants living in the northern part of the Netherlands.

MATERIALS AND METHODS

Study Design and Population

The Lifelines Cohort Study is a large observational population-based cohort study and Biobank which examines the health and health-related behaviors of more than 167,000 persons [23]. The participants were recruited from the three Northern provinces of the Netherlands between 2006 and 2013. A more detailed description of the Lifelines Cohort study can be found elsewhere [24,25]. In short, the first group of participants were recruited via local general practices. Participants could indicate whether family members were interested as well. In addition, individuals who were interested in the study had the possibility to register via an online self-registration. Individuals with insufficient knowledge of the Dutch language, with severe psychiatric or physical illness, and those with limited life expectancy (<5 years) were excluded from the study. Participants (>18 years old) completed several questionnaires, including topics such as occurrence of diseases, general health, medication use, diet, physical activity and personality. Participants were invited to the Lifelines Research sites for a comprehensive health assessment. A randomly selected amount of 864 participants from the baseline cross-sectional database were included in the current study. All participants provided written consent. The Lifelines Cohort Study was conducted according to the principles of the Declaration of Helsinki and approved by the Medical ethical committee of the University Medical Center Groningen, The Netherlands.

Dietary Intake

To assess dietary intake in the Lifelines Cohort, a 110-item semi-quantitative baseline food frequency questionnaire (FFQ) assessing food items over the previous month was developed and validated by Wageningen University using the Dutch FFQTOOL™, in which food items were selected based on the Dutch national Food Consumption Survey of 1997/1998 [26]. Seven answers categories were used to assess consumption frequency, ranging from 'not this month' to '6–7 days a week'. Portion size was estimated by fixed portion sizes (e.g., slices of bread, pieces

of fruit) and commonly used household measures (e.g., cups, spoons). Energy and macronutrient intake was estimated from the FFQ data by using the Dutch food composition database of 2011 (NEVO). This study specifically looked at total dairy fat intake and total fish fat intake. Total dairy fat intake consisted of fat from cheese, milk, buttermilk, yoghurt, sweetened yoghurt drinks, custard, curd cheese, ice cream, whipped cream and porridge. Total fish fat intake consisted of fat from lean and fatty fish, including types such as salmon, herring and codfish.

Data on Education, Smoking Habits and General Health

Information about education, smoking and general health was collected from the self-administered questionnaire. Educational level was categorized in four groups (1. Never been to school or elementary school only, 2. Lower vocational or secondary schooling, 3. Intermediate vocational schooling or intermediate/higher secondary schooling or 4. Higher vocational schooling or university). Additionally, subjects were classified according to their smoking habits (non-smokers, former smokers or current smokers).

Clinical Measurements

Anthropometric measurements (weight, height, and waist circumference) and blood pressure were measured by well-trained staff. The anthropometric measurements were measured without shoes. Body weight was measured to the nearest 0.1 kg. Height and waist circumferences were measured to the nearest 0.5 cm. Height was measured with a stadiometer placing their heels against the rod and the head in Frankfort Plane position. Waist circumference was measured in standing position with a tape measure all around the body, at the level midway between the lower rib margin and the iliac crest. BMI was calculated as weight (kg) divided by height squared (m^2). Systolic and diastolic blood pressures were measured 10 times during a period of 10 minutes, using an automated Dinamap Monitor (GE Healthcare, Freiburg, Germany). The average of the final three readings was used for each blood pressure parameter.

Biochemical Measurements

For analysis of lipids, glucose, uric acid, creatinine and the inflammation marker high sensitivity C-reactive protein (hs-CRP), blood samples were drawn in the morning between 8:00 and 10:00 am after a period of overnight fasting. Serum levels of total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with an enzymatic colorimetric method, low-density lipoprotein (LDL) cholesterol with an enzymatic method and total triglycerides with a colorimetric UV method, all on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). Fasting blood glucose was measured using a hexokinase method. HbA1c was determined in whole blood (EDTA-anticoagulated) by means of turbid metric inhibition immunoassay on a Cobas Integra 800 CTS analyzer (Roche Diagnostics Netherland BV, Almere, The Netherlands). Insulin resistance was measured by calculating the ratio total triglycerides/HDL cholesterol [27]. Serum

uric acid and creatinine were measured on a Roche Modular P chemistry analyzer (Roche, Basel, Switzerland). The hs-CRP was determined by nephelometry (BN II system Siemens, Marburg, Germany).

Fatty Acid Analyses

EDTA-plasma samples were collected at baseline and stored at -80°C until analyses of fatty acids were carried out. Analyses of fatty acids were performed at the Department of Laboratory Medicine of the University Medical Center Groningen, The Netherlands using the methodology as described by Hoving et al. [28]. In short, total lipids were extracted by the method of Folch et al., using 6 mL of chloroform-methanol (2:1) and a 200 μL EDTA-plasma sample [29]. Additionally, a shortened version of the method of Kaluzny et al. was used to isolate plasma cholesterol esters (CE), triglycerides (TG) and phospholipids (PL), using aminopropyl SPE columns for the separation (Isolute, Biotage) [30]. Fatty acids were transmethyalted with methanolic-HCL into fatty acid methyl esters (FAME). The samples were extracted with hexane and eventually redissolved into 100 μL hexane. 100 μL of an internal standard for the quantification of fatty acids in CE (17:0) (50.1 mg/100 mL chloroform-methanol, 2:1 v/v), and 100 μL of an internal standard for the quantification of fatty acids in TG (19:0) (19.9 mg/100 mL chloroform-methanol, 2:1 v/v), both obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), were added before isolation of classes. For the quantification of fatty acids in PL, 100 μL of free fatty acid 19:0 (50.0 mg/100 mL methanol), obtained from Larodan (Solna, Sweden), was added after isolation of classes. 100 μL Butylated Hydroxytoluene (1 g/100 mL methanol) from Sigma-Aldrich (Zwijndrecht, The Netherlands) was added to prevent fatty acid oxidation.

Aliquots of 2 μL were injected into an Agilent model 6890 gas chromatography equipped with a 200 m x 0.25 mm polar column (CP Select for FAME) and detected with an Agilent 7683 series flame ionization detector. FAME were identified by comparing retention times with those of known standards (Supelco 37 component FAME mix (Sigma-Aldrich)). Fatty acids were calculated into mol%. Firstly, fatty acids were measured in a pilot of 96 samples to investigate whether the fatty acids of interest were detectable in plasma CE, TG and PL. Compared to plasma TG and PL, potentially interesting fatty acids were less detectable in plasma CE. Subsequently, we decided to only move forward with plasma TG and PL. The precision of the measurements was tested by calculating the variation coefficient from 10 replicate samples using quality-control samples (pooled plasma samples). The circulating fatty acids reported in this paper had a variation coefficient $\leq 15\%$.

Statistical Analyses

All analyses were performed using IBM SPSS Statistics, version 22.0 for Windows software (IBM, Armonk, NY, USA) and GraphPad Prism, version 5.03 for Windows software (Graphpad Software, La Jolla, CA, USA). At the start of the study, plasma circulating fatty acids were measured in 864

lifelines participants, whereof 776 had complete FFQ data. Cases with missing data either on circulating fatty acids or dietary intake were removed before analysis leaving 769 participants in the lifelines cohort with complete data. For illustrative purposes, baseline data are presented for the total population, and separately for males and females. Baseline data are presented as mean $\pm$ SD (normally distributed data), median (25th–75th percentile) (non-normally distributed data) or as number (%) (categorical data). Differences in baseline data between males and females were tested with an independent t-test (continuous data), Mann-Whitney U test (non-normally distributed data) or Chi-square test (categorical data). Multivariate linear regression analyses were carried out to investigate the association of circulating fatty acids in plasma TG and PL with dairy fat intake. Non-normal data were transformed before analyses. Models were adjusted for total energy intake (model 2), followed by further adjustments for age, education, smoking habits, BMI, total carbohydrates and total fat intake, fish fat intake, and total serum triglycerides and serum cholesterol (model 3–7). The same analyses were carried out to examine the association between circulating fatty acids and fish fat intake, except that the additional adjustments now included dairy fat intake instead of fish fat intake. As a sensitivity analysis, we also examined the association of EPA and DHA in plasma TG and PL with fish fat intake. To investigate our second purpose, multivariate linear regression analyses were used to examine the cross-sectional association of circulating fatty acids in plasma TG and PL with cardiovascular risk factors. Associations were adjusted for age and sex (model 2). In secondary analyses, models were additionally adjusted for education and smoking habits, total serum triglycerides, total serum cholesterol (only plasma PL), and fatty acids from de novo lipogenesis (C14:0 + C16:0 + C16:1 (*n*-7) from plasma TG). Lastly, the association between dairy fat intake and cardiovascular risk factors, and the association between fish fat intake and cardiovascular risk factors was investigated. Models were adjusted for total energy intake, age, sex, education, smoking habits, BMI, total carbohydrates and total fat intake, total fish fat intake/total dairy fat intake, and total serum triglycerides and serum cholesterol. All reported probability values are two-tailed, and a $P \leq 0.05$ was considered statistically significant.

RESULTS

The Lifelines Population

The characteristics of the lifelines population (total population and separately for males ($n = 404$) and females ($n = 365$)) can be found in **Table 1**. The total population had a mean age of 53.0 $\pm$ 15.5 years and a BMI of 26.0 $\pm$ 4.0 kg/m². Differences in dietary intake were observed between males and females, i.e., the total energy intake, and the carbohydrate, protein and fat intake were significantly higher in males compared to females. However, after correction for individual energy intake (intake calculated as En%), only protein intake remained significantly higher in

males compared to females (**Table 1**). Concerning intake of dairy fat and fish fat, if corrected for individual energy intake, dairy fat intake did not differ between males and females, while fish fat intake was higher in females compared to males (**Table 1**).

The circulating fatty acids C14:0, C15:0, C17:0, trans-C16:1 (*n*-7), trans-C18:1 (*n*-7) and CLA were measured in plasma TG and PL. Overall, the circulating fatty acids in plasma TG were higher compared to plasma PL. The quantitatively most abundant investigated marker was C14:0 (Total TG: 1.87 mol%, PL: 0.49 mol%), followed by C17:0 and C15:0 in both fractions.

Table 1: Characteristics of the total Lifelines Population, separate for males and females.

Baseline Characteristics	Total N = 769	Males N = 404	Females N = 365	P-value
General				
Age visit 1, years	53.0 ± 15.5	53.3 ± 15.2	52.7 ± 16.0	0.60
Weight, kg	79.3 ± 14.2	85.7 ± 11.9	72.3 ± 13.2	<0.001
BMI, kg/m ²	26.0 ± 4.0	26.3 ± 3.4	25.8 ± 4.6	0.08
Smoking, yes (%)	115 (15.0%)	68 (16.8%)	47 (12.9%)	0.13
SBP, mmHg	126.5 ± 17.0	129.5 ± 15.1	123.1 ± 18.2	<0.001
DBP, mmHg	73.7 ± 9.7	76.6 ± 9.8	70.5 ± 8.4	<0.001
Laboratory measurements				
Total Cholesterol, mmol/L	5.2 ± 1.0	5.1 ± 1.0	5.3 ± 1.1	0.03
HDL cholesterol, mmol/L	1.5 ± 0.4	1.3 ± 0.3	1.7 ± 0.4	<0.001
LDL cholesterol, mmol/L	3.3 ± 0.9	3.3 ± 0.9	3.3 ± 1.0	0.27
Total Triglycerides, mmol/L	1.0 (0.8–1.5)	1.2 (0.9–1.6)	1.0 (0.7–1.3)	<0.001
Total TG/HDL-c ratio	0.72 (0.45–1.17)	0.92 (0.60–1.43)	0.56 (0.38–0.88)	<0.001
Glucose, mmol/L	5.1 ± 0.7	5.2 ± 0.6	5.0 ± 0.7	0.003
HbA1c, %	5.6 ± 0.4	5.6 ± 0.4	5.6 ± 0.4	0.99
Creatinine, umol/L	76.0 ± 13.6	83.6 ± 12.3	67.6 ± 9.3	<0.001
Hs-CRP, mg/L ¹	1.0 (0.5–2.2)	0.9 (0.4–1.9)	1.1 (0.5–2.9)	0.05
Uric acid, mmol/L ¹	0.31 ± 0.1	0.34 ± 0.1	0.28 ± 0.1	<0.001
Dietary intake (FFQ)				
Energy intake, Kcal/day	1971 ± 624	2206 ± 665	1711 ± 449	<0.001
Total carbohydrates, g/day	228.8 ± 76.7	254.4 ± 80.7	200.4 ± 60.6	<0.001
Total carbohydrates, En%	46.4 ± 6.0	46.2 ± 5.7	46.7 ± 6.2	0.08
Total protein, g/day	74.8 ± 20.9	81.5 ± 22.6	67.3 ± 15.8	<0.001
Total protein, En%	15.5 ± 2.4	15.0 ± 2.1	16.0 ± 2.6	0.05
Total fat, g/day	77.2 ± 30.0	87.1 ± 32.7	66.2 ± 22.0	<0.001
Total fat, En%	34.9 ± 5.1	35.1 ± 5.0	34.6 ± 5.1	0.82
Total Dairy intake, g/day	322 (209–447)	315 (202–444)	324 (216–447)	0.99

Table 1: Continued

Baseline Characteristics	Total N = 769	Males N = 404	Females N = 365	P-value
Total Dairy fat intake, g/day	12.3 (8.4–17.4)	13.5 (9.0–18.3)	11.3 (8.0–15.6)	<0.001
Total Dairy fat intake (En%)	6.0 (4.3–7.8)	5.7 (3.4–7.4)	6.2 (4.5–8.1)	0.90
Total Fish intake, g/day	11.7 (5.6–17.5)	12.0 (6.8–17.4)	11.6 (5.2–17.5)	0.87
Total Fish fat intake, g/day	1.14 (0.53–1.75)	1.16 (0.58–1.74)	1.10 (0.48–1.77)	0.50
Total Fish fat intake (En%)	0.52 (0.24–0.88)	0.49 (0.23–0.77)	0.57 (0.26–1.01)	0.01
Fatty acid status in plasma TG				
Myristic acid, mol%	1.87 (1.40–2.48)	1.98 (1.49–2.68)	1.74 (1.35–2.35)	<0.001
Pentadecanoic acid, mol%	0.30 (0.25–0.35)	0.29 (0.24–0.35)	0.30 (0.25–0.35)	0.27
Heptadecanoic acid, mol%	0.49 (0.41–0.59)	0.48 (0.39–0.58)	0.52 (0.44–0.62)	<0.001
Trans-Palmitoleic acid, mol%	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.05
Vaccenic acid, mol%	0.10 (0.06–0.14)	0.10 (0.07–0.15)	0.10 (0.07–0.14)	0.30
Conjugated Linoleic acid, mol%	0.08 (0.05–0.10)	0.07 (0.05–0.10)	0.07 (0.05–0.10)	0.24
Fatty acid status in plasma PL				
Myristic acid, mol%	0.49 (0.41–0.57)	0.47 (0.40–0.55)	0.50 (0.43–0.58)	<0.001
Pentadecanoic acid, mol%	0.28 (0.24–0.33)	0.28 (0.24–0.33)	0.29 (0.25–0.33)	0.09
Heptadecanoic acid, mol%	0.40 (0.36–0.44)	0.39 (0.35–0.44)	0.40 (0.36–0.45)	0.08
Trans-Palmitoleic acid, mol%	0.02 (0.01–0.03)	0.02 (0.01–0.02)	0.02 (0.02–0.03)	0.002
Vaccenic acid, mol%	0.09 (0.07–0.12)	0.09 (0.06–0.11)	0.09 (0.07–0.12)	0.002
Conjugated Linoleic acid, mol%	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.01

Data are presented as mean $\pm$ SD, median (25–75th percentile) or number (%). Differences between gender were tested by independent t-test, Mann-Whitney U or Chi-square test. Abbreviations: SD, standard deviation;; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, Triglycerides; TG/HDL-c ratio, Total Triglycerides/HDL cholesterol ratio; HbA1C, Hemoglobin A1C; hs-CRP, High sensitivity C-reactive protein; FFQ, Food Frequency Questionnaire; Kcal, Kilocalories; PL, Phospholipids. ¹Hs-Crp was available in $n = 412$, uric acid was available in $n = 348$.

Biomarkers for Dairy Fat or Fish Fat

To investigate whether the fatty acids in plasma TG and PL were dairy fat biomarkers, fish fat biomarkers or both, linear regression analyses were carried out. Firstly, the association with dairy fat intake was explored. After adjustment for energy intake, fatty acids in both plasma TG and PL, except C17:0 in plasma TG, were significantly associated with dairy fat intake (**Figure 1**). In plasma TG, C15:0 and trans-C18:1 ($n=7$) had the strongest association with dairy fat intake (both $\text{std.}\beta = 0.25$, $P < 0.001$). In plasma PL, trans-C18:1 ($n=7$) and CLA had the strongest association with dairy fat intake (both $\text{std.}\beta = 0.24$, $P < 0.001$), followed by C14:0 and C15:0 ($\text{std.}\beta = 0.20$, $P < 0.001$; $\text{std.}\beta = 0.19$, $P < 0.001$). Additional adjustments for age, sex, education, smoking habits, BMI, total carbohydrate and total fat intake, total fish fat intake, and total serum triglycerides

and cholesterol did not alter the associations between circulating fatty acids and dairy fat intake (**Supplemental Table 1**). If dairy fat intake was expressed as En%, associations with fatty acid biomarkers did not materially differ from associations found for dairy fat intake with adjustment for energy intake by means of linear regression.

Figure 1: Association between circulating fatty acids from plasma triglycerides and phospholipids and dairy fat intake, adjusted for energy intake. *Equal or below significance level $P \leq 0.05$. Abbreviations: β , beta; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1 (*n*-7), Trans-Palmitoleic acid; T-C18:1 (*n*-7), Vaccenic acid; CLA, Conjugated Linoleic acid; TG, Triglycerides; PL, Phospholipids.

Secondly, the association with fish fat intake was explored. After adjustment for energy intake, only C14:0, C17:0 and CLA in plasma TG were significantly associated with fish fat intake (**Figure 2**). C14:0 and CLA were inversely associated (std. β = -0.10, P = 0.01; std. β = -0.09, P = 0.01, respectively), while C17:0 was positively associated with fish fat intake (std. β = 0.08, P = 0.03). The association with C14:0 and C17:0 disappeared after additional adjustments for age, sex, BMI, and total serum triglycerides and cholesterol (**Model 7, Supplemental Table 2**). After adjustment for energy intake, fatty acids measured in plasma PL were not associated with fish fat intake. As a sensitivity analysis, the association of EPA and DHA with fish fat intake was explored as well. After adjustment for energy intake, age, sex, education and smoking habits, EPA and DHA in both plasma TG and PL were significantly associated with fish fat intake (plasma TG EPA: std. β = 0.27 and DHA: std. β = 0.45; plasma PL EPA: std. β = 0.25 and DHA: std. β = 0.46; P < 0.001) (**Supplemental Table 3**). If fish fat intake was expressed as En%, associations with fatty acid biomarkers did not materially differ from associations found for fish fat intake with adjustment for energy intake by means of linear regression.

Figure 2:. Association between circulating fatty acids from plasma triglycerides and phospholipids and fish fat intake, adjusted for energy intake. *Equal or below significance level $P \leq 0.05$. Abbreviations: β , beta; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; T-C16:1 ($n-7$), Trans-Palmitoleic acid; T-C18:1 ($n-7$), Vaccenic acid; CLA, Conjugated Linoleic acid; TG, Triglycerides; PL, Phospholipids.

Circulating Fatty Acids in Plasma TG and Cardiovascular Risk Factors

The second aim of the study was to investigate whether the circulating fatty acids were associated with cardiovascular risk factors. Overall, C14:0 and C17:0 showed the strongest associations with cardiovascular risk factors (**Table 2**). C14:0 was positively associated with weight, waist circumference and BMI, while C15:0 and C17:0 showed an inverse association with weight, waist circumference and BMI. Additionally, C14:0 was positively associated with glucose and HbA1c% (std. β = 0.14, $P < 0.001$; std. β = 0.12, $P < 0.01$, respectively), while C17:0 was inversely associated with glucose (std. β = -0.13, $P < 0.001$). Furthermore, C15:0 and C17:0 were inversely associated with serum hs-CRP (std. β = -0.11, $P < 0.05$; std. β = -0.17, $P < 0.01$; respectively) and uric acid (std. β = -0.14, $P < 0.05$; std. β = -0.25, $P < 0.001$; respectively), and remained associated after adjustment for age and sex. Trans-C16:1 ($n-7$) and trans-C18:1 ($n-7$) became inversely associated with uric acid after adjustment for age and sex (std. β = -0.12, $P < 0.05$; std. β = -0.11; $P < 0.05$; respectively). C14:0 and C17:0 had a strong positive association with total triglycerides (std. β = 0.40, $P < 0.001$; std. β = -0.56, $P < 0.001$; respectively), and these associations remained after adjustment for age and sex. The age and sex adjusted analyses were therefore also adjusted for total triglycerides (**Supplemental Table 4**). The association with weight, waist circumference and BMI remained for C15:0, but disappeared for C14:0 and C17:0. Additionally, the association with hs-CRP remained for C15:0 and C17:0, and the inverse association with uric acid remained for C15:0 and trans-C18:1 ($n-7$), but disappeared for C17:0, and became visible for C14:0.

Table 2: Association between circulating fatty acid markers from plasma triglycerides (TG) and cardiovascular risk factors.

Dependent Variable	C14:0		C15:0		C17:0		Trans-C16:1 (n-7)		Trans-C18:1 (n-7)		CLA	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Age (years)	0.063		0.071*		-0.027		0.022		0.088*		0.047	
Sex	-0.129***		0.023		0.152***		0.066		-0.058		0.037	
Weight (kg)	0.132***	0.070*	-0.091*	-0.083*	-0.187***	-0.117***	-0.073	-0.043	0.030	0.000	-0.024	-0.008
Waist circumference	0.146***	0.084*	-0.096*	-0.112***	-0.201***	-0.144***	-0.076	-0.061	0.048	0.000	-0.020	-0.022
BMI (kg/m ²)	0.091*	0.071*	-0.117**	-0.132***	-0.181***	-0.170***	-0.073	-0.075	-0.008	-0.031	-0.022	-0.030
SBP (mmHg)	0.075*	0.024	-0.042	-0.069*	-0.103*	-0.064*	-0.049	-0.047	0.019	-0.031	-0.043	-0.057
DBP (mmHg)	0.128***	0.068*	-0.053	-0.070*	-0.147***	-0.093*	-0.028	-0.015	0.054	0.005	0.014	0.010
Total C (mmol/L)	0.152***	0.147***	0.000	-0.024	-0.296***	-0.308***	0.051	0.039	0.128***	0.108**	0.079*	0.062
HDL-c (mmol/L)	-0.262***	-0.213***	-0.035	-0.052	0.220***	0.157***	0.051	0.019	-0.060	-0.041	-0.057	-0.078*
LDL-c (mmol/L)	0.172***	0.154***	0.017	-0.001	-0.277***	-0.270***	0.029	0.025	0.119**	0.095*	0.070*	0.059
Total TG	0.401***	0.370***	-0.012	-0.018	-0.563***	-0.537***	-0.077	-0.066	0.127***	0.103**	0.124**	0.126***
TG/HDL-c ratio	0.396***	0.354***	0.003	0.005	-0.503***	-0.459***	-0.076	-0.055	0.120**	0.095**	0.114**	0.123***
Glucose (mmol/L)	0.136***	0.104**	-0.031	-0.054	-0.128**	-0.105**	-0.031	-0.033	0.063	0.026	0.019	0.006
HbA1c (%)	0.119**	0.088*	-0.002	-0.041	-0.040	-0.027	-0.018	-0.031	0.041	-0.006	-0.014	-0.040
Hs-CRP (mg/L) ¹	0.021	0.039	-0.109*	-0.118*	-0.168**	-0.203***	-0.057	-0.076	-0.063	-0.057	-0.019	-0.027
Creatinine (umol/L)	0.100*	0.016	-0.029	-0.025	-0.147***	-0.055	-0.016	0.020	0.052	0.005	-0.011	0.005
Uric acid (mmol/L) ¹	0.131*	0.082	-0.139*	-0.160***	-0.249***	-0.233***	-0.096	-0.123*	-0.087	-0.108*	-0.042	-0.038
Ureum (mmol/L) ¹	0.031	0.007	0.146**	0.122*	0.090	0.086	0.113	0.096	0.150**	0.137**	0.105*	0.080

Model 1: Crude model; **Model 2:** Model 1 + additional adjustments for age and sex. Dependent: cardiovascular risk factors, independent: circulating fatty acids.

Associations between fatty acid status and cardiovascular risk factor are reported as standardized β 's. P -value: * = ≤ 0.05 , ** = ≤ 0.01 , *** = ≤ 0.001 . The significant values are highlighted in bold. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C; total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitivity C-reactive protein. ¹ Hs-CRP data was available in $n = 412$, Uric acid and ureum data were available in $n = 348$.

As an extra analyses, we adjusted the age and sex adjusted analyses for fatty acids from the *de novo* lipogenesis DNL instead of for total triglycerides. Interestingly, trans-C16:1 (*n*-7) and trans-C18:1 (*n*-7) became inversely associated with BMI (std.β = -0.11 and std.β = 0.10, both *P* < 0.05; respectively) and waist circumference (std.β = -0.101 and std.β = -0.07, both *P* < 0.05; respectively).

Circulating Fatty Acids in Plasma PL and Cardiovascular Risk Factors

Besides fatty acids in plasma TG, the association between fatty acids from plasma PL and cardiovascular risk factors were also investigated. Overall, most associations were found with C17:0 and trans-C18:1 (*n*-7) (**Table 3**). C15:0, C17:0, trans-C16:1 (*n*-7) and trans-C18:1 (*n*-7) were inversely associated with weight and waist circumference. Additionally, C17:0 and trans-C18:1 (*n*-7) were inversely associated with BMI and glucose (std.β = -0.10, *P* < 0.05; std.β = -0.08, *P* < 0.05, respectively). Furthermore, C17:0 and trans-C18:1 (*n*-7) were inversely associated with hs-CRP (std.β = -0.22, *P* < 0.001; std.β = -0.16, *P* < 0.01, respectively), and all fatty acids, except for CLA, were inversely associated with uric acid, for which the strongest associations were found for C17:0 (std.β = 0.27, *P* < 0.001) and trans-C18:1 (*n*-7) (std.β = -0.24, *P* < 0.001). Associations remained after adjustments for age and sex.

C15:0, C17:0, trans-C16:1 (*n*-7) and trans-C18:1 (*n*-7) were inversely associated with total triglycerides (std.β = -0.16, *P* < 0.001; std.β = -0.24, *P* < 0.001; std.β = -0.15, *P* < 0.001; std.β = -0.10, *P* < 0.01, respectively), and these associations remained after adjustment for age and sex. To stay in line with the analyses carried out for plasma TG, the age and sex adjusted analyses were also adjusted for total triglycerides. Additionally, we adjusted the analyses for total cholesterol, since it is known that the fatty acids from PL are closely related to the fatty acids of plasma CE [31] (**Supplemental Table 5**). The inverse association of C17:0 and Trans-C18:1 (*n*-7) with waist circumference and BMI remained. The association between CLA and BMI became inversely associated (std.β = -0.09, *P* < 0.05). C17:0 and trans-C18:1 (*n*-7) remained inversely associated with hs-CRP, while trans-C16:1 (*n*-7) became inversely associated with hs-CRP. Only C14:0, C17:0 and trans-C18:1 (*n*-7) remained inversely associated with uric acid. As an extra analyses, we adjusted the age and sex adjusted analyses for fatty acids from the DNL instead of for total triglycerides, but the associations did not change.

Table 3: Association between circulating fatty acid markers from plasma Phospholipids (PL) and cardiovascular risk factors.

Dependent Variable	C14:0		C15:0		C17:0		Trans-C16:1 (n-7)		Trans-C18:1 (n-7)		CLA	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
Age (years)	0.071*		0.070*		0.078*		0.008		0.071*		0.061	
Sex	0.141***		0.066		0.063		0.110**		0.102*		0.103**	
Weight (kg)	-0.071*	-0.008	-0.084*	-0.056	-0.173***	-0.148***	-0.071*	-0.020	-0.114**	-0.069*	-0.046	0.000
Waist circumference	-0.037	-0.012	-0.092*	-0.094**	-0.176***	-0.182***	-0.102**	-0.068*	-0.102*	-0.092**	-0.040	-0.025
BMI (kg/m ²)	-0.022	-0.030	-0.057	-0.069*	-0.170***	-0.185***	-0.069	-0.065	-0.107**	-0.119**	-0.037	-0.044
SBP (mmHg)	-0.029	-0.036	-0.024	-0.044	-0.045	-0.069*	-0.078*	-0.062*	-0.081*	-0.095**	0.055	-0.064*
DBP (mmHg)	-0.029	-0.010	-0.063	-0.068*	-0.121**	-0.130***	-0.075*	-0.044	-0.072*	-0.066*	-0.003	0.008
Total C (mmol/L)	0.153***	0.123***	-0.058	-0.085*	-0.212***	-0.243***	-0.034	-0.047	0.047	0.017	0.173***	0.148***
HDL-c (mmol/L)	0.068	-0.001	0.072*	0.036	-0.015	-0.050	0.053	0.003	0.093*	0.042	0.084*	0.033
LDL-c (mmol/L)	0.137***	0.127***	-0.054	-0.071*	-0.157***	-0.178***	-0.022	-0.021	0.033	0.018	0.121**	0.110**
Total TG	0.018	0.040	-0.155***	-0.153***	-0.235***	-0.235***	-0.151***	-0.130***	-0.098*	-0.088*	0.091*	0.106**
TG/HDL-c ratio	-0.015	0.028	-0.142***	-0.127***	-0.169***	-0.135***	-0.131***	-0.095**	-0.108**	-0.080*	0.033	0.064
Glucose (mmol/L)	0.033	0.025	-0.048	-0.065	-0.100*	-0.121***	-0.035	-0.027	-0.077*	-0.095*	-0.036	-0.048
HbA1c (%)	0.014	-0.027	-0.001	-0.040	-0.008	-0.052	-0.023	-0.029	-0.057	-0.099**	0.006	-0.029
Hs-CRP (mg/L) ¹	-0.030	-0.055	-0.074	-0.087	-0.223***	-0.233***	-0.089	-0.117*	-0.160**	-0.179***	0.023	0.005
Creatinine (umol/L)	-0.112**	-0.040	-0.029	0.000	-0.107**	-0.082*	-0.059	0.005	-0.093*	-0.044	-0.062	-0.010
Uric acid (mmol/L) ¹	-0.140*	-0.096	-0.157**	-0.130*	-0.270***	-0.258***	-0.140*	-0.131*	-0.243***	-0.208***	-0.029	-0.065
Urem (mmol/L) ¹	0.021	0.007	0.188***	0.186	0.109*	0.089	0.097	0.096	0.105*	0.099	0.032	0.026

Model 1: Crude model; **Model 2:** Model 1 + additional adjustments for age and sex. Dependent: cardiovascular risk factors, independent: circulating fatty acids. Associations between fatty acid status and cardiovascular risk factor are reported as standardized β 's. *P*-value: * = ≤ 0.05 , ** = ≤ 0.01 , *** = ≤ 0.001 . The significant values are highlighted in bold. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C; total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitivity C-reactive protein. ¹ Hs-CRP data was available in $n = 412$, Uric acid and ureum data were available in $n = 348$.

Dairy Fat Intake, Fish Fat Intake and Cardiovascular Risk Factors

The association of dairy fat, fish fat and cardiovascular risk factors was investigated as an extra analysis. Dairy fat intake was significantly associated with age (std.β = 0.36, $P < 0.001$) and sex (std.β = 0.08, $P < 0.05$) (**Supplemental Table 6**). Furthermore, dairy fat intake was significantly associated with waist circumference, systolic and diastolic blood pressure, total, HDL and LDL cholesterol, glucose and HbA1c%. However, these associations disappeared after adjustment for age and sex. Dairy fat intake was inversely associated with uric acid, even after adjustment for age, sex, education, smoking habits, BMI, fish fat intake, and total carbohydrate and total fat intake. In addition to dairy fat intake, fish fat intake was also significantly associated with age (std.β = 0.08, $P \leq 0.05$), but not with sex (**Supplemental Table 7**). Furthermore, fish fat intake was positively associated with HDL cholesterol (std.β = 0.10, $P < 0.01$), and inversely associated with total triglycerides (std.β = -0.12, $P < 0.01$) and the TG/HDL-c ratio. The associations remained after adjustments for age, sex, education, smoking habits, BMI, total dairy fat intake, total carbohydrate and total fat intake.

DISCUSSION

The current paper showed that C14:0, C15:0, C17:0, trans-C16:1 ($n-7$), trans-C18:1 ($n-7$) and CLA were solid biomarkers to predict dairy fat intake in the general population, while in contrast, C15:0 and C17:0 were not confirmed as strong biomarkers for fish fat intake. The dairy fatty acids C15:0 and C17:0 in plasma TG and C15:0, C17:0 and trans-C18:1 ($n-7$) in plasma PL had an inverse association with cardiovascular risk factors such as weight, waist circumference and BMI. Additionally, dairy fat biomarkers were also inversely associated with diabetes-related outcomes, the inflammatory marker hs-CRP and the kidney marker uric acid.

For many years now the saturated fatty acids C15:0 and C17:0, and to a lesser extend C14:0 have been described as biomarkers to predict dairy and dairy fat intake [5,12,15,32–36]. Additionally, the trans fatty acids trans-C16:1 ($n-7$), trans-C18:1 ($n-7$) and CLA were added to the list of biomarkers [5,15,36–38]. In line with the previous studies, we also found that the current mentioned biomarkers are predictors of dairy fat intake in the general population, of which the strongest associations were found with TG C15:0, TG trans-C18:1 ($n-7$), PL trans-C18:1 ($n-7$) and PL CLA. Associations of dairy fat intake with trans-C18:1 ($n-7$) and CLA have hardly been explored. One study investigated the association between dairy fat intake and erythrocyte trans-C18:1 ($n-7$), but found no significant association [36]. Furthermore, associations between CLA and dairy fat intake are more commonly investigated in adipose tissue. A case-control study that investigated the association between CLA and the risk of myocardial infarction demonstrated that CLA was significantly associated with dairy intake in the Costa Rican population ($r = 0.31$) [37]. In contrast to trans-C18:1 ($n-7$) and CLA, the association between dairy fat intake and plasma C15:0 has been investigated and confirmed in several studies [12–15]. In a cross-sectional

study of Lund-Blix et al., it was for example found that plasma PL C15:0 and total dairy fat were significantly associated with an r of 0.39 [14].

Besides dairy fat, the saturated fatty acids C15:0 and C17:0 have also been described as potential fish fat biomarkers. The fatty acids can be found in marine water fish and have recently found to be related to DHA [18,19]. C15:0 and C17:0 were therefore presumed to be invalid dairy fat biomarkers in a population with a high fish consumption. Participants from the current study showed a low intake of fish (11.7 g/day) and a high intake of dairy (322 g/day). Subsequently, C15:0 and C17:0, and the other dairy fat biomarkers were not confirmed as strong biomarkers for fish fat intake. C15:0, C17:0 and the other circulating fatty acids are therefore valid dairy fat biomarkers for the general population in the northern part of the Netherlands.

In the current study, significant associations were found between dairy fat biomarkers and cardiovascular risk factors. The association between the circulating dairy fat biomarkers C14:0, C15:0, C17:0 and trans-C16:1 ($n=7$) and cardiovascular health has been investigated before and an illustrative overview of papers investigating this association can be found in **Supplemental Table 8**. One of the main interesting outcomes of the current paper is the inverse association of the saturated fatty acids C15:0 and C17:0 with weight, waist circumference and BMI. A Swedish prospective study regarding risk factors for ischemic heart disease in 62 70-year old men found data that was in line with the current findings. Researchers showed an inverse association between plasma CE C15:0 and weight ($r^2 = -0.36$), waist circumference ($r^2 = -0.28$) and BMI ($r^2 = -0.39$) [32]. A more recent Swedish study on the effect of dairy fat biomarkers and the risk to develop myocardial infarction in an adult population demonstrated an inverse association of plasma PL C15:0, PL C17:0 and PL C15:0 + C17:0 with BMI ($r = -0.08$, $r = -0.14$ and $r = -0.14$ respectively) [6]. Additionally, serum PL C15:0 and C17:0 were inversely associated with abdominal obesity ($r = -0.22$ and $r = -0.30$, respectively) as was found in a cross-sectional study with 301 healthy 63-year old men [39]. The inverse association between the saturated fatty acids and adiposity markers are in line with a recent published paper on dairy and overweight [40]. Moreover, the authors used data from the Lifelines Cohort also. However, while we investigated the association between dairy fat biomarkers and adiposity, they investigated the association between dairy fat products and adiposity. The investigators found an inverse association between full-fat dairy product intake and BMI-defined overweight ($\geq 25-30$ kg/m²) and obesity (≥ 30 kg/m²). Additionally, age and sex adjusted multivariate linear regression analyses identified inverse associations of full-fat dairy product intake (per 100g) with waist circumference ($\beta = -0.39$) and BMI ($\beta = -0.23$). In line with this paper, a systematic review of observational studies on the relationship between dairy intake and obesity, cardiovascular, and metabolic disease found an inverse association between high fat dairy consumption and measurements of adiposity in 11 out of 16 studies [41].

The ruminant derived trans fatty acids were also found to be related to weight, waist circumference and BMI in the Lifelines Cohort. The plasma PL trans fatty acids trans-C16:1 (*n*-7) and trans-C18:1 (*n*-7) were inversely related to weight and waist circumference. Additionally, trans-C18:1 (*n*-7) was also inversely associated with BMI. In secondary analyses, plasma TG trans fatty acids trans-C16:1 (*n*-7) and trans-C18:1 (*n*-7) became inversely associated with waist circumference and BMI after adjustment for the fatty acids from DNL. The cardiovascular health study, a prospective cohort study from the U.S. including 3736 adult participants investigated the association between trans-C16:1 (*n*-7) and metabolic risk and diabetes type 2 [42]. The researchers found a significant reduction of 1.8% (across quintiles) in BMI and waist circumference. Furthermore, a study in which the intake of ruminant derived trans fat was measured by FFQs also suggested a favorable effect on cardiovascular health. Results from this cohort showed an inverse association between intake of ruminant derived trans fat and changes in weight [43].

Concerning the effect on diabetes-related outcomes, plasma TG C14:0 was positively associated with glucose and HbA1c%, however this association did not remain after adjustment for total serum triglycerides. Plasma TG C17:0, PL C17:0 and PL trans-C18:1 (*n*-7) were inversely associated with glucose. Additionally, trans-C18:1 (*n*-7) was inversely associated with HbA1c%. All other dairy fat biomarkers were not associated with the diabetes-related outcomes. In a cross-sectional study with 17 men and women without nonalcoholic fatty liver disease, it was found that serum PL C15:0, C17:0 and trans-C16:1 (*n*-7), and free fatty acid C15:0 and C17:0 were inversely related to fasting plasma glucose, the area under the curve for glucose during an oral glucose tolerance test (OGTT) and liver fat [44]. Additionally, a prospective, case-control study with Swedish adults demonstrated that serum PL C17:0 and C15:0 + C17:0, but not C15:0, were inversely associated with fasting glucose ($r = -0.13$ and $r = -0.16$, respectively) [6]. Moreover, a cross-sectional study with 795 elderly men showed an inverse association between adipose tissue C17:0 and insulin sensitivity, but not between adipose tissue C15:0 and insulin sensitivity [45]. While data from the Lifelines Cohort demonstrated an inverse association between dairy fat biomarkers and diabetes-related outcomes, an opposite association of high-fat dairy product intake with pre-diabetes and newly diagnosed type 2 diabetes was actually found in the same cohort [46]. The authors suggested that the positive association between dairy fat intake and diabetes-related outcomes may potentially not depend on the 'fat content' of a dairy product, but to the individual dairy product intake.

Little is known about the mechanism behind the inverse association between dairy fat biomarkers and cardiovascular risk factors such as weight, waist circumference and BMI. One potential mechanism by which dairy fat may exert beneficial effects on cardiovascular risk factors, may be via reducing chronic inflammation and lipid peroxidation [41,47]. This mechanism was suggested by a cross-sectional study among 305 adolescents, in which it was demonstrated

that serum C15:0 and C17:0 were inversely associated with inflammation markers and oxidative stress in the overweight group [47]. The inverse association between dairy fat biomarkers and inflammation was confirmed in the current study, and in earlier published papers [47,48]. While it is commonly known that inflammatory markers and oxidative stress are positively related with cardiovascular risk factors [47,49–51], dairy fat biomarkers may actually reduce inflammatory markers, and subsequently cardiovascular risk factors.

In addition to the potential mechanism ascribed above, uric acid may also be involved in this process. Uric acid was found to be inversely related with dairy fat biomarkers in plasma TG and PL. The strongest associations were found with plasma PL C17:0 and PL trans-C18:1(*n-7*). Inverse associations between these dairy fat biomarkers and uric acid remained after adjustment for age, sex, education, smoking habits, total serum triglycerides and serum cholesterol. In addition to the dairy fat biomarkers, there was also an inverse association observed between dairy fat intake measured by FFQ, and uric acid and has been observed in the literature before [52]. While an increased uric acid level may increase the risk for the development of cardiovascular diseases [53,54], perhaps by activating a complex mechanism involving inflammatory and oxidative related mechanisms [55,56], it can be hypothesized that the opposite may actually reduce the risk for the development of cardiovascular risk. Overall, more research warrants an investigation of the potential mechanism between dairy fat biomarkers, uric acid levels, inflammation and cardiovascular health.

Interestingly, the associations between circulating fatty acids and cardiovascular risk factors were less strong in plasma TG compared to plasma PL. This might probably be due to the fact that the association between plasma TG trans fatty acids and cardiovascular risk factors can be influenced by recent dietary intakes and de novo lipogenesis, whereas this is less for plasma PL [57]. In our cohort, the association between fatty acids from de novo lipogenesis (i.e., C14:0, C16:0, C16:1 (*n-7*)) measured in plasma TG) were significantly associated with total triglycerides. Additionally, adjusting for the fatty acids from de novo lipogenesis changed associations between circulating dairy fat biomarkers and cardiovascular risk factors. Our results therefore also suggest that it is preferable to measure fatty acids in phospholipids to investigate associations with cardiovascular risk factors.

A main strength of this paper is the inclusion of the relatively large amount of participants. Furthermore, the association between circulating fatty acids and cardiovascular risk factors were investigated. Lifelines is building a biobank and database and for this purpose, Lifelines has collected and is still collecting information via questionnaires, physical examinations and biological samples, including information on several cardiovascular risk factors. This huge collection of data and samples allowed us to investigate a broad range of cardiovascular health aspects in relation to dairy fat biomarkers, dairy fat intake and fish fat intake. A third strong point is that we measured the fatty acids in two different compartments, i.e., plasma TG and

PL, providing the opportunity to investigate whether the association of dairy fat biomarkers with cardiovascular risk factors were consistent in plasma lipid classes with widely differing functions and half-lives.

Some limitations of this study should be addressed. First, given the observational nature of this study, it is impossible to draw a definite conclusion about the causality of the association of the circulating fatty acids with dairy fat, fish fat and cardiovascular risk factors. Secondly, the Lifelines cohort study uses an FFQ to measure dietary intake of the participants which is a method based on self-report, and therefore subject for recall bias. It is possible that the intakes of dairy and fish fat are over- or underreported. However, since there is no gold standard for measuring dietary intakes so far, FFQs are still seen as one of the best methods since they are able to capture usual, individual, long-term dietary intakes. Additionally, the burden for the participant is low [58,59]. Thirdly, we used Hoving's method for the fatty acid analyses. The coefficients of variation for measuring circulating fatty acids in plasma was relatively high. Despite this limitation, we still found several associations between circulating fatty acids and cardiovascular risk factors. Potentially, using a method that allows for measurement of circulating fatty acids with lower coefficients of variation, such as Glaser's method, might have given stronger associations between circulating fatty acids and cardiovascular risk factors [60].

CONCLUSIONS

This paper confirmed that the current known dairy fatty acid biomarkers are solid biomarkers to predict dairy fat intake in the general population of the Netherlands. Additionally, the fat biomarkers were inversely associated with adiposity, diabetes and inflammatory outcomes, suggesting that dairy fat intake may have a beneficial effect on cardiovascular health. Longitudinal studies are needed to further investigate the influence of dairy fat intake on cardiovascular risk and overall health.

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The manuscript is based on data from the LifeLines Cohort Study. LifeLines adheres to standards for open data availability. The data catalogue of LifeLines is publicly accessible on

www.LifeLines.net. All international researchers can apply for data at the LifeLines research office (LLscience@umcg.nl). The LifeLines system allows access for reproducibility of the study results.

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Supplemental Table 1: Cross-sectional association between circulating fatty acids and dairy fat intake

Total dairy fat (g/d)	Fatty acids									
	C14:0		C15:0		C17:0		Trans-C16:1(n-7)		Trans-C18:1(n-7)	
	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value
<i>Plasma TG</i>										
Model 1	0.222	<0.001	0.257	<0.001	0.030	0.43	0.188	<0.001	0.261	<0.001
Model 2	0.175	<0.001	0.250	<0.001	0.048	0.15	0.205	<0.001	0.248	<0.001
Model 3	0.162	<0.001	0.231	<0.001	0.043	0.18	0.189	<0.001	0.229	<0.001
Model 4	0.167	<0.001	0.230	<0.001	0.040	0.21	0.194	<0.001	0.230	<0.001
Model 5	0.183	<0.001	0.240	<0.001	0.040	0.20	0.191	<0.001	0.218	<0.001
Model 6	0.168	<0.001	0.230	<0.001	0.041	0.20	0.195	<0.001	0.229	<0.001
Model 7	0.204	<0.001	0.231	<0.001	0.042	0.27	0.193	<0.001	0.236	<0.001
<i>Plasma PL</i>										
Model 1	0.198	<0.001	0.192	<0.001	0.127	<0.001	0.085	0.02	0.232	<0.001
Model 2	0.202	<0.001	0.189	<0.001	0.119	<0.001	0.118	<0.001	0.239	<0.001
Model 3	0.170	<0.001	0.162	<0.001	0.092	0.003	0.105	0.001	0.219	<0.001
Model 4	0.174	<0.001	0.164	<0.001	0.090	0.004	0.110	<0.001	0.214	<0.001
Model 5	0.182	<0.001	0.165	<0.001	0.127	<0.001	0.101	0.001	0.197	<0.001
Model 6	0.174	<0.001	0.164	<0.001	0.090	0.003	0.111	<0.001	0.214	<0.001
Model 7	0.177	<0.001	0.166	<0.001	0.094	0.003	0.109	<0.001	0.214	<0.001

Model 1: Crude model; **Model 2:** Adjusted for energy intake; **Model 3:** Adjusted for Model 2 + age, sex, education and smoking; **Model 4:** Adjusted for model 2 + age, sex and BMI; **Model 5:** Adjusted for model 4 + total carbohydrates and fat; **Model 6:** Adjusted for Model 4 + fish fat intake; **Model 7:** Adjusted for model 4 + Total Triglycerides + Total Cholesterol

Dependent: Dairy fat intake, independent: Circulating fatty acids. Associations between fatty acid status and dairy fat intake are reported as standardized β 's.

Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), Trans-Palmitoleic acid; Trans-C18:1(n-7), Vaccenic acid; CLA, Conjugated Linoleic acid; TG, Triglycerides; PL, Phospholipids; BMI, Body Mass Index

Supplemental Table 2: Cross-sectional association between circulating fatty acids and fish fat intake

Total fish Fat (g/d)	Fatty acids																					
	C14:0				C15:0				C17:0				Trans-C16:1(n-7)				Trans-C18:1(n-7)				CLA	
	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value	β	p-value		
Plasma TG																						
Model 1	-0.094	0.01	-0.045	0.22	0.080	0.04	0.022	0.60	-0.018	0.61	-0.093	0.01										
Model 2	-0.101	0.01	-0.045	0.21	0.082	0.03	0.024	0.57	-0.020	0.58	-0.091	0.01										
Model 3	-0.118	0.001	-0.069	0.06	0.093	0.02	0.001	0.98	-0.045	0.23	-0.106	0.004										
Model 4	-0.104	0.01	-0.062	0.09	0.075	0.05	0.017	0.69	-0.030	0.42	-0.097	0.01										
Model 5	-0.095	0.01	-0.045	0.21	0.087	0.02	0.019	0.63	-0.032	0.38	-0.101	0.004										
Model 6	-0.104	0.01	-0.062	0.10	0.076	0.05	0.022	0.61	-0.027	0.48	-0.098	0.01										
Model 7	-0.068	0.09	-0.058	0.11	0.023	0.61	0.008	0.85	-0.019	0.61	-0.082	0.02										
Plasma PL																						
Model 1	-0.064	0.07	-0.008	0.83	0.067	0.07	0.034	0.35	-0.065	0.89	-0.070	0.05										
Model 2	-0.064	0.08	-0.008	0.83	0.066	0.07	0.039	0.29	-0.004	0.91	-0.068	0.06										
Model 3	-0.087	0.02	-0.016	0.65	0.063	0.09	0.026	0.49	-0.024	0.53	-0.089	0.02										
Model 4	-0.076	0.04	-0.020	0.59	0.049	0.19	0.034	0.36	-0.020	0.60	-0.078	0.03										
Model 5	-0.068	0.06	-0.007	0.84	0.098	0.01	0.041	0.26	-0.014	0.72	-0.097	0.01										
Model 6	-0.076	0.04	-0.017	0.65	0.051	0.17	0.037	0.33	-0.016	0.67	-0.079	0.04										
Model 7	-0.077	0.04	-0.036	0.33	0.037	0.34	0.020	0.59	-0.029	0.44	-0.070	0.06										

Model 1: Crude model; **Model 2:** Adjusted for energy intake; **Model 3:** Adjusted for Model 2 + age, sex, education and smoking; **Model 4:** Adjusted for Model 2 + age, sex and BMI; **Model 5:** Adjusted for model 4 + total carbohydrates and Fat; **Model 6:** Adjusted for Model 4 + dairy fat intake; **Model 7:** Adjusted for model 4 + Total Triglycerides + Total Cholesterol

Dependent: fish fat intake, independent: fatty acids. Associations between fatty acid status and fish fat intake are reported as standardized β 's. Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; Trans-C16:1(n-7), Trans-Palmitoleic acid; Trans-C18:1(n-7), Vaccenic acid; CLA, Conjugated Linoleic acid; TG, Triglycerides; PL, Phospholipids; BMI, Body Mass Index

Supplemental Table 3: Association between the circulating fatty acids EPA and DHA and fish fat intake

Total fish Fat (g/d)	Omega-3 fatty acids			
	EPA		DHA	
	β	p-value	β	p-value
Plasma TG				
Model 1	0.277	<0.001	0.425	<0.001
Model 2	0.283	<0.001	0.440	<0.001
Model 3	0.272	<0.001	0.445	<0.001
Plasma PL				
Model 1	0.256	<0.001	0.457	<0.001
Model 2	0.258	<0.001	0.465	<0.001
Model 3	0.248	<0.001	0.464	<0.001

Model 1: Crude model; **Model 2:** Adjusted for model 1 + energy intake; **Model 3:** Adjusted for Model 2 + age and sex, education and smoking

Dependent: fish fat intake, independent: fatty acids. Associations between fatty acid status and fish fat intake are reported as standardized β 's.

Abbreviations: DHA, docosahexaenoic acid; EPA: eicosapentaenoic acid; PL, Phospholipids; TG, Triglycerides

Supplemental Table 4: Association between fatty acid markers from plasma triglycerides (TG) and cardiovascular risk factors

Dependent variable	C14:0		C15:0		C17:0		Trans-C16:1(n-7)		Trans-C18:1(n-7)		CLA	
	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2
Weight (kg)	0.074*	-0.025	-0.079*	-0.079	-0.116**	0.022	-0.032	-0.027	0.011	-0.026	0.000	-0.040
Waist circumference	0.094**	-0.041	-0.096**	-0.107***	-0.144***	0.039	-0.035	-0.040	0.023	-0.033	-0.007	-0.064*
BMI (kg/m ²)	0.081*	-0.066	-0.121**	-0.126***	-0.173***	0.022	0.078	-0.052	-0.010	-0.067*	-0.015	-0.075*
SBP (mmHg)	0.032	-0.047	-0.059	-0.066*	-0.074*	0.039	-0.024	-0.036	-0.013	-0.050	-0.044	-0.080
DBP (mmHg)	0.073*	0.001	-0.064*	-0.067*	-0.088*	0.005	-0.008	-0.003	0.014	-0.013	0.015	-0.013
Total C (mmol/L)	0.145***	0.009	-0.025	-0.017	-0.311***	-0.147***	0.028	0.063	0.104**	0.070*	0.057	0.015
HDL-c (mmol/L)	-0.222***	-0.057	-0.072*	-0.060*	0.156***	-0.113**	-0.007	-0.010	-0.063	0.005	-0.093**	-0.023
LDL-c (mmol/L)	0.150***	0.039	-0.001	0.005	-0.273***	-0.133**	0.016	0.047	0.093*	0.062	0.056	0.019
Total TG	0.384***	-	0.004	-	-0.543***	-	-0.042	-	0.126***	-	0.141***	-
TG/HDL-c ratio	0.368***	0.016	0.030	0.022*	-0.462***	0.049**	-0.027	0.006	0.121***	0.000	0.140***	0.007
Glucose (mmol/L)	0.106**	0.014	-0.044	-0.049	-0.100**	0.038	-0.027	-0.016	0.035	0.000	0.017	-0.025
HbA1c (%)	0.093**	0.034	-0.020	-0.039	-0.023	0.080	-0.013	-0.021	0.011	-0.022	-0.023	-0.060
Hs-CRP (mg/L) ¹	0.053	-0.038	-0.103*	-0.110*	-0.188***	-0.119*	-0.060	-0.070	-0.047	-0.085	-0.018	-0.065
Creatinine (μmol/L)	0.015	-0.038	-0.039	-0.023	-0.067*	0.022	0.018	0.028	0.001	-0.008	-0.001	-0.012
Uric acid (mmol/L) ¹	0.084	-0.134*	-0.150**	-0.163***	-0.230***	0.052	-0.090	-0.085	-0.091	-0.140**	-0.027	-0.079
Urem (mmol/L) ¹	0.016	0.001	0.130**	0.121*	0.087	0.138*	0.109	0.097	0.143**	0.137*	0.082	0.079

Model S1: Adjusted for age, sex, education and smoking; **Model S2:** Adjusted for age, sex and triglycerides.

Dependent: cardiovascular risk factors, independent: fatty acid status. Associations between fatty acid status and cardiovascular risk factor are reported as standardized

β's. P-value: * = ≤ 0.05, ** = < 0.01, *** = < 0.001. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C; total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-CRP data was available in n = 412, Uric acid and ureum data were available in n = 348

Supplemental Table 5: Association between fatty acid markers from plasma phospholipids (PL) and cardiovascular risk factors.

Dependent variable	C14:0		C15:0		C17:0		Trans-C16:1(n-7)		Trans-C18:1(n-7)		CLA	
	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2	Model S1	Model S2
Weight (kg)	-0.002	-0.015	-0.056	-0.019	-0.150***	-0.102**	-0.011	0.012	-0.056	-0.047	0.012	-0.024
Waist circumference	0.001	-0.023	-0.090**	-0.047	-0.176***	-0.120***	-0.052	-0.027	-0.070*	-0.063*	-0.008	-0.058
BMI (kg/m ²)	-0.018	-0.040	-0.087	-0.017	-0.186***	-0.120**	-0.049	-0.021	-0.099*	-0.087*	-0.025	-0.079*
SBP (mmHg)	-0.026	-0.046	-0.043	-0.017	-0.074*	-0.026	-0.049	-0.041	-0.087*	-0.083*	-0.048	-0.088*
DBP (mmHg)	-0.005	-0.030	-0.067*	-0.038	-0.128***	-0.078*	-0.039	-0.021	-0.063	-0.057	0.012	-0.023
Total C (mmol/L)	0.117**	-	-0.087*	-	-0.253***	-	-0.052	-	0.006	-	0.136***	-
HDL-c (mmol/L)	-0.012	-0.022	0.030	-0.022	-0.063	-0.109***	-0.015	-0.057*	0.022	-0.015	0.018	0.043
LDL-c (mmol/L)	0.120**	0.010	-0.073*	0.005	-0.184***	0.051***	-0.025	0.019	0.011	-0.001	0.100**	-0.028*
Total TG	0.053	-	-0.146***	-	-0.232***	-	-0.114**	-	-0.073*	-	0.120**	-
TG/HDL-c ratio	0.042	0.007	-0.119***	0.010	-0.147***	0.043***	-0.076*	0.025*	-0.061	0.008	0.080*	-0.018
Glucose (mmol/L)	0.029	0.026	-0.060	-0.031	-0.107**	-0.085*	-0.023	0.005	-0.084*	-0.069*	-0.044	-0.065*
HbA1c (%)	-0.019	-0.024	-0.030	-0.019	-0.029	-0.031	-0.018	-0.009	-0.084*	-0.081**	-0.023	-0.037
Hs-CRP (mg/L) ¹	-0.037	-0.056	-0.081	-0.051	-0.222***	-0.200***	-0.110*	-0.102*	-0.172***	-0.168*	0.015	-0.028
Creatinine (umol/L)	-0.041	-0.044	-0.005	0.020	-0.109***	-0.060*	0.002	0.022	-0.055	-0.032	-0.006	-0.022
Uric acid (mmol/L) ¹	-0.087	-0.106*	-0.119*	-0.076	-0.265***	-0.197***	-0.108*	-0.039	-0.197***	-0.128**	-0.047	-0.073
Ureum (mmol/L) ¹	0.012	0.020	0.188**	0.188***	0.091	0.088	0.096	0.104	0.093	0.105	0.028	0.037

Model S1: Adjusted for age, sex, education and smoking; **Model S2:** Adjusted for age, sex, triglycerides and cholesterol.

Dependent: cardiovascular risk factors; independent: fatty acid status. Associations between fatty acid status and cardiovascular risk factor are reported as standardized

β's. P-value: * = ≤ 0.05, ** = < 0.01, *** = < 0.001. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C; total cholesterol;

HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c,

Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-CRP data was available in n = 412, Uric acid and ureum data were available in n = 348

Supplemental Table 6: Association between dairy fat intake and cardiovascular risk factors

	Dairy fat intake (g/d)					
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Age (years)	0.355***					
Sex	0.083*					
Weight (kg)	-0.003	0.022	0.043	0.037	0.043	0.042
Waist circumference	0.094*	0.007	0.023	0.014	0.023	0.025
BMI (kg/m ²)	0.048	-0.022				
SBP (mmHg)	0.103*	-0.043	-0.048	-0.044	-0.048	-0.046
DBP (mmHg)	0.108**	0.021	0.023	0.012	0.024	0.024
Total C (mmol/L)	0.118**	-0.003	0.006	0.000	0.006	
HDL-c (mmol/L)	0.112**	0.051	0.051	-0.004	0.053	0.036
LDL-c (mmol/L)	0.086*	-0.015	-0.003	0.012	-0.003	-0.011
Total TG	0.009	-0.024	-0.026	-0.009	-0.028	
TG/HDL-c ratio	-0.037	-0.038	-0.039	-0.006	-0.041	-0.014
Glucose (mmol/L)	0.085*	-0.036	-0.024	-0.055	-0.024	-0.018
HbA1c (%)	0.215***	0.020	0.027	0.030	0.027	0.031
Hs-CRP (mg/L) ¹	0.001	0.005	-0.008	0.004	-0.007	-0.012
Creatinine (umol/L)	-0.030	-0.020	-0.026	-0.030	-0.027	-0.023
Uric acid (mmol/L) ¹	-0.172**	-0.122*	-0.107*	-0.106*	-0.101	-0.058
Ureum (mmol/L) ¹	0.188**	0.183**	0.186**	0.184**	0.186**	0.184**

Model 1: Adjusted for energy intake; **Model 2:** Model 1 + age, sex, education and smoking; **Model 3:** Model 1 + age, sex and BMI; **Model 4:** Model 3 + total carbohydrates and total fat; **Model 5:** Model 3 + total fish fat intake; **Model 6:** Model 3 + total triglycerides and total cholesterol.

Dependent: cardiovascular risk factors, independent: dairy fat intake. Associations between dairy fat intake and cardiovascular risk factor are reported as standardized β 's. P-value: * = ≤ 0.05 , ** = < 0.01 , *** = < 0.001 . Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-CRP data was available in n = 412, Uric acid and ureum data were available in n = 348

Supplemental Table 7: Association between fish fat intake and cardiovascular risk factors

	Fish fat intake (g/d)					
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Age (years)	0.077*					
Sex	0.004					
Weight (kg)	-0.041	-0.036	0.007	0.003	0.007	0.003
Waist circumference	-0.022	-0.035	0.000	-0.001	0.001	0.007
BMI (kg/m ²)	-0.045	-0.048				
SBP (mmHg)	0.032	0.014	0.013	0.013	0.012	0.022
DBP (mmHg)	0.041	0.020	0.029	0.012	0.029	0.038
Total C (mmol/L)	0.019	-0.015	0.001	-0.011	0.002	
HDL-c (mmol/L)	0.100**	0.080*	0.075*	0.042	0.076*	0.018
LDL-c (mmol/L)	0.018	0.043	0.007	0.009	0.007	-0.001
Total TG	-0.117**	-0.120**	-0.109**	-0.110**	-0.109**	
TG/HDL-c ratio	-0.126***	-0.121***	-0.110***	-0.098**	-0.111***	-0.006
Glucose (mmol/L)	0.000	-0.030	-0.006	-0.014	-0.006	0.016
HbA1c (%)	0.016	-0.026	-0.013	-0.004	-0.012	0.001
Hs-CRP (mg/L) ¹	-0.085	-0.079	-0.047	-0.047	-0.047	-0.033
Creatinine (umol/L)	-0.013	-0.012	-0.015	-0.021	-0.016	-0.002
Uric acid (mmol/L) ¹	0.097	0.102*	0.081	0.079	0.076	0.102**
Ureum (mmol/L) ¹	-0.030	-0.019	-0.012	-0.015	-0.001	-0.011

Model 1: Adjusted for energy intake; **Model 2:** Model 1 + age, sex, education and smoking; **Model 3:** Model 1 + age, sex and BMI; **Model 4:** Model 3 + total carbohydrates and total fat; **Model 5:** Model 3 + total dairy fat intake; **Model 6:** Model 3 + total triglycerides and total cholesterol.

Dependent: cardiovascular risk factors, independent: fish fat intake. Associations between dairy fat intake and cardiovascular risk factor are reported as standardized β 's. P-value: * = ≤ 0.05 , ** = < 0.01 , *** = < 0.001 . Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Total C; total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol; Total TG, total triglycerides; TG/HDL-c ratio, total triglycerides/HDL cholesterol; HbA1c, Hemoglobin A1C; Hs-CRP, High sensitive C-reactive protein. ¹ Hs-Crp data was available in n = 412, Uric acid and ureum data were available in n = 348

Supplemental Table 8A: Papers investigating the association between dairy fat biomarkers and cardiovascular risk factors (based on an illustrative search)

Fraction	Plasma/ Serum	Paper	Population	n	Outcome	+/- ³	Biomarkers ^{1,2}				
							C14:0	C15:0	C15:0+ C17:0	C17:0	Trans-C16:1(n-7)
CE+TG+PL+FFA	Plasma Serum	Santaren et al. (2014) ¹	Hispanic, African American and Non-Hispanic white adults 40-60 yr	659	-						
					Insulin resistance by intravenous glucose-tolerance test (insulin sensitivity index)	r +		0.14***			-0.12**
					β	+		0.84*			-0.42
					β-cell dysfunction by intravenous glucose-tolerance test (product of the insulin sensitivity index and acute insulin response)	r +		0.11**			-
CE	Plasma Serum	Smedman et al. (1999) ²	70-year old men	66	-						
					Weight	r +		- 0.36**			-
					BMI	r +		- 0.39***			
					WC	r +		- 0.28*			
					HC	r +		- 0.32**			
					HDL triacylglycerol	r +		- 0.23			
					Insulin sensitivity by clamp (insulin sensitivity index (glucose disposal/actual insulin concentration) *100)	r +		0.21			
TG	Plasma/ Serum	-	-	-	-	-	-	-	-	-	-

Supplemental Table 8A: Continued

Fraction	Plasma/ Serum	Paper	Population	n	Outcome	+/- ³	Biomarkers ^{1,2}				
							C14:0	C15:0	C15:0+ C17:0	C17:0	Trans-C16:1(n-7)
PL	Plasma	Mozaffarian et al. (2010) ³	Cardio vascular Health study, adults from 4 US communities	3736	BMI	Δ% ^{a,c}	+				-1.8%*
					WC	Δ%	+				-1.8%***
					LDL	Δ%	0				+2.4%
					HDL	Δ%	+				+1.9%*
					Total Triglycerides	Δ%	+				-19.0%***
					Total:HDL ratio	Δ%	+				-4.7%***
					CRP	Δ%	+				-13.8%*
					Fasting glucose	Δ%	0				-0.96%
					Fasting insulin	Δ%	+				-13.3%***
					Insulin resistance by HOMA-IR	Δ%	+				-16.7%**
		Mozaffarian et al. (2013) ⁴	US adults (45-84 yr) from the Multi-ethnic study of atherosclerosis	2617	SBP	Δ% ^{a,c}	+				-2.4%*
					DBP	Δ%	+				-2.9%***
					LDL	Δ%	-				+6.4%***
					HDL	Δ%	0				-1.19
					Total Triglycerides	Δ%	+				-19.1%***
					Total:HDL ratio	Δ%	0				+1.5%
		Nestel et al. (2014) ⁵	Overweight and obese subjects with metabolic syndrome	86	Fasting glucose	Δ%	0				-1.2%
					Fasting insulin	Δ%	+				-9.1%***
					insulin sensitivity index by OGTT (Matsuda index)	β	+			0.30*	0.02
					Insulin resistance (Fasting Insulin) by OGTT	β	+			-2.41*	-0.76
					Insulin resistance (HOMA-IR) by OGTT	β	+			-0.70*	-0.17

Supplemental Table 8A: Continued

Fraction	Plasma/ Serum	Paper	Population	n	Outcome	+/- ³	Biomarkers ^{1,2}				
							C14:0	C15:0	C15:0+ C17:0	C17:0	Trans-C16:1(n-7)
Serum		Oliveira et al. (2013) ⁶	US adults (45-84 yr) from the Multi-ethnic study of atherosclerosis	2837	SBP	Δ% ^{1,c}	+	+2.5%*	-4.0%***		-1.18%*
					DBP	Δ%	+	+1.4%*	-4.1%***		-2.1%***
					LDL	Δ%	0	+1.9%	-1.11%		+4.4*
					HDL	Δ%	0	-0.2%	+0.4%		-0.6%
					Total: HDL ratio	Δ%	0	+5.0%***	-3.4%		+1.0%
					Total Triglycerides	Δ%	+	+29.5%***	-15.0%***		-17.7%***
					CRP	Δ%	0	0%	0%		+2.8%
					Fasting glucose	β	+		-1.84*	-0.80*	-1.31*
					AUC glucose by OGTT	β	+		-34.27*	-23.94*	-36.02*
					Incremental AUC glucose by OGTT	β	+		-9.9	-13.26*	-18.68
					Liver fat (liver-spleen ratio)	β	+		2.39	2.13*	3.56*
					Hepatic insulin resistance (basal hepatic glucose * fasting plasma insulin)	β	+		-1.21	-2.68	-5.95*
					Systemic insulin resistance (LL)	β	0		-0.60	5.93	11.2*
					Systemic insulin resistance (HL)	β	0		-0.78	5.72	10.97*
		Rosell et al. (2004) ⁸	Healthy men (63-year old)	299	Pancreatic β-cell function (AIRg)	β	0	2.12		4.35	-1.67
					Pancreatic β-cell function (oral DI)	β	0	2.73		3.82	4.34
					Pancreatic β-cell function (IV DI)	β	0	1.68		6.24	1.24
					Abdominal obesity	r	+	0.09	-0.22***	-0.30***	
					Weight	r	0		-0.21		
					HC	r	+		-0.25*		
					Apo-B	r	0		-0.27		

Supplemental Table 8A: Continued

Fraction	Plasma/ Serum	Paper	Population	n	Outcome	+/- ³	Biomarkers ^{1,2}				
							C14:0	C15:0	C15:0+ C17:0	C17:0	Trans-C16:1(n-7)
FFA	Wahrensjö et al. (2010) ⁹	Swedish general population	1000		SBP	+		-0.05	-0.10***	-0.11***	
					DBP	+		-0.06	-0.13***	-0.16**	
					BMI	+		-0.08*	-0.14**	-0.14**	
					Total cholesterol	+		-0.10***	-0.04	0.008	
					Total Triglycerides	+		-0.18**	-0.23**	-0.22*	
					Fasting glucose	+		-0.05	-0.13**	-0.16*	
	Kratz et al. (2014) ⁷	Population without fatty liver disease	32		Fasting glucose	+		-1.09*		-0.54	-1.23
					AUC glucose by OGTT	+		-32.43*		-18.07*	-35.25
					Incremental AUC glucose by OGTT	+		-18.00*		-10.96*	-18.95
					Liver fat (liver-spleen ratio)	+		2.55*		1.80*	3.20
					Hepatic insulin resistance (basal hepatic glucose * fasting plasma insulin)	0		-2.21		-0.74	-4.79
					Systemic insulin resistance (LL)	0		7.24		1.15	7.95
Erythrocytes					Systemic insulin resistance (HL)	0		6.89		1.10	8.14
					Pancreatic β -cell function (AIRg)	0		-2.32		2.29	-3.49
					Pancreatic β -cell function (oral DI)	0		3.75		1.30	-1.82
					Pancreatic β -cell function (IV DI)	0		2.15		4.05	4.82

Supplemental Table 8A: Continued

Fraction	Plasma/ Serum	Paper	Population	n	Outcome	+/- ³	Biomarkers ^{1,2}				
							C14:0	C15:0	C15:0+ C17:0	C17:0	Trans-C16:1(n-7)
AT	-	Castro-Webb et al. (2012) ¹⁰	Costa-Rican adults	1744 (232 cases)	Type 2 diabetes	PR ^b 0			1.33 (0.88-2.11)		1.05 (0.66-1.66)
		Iggman et al. (2010) ¹¹	Elderly men	795	Insulin sensitivity by clamp (glucose infusion rate)	r	0.32***	0.07		0.21***	
							0.35**	0.04		3.5*	
		Rosell et al. (2004) ¹²	Healthy men (63-year old)	297	Abdominal obesity	r	-0.27***	-0.04		-0.21***	
							-0.10*	0.10		-1.4***	
							-0.30***	-0.20***		-0.31***	
		Smit et al. (2010) ¹³	Costa-Rican Adults	1785	BMI	β					0.37***
											0.95***
											-2.27***
											-1.38***
											-1.67***

Significance level: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

^a. Relationships of the dairy fatty acid with cardiovascular risk factors were evaluated using linear regression; ^b. Highest versus lowest quintile; ^c. Δ% is calculated by the authors of this manuscript, using data from the study.

¹. r = correlation coefficient, Δ% = difference between low and high fatty acid biomarker, β = beta-coefficient, PR = prevalence ratio

². Adjustments: Santaren (2014) = demographics, lifestyle, dietary variables; Mozaffarian (2010) = age, sex, diabetes, smoking, coronary heart disease, physical activity, dietary intake (BMI, waist circumference); Mozaffarian (2013) = age, sex, race, education, smoking, alcohol intake, physical activity, prevalent diabetes, waist circumference; Nestel (2014) = age, systolic blood pressure, waist:hip ratio; Oliveira (2013) = demographics, lifestyle, prevalent diabetes, medication; Kratz (2014) = age, sex, BMI; Castro-Webb (2012) = demographics, lifestyle, several fatty acids; Iggman (2010) = BMI, smoking, alcohol intake, physical activity, prevalence of diabetes, all trans-fatty acids, smoking, physical activity, income, diabetes, hypertension, energy intake and adipose tissue alpha-linolenic acid

³. + = An increase of the biomarker reduces the cardiovascular risk factor. - = An increase in the biomarker increases the cardiovascular risk factor. 0 = No significant effect

Abbreviations: A1Rg, acute insulin response to glucose; AT, Adipose tissue; AUC, area under the curve for glucose; BMI, Body mass index; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; CE, Cholesterol esters; CRP, C-reactive protein; DBP, diastolic blood pressure; FFA, Free fatty acids; HC, Hip circumference; HDL, High density lipoprotein; HL, High level insulin infusion; HOMA-IR, homeostatic model assessment-estimated insulin resistance; IV DI, intravenous disposition index; LDL, Low density lipoprotein; LL, low-level insulin infusion; OGTT, oral glucose tolerance test; oral DI, oral disposition index; PL, Phospholipids; SBP, systolic blood pressure; SS, Subscapular Skinfold; SUS, Suprailiac skinfold; TG, Triglycerides; Trans-C16:1(n-7), Trans-Palmitoleic acid; TS, Triceps skinfold; WC, waist circumference.

Supplemental Table 8B: Papers investigating the association of dairy fatty acid biomarkers with the development of cardiovascular diseases (based on an illustrative search)

Paper	Population	n	Outcome	Blood Sample	Lipid Fraction	+/- ³	Biomarkers ^{1,2} (Risk (95%CI))			
							C14:0	C15:0	C17:0	Trans-C16:1(n-7)
Iggman et al. (2016) ¹⁴	Uppsala Longitudinal Study of Adult Men (ULSAM)	535 (251 cases)	Coronary Heart Disease Cardiovascular diseases	Adipose Tissue		HR ^a	0.95 (0.79–1.15)	1.03 (0.86–1.23)	0.98 (0.82–1.16)	
Khaw et al. (2012) ¹⁵	Men and women from the EPIC cohort study	2424 cases, 4930 controls	Incidence of coronary heart disease	Plasma	PL	OR ^a	0.97 (0.87–1.08)	0.98 (0.89–1.09)	0.91 (0.82–1.01)	
Matthan et al. (2014) ¹⁶	Post-menopausal women	2448 (1224 cases, 1224 controls)	Coronary heart disease	Plasma	PL	OR ^a	1.05 (0.70–1.56)	1.02 (0.61–1.72)		
Oliveira et al. (2013) ⁶	US adults (45–84yr) from the Multi-ethnic study of atherosclerosis	2837 (189 cases)	Incidence of cardiovascular disease	Plasma	PL	HR ^b	1.06 (0.65–1.74)	0.55 (0.32–0.95)		0.99 (0.58–1.69)
						HR ^a	1.02 (0.87–1.20)	0.81* (0.68–0.98)		0.97 (0.82–1.13)
						HR ^b	1.13 (0.63–2.03)	0.41* (0.22–0.78)		0.82 (0.45–1.49)
						HR ^a	1.01 (0.84–1.21)	0.74* (0.60–0.92)		0.91 (0.75–1.10)
Sun et al. (2007) ¹⁷	Female nurses, 30–55 years old	166 cases, 327 controls	Ischemic heart disease	Plasma	Total	RR ^c		2.36* (1.16–4.78)	1.25 (0.68–2.30)	0.79 (0.44–1.42)
				Erythrocytes		RR ^c		0.93 (0.42–2.05)	0.72 (0.38–1.38)	0.98 (0.53–1.83)
Warensjö et al. (2010) ⁹	Swedish general population	444 cases (307 men), 556 controls (308 men)	First myocardial infarction	Plasma	PL	OR ^a (men)		0.92 (0.78–1.10)	0.92 (0.78–1.10)	
				Plasma	PL	OR ^a (wo-men)		0.78* (0.61–0.99)	0.74* (0.58–0.95)	
Warensjö et al. (2009) ¹⁸	Northern Swedish general population	108 stroke cases, 216 controls	Stroke First stroke	Plasma	PL	OR ^a		0.81* (0.60–1.10)	0.71* (0.52–0.79)	

Supplemental Table 8B: Continued

Paper	Population	n	Outcome	Blood Sample	Lipid Fraction	+/- ³	Biomarkers ^{1,2} (Risk (95%CI))				
							C14:0	C15:0	C17:0	Trans-C16:1(n-7)	
Yaemsiri et al. (2013) ¹⁹	Womens Health Initiative Observational Study (WHI-OS)	964 cases, 964 controls	Incident ischemic stroke	Serum	PL	OR ^{a,d}	0	1.09 (0.86-1.37)	1.07 (0.87-1.13)	1.01 (0.85-1.21)	0.94 (0.76-1.16)
Yakoob et al. (2014) ²⁰	Women in the Nurses' Health study (NHS) and men in the Health professionals study (HPFS)	472 cases NHS, 122 cases HPFS, 594 controls	Incident stroke	Plasma	Total	HR ^e	0	1.05 (0.62-1.78)	0.85 (0.54-1.33)	0.99 (0.67-1.49)	0.89 (0.55-1.45)
				Erythrocytes		HR ^e	0	1.31 (0.73-2.36)	0.88 (0.53-1.45)	0.87 (0.42-1.76)	1.07 (0.61-1.88)
Yamagishi et al. (2013) ²¹	Men and Women from the Minneapolis Field Center of the Athero-sclerosis Risk in Communities Study (ARIC)	3870 (168 cases)	Incident Ischemic stroke	Plasma	CE	HR ^e	0	1.43 (0.92-2.22)	0.83 (0.55-1.25)		
				PL		HR ^e	0	1.29 (0.83-2.01)	0.89 (0.60-1.33)		
Heart Failure											
Matsumoto et al. (2013) ²²	Physicians Health Study (PHS)	788 cases, 788 controls	Incident heart failure	Plasma	PL	OR ^e	0	1.03 (0.65-1.64)	0.73 (0.44-1.12)		
					OR ^a	0	1.02 (0.86-1.20)	0.91 (0.76-1.07)			
Tokede et al. (2013) ²³	Physicians Health Study (PHS)	788 cases, 788 controls	Incident heart failure	Plasma	PL	OR ^b	0				0.74 (0.49-1.10)
					OR ^a	0				0.91 (0.80-1.04)	

Supplemental Table 8B: Continued

Paper	Population	n	Outcome	Blood Sample	Lipid Fraction	HR ^e	+/- ³	Biomarkers ^{1,2} (Risk (95%CI))			
								C14:0	C15:0	C17:0	Trans-C16:1(n-7)
Yamagishi et al. (2008) ^{2a}	Men and Women from the Minneapolis Field Center of the Athero-sclerosis Risk in Communities Study (ARIC)	3592 (197 cases)	Incident heart failure	Plasma	CE	HR ^e	0	1.70** (1.06-2.71)	0.80 (0.50-1.28)	0.81 (0.52-1.26)	
				Plasma	PL	HR ^e	+	1.29 (0.80-2.08)	0.62* (0.38-1.02)	0.55** (0.35-0.85)	

Significance level: * = p < 0.05, ** = p < 0.01, *** = p < 0.001. One asterisk was ascribed when the risk was significant, but the level unknown.

^a. Evaluated as continuous variables (per 1 SD unit difference)^b. Highest versus lowest quintile ^c. Highest versus lowest tertile ^d. CI is 99.9% instead of 95%, ^e Highest versus lowest quartile.

¹. RR= Relative risk, HR= hazard ratio, OR= Odds ratio

². Adjustments: Iggman (2016)= age, sex and cardiovascular risk factors; Kwah (2012)= age, sex, polyunsaturated fatty acids, BMI, smoking, alcohol intake, physical activity, social class, education, blood pressure; Matthan (2014)= demographics, BMI, systolic blood pressure, smoking, education, medication and hormone use, family history of cardiovascular disease/stroke/myocardial infarction/type 2 diabetes, physical activity, dietary intake; de Oliveira (2013)= demographics, lifestyle, dietary variables; Sun (2007)= age, smoking, fasting status, BMI, postmenopausal status and hormone use, physical activity, alcohol intake, aspirin intake, history of hypertension, hypercholesterolemia, diabetes, linoleic acid, total trans fatty acids; Warensjö (2009) = BMI, serum-cholesterol, tobacco use, systolic blood pressure, diastolic blood pressure; Yaemsiri (2013)= BMI, smoking, diabetes, aspirin use, systolic blood pressure, antihypertensive medication, total cholesterol, HDL cholesterol and triglycerides; Yakoob (2014)= age, race, month of blood collection, smoking status, physical activity, alcohol, family history of diabetes, myocardial infarction, menopausal status, postmenopausal hormone use, food intake, energy intake, plasma/erythrocytes trans 18:1, trans 18:1, 16:0, 18:0; Yamagishi (2008 & 2013)= Age and sex; Matsumoto (2013) and Tokede (2013)= age, race, year of birth, blood sample collection, BMI, smoking, exercise, alcohol intake, history of diabetes and atrial fibrillation and saturated fatty acids.

³. + = An increase of the biomarker decreases the risk of diabetes type 2 and/or cardiovascular diseases. - = An increase in the biomarker increases the risk of diabetes type 2 and/or cardiovascular diseases. 0 = No significant effect

Abbreviations: AT, Adipose tissue; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; CE, Cholesterol esters; FFA, Free fatty acids; PL, Phospholipids; TG, Triglycerides; Trans-C16:1(n-7), Trans-Palmitoleic acid.

Supplemental Table 8C: Papers investigating the association of dairy fatty acid biomarkers with the development of diabetes type 2 (based on an illustrative search)

Paper	Population	n	Blood Sample	Lipid Fraction	HR ^a	+/- ³	Biomarkers ^{1,2} (Risk (95%CI))			
							C14:0	C15:0	C17:0	Trans-C16:1(n-7)
Forouhi et al. (2014) ²⁵	Adults in the EPIC cohort study	27296 (12132 cases, 15164 controls)	Plasma	PL	HR ^a	+	1.15* (1.09-1.22)	0.79* (0.73-0.85)	0.67* (0.63-0.71)	
Hodge et al. (2007) ¹⁶	Adults in the Melbourne collaborative cohort study	364 cases, 3391 controls	Plasma	PL	OR ^b	+		0.26*** (0.17-0.40)		
Krachler et al. (2008) ²⁷	Adults in the VIP study	159 cases, 291 controls	Erythrocytes		OR ^a	+	1.25* (1.01-1.53)	0.65** (0.50-0.85)	0.47*** (0.35-0.65)	
Mozaffarian et al. (2010) ³	Cardio-vascular Health study, adults	2985 (304 cases)	Plasma	PL	HR ^b	+				0.38*** (0.24-0.62)
					HR ^c	0		0.99 (0.89-0.10)	0.98 (0.85-1.13)	
Mozaffarian et al. (2013) ⁴	US adults (45-84yr) from the Multi-ethnic study of atherosclerosis	2281 (205 cases)	Plasma	PL	HR ^b	+	0.98 (0.63-1.53)	0.70 (0.42-1.15)		0.52* (0.32-0.85)
Santaren et al. (2014) ¹	Hispanic, African American and Non-Hispanic white adults 40-60 yr	659	Serum	Total	OR ^a	+		0.73* (0.56-0.95)		

Supplemental Table 8C: Continued

Paper	Population	n	Blood Sample	Lipid Fraction	HR ^d	+/- ³	Biomarkers ^{1,2} (Risk (95%CI))			
							C14:0	C15:0	C17:0	Trans-C16:1(n-7)
Yakoob et al. (2016) ²⁸	Women in the Nurses' Health study (NHS) and men in the Health professionals study (HPFS)	3333 (184 cases NHS study, 93 cases HPFS study)	Plasma	Total	HR ^d	+	1.03 (0.65-1.64)	0.56* (0.37-0.86)	0.57* (0.39-0.83)	0.48*** (0.33-0.70)
							1.56 (0.98-2.49)	0.83 (0.55-1.25)	0.54*** (0.40-0.73)	0.78* (0.51-1.18)

Significance level: * = p < 0.05, ** = p < 0.01, *** = p < 0.001. One asterisk was ascribed when the risk was significant, but the level unknown.

^a. Evaluated as continuous variables (per 1 SD unit difference), ^b. Highest versus lowest quintile, ^c. HR's evaluated per 0.05% points of total fatty acids^d. Highest versus lowest quartile

¹. RR= Relative risk, HR= hazard ratio, OR= Odds ratio

². Adjustments: Forouhi (2014), Santaren (2014), Mozaffarian (2010, 2013)= demographics, lifestyle, dietary variables; Hodge (2007)= demographic, diabetes history, physical activity, alcohol intake; Krachler (2008)= alcohol intake; Yakoob (2016)= age, race, month of blood collection, smoking status, physical activity, alcohol, family history of diabetes, myocardial infarction, menopausal status, postmenopausal hormone use, food intake, energy intake, plasma/erythrocytes trans 18:1, trans 18:1, 16:0, 18:0;

³. + = An increase of the biomarker decreases the risk of diabetes type 2 and/or cardiovascular diseases. - = An increase in the biomarker increases the risk of diabetes type 2 and/or cardiovascular diseases. 0 = No significant effect

Abbreviations: AT, Adipose tissue; C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; CE, Cholesterol esters; FFA, Free fatty acids; PL, Phospholipids; TG, Triglycerides; Trans-C16:1(n-7), Trans-Palmitoleic acid.

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CHAPTER 6

Intake of omega-3 fatty acids and long-term outcome in renal transplant recipients: A post-hoc analysis of a prospective cohort study

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ABSTRACT

Supplementation with n-3 fatty acids may improve long-term outcomes of renal transplant recipients (RTR). Recent evidence suggests that eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have different outcomes compared with α -linolenic acid (ALA). We examined the prospective associations of EPA-DHA and ALA intakes with graft failure and all-cause mortality in 637 RTR. During 3.1 years (Interquartile range (IQR) 2.7, 3.8) of follow-up, 41 developed graft failure and 67 died. In age and sex adjusted analyses, EPA-DHA and ALA intakes were not associated with graft failure. EPA-DHA intake was not significantly associated with mortality (Hazard ratio (HR)=0.79; 95% CI 0.54, 1.15 per 0.1 Energy% difference). ALA intake was significantly associated with mortality (HR=1.17; 95% CI 1.04, 1.31 per 0.1 Energy% difference). This association remained following adjustments for BMI, proteinuria and intakes of fat, carbohydrate and protein. RTR in the highest tertile of ALA intake exhibited an about two fold higher mortality risk (HR=2.21; 95% CI 1.23, 3.97) compared to the lowest tertile. In conclusion, ALA intake may be associated with increased mortality in RTR. Future RCTs are needed to confirm these results.

INTRODUCTION

The prevalence of end-stage renal disease (ESRD) is increasing worldwide [1]. In the United States the incidence of ESRD in 2012 was as high as 400 cases per million residents per year [2]. The preferred treatment for most ESRD patients is renal transplantation. Compared to dialysis treatment it is less expensive, improves quality of life and increases survival [3,4]. Although 90% of the allografts survives at least one year [5,6], more than half of these grafts do not last longer than 10-12 years [6]. This is largely due to graft failure and premature mortality [6]. Thus there is a great need for approaches to improve survival.

Many randomized controlled trials have investigated whether n-3 fatty acid supplements reduce cardiovascular risk and improve graft survival in renal transplant recipients (RTR). A meta-analysis from 2007 concluded that it cannot be excluded that RTR may benefit from such supplements [7]. In this respect it is important to realize that the effects of the marine-derived n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the plant-derived n-3 fatty acid α -linolenic acid (ALA) may differ. For example, in the general population it has been found that supplementation with EPA and DHA was protective, whereas supplementation with ALA was not [8]. Importantly, in a large Swedish study in more than 2000 men aged 50 years or older, it was suggested that exposure to ALA has an adverse effect on mortality [9]. In RTR, it is unclear whether marine-derived and plant-derived n-3 fatty acids differ in effects on long-term outcome.

In the present study, we aimed to investigate the association of marine-derived and plant-derived n-3 fatty acids with long-term outcome in stable outpatient RTR. For this purpose, we examined EPA-DHA and ALA intakes separately, and analyzed whether the intakes of these n-3 fatty acids are associated with graft failure and all-cause mortality.

METHODS

Design and study population

We performed a post-hoc analysis in an existing prospective cohort study on RTR in which baseline food frequency questionnaire data were available that were suitable to address our research question. The study took place at the University Medical Center Groningen between November 2008 and March 2011 [10]. All RTR (≥ 18 years of age) had a functioning graft for at least 1 year, no alcohol or drug addiction and sufficient knowledge of the Dutch language. In total, 817 RTR were invited for the study, of whom 707 (87%) provided written consent to participate. In the current study, patients with missing dietary data were excluded from the analyses, resulting in 637 RTR for analysis. The study was approved by the Institutional Review Board (METc 2008/186). Dietary intake and clinical parameters were assessed once at baseline. Outcome assessors were blinded to dietary intake measurements and other baseline parameters.

RTR did not receive dietary recommendation regarding n-3 fatty acid intake. Patients were taking standard immunosuppressive and antihypertensive drugs.

Outcome parameters

The primary long-term outcomes of the study were graft failure and all-cause mortality. Graft failure was defined as return to dialysis or re-transplantation. The patients were followed until 30 April 2013, with no loss to follow-up.

Dietary assessment

Dietary intake was assessed with a semi-quantitative validated Food Frequency Questionnaire (FFQ) that was developed at Wageningen University [11]. The questionnaire consisted of 177 food items to record intake during the last month, expressed in times per day, week or month. The number of servings was recorded in natural units (for example, slice of bread or apple) or household measures (for example, cup or spoon). Patients had to fill in the questionnaire at home. All FFQs were checked by a trained researcher and verified with the patients in case of inconsistent answers. The results of the FFQ were converted into total energy and nutrient intake by using the Dutch Food Composition Table of 2006 [12].

Clinical parameters

All measurements were performed during a morning visit to the outpatient clinic. Patients were instructed to collect a 24-hour urine sample the day before the visit. In the morning, blood was drawn after a fasting period of 8-12 hours. Body weight and height were measured with patients wearing indoor clothing without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2), and body surface area (BSA) was measured as meters squared (m^2). Diabetes was defined as use of antidiabetic medication or fasting serum glucose ≥ 7.0 mmol/L [13]. Fasting blood pressure, heart rate and mean arterial pressure (MAP) were determined with a semi-automatic device (Dinamap 1846; Critikon). Blood pressure and heart rate were measured every minute for 15 minutes, and the last three were averaged [14]. Renal function was assessed by the eGFR (estimated glomerular filtration rate) [15]. Serum creatinine levels were measured with a modified version of the Jaffé method (MEGA AU 510; Merck Diagnostica). Total urinary albumin concentration was analyzed by nephelometry (Dade Behring Diagnostics), while total urinary protein concentration was determined using the Biuret reaction (MEGA AU 150; Merck Diagnostica). Serum cholesterol, glycated hemoglobin (HbA1C) and high-sensitive C-reactive protein (hs-CRP) were measured according routine laboratory methods. Proteinuria was defined as urinary protein excretion ≥ 0.5 g/24 h.

Information on patient health status, medical history, and medication use was obtained from patient records. Information on smoking habits and alcohol intake was collected as well. Patients were divided into current smokers, former smokers or never smokers. In addition,

patients were also divided into four groups according to their alcohol intake (g ethanol/d) (no alcohol, 0-10 g/d, 10-30 g/d and >30g/d). Smoking behavior and alcohol intake were based on self-report.

Statistical analyses

Data analysis was performed using SPSS version 22.0 software (SPSS, Inc.) and R version 3.2.3. Data are presented as the mean (SD) or median (Inter Quartile range (IQR)) unless stated otherwise. N-3 fatty acid intake was measured as percentage of the total energy intake (En%). For initial exploration, EPA-DHA and ALA intake were divided into tertiles, which were compared by means of ANOVA, Kruskal-wallis or Chi-square test depending on whether the distribution of data was normal, skewed or categorical respectively. Linear regression analyses with intake of n-3 fatty acids as a dependent variable were carried out to compare strengths of associations of different baseline variables with n-3 fatty acids. For these analyses, categorical variables were divided into 2 or more dummy variables. Baseline characteristics with a P-value < 0.10 for the association with intake of n-3 fatty acids were selected as potential explanatory variables in subsequent Cox regression analyses. Cox regression analyses were also used to test whether there were interactions between n-3 fatty acid intake and potential explanatory variables (age, sex, BMI, smoking habits, alcohol intake, intake of protein, fat and carbohydrates, proteinuria, blood pressure). In prospective analyses, we investigated the associations EPA-DHA and ALA intake with graft failure and all-cause mortality in RTR. Analyses were performed with adjustment for age and sex (model 1) and further cumulative adjustments for BMI (model 2), alcohol intake, smoking behavior (model 3), total fat intake, total protein intake, total carbohydrate intake (model 4), and proteinuria and diastolic blood pressure (DBP) (model 5). Analyses were each time initially performed for EPA-DHA and ALA intake as continuous variables and repeated for EPA-DHA and ALA intake as tertiles. Cox regression analyses with restricted cubic splines was carried out to show the association between ALA intake and all-cause mortality. A two-sided P-value < 0.05 was considered statistically significant. Since there were only a small number of statistical tests related to our a priori hypotheses, correction for multiple testing is not required [16,17].

In secondary analyses, n-3 fatty acid intake was measured in gram per day (g/d) instead of En%. For this analysis, n-3 fatty acid intake was adjusted for total energy intake according to the residual method, which is based on the isoenergetic principle [18]. In addition, we calculated both the intake of EPA-DHA and the intake of ALA as a proportion of the total intake of fatty acids. As sensitivity analyses, we repeated the Cox regression analyses after elimination of patients with extreme intakes of n-3 fatty acids outside of 3 SD from the mean value, i.e. an EPA-DHA intake > 0.30 En% or an ALA intake > 1.10 En%.

RESULTS

Population characteristics of RTR are presented in **Table 1**. Median time after transplantation was 5.6 (1.8 - 12.1) years. The mean age of the study population was 53 (SD 13) years, and 57% was male. The participants were slightly overweight (mean BMI of 26.6) and 24% had diabetes. Mean blood pressure of RTR was within normal boundaries (136/83 mmHg). It should, however, be noted that 88% of the 637 participants were using antihypertensive drugs. Mean serum total cholesterol was 5.1 (SD 1.1) mmol/l, while statins were taken by 53% of the recipients. Calcineurin inhibitors were used in 57.1% of the RTR, and 83.3% were using proliferation inhibitors. Mean eGFR was 49 ml/min per 1.73m². The median urinary protein excretion was 0.19 (0-8.91) mg/24h, with 139 (22%) RTR having proteinuria.

Table 1: Baseline characteristics of 637 RTR.

Characteristics	Mean	SD	Range (min-max)
<i>Demographics</i>			
Age, yr	53	13	18 - 78
Gender (% male)	57		
BMI, kg/m ²	26.6	4.8	15.7 - 45.0
BSA, m ²	1.9	0.2	1.1 - 2.8
Smoking behavior (%)			
Not	41.3		
Former	45.8		
Current	12.9		
Diabetes (yes)			
n	154		
%	24.2		
<i>Haemodynamic parameters</i>			
SBP, mmHg	136	17	80 - 200
DBP, mmHg	83	11	54 - 125
MAP, mmHg	101	12	66 - 145
Heart rate, bpm	69	12	41 - 122
<i>Medication use</i>			
Antihypertensives (%)	87.9		
Statins (%)	52.8		
CNI (%)	57.1		
Proliferation inhibitors (%)	83.8		
mTor (%)	1.6		
Prednisolone dose, mg/d			
Median	10		0 - 20.0
IQR	7.5-10		

Table 1: Continued

Characteristics	Mean	SD	Range (min-max)
<i>Renal function parameters</i>			
Time after transplantation, yr			0.2-40
Median	5.6		
IQR	1.8-12.1		
Serum creatinine, $\mu\text{mol/l}$			50 - 591
Median	124		
IQR	100-160		
eGFR, ml/min per 1.73 m ²	49	20	7-115
Proteinuria ≥ 0.5 g/d (%)			
n	139		
%	22		
Protein excretion, mg/24h			0.00-8.91
Median	0.19		
IQR	0.02-0.35		
Albumin excretion, mg/24h			0.02 - 6258
Median	42.5		
IQR	10-172		
<i>Serum parameters</i>			
Albumin, g/l	43.0	3.0	28 - 51
Cholesterol, mmol/l	5.1	1.1	2.3 - 9.7
LDL cholesterol, mmol/l	2.9	0.9	0.7 - 6.6
HDL cholesterol, mmol/l	1.4	0.5	0.4 - 3.5
Triglycerides, mmol/l			
Median	1.7		0.3 - 8.5
IQR	1.2-2.3		
HbA1c, %	6.0	0.8	4.5 - 11.8
Hs-CRP, mg/l			
Median	1.6		0.1 - 114.0
IQR	0.7-4.5		
<i>Dietary intake (FFQ)</i>			
Energy intake			
Kcal/d	2175	637	657 - 6575
KJ/d	9117	2664	2770 - 27428
Total fat intake, g/d	88	34	21 - 412
Total protein intake, g/d	82	20	25 - 177
Total carbohydrates, g/d	249	78	16 - 539
Alcohol intake, g/d			
Median	2.6		0.0-75.8
IQR	0.03-11.2		
<i>N-3 fatty acid intake</i>			
EPA-DHA intake, mg/d			0.0 - 1370
Median	92.1		
IQR	28.2-204.8		
Relative amount (En%)	0.04		
ALA intake, g/d			0.3 - 6.4
Median	1.2		
IQR	0.9-1.7		
Relative amount (En%)	0.5		

Abbreviations: BMI, body mass index; BSA, body surface area; SBP, systolic blood pressure; DBP, Diastolic blood pressure; MAP, mean arterial pressure; CNI, calcineurin inhibitor; mTor, mammalian target of rapamycin; FFQ: Food Frequency Questionnaire; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, High density lipoprotein; HbA1c, glycated hemoglobin ; Hs-CRP, High-sensitive C-reactive protein; EPA-DHA, eicosapentaenoic acid-docosahexaenoic acid; En%, Energy Percentage, ALA, α -linolenic acid

Energy intake was 9117 (SD 2664) KJ/d (2175 (SD 637) kcal/d). Of the total intake, 36 en% was derived from fat (saturated fat: 13 en%; monounsaturated fat: 12 en%; polyunsaturated fat: 8 en%), 15 en% from proteins and 46 en% from carbohydrates. Median intakes of EPA-DHA and ALA were 92 (28-205) mg/d (0.04 (0.01-0.09) En%) and 1.2 (0.9-1.7) g/d (0.5 (0.4-0.7) En%) respectively. According to the European Food Safety Authority (EFSA), intake of EPA and DHA was below the recommended combined intake of 250 mg of EPA plus DHA per day in 524 RTR (82%), while intake of ALA was in accordance with the guidelines (0.5 En%) in 352 RTR (55%) [19]. Patient characteristics according to tertiles EPA-DHA and ALA intake (En%) are shown in **Supplementary Table 1** and **Supplementary Table 2** respectively.

The associations of baseline characteristics with intake of n-3 fatty acids are shown in **Table 2**. Age was found to be positively associated with the marine-derived n-3 fatty acids ($\beta = 0.094$, $p = 0.02$), while age was inversely associated with intake of plant-derived ALA ($\beta = -0.093$, $p = 0.02$). In addition, moderate alcohol intake ($\beta = 0.197$ $p < 0.001$) and former smoking habits ($\beta = 0.128$ $p = 0.01$) were positively associated with EPA-DHA intake, while there was no association with ALA intake. Furthermore, total fat intake ($\beta = -0.157$ $p < 0.001$) and total carbohydrate intake ($\beta = -0.272$, $p < 0.001$) were inversely associated with EPA-DHA intake, while total fat intake ($\beta = 0.318$, $p < 0.001$) had a positive association with ALA intake. Carbohydrate intake was not associated with ALA intake. We found no significant interactions of n-3 fatty acid intake with potential explanatory variables for the association with graft failure and all-cause mortality.

For prospective analyses, median follow-up was 3.1 (2.7-3.8) years. During the follow-up period, 41 RTR developed graft failure, while 67 died.

Results for Cox regression analyses for graft failure are shown in **Table 3**. Neither EPA-DHA nor ALA intake appeared to be associated with graft failure (hazard ratio (HR)= 0.83; 95% CI 0.50, 2.27; and HR=0.90; 95% CI 0.74, 1.09 respectively per 0.1 En% difference) while treating intakes as continuous variables. Findings were similar when intakes were treated as tertiles.

Results of Cox regression analyses for all-cause mortality are shown in **Table 4**. In age and sex adjusted analyses, with EPA-DHA intake treated as a continuous variable, there was no association with all-cause mortality (HR=0.79; 95% CI 0.54, 1.15 per 0.1 En% difference), while ALA intake was significantly associated with increased all-cause mortality risk (HR=1.17; 95% CI 1.04, 1.31 per 0.1 En% difference) (Model 1) (**Figure 1A**). Moreover, RTR in the highest tertile of ALA intake (> 0.60 En%) were at 2-fold higher risk of all-cause mortality (HR=2.21; 95% CI 1.23, 3.97) than those in the lowest tertile (reference tertile) (< 0.45 En%). Associations remained materially unchanged after cumulative adjustment for potential explanatory variables, including BMI, diastolic blood pressure, alcohol intake, smoking behavior, fat, protein and carbohydrate intake, and proteinuria.

Table 2: Regression coefficients for the association between baseline EPA-DHA and ALA intakes with baseline parameters. (β -coefficients per standard deviation of EPA-DHA and ALA intakes)

Dependent Variable	EPA-DHA		ALA	
	β	P	β	P
<i>Demographics</i>				
Age, yr	0.094	0.02	-0.093	0.02
Gender	0.033	0.41	-0.068	0.09
BMI, kg/m ²	0.068	0.09	0.065	0.10
BSA, m ²	-0.025	0.53	0.095	0.02
Smoking habits				
Former	0.128	0.003	-0.017	0.69
Current	0.062	0.15	-0.061	0.16
Diabetes (%yes)	0.005	0.90	-0.002	0.96
<i>Haemodynamic factors</i>				
SBP, mmHg	-0.045	0.26	-0.032	0.42
DBP, mmHg	0.002	0.96	0.073	0.07
MAP, mmHg	-0.036	0.37	0.005	0.89
Heart rate, bpm	0.063	0.12	0.061	0.14
<i>Dietary intake (FFQ)</i>				
Total fat intake, g/d	-0.157	<0.001	0.318	<0.001
Total protein intake, g/d	-0.035	0.38	0.066	0.09
Total carbohydrates, g/d	-0.273	<0.001	0.009	0.83
Alcohol intake, g/d				
0-10	0.116	0.05	0.149	0.01
10-30	0.197	0.001	0.064	0.27
>30	0.113	0.01	0.048	0.29
<i>Medication use</i>				
Antihypertensives (% yes)	-0.015	0.70	0.019	0.63
Statines (% yes)	0.045	0.26	-0.062	0.12
<i>Renal function factors</i>				
Serum creatinine, μ mol/l	0.021	0.59	0.004	0.91
eGFR, ml/min per 1.73m ²	-0.063	0.11	0.039	0.33
Albuminuria ^a , mg/24h	0.008	0.84	-0.082	0.04
Proteinuria ^a , ≥ 0.5 g/d	-0.004	0.91	-0.072	0.07
<i>Serum parameters</i>				
Albumin, g/l	-0.022	0.58	0.011	0.79
Cholesterol, mmol/l	0.048	0.23	-0.022	0.58
Triglycerides, mmol/l	0.004	0.91	0.002	0.95
Hs-CRP, mg/L ^a	0.068	0.10	0.052	0.21
HbA1C, %	0.042	0.30	0.007	0.86

Abbreviations: BMI, body mass index; BSA, body surface area; SBP, systolic blood pressure; DBP, Diastolic blood pressure; MAP, mean arterial pressure; CNI, calcineurin inhibitor; mTor, mammalian target of rapamycin; FFQ: Food Frequency Questionnaire; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, High density lipoprotein; HbA1c, glycated hemoglobin; Hs-CRP, High-sensitive C-reactive protein; EPA-DHA, eicosapentaenoic acid-docosahexaenoic acid; ALA, α -linolenic acid

^aLog-transformed for analysis

Table 3: Cox-regression analyses for prediction of graft failure based on n-3 fatty acid intakes (En%).
(Hazard ratios and 95% confidence intervals)

Model*	Tertiles of n-3 fatty acid intake (En%)									
	I			II			III			Continuous (En% per 0.1 En% difference)
	Reference Value	HR	95% CI	P	HR	95% CI	P	HR	95% CI	
EPA-DHA intake										
1	1.00	1.08	0.53, 2.20	0.84	0.79	0.36, 1.73	0.56	0.83	0.50, 2.27	0.46
2	1.00	1.09	0.53, 2.24	0.81	0.81	0.37, 1.77	0.59	0.98	0.92, 1.05	0.53
3	1.00	1.29	0.61, 2.72	0.51	0.95	0.42, 2.13	0.89	0.92	0.56, 1.49	0.72
4	1.00	1.27	0.60, 2.69	0.54	0.91	0.40, 2.11	0.83	0.89	0.53, 1.49	0.65
5	1.00	1.02	0.47, 1.23	0.96	0.72	0.30, 1.72	0.45	0.77	0.43, 1.39	0.39
ALA intake										
1	1.00	0.97	0.45, 2.09	0.95	1.10	0.51, 2.36	0.81	0.90	0.74, 1.09	0.27
2	1.00	0.99	0.46, 2.12	0.98	1.13	0.52, 2.44	0.75	0.90	0.75, 1.09	0.29
3	1.00	1.26	0.56, 2.83	0.58	1.47	0.65, 3.28	0.35	0.95	0.78, 1.14	0.56
4	1.00	1.34	0.58, 3.08	0.49	1.79	0.73, 4.39	0.20	0.96	0.77, 1.19	0.70
5	1.00	1.16	0.47, 2.85	0.75	1.99	0.80, 4.98	0.14	1.02	0.81, 1.30	0.86

Abbreviations: En%, Energy Percentage; EPA-DHA, Eicosapentaenoic acid-Docosahexaenoic acid; ALA, α -linolenic acid

***Model 1**, adjusted for age and sex; **Model 2**, as model 1, additionally adjusted for BMI; **Model 3**, as model 2, additionally adjusted for alcohol intake and smoking habits; **Model 4**, as model 3, additionally adjusted for fat, protein and carbohydrate intake; **Model 5**, as model 4, additionally adjusted for proteinuria and diastolic blood pressure. Proteinuria was log-transformed for analyses.

Table 4: Cox-regression analyses for prediction of all-cause mortality based on n-3 fatty acid intakes (En%). (Hazard ratios and 95% confidence intervals)

Model*	Tertiles of n-3 fatty acid intake (En%)										Continuous (En% per 0.1 En% difference)		
	I			II			III						
	Reference Value	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P			
EPA-DHA intake													
1	1.00	1.25	0.71, 2.20	0.44	0.72	0.39, 1.35	0.31	0.79	0.54, 1.15	0.22			
2	1.00	1.35	0.71, 2.20	0.44	0.72	0.39, 1.35	0.31	0.79	0.54, 1.16	0.23			
3	1.00	1.41	0.77, 2.54	0.26	0.89	0.46, 1.71	0.71	0.88	0.61, 1.29	0.52			
4	1.00	1.56	0.86, 2.86	0.15	1.02	0.52, 2.00	0.97	0.95	0.64, 1.40	0.79			
5	1.00	1.56	0.85, 2.86	0.15	0.96	0.48, 1.93	0.91	0.90	0.60, 1.34	0.60			
ALA intake													
1	1.00	1.06	0.55, 2.07	0.86	2.21	1.23, 3.97	0.01	1.17	1.04, 1.32	0.01			
2	1.00	1.07	0.55, 2.07	0.85	2.22	1.24, 3.98	0.01	1.17	1.04, 1.32	0.01			
3	1.00	1.13	0.56, 2.25	0.74	2.20	1.20, 4.03	0.01	1.17	1.03, 1.32	0.02			
4	1.00	1.05	0.52, 2.14	0.88	2.08	1.07, 4.05	0.03	1.16	1.01, 1.35	0.04			
5	1.00	0.92	0.45, 1.90	0.83	2.04	1.05, 3.98	0.04	1.19	1.02, 1.38	0.03			

Abbreviations: En%, Energy Percentage; EPA-DHA, Eicosapentaenoic acid-Docosahexaenoic acid; ALA, α -linolenic acid***Model 1**, adjusted for age and sex; **Model 2**, as model 1, additionally adjusted for BMI; **Model 3**, as model 2, additionally adjusted for alcohol intake and smoking habits; **Model 4**, as model 3, additionally adjusted for fat, protein and carbohydrate intake; **Model 5**, as model 4, additionally adjusted for proteinuria and diastolic blood pressure. Proteinuria was log-transformed for analyses.

In secondary analyses, the association of ALA intake with all-cause mortality was analyzed using g/d instead of En%. In age- and sex-adjusted analyses, RTR in the highest tertile of ALA intake (> 1.45 g/d) were at almost 2-fold higher risk of all-cause mortality (HR=1.90 95% CI 1.07, 3.37) compared to the lowest tertile (reference tertile) (< 1.14 g/d). Our findings did not change after further cumulative adjustment for potential explanatory variables. We also calculated the intake of ALA as a proportion of the total intake of fatty acids. It remained that there was no association of ALA intake (now expressed as proportion of total fatty acid intake) with graft failure (HR=0.93; 95% CI 0.80, 1.08 per 0.1% difference). It also remained that there was an association of ALA intake (now expressed as proportion of total fatty acid intake) with increased risk for mortality (HR=1.14; 95% CI 1.02, 1.27 per 0.1% difference, $p = 0.02$).

In sensitivity analyses, we excluded patients with extreme intakes of n-3 fatty acids. Hazard ratios of EPA-DHA and ALA intakes for graft failure and all-cause mortality remained materially unchanged, with no associations for graft failure, and HR=0.78; 95% CI 0.50, 1.22 and HR=1.21; 95% CI 1.06, 1.37 per 0.1 En% difference for the associations of EPA-DHA and ALA intake with all-cause mortality respectively (**Figure 1B**).

DISCUSSION AND CONCLUSION

Our study is, to the best of our knowledge, the first to separately investigate the associations of the marine-derived n-3 fatty acids EPA and DHA and the plant-derived n-3 fatty acid ALA with long-term outcome in a large cohort of RTR. Both EPA-DHA and ALA intake were not associated with graft failure. Additionally, EPA-DHA intake was not associated with all-cause mortality, while ALA intake was positively associated with all-cause mortality.

The n-3 fatty acids EPA, DHA and ALA are long-chain polyunsaturated fatty acids (PUFAs). EPA and DHA are mainly found in fatty fish, whereas vegetable oils, such as linseed, soybean and rapeseed oils, are main sources of ALA. [20,21]. According to the EFSA, the combined intake of EPA plus DHA was found to be below the recommended intake in 82% of RTR, while 45% had an inadequate intake of ALA. Various recommendations for PUFA intakes are circulating around the world [22]. For instance, the ALA intake recommendation in Australia is 2.0 g/d (1 En%), while in the USA and Canada the recommended ALA intake is 1.35 g/d (0.7 En%) [23]. According to these international guidelines the number of RTR with an ALA intake below the recommended daily intake increases enormously.

Figure 1A: All-cause Mortality in 637 RTR **Figure 1B: All-cause Mortality in 632 RTR**

Figure 1: Restricted cubic spline showing the association between ALA intake (En%) and all-cause mortality in 637 RTR (a) and 632 RTR (b). The line in the graph represents the age and sex adjusted risk for all-cause mortality. The 95% CI of the HR is represented by the grey area.

At least two meta-analyses of randomized controlled trials have investigated whether fish oil supplementation may improve renal function, rejection rates, and patient and graft survival in renal transplant recipients [7,24]. Both these meta-analyses reported that there was insufficient evidence to conclude that fish oil has a beneficial effect on either of these outcomes. It was mentioned that the meta-analyses were hampered by methodological problems, including different doses of fish oils, different duration and timing of treatment, studies with small numbers of patients, inadequate randomization and lack of blinding [7]. Recently, two studies in a large observational cohort, with 569 allograft failures and 406 deaths in 1990 RTR that were followed for 6.8 years, showed significant inverse associations of plasma phospholipid concentrations of marine derived n-3 fatty acids with graft failure and mortality in renal transplant recipients [25,26]. Interestingly, in a large systematic review of the effects of n-3 fatty acids on cardiovascular disease in the general population it was concluded that EPA-DHA intake reduces the rates of all-cause mortality, cardiac and sudden death and possibly stroke [8]. These authors analyzed ALA separately from EPA-DHA and found that ALA was not associated with these positive findings [7,8]. In another systematic review, it was concluded that supplementation of EPA and DHA or fish oil has a consistent beneficial effect on serum lipids, while this is not the case for supplementation with ALA [27]. This pattern of decrease in risk associated with high intake of EPA-DHA, and increase in risk associated with high intake of ALA seems also present in studies including biomarkers. A recent study in which circulating levels of EPA, DHA and ALA were measured in 60-year old adults showed that high circulating concentrations of EPA and DHA were found to be associated with low risk of all-cause mortality, while it was found that there was a trend towards an association of high circulating levels of ALA with an increased risk for all-cause mortality [28].

In our cohort, there was no association of EPA-DHA intake with all-cause mortality, while a high ALA intake was associated with a significantly increased risk of all-cause mortality. The fact that the association between the marine-derived n-3 fatty acid intakes and all-cause mortality did not reach significance may have been the consequence of the relatively small number of cases (67 deaths in a cohort of 637 RTR during a follow-up period of 3.1 years). Indeed, in a recent analysis of a cohort study, with 406 deaths in 1990 RTR that were followed for 6.8 years, it was found that circulating levels of marine n-3 fatty acid in plasma phospholipids were inversely associated with all-cause and cardiovascular mortality after renal transplantation [26]. Other than in size and duration of follow-up, this study differed from our study with respect to percentage of males (67 in de study by Eide et al. vs 57% in our study), percentage of diabetics (18.2 vs 24.2%), percentage of former smokers (36.2 vs 45.8%) and eGFR (57 vs 49 ml/min per 1.73m²), while age (52 vs 53 years), BMI (24.8 vs 26.6 kg/m²) and percentage of current smokers (16.0 vs 12.9%) were less dissimilar. Another difference between the studies is the way in which

n-3 fatty acid exposure was assessed (i.e. a status marker (n-3 fatty acids in plasma phospholipids) in the study by Eide et al. vs intake (FFQ) in our study).

Results regarding the effect of EPA-DHA and ALA intake on graft failure are inconsistent. As mentioned before, meta-analyses of randomized-controlled trials on this subject found no effect of EPA-DHA on graft failure [7,24], what is in line with our finding of a virtual absence of an association of EPA-DHA and ALA intakes with graft failure in RTR. An intervention study in 2344 post-myocardial infarction patients suggests, however, that a combined intake of 400 mg/d of EPA and DHA provides a small beneficial effect on renal function, while this was not the case for ALA [29]. A positive effect of EPA-DHA was also suggested by a study of circulating levels of EPA and DHA. In an observational cohort study from Norway it was described that high levels of marine derived n-3 fatty acids in plasma phospholipids were associated with better allograft survival. The authors showed an 11% reduction in risk of graft loss for every 1.0 wt% increase in EPA-DHA level [25].

Recently, two authoritative meta-analyses in the field of n-3 fatty acid intake and mortality investigated whether there is a threshold for the relation of EPA-DHA intake with outcome [30,31]. These studies found that there was a decline in CHD [30,31] and total mortality [31] with increasing EPA-DHA intake in the range of EPA-DHA intake from zero to 250 mg/d, while there was no further decline in CHD and total mortality with increasing intake beyond 250 mg/d. In our cohort, the median EPA-DHA intake was 92.1 (28.2-204.8) mg/d, with only 18% of the population consuming more than 250 mg of EPA-DHA per day. So, the EPA-DHA intake in our cohort is rather low compared to the threshold determined in the previous meta-analyses.

The reason why a higher ALA intake is associated with increased risk of all-cause mortality in RTR is unknown. ALA can be metabolized via at least two pathways. First, ALA is a preferred substrate for β -oxidation, with either complete oxidation to CO_2 and water or incomplete conversion to intermediates that may subsequently be employed for the synthesis of fatty acids and cholesterol [32,33].

A second route is by conversion of ALA into EPA and subsequently into DHA [32,34]. Rate limiting in this pathway are the activities of $\Delta 5$ and $\Delta 6$ desaturases [35,36]. In renal transplant recipients, this route, but also β -oxidation, could be affected by glucocorticoids, a class of immunosuppressive drugs used to protect the transplanted kidney from rejection [37,38,38]. In our cohort, 99% of the renal transplant recipients were treated with prednisolone. Glucocorticoids can depress the activities of $\Delta 5$ and $\Delta 6$ desaturases, giving rise to altered fatty acid precursor/metabolic ratios, possibly including the ALA/EPA-DHA ratio [35,39].

Finally, it is also possible that the association of ALA intake with high mortality risk might not be through the intake of ALA itself but rather via its decomposed products produced during the baking or frying of ALA rich food [40]. ALA can be found in various widely used vegetable oils and is highly susceptible to peroxidation during heating [20,40]. The degraded products are

hazardous to health and adverse effects may possibly become amplified in susceptible patients, such as renal transplant recipients [40].

Strength of the current study is the large population included in the analyses. In total, data of 637 RTR were used for analyses what made the results reliable. Another strength of the study is the prospective analyses with Cox regression in RTR using 3 year follow-up information of patients. Currently, studies on the long-term effects of n-3 fatty acids are especially carried out in the general population. In our study we were able to identify specific long-term association in our target group that nevertheless needs further investigations of causality. Third strong point is the inclusion of intake of ALA in the prospective study. Most studies in the field of n-3 fatty acids predominantly focus on EPA-DHA intake, while ALA intake might be important as well. We analyzed the EPA-DHA and ALA intakes separately, to investigate the specific effects of the marine-derived and plant-derived n-3 fatty acids and found a difference between the two.

We acknowledge that our study also has some limitations. The study is an observational epidemiological study which may only suggest biological significance. Another limitation of our study is the use of an FFQ for measuring dietary intake. Measurements of intake were based on self-report which may lead to misclassification, underreporting and overreporting. Furthermore, intakes were only measured once at baseline, while changes in intake and clinical parameters may appear during the 3-years of follow-up. Last, dietary intake and status form complementary information on n-3 fatty acid exposure. The current study made use of dietary n-3 fatty acid intake, but not status. A next study should therefore focus on n-3 fatty acid biomarkers also.

In conclusion, in RTR, the EPA-DHA and ALA intakes showed no clear association with graft failure. The EPA-DHA intake showed no association with all-cause mortality, while the ALA intake was associated with increased all-cause mortality. Although the association between ALA intake and all-cause mortality was strong, a causal relationship in RTR is as yet questionable. Further investigation on the long-term outcome of n-3 fatty acids, particularly ALA, in RTR is warranted.

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Supplemental Table 1: Patient characteristics of 637 RTR across tertiles of EPA-DHA intake (En%)

Characteristics	Tertile I N=212			Tertile II N=213			Tertile III N=212		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P
<i>N-3 fatty acid intake</i>									
EPA-DHA intake, En%									
Median		0.01			0.04		0.11		<0.001
IQR		0.00-0.01			0.03-0.05		0.09-0.16		
EPA-DHA intake cut-off points, En%		<0.02			0.02-0.07		>0.07		
EPA-DHA intake, mg/d									
Median		13.9			92.3		258.1		<0.001
IQR		0.0-29.9			57.3-124.3		196.4-348.5		
<i>Demographics</i>									
Age, yr	51	13	53	12	54	12			0.04
Gender (% male)	60			57		54			0.42
BMI, kg/m ²	26	5	27	5	27	5			0.17
BSA, m ²	1.9	0.2	1.9	0.2	2.0	0.2			0.72
Smoking behavior (current) n (%)									0.01
Never		51		38		35			
Ever		35		51		52			
Current		14		11		14			
Diabetes (yes)									0.68
n		49		56		49			
%		23		26		23			
<i>Haemodynamic parameters</i>									
SBP, mmHg	135	16	137	17	136	19			0.30
DBP, mmHg	82	10	83	11	83	11			0.22
MAP, mmHg	100	11	101	12	101	13			0.22
Heart rate, bpm	69	13	69	11	68	12			0.92
<i>Dietary intake (FFQ)</i>									
Energy intake									
Kcal/d	2303	731	2185	578	2039	565			<0.001
KJ/d	9646	3061	9157	2411	8548	2362			<0.001
Total fat intake, g/d	95	41	88	30	82	29			<0.001
Total protein intake, g/d	82	21	83	20	82	19			0.76
Total carbohydrates, g/d	269	88	251	69	227	70			<0.001
Alcohol intake (%)									0.02
0 g/d		13		9		7			
0-10 g/d		68		62		60			
10-30 g/d		14		24		28			
>30 g/d		5		4		5			

Supplemental Table 1: Continued

Characteristics	Tertile I N=212		Tertile II N=213		Tertile III N=212		P
	Mean	SD	Mean	SD	Mean	SD	
<i>Medication use</i>							
Antihypertensives, (%)	89		87		87		0.80
Statins, (%)	51		49		59		0.25
<i>Renal function parameters</i>							
Serum creatinine, $\mu\text{mol/l}$							
Median	122		126		126		0.56
IQR	98-162		101-159		103-160		
eGFR, ml/min per 1.73 m ²	55		53		50		0.06
Proteinuria ≥ 0.5 g/d							
n	49	21	46		44	19	0.85
%	23		22		21		0.27
Albumin excretion, mg/24h							
Median	37		50		41		
IQR	9-186		10-184		11-128		
<i>Serum parameters</i>							
Albumin, g/l	43	3	43	3	43	3	0.86
Cholesterol, mmol/l	5.0	1.1	5.1	1.1	5.2	1.2	0.12
LDL cholesterol, mmol/l	2.9	0.9	3.0	0.9	3.0	1.0	0.39
HDL cholesterol, mmol/l	1.4	0.5	1.4	0.5	1.4	0.5	0.23
Triglycerides, mmol/l							
Median	1.7		1.7		1.7		0.92
IQR	1.3-2.3		1.2-2.3		1.2-2.2		
HbA1c, %							
Hs-CRP, mg/l	5.9	0.7	6.0	0.9	6.0	0.8	0.35
Median	1.4		1.6		1.7		0.26
IQR	0.6-3.8		0.6-4.5		0.8-5.0		

Abbreviations: EPA-DHA, eicosapentaenoic acid-docosahexaenoic acid; En%, Energy Percentage; BMI, Body mass index; BSA, Body surface area; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FFQ, Food Frequency Questionnaire; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, High density lipoprotein; HbA1c, glycated hemoglobin; Hs-CRP, High-sensitive C-reactive protein

Supplemental Table 2: Patient characteristics of 637 RTR across tertiles of ALA intake (En%)

Characteristics	Tertile I N=212		Tertile II N=213		Tertile III N=212		P
	Mean	SD	Mean	SD	Mean	SD	
<i>N-3 fatty acid intake</i>							
EPA-DHA intake, En%							
Median	0.38		0.52		0.71		<0.001
IQR	0.33-0.42		0.48-0.55		0.65-0.81		
EPA-DHA intake cut-off points, En%	<0.45		0.45-0.60		>0.60		
EPA-DHA intake, mg/d							
Median	0.81		1.19		1.83		<0.001
IQR	0.65-0.98		0.97-1.50		1.43-2.20		
<i>Demographics</i>							
Age, yr	55	12	51	12	52	13	0.01
Gender (% male)	60		56		64		0.03
BMI, kg/m ²	26	4	26	5	27	5	0.11
BSA, m ²	1.9	0.2	1.9	0.2	2.0	0.2	0.02
Smoking behavior (current) n (%)							0.49
Never	40		40		45		
Ever	45		47		46		
Current	15		14		10		
Diabetes (yes)							0.25
n	55		43		56		
%	26		20		26		
<i>Haemodynamic parameters</i>							
SBP, mmHg	137	18	136	17	136	17	0.71
DBP, mmHg	82	11	83	11	84	11	0.10
MAP, mmHg	100	12	101	12	101	12	0.64
Heart rate, bpm	68	12	68	12	70	12	0.38
<i>Dietary intake (FFQ)</i>							
Energy intake							
Kcal/d	2056	546	2152	581	2318	742	<0.001
KJ/d	8619	2286	9020	2436	9713	3097	<0.001
Total fat intake, g/d	77	26	86	27	102	42	<0.001
Total protein intake, g/d	81	19	80	19	85	22	0.03
Total carbohydrates, g/d	245	76	251	80	251	78	0.66
Alcohol intake (%)							0.01
0 g/d	16		3		10		
0-10 g/d	57		69		65		
10-30 g/d	23		24		19		
>30 g/d	5		4		6		

Supplemental Table 2: Continued

Characteristics	Tertile I N=212		Tertile II N=213		Tertile III N=212		P
	Mean	SD	Mean	SD	Mean	SD	
Medication use							
Antihypertensives, (%)							
Statins, (%)			86		90		0.38
			56		51		0.69
Renal function parameters							
Serum creatinine, $\mu\text{mol/l}$							
Median			125		124		0.85
IQR			99-168		103-155		
eGFR, ml/min per 1.73 m ²	51		53		53		0.59
Proteinuria ≥ 0.5 g/d				20			
n		19				21	0.43
%		22				19	
Albumin excretion, mg/24h							0.13
Median		44		51		32	
IQR		10-165		13-214		8-130	
Serum parameters							
Albumin, g/l	43		43		43		0.59
Cholesterol, mmol/l	5.2	1.2	5.1	1.1	5.1	1.1	0.74
LDL cholesterol, mmol/l	3.0	1.0	3.0	0.9	3.0	0.9	0.83
HDL cholesterol, mmol/l	1.5	0.5	1.4	0.5	1.3	0.4	0.01
Triglycerides, mmol/l							
Median	1.7		5.9		1.7		0.64
IQR	1.2-2.2		1.2-2.3		1.3-2.5		
HbA1c, %	6.0				6.0		0.24
Hs-CRP, mg/l		0.9		0.7		0.8	
Median	1.4		1.7		1.6		0.40
IQR	0.6-3.5		0.7-5.0		0.7-5.1		

Abbreviations: ALA, α -linolenic acid; En%, Energy Percentage; BMI, Body mass index; BSA, Body surface area; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; FFQ, Food Frequency Questionnaire; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein; HDL, High density lipoprotein; HbA1c, glycated hemoglobin; Hs-CRP, High-sensitive C-reactive protein

CHAPTER 7

Intake of marine-derived polyunsaturated fatty acids and mortality in renal transplant recipients

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ABSTRACT

The effect of marine-derived omega-3 polyunsaturated fatty acids (n-3 PUFA) on long-term outcome in renal transplant recipients (RTR) remains unclear. We investigated whether marine-derived n-3 PUFA intake is associated with all-cause and cardiovascular (CV) mortality in RTR. Intake of eicosapentaenoic acid plus docosahexaenoic acid (EPA-DHA) was assessed using a validated Food Frequency Questionnaire. Cox regression analyses were performed to evaluate the associations of EPA-DHA intake with all-cause and CV mortality. We included 627 RTR (age 53 ± 13 years). EPA-DHA intake was 102 (42–215) mg/day. During median follow-up of 5.4 years, 130 (21%) RTR died, with 52 (8.3%) due to CV causes. EPA-DHA intake was associated with lower risk of all-cause mortality (Hazard Ratio (HR) 0.85; 95% confidence interval (95% CI) 0.75–0.97). Age ($p = 0.03$) and smoking status ($p = 0.01$) significantly modified this association, with lower risk of all-cause and CV mortality particularly in older (HR 0.75, 95% CI 0.61–0.92; HR 0.68, 95% CI 0.48–0.95) and non-smoking RTR (HR 0.80, 95% CI 0.68–0.93; HR 0.74, 95% CI 0.56–0.98). In conclusion, marine-derived n-3 PUFA intake is inversely associated with risk of all-cause and CV mortality in RTR. The strongest associations were present in subgroups of patients, which adds further evidence to the plea for EPA-DHA supplementation, particularly in elderly and non-smoking RTR.

INTRODUCTION

Renal transplantation offers superior survival, quality of life, and cost-effectiveness in comparison with chronic dialysis treatment [1-8]. Thus, it is considered the “gold standard” treatment for most patients with end-stage renal disease. However, compared to age-matched controls in the general population, the survival of renal transplant recipients (RTR) continues to be significantly lower [9], with cardiovascular (CV) disease as the most important contributor to excess of mortality in RTR [10,11].

The consumption of polyunsaturated fatty acids may reduce the CV risk profile. Consequently, several countries and organizations such as the World Health Organization have made population-based diet recommendations for omega-3 polyunsaturated fatty acids (n-3 PUFA) intake. Fish are the major food source of the n-3 PUFAs docosahexaenoic acid (DHA, C22:6 n-3) and eicosapentaenoic acid (EPA, C20:5 n-3). Thus, recommendations of EPA-DHA intake can easily be met by following dietary guidelines such as those of the American Heart Association, which advises consumption of at least two fish meals per week [11,12].

Evidence from both epidemiologic and interventional studies have shown that EPA and DHA may exert beneficial effects on all-cause and CV mortality in both patients with pre-existing CV disease as well as in healthy populations [13-19]. Prospective epidemiological studies have shown that weekly fish intake is associated with lower rates of coronary heart disease (CHD) mortality in men [20-22]. It has also been shown in women that intake of fish and n-3 PUFA are inversely associated with risk of CHD death [23]. Furthermore, in a study conducted across 36 countries, fish intake was associated with a reduced risk from all-cause and ischemic heart disease mortality [24]. In addition, interventional studies have reported a reduction in all-cause mortality in male survivors of a myocardial infarction (MI), who were advised to increase their dietary intake of oily fish [25]. The largest randomized control trial testing the efficacy of n-3 PUFA for secondary prevention of CHD so far included 11,324 patients. It showed a 15% reduction in the primary end point of death, nonfatal MI, and nonfatal stroke, and a 20% reduction in all-cause mortality in patients receiving n-3 PUFA supplementation [26].

In RTR, a recent observational cohort study performed in a total of 1990 Norwegian patients found that high levels of plasma phospholipid marine n-3 PUFA were independently associated with better long-term survival late after renal transplantation [27]. However, this study was on plasma biomarker status and not on intake per se. Hence, a recent systematic report from The Cochrane Collaboration concluded that there is still insufficient evidence to recommend fish oil supplementation to improve survival of RTR [28]. Nonetheless, in a recent study with relatively short follow-up, we found a trend towards an inverse association of intake of marine-derived n-3 PUFA with all-cause mortality in RTR [29].

We therefore set out to update data on intake of marine-derived n-3 PUFA and to extend follow-up to increase power for prospective evaluation of the association of marine-derived

n-3 PUFA intake with all-cause and CV mortality in the cohort of stable outpatient RTR. For this purpose, we investigated the intake of EPA and DHA, and evaluated its association with risk of all-cause and CV mortality in RTR.

METHODS

Study Design

An observational prospective study was conducted in a large single center RTR cohort [30]. All adult (≥ 18 years old) RTR with a functioning allograft for at least one year after transplantation and without known or apparent systemic illnesses (i.e., malignancies, opportunistic infections) who visited our outpatient clinic between November 2008 and March 2011 were invited to participate in this prospective cohort study. From a total of 817 RTR invited to be enrolled, 707 (87%) patients signed informed consent. We excluded all patients missing dietary or laboratory data, resulting in 627 RTR eligible for the statistical analysis. The present study was conducted according to the guidelines settled in the Declaration of Helsinki, and the Institutional Review Board approved the study protocol (METc 2008/186).

The primary long-term endpoints of the current study were all-cause and CV mortality in RTR. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status. We contacted general practitioners or referring nephrologists in cases the status of a patient was unknown. The patients were followed until 30 September 2015, with no loss to follow-up.

Renal Transplant Characteristics

RTR were all transplanted in the University Medical Center Groningen and had no history of drug or alcohol addiction according to their patient files. RTR were on standard antihypertensive and immunosuppressive therapy. Except for discouraging excess sodium intake and encouraging losing weight in overweight individuals, no specific dietary counseling was included as a routine regimen, nor was dietary recommendation regarding n-3 PUFA intake advised to participants. Relevant characteristics including recipient age, gender, CV history, and transplant information were extracted from patient records. Self-report questionnaires were used to obtain information on smoking behavior and alcohol intake. Physical activity was assessed using the Short Questionnaire to Assess Health-Enhancing Physical Activity (SQUASH) score in time multiplied by intensity [31].

Dietary Assessment

Dietary intake was assessed with a semi-quantitative validated food frequency questionnaire (FFQ) that was developed at Wageningen University [32]. The questionnaire inquired about intake of 177 food items during the last month. For each item, the frequency was documented in times

per day, week, or month. The number of servings was recorded in natural units (for example, slice of bread or apple) or household measures (for example, cup or spoon). The questionnaire was self-administered and filled in at home. All FFQ were checked for completeness on the day of the visit to the outpatient clinic by a trained researcher. The results of the FFQ were converted into total energy and nutrient intake by using the Dutch Food Composition Table of 2006 [33].

Measurements

All measurements were performed once at baseline during a morning visit to the outpatient clinic. Body weight and height were measured with patients wearing indoor clothing without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2), and body surface area (BSA) was estimated in meters squared (m^2) [34]. Blood pressure, heart rate, and mean arterial pressure were measured according to a strict protocol and determined with a semi-automatic device (Dinamap 1846, Critikon, Tampa, FL, USA). Blood pressure and heart rate were measured every minute for 15 min, and the last three measurements were averaged [35].

Blood was drawn after a fasting period of 8–12 h, which included no medication intake. Diabetes was defined as use of antidiabetic medication, fasting plasma glucose ≥ 7.0 mmol/L [36] or glycated hemoglobin (HbA_{1c}) higher than 6.5 as proposed by Shabir et al. [37]. Serum creatinine levels were measured with a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Serum cystatin C concentrations were measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics, Mannheim, Germany). Renal function was assessed by the estimated glomerular filtration rate (eGFR) based on the CKD-EPI equation of creatinine/cystatine C developed as proposed by Terpos et al. [38].

Serum albumin, high-sensitivity C-reactive protein (hs-CRP), HbA_{1c} , triglycerides, low-density lipoprotein (LDL)-, high-density lipoprotein (HDL)-, and total cholesterol were measured according to routine laboratory methods. According to a strict protocol, all RTR were asked to collect a 24-h urine sample during the day before their visit to the outpatient clinic. Total urinary protein concentration was determined using the Biuret reaction (MEGA AU 150, Merck Diagnostica, Darmstadt, Germany). Proteinuria was defined as urinary protein excretion ≥ 0.5 g/24 h.

Statistical Analyses

Data analysis was performed using SPSS version 22.0 software (IBM Corp., Armonk, NY, USA), STATA version 13 (StataCorp LP, College Station, TX, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). In all analyses, a two-sided $p < 0.05$ was considered significant. Continuous variables were summarized using mean (standard deviation, SD) for normally distributed data, whereas skewed distributed variables are given as median

(interquartile range (IQR)); percentages were used to summarize categorical variables. Marine-derived n-3 PUFA intake was accounted as the sum of EPA and DHA intake (100 mg/day) adjusted for total energy intake (kJ/day) according to the residual method [39].

Linear regression analyses were performed to evaluate the association of marine-derived n-3 PUFA intake with baseline characteristics. Natural log transformation was used for analyses of variables with a skewed distribution. Schoenfeld residuals of EPA-DHA intake (per 100 mg) were checked: the assumption of proportional hazards was not violated ($p = 0.65$).

In prospective analyses, Cox-proportional hazard regression analyses were performed to investigate the associations of marine-derived n-3 PUFA intake as continuous variable, with all-cause and CV mortality in RTR. Analyses were performed with adjustment for age and sex (Model 1) and additional adjustment for eGFR, proteinuria, and time between transplantation and baseline measurement (Model 2). To avoid the inclusion of too many variables for the number of events, further models were performed with additive adjustments to model 2. We performed additional adjustment for smoking status, alcohol use, and physical activity (Model 3); for BMI, diabetes mellitus, and CV history (Model 4); for LDL-, total cholesterol, triglycerides concentration, and systolic blood pressure (Model 5); for hs-CRP and albumin concentration (Model 6). Hazard ratios (HR) of Cox-regression models are reported with 95% confidence interval (CI).

In secondary analyses, we performed stratified analyses according to subgroups of age, sex, BMI, diabetes, cardiovascular history, renal function, smoking status, alcohol use, and physical activity. We also tested for interaction to identify potential effect modification.

RESULTS

Baseline Characteristics

Baseline characteristics of RTR are shown in **Table 1**. A total of 627 patients were included. At a median (IQR) of 5.7 (2.0-12.2) years after transplantation, participants were 53 ± 13 years old and 56.3% were male. The patients were slightly overweight (BMI 26.6 ± 4.7), and 152 (24.2%) had diabetes. Mean blood pressure of RTR was within normal boundaries ($136 \pm 17/83 \pm 11$ mmHg). It should, however, be noted that 552 (88%) of the 627 participants were using antihypertensive drugs. Mean eGFR was 45 ± 19 mL/min/1.73 m² and 139 (22.2%) patients had proteinuria. Mean serum total cholesterol was 5.1 ± 1.1 mmol/L, while statins were taken by 333 (53.1%) of the RTR. Eighty-seven percent of RTR reported the use of alcohol and 12.3% were current smokers. Smoking status was unknown in 25 (4.0%) of RTR. Total energy intake was 8756 (7224–10,636) kJ/day. EPA, DHA, and DHA-EPA intake were 39 (13–85), 60 (28–129), and 102 (42–215) mg/day, respectively.

Table 1: Baseline characteristics of RTR, and association of energy adjusted EPA-DHA intake across clinical parameters.

Clinical Variables	All Patients	EPA-DHA Intake (100 mg/Day)	
		Std. β	P
No. of patients	627	-	-
DHA, mg/day	60 (28 - 129)	-	-
EPA, mg/day	39 (13 - 85)	-	-
EPA-DHA, mg/day	102 (42 - 215)	-	-
Demographics			
Age, years	53 $\pm$ 13	0.13	0.001
Ethnicity (Caucasian), n (%)	625 (99.7)	0.03	0.46
Sex (male), n (%)	353 (56.3)	0.04	0.39
Body mass index, kg/m ²	26.6 $\pm$ 4.7	0.08	0.04
Body surface area, m ²	1.9 $\pm$ 0.2	0.01	0.78
Cardiovascular history, n (%)	251 (40.0)	0.04	0.28
Renal transplantation characteristics			
Pre-emptive transplantation, n (%)	102 (16.3)	0.003	0.93
Time between transplantation and baseline, years	5.7 (2.0 – 12.2)	- 0.002	0.95
Hemodynamic parameters			
Systolic blood pressure, mmHg	136 $\pm$ 17	0.001	0.98
Diastolic blood pressure, mmHg	83 $\pm$ 11	0.03	0.41
Mean arterial pressure, mmHg	108 $\pm$ 15	0.02	0.71
Heart rate, beats per minute	69 $\pm$ 12	0.01	0.78
Antihypertensives, n (%)	552 (88.0)	- 0.04	0.34
Renal function parameters			
Creatine, μ mol/L	123 (99 - 159)	0.005	0.91
Cystatine-C, mg/L	1.7 (1.3 – 2.2)	- 0.06	0.13
eGFR, ml/min/1.73 m ²	45 19	- 0.01	0.83
Proteinuria $\geq$ 0.5 g/day, n (%)	139 (22.2)	- 0.02	0.55
Glucose homeostasis			
Glucose, mmol/L	5.3 (4.8 – 6.0)	0.06	0.14
HbA1c, %	5.8 (5.5 – 6.2)	0.05	0.27
Diabetes, n (%)	152 (24.2)	0.02	0.60
Antidiabetic medication, n (%)	98 (15.6)	0.02	0.67
Serum parameters			
Albumin, g/L	43 $\pm$ 3.0	- 0.02	0.67
Hs-CRP, mg/L	1.6 (0.7 – 4.5)	0.06	0.13

Table 1: Continued

Clinical Variables	All Patients	EPA-DHA Intake (100 mg/Day)	
		Std. β	P
Lipids			
Total cholesterol, mmol/L	5.1 $\pm$ 1.1	0.08	0.06
LDL cholesterol, mmol/L	3.0 $\pm$ 0.9	0.06	0.13
HDL cholesterol, mmol/L	1.3 (1.1 – 1.7)	0.07	0.10
Triglycerides, mmol/L	1.7 (1.2 – 2.3)	- 0.01	0.75
Statin use, n (%)	333 (53.1)	0.02	0.57
Health lifestyle			
Current smoker, n (%)	77 (12.3)	- 0.008	0.85
Alcohol consumer, n (%)	547 (87.2)	- 0.14	< 0.001
Physical activity, intensity x hours	5250 (2400 - 8160)	0.03	0.41
Total energy intake, kj/day	8756 (7224 – 10,636)	-	-

Abbreviations: RTR, renal transplant recipients; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; eGFR, estimated glomerular filtration rate; HbA1C, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; kJ, kilojoule.

Association between Intake of Marine-Derived n-3 PUFA with Clinical Baseline Characteristics

The association between EPA-DHA intake with clinical baseline characteristics is shown in **Table 1**. EPA-DHA intake was positively associated with age (std. β = 0.13, p = 0.001) and BMI (std. β = 0.08, p = 0.04), and was negatively associated with alcohol use (std. β = -0.14, p < 0.001).

Results of multivariate Cox-proportional hazard regression analyses are shown in **Table 2**. During follow-up of 5.4 (4.9–6.0) years, 130 (20.7%) RTR died. EPA-DHA intake was associated with lower risk of all-cause mortality (Model 2: HR 0.85; 95% CI 0.75–0.97, p = 0.02) independent of potential confounders including age, sex, eGFR, proteinuria, and time between transplantation and baseline. This inverse association remained significant after additional adjustment for other potential confounders. The association of EPA-DHA intake with CV mortality was of similar magnitude, but did not reach statistical significance (Model 2: HR 0.83, 95% CI 0.68–1.02, p = 0.08).

Table 2: Prospective analysis of EPA-DHA intake (100 mg/day) on all-cause and CV mortality in RTR.

Model	EPA-DHA Intake, 100 mg/Day	
	HR (95% CI)	P
All-Cause Mortality		
Model 1	0.87 (0.77 – 0.99)	0.03
Model 2	0.85 (0.75 – 0.97)	0.02
Model 3	0.87 (0.77 – 1.00)	0.04
Model 4	0.87 (0.76 – 0.99)	0.03
Model 5	0.84 (0.73 – 0.96)	0.01
Model 6	0.85 (0.74 – 0.97)	0.02
CV Mortality		
Model 1	0.85 (0.69 – 1.05)	0.13
Model 2	0.83 (0.68 – 1.02)	0.08
Model 3	0.86 (0.70 – 1.07)	0.18
Model 4	0.84 (0.68 – 1.03)	0.10
Model 5	0.81 (0.64 – 1.01)	0.06
Model 6	0.82 (0.65 – 1.03)	0.08

Abbreviations: RTR, renal transplant recipients; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CV, cardiovascular.

Model 1: adjustment for age and sex. Model 2: model 1 + adjustment for estimated glomerular filtration Rate, proteinuria, and time between transplantation and baseline measurement. Model 3: model 2 + adjustment for smoking status, alcohol use, and physical activity. Model 4: model 2 + adjustment for body mass index, diabetes mellitus and cardiovascular history. Model 5: model 2 + adjustment for total cholesterol, low-density lipoprotein-cholesterol, triglycerides concentration, and systolic blood pressure. Model 6: model 2 + adjustment for high-sensitivity C-reactive protein and albumin concentration.

In secondary analyses, we found effect modification of age ($p = 0.03$) and smoking status ($p = 0.01$) on the association of EPA-DHA intake with all-cause mortality, independent of age and sex (**Figure 1**). Consequently, we proceeded with further prospective stratified-analyses by subgroups of age ($<$ or ≥ 63 years old) and smoking status (non-smokers or smokers), as depicted in **Supplemental Table 1 and Table 2**, respectively.

Figure 1. Stratified-analysis of the association of EPA-DHA intake with all-cause mortality in RTR. Hazard ratios adjusted for age and sex are shown. Subgroups with *p*-interaction < 0.05 were considered effect modifiers on the association of EPA-DHA intake with all-cause mortality.

EPA-DHA intake was associated with lower risk of all-cause mortality within the older subgroup of patients (Model 2: HR 0.75; 95% CI 0.61–0.92, *p* = 0.01) but not within the younger subgroup of RTR (Model 2: HR 0.98; 95% CI 0.85–1.18, *p* = 0.8). Likewise, EPA-DHA intake was associated with lower risk of CV mortality within the older subgroup of RTR (Model 2: HR 0.68; 95% CI 0.48–0.95, *p* = 0.02), but not in the younger subgroup of patients (Model 2: HR 1.02; 95% CI 0.80–1.29, *p* = 0.9).

DISCUSSION

In this study, we show that marine-derived n-3 PUFA intake is inversely associated with risk of all-cause mortality in a large cohort of stable outpatients RTR, and we show an association of similar magnitude—though not statistically significant—of marine-derived n-3 PUFA intake on the risk of CV mortality in RTR. We also show that age and smoking status are effect-modifiers of the association of marine-derived n-3 PUFA intake with all-cause mortality. Accordingly, further prospective age-stratified analysis shows that marine-derived n-3 PUFA intake is inversely associated with risk of all-cause and CV mortality within the older (cut-off point 63 years old), but not the younger subgroup of RTR. Likewise, prospective smoking status-stratified analysis shows that marine-derived n-3 PUFA intake is inversely associated with risk of all-cause and CV mortality within the non-smoker patient subgroup, but not within the subgroup of smoker RTR.

In the general population, it has been largely shown that marine-derived n-3 PUFA intake may exert beneficial effects on both CV endpoints and all-cause mortality [13-19]. The mechanisms by which increased ingestion of n-3 PUFAs exert favorable effects on CV health are not fully known; however, it has been proposed that reducing susceptibility of the heart to ventricular arrhythmia, retarding growth of atherosclerotic plaques, reducing adhesion molecule expression, reducing platelet-derived growth factor, promoting nitric oxide-induced endothelial relaxation and anti-inflammatory effects may be underlying mechanisms involved [13].

Up-to-date results regarding their potential beneficial effects on survival after renal transplantation remain controversial thus far. Previously, Eide and colleagues reported that in a cohort of 1990 Norwegian RTR with 406 deaths events at a median follow-up of 6.8 years, higher plasma phospholipid marine n-3 PUFA levels were independently associated with better patient survival in RTR [40]. Importantly, this study was not performed on intake per se, but on plasma biomarker status of marine-derived n-3 PUFA, which is a resultant of intake and metabolism. However, a recent systematic report from the Cochrane Collaboration concluded that there is still insufficient evidence to recommend fish oil supplementation to improve survival of RTR [28]. On the basis of the latter statement, it seems that the beneficial effect of marine-derived n-3 PUFA on survival of RTR remains under discussion. Importantly, it is critical to consider that inconsistent results across the three randomized control trials accounted in this systematic review (for the outcome of mortality in RTR) may arise from methodological problems such as differing doses of fish oils, duration and timing of treatment, small number of patients, and a number of events too low to establish definitive conclusions. Previously, we reported a trend towards an inverse association of intake of marine-derived n-3 PUFA with all-cause mortality in RTR, with relatively short follow-up of RTR [29]. The current study updated data on n-3 PUFA intake and extended follow-up of our cohort. The findings of the current study support the notion [17,18,19,27] that marine-derived n-3 PUFAs exert beneficial effects on long-term survival after renal transplantation. Moreover, our findings support that favorable outcomes offered by marine-derived n-3 PUFA intake may vary among different subgroups of RTR according to age and smoking status.

It is not completely understood which are the underlying mechanisms that explain the effect modification of age on the association of n-3 PUFA intake with mortality. Despite the fact that chronic low-grade inflammation remains after renal transplantation and that many of the beneficial effects of n-3 PUFA have been attributed to their anti-inflammatory properties [41], we did not find an association of n-3 PUFA intake with inflammatory biomarkers. However, it has been shown that independently of any influence on inflammation, n-3 PUFA may be an effective intervention against anabolic resistance [42-45], which is known to be one of the main causes of age-related sarcopenia. Animal studies have shown that n-3 PUFAs improve whole-body- as well as muscle insulin-sensitivity [46]. Moreover, n-3 PUFA supplementation helps to

maintain whole-body protein metabolism via mTOR signaling pathways [47]. In healthy young adults, n-3 PUFA supplementation has been shown to increase the anabolic response [43], and particularly in older adults, an increase of muscle protein synthesis has been reported [42]. Thus, one might speculate that the effect modification of age on the association of n-3 PUFAs with mortality could be at least partly explained through the beneficial effect of n-3 PUFAs on muscle protein synthesis.

Regarding smoking status as an effect modifier, it should be noted that it has previously been suggested that oral supplementation of marine-derived n-3 PUFA may have effects that are distinct between smokers and non-smokers [48]. Research regarding biomarkers of n-3 PUFA has already shown that n-3 PUFA biomarkers are lower in smokers. It is noteworthy that this relationship was independent of dietary intake of n-3 PUFAs [49]. Furthermore, it has been suggested that smoking may affect fatty acid absorption and metabolism [50], including increased n-3 lipid peroxidation in smokers [51,52]. Future studies are needed to better understand the effect of smoking on the metabolism of n-3 PUFAs.

The strengths of our study include a median follow-up of 5.4 years with all-cause and CV mortality as clinically relevant endpoints, without loss of participants due to follow-up. Furthermore, our study included a large sample size of the specific setting of stable outpatient RTR. Moreover, data was extensively collected, which allowed adjustment for many potential confounders of the main results. On the other hand, we acknowledge that the current study has several limitations; for example, marine-derived n-3 PUFA intake was measured using a self-reporting FFQ, which could lead to possible over- or under-reporting of dietary intake. Moreover, dietary intake was only measured on baseline and therefore changes in dietary intake during the follow-up period might not be accounted for in our analysis. The higher the intra-individual variation of n-3 PUFA intake along the follow-up period would be, the greater one would expect the benefit of repeated measurement for prediction of outcomes [53,54]. Plasma biomarkers of n-3 PUFA were not available in this cohort study. Data regarding plasma status of marine-derived n-3 PUFA could have provided better understanding of the relation between dietary intake with plasma levels of marine-derived n-3 PUFA biomarkers. Further, CV complications or interventions were not documented; therefore, we were unable to assess the effect of dietary intake of n-3 PUFA on non-fatal CV-events. Additionally, our study population consisted predominantly of Caucasian people, which calls for prudence to extrapolate our results to different populations with regard to ethnicity. Finally, since the current study is observational by design, no conclusions of causality can be drawn from our results.

CONCLUSIONS

In conclusion, marine-derived n-3 PUFA intake is inversely associated with a lower risk of all-cause mortality in RTR. Furthermore, according to effect modifiers of this association, the beneficial effects of marine-derived n-3 PUFA intake are different over subgroups of RTR. Thus, marine-derived n-3 PUFA intake is inversely associated with all-cause and CV mortality in the older but not the younger subgroup of patients. In turn, marine-derived n-3 PUFA intake is associated with lower risk of all-cause and CV mortality in the non-smoker but not in the smoker subgroup of RTR. Our findings support the protective effect of marine-derived n-3 PUFA intake on all-cause and CV mortality that has been previously reported. We provide relevant data on intake complementary to existing data on plasma biomarkers, further adding to the evidence that they are protective. However, due to methodological heterogeneity across different studies, further design of multicenter, randomized, double-blind, placebo-controlled trials aimed at testing the beneficial effects of marine-derived n-3 PUFA supplementation on mortality of RTR are still lacking.

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Supplemental Table 1: Prospective age-stratified analysis of EPA-DHA intake (100 mg/day) on all-cause and CV mortality in RTR.

Type	EPA-DHA Intake, 100 mg/day			
	<63 years old		≥63 years old	
No. of subjects	474		153	
	HR (95 % CI)	<i>p</i>	HR (95 % CI)	<i>p</i>
No. of events	65		65	
Model 1	0.98 (0.84–1.14)	0.8	0.77 (0.63–0.95)	0.01
Model 2	0.98 (0.84–1.15)	0.8	0.75 (0.61–0.91)	0.01
Model 3	0.98 (0.86–1.17)	0.9	0.78 (0.63–0.96)	0.02
Model 4	0.98 (0.84–1.15)	0.8	0.75 (0.61–0.93)	0.01
Model 5	0.97 (0.82–1.14)	0.7	0.73 (0.59–0.90)	0.004
Model 6	0.98 (0.83–1.17)	0.8	0.73 (0.58–0.91)	0.004
No. of events	25		27	
Model 1	1.01 (0.80–1.27)	0.9	0.70 (0.50–1.00)	0.05
Model 2	1.02 (0.80–1.29)	0.9	0.68 (0.48–0.95)	0.02
Model 3	1.04 (0.82–1.32)	0.8	0.68 (0.47–0.99)	0.05
Model 4	1.01 (0.80–1.26)	0.9	0.66 (0.47–0.95)	0.02
Model 5	0.97 (0.74–1.26)	0.8	0.67 (0.46–0.98)	0.04
Model 6	1.02 (0.78–1.31)	0.9	0.65 (0.44–0.96)	0.03

Abbreviations: RTR, renal transplant recipients; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; CV, cardiovascular. Model 1: adjustment for age and sex. Model 2: model 1 + adjustment for estimated Glomerular Filtration Rate, proteinuria and time between transplantation and baseline measurement. Model 3: model 2 + adjustment for smoking status, alcohol use, and physical activity. Model 4: model 2 + adjustment for body mass index, diabetes mellitus and cardiovascular history. Model 5: model 2 + adjustment for total cholesterol, low-density lipoprotein-cholesterol, triglycerides concentration, and systolic blood pressure. Model 6: model 2 + adjustment for high-sensitivity C-reactive protein and albumin concentration.

Supplemental Table 2: Prospective smoking status-stratified analysis of EPA-DHA intake (100 mg/day) on all-cause and CV mortality in RTR.

Type	EPA-DHA Intake, 100 mg/day			
	Non-smokers		Smokers	
No. of subjects	525		77	
	HR (95 % CI)	<i>p</i>	HR (95 % CI)	<i>p</i>
All-cause mortality				
No. of events	104		18	
Model 1	0.81 (0.69–0.95)	0.01	1.12 (0.89–1.41)	0.3
Model 2	0.80 (0.68–0.93)	0.01	1.15 (0.89–1.48)	0.3
Model 3	0.81 (0.70–0.96)	0.01	1.15 (0.88–1.50)	0.3
Model 4	0.80 (0.68–0.94)	0.01	1.18 (0.88–1.57)	0.3
Model 5	0.80 (0.68–0.94)	0.01	1.11 (0.82–1.50)	0.5
Model 6	0.78 (0.66–0.92)	0.003	1.23 (0.91–1.66)	0.2
CV mortality				
No. of events	38		9	
Model 1	0.76 (0.57–1.01)	0.05	1.15 (0.85–1.57)	0.4
Model 2	0.74 (0.56–0.98)	0.04	1.16 (0.80–1.69)	0.4
Model 3	0.76 (0.57–1.00)	0.05	1.24 (0.83–1.84)	0.3
Model 4	0.73 (0.55–0.97)	0.03	1.17 (0.79–1.73)	0.4
Model 5	0.76 (0.57–1.02)	0.06	1.03 (0.64–1.67)	0.9
Model 6	0.68 (0.50–0.94)	0.02	1.21 (0.76–1.92)	0.4

Abbreviations: RTR, renal transplant recipients; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; CV, cardiovascular. Model 1: adjustment for age and sex. Model 2: model 1 + adjustment for estimated Glomerular Filtration Rate, proteinuria and time between transplantation and baseline measurement. Model 3: model 2 + adjustment for alcohol use, and physical activity. Model 4: model 2 + adjustment for body mass index, diabetes mellitus and cardiovascular history. Model 5: model 2 + adjustment for total cholesterol, low-density lipoprotein-cholesterol, triglycerides concentration, and systolic blood pressure. Model 6: model 2 + adjustment for high-sensitivity C-reactive protein and albumin concentration.

CHAPTER 8

Influence of prednisolone on parameters of de novo lipogenesis and indices for steaoryl-CoA and $\Delta 6$ -desaturase activity in healthy males: A post-hoc analysis of a randomized, placebo-controlled, double-blind trial

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ABSTRACT

Background: Glucocorticoid treatment decreases liver insulin sensitivity and may modify fatty acid metabolism. We investigated the influence of oral prednisolone on indices for *de novo* lipogenesis (DNLi), stearoyl-CoA desaturase (SCDi) and $\Delta 6$ -desaturase (D6Di) activity in healthy males. In addition, we explored whether the changes may be associated with prednisolone-induced changes in glucose and lipid metabolism and insulin sensitivity.

Methods: Thirty-two healthy young males (mean $\pm$ SD age 22 ± 3 years, BMI 22.4 ± 1.7 kg/m²) were allocated to receive prednisolone 7.5 mg/day (PRED7.5; n=12), prednisolone 30 mg/day (PRED30; n=12), or placebo (n=8) in a randomized double-blind fashion for 2 weeks. Fatty acid compositions of plasma cholesteryl esters (CE), phospholipids (PL) and triglycerides (TG) were measured at baseline and on day 14. DNLi, SCDi and D6Di were estimated from product/precursor ratios in CE, with DNLi primary deriving from 16:1 ω 7/18:2 ω 6, SCDi from 16:1 ω 7/16:0 and D6Di from 22:6 ω 3/20:5 ω 3. Ratios were also assessed in PL and TG.

Results: In CE, PRED30 increased DNLi by 51.2 [95%CI 14.8 ; 87.6], increased SCDi by 48.6 [18.7 ; 78.5]%, and decreased D6Di by 57.7 [-91.8 ; -23.5]% ($p \leq 0.01$ for all, compared to placebo). The prednisolone-induced increases in DNLi and SCDi were positively correlated with insulin sensitivity ($r = 0.35$ and 0.50 , respectively). Similar results were found in PL and TG.

Conclusion: Prednisolone dose-dependently increases DNLi and SCDi and decreases D6Di in plasma CE, PL and TG in healthy males after 2 weeks. The observed unfavorable effects on fatty acid metabolism were related to the induction of glucocorticoid-induced insulin resistance.

INTRODUCTION

Glucocorticoids (such as prednisolone) are anti-inflammatory drugs commonly used to treat both acute and chronic inflammatory diseases, including gout, pulmonary infection, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and psoriasis [1,2]. Intake of glucocorticoids is high, especially in the US, where the prevalence of oral glucocorticoids in the general population is around 1.2% [3]. Glucocorticoid treatment is associated with multiple adverse effects, including systemic hypertension, impaired glucose metabolism and overt diabetes, dyslipidemia, osteoporosis, muscle and skin atrophy and increased susceptibility to infections [4-9]. Furthermore, chronic exposure to increased glucocorticoid levels, e.g. as observed in patients with Cushing's disease, causes unfavorable body fat distribution and increased cardiometabolic risk [10].

Glucocorticoids alter metabolic processes. Our previous study indicated that even short term use of low-dose glucocorticoids may impair insulin sensitivity in the liver, and modify fatty acid metabolism in healthy subjects [11]. Glucocorticoids also stimulate gluconeogenesis which could eventually result in an increase in hepatic *de novo* lipogenesis (DNL) [12-14]. Animal studies suggested that glucocorticoids influence other processes of the fatty acids acid metabolism as well, i.e. an increase in the activity of stearoyl-CoA desaturase (SCD; also named $\Delta 9$ desaturase) and a decrease in the activity of $\Delta 6$ -desaturase (D6D) [15,16]. Whether this also occurs in humans is unknown.

DNL via fatty acid synthase ends in the production of palmitic acid (16:0), which can subsequently become elongated to stearic acid (18:0) [17]. 16:0 and 18:0 may subsequently become desaturated to palmitoleic (16:1 ω 7), oleic (18:1 ω 9) and vaccenic (18:1 ω 7) acids (**Figure 1a**) [18,19]. SCD catalyzes the desaturation of 16:0 and 18:0 to 16:1 ω 7 and 18:1 ω 9, respectively [18]. D6D is a key enzyme in the conversion of 18:2 ω 6 to arachidonic acid (20:4 ω 6) and of alpha-linolenic acid (18:3 ω 3) to eicosapentaenoic (20:5 ω 3) and docosahexaenoic (22:6 ω 3) acids (**Figure 1b**) [15,16]. An indirect manner to estimate DNL, and the activities of SCD and D6D is by calculating their product/precursor ratios, which provide indices for their respective activities.

Studies investigating the effects of oral glucocorticoids on fatty acid metabolism in humans have mainly focused on adipose tissue and lipolysis [14,20-22]. To the best of our knowledge, there are few studies investigating the effects of glucocorticoids on lipogenesis (DNLindex; DNLi) by measuring whole body DNL using indirect calorimetry, but there are no reports from human studies on the SCD index (SCDi) and the D6D index (D6Di). In this study, we conducted a *post-hoc* analysis of a 2-week randomized, placebo-controlled, double-blind trial, in which the dose-dependent effect of oral prednisolone on fatty acid metabolism was investigated in healthy young males. As a secondary aim, we investigated whether the changes in fatty acid metabolism may be associated with prednisolone-induced changes in fasting metabolic parameters (circulating triglycerides (cTG), insulin, glucose) and insulin sensitivity reported in our previous study [11].

Figure 1a: Major saturated and monounsaturated fatty acids produced by the *de novo* lipogenesis (DNL).

¹16:0, 18:0, 16:1ω7, 18:1ω7 and 18:1ω9 dilute the pool of 18:2ω6.

Primary measurements and data analyses were done in plasma CE (DNLi: 16:1ω7/18:2ω6, SCDi: 16:1ω7/16:0), while secondary measurements and data analyses were performed in plasma PL and TG. Extra analyses (CE, PL, TG) DNLi: 16:0/18:2ω6, 18:0/18:2ω6, 18:1ω7/18:2ω6, 18:1ω9/18:2ω6; SCDi: 18:1ω7/16:0 and 18:1ω9/18:0.

Abbreviations: Acetyl CoA, Acetyl-coenzyme A; CE, cholesteryl esters; DNL, De novo lipogenesis index; PL, phospholipids; TG, triglycerides; SCDi, Stearoyl Coenzyme A index.

Figure 1b: Elongations and desaturation of polyunsaturated fatty acids by D5D and D6D activities.

Primary measurements and data analyses were done in plasma CE (D6Di: 22:6ω3/20:5ω3), while secondary measurements and data analyses were performed in plasma PL and TG. Extra analyses (CE, PL, TG) D6Di: 18:3ω6/18:2ω6; D5Di: 20:4ω6/20:3ω6. Abbreviations: AA, Arachidonic acid; ALA, α-Linolenic acid; CE, cholesteryl esters; D5Di, Delta-5-Desaturase index; D6Di, Delta-6-Desaturase index; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic acid; PL, phospholipids; TG, triglycerides.

PATIENTS AND METHODS

Study design and population

A *post-hoc* analysis was performed of a randomized placebo-controlled, parallel-group, double-blind dose-response intervention study, conducted at the Diabetes Center of the VU University Medical Center, and the Metabolic Research Unit of the Academic Medical Center in Amsterdam, The Netherlands [11]. Briefly, 32 healthy Caucasian young men were recruited via local advertisements. Inclusion criteria included: age 18-35 years; BMI 20-25 kg/m²; and normal glucose metabolism defined as fasting plasma glucose (FPG) < 5.6 mmol/l and 2 h glucose < 7.8 mmol/l following a 75-g oral glucose tolerance test (OGTT). Subjects with a history of any disease, use of medication, first-degree relative with type 2 diabetes, smoking history or current smoking, shift work, a history of glucocorticoid use and sporting activities more than twice/week were excluded from the study. Furthermore, participants with unstable weight in the past 3 months were also excluded.

Participants were allocated to receive either prednisolone 7.5 mg once-daily (PRED7.5; n=12), prednisolone 30.0 mg once-daily (PRED30; n=12), or placebo (n=8) for a period of 2 weeks using block randomization. Before randomization and on day 13 of drug-exposure, body composition, body fat distribution and liver fat content were quantified. At baseline and on day 14 of drug-exposure, glucose kinetics and lipolysis were measured. All measurements were conducted following a 12 h overnight fast. Participants refrained from drinking alcohol for a period of 24 h before testing and did not perform strenuous exercise for a period of 48 h before the study days. The study protocol was approved by an independent ethics committee and conducted in accordance with the Declaration of Helsinki. This trial is registered in the ISRCTN registry (ID: ISRCTN83991850). All participants provided written consent before participation.

Study Medication

Prednisolone tablets were purchased from Pfizer (Sollentuna, Sweden) and matching placebo tablets were obtained from Xendo Drug Development (Groningen, The Netherlands). The tablets were encapsulated in order to allow the treatment to be blinded. Study medication was taken orally at 08:00 h for 2-weeks, except on days 13 and 14, when it was ingested at 06:00 h.

Clinical parameters

Bodyweight and height were measured at baseline. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m²). Systolic and diastolic blood pressure were measured manually by a single experienced investigator following a 30 min acclimatization period. The average of three consecutive blood pressure measurements,

performed with 5 min intervals, was used for further analyses. Body composition was measured by dual energy X-ray absorptiometry (DEXA) scans (Delphi A; Hologic, Waltham, MA, USA) [23,24].

Metabolic variable analyses

Fasting state blood samples were drawn for the determination of basal glucose, non-esterified fatty acid (NEFA), insulin and glucagon concentrations. Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line Plus glucose analyser (EKF Diagnostics, Barleben/Magdeburg, Germany). Plasma NEFA concentrations were measured with an enzymatic colorimetric method (NEFA-C test kit; Wako Chemicals, Neuss, Germany). Insulin was determined with an Immulite 2000 system (Diagnostic Products, Los Angeles, CA, USA) that employs a chemiluminescent immunometric assay. Glucagon was determined with the Linco ¹²⁵I radioimmunoassay (St Charles, MO, USA). Homeostatic model assessment – Insulin resistance (HOMA-IR) was calculated to estimate insulin resistance by using the following equation: (fasting glucose (mmol/l) x fasting insulin (μU/l))/22.5 [25].

Fatty acid analyses

EDTA-plasma samples were collected at baseline and after 2-weeks drug-exposure. Analyses of fatty acids were performed at the Department of Laboratory Medicine of the University Medical Center Groningen, The Netherlands using the methodology as described by Hoving *et al.* [26]. Total lipids were extracted by the method of Folch *et al.*, using 3.75 ml of chloroform-methanol (2:1) and a 100 μl EDTA-plasma sample [27]. A shortened version of the method of Kaluzny *et al.* was used to isolate cholesteryl esters (CE), phospholipids (PL) and triglycerides (TG), using aminopropyl SPE columns for the separation of the plasma lipid classes (Isolute, Biotage) [28]. Fatty acids were transmethyalted with methanolic-HCL into fatty acid methyl esters (FAME). The samples were extracted with hexane and eventually redissolved into 100 μl hexane. 100 μl of an internal standard for the quantification of fatty acids in CE (17:0) (50.1 mg/100 ml chloroform-methanol, 2:1 v/v) and TG (19:0) (19.9 mg/100 ml chloroform-methanol, 2:1 v/v), both obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), were added before isolation of classes. For the quantification of fatty acids in PL, 100 μl of free fatty acid 17:0 (50.0 mg/100 ml methanol), obtained from Larodan (Solna, Sweden), was added after isolation of classes. 100 μl Butylated Hydroxytoluene (1 g/100 ml methanol) from Sigma-Aldrich (Zwijndrecht, The Netherlands) was added to prevent fatty acid oxidation.

Aliquots of 2 μl were injected into an Agilent model 6890 gas chromatography equipped with a 50 m x 0.2 mm x 0.11 μm apolar column (CMS WCOT capillary column) and detected with an Agilent 7683 series flame ionization detector. FAME were identified by comparing retention times with those of known standards. Fatty acids were eventually calculated into mol%. DNLi, SCDi and D6Di were primary assessed in plasma CE and secondary in plasma PL and TG.

Parameters of DNLI, SCDi and D6Di

DNLI, SCDi and D6Di at baseline and after 2-weeks drug-exposure were estimated from their product/precursor ratios (PPR) or the ratio of the product to a fatty acid with relatively stable status. DNLI was primary assessed as 16:1 ω 7/18:2 ω 6. 16:1 ω 7 is one of the products of DNL and might be a more specific marker than 16:0, which is the end product of fatty acid synthase activity commonly used for PPR calculations reflecting DNL [22]. An increase in DNL may be expected to increase 16:1 ω 7 and other DNL-derived fatty acids and thereby dilute the relative amount of the essential fatty acid 18:2 ω 6. SCDi was primary assessed as the 16:1 ω 7/16:0 ratio, a PPR commonly used as a marker of SCDi [22,29,30]. D6Di was primary assessed as the 22:6 ω 3/20:5 ω 3 ratio. Although this is not a *direct* marker for D6Di, D6D is widely considered to be rate limiting in the conversion of 20:5 ω 3 to 22:6 ω 3 [16,31,32].

In additional analyses, DNLI, was calculated as 16:0/18:2 ω 6, 18:0/18:2 ω 6, 18:1 ω 7/18:2 ω 6 and 18:1 ω 9/18:2 ω 6, SCDi as 18:1 ω 7/16:0 and 18:1 ω 9/18:0, and D6Di as 20:3 ω 6/18:2 ω 6 and 18:3 ω 6/18:2 ω 6 [32]. Furthermore, another desaturase activity enzyme (Delta-5-desaturase index; D5Di), operational in e.g. AA (20:4 ω 6) synthesis, was calculated as 20:4 ω 6/20:3 ω 6 [16,30,33].

Statistical analyses

Data analyses were performed using SPSS statistics (Version 22.0; IBM SPSS Inc. Chicago, IL) and GraphPad Prism (Version 5.03; Graphpad Software, La Jolla, CA). Data are presented as mean $\pm$ SD or median (interquartile range (IQR)) in case of skewed data. The change in plasma fatty acids (mol%) after 2-week prednisolone or placebo was calculated as Delta% ([Fatty acid study end - Fatty acid baseline]/[Fatty acid Baseline] * 100%). The change in DNLI, SCDi and D6Di after 2-week prednisolone or placebo was also calculated as Delta% ([PPR study end – PPR baseline]/[PPR baseline] * 100%). Between group changes of fatty acids (mol%) and PPRs were tested with ANCOVA, with change in fatty acids or PPR (Delta%) as continuous variable and baseline fatty acids or PPR as covariate. PRED7.5 and PRED30 were compared against placebo by post-hoc testing. Notable outliers (defined as: 1) values < Quartile 1 (Q1) – 3 * IQR, or 2) values > Quartile 3 (Q3) + 3 * IQR) were removed before analyses. Between group changes in fasting plasma glucose, insulin, glucagon, NEFA, circulating TG (cTG) and HOMA-IR were calculated and tested using the same statistical methods. Univariate correlations between change in fasting plasma glucose, insulin, glucagon, NEFA, cTG and HOMA-IR, and change in DNLI, SCDi and D6Di were assessed using Spearman correlation. All primary analyses were done with data from plasma CE, while secondary analyses were performed with data from plasma PL and TG. A P-value < 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics were generally well balanced between groups at inclusion (**Table 1**). Lean body mass (LBM) and total fat mass percentages were on average $81.2 \pm 6.1\%$ and $15.8 \pm 3.6\%$, respectively. No treatment-related side effects were reported.

In plasma CE, prednisolone did not affect the saturated fatty acids 16:0 and 18:0 (**Supplemental Table 1A**). Prednisolone affected the fatty acids 16:1 ω 7, 18:3 ω 6 and 20:5 ω 3. Both administration of PRED7.5 and PRED30 resulted in increases of 16:1 ω 7 by 35.1 [5.0 ; 65.1]% ($p = 0.02$) and 59.9 [95%CI 28.5 ; 90.1]% ($p = 0.004$), respectively. PRED30, but not PRED7.5, resulted in an increase of 18:3 ω 6 by 60.0 [25.6 ; 94.3]% ($p = 0.001$) and an increase of 20:5 ω 3 by 55.9 [23.2 ; 88.7]% ($p = 0.002$). In secondary analyses, PRED30, but not PRED7.5, increased both 16:0 and 18:0 in plasma TG and 16:1 ω 7 in plasma PL and TG, while 20:5 ω 3 in plasma PL was increased by both PRED7.5 and PRED30 (**Supplemental Table 1B, 1C**).

Table 1: Baseline characteristics

Characteristics	Placebo	PRED7.5	PRED30
<i>n</i>	8	12	12
Age (years)	22 $\pm$ 3	22 $\pm$ 2	22 $\pm$ 3
Weight (kg)	76.9 $\pm$ 8.7	74.8 $\pm$ 7.2	77.0 $\pm$ 5.4
BMI (kg/m ²)	22.7 $\pm$ 2.1	22.2 $\pm$ 1.9	22.4 $\pm$ 1.2
LBM (%)	78.6 $\pm$ 7.9	82.1 $\pm$ 3.9	80.7 $\pm$ 2.5
Fat Mass (%)	16.9 $\pm$ 4.7	15.9 $\pm$ 2.8	14.8 $\pm$ 3.5
Systolic BP (mmHg)	117 $\pm$ 11	125 $\pm$ 9	127 $\pm$ 9
Diastolic BP (mmHg)	74 $\pm$ 14	72 $\pm$ 14	79 $\pm$ 13
FPG (mmol/L)	5.1 $\pm$ 0.3	5.0 $\pm$ 0.2	5.0 $\pm$ 0.4
2 h OGTT (mmol/L)	4.2 $\pm$ 0.8	4.7 $\pm$ 0.9	4.2 $\pm$ 1.1
Cholesterol (mmol/L)	4.1 $\pm$ 0.9	4.3 $\pm$ 0.8	4.1 $\pm$ 0.8
HDL-cholesterol (mmol/L)	1.6 $\pm$ 0.4	1.4 $\pm$ 0.3	1.4 $\pm$ 0.3
LDL-cholesterol (mmol/L)	2.1 $\pm$ 0.6	2.5 $\pm$ 0.9	2.4 $\pm$ 0.9
Triglycerides (mmol/L)	0.9 (0.6-1.0)	0.6 (0.5-1.0)	0.6 (0.5-0.7)

Data are mean $\pm$ SD or median (IQR). Abbreviations: BP, blood pressure; BMI, body mass index; FPG, fasting plasma glucose; HDL, high density lipoprotein; IQR, interquartile range; LBM, lean body mass; LDL, low density lipoprotein; OGTT, oral glucose tolerance test; PRED7.5, prednisolone 7.5 mg group; PRED30, Prednisolone 30 mg group; *n*, amount.

Prednisolone resulted in a dose-dependent increase in DNLI ($p = 0.03$), an increase in SCDi ($p = 0.01$) and a decrease in D6Di ($p = 0.01$) in plasma CE (**Table 2**). PRED30 resulted in an increase of DNLI by 51.2 [14.8 ; 87.6]% ($p = 0.01$) and an increase of SCDi by 48.6 [18.7 ; 78.5]% ($p = 0.003$). PRED7.5 did cause a numerical increase in DNLI and SCDi, however, this trend was not significantly

different compared to placebo. PRED7.5 and PRED30 resulted in dose-dependent decreases of D6Di by -44.4 [-77.3 ; -11.5]% ($p = 0.01$) and -57.7 [-91.8 ; -23.5]% ($p = 0.002$), respectively. DNLI and SCDi were positively correlated ($r = 0.83$, $p < 0.001$). DNLI and SCDi were each inversely correlated with D6Di ($r = -0.71$ and -0.63 , respectively, both $p < 0.001$). In secondary analyses, the increase in DNLI and decrease in D6Di were also found in PL and TG. The increase in SCDi was found in PL, but not in plasma TG (**Supplemental Table 2(A-C)**).

DNLI, SCDi and D6Di were additionally evaluated from other PPRs (**Supplemental Table 2(A-C)**). PRED30, but not PRED7.5, resulted in increases in DNLI in TG, as observed from the PPRs 16:0/18:2 ω 6 and 18:0/18:2 ω 6. Only PRED30 resulted in an increase of 18:3 ω 6/18:2 ω 6 in CE, while only a trend towards an increase was found in PL and TG. Prednisolone did not influence D5Di in plasma CE, PL and TG.

Overall between-group differences in metabolic variables were observed for FPG, FPI, fasting glucagon and cTG (**Table 3**). PRED7.5 and PRED30 increased FPG by 5.9 [0.9 ; 10.9]% ($p = 0.02$) and 15.0 [9.6 ; 20.4]% ($p < 0.001$) respectively. PRED30, but not PRED7.5, significantly increased FPI by 127.3 [54.4 ; 200.2]% ($p < 0.001$), fasting glucagon by 37.0 [9.8 ; 64.3]% ($p = 0.01$) and cTG by 65.2 [15.5 ; 114.8]% ($p = 0.01$). After a 2-week drug-exposure an overall difference was found between the three groups in HOMA-IR ($p < 0.001$). HOMA-IR increased in the PRED30 by 172.3 [112.9 ; 231.9]% ($p < 0.001$).

The prednisolone-induced changes in DNLI, SCDa and D6Da in plasma CE, PL and TG were related to prednisolone-induced changes in several metabolic variables (**Supplemental Table 3**). In CE, DNLI and SCDi were positively correlated with FPG (DNLI: $r = 0.48$, $p = 0.01$; SCDa: $r = 0.60$, $p < 0.001$) (**Figures 2a and 2b**), while D6Di correlated inversely with FPG ($r = -0.37$; $p = 0.05$) (**Figure 2c**). FPI was only significantly correlated with SCDi (SCD: $r = 0.49$, $p = 0.01$). DNLI and SCDi were positively correlated with cTG (DNLI: $r = 0.38$, $p = 0.03$; SCDa: $r = 0.42$, $p = 0.02$) (**Figures 2d and 2e**), while the D6Da correlated inversely with cTG ($r = -0.37$, $p = 0.05$) (**Figure 2f**). DNLI and SCDi were positively correlated with HOMA-IR (DNLI: $r = 0.35$, $p = 0.05$; SCDi: $r = 0.50$, $p = 0.01$) (**Figures 2g and 2h**), while D6Di did not correlate with HOMA-IR ($r = -0.27$, $p = 0.17$) (**Figure 2i**). Correlations with the DNLI, SCDi and D6Di in PL and TG showed the same overall results. D6Di in PL showed an even stronger association with FPG and HOMA-IR compared to the activity in CE and TG ($r = -0.56$; $p < 0.001$ and $r = -0.35$; $p = 0.05$, respectively).

Table 2: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on DNLI, SCDi and D6Di in plasma cholesteryl esters in healthy young males

	Baseline			Mean difference (95%CI)				ANCOVA <i>P-value</i> ^a	Post-hoc <i>P-value</i> ^b	Post-hoc <i>P-value</i> ^c
	Placebo (n = 8)	PRED7.5 (n = 12)	PRED30 (n = 12)	PRED7.5 vs Placebo	PRED30 vs Placebo					
DNLI (16:1ω7/18:2ω6)	4.0 (3.2 ; 5.2)	4.1 (2.9 ; 4.6)	3.5 (2.7 ; 3.8)	26.6 (-9.7 ; 62.9)	51.2 (14.8 ; 87.6)			0.03	0.14	0.01
SCDi (16:1ω7/16:0)	17.3 (14.7 ; 20.0)	16.2 (11.7 ; 21.5)	14.7 (13.2 ; 16.1)	23.8 (-5.6 ; 53.3)	48.6 (18.7 ; 78.5)			0.01	0.11	0.003
D6Di (22.6ω3/20.5ω3)	36.1 (29.2 ; 71.4)	44.4 (28.5 ; 67.2)	64.2 (38.3 ; 85.8)	-44.4 (-77.3 ; -11.5)	-57.7 (-91.8 ; -23.5)			0.01	0.01	0.002

Baseline data are median product/precursor ratios (IQR). Baseline data are presented as x10⁻². (a) Differences between groups were tested with ANCOVA, with delta% of the product/precursor ratios as continuous variable and baseline product/precursor ratio as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; D6Di, Delta-6 desaturase index; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; DNLI, de novo lipogenesis index; IQR, interquartile range; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SCDi, stearoyl-CoA desaturase index.

Table 3. Effects of 2-weeks prednisolone (7.5 mg or 30 mg once daily) or placebo on metabolic variables and insulin sensitivity in healthy young males

	Baseline			After treatment					ANCOVA P-value ^a	Post-Hoc P-value ^b	Post-hoc P-value ^c
	Placebo (n = 8)	PRED7.5 (n = 12)	PRED30 (n = 12)	Placebo (n = 8)	PRED7.5 (n = 12)	PRED30 (n = 12)					
FPG (mmol/l)	5.0 ± 0.2	4.9 ± 0.2	4.7 ± 0.2	4.8 ± 0.3	5.0 ± 0.3	5.4 ± 0.3			< 0.001	0.02	< 0.001
FPI (pmol/l)	30 (16 ; 51)	29 (11 ; 40)	19 (8 ; 27)	28 (11 ; 36)	19 (8 ; 42)	34 (23 ; 51)			< 0.001	0.52	< 0.001
Fasting glucagon (ng/l)	49 (39 ; 55)	65 (53 ; 74)	48 (41 ; 59)	41 (39 ; 48)	62 (54 ; 74)	62 (53 ; 80)			0.02	0.32	0.01
Fasting NEFA (mmol/l)	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1			0.23	0.53	0.10
Fasting cTG (mmol/l)	0.5 (0.4 ; 0.9)	0.8 (0.6 ; 1.0)	0.6 (0.4 ; 0.7)	0.5 (0.3 ; 0.7)	0.7 (0.6 ; 0.9)	0.7 (0.6 ; 0.9)			0.03	0.43	0.01
HOMA-IR	1.1 (0.6 ; 1.9)	1.0 (0.4 ; 1.5)	0.7 (0.3 ; 0.9)	0.9 (0.4 ; 1.3)	0.7 (0.3 ; 1.6)	1.3 (0.9 ; 2.0)			< 0.001	0.71	< 0.001

Data are mean ± SD or median (IQR). (a) Differences between groups were tested with ANCOVA, with delta% of the metabolic variables as continuous variable and baseline variable as covariate. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: cTG, circulating triglycerides; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; FPG, fasting plasma glucose; FPI, fasting plasma insulin; HOMA-IR, homeostatic model assessment – insulin resistance; IQR, interquartile range; NEFA, non-esterified fatty acids.

Figure 2: Correlations of prednisolone-induced DNLI, SCDi, and D6Di in plasma CE with alterations in fasting metabolic parameters and insulin sensitivity. Figures a-c represent correlations between FPG and DNLI (a), SCDi (b) and D6Di (c). Figures d-f represent correlations between fasting circulating triglycerides (cTG) and DNLI (d), SCDi (e) and D6Di (f). The figures g-i represent correlations between insulin sensitivity measured as HOMA-IR and DNLI (a), SCDi (b) and D6Di (c). *Abbreviations:* CE, plasma cholesteryl esters; cTG, fasting circulating triglycerides; D6Di, Delta-6 desaturase index; DNLI, de novo lipogenesis index; FPG, fasting plasma glucose; HOMA-IR, homeostatic assessment model – Insulin resistance; PL, plasma phospholipids; SCDi, stearoyl-CoA desaturase index.

DISCUSSION AND CONCLUSIONS

To the best of our knowledge, this is the first study that has investigated the effect of glucocorticoids on specific parameters of fatty acid metabolism in humans. We determined the effects of two doses of oral prednisolone for two weeks on DNLI, SCDi and D6Di in healthy young males. Prednisolone administration caused increases in both DNLI and SCDi, while D6Di decreased. The increase in DNLI and the decrease in D6Di were observed in plasma CE, PL and TG, while the increase in SCDi was only found in CE and PL. The observed prednisolone-induced changes in fatty acid metabolism were correlated to alterations in metabolic parameters and estimated insulin sensitivity. This study shows that glucocorticoids influence human fatty acid metabolism, probably as a consequence of the glucocorticoid-induced changes in metabolic parameters.

Not only at high doses, but also at low doses, glucocorticoid therapy has been shown to influence fatty acid metabolism. Prednisolone administration was dose-dependently associated with increases in DNLI and SCDi, and a decrease in D6Di. The increases in DNLI and SCDi were primarily assessed in plasma CE, and confirmed in plasma PL, while plasma TG showed an increase in DNLI only. Additional analyses revealed that DNLI in TG also increased, as derived from the ratios 16:0/18:2 ω 6 and 18:0/18:2 ω 6. Literature data on the effects of glucocorticoids on DNL and SCD are scarce. In animals, injections with the synthetic glucocorticoid dexamethasone influenced lipid metabolism in broiler chickens by increasing hepatic DNL, as compared to saline injection [34]. Two other preclinical experiments showed an increase in SCD following exogenous glucocorticoid administration [35,36]. A glucocorticoid-induced increase in SCD was also demonstrated in liver microsomal preparations of rats by means of increased conversion of 16:0 to 16:1 ω 7 [37].

We demonstrated that 2-week prednisolone reduces the 22:6 ω 3/20:5 ω 3 ratio in CE, which was confirmed by subsequent analyses in PL and TG. Experiments performed as early as in 1979 showed that synthetic glucocorticoids, such as triamcinolone and dexamethasone, depress D6D in liver microsomes [37]. More recent studies in rat and cell cultures confirmed that D6D can be inhibited by this type of medication [15,36,38,39].

Our findings that prednisolone-induced changes in fatty acid metabolism were strongly correlated with alterations in fasting metabolic parameters and changes in insulin sensitivity suggests that administration of glucocorticoids may lead to changes in fatty acid metabolism through alterations in metabolic parameters. The link between fatty acid metabolism and metabolic parameters is in line with several animal and human studies. In an animal study conducted in 2004, it was suggested that a local increase of glucocorticoids promotes insulin resistance as well as hepatic steatosis and hepatic lipid synthesis [39]. An experiment in broiler chickens has been carried out to investigate the influence of dexamethasone on hepatic lipid metabolism and the role of insulin in this process. The authors concluded that glucocorticoids together with the induced hyperinsulinemia are responsible for the up-regulated hepatic lipogenesis [34,40]. In addition, it was shown that an inverse correlation between intrahepatic TG content and hepatic insulin sensitivity in 42 non-diabetic obese subjects existed [41]. Another study in overweight adolescents showed that elevated intrahepatic TG content is associated with insulin resistant glucose metabolism in the liver [42].

Glucocorticoids influence metabolic processes, such as gluconeogenesis, insulin sensitivity and insulin secretion [43]. Glucocorticoids may increase gluconeogenesis by stimulating the production of the gluconeogenic precursor glycerol through enhanced lipolysis, and by stimulating amino acid release through enhanced proteolysis [11,14,43-45]. Furthermore, glucocorticoids stimulate the transport and hepatic uptake of these gluconeogenesis precursors, induce synthesis and modulate the activities of gluconeogenic enzymes, and regulate the effects of other

hormones on gluconeogenesis [43]. The increased gluconeogenesis in the liver causes excessive glucose production, resulting in stimulated hepatic DNL [14]. Concomitant upregulation of SCD leads to the production of 16:1 ω 7, 18:1 ω 7 and 18:1 ω 9 [19,46]. Upregulated DNL and SCD are thus ultimately responsible for lipid accumulation in the liver. Glucocorticoids may increase insulin secretion and reduce insulin sensitivity directly, or indirectly by stimulated gluconeogenesis [43]. Reduced insulin sensitivity is among the diabetogenic effects of glucocorticoids and might eventually, in combination with beta-cell failure, result in overt diabetes [44].

Administration of glucocorticoids in this study was found to decrease D6Di, as derived from the ratio 22:6 ω 3/20:5 ω 3, suggesting lower conversion of EPA to DHA (**Figure 1b**). However, other product/precursor ratios (i.e. 18:3 ω 6/18:2 ω 6), that may also reflect D6Di, did not confirm this result. The discrepancy might point at the omission that the conversion of EPA to DHA does not merely reflect D6Di, but also elongation and β -oxidation, while the conversion of 18:2 ω 6 to 18:3 ω 6 depends on D6Da only. In our study, a 2-week prednisolone treatment resulted in an increase of EPA in all three compartments, while DHA did not change. Based on the observed reduction of the 22:6 ω 3/20:5 ω 3 ratio and the accumulation of EPA, we speculate that glucocorticoids may influence elongation and/or β -oxidation.

This study has some limitations. Plasma fatty acid levels fluctuate in parallel with the feed/fast cycle. A high or low fat diet consumed on the day before or during the study may differentially influence fasting levels of fatty acids in plasma at baseline and study end. One limitation of the study is therefore that we did not standardize food intake, or assessed fatty acid intake, on the days before testing. However, we did statistically correct for baseline fatty acid status by using the ANCOVA test. Another limitation of the study is the use of healthy volunteers. The results of this study may therefore not be generalizable to a patient population. A third limitation is that we measured DNL and indices of SCD and D6D by calculating the product/precursor ratios rather than by measuring activities of DNL, SCD and D6D by methods in which labeled fatty acids are added. The indices have been criticized since they can be influenced by factors like diet (e.g. MUFA/SFA ratio), lifestyle factors (e.g. exercise training) or hormones (e.g. estrogens) [47]. Nevertheless, fatty acid indices are considered a reasonable alternative to investigate changes in DNL, SCD and D6D when direct measurements are not feasible [22,48].

Prednisolone-induced changes in fatty acid metabolism were less pronounced in plasma TG compared to plasma CE and PL, which may be contra-intuitively. We speculate that this could be due to the use of blood samples that were drawn following a 12 h fast. In the postprandial state, VLDL-TG carry dietary- and *de novo* synthesized fatty acids from the liver to peripheral tissues resulting in higher VLDL-TG levels. As the half-life of VLDL is about 3- to 4 h [49], these higher VLDL-TG levels have normalized after an overnight fast, when residing VLDL-TG fatty acids might predominantly derive from adipose tissue. Consequently, postprandial DNL and other parameters of hepatic lipid metabolism might be better demonstrable from non-fasting TG.

The study has several strong points. Most studies in this field investigated the acute or chronic effects of prednisolone treatment in patients using a single drug-dose. In the current study we exposed healthy young volunteers to two different doses of prednisolone with a placebo control, allowing us to investigate the specific effects of prednisolone on metabolic parameters and fatty acid metabolism at both low and high doses without any background of pathophysiological effects. Another strong point of our study is that we measured fatty acid metabolism in three different compartments (i.e. plasma CE, PL and TG), providing the opportunity to investigate whether the prednisolone-induced changes in fatty acid metabolism were consistent in plasma lipid classes with widely differing functions and half-lives.

In conclusion, 2-week prednisolone administration in healthy young males dose-dependently increased DNLI and SCDi, and decreased D6Di. In addition, we demonstrated that the prednisolone-induced changes in fatty acid metabolism are associated with glucocorticoid-induced alterations of the metabolic parameters. Further research is necessary to confirm our results and understand this complex mechanism in more detail.

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Supplemental Table 1A: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on fatty acids (mol%) measured in plasma cholesteryl esters (CE) of healthy young males.

	Baseline		Mean Difference (95% CI)					ANCOVA P-value ^a	Post-hoc P-value ^b	Post-hoc P-value ^c
	Placebo		PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo				
SFA	16:0	12.1 (11.1 ; 13.0)	12.3 (11.2 ; 14.2)	12.1 (11.2 ; 12.9)	3.4 (-11.9 ; 18.7)	-1.3 (-16.0 ; 13.3)	0.77	0.65	0.85	
	18:0	1.4 (1.3 ; 1.5)	1.6 (1.3 ; 2.2)	1.7 (1.4 ; 1.9)	-104.4 (-263.8 ; 55.1)	-126.5 (-270.7 ; 17.6)	0.21	0.19	0.08	
MUFA	16:1ω7	2.1 (1.7 ; 2.6)	2.1 (1.5 ; 2.5)	1.8 (1.5 ; 2.0)	35.1 (5.0 ; 65.1)	59.3 (28.5 ; 90.1)	0.002	0.02	0.001	
	18:1ω7	1.3 (1.2 ; 1.5)	1.2 (1.1 ; 1.4)	1.4 (1.3 ; 1.6)	-1.3 (-11.8 ; 9.2)	-3.4 (-13.6 ; 6.7)	0.78	0.81	0.49	
PUFAω6	18:1ω9	21.8 (20.3 ; 23.9)	20.2 (18.4 ; 21.5)	20.2 (18.7 ; 22.7)	0.2 (-10.4 ; 10.8)	3.9 (-6.1 ; 13.9)	0.62	0.97	0.43	
	18:2ω6	51.5 (50.2 ; 54.8)	50.8 (48.9 ; 55.4)	52.5 (51.8 ; 55.1)	-0.8 (-8.7 ; 7.0)	-1.7 (-9.5 ; 6.0)	0.90	0.84	0.65	
PUFAω3	18:3ω6	0.9 (0.7 ; 1.3)	0.9 (0.7 ; 1.3)	0.6 (0.6 ; 0.8)	22.2 (-9.9 ; 54.3)	60.0 (25.6 ; 94.3)	0.004	0.17	0.001	
	20:3ω6	0.8 (0.7 ; 0.9)	0.8 (0.8 ; 0.8)	0.8 (0.8 ; 0.8)	-11.0 (-28.9 ; 6.8)	-6.4 (-24.2 ; 11.5)	0.46	0.22	0.47	
PUFAω3	20:4ω6	5.3 (4.6 ; 5.9)	6.1 (4.6 ; 8.8)	5.6 (5.1 ; 6.5)	-1.1 (-16.0 ; 13.8)	-2.5 (-16.7 ; 11.7)	0.94	0.88	0.73	
	20:5ω3	0.4 (0.3 ; 0.7)	0.9 (0.6 ; 1.0)	0.3 (0.3 ; 0.4)	31.4 (-7.2 ; 69.9)	55.9 (23.2 ; 88.7)	0.01	0.11	0.002	
PUFAω3	22:6ω3	0.2 (0.2 ; 0.2)	0.3 (0.2 ; 0.5)	0.2 (0.2 ; 0.4)	13.3 (-32.4 ; 59.1)	-13.3 (-53.7 ; 27.1)	0.38	0.55	0.50	

Baseline data are median fatty acid status (IQR). (a) Differences between groups were tested with ANCOVA, with delta% of the fatty acids as continuous variable and baseline fatty acid as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; IQR, interquartile range; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SFA, saturated fatty acids.

Supplemental Table 18: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on fatty acids (mol%) measured in plasma phospholipids (PL) of healthy young males.

Baseline		Mean Difference (95% CI)					ANCOVA P-value ^a	Post-hoc P-value ^b	Post-hoc P-value ^c
	Placebo	PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo				
SFA	16:0	29.9 (28.3 ; 30.9)	30.1 (28.1 ; 31.1)	30.0 (28.4 ; 30.9)	7.9 (0.4 ; 15.4)	6.0 (-1.4 ; 13.3)	0.10	0.04	0.11
	18:0	14.3 (13.6 ; 14.7)	13.8 (12.9 ; 14.9)	14.5 (13.9 ; 15.10)	-2.4 (-9.1 ; 4.2)	-10.4 (-16.8 ; -3.9)	0.01	0.46	0.003
MUFA	16:1ω7	0.4 (0.3 ; 0.5)	0.4 (0.3 ; 0.5)	0.4 (0.3 ; 0.5)	24.9 (-1.0 ; 50.8)	34.2 (8.9 ; 59.4)	0.03	0.06	0.01
	18:1ω7	1.4 (1.6 ; 1.7)	1.4 (1.3 ; 1.6)	1.6 (1.4 ; 1.7)	4.0 (-7.6 ; 15.7)	-7.0 (-18.5 ; 4.5)	0.14	0.48	0.23
	18:1ω9	10.1 (8.9 ; 11.4)	9.2 (8.4 ; 10.3)	9.1 (8.6 ; 9.6)	0.6 (-10.5 ; 11.7)	1.9 (-9.1 ; 12.9)	0.93	0.92	0.73
PUFAω6	18:2ω6	21.3 (20.4 ; 22.5)	21.9 (19.4 ; 22.8)	20.3 (19.2 ; 21.3)	-3.0 (-12.3 ; 6.4)	1.7 (-7.8 ; 11.2)	0.54	0.52	0.72
	18:3ω6	0.1 (0.1 ; 0.1)	0.1 (0.0 ; 0.1)	0.1 (0.0 ; 0.1)	-21.2 (-90.2 ; 47.7)	66.6 (-3.8 ; 136.9)	0.03	0.53	0.06
	20:3ω6	2.9 (2.6 ; 3.0)	3.0 (2.4 ; 3.3)	2.8 (2.5 ; 3.3)	-6.9 (-18.8 ; 5.0)	-0.4 (-12.1 ; 11.4)	0.38	0.25	0.95
PUFAω3	20:4ω6	8.6 (7.7 ; 8.8)	9.3 (8.0 ; 11.0)	9.6 (9.1 ; 10.8)	-9.7 (-22.2 ; 2.9)	-11.5 (-24.0 ; 0.9)	0.17	0.13	0.07
	20:5ω3	0.6 (0.5 ; 1.1)	1.0 (0.6 ; 1.3)	0.5 (0.4 ; 0.7)	41.3 (0.6 ; 82.0)	45.1 (6.3 ; 83.9)	0.05	0.05	0.02
	22:6ω3	2.1 (1.6 ; 2.6)	2.7 (2.3 ; 3.4)	2.6 (2.2 ; 3.0)	-20.5 (-51.8 ; 10.8)	-23.0 (-53.2 ; 7.2)	0.29	0.19	0.13

Baseline data are median fatty acid status (IQR). (a) Differences between groups were tested with ANCOVA, with delta% of the fatty acids as continuous variable and baseline fatty acid as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; IQR, interquartile range; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SFA, saturated fatty acids.

Supplemental Table 1C: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on fatty acids (mol%) measured in plasma triglycerides (TG) of healthy young males.

	Baseline		Mean Difference (95% CI)					ANCOVA P-value ^a	Post-hoc P-value ^b	Post-hoc P-value ^c
	Placebo		PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo				
SFA	16:0	20.1 (17.5 ; 21.1)	21.7 (19.4 ; 25.1)	19.2 (16.7 ; 20.6)	13.3 (-5.6 ; 32.1)	23.1 (4.6 ; 41.5)	0.05	0.16	0.02	
	18:0	3.1 (2.9 ; 3.4)	3.2 (2.8 ; 3.7)	2.7 (2.5 ; 3.3)	-3.6 (-26.8 ; 19.5)	23.6 (0.7 ; 46.4)	0.03	0.75	0.04	
MUFA	16:1 ω 7	2.3 (1.7 ; 2.9)	2.6 (2.1 ; 3.2)	2.5 (1.9 ; 2.9)	13.6 (-13.3 ; 40.4)	32.9 (7.1 ; 58.6)	0.04	0.31	0.01	
	18:1 ω 7	2.7 (2.3 ; 2.9)	2.5 (2.3 ; 3.0)	2.8 (2.3 ; 2.9)	-4.6 (-16.2 ; 6.9)	-1.9 (-13.3 ; 9.5)	0.71	0.42	0.74	
	18:1 ω 9	37.0 (32.0 ; 39.8)	36.5 (34.6 ; 38.2)	32.2 (30.2 ; 38.0)	-5.6 (-17.0 ; 5.8)	0.1 (-11.6 ; 11.7)	0.47	0.32	0.99	
PUFA ω 6	18:2 ω 6	24.8 (23.2 ; 26.7)	23.5 (20.6 ; 25.7)	28.5 (25.0 ; 30.7)	-4.6 (-21.6 ; 12.4)	-18.5 (-36.0 ; -1.1)	0.10	0.58	0.04	
	18:3 ω 6	0.6 (0.5 ; 0.7)	0.6 (0.43 ; 0.84)	0.6 (0.5 ; 0.7)	-1.7 (-25.9 ; 22.5)	0.3 (-24.4 ; 25.0)	0.98	0.89	0.98	
	20:3 ω 6	0.5 (0.5 ; 0.6)	0.5 (0.4 ; 0.6)	0.5 (0.5 ; 0.6)	-18.8 (-41.2 ; 3.6)	-16.5 (-38.6 ; 5.5)	0.20	0.10	0.14	
20:4 ω 6	3.5 (2.7 ; 4.6)	3.3 (2.3 ; 4.0)	4.3 (3.4 ; 6.1)	3.0 (-29.2 ; 35.2)	-26.1 (-58.8 ; 6.6)	0.15	0.84	0.11		
PUFA ω 3	20:5 ω 3	0.6 (0.4 ; 0.8)	0.8 (0.4 ; 0.9)	0.5 (0.4 ; 0.7)	64.8 (15.4 ; 114.2)	16.2 (-31.5 ; 63.9)	0.03	0.01	0.49	
	22:6 ω 3	0.6 (0.5 ; 0.8)	0.9 (0.5 ; 1.1)	0.8 (0.5 ; 1.2)	-17.4 (-58.4 ; 23.4)	-34.2 (-74.6 ; 6.3)	0.24	0.39	0.10	

Baseline data are median fatty acid status (IQR). (a) Differences between groups were tested with ANCOVA, with delta% of the fatty acids as continuous variable and baseline fatty acid as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; IQR, interquartile range; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SFA, saturated fatty acids.

Supplemental Table 2A: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on DNLI, SCDi and D6Di in plasma cholesteryl esters (CE) of healthy young males.

	Baseline		Mean Difference (95% CI) %			
	Placebo		PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo
DNL	16:0/18:2ω6 ¹	22.9 (20.9 ; 25.9)	22.4 (21.4 ; 29.1)	22.6 (19.9 ; 24.1)	-0.3 (-21.7 ; 21.1)	-0.9 (-21.1 ; 19.4)
	18:0/18:2ω6 ¹	2.6 (2.5 ; 2.6)	3.2 (2.5 ; 4.0)	3.0 (2.7 ; 3.5)	-121.4 (-297.3 ; 54.5)	-143.5 (-300.3 ; 13.2)
	18:1ω7/18:2ω6 ¹	2.6 (2.4 ; 2.9)	2.5 (1.9 ; 2.8)	2.7 (2.3 ; 3.0)	0.5 (-10.7 ; 11.7)	-2.1 (-13.0 ; 8.8)
	18:1ω9/18:2ω6 ¹	42.5 (37.8 ; 45.6)	39.9 (34.4 ; 43.4)	38.0 (34.4 ; 44.2)	3.3 (0.6 ; 0.5)	6.5 (-5.6 ; 18.6)
SCD	18:1ω7/16:0 ¹	12.0 (10.5 ; 12.3)	9.6 (8.3 ; 11.7)	12.7 (11.3 ; 13.2)	-8.5 (-29.3 ; 12.3)	-1.5 (-20.7 ; 17.7)
	18:1ω9/18:0	16.3 (13.9 ; 17.6)	13.0 (8.8 ; 16.6)	13.3 (10.6 ; 15.3)	-24.2 (-70.1 ; 21.6)	-2.6 (-46.8 ; 41.6)
D5D	20:4ω6/20:3ω6	6.7 (6.4 ; 7.5)	7.6 (6.1 ; 11.0)	7.2 (5.7 ; 9.2)	2.4 (-20.5 ; 25.3)	-1.4 (-23.4 ; 20.5)
D6D	20:3ω6/18:2ω6 ¹	1.4 (1.2 ; 1.7)	1.5 (1.4 ; 1.7)	1.5 (1.2 ; 1.7)	-8.6 (-25.2 ; 8.1)	-4.4 (-20.7 ; 12.0)
	18:3ω6/18:2ω6 ¹	1.7 (1.3 ; 2.48)	1.7 (1.3 ; 2.4)	1.2 (1.1 ; 1.5)	24.8 (-11.0 ; 60.5)	62.4 (24.2 ; 101.0)

Baseline data are median product/precursor ratios (IQR). 1. Baseline data are presented as x10⁻². (a) Differences between groups were tested with ANCOVA, with delta% of the product/precursor ratios as continuous variable and baseline product/precursor ratio as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; D6D, Delta-6 desaturase; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; DNLI, de novo lipogenesis; IQR, interquartile range; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SCD, stearoyl-CoA desaturase.

Supplemental Table 2B: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on DNLi, SCDi and D6Di in plasma phospholipids (PL) of healthy young males.

	Baseline		Mean Difference (95% CI)				ANCOVA P-value ^a	Post-hoc P-value ^b	Post-hoc P-value ^c
	Placebo		PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo			
DNL	16:0/18:2ω6	1.4 (1.3 ; 1.5)	1.4 (1.2 ; 1.6)	1.5 (1.3 ; 1.6)	12.5 (-1.0 ; 26.0)	5.8 (-7.6 ; 19.3)	0.18	0.07	0.38
	18:0/18:2ω6 ¹	66.3 (62.4 ; 68.6)	64.4 (58.9 ; 71.0)	73.2 (65.3 ; 78.2)	2.6 (-7.6 ; 12.7)	-10.4 (-20.8 ; 0.1)	0.03	0.61	0.05
	16:1ω7/18:2ω6 ¹	1.7 (1.5 ; 2.2)	1.7 (1.4 ; 2.2)	2.1 (1.6 ; 2.3)	28.9 (1.1 ; 56.7)	29.0 (1.9 ; 56.1)	0.07	0.04	0.04
	18:1ω7/18:2ω6 ¹	6.6 (5.8 ; 7.8)	7.0 (5.6 ; 7.5)	7.7 (6.6 ; 9.0)	3.6 (-11.5 ; 18.6)	-10.1 (-25.2 ; 5.0)	0.15	0.63	0.18
	18:1ω9/18:2ω6 ¹	48.4 (44.0 ; 52.0)	42.2 (39.4 ; 47.1)	47.3 (42.0-49.7)	5.1 (-9.0 ; 19.2)	0.9 (-12.7 ; 14.5)	0.70	0.46	0.89
SCD	16:1ω7/16:0 ¹	1.2 (1.1 ; 1.5)	1.2 (1.1 ; 1.7)	1.4 (1.1 ; 1.6)	15.3 (11.6 ; 63.4)	27.3 (7.1 ; 47.4)	0.03	0.14	0.01
	18:1ω7/16:0 ¹	5.0 (4.6 ; 5.4)	4.7 (4.6 ; 5.0)	5.3 (4.8 ; 5.5)	-7.5 (-24.4 ; 9.5)	-13.1 (-29.8 ; 3.5)	0.29	0.38	0.12
	18:1ω9/18:0	74.2 (61.7 ; 80.4)	66.9 (56.8 ; 77.7)	64.7 (55.0 ; 69.7)	3.5 (-8.8 ; 15.9)	15.3 (2.7 ; 27.8)	0.04	0.56	0.02
	20:4ω6/20:3ω6	3.0 (2.6 ; 3.3)	2.9 (2.5 ; 4.8)	3.5 (2.9 ; 4.2)	2.4 (-15.8 ; 20.6)	-9.4 (-27.4 ; 8.4)	0.29	0.79	0.29
D6D	20:3ω6/18:2ω6 ¹	12.8 (12.0 ; 14.5)	14.3 (10.8 ; 15.1)	13.9 (11.5 ; 17.4)	-2.9 (-18.0 ; 12.2)	-1.0 (-15.9 ; 13.9)	0.92	0.69	0.89
	18:3ω6/18:2ω6 ¹	0.3 (0.2 ; 0.6)	0.5 (0.2 ; 0.6)	0.2 (0.2 ; 0.4)	-1.4 (-66.2 ; 63.3)	55.4 (-11.5 ; 122.3)	0.13	0.96	0.10
	22:6ω3/20:5ω3	3.2 (2.2 ; 4.2)	2.7 (2.2 ; 3.4)	4.4 (3.6 ; 6.0)	-51.1 (-85.6 ; -16.6)	-63.6 (-100.2 ; -27.1)	0.003	0.01	0.001

Baseline data are median product/precursor ratios (IQR). 1. Baseline data are presented as x10-2. (a) Differences between groups were tested with ANCOVA, with delta% of the product/precursor ratios as continuous variable and baseline product/precursor ratio as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; D6D, Delta-6 desaturase; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; DNL, de novo lipogenesis; IQR, interquartile range; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SCD, stearoyl-CoA desaturase.

Supplemental Table 2C: Effects of 2-week prednisolone (7.5 mg or 30 mg once-daily) or placebo on DNLI, SCDi and D6Di in plasma triglycerides (TG) of healthy young males.

Baseline		Mean Difference (95% CI)					ANCOVA P-value ^a	Post-hoc P-value ^b	Post-hoc P-value ^c
Placebo		PRED7.5	PRED30	PRED7.5 vs Placebo	PRED30 vs Placebo				
DNL	16:0/18:2ω6 ¹	83.2 (69.8-86.7)	65.0 (54.7-82.7)	20.1 (-16.6 ; 56.8)	58.0 (22.2 ; 93.8)		0.01	0.27	0.003
	18:0/C18:2ω6 ¹	12.6 (11.0-14.2)	9.9 (8.4-12.2)	2.1 (-40.4 ; 44.6)	52.9 (9.8 ; 97.1)		0.03	0.92	0.02
	16:1ω7/18:2ω6 ¹	9.0 (6.7-12.3)	8.6 (6.7-11.3)	21.1 (-26.2 ; 68.5)	66.9 (21.7 ; 112.1)		0.01	0.37	0.01
	18:1ω7/18:2ω6 ¹	11.3 (8.6-12.7)	9.9 (7.5-11.6)	1.5 (-18.3 ; 21.3)	14.4 (-4.9 ; 33.7)		0.24	0.87	0.14
	18:1ω9/18:2ω6	1.6 (1.3-1.67)	1.1 (1.0-1.6)	3.9 (-19.3 ; 27.1)	22.4 (-1.1 ; 45.9)		0.14	0.73	0.06
SCD	16:1ω7/16:0 ¹	11.2 (9.3-14.5)	12.5 (10.4-14.9)	-5.1 (-20.3 ; 9.9)	8.1 (-6.7 ; 22.9)		0.14	0.49	0.27
	18:1ω7/16:0 ¹	14.2 (11.5-15.8)	14.5 (13.6-15.9)	-16.8 (-42.1 ; 8.4)	-12.1 (-35.9 ; 11.7)		0.38	0.18	0.31
	18:1ω9/18:0	12.2 (11.4-13.0)	12.1 (10.6-14.4)	-8.7 (-30.1 ; 12.7)	-9.3 (-30.2 ; 11.6)		0.63	0.41	0.37
	20:4ω6/20:3ω6	7.4 (5.7-8.4)	8.3 (6.9-9.6)	18.0 (-13.6 ; 49.5)	-17.7 (-49.7 ; 14.2)		0.07	0.25	0.27
D5D	20:3ω6/18:2ω6 ¹	2.0 (1.9-2.5)	2.0 (1.8-2.1)	-9.4 (-26.0 ; 7.0)	6.5 (-9.7 ; 22.7)		0.11	0.25	0.42
D6D	18:3ω6/18:2ω6 ¹	2.5 (1.9-2.8)	2.0 (1.7-2.2)	4.9 (-24.8 ; 34.5)	27.1 (-4.1 ; 58.2)		0.17	0.74	0.09
	22:6ω3/20:5ω3	1.2 (0.8-1.7)	1.4 (1.2-1.8)	-45.4 (-74.4 ; -16.4)	-45.0 (-73.8 ; -15.8)		0.01	0.003	0.004

Baseline data are median product/precursor ratios (IQR). 1. Baseline data are presented as x10-2. (a) Differences between groups were tested with ANCOVA, with delta% of the product/precursor ratios as continuous variable and baseline product/precursor ratio as covariates. (b) Differences between PRED7.5 and placebo and (c) between PRED30 and placebo were tested with a post-hoc analysis. Abbreviations: CI, confidence interval; D6D, Delta-6 desaturase; Delta%, (Treatment End – Baseline) / (Baseline) * 100%; DNLI, de novo lipogenesis; IQR, interquartile range; PRED7.5, prednisolone 7.5 mg group; PRED30, prednisolone 30.0 mg group; SCD, stearoyl-CoA desaturase.

Supplemental Table 3: Correlation between prednisolone induced changes in fatty acid metabolism and alterations in fasting metabolic parameters and insulin sensitivity

	DNLI (16:1 ω 7/18:2 ω 6) ¹			SCDi (16:1 ω 7/16:0) ¹			D6Di (22:6 ω 3/20:5 ω 3) ¹		
	CE	PL	TG	CE	PL	TG	CE	PL	TG
FPG (mmol/l)	0.48**	0.40*	0.47**	0.60**	0.34*	0.03	-0.37*	-0.56***	-0.21
FPI (pmol/l)	0.33	0.07	0.38*	0.49**	0.24	0.24	-0.26	-0.32*	-0.16
Fasting glucagon (ng/l)	0.25	0.22	0.27	0.32	0.22	0.01	-0.26	-0.27	-0.18
Fasting NEFA (mmol/l)	-0.01	0.13	-0.10	-0.08	0.08	0.06	0.15	0.11	0.15
Fasting cTG	0.38*	0.39*	0.58**	0.42*	0.53***	0.45*	-0.37*	-0.37*	-0.17
HOMA-IR	0.35*	0.08	0.38*	0.50**	0.20	0.16	-0.27	-0.35*	-0.16

Spearman rho's were used for analyses. 1 Prednisolone-induced changes in DNLI, SCDi and D6Di (Delta%) were used for analyses. *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: CE, plasma cholesterol esters; cTG, circulating triglycerides; D6D, delta-6 desaturase; DNLI, de novo lipogenesis; FPG, fasting plasma glucose; FPI, fasting plasma insulin; HOMA-IR, homeostatic model assessment – insulin resistance; NEFA, non-esterified fatty acids; PL, plasma phospholipids; SCD, stearoyl-CoA desaturase; TG, plasma triglycerides.

CHAPTER 9

General discussion and conclusions

Dairy intake has been suggested to have a positive effect on various cardiovascular risk factors, including BMI, fasting glucose and insulin resistance[1,2]. High intake of dairy has also been linked to a reduced risk of cardiovascular disease. Cardiovascular disease is still a common cause of death worldwide[3,4]. It is well recognized that multiple dietary factors contribute[5]. Fatty acids have been proposed as one of these dietary factors. Examples are the industrially produced trans fatty acids, which can for instance be found in baked goods and fast food. Fatty acids can, however, also have a positive influence on cardiovascular health. For instance, saturated odd-chain fatty acids from dairy products have been suggested to improve cardiovascular health[6-9]. Dairy fat is therefore an interesting target for further research to reduce cardiovascular risk in the general population.

One difficult issue concerning investigation of the association between dairy fat intake and cardiovascular health is that a perfectly objective measure for dairy fat intake is missing. Usually, investigations make use of dietary assessment tools such as food frequency questionnaires, which are not fully objective and prone to bias. Fatty acid biomarkers measured in blood might be a solution, since these are more objective. However, these methods have not been fully investigated. This is especially the case for the trans fatty acid biomarkers trans-C18:1(n-7) and CLA. A second issue is that some of the potential dairy fat biomarkers have also been proposed as fish fat biomarkers (i.e. C15:0 and C17:0). An important part of this thesis therefore concerns the investigation of potential dairy fat biomarkers in the general population. We explored the literature for existing biomarkers and investigated a potential new method to be able to measure the less common biomarkers. We also explored whether the association between dairy fat biomarkers and dairy fat intake was independent of fish fat intake, so that the potential reduction in cardiovascular risk factors can mainly be attributed to dairy fat intake. Since former studies especially described a reduction in cardiovascular risk due to fish fat intake and the omega-3 fatty acid supplements EPA and DHA, a minor part of this thesis concerns the effects of the fish fat biomarkers on cardiovascular disease, with special attention to the effects in renal transplant recipients, which is a patient group with a particularly high risk of cardiovascular disease.

MEASURING DAIRY FAT INTAKE

The traditional way of measuring dairy fats is by using dietary assessment tools such as food frequency questionnaires (FFQs), food records or 24-h recalls. The FFQ is the most convenient one, since it can capture usual, individual, long-term dietary intakes[10,11]. The use of dietary assessment tools remains popular to date, since they are considered easy measurement tools, with a low burden for participants[11]. Although these methods provide specific information on dairy fat intake, the data are based on self-reporting, and therefore subjective and often biased. The methods also rely on memory and may be subject to socially desirable answers[11,12]. This may lead to misclassification, underreporting and/or overreporting of dietary fat intake[11,13].

Therefore, in **Chapter 2-5** of this thesis, we explored alternative or complementary methods for measuring dairy fat intake. In these chapters, we focused on fatty acids as biomarkers for dairy fat intake. These biological markers are considered to provide more objective information of the dairy fat intake.

In previous years, several papers have reported that the fatty acids C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) and CLA may be useful biomarkers to predict dairy fat intake[14-16]. However, up to now, a clear overview of fatty acids that can be used as biomarkers of dairy and dairy fat intake was lacking. Therefore, in **Chapter 2**, we carried out a systematic review and meta-analysis to examine which fatty acids are suitable as biomarkers of dairy and dairy fat intake in the general population. We reviewed observational studies that assessed the association of dairy fatty acids measured in plasma/serum, erythrocytes and adipose tissue with dairy and dairy fat intake by a traditional dietary assessment tool (FFQ, Food record, 24-recall). The systematic review identified C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) and CLA in plasma/serum, erythrocytes and adipose tissue as potential dairy fat biomarkers in the general population. Additionally, the meta-analysis confirmed C14:0, C15:0, C17:0 and trans-C16:1(n-7) as biomarkers of dairy and dairy fat intake. C15:0 was found to be one of the strongest markers among the set of biomarkers investigated in this review. Stronger associations were found with dairy fat intake compared to dairy intake as a whole.

In **Chapter 2** we identified several limitations concerning the use of fatty acid biomarkers for estimation of dairy intake. Firstly, we found that the fatty acids trans-C18:1(n-7) and CLA were hardly explored as biomarkers of dairy and dairy fat intake, while these are also thought to mainly originate from dairy derived products[17]. Potentially, this might be due to suboptimal measurement techniques that are less capable of separating naturally occurring trans-C18:1(n-7) from other, often industrially produced, trans-C18:1 fatty acids. Secondly, we found that the correlations of dairy fat biomarkers with dairy intake and dairy fat intake were between the 0.10 and 0.33, which is adjudicated as low. Thirdly, C15:0 and C17:0 can also be found in marine water fish and have recently found to be related to DHA, a common fatty acid found in fatty fish[18,19]. C15:0 and C17:0 might therefore be invalid biomarkers of dairy fat intake in a population with a high fish consumption.

In **Chapters 3-5**, we describe and use a method that we developed to improve the measurement of commonly and less commonly investigated biomarkers. Furthermore, we investigated whether the dairy fat biomarkers (single markers or a combination) were associated with dairy fat intake, independently of fish fat intake. To do so, we used data from the Lifelines Biobank and Cohort study, an observational study with initially healthy participants of the Northern three provinces of the Netherlands. We measured the commonly and less commonly

used fatty acids as biomarkers of dairy fat intake in plasma from a random subset of 864 participants[20].

Development of a new method to measure dairy fat biomarkers

In **Chapter 3**, we described the development of a new method to measure both commonly investigated biomarkers and less commonly investigated biomarkers (i.e. trans-C18:1(n-7) and CLA) in plasma CE, TG and PL. Up to now, dairy fat biomarkers were mostly measured with a polar column with a length of up to 100 m. Such a column does, however, not have the separation efficiency to prevent co-elution of trans-C18:1(n-7) acid with other trans fatty acids, rendering an uncontaminated measurement of the potential dairy fat biomarker Trans-C18:1(n-7) impossible and associations with dairy fat intake impossible to calculate. We therefore developed a new method that consisted of a 200 m polar column. These columns are specifically developed to allow for better separation between trans-C18:1 fatty acids (i.e. trans-C18:1(n-9))[21]. Now, it was possible to measure both commonly and less commonly investigated dairy fat biomarkers within a single run. In **Chapter 3**, we also investigated whether the potential dairy fat biomarkers, and especially the less commonly investigated fatty acids trans-C18:1(n-7) and CLA, could be detected in all three fractions (plasma CE, TG and PL). In the literature, plasma PL was the most recommended fraction to measure fatty acids biomarkers[1,8,9,22-29], while, plasma TG was only used in one study[23]. In the LifeLines Cohort, we found that the less commonly investigated biomarkers were well-detectable both in plasma TG and plasma PL.

Although the new method has several strengths, it also has drawbacks. For instance, the runtime for one sample is almost two hours and analyzing both plasma TG and plasma PL of more than 864 samples was therefore a challenge. Furthermore, we measured the dairy fat biomarkers in both plasma TG and PL, but not in CE, since the biomarkers of interest were only detectable in the former and not in plasma CE. In literature, stronger associations between fatty acid intakes and fatty acid biomarkers have been suggested to be present for plasma CE and PL compared to plasma TG[27-29]. This might be due to the fact that plasma TG can be influenced by recent dietary intakes and *de novo* lipogenesis, whereas this is less true for plasma CE and PL[30]. Despite these limitations, there is up to now no better method to measure the commonly and less commonly investigated dairy fat biomarkers in plasma.

An alternative to plasma fatty acids biomarkers are fatty acids measured in adipose tissue. In literature, stronger associations between intake and biomarkers can be found when they are measured in this tissue[23]. Fatty acid biomarkers measured in adipose tissue reflect long-term intake, while fatty acid biomarkers measured in plasma are likely more influenced by short-term changes in the diet, especially plasma TG[31]. Given the fact that FFQs capture usual, long-term dietary intake, adipose tissue might be a better match compared to plasma. Adipose tissue

is, however, not always available in studies, as was also the case in the LifeLines Biobank and Cohort study.

Dairy fat biomarkers in the LifeLines BioBank and Cohort Study

In **Chapter 4**, we investigated the biomarkers trans-C18:1(n-7) and CLA as potential biomarkers of dairy and dairy fat intake and compared them with the more commonly used biomarkers. Secondly, we investigated whether a combination of biomarkers was a better predictor for dairy fat intake compared to single biomarkers.

C15:0, measured in both plasma TG and PL, was found to be one of the best biomarkers from the set of investigated biomarkers for the prediction of dairy fat intake, with standardized beta's for the association with dairy fat intake of 0.29 in plasma TG and 0.22 in plasma PL. Significant associations with comparable magnitude have been found in the literature before[9,32]. For example, a study with more than 3,000 subjects found a correlation of 0.29 between plasma C15:0 and dairy fat intake[32]. An even higher correlation was reported in a study of 110 children[8]. The researchers reported a Spearman rank correlation of 0.43 between the weighted percentage of plasma PL C15:0 and dairy fat intake. Significant associations between C15:0 and dairy fat intake have been confirmed in fractions such as erythrocytes and adipose tissue. Where the association in erythrocytes were slightly lower compared to plasma TG and PL[32,33], the associations found in adipose tissue were higher compared to plasma TG and PL[7,24,26]. For instance, one study even reported a correlation of 0.71 between adipose tissue C15:0 and dairy fat intake[26]. Unfortunately, adipose tissue samples were not available in the LifeLines Biobank and Cohort Study.

With regard to trans-C18:1(n-7), until now, only one study, apart from ours, investigated the association between plasma trans-C18:1(n-7) and dairy fat intake[14]. This other study found, however, no significant association between plasma trans-C18:1(n-7) and dairy fat intake. Most other studies used a cluster of trans-C18:1 fatty acids, which is a combination of the rumen-derived (trans-C18:1(n-7)) and the industrially-derived trans fatty acid (trans-C18:1(n-9)), rather than trans-C18:1(n-7) alone[15,34,35]. These studies provided contradicting results, with some studies reporting positive correlations with dairy intake[34,35], and others reporting absence of a correlation with dairy intake[15].

Plasma CLA may be a predictor of dairy fat intake, alone or in combination with other biomarkers. In The LifeLines Biobank and Cohort study, CLA was associated with dairy fat intake, with associations stronger for plasma PL than for plasma TG. This is in line with the current literature, which mainly consists of research carried out with adipose tissue CLA. For instance, a study with 123 participants demonstrated that adipose tissue CLA was associated with milk fat intake ($r = 0.43$)[15]. Furthermore, a more recent study with 3,630 participants investigated the association between dairy fat biomarkers and the risk of myocardial infarction,

and demonstrated that adipose tissue CLA was associated with energy-adjusted dairy intake ($r = 0.24$, $P < 0.001$)[36].

Although in this chapter, we revealed several dairy fat biomarkers, there is also a critical note on how we identified these biomarkers. A common way to explore potential biomarkers is by comparing the biomarkers against a traditional dietary assessment. Like previous researchers, we associated fatty acid biomarkers in plasma with fat intake from the FFQ, which usually results in a relatively low correlation between the two[8,14,23,32,37,38]. Whether this is the consequence of the biomarker providing a poor reflection of intake, or the dietary assessment tool providing a poor reflection of intake, is not completely clear. In addition, it has been shown that some of the fatty acids in plasma are not only influenced by dairy fat intake, but can also be affected by metabolic processes (e.g. trans-C16:1(n-7) can be influenced by β -oxidation of trans-C18:1(n-7)), or by the intake of dietary fibers (e.g. C17:0 can be influenced by inulin), meat and coconut oil (e.g. C14:0) [39-42]. It should also be noted that the fatty acid composition of dairy fat can vary depending on factors such as cow genetics, forage content, production level, duration of lactation, physiological status of the animal, and seasonal variation[43-46]. Nevertheless, fatty acid biomarkers can provide an objective estimation of dairy fat intake, while this is not the case with the FFQ.

Dairy intake versus Dairy fat intake; unadjusted versus energy-adjusted dairy fat intakes

In **Chapter 4**, it was found that the biomarkers were more strongly associated with dairy fat intake compared to dairy intake. This is in line with recent studies. For instance, in a study with 114 children serum C14:0, C15:0 and C17:0 were all more strongly related to dairy fat intake than to dairy intake as a whole[47]. Moreover, a study among 313 participants also demonstrated that C14:0, C15:0, C17:0 and trans-C16:1(n-7) were more strongly associated with dairy fat intake than with dairy intake as a whole[14]. In Chapter 4, we also found that the biomarkers were more strongly associated with energy-adjusted intakes compared to unadjusted intakes (dairy fat intake adjusted for energy-intake). That energy-adjusted intakes better relate to dairy and dairy fat intake may potentially be due to the fact that energy intake can operate as a confounding variable[48].

Are dairy fat biomarkers solid markers for dairy fat intake?

It has been doubted by some researchers whether the association between dairy (fat) intake and dairy fat biomarkers is always entirely attributable to dairy intake, because some of the dairy fatty acids can also be found in other foods. For example, C15:0 and C17:0 are fatty acids that can also be found in fish[19]. **Table 1** shows the composition of some of the fatty acids that can be found in milk and fish according to the Dutch NEVO table. The question was therefore whether C15:0 and C17:0, and other dairy fat biomarkers are reliable solid markers for the

prediction of dairy fat intake in the LifeLines population. In **Chapter 5**, we explored whether C15:0 and C17:0 were solid markers of dairy fat intake in the general population. We therefore investigated whether the association between the dairy fat biomarkers and total dairy fat intake (according to the FFQ) was independent of fish fat intake. We repeated these analyses for other biomarkers that are considered dairy fat biomarkers (C14:0, trans-C16:1(n-7), trans-C18:1(n-7)). We demonstrated that C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) and CLA were biomarkers for the prediction of dairy fat intake, while C15:0 and C17:0 appeared unrelated to fish fat intake. The same was true for the other investigated dairy fat biomarkers. The biomarkers therefore seem valid markers for dairy fat intake in the LifeLines population. Importantly, we found these results in a population with a high dairy and low fish fat intake. According to the Dutch Food Center (Voedingscentrum), the median intake of fish is approximately 70 g/week[49]. This reflects the intake of around 1x fish per two weeks. In the LifeLines population, we found a median intake of around 81.9 g/week, which was slightly higher compared to the numbers of the Dutch Food Center. The Dutch Food Center currently recommends the intake of one portion of fish per week. Potentially, our conclusion would have been different if we had chosen to investigate the fatty acids in plasma of populations eating large amounts of fish, such as the habitants of Greenland and Japan. The outcome of this study may therefore only apply for populations low in fish intake and high in dairy intake.

Table 1: Composition of fat from milk and fish (i.e. salmon and Herring) in the Netherlands

	Milk			Fish	
	Full-Fat	Semi-Skimmed	Skimmed	Salmon	Herring
g/100g					
Total fat	3.4	1.5	0.1	10.8	10.01
SFA	2.2	1.0	0.1	2.5	2.5
MUFA	0.7	0.3	0	5.4	5.1
PUFA	0.1	0	0	2.1	1.8
TFA	0.1	0	0	0	0
% of total fat					
C14:0		11.3		5.4	7.8
C15:0		1.0		0.4	0.5
C17:0		0.6		0.2	0.3
T-C16:1		0.2		0	0
T-C18:1		1.7		0	0
EPA		0.1		4.7	5.6
DHA		0		7.9	6.0

The fatty acid composition of the most common potential fat biomarkers in milk and fish. In this table, full-fat, semi-skimmed and skimmed milk are compared to the two most common types of fish that are eaten in the Netherlands; i.e. salmon and herring. Numbers are from the online NEVO table 2016 (<https://nevo-online.rivm.nl>).

Abbreviations: C14:0, Myristic acid; C15:0, Pentadecanoic acid; C17:0, Heptadecanoic acid; EPA, Eicosapentaenoic acid, DHA, Docosahexaenoic acid.

DAIRY FAT INTAKE AND CARDIOVASCULAR HEALTH

Intake of dairy has been suggested to reduce cardiovascular disease and its risk factors. For example, a narrative review concluded that dairy consumption, despite its high saturated fat content, may have positive or neutral effects on cardiometabolic risk factors and cardiovascular diseases[50]. Additionally, evidence from a prospective cohort study with more than 42,000 participants demonstrated that the intake of dairy products may reduce the risk of cardiovascular mortality[51]. In addition to total dairy intake, fat from dairy products may have beneficial effects on cardiovascular risk factors also, including saturated and trans fatty acids. A study with Swedish adults demonstrated that C15:0 was inversely associated with weight, waist circumference and BMI[1]. Two papers with data from 3,736 US adults found that trans-C16:1(n-7) was associated with higher insulin sensitivity, and lower levels of atherogenic dyslipidemia, circulating triglycerides, blood pressure and lower incidence of diabetes[52,53]. To investigate whether the fatty acids from dairy intake were also associated with a beneficial cardiovascular health profile in the Dutch population, we explored the association of commonly and less commonly investigated biomarkers with several cardiovascular risk factors in the Lifelines Biobank and Cohort study in **Chapter 5** of this thesis.

Besides trans-C18:1(n-7), the dairy fatty acids C15:0 and C17:0 in both plasma TG and PL exhibited inverse associations with cardiovascular risk factors, especially waist circumference and BMI. Our results suggest that waist circumference and BMI may have a mediating effect. Furthermore, dairy fat biomarkers may improve cardiovascular health by reducing chronic inflammation and uric acid as well. Both hs-CRP and uric acid had strong associations with dairy fat biomarkers.

Recently, two other papers have been published with data from the LifeLines Biobank and Cohort study. Where we investigated the association between dairy fat biomarkers and cardiovascular risk factors, they investigated the effects of dairy product intake on overweight, pre-diabetes and newly diagnosed type 2 diabetes[54,55]. The investigators found an inverse association between full-fat dairy product intake and overweight (BMI ≥ 25 -30 kg/m²) and obesity (BMI ≥ 30 kg/m²)[55], which is actually in line with our findings for C15:0 and C17:0. Interestingly, compared to our own data, the authors found an opposite association of high-fat dairy product intake with pre-diabetes and newly diagnosed type 2 diabetes[54]. While we found an inverse association between dairy fat biomarkers and diabetes related outcomes (including HbA1c and fasting glucose), they found an increase on the risk of pre-diabetes and newly diagnosed type 2 diabetes with intake of high-fat dairy products. The authors suggested that the association between high fat dairy products and diabetes-related outcomes may potentially not depend on the 'fat content' of a dairy product, but could be related to other attributes of the products in the high fat dairy category.

FISH INTAKE, FISH FAT BIOMARKERS AND (CARDIOVASCULAR) HEALTH

In the general population, there is abundant evidence that fat from fish may reduce the risk to develop cardiovascular diseases[56-59], by reducing inflammatory markers[60]. Potentially, this could have been due to a higher intake of C15:0 and C17:0 as described in **Chapter 5**. However, both odd-chain fatty acids proved unrelated to fish fat intake and to fish intake as a whole in the LifeLines Cohort. Instead, a strong association was found of EPA and DHA with fish intake and fish fat intake. These results are in line with previous studies. In the NQplus study, another Dutch cohort with more than 350 adults included, it was found that the omega-3 fatty acids were significantly associated with fish intake[11]. Furthermore, EPA and DHA were also associated with fish in a cross-sectional study with 110 young children (age 3-10 years)[8]. In addition, a study with 192 Asian participants demonstrated that plasma EPA+DHA was significantly associated with fish and seafood[61]. Moreover, randomized controlled trials showed that the intake of fish oil supplements (which are rich in EPA and DHA) may increase the amount of EPA and DHA that incorporates into body compartments such as plasma phospholipids and erythrocytes[62-65]. Based on previous and current findings, it is safe to state that up to now, EPA and DHA are the best biomarkers of fish fat intake in a high and low fish eating population.

Intake of fish, omega-3 fatty acids and (cardiovascular) health in renal transplant recipients

It has been suggested that omega-3 fatty acids may also have beneficial effects in a patient population such as renal transplant recipients (RTR). For instance, EPA and DHA supplements have been proposed to improve renal function, rejection rates and patient survival in RTR, but convincing evidence whether it may also improve survival is still lacking[66,67][68]. Besides the marine-derived omega-3 fatty acids EPA and DHA, there is also the plant-derived omega-3 fatty acid, called α -linolenic acid (ALA)[69], which has only been investigated sporadically. Up to now, it was unknown whether both the marine-derived and plant-derived omega-3 fatty acids may reduce all-cause and cardiovascular mortality in RTR. In **Chapter 6 and 7**, we therefore aimed to investigate the association between the intake of EPA+DHA and ALA with all-cause and cardiovascular mortality in stable RTR patients.

In **Chapter 6**, EPA-DHA and ALA intakes were estimated separately, so that both the marine-derived and plant-derived omega-3 fatty acids could be investigated. Firstly, we found a positive association between ALA intake and all-cause mortality. The reason why a higher ALA intake is associated with increased risk of all-cause mortality in RTR is unknown. However, we know that ALA may convert into EPA and subsequently into DHA[70,71]. Rate limiting in this pathway are the activities of $\Delta 5$ and $\Delta 6$ desaturases [72,73]. In renal transplant recipients, this route could be affected by glucocorticoids, a class of immunosuppressive drugs used to protect the transplanted kidney from rejection [74,75,75].

Secondly, we found a trend towards an inverse association between EPA-DHA intake and all-cause mortality in RTR. The contrast between the intake of marine and plant-derived omega-3 fatty acids is in line with findings reported for the general population[76]. However, the absence of an association between EPA-DHA intakes and all-cause mortality is not in line with literature on RTR[77,78]. Two observational studies showed a significant inverse association between EPA-DHA intake or status and all-cause mortality in RTR. The studies included 1,990 Norwegian RTR and demonstrated that high levels of plasma phospholipid omega-3 fatty acids were inversely associated with mortality. Importantly, these studies were not on omega-3 fatty acid intake per se, but on plasma biomarker status of omega-3 fatty acids, which is the outcome of intake and metabolism. Furthermore, these studies had a longer follow-up period (6.8 years) with a large number of cases (569 developed graft failure and 406 died in a population of 1,990 RTR) compared to our study (follow-up period of 3.1 years, during which 41 developed graft failure and 67 died in a population of 637 RTR)[78,79]. Potentially, the association between omega-3 fatty acid intake and all-cause mortality did not reach significance in the current study due to the relatively short follow-up period and small number of cases. Therefore, in **Chapter 7**, we updated our database on the intake of omega-3 fatty acids and extended the follow-up period (130 deaths in a cohort of 627 with a follow-up period of 5.4 years) to increase the power for the association of omega-3 fatty acid intake with all-cause and cardiovascular mortality.

In **Chapter 7**, we did demonstrate that intake of the omega-3 fatty acids EPA and DHA is inversely associated with all-cause mortality in stable outpatient RTR. Additionally, we showed an inverse, albeit not significant, association with cardiovascular mortality. This is in line with the Norwegian study in 1,990 RTR, which found a weak inverse association between plasma EPA-DHA levels and cardiovascular mortality[80]. Additionally, they showed that higher levels of plasma EPA-DHA were associated with lower levels of cardiovascular risk markers, including fasting plasma glucose and plasma triglycerides.

Influence of drugs on omega-3 fatty acids

As previously mentioned in the literature, supplementation of the omega-3 fatty acids EPA and DHA increases the omega-3 status in humans and may thereby reduce cardiovascular risk[62-65]. In addition, in this thesis, we discovered an inverse association between EPA and DHA status and cardiovascular risk. For instance, an increase in the status of omega-3 fatty acid has been suggested to decrease inflammation, and related chronic diseases[81-84]. Secondly, we found a positive association between the ALA status and the risk of mortality in RTR. It has been suggested that drugs such as glucocorticoids (i.e. prednisolone) may have influence on the fatty acid ratios in the body. Glucocorticoids may depress the conversion of ALA into EPA and DHA, which results in an inhibition of the EPA+DHA status and accumulation of ALA. Both may be related to an increase in cardiovascular risk factors. In addition, glucocorticoid treatment is

associated with impaired insulin sensitivity in the liver, diabetes, dyslipidemia and an unfavorable body fat distribution[85-88].

From animal studies, it has been suggested that glucocorticoids may decrease delta-6-desaturase activity (D6D), and thereby decrease the formation of omega-3 fatty acids[72,89]. Whether this is also the case in humans is unknown. In **Chapter 8**, we therefore conducted a *post-hoc* analysis of a 2-week randomized, placebo-controlled, double-blind trial in which we investigated the dose-dependent influence of prednisolone on a surrogate marker of D6D activity in healthy young males. As a secondary aim, we investigated whether the changes in surrogate D6Da activity were related with prednisolone induced changes in fasting metabolic parameters and insulin sensitivity.

In **Chapter 8**, we demonstrated as one of the first investigators that a 2-weeks trial of prednisolone administration in healthy young males resulted in a dose-dependent decrease in surrogate D6D activity. In addition, we demonstrated that the prednisolone-induced changes in surrogate D6D activity were related to prednisolone-induced unfavorable alterations in metabolic parameters, including circulating triglycerides, fasting glucose and insulin resistance (HOMA-IR). The association between prednisolone-induced changes in fatty acid metabolism and metabolic parameters was in accordance with other human studies. First of all, a study carried out in 2008 showed an inverse correlation between intrahepatic TG content and hepatic insulin sensitivity in 42 non-diabetic obese subjects[90]. In addition, a study in overweight adolescents showed that an elevated intrahepatic TG content was associated with insulin resistant glucose metabolism in the liver[91]. The difference between these studies and our study is that they were performed in overweight and obese subjects, while we investigated the association in healthy young males.

A limitation of **Chapter 8** is that the fatty acid ratios (product/precursor ratios) are not the perfect parameters for genuine DNL, SCD and D6D activities. The ratios have been criticized since they can be influenced by factors like diet, hormones and lifestyle factors (e.g. exercise) [92]. An alternative for measuring proxies for the DNL, SCD and D6D activities is by using in vivo methods in which stable isotopically labeled fatty acids are employed, in conjunction with ex vivo stable-isotope techniques[93]. However, since this was not available in our dataset, calculation of product/precursor ratios was considered a reasonable surrogate[94].

CONCLUSIONS AND FUTURE PERSPECTIVES

Cardiovascular disease is a common cause of death in the general as well as diseased population. Several dietary components, such as fatty acids, may play a huge role in this. Particularly fatty acids from dairy and fish have been proposed to reduce the risk of cardiovascular disease. In this thesis we explored the association of fatty acids from dairy with overall and cardiovascular health. Firstly, we investigated the commonly used and the less explored biomarkers of dairy

fat intake. Additionally, we tried to link these biomarkers to cardiovascular health and mortality. As a minor part of this thesis, we also investigated the association between omega-3 fatty acids and cardiovascular health.

In the LifeLines Dataset, we found that C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) and CLA measured in plasma TG and PL can be useful biomarkers of dairy fat intake for the general population. The associations with dairy biomarkers were independent of fish fat. Furthermore, a combination of fatty acids, including at least C15:0 and trans-C18:1(n-7), may predict dairy intake even better. We therefore recommend to further investigate a combination of dairy fat biomarkers. Furthermore, future research should preferably include analyses of fatty acid biomarkers in adipose tissue since the amount of fatty acids in adipose tissue is often higher than can be found in plasma or erythrocytes which may therefore show even stronger associations between dairy fat intake and fatty acid biomarkers. Thirdly, we would recommend to further investigate the laboratory methodology of the dairy fat biomarkers to optimize and shorten the analyses. Potentially, measuring the fatty acid in adipose tissue on a 100 m column would improve and shorten the analyses due to higher output compared to the current method. Furthermore, use of a GC-MS (alone or in combination with a GC-FID) equipped with a 200 m column might be a good alternative for the current method. In comparison to the dairy fat biomarkers, C15:0 and C17:0 were not found to be strong biomarkers for fish and fish fat intake in the present cohort, but we confirmed EPA and DHA to be strong biomarkers for fish and fish fat intakes.

In this thesis, using data from the LifeLines Biobank and Cohort study, we found that an increase in dairy fat biomarkers may be related to a decrease in cardiovascular risk factors. Associations were especially found for the dairy fat biomarkers C15:0, trans-C18:1(n-7) and CLA with BMI and waist circumference. An important limitation of the Lifelines Cohort is that the data are observational in nature and that other nutrients in milk and milk products (i.e. lactose, milk proteins and micronutrients), total energy intake and expenditure, percentage milk fat of total fat intake, and all potential interactions, might also explain some of the current results. It is therefore not possible to draw definite conclusions about the causality of the association of the fatty acids with cardiovascular risk factors. Furthermore, Lifelines only contained cross-sectional data which means that data were collected at a single point in time. At the moment, LifeLines is collecting prospective data. It is expected that, based on prospective data from other cohort studies, that the inverse association between dairy fat biomarkers and risk to develop cardiovascular diseases would even be stronger in the prospective dataset of LifeLines.

In addition to the dairy fat biomarkers, literature suggests that the traditional fish fat biomarkers EPA and DHA are related to a beneficial cardiovascular health profile. In addition, we found associations in a patient population such as renal transplant recipients. This was shown in a large single center RTR cohort in the University Medical Center Groningen. The results of

EPA-DHA were in contrast to the results found with ALA. In addition, earlier research suggested that in the general population ALA does not have a beneficial effect. Future studies should preferably investigate EPA-DHA separately of ALA because of the discrepancy between EPA-DHA and ALA found, otherwise this might have influence on the overall outcome. Interestingly, this thesis shows that high intake of ALA may even be associated with an increased risk of premature mortality in renal transplant recipients. What the mechanism is behind the inverse association is not known, however it may be suggested that the inverse association is not directly due to ALA status or intake. An explanation might be that the inverse association is actually due to the high intake of glucocorticoids. Glucocorticoids may have a negative influence on the ALA, EPA and DHA status; i.e. reduced conversion of ALA into EPA and DHA causing accumulation of ALA. Further research in this field is therefore necessary to unravel the underlying mechanism between the association of ALA status and (cardiovascular) mortality risk in RTR. It is also suggested that the inverse association might actually be due to oxidation of ALA during baking or frying of ALA rich foods (i.e. oils rich in ALA). Future studies should therefore also investigate the source of ALA, and whether this is used for baking or frying of products.

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NEDERLANDSE SAMENVATTING

In de algemene bevolking komen veel mensen te overlijden door hart- en vaatziekten. Voedingscomponenten zijn belangrijke factoren bij de ontwikkeling van en bescherming tegen het ontstaan van hart- en vaatziekten. In dit kader worden vetzuren gezien als een belangrijke voedingscomponent. Vetzuren zijn onderdeel van een grote diversiteit aan voedselproducten, waaronder zuivel, vis en plantaardige oliën. Hiervan zijn vetzuren die voornamelijk in zuivel voorkomen bijzonder, omdat in zuivel de diversiteit van de vetzuren die er in voorkomen erg hoog is. Daarnaast worden verschillende van de vetzuren die er in voorkomen gezien als uniek voor deze voedingsbron. Het gaat hier vooral om twee vetzuren met een oneven ketenlengte (C15:0 en C17:0), en de natuurlijke transvetzuren Vacceenzuur (Trans-C18:1(n-7)) en Linolzuur (CLA). Eén van de redenen waarom het moeilijk is om het verband tussen inname van zuivelvet en cardiovasculaire gezondheid te onderzoeken, is omdat een objectieve meetmethode om de inname van zuivelvet te meten momenteel ontbreekt. Tot op de dag van vandaag maken onderzoekers voornamelijk gebruik van methodes zoals voedselfrequentievragenlijsten of voedingsdagboekjes. Deze methodes zijn over het algemeen niet objectief, en kunnen daarom een vertekend beeld geven. Het meten van vetzuur biomarkers in het bloed geeft weliswaar geen directe informatie over de inname, maar geeft wel objectieve informatie. Tot op heden is deze methode nog maar weinig onderzocht. Een andere reden daarvan kan zijn dat biomarkers die met zuivelvet worden ingenomen, potentieel ook gezien kunnen worden als biomarkers voor visvet inname (vooral C15:0 en C17:0).

In dit proefschrift was ons eerste doel om reeds eerder geëvalueerde (C14:0, C15:0, C17:0 en Trans-C16:1(n-7)) alsmede nog nauwelijks onderzochte biomarkers (Trans-C18:1(n-7) en CLA) voor het voorspellen van zuivel en zuivelvet inname in de algemene bevolking te onderzoeken. Hiervoor hebben we een nieuwe methode ontwikkeld waarmee we alle mogelijke biomarkers konden meten. Daarnaast hebben we onderzocht of het verband tussen de zuivelvet biomarkers en zuivelvet inname onafhankelijk was van visvet inname. Ons tweede doel van dit proefschrift was onderzoek te doen naar het verband tussen zuivelvet inname en risicofactoren voor de ontwikkeling van hart- en vaatziekten. Daarnaast hebben we ook onderzoek gedaan naar het verband tussen visvet inname en risicofactoren voor de ontwikkeling van hart- en vaatziekten. Dit hebben we gedaan, om te onderzoeken of een effect van vetzuren in zuivel eigenlijk het gevolg zou kunnen zijn van een verhoging van vetzuren in visvet, omdat eerdere publicaties vooral een reductie in risicofactoren van hart- en vaatziekten lieten zien naarmate men meer visvet at. We hebben hierbij niet alleen gericht op de algemene bevolking, maar ook op niertransplantatiepatiënten, omdat dit een patiëntengroep is waarbij het risico op hart- en vaatziekten zeer hoog is.

Zuivelvet biomarkers

Om te kijken of vetzuren in bloed kunnen dienen als biomarkers voor de inname van zuivel en zuivelvet in de algemene bevolking, hebben we in **hoofdstuk 2** een systematische review en meta-analyse van observationele studies uitgevoerd. Hierbij werden observationele studies meegenomen welke het verband bekeken van zuivelvetzuren gemeten in plasma/serum, erythrocyten en vetweefsel met zuivel en zuivelvet gemeten met meer traditionele methoden (voedselfrequentievragenlijsten, voedingsdagboekjes en 24-uurs “recalls”). De systematische review identificeerde C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) en CLA in plasma/serum, erythrocyten en vetweefsel als mogelijke biomarkers voor zuivelvet inname in de algemene bevolking. Daarnaast bevestigde de meta-analyse dat C14:0, C15:0, C17:0 en trans-C16:1(n-7) als biomarkers voor de inname zuivel en zuivelvet kunnen worden gebruikt. C15:0 werd geïdentificeerd als één van de sterkste biomarkers.

In het review waren er ook een aantal tekortkomingen die naar voren kwamen aangaande het gebruik van vetzuren als biomarkers voor het meten van de inname van zuivelvet. Als eerste kwamen we er achter dat trans-C18:1(n-7) en CLA amper waren onderzocht, mogelijk doordat de gebruikte meetmethode niet toeliet ze apart te identificeren. De meeste methodes voor het meten van vetzuren kunnen namelijk het natuurlijk transvetzuur (trans-C18:1(n-7)) niet onderscheiden van andere, voornamelijk industrieel gevormde C18:1 transvetzuren. Ten tweede zagen we dat de sterktes van de verbanden tussen de zuivelvetzuren en inname van zuivelvet op een schaal van -1.00 tot 1.00 lagen tussen de 0.10 en 0.33, wat gezien word als relatief zwakke verbanden. Een van de mogelijke oorzaken is dat biomarkers vaak afzonderlijk worden onderzocht, terwijl combinaties mogelijk sterkere verbanden laten zien. Ten derde kunnen C15:0 en C17:0 ook gevonden worden in vis. Beide biomarkers werden daarom aangemerkt als minder valide biomarkers voor de inname van zuivelvet in een populatie met hoge vis consumptie.

Door de observaties die wij hebben gedaan bij het maken van het review, zijn we in **hoofdstuk 3** begonnen met het ontwikkelen van een nieuwe methode voor het meten van zowel de reeds bekende zuivelvetzuren alsmede het meten van de nog veel minder onderzochte zuivelvetzuren trans-C18:1(n-7) en CLA in plasma cholesterol esters (CE), triglyceriden (TG) en fosfolipiden (PL). De metingen werden uitgevoerd in samples afkomstig uit de Lifelines Biobank. In dit hoofdstuk wordt de methode beschreven die we hebben ontwikkeld waarmee trans-C18:1(n-7) van de andere transvetzuren onderscheiden kan worden. Dit kon door gebruik te maken van een polaire gas chromatografie kolom met een lengte van 200 meter in plaats van de normaal gebruikte apolaire kolom met een lengte van 100 meter. De betreffende vetzuren bleken alleen meetbaar in plasma triglyceriden en fosfolipiden.

De nieuwe methode die beschreven werd in hoofdstuk 3, is toegepast in **hoofdstuk 4**. In dit hoofdstuk hebben we onderzoek gedaan naar mogelijke biomarkers voor zuivel en zuivelvet inname in de algemene bevolking. In dit hoofdstuk werden zowel de bekende als de tot nu

toe minder onderzochte biomarkers meegenomen. Ook in dit hoofdstuk is er gebruik gemaakt van samples en data van het Lifelines cohort, waarvan de deelnemers afkomstig zijn uit de noordelijke drie provincies van Nederland. Van de reeds eerder onderzochte biomarkers bleek C15:0 de sterkste enkelvoudige biomarker voor het voorspellen van de inname van zuivelvet in de algemene bevolking. De sterkte van het verband van C15:0 met de inname van zuivelvet, wederom afgemeten op een schaal die loopt van -1.00 tot 1.00, was 0.29 voor plasma TG en 0.22 voor plasma PL. De minder onderzochte biomarkers, plasma trans-C18:1(n-7) en CLA, waren beide goede vetzuur biomarkers voor het voorspellen van zuivelvet inname (trans-C18:1(n-7): 0.29 voor plasma TG en 0.27 voor plasma PL; CLA: 0.22 voor plasma TG en 0.27 voor plasma PL). Een combinatie van de bekende vetzuren samen met de tot nu toe minder onderzochte vetzuren leverde een betere voorspelling voor de inname van zuivelvet in de algemene bevolking dan enkelvoudige biomarkers.

Omdat sommige van de vetzuren die worden gezien als biomarkers voor de inname van zuivel ook kunnen voorkomen in vis (dit geldt vooral voor C15:0 en C17:0) en daarom soms gezien worden als visvet biomarkers, hebben we in **hoofdstuk 5** onderzocht of het verband tussen inname van zuivelvet en biomarkers voor zuivelvet compleet toe te schrijven was aan inname van zuivelvet, of dat sommige biomarkers toch meer de inname van visvet zouden voorspellen. C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) en CLA bleken goede voorspellers voor de inname van zuivelvet, omdat het verband tussen de biomarkers en de inname van zuivelvet onafhankelijk was van de inname van visvet. C15:0 en C17:0 bleken geen goede voorspellers voor de inname van visvet.

Zuivelvet biomarkers en cardiovasculaire gezondheid

In de literatuur is terug te vinden dat vet afkomstig uit zuivelproducten risicofactoren van hart- en vaatziekten mogelijk kunnen verminderen. Risicofactoren voor het ontstaan van hart- en vaatziekten zijn onder meer gewicht, “body mass index”, bloeddruk en cholesterol, maar ook meer diabetes gerelateerde risicofactoren en de minder traditionele risicofactoren Hs-CRP (inflammatie marker) en urinezuur. In **hoofdstuk 5** hebben we dan ook gekeken naar het verband tussen de zuivelvet biomarkers en verschillende traditionele, diabetes gerelateerde en minder traditionele risicofactoren voor hart- en vaatziekten. Er was duidelijk een gunstig verband gevonden tussen de zuivelvet biomarkers C15:0 en C17:0 in plasma TG en C15:0, C17:0 en trans-C18:1(n-7) in plasma PL met traditionele risicofactoren zoals gewicht, buikomvang, en de “body mass index”. Daarnaast zagen we dat er een gunstig verband was tussen zuivelvet biomarkers en diabetes gerelateerde risicofactoren, zoals bloedglucose en HbA1C, alsmede met de minder traditionele risicofactoren.

Vis inname en (cardiovasculaire) gezondheid

Er is overvloedig bewijs dat een hoge inname van visvet het risico op hart- en vaatziekten in de algemene bevolking verlaagd. Zoals aangegeven in **hoofdstuk 5**, kon dit mogelijk het geval zijn door een gelijktijdige hoge inname van C15:0 en C17:0. Voor beide vetzuren werd een dergelijk verband tussen de vetzuren C15:0 en C17:0 met visvet inname echter niet gevonden in het Lifelines Cohort, en was het verband tussen de vetzuren en cardiovasculaire risicofactoren niet toe te schrijven aan visvet inname. Daarentegen was er een sterk verband tussen de omega-3 vetzuren eicosapentaëenzuur (EPA) en docosahexaëenzuur (DHA), en de inname van vis en visvet. In de literatuur is eerder naar voren gekomen dat EPA en DHA mogelijk een positief effect hebben op de cardiovasculaire gezondheid. Dit is niet alleen het geval in de algemene bevolking, maar meer nog in patiëntengroepen zoals niertransplantatiepatiënten. Naast de omega-3 vetzuren uit vis, bestaat er ook een plantaardig omega-3 vetzuur α -Linoleenzuur (ALA), waar maar sporadisch afzonderlijk onderzoek naar is gedaan. Tot op heden was het onbekend of zowel de omega-3 vetzuren uit vis, alsmede het plantaardige omega-3 vetzuur het risico op hart- en vaatziekten bij niertransplantatiepatiënten zou kunnen verkleinen. In de **hoofdstukken 6 en 7** hebben we daarom onderzoek gedaan naar het verband tussen de inname van EPA+DHA en ALA met sterfte aan hart- en vaatziekten bij stabiele niertransplantatiepatiënten.

In **hoofdstuk 6** werd de inname van EPA+DHA en ALA apart vastgelegd, zodat beide typen omega-3 vetzuren apart konden worden onderzocht. Ten eerste vonden we een positief verband tussen de inname van ALA en sterfte. Ten tweede zagen we een neiging naar een beschermend verband tussen de inname van EPA+DHA en sterfte. Mogelijk vonden we hier geen significant verband, doordat de patiënten nog relatief kort gevolgd waren (gemiddeld 3,1 jaar), wat er voor zorgde dat er maar weinig te onderzoeken gevallen waren (67 doden in een cohort van 637 patiënten). Voor **hoofdstuk 7** hebben we daarom de database aangevuld; de patiënten werden langer gevolgd (gemiddeld 5,4 jaar), wat er toe leidde dat er meer te onderzoeken gevallen waren (130 doden in een cohort van 627). Het verband tussen de inname van omega-3 vetzuren EPA+DHA en de kans op sterfte kreeg hierdoor meer kracht. We hebben in dit hoofdstuk dan ook gevonden dat er een beschermend verband was tussen de inname van de omega-3 vetzuren EPA en DHA en sterfte in stabiele niertransplantatiepatiënten. Verder hebben we in dit hoofdstuk een neiging naar een beschermend effect voor het optreden van sterfte aan hart- en vaatziekten gevonden.

De reden waarom een hogere ALA inname in niertransplantatiepatiënten is verbonden met een verhoogde kans op voortijdig overlijden is niet bekend. Het is wel bekend dat ALA kan worden omgezet naar EPA, en vervolgens naar DHA. In niertransplantatiepatiënten kan deze omzettingroute mogelijk worden geblokkeerd door het chronisch gebruik van glucocorticoiden. Glucocorticoiden hebben mogelijk een negatieve beïnvloeding op de EPA en DHA status, wat weer kan resulteren in een verslechtering van de cardiovasculaire gezondheid. Uiteindelijk kan

dit dan leiden tot een hogere kans op voortijdig overlijden. In **hoofdstuk 8** hebben we onderzocht wat voor effect glucocorticoïden hebben op de EPA en DHA status bij gezonde jonge mannen. Daarnaast hebben we het verband tussen eventuele veranderingen en risicofactoren voor hart- en vaatziekten onderzocht. Informatie van dierexperimenten suggereert dat glucocorticoïden de activiteit van het enzym delta-6-desaturase negatief beïnvloeden, wat kan leiden tot een verminderde omzetting van ALA naar EPA en DHA. We hebben in een 2-weken durend onderzoek naar het effect van inname van het glucocorticoïd prednisolon bij gezonde jonge mannen inderdaad gevonden dat er een afname was van de activiteit van dit enzym. Deze afname was dosisafhankelijk, waarbij de hoogste dosis de grootste afname veroorzaakte. Er was een verband tussen de afname van de delta-6-desaturase activiteit en ongunstige veranderingen in risicofactoren voor hart- en vaatziekten en suikerziekte.

Conclusie en toekomstig onderzoek

In het eerste gedeelte van dit proefschrift hebben we gevonden dat C14:0, C15:0, C17:0, trans-C16:1(n-7), trans-C18:1(n-7) en CLA, gemeten in plasma TG en PL, goede biomarkers zijn voor het meten van de inname van zuivelvet in de algemene bevolking. Het verband tussen inname van zuivelvet en biomarkers voor zuivelvet was onafhankelijk van de inname van visvet. Daarnaast hebben we gevonden dat een combinatie van vetzuren, met tenminste de vetzuren C15:0 en trans-C18:1(n-7), de inname van zuivelvet nog beter kan voorspellen dan enkelvoudige biomarkers. We raden toekomstige onderzoekers dan ook aan om een combinatie van vetzuren te gebruiken voor een optimale voorspelling van inname van zuivelvet. Daarnaast raden we aan om ook vetzuren te onderzoeken in vetweefsel, omdat mogelijk sterkere verbanden kunnen worden gevonden tussen inname van zuivelvet en biomarkers gemeten in vetweefsel dan in plasma. Als derde, zouden we willen aanraden om de meetmethode nog verder te optimaliseren, bijvoorbeeld door het gebruik van massaspectrometrie.

In het tweede gedeelte van dit proefschrift hebben we geprobeerd om de vetzuur biomarkers te verbinden aan gezondheid van hart- en bloedvaten. Hiervoor hebben we gebruik gemaakt van gegevens van de Lifelines Biobank en Cohort studie. Er was voornamelijk een gunstig verband van de vetzuren C15:0, trans-C18:1(n-7) en CLA met risicofactoren voor hart- en vaatziekten, waaronder de “body mass index” en de buikomvang. Naast een gunstig verband voor de biomarkers voor zuivelvet met risicofactoren voor hart- en vaatziekten, wordt dit ook veelvuldig gesuggereerd voor de traditionele visvetten EPA en DHA. In het laatste gedeelte van dit proefschrift hebben we gevonden dat de inname van EPA en DHA in een populatie niertransplantatiepatiënten mogelijk kan leiden tot een betere gezondheid met een verminderde kans op voortijdig overlijden. Daarentegen was de inname van ALA juist verbonden met een verhoogde kans op voortijdig overlijden. Toekomstige studies doen er daarom goed aan om de inname van EPA+DHA en ALA apart te onderzoeken. Het mechanisme dat verantwoordelijk is

voor het verhoogde risico op voortijdig overlijden bij een relatief grote inname van ALA is niet bekend. Mogelijk speelt de inname van glucocorticoïden bij de groep patiënten die is onderzocht een grote rol. Glucocorticoïden kunnen mogelijk leiden tot een verlaging van EPA en DHA, wat weer kan resulteren in een verslechtering van de gezondheid op het gebied van hart- en vaatziekten. Uiteindelijk kan dit dan leiden tot een hogere kans op voortijdig overlijden. Meer onderzoek is nodig om uit te zoeken wat precies de reden is waarom een verhoogde inname van ALA kan leiden tot een verhoogde kans op voortijdig overlijden.

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-Ilse-

ABOUT THE AUTHOR

Ilse Geertje Pranger was born on October 30th, 1988 in Dronrijp (Friesland, The Netherlands). She attended high school at CSG Comenius in Leeuwarden, and received her VWO diploma (profile Nature & Health) in 2007 at the Luzac College in Groningen. In the same year, she started to study Nutrition & Health at the Wageningen University and Research Centre (WUR). In 2010, she started with the Master specialization 'Nutritional Physiology and Health Status. As part of her Master she studied at the University of Wisconsin-Madison, USA. There, she followed several courses in the field of Dairy science, Physiology and Cardiovascular Health. In 2011/2012 she successfully performed a Master Thesis at the University Medical Center Nijmegen under supervision of Manon van den Berg (UMCN), Ellen Rasmussen (WUR) and Jeanne de Vries (WUR). She investigated whether a combined therapy of individual dietary counseling and swallowing therapy improved the quality of life in head- and neck cancer patients who received (adjuvant) (chemo)radiotherapy. As part of her Master, she also did an internship at the Research and Development Department of FrieslandCampina, where she investigated the nutritional and physiological status of several sugars and fibers in infant formulas.

After she obtained her Master's Degree in 2012, she first worked as a Research Assistant for nutritional studies (e.g. NQPLUS, ENOF) at the WUR, before starting with her PhD in the field of Nutrition and Epidemiology in March 2014 at the UMCG. Her PhD focused on fatty acids as biomarkers for health status and nutritional intake. The main project of the thesis was the identification of dairy fat biomarkers and their association with cardiovascular risk factors. The research was performed in collaboration with FrieslandCampina. During her PhD she followed the Advanced Epidemiology Programme for PhD at the UMCG.

In May 2018, Ilse started working at PPD (Bennekom, The Netherlands) in the field of clinical research. There, she is following a programme to become an (in-house) CRA (Clinical Research Associate).

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