Importance of dairy foods as a contributor to dietary saturated fat intake and impact on cardiometabolic disease risk

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A thesis is part fulfilment of the requirement for the degree of

Doctor of Philosophy

Department of Food and Nutritional Sciences

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July, 2022

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Laury Sellem,

Reading, July 2022.

Acknowledgments

First of all, I would like to express my immense gratitude to my supervisors, Prof Julie Lovegrove and Dr Kim Jackson. Their continuous support, patience, knowledge, and guidance have helped me to get through the good and the difficult times of this five-year journey. I could not have imagined having better advisors and mentors for my PhD studies.

I would also like to thank the members of the RISSCI-1 study team Thanasis Koutsos, Ezgi Ozen, Gloria Wong, Rona Antoni, and Bruce Griffin, for their great team work, guidance, and perseverance. I also sincerely thank the members of the ILSI task force on dietary SFAs Matthieu Flourakis, Peter Joris, Szimonetta Lohner, Ronald Mensink, and Sabita Soedamah-Muthu, for their guidance, patience, and for everything they taught me along the way. I would also like to thank our collaborators from the German Institute of Human Nutrition, Fabian Eichelmann, Clemens Wittenbecher, and Matthias Schulze, for their team spirit and support for the analysis of lipidomics data, and Andreas Wernitz for his contribution to the analysis of samples from the RISSCI-1 study. Finally, I would like to thank our collaborators from the Sorbonne Paris Nord University, especially Mathilde Touvier and Bernard Srour, who gave me not only the opportunity to work together on the NutriNet-Santé cohort, but also supported me for the past year by welcoming me in their research team in Paris while finishing my PhD studies.

I would also like to express my gratitude to all the staff within the Hugh Sinclair Unit of Human Nutrition who participate in the smooth running of human intervention studies on a daily basis: research nurses Rada Mihaylova and Karen Jenkins, unit managers Kelly Jarett and Sarah Hargreaves. I also thank current and previous researchers in the Nutrition Research Group and the School of Agriculture Kirsty Kliem, Michelle Weech, Arife Yilmaz, Oonagh Markey, Kumari Rathnayake, Ian Givens, and Yianna Chatzidiakou for all their contributions to our research projects, advice, and useful discussions over the past five years. I am also very grateful to the research students who contributed to the RISSCI study, along with James Lumley and Laura Paper who participated in the successful publication of our research projects.

Finally, I am infinitely grateful to my friends and family for their endless support throughout this journey. I want to give a special thanks to Ezgi, who was the best PhD buddy one could hope for, and to Tom, who is my greatest supporter.

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List of abbreviations

%TE, % total energy

%wt, % weight

Ach, acetylcholine

AOAC, Association of Analytical Chemists

Apo, apolipoprotein

AU, arbitrary units

BMI, body mass index

CE, cholesteryl esters

CER, ceramides

CHD, coronary heart disease

CI, confidence interval

CLP, Complex Lipid Platform

CMD, cardiometabolic disease

CRP, C-reactive protein

CV, cross-validation

CVD, cardiovascular disease

DAG, diacylglycerol

DBP, diastolic blood pressure

DHA, docosahexaenoic acid

DIVAS, Dietary Intervention and VAScular function

ENR, elastic-net regression

EPIC, European Prospective Investigation into Cancer and Nutrition

FAMEs, fatty acid methyl esters

FAs, fatty acids

FFQ, food frequency questionnaire

FID, flame ionization detector

FMD, flow-mediated dilation

GC, gas chromatograph

HCER, hexosylceramides

HDL-C, high-density lipoprotein cholesterol

HKSJ, Knapp-Hartung-Sidik-Jonkman

HOMA-IR, homeostatic model assessment for insulin resistance

HR, hazard ratio

ICAM-1, intercellular adhesion molecule 1

ICD-CM, International Classification of Diseases-Clinical Modification codes

IL-6, interleukin 6

IPAQ, international physical activity questionnaire

LDI, laser doppler imaging

LDL-C, low-density lipoprotein cholesterol

Lp(a), lipoprotein (a)

LPC, lysophosphatidylcholine

LPE, lysophosphatidylethanolamine

MAG, monoacylglycerol

MI, myocardial infarction

MUFAs, monounsaturated fatty acids

N/A, not applicable

NDNS, National Diet and Nutrition Survey

NEFAs, non-esterified fatty acids

NOx, nitrogen oxides

NS, not specified/not significant

PAI-1, plasminogen activator inhibitor-1

PAL, physical activity levels

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PEP, phosphatidylethanolamine plasmalogen

PhA, phytanic acid

PL, phospholipid

PNNS, French National Health and Nutrition Programme

PP, pulse pressure

PUFAs, polyunsaturated fatty acids

QUICKI, quantitative insulin sensitivity check index

RCT, randomised controlled trial

REML, restricted maximum likelihood

RESET, Replacement of Saturated fat in dairy on Total cholesterol

rQUICKI, revised quantitative insulin sensitivity index

RR, relative risk/risk ratio

SACN, Scientific Advisory Committee on Nutrition

SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation

SBP, systolic blood pressure

SD, standard deviation

SE, standard error

SFAs, saturated fatty acids

SLR, systematic literature review

SNP, sodium nitroprusside

T2D, type 2 diabetes

TAG, triacylglycerol

TC, total cholesterol

TFAs, *trans* fatty acids

TIA, transient ischemic attack

TNF-α, tumour necrosis factor-α

tPA, *trans*-palmitoleic acid (Chapter 1), tissue plasminogen activator (Chapter 3)

tVA, vaccenic acid

UFAs, unsaturated fatty acids

VCAM-1, vascular cell adhesion protein 1

VLDL-C, very low-density lipoprotein cholesterol

WMD, weighted mean difference

List of publications and presentations

Original research manuscripts published as a first author

Sellem L, Antoni R, Koutsos A, Ozen E, Wong G, Ayyad H, Weech M, Schulze MB, Wernitz A, Fielding BA, Robertson MD, Jackson KG, Griffin BA, Lovegrove JA. Impact of a Food-Based Dietary Fat Exchange Model for Replacing Dietary Saturated with Unsaturated Fatty Acids in Healthy Men on Plasma Phospholipids Fatty Acid Profiles and Dietary Patterns. *European Journal of Nutrition*, 6 June 2022. https://doi.org/10.1007/s00394-022-02910-2.

Sellem L, Jackson KG, Paper L, Givens ID, Lovegrove JA. Can Individual Fatty Acids Be Used as Functional Biomarkers of Dairy Fat Consumption in Relation to Cardiometabolic Health? A Narrative Review. *The British Journal of Nutrition*, 28 January 2022, 1-38. https://doi.org/10.1017/S0007114522000289.

Sellem L, Flourakis M, Jackson KG, Joris P, Lumley J, Lohner S, Mensink RP, Soedamah-Muthu SS, Lovegrove JA. Impact of Replacement of Individual Dietary SFAs on Circulating Lipids and Other Biomarkers of Cardiometabolic Health: A Systematic Review and Meta-Analysis of Randomized Controlled Trials in Humans. *Advances in Nutrition*, 25 November 2021. https://doi.org/10.1093/advances/nmab143.

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Sellem L, Kliem KE, Weech M, Yilmaz A, Minihane AM, Jackson KG, Lovegrove JA. Identification of plasma phospholipid fatty acids associated with chronic dairy consumption in UK adults: a secondary analysis of controlled dietary intervention studies. For submission to the *American Journal of Clinical Nutrition*.

Abstracts peer-reviewed and published in conference proceedings as a first author

Sellem L, Eichelmann F, Weech M, Jackson KG, Schulze M, Lovegrove JA. The impact of dietary saturated fat replacement with unsaturated fat on the plasma lipidome and cardiometabolic disease risk. *Proceedings of the Nutrition Society*. Cambridge University Press; 2022;81(OCE1):E51.

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Oral presentations

Nutrition Society Summer Conference, July 2021 (online):

- "Plasma phospholipid fatty acid profiles in response to a dietary exchange of saturated with unsaturated fat in healthy men using full-fat or lower-fat dairy foods"
- "Evaluation of the role of individual dietary fatty acids and food matrix on cardiometabolic disease risk"

Food and Nutritional Sciences Postgraduate Symposium, November 2020 (online): "The impact of exchanging dietary saturated with unsaturated fat on dietary patterns in healthy men"

Food and Nutritional Sciences Postgraduate Symposium, November 2019 (online): "Consumption of dairy products and cardiovascular disease risk: results from the French prospective cohort NutriNet-Santé"

13th European Nutrition Conference (FENS), October 2019 (Dublin, Ireland): "Consumption of dairy products and cardiovascular disease risk: results from the French prospective cohort NutriNet-Santé"

Rank Prize Funds Mini-Symposium on Dairy Products and the Life Cycle – Emerging Evidence, September 2018 (Grasmere, UK): "Dairy fatty acids as biomarkers of cardiometabolic health"

Institute of Food Science and Technology – UK Postgraduate Young Scientist Competition, May 2018 (London, UK): "A food-chain approach to reduce saturated fat in dairy products: insights from the RESET human study"

Hugh Sinclair Unit of Human Nutrition Postgraduate Symposium, February 2018 (Reading, UK): "Dairy fatty acids as biomarkers of cardiometabolic health"

Prizes and Awards

Nutrition Society, Postgraduate Competition Winning Award (July 2021)

Yakult, Student Award for the "Best student in the Department of Food and Nutritional Sciences" (December 2018)

Institute of Food Science and Technology, UK Postgraduate Young Scientist Competition Winning Award (May 2018)

Food and Nutritional Sciences Postgraduate Symposia (2018-2020):

- 2nd place Award for the "Best 5-min oral communication" (November 2019)
- Winning Award for the "Best abstract" (February 2019)
- Winning Award for the "Best 3-minute thesis presentation" (February 2018)

List of original research manuscripts and abstracts published as a co-author

Original research manuscripts published in peer-reviewed journals

Eichelmann F, **Sellem L**, Wittenbecher C, Jäger S, Kuxhaus O, Prada M, Cuadrat R, Jackson KG, Lovegrove JA, Schulze MB. Deep Lipidomics in Human Plasma - Cardiometabolic Disease Risk and Effect of Dietary Fat Modulation. *Circulation*. 2022 Apr 15. DOI: 10.1161/CIRCULATIONAHA.121.056805. Epub ahead of print. PMID: 35422138.

Contribution: I organised the lipidomics analysis in samples from the DIVAS randomised controlled trial and assisted FE (first author) with the statistical analyses in the DIVAS dataset. Full-text of the published manuscript is included in **Appendix 2**.

Witard OC, Bath SC, Dineva M, **Sellem L**, Mulet-Cabero AI, van Dongen LH, Zheng JS, Valenzuela C, Smeuninx B. Dairy as a Source of Iodine and Protein in the UK: Implications for Human Health Across the Life Course, and Future Policy and Research. *Front Nutr*. 2022 Feb 10;9:800559. DOI: 10.3389/fnut.2022.800559. PMID: 35223949.

Contribution: I participated in the writing of the paragraphs on "biomarkers of dairy fat" and "untargeted approaches to identify novel biomarkers: metabolomics and lipidomics". I also participated in the revisions of the manuscript for publication.

Abstracts peer-reviewed and published in conference proceedings

Koutsos A, Antoni R, Wong G, **Sellem L**, Ozen E, Ayyad H, et al. Reproducibility of the Reading Imperial Surrey Saturated fat Cholesterol Intervention (RISSCI-1 and 2) study. *Proceedings of the Nutrition Society.* Cambridge University Press; 2021;80(OCE5):E219.

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Koutsos A, Antoni R, Ozen E, Wong G, **Sellem L**, Jin L, et al. Determination of variability in serum low density lipoprotein cholesterol response to the replacement of dietary saturated fat with unsaturated fat, in the Reading, Imperial, Surrey Saturated fat Cholesterol Intervention ('RISSCI') project. *Proceedings of the Nutrition Society.* Cambridge University Press; 2020;79(OCE1):E6.

Antoni R, **Sellem L**, Koutsos A, Weech M, Zhong X, Wong G, et al. A dietary exchange model to study inter-individual variation in serum low-density lipoprotein cholesterol response to dietary saturated fat intake. *Proceedings of the Nutrition Society*. Cambridge University Press; 2019;78(OCE1):E9.

General abstract

Public dietary guidelines worldwide recommend that dietary saturated fatty acids (SFAs) do not exceed 10% total energy (%TE), and their replacement with unsaturated fatty acids (UFAs) has been shown to help prevent cardiometabolic disease (CMD). However, research on the impact of dietary SFAs on CMD risk mostly relies on indirect evidence either from intervention studies measuring biomarkers of disease risk, such as fasting lipid profiles, or from observational prospective cohort studies with disease outcomes. In addition, emerging research suggests the impact of dietary SFAs on cardiometabolic health might be modulated by novel CMD risk markers identified by omics approaches, differential effects of individual SFAs, and/or specific food matrix effects. In particular, dairy products contribute to 21% of dietary SFAs intakes in UK adults, but their consumption does not seem to be associated with increased CMD risk according to epidemiological studies. In this context, this PhD thesis aimed to (i) investigate the impact of overall and individual dietary SFAs on medium-term CMD risk markers and long-term CMD risk, and (ii) assess the utility of dairy foods, and more particularly dairy fat, for CMD prevention at a population level.

I first assessed the plasma lipidome-mediated impact of isoenergetically replacing dietary SFAs with monounsaturated fatty acids (MUFAs) or a mixture of MUFAs and polyunsaturated fatty acids (PUFAs) on CMD risk markers and long-term CMD risk (Chapter 2). To achieve this, joint lipidomics analyses in a subset of n=113 participants from the DIVAS randomised controlled trial (RCT) and a sub-cohort from the EPIC-Potsdam prospective cohort study (specific case-cohorts: n=1,707 and n=775 cases for type 2 diabetes, n=1,886 and n=551 cases for cardiovascular disease [CVD]) were completed. This secondary analysis showed that UFA-rich diets implemented over 16 weeks to reduce dietary SFAs in the DIVAS RCT significantly reduced the plasma concentrations of SFA-containing glycerolipids (i.e. mono-, di-, and triacylglycerols) and sphingolipids which were associated with long-term CVD risk in the EPIC-Potsdam cohort study. In addition, I identified that increased serum concentrations of low-density lipoprotein cholesterol (LDL-C), an established CMD risk marker, were associated with higher plasma levels of glycerolipids containing lauric (12:0) and stearic acids (18:0).

The impact of individual SFAs was further assessed at the dietary level by conducting a systematic literature review and meta-analysis of 44 RCTs which substituted individual dietary SFAs with another fatty acid (FA) or a mixture of UFAs (Chapter 3). In quantitative meta-analyses, I observed reductions in LDL-C concentrations after the replacement of palmitic acid (C16:0) with UFAs (-0.36 mmol/L, 95%CI -0.50 to -0.21, I^2=96.0%, n=18 RCTs) or oleic acid (C18:1) (-0.16 mmol/L, 95% CI -0.28 to -0.03, I^2=89.6%, n=9 RCTs), with a similar impact on total cholesterol and apolipoprotein B concentrations. Furthermore, I identified important research gaps regarding the impact of individual dietary SFAs on

novel CMD risk markers (e.g. markers of inflammation, endothelial activation, and glycaemic control) and the specific effect of short-chain SFAs, lauric acid (12:0), and myristic acid (14:0).

To assess the practical applications of reducing dietary SFAs in free-living UK adults, we developed a food-based dietary fat exchange model (the RISSCI-1 study), which aimed to replace dietary SFAs with UFAs by replacing high-fat dairy and high-SFA snacks with commercially available lower-fat dairy foods along with high-UFA cooking oil and snacks into the habitual diets of n=109 UK adult men for 4 weeks (Chapter 4). Participants successfully exchanged 10.4%TE of dietary SFAs with 9.7%TE UFAs, with minimal impact on other nutrients. In addition, participants incorporated the intervention food items without changing their overall dietary habits. Importantly, the analysis of plasma phospholipid fatty acids (PL FAs) in the RISSCI-1 study, along with those performed in the SATgenε, DIVAS, and RESET dietary fat intervention studies (Chapter 5), revealed contrasted results on the validity of individual plasma PL FAs as proxies for dairy consumption. In particular, circulating odd-chain SFAs and ruminant *trans* FAs have been commonly use as biomarkers of intakes in epidemiological studies so far, but only modestly correlate with overall dairy intakes in RCTs and do not seem to accurately capture the intakes of low-fat dairy foods (Chapter 1).

Finally, a prospective analysis of the NutriNet-Santé cohort study did not reveal statistically significant associations between overall and specific dairy consumption and overall CVD (n=1,952 cases) or coronary heart disease risk (n=1,219 cases) among n=104,805 French adults (Chapter 6). However, we observed a 19% reduction (HR=0.81, 95%CI 0.66 to 0.98, p-trend=0.01) in cerebrovascular disease risk (n=878 cases) associated with higher intakes (i.e. at least 160 g/d) of fermented dairy foods (i.e. yogurt, cheese, and fermented milk) compared to low intakes (i.e. below 57 g/d). Despite being observational, these results generated new hypotheses on the potential beneficial effects of specific dairy food matrices on CMD risk, which may stem from bioactive peptides, calcium, and the fermentation process.

Overall, results from this PhD thesis concur with current dietary guidelines on the reduction of dietary SFAs in favour of UFAs and suggest that their deleterious impact on CMD risk may be modulated by their individual structure and/or their effect on the plasma lipidome. Moreover, these findings provide further evidence supporting the presence of beneficial compounds within the dairy food matrix, which may counterbalance the potential deleterious effects of their SFA content. Nonetheless, further interventional and observational studies are warranted to validate these conclusions, and future research is needed to elucidate the physiological mechanisms underlying the complex interactions between dietary SFAs, the dairy food matrix, and the physiological response to dairy consumption.

Chapter 1: General introduction and literature review

1.1 General introduction

Cardiovascular diseases (CVD) and type 2 diabetes are two of the main causes of deaths in the UK and worldwide [1,2]. The aetiology of these cardiometabolic diseases (CMD) is complex, and often results from a combination of non-modifiable risk factors (e.g. age, sex, and genetic predispositions), modifiable factors (e.g. physical activity, smoking status, and dietary habits), and the presence of concomitant metabolic disorders (e.g. metabolic syndrome, hypertension, and hyperlipidaemia) [3].

Among the public health strategies implemented to help prevent CVD risk at a population level, the reduction of dietary saturated fatty acids (SFAs) has been a cornerstone of dietary guidelines around the world for the past four decades [4]. In this respect, most countries advocate a maximum of 10% total energy from dietary SFAs and epidemiological evidence suggests greater CMD risk reduction when SFAs are replaced with unsaturated fatty acids (UFAs) [5,6]. These recommendations mostly derive from research showing direct causal links between high intakes of SFAs and high circulating levels of low-density lipoprotein cholesterol (LDL-C) [6], the latter being an established risk factor for atherosclerosis [7]. In addition, a 2020 meta-analysis of 13 long-term randomised controlled trials reported a 17% decrease in risk from CVD events associated with a reduction of dietary SFAs, suggesting a potential direct causal link between dietary SFAs and CVD risk [8]. Despite the extensive body of evidence on overall dietary SFAs and CMD risk available to date, we identified two emerging areas of research which may contribute to better elucidate the role of dietary SFAs in CMD aetiology. First, omics approaches such as lipidomics have been generating novel hypotheses around the role of plasma lipid metabolites in the cardiometabolic health status, but little is known about the impact of dietary fat quality on the plasma lipidome [9–11]. Second, most public dietary guidelines consider dietary SFAs as a whole group of nutrients, despite emerging, but sparse evidence that individual SFAs may exert differential effects on health [12–14].

From a food perspective, dairy products contribute to 21% of dietary SFA intakes in UK adults according to the most recent National Diet and Nutrition Survey (2017-2019) [15], which raised the question of their potential impact on cardiometabolic health. Nonetheless, most epidemiological evidence so far has reported null or inverse associations between dairy consumption and CMD risk [16,17]. While these results may be explained by the hypothesis that dairy fat itself is not detrimental to cardiometabolic health, a more likely explanation may be that other compounds of the dairy food matrix, such as bioactive peptides and calcium, exert beneficial effects on CMD risk markers [18]. In line with the latter hypothesis, beneficial effects of dairy intakes tend to be observed after the

consumption of cheese and other fermented dairy products, but not butter which mostly contains fat [19–21]. In this context, lower-fat dairy products may appear as an interesting alternative to high-fat dairy foods in order to limit the entry of SFAs in the food chain while retaining the potential beneficial health effects of the dairy food matrix. However, few intervention studies have explored the feasibility and compliance to an isoenergetic replacement of dietary SFAs with UFAs relying on dairy products to achieve nutritional targets.

Importantly, improving the accuracy of dairy intake assessment seems crucial to strengthening study designs in this area. Since an increasing number of epidemiological studies rely on circulating fatty acids as proxies of dairy consumptions, I identified the need for an assessment of their utility and validity as objective biomarkers of intakes. The report from this review of the literature is presented in the following part of this introduction (1.2) [22].

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1.2 Can individual fatty acids be used as functional biomarkers of dairy fat consumption in relation to cardiometabolic health? A narrative review.

Contribution towards PhD thesis: My responsibilities included the definition of the subject and scope of this narrative literature review, along with conducting the initial literature search. An updated literature search was then performed with the assistance of Laura Paper, an undergraduate intern student working under the supervision of myself and Julie Lovegrove. Finally, I prepared the initial draft of the manuscript for publication, and finalised the published manuscript presented below after including the feedback and comments received from co-authors and journal reviewers.

Manuscript published in the *British Journal of Nutrition* (January 2022). DOI: 10.1017/S0007114522000289

Can individual fatty acids be used as functional biomarkers of dairy fat consumption in relation to cardiometabolic health? A narrative review.

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Short title: Fatty acids as biomarkers of dairy fat consumption

Author contributions towards manuscript: LS and LP conducted the literature searches. LS interpreted the findings and drafted the manuscript. LP, IDG, KGJ and JAL contributed to the conception of the manuscript and revised each draft for important intellectual content. KGJ and JAL had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

Financial support: Laury Sellem was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Joint Programme Initiatives (JPI) "HDHL Biomarkers: Fatty Acid Metabolism - Interlinking Diet with Cardiometabolic Health (FAME)" (Project Reference: BB/P028217/1).

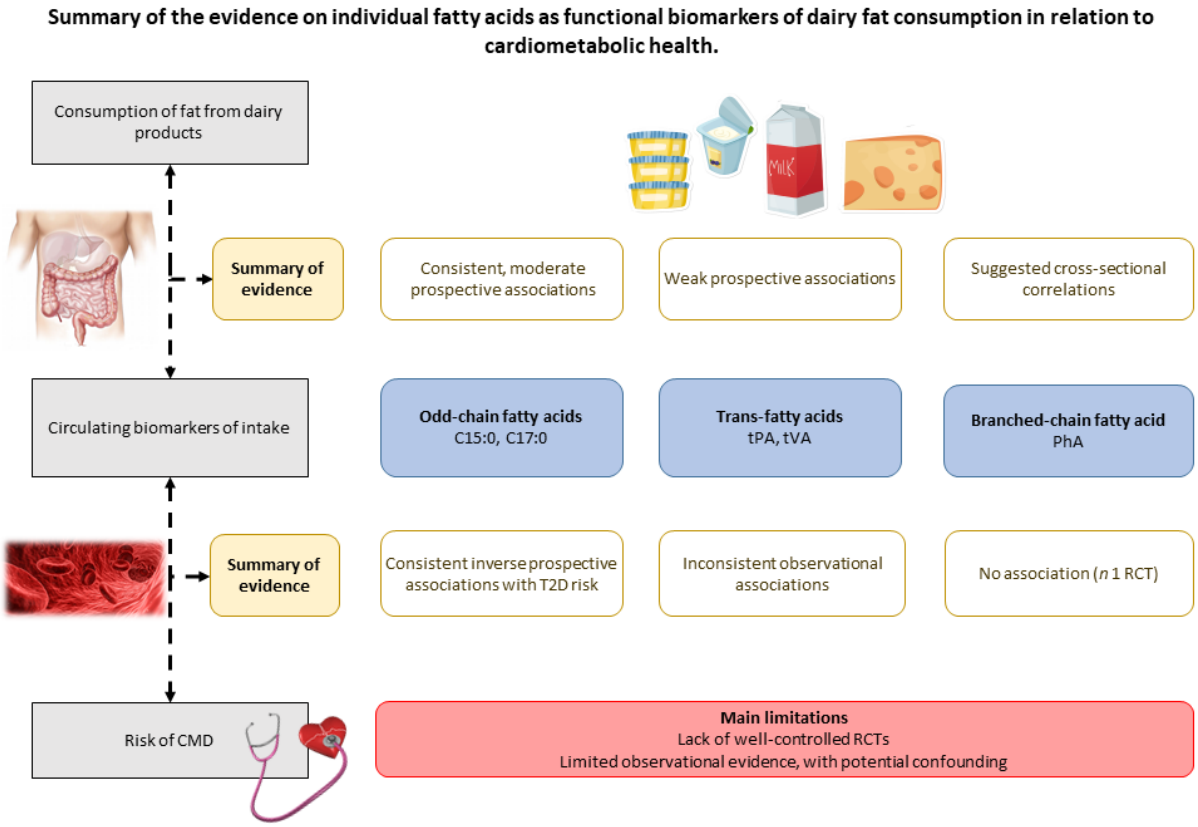
Conflicts of interest: JAL is Deputy Chair of the UK Scientific Advisory Committee for Nutrition (SACN) and was an expert on SACN's saturated fats working group. JAL (Chair), LS, and KGJ are part of the International Life Science Institute (ILSI) Europe expert group on "Update on health effects of different dietary saturated fats".

Abstract

In epidemiological studies, dairy food consumption has been associated with minimal effect or decreased risk of some cardiometabolic diseases (CMD). However, current methods of dietary assessment do not provide objective and accurate measures of food intakes. Thus, the identification of valid and reliable biomarkers of dairy intake is an important challenge to best determine the relationship between dairy consumption and health status. This review investigated potential biomarkers of dairy fat consumption, such as odd-chain, *trans*- and branched-chain fatty acids, which may improve the assessment of full-fat dairy product consumption. Overall, the current use of serum/plasma fatty acids as biomarkers of dairy fat consumption is mostly based on observational evidence, with a lack of well-controlled, dose-response intervention studies to accurately assess the strength of the relationship. Circulating odd-chain saturated fatty acids and *trans*-palmitoleic acid are increasingly studied in relation to CMD risk and seem to be consistently associated with a reduced risk of type 2 diabetes in prospective cohort studies. However, associations with cardiovascular diseases are less clear. Overall, adding less studied fatty acids such as vaccenic and phytanic acids to the current available evidence may provide a more complete assessment of dairy fat intake and minimise potential confounding from endogenous synthesis. Finally, the current evidence base on the direct effect of dairy fatty acids on established biomarkers of CMD risk (e.g. fasting lipid profiles and markers of glycaemic control) mostly derives from cross-sectional, animal, and *in vitro* studies, and should be strengthened by well-controlled human intervention studies.

Keywords: dairy biomarkers, dairy fatty acids, dairy foods, heptadecanoic acid, pentadecanoic acid, phytanic acid, *trans*-palmitoleic acid, vaccenic acid

Graphical abstract



Abbreviations: C15:0, pentadecanoic acid, C17:0 heptadecanoic acid; CMD, cardiometabolic disease; CVD, cardiovascular disease; PhA, phytanic acid; RCT: randomised controlled trial; T2D, type 2 diabetes; tPA, trans-palmitoleic acid; tVA, vaccenic acid.

1.2.1 Introduction

Cardiovascular diseases (CVD) are responsible for 26% of deaths in the UK, and healthcare costs related to CVD represent a £9 billion economic burden annually [1]. In parallel, metabolic disorders such as type 2 diabetes (T2D), metabolic syndrome and non-alcoholic fatty liver disease are associated with increased risk of CVD in epidemiological studies [2–4]. With 7 million people living with CVD and 4.6 million with T2D in the UK, prevention of these cardiometabolic diseases (CMD) is currently one of the biggest modern challenges for public health [1,5]. Whilst some CMD risk factors are not modifiable (e.g. age, sex, or genetic makeup), modifying dietary behaviours may constitute an effective strategy for disease risk reduction. In particular, dietary intakes of saturated fat, sodium, and fruits and vegetables have been extensively studied in relation to CMD prevention and have been targeted by public health recommendations [6]. In the UK, the first recommendation on dietary SFAs (< 10%TE) intake was initially implemented by the National Advisory Committee on Nutritional Education (NACNE) in 1983 [7,8] and was reiterated in the 2019 Scientific Advisory Committee in Nutrition (SACN) report on saturated fat and health which recommended a replacement of SFAs with poly- (PUFAs) or monounsaturated fatty acids (MUFAs) for CVD prevention [9]. Furthermore, recent epidemiological evidence suggested the potential different associations of individual SFAs on CMD risk, which might reflect their different chain-lengths or food matrices in which they are incorporated [10,11].

From a food perspective, dairy products are an interesting food group for the management of SFA entry in the food chain. Dairy products contribute up to 21% of total SFA intake among British adults, and their consumption (particularly cheese and yogurt) seems to have beneficial associations with some risk markers for CMD such as blood pressure and arterial stiffness [12,13]. However, most of the currently available evidence on the consumption of dairy foods and/or their fat content and CMD risk is limited to observational studies which often rely on imprecise methods of dietary assessment. Indeed, the reported intakes from food frequency questionnaires (FFQs), diet-diaries or dietary recalls may be subject to underestimation, recall bias, and systematic errors from food composition tables, which may impact on the associations between dairy intakes and CMD risk in prospective cohort studies. However, validation studies of FFQs against dietary records or blood and urinary biomarkers, the use of repeated assessments of dietary intakes in longitudinal studies, and the integration of novel technological tools, have contributed to improving the accuracy of these traditional methods of dietary assessment [14,15]. Limited precision when assessing dairy foods consumed within composite dishes (e.g. pizza, bakery items, or coffee drinks), which might independently impact CMD risk, is a further shortcoming of these dietary assessment methods that may be overcome by deconstructing dairy-derived ingredients from composite dishes and re-allocating them to dairy food categories, as

illustrated in the Prospective Urban Rural Epidemiology (PURE) study [16]. Whilst these methodological limitations are not restricted to dairy fat intakes and are commonplace in nutritional science, the integration of data from traditional methods of dietary assessment with more objective biomarkers of intake may improve the accuracy of dietary intake assessments and prediction of disease risk. In particular, reliable circulating biomarker candidates need to be: 1) specific to the food source of interest, 2) easy to quantify in the organism, 3) exclusively derived from dietary intake rather than endogenous synthesis, and 4) highly correlated to dietary intakes. For example, blood concentrations of vitamin C and carotenoids have been widely used as a proxy for fruit and vegetable intakes and linked with decreased risks of cancer in observational studies [17]. Therefore, the identification of biomarkers for the consumption of other food groups, such as fats derived from dairy foods, might provide useful insights into their role in cardiometabolic health by complementing the extensive evidence base already available [18].

The following article will thus aim to review the evidence for a range of FAs present in milk and dairy foods as biomarker candidates for dairy fat intake: odd-chain SFAs (C15:0 and C17:0), which are traditionally used in nutritional epidemiology, and more novel biomarkers such as ruminant *trans*-FAs (*trans*-palmitoleic acid C16:1 *trans*-9 and vaccenic acid C18:1 *trans-11*) and one branched-chain FA (phytanic acid). The physiological role of those FAs in the context of cardiometabolic health and possible mechanisms of action will also be discussed.

1.2.2 Identification of individual fatty acids as biomarkers of dairy fat intake

Odd-chain FAs: pentadecanoic (C15:0) and heptadecanoic (C17:0) acids

Presence in dairy milk and other food group

In dairy cows and other ruminants, odd-chain FAs are produced by bacteria in the rumen and by post-absorptive *de novo* lipogenesis using short-chain FAs like propionic acid (C3:0) as a substrate, or by α-oxidation which converts C16:0 or C18:0 into C15:0 or C17:0 respectively via the elimination of the α-carbon [19–21] (*Figure 1.1*). The produced odd-chain FAs are then utilised in the mammary gland for the production of milk fat, although their contribution remains minor in comparison to even-chain FAs which are produced via *de novo* lipogenesis at much higher levels [22]. Although there are small seasonal variations, SFAs account for 67-72% of total FAs in milk, and include mostly palmitic (C16:0, 30-33% total FAs), myristic (C14:0, 10-11% total FAs), stearic (C18:0, 9-10% total FAs) acids and short-/ medium-chain FAs (C4:0-C12:0, < 4% total FAs), while odd-chain FAs such as pentadecanoic (C15:0) and heptadecanoic (C17:0) represent 1% and 0.5% total FAs respectively [23]. Thus, one serving of 200

ml of semi-skimmed milk would provide 2 g SFA, of which 20 mg were from C15:0 and 10 mg from C17:0.

Non-dairy dietary sources of C15:0 and C17:0 include ruminant meats and fish, although the proportions of C15:0 from these sources are lower than of C17:0 [24]. With meat and meat products contributing to as much dietary SFA as dairy foods in British adults (about 21% of total dietary SFA) [12], the sole use of odd-chain SFAs as biomarkers of dairy fat consumption may be questionable especially in the context of diets rich in ruminant meat. However, very limited evidence from the EPIC-Potsdam [25] and EPIC-E3N [26] prospective studies has suggested that circulating C15:0 or C17:0 and red meat consumption might be inversely correlated or not correlated, respectively. Moreover, the analysis of the FA content of 27 freshwater fish species revealed that C15:0 and C17:0 represented 0.4% and 0.6% total FAs respectively [27], which was also reflected in the analysis of nineteen different European brands of fish oil supplements [28]. Thus, further concerns could be raised on the validity of odd-chain SFAs as biomarkers of dairy fats in populations consuming high-fish, low-dairy diets. This is not the case for most Western populations, including British adults, among which fish consumption is relatively low (22 g/day on average or 3%TE for British adults aged between 19-64 years old) [12], but does not account for the intake of fish oil supplements consumed by an estimated 11% of British adults in 2012 [29].

Correlations between circulating levels and dietary intakes

Despite their low contribution to total dietary SFAs, odd-chain SFAs are detectable in low concentrations in human plasma samples but were traditionally used as internal standards in gas chromatography (GC) analytical methods which masked any possible quantitative assessment [30,31]. However, with the growing interest in their utility as biomarkers of dairy fat intake, circulating levels are more routinely measured in a number of plasma FA fractions, such as non-esterified FAs or phospholipids [32].

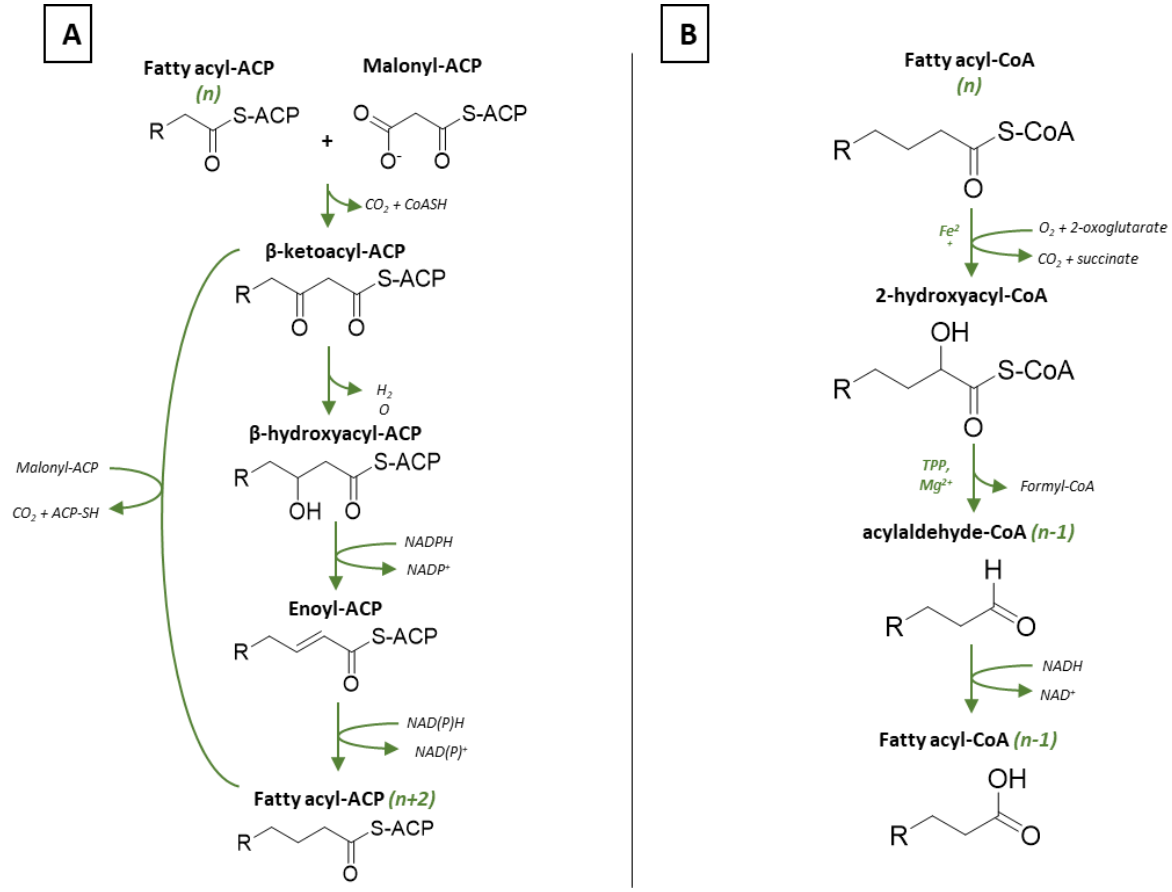


Figure 1.1. Possible mechanisms for the synthesis of odd-chain SFAs. Adapted from Jansen (2006) and Jenkins (2015) [21,32]*. A – Metabolic pathway for the synthesis and elongation of even-chain FAs (e.g. 16:0, 18:0), starting with the condensation of malonyl-CoA with a fatty acyl-ACP of n carbons. Odd-chain SFAs may be produced via the same route, using propionyl-CoA as a precursor instead of malonyl-CoA. B – Main steps of the α-oxidation of odd-chain fatty acids, involving the decarboxylation of the α-carbon to allow further oxidation of the acyl chain. Even-chain SFAs may undergo the same decarboxylation reaction, leading to the formation of odd-chain SFAs.*

To date, C15:0 and C17:0 have formed the basis for much of the research on biomarkers of dairy fat and specific dairy food group consumption. In particular, findings from two cross-sectional studies indicated a better correlation between circulating C15:0 and high-fat dairy products or total dairy fat compared to lower-fat dairy foods [33,34]. In a cross-sectional study of n = 72 participants, C15:0 in plasma phospholipids was positively correlated with fat from both milk and cream (r = 0.34) and total dairy fat (r = 0.34), but not with butter, ice cream, or fat from either [33]. More recently, findings from the Food4Me study (n = 1054 participants) suggested stronger correlations between high-fat dairy food consumptions and C15:0 in dried blood spots compared to C17:0 [34]. The authors reported positive associations between C15:0 (% change in blood) and consumed daily portions of total dairy (regression coefficient β = 1.02, 95% CI [0.14-1.91]), high-fat dairy (β = 0.32, 95%CI [0.05-0.58]), cheese (β = 1.77, 95%CI [0.52-3.02]), and butter (β = 3.34, 95%CI [1.34-5.35]). In contrast, C17:0 was associated with intakes of cream (β = 9.42, 95%CI [3.43-15.4]) and high-fat dairy albeit to a lesser

extent than C15:0 (β = 0.27, 95%CI [0.1-0.45]), but not with total dairy, cheese, or butter. Finally, the authors did not report any associations between C15:0 or C17:0 and consumption of low-fat dairy foods, milk, or yogurts [34]. The discrepancy observed between C15:0 and C17:0 might be partly explained by the biological matrix used for analysis, as suggested by findings from the LifeLines Cohort study (n = 864 participants) where total dairy intakes were positively related with both C15:0 and C17:0 in plasma phospholipids, but only C15:0 in plasma triacylglycerol (TAG) [35]. Overall, findings from observational studies tend to support consistent, albeit weak, correlations between dairy fat intakes and plasma/serum C15:0 (r = 0.33, 95%CI [0.27 – 0.39]) and to a lesser extent C17:0 (r = 0.19, 95%CI [0.14 – 0.25]), as summarised in a 2019 meta-analysis of 18 cross-sectional and prospective studies [36]. However, these results need to be interpreted in the light of their observational and cross-sectional nature, which prevent causal links between dairy fat consumption and circulating C15:0 or C17:0 from being inferred [37].

In line with findings from observational studies, findings from RCTs suggest that circulating concentrations of C15:0 are more responsive to dairy fat consumption than concentrations of C17:0 [38–40] (*Table 1*). However, the correlations between total dairy intake and circulating levels of C15:0 and C17:0 are generally weak, as observed in a 12-month RCT in 76 adolescents which reported modest correlations between total dairy consumptions (in servings per day) and C15:0 (r = 0.27) or C17:0 (r = 0.25) measured in erythrocytes [41]. Importantly, the designs of the RCTs included in this review suggest that most often the hypothesis being tested is whether specific FAs, such as C15:0 and C17:0, are biomarkers of dairy food consumption, rather than dairy fat. As suggested by the poor or null correlations observed between C15:0 or C17:0 and intakes of low-fat dairy foods [33,34], this approximation seems misplaced, as the circulating levels of FAs would not accurately capture the consumption of dairy foods with a minimal fat content and thus would not reflect the consumption of total dairy accurately.

Potential limitations for use of C15:0 and C17:0 as biomarkers of dairy fat consumption

In phospholipid FAs, levels of C15:0 and C17:0 range from 0.15-0.23% (n = 4 studies) and 0.33-0.41% total phospholipid FAs (n = 3 studies) respectively [32]. Therefore, although C15:0 is more abundant in milk than C17:0, plasma phospholipid levels seem to indicate an inverse abundance of C15:0 and C17:0 in human plasma, which raises the question of possible alternative pathways for endogenous synthesis and/or oxidation of odd-chain SFAs in humans. Proposed mechanisms suggest the human gut microbiota might be involved in the elongation of short-chain FAs and/or decarboxylation of even-numbered very-long chain FAs [32,42].

In vivo studies in rats suggest the need for a distinction between circulating levels of C15:0, which would be mostly of dietary origin and inversely correlated with total dietary fat, and circulating levels of C17:0, which seem unaffected by total dietary fat and may be endogenously synthesised [43]. This hypothesis is in line with recent *in vitro* findings which suggested that the main human elongation of very long chain fatty acids (ELOVL) enzymes could not only elongate even-chain SFAs, but could also act on odd-chain FAs by catalysing the elongation of C13:0 to C15:0 and C15:0 to C17:0 [44]. To date, the metabolic mechanisms related to the elongation of odd-chain SFAs in humans are not fully elucidated and their contribution to circulating levels have not been precisely quantified.

In addition to potential endogenous synthesis and gut microbiome fermentation, the interactions between odd-chain SFAs and other dietary nutrients such as fibre are unclear [45]. In particular, in a human RCT of 16 healthy participants, the consumption of 30 g/day of inulin or 6 g/day of propionate for 7 days increased plasma phospholipid concentrations of C15:0 by 17% and 13%, and concentrations of C17:0 by 11% and 13% [46]. The authors suggested that these results may be explained by the activation of odd-chain SFA metabolic pathways in the gut microbiota [46]. A potential endogenous synthesis of odd-chain SFAs in response to dietary fibre and in the absence of dairy fat consumption would be in line with observations from an Austrian cross-sectional study which reported similar circulating levels of C17:0 in vegan (n = 37) to omnivore (n = 23), semi-omnivore (n = 13), and vegetarian (n = 25) participants [47].

Table 1.1. Human RCTs investigating the correlations between dairy consumption and circulating levels of odd-chain, *trans*, and/or branched-chain fatty acids.

Reference	Fatty acid(s) of interest	Biological fraction measured	Subject group n, sex, mean age, BMI	study design and duration	Intervention(s)	Comparison(s)	Intervention effect(s)
Vissers et al. 2020 [39]	C15:0, C17:0	Plasma cholesteryl esters	n = 30 (13M/17F) Age: 22 y BMI: 21.6 kg/m^2	Crossover, 1 week	High-dairy diet (14% dietary protein from dairy foods)	High-meat diet (14% dietary protein from pork, beef, and chicken), High-grain diet (14% dietary protein from wheat, bran, rice, maize, and legumes)	↑ C15:0 vs. high-meat ($p = 1.2 \times 10^{-5}$) or high-grain diets ($p = 1.2 \times 10^{-2}$) ↔ C17:0 vs. high-meat or high-grain diets
Slim et al. 2019 [41]	C15:0, C17:0, tPA	Plasma total lipids, erythrocytes	**Intervention** n = 29 (8M/21F) Age: 16.5 y BMI: 21.7 kg/m^2 **Control (CTL)** 33 (8M/25F) Age: 16.6 y BMI: 21.7 kg/m^2	Parallel, 12 months	≥ 4 serv/d of dairy food of choice	≤ 2 serv/d of dairy food of choice (CTL)	**Plasma total lipids:** ↔ C15:0, C17:0, tPA vs. CTL Correlations with dairy consumption: NS **Erythrocytes:** ↑ C15:0 vs. CTL ($p < 0.05$) ↔ C17:0, tPA vs. CTL Spearman Correlations with dairy consumption: (+) C15:0 ($r = 0.27$), C17:0 ($r = 0.25$) NS tPA
O'Connor et al. 2019 [40]	C15:0, C17:0	Serum total lipids	n = 26 (19M/7F) Age: 54.9 y BMI: 21.9 kg/m^2	Crossover, 18 weeks	≥ 4 serv/d of dairy food of choice	≤ 2 serv/d of dairy food of choice (CTL)	**Change from baseline in intervention:** ↑ C15:0 ($p = 0.04$) ↔ C17:0 **Intervention vs. CTL:** ↑ C17:0 ($p = 0.045$) ↔ C15:0
Abdullah et al. 2015 [38]	C15:0, C17:0, tPA	Plasma total lipids	n = 124 (40M/84F) Age: 39.3 y BMI: 25.7 kg/m^2	Crossover, 4 weeks	3 serv/d of dairy: 375 ml of 1% fat milk 175 g of 1.5% fat yogurt 30 g of 34% fat cheese	Energy-equivalent control (CTL): 290 ml of fruit and vegetable juice 20 g of cashews 39 g of cookie	↑ C15:0, C17:0 vs. CTL ($p < 10^{-4}$) ↔ tPA vs. CTL
Werner et al. 2013 [65]	C15:0, PhA	Plasma total lipids	**Intervention** n = 20 (7M /13F) Age: 61.9 y BMI: 25.4 kg/m^2 **Control (CTL)** 18(8M/10F) Age: 60.7 y BMI: 26.5 kg/m^2	Parallel, 12 weeks	39 g/d of fat from high-PhA butter, made from milk of mountain-pasture grazing cows	39 g/d of fat from butter lower in PhA, made from milk of cows fed conventional winter fodder (CTL)	↔ PhA vs. CTL ↑ C15:0 vs. baseline in both intervention and CTL (p-value not reported)
Werner et al. 2011 [64]	PhA	Plasma total lipids	**Intervention** n = 9 (3M/6F) Age: 28.3 y BMI: 22.2 kg/m^2 **Control (CTL)** n = 5 (3M/2F) Age: 31.6 y BMI: 22.4 kg/m^2	Parallel, 4 weeks	45 g/d of fat from test butter and cheese with 0.24 wt% PhA	45 g/d of fat from test butter and cheese with 0.13 wt% PhA (CTL)	↔ PhA vs. CTL ↑ PhA vs. baseline in both intervention and CTL ($p < 0.05$)

Abbreviations: C15:0, pentadecanoic acid; **C17:0**, heptadecanoic acid; **tPA**, *trans*-palmitoleic acid; **PhA** phytanic acid; **M**, male; **F**, female; **y**, year; **serv/d**, serving per day; **g/d**, gram per day; **%wt**, weight percent; **CTL**, control; **↑**, increase; **↔**, no change; **NS**, not significant; **(+)**, positive.

Trans-fatty acids of ruminant origins: trans-palmitoleic acid (C16:1* trans*-9) and vaccenic acid (C18:1 trans-11)

Presence in dairy milk and other food groups

In ruminant animals, dietary FAs such as oleic, linoleic, or α-linolenic acids, are utilised by rumen microbiota and undergo a series of biohydrogenation reactions leading to the synthesis of *trans*-FAs (*Figure 1.2*) [48]. *Trans*-FAs represent 4.5-6.5% total FAs in retail milk, with *trans*-vaccenic acid (tVA, C18:1 *trans*-11) being the major contributor (0.8-1.5% total FAs), followed by *trans*-palmitoleic acid (tPA, C16:1 *trans*-9) representing 0.35-0.45% total FAs [23]. Thus, one serving of 200 ml semi-skimmed milk would provide low doses of tVA and tPA – up to 84 mg total *trans*-FAs, including 27 mg tVA and 8.1 mg tPA [23]. Similar to odd-chain SFAs, tPA and tVA are not entirely specific to dairy foods. However, they belong to the distinct category of ruminant *trans*-FAs, as opposed to industrially processed *trans*-FAs which derive from the partial hydrogenation of vegetable oils such as elaidic acid (C18:1 *trans*-9).

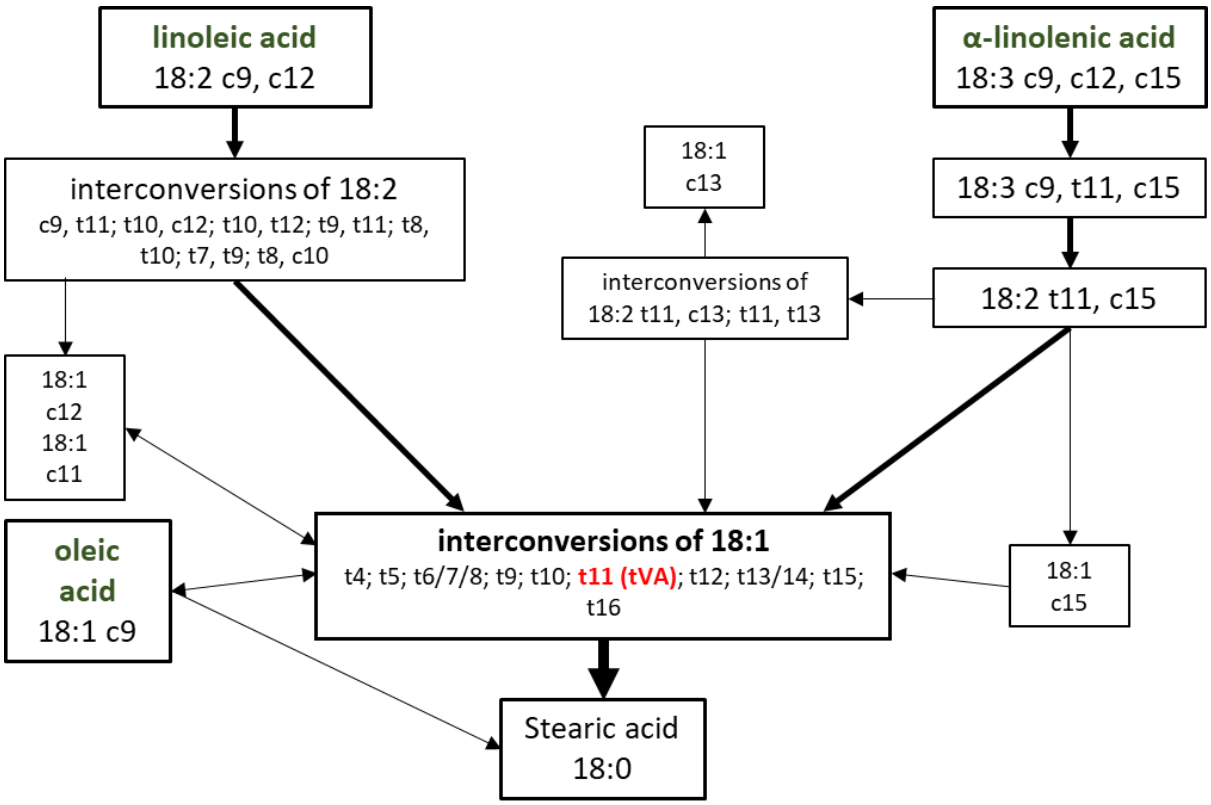


Figure 1.2. Major biohydrogenation pathways of oleic, linoleic, and α-linolenic acids into trans-fatty acids in ruminants. Adapted from Enjalbert (2012) [48].

Correlations between circulating levels and dietary intakes

Circulating tPA measured by GC represents less than 1% total phospholipid FAs (0.18 ± 0.05% total FAs) [49], although most studies only tend to report concentrations of total *trans*-FAs or elaidic acid [50,51].

However, one cross-sectional study of 210 healthy Canadian adults reported that tPA and tVA each represented less than 0.20% of total plasma phospholipid FAs [52]. Circulating tPA levels in this study were weakly correlated with dairy intakes estimated by a FFQ ($r = 0.15$, $p = 0.04$) but tVA levels were not [52]. In contrast, a more recent cross-sectional study from the Netherlands described that circulating tVA in plasma phospholipids had a stronger correlation with dairy fat consumption than odd-chain SFAs [35]. Overall, very few well-controlled RCTs have investigated the associations between dairy fat consumption and circulating ruminant *trans*-FAs, and the few that have only reported results on tPA, but not tVA (*Table 1*).

Several epidemiological studies, which date from the 1990s, observed significant correlations between circulating levels of tPA and non-dairy fat dietary sources such as cakes, cookies, and pies rather than dairy fat [53,54]. At the time, such products contained hidden sources of dairy fat such as milk powder, butter, and chocolate, but also partially hydrogenated oils. Since then, *trans*-fat has mostly been eliminated from the European and American food chains due to the significant link with increased CVD risk [55]. Therefore, dietary tPA and tVA now almost entirely originate from dairy foods and ruminant fats, as described by a wide market basket analysis of French foods [56].

Potential limitations for use as biomarkers of dairy fat consumption

Findings from a human parallel RCT, which showed a significant increase in tPA circulating levels following dietary intake of tVA (2.9 g/day for 6 weeks), suggested that dietary tVA could be converted into tPA endogenously, with a conversion rate of approximately 17% [57]. These results were in line with *in vitro* studies, suggesting that tVA could be converted into tPA via peroxisomal β-oxidation pathways [58]. Of note, these findings were challenged in an editorial from Mozaffarian, on the basis that one of the genome-wide association studies his group led failed to reveal significant genetic determinants for the synthesis of tPA, suggesting that this endogenous synthesis would be negligible [59,60]. Overall, the limited evidence on potential endogenous synthesis of tPA and tVA raises questions about their suitability as biomarkers of dairy fat consumption.

Branched-chain fatty acids: phytanic acid (PhA)

Presence in dairy milk and other food groups

Phytanic acid (PhA) is a branched-chain FA with a main chain of 16 carbons and 4 methyl groups (3,7,11,15-tetramethylhexadecanoic acid). As with previous FAs described in this review, it is found in relatively low quantities in milk (200 ml semi-skimmed milk provides approximately 9.8 mg of PhA), with levels found to be 10 times higher in hard cheese (98.9 mg per 100 g cheese) [61]. However, PhA is also present in relatively high quantities in beef (up to 326 mg/100 g food) and fish (100 mg/ 100 g

halibut or capelin) [61]. Furthermore, PhA is the degradation product of phytol, which derives from chlorophyll. Thus, its content in animal derived products widely varies according to the farming method and animal diet, with a diet rich in grass leading to a higher PhA content in meat and dairy foods. In humans, PhA cannot be endogenously synthesised *de novo* but can be produced in the liver via the conversion of pristanic acid, a branched-chain FA involved in peroxisomal metabolism, although this pathway seems to be minimal [62].

Correlations between circulating levels and dietary intakes

A 2010 prospective study from the EPIC cohort revealed that although PhA levels correlated with dairy intakes ($r = 0.49$, $p < 10^{-4}$), this correlation was influenced by external factors such as age, country of residence and duration of fasting at blood collection [63]. To date, very few RCTs investigated the correlations between dairy fat intake and circulating PhA levels (*Table 1*). In 2011, a dietary RCT in humans revealed significant increases in PhA levels after consumption of milk, butter and cheese [64]. The authors also reported a significant correlation between the consumption of butter from mountain-pasture grazing cows or cows fed conventional winter fodder and circulating PhA levels [65].

Summary of findings

Most of the evidence available to identify individual FAs as biomarkers of dairy fat intake focused on odd-chain SFAs, while findings on tPA, tVA and PhA are much more limited. The lack of well-controlled dose-response studies designed to validate biomarkers of dairy consumption represents an important limitation to their use in epidemiological studies.

1.2.3 Relevance to cardiometabolic disease risk and potential mechanisms

Circulating FAs are often used as proxies for assessing the associations between dairy consumption and cardiometabolic health in observational epidemiological studies, although their physiological role in metabolism and cardiometabolic health is still unclear.

Odd-chain FAs: C15:0 and C17:0

Associations with markers of cardiometabolic health

At least six cross-sectional studies observed significant inverse associations between circulating odd-chain SFAs, particularly C17:0, and lipid profiles, markers of inflammation, or glucose metabolism, although these correlations seem to be observed mostly in overweight and obese participants [36,66–70]. Two cross-sectional studies also suggested potential influences of other nutrients such as alcohol or different dietary patterns on the relationship between circulating odd-chain SFAs and lipid profiles [69,71]. Stronger evidence from well-controlled intervention studies investigating the specific role of odd-

chain SFAs is still very limited. One recent dairy-feeding RCT reported concomitant increases in odd-chain SFA levels and LDL-C concentrations after the consumption of 3 servings of dairy per day for 4 weeks compared to an energy-matching control (290 ml of fruit and vegetable juice, 20 g of cashew nuts and a 39 g-cookie) [38].

Associations with CMD risk and mortality

So far, meta-analyses of prospective observational studies on the associations between circulating odd-chain SFAs and CVD risk and mortality have yielded contrasting results, suggesting either neutral or inverse associations. Chowdury and colleagues observed a 23% CHD risk reduction (relative risk RR = 0.77 [0.63 – 0.93], n = 2,283 cases / 5,490 participants) associated with higher circulating levels of C17:0. This association was consistent, albeit not statistically significant, when adding circulating C15:0 (RR = 0.81 [0.62 – 1.06], n = 2,283 cases / 5,490 participants) [72]. However, these findings were not supported by data from a more recent meta-analysis of 12 European and North American prospective studies which included 7,680 CVD cases [73]. The authors failed to observe a significant association between circulating odd-chain SFAs and overall CVD or subtypes of CVD risks, but did reveal a 28% heart failure risk reduction associated with circulating C15:0 (RR = 0.72 [0.55–0.95], n = 983 cases from two studies) [73]. Further prospective human studies that investigated these associations but were not included in the above meta-analyses are summarised in *Table 2*. Briefly, findings from the prospective Cardiovascular Health Study revealed no changes in CVD risk, but a 33% reduction in CVD mortality risk (hazard ratio HR = 0.77 [0.61 – 0.98], n = 833 CVD deaths / 1,595 non-CVD deaths) associated with the highest quintile of circulating C17:0 compared to the lowest quintile (median concentration 0.48% versus 0.31% total plasma FA, respectively) after a 22-year follow-up. However, circulating C15:0 was not associated with CVD risk or mortality in this cohort [74]. In contrast, three prospective studies failed to observe associations between circulating C17:0 and CVD risk but did report inverse associations between C15:0 and risks of myocardial infarction and stroke [70,74,75].

Many prospective cohorts have assessed the relationships between circulating odd-chain SFAs and risk of T2D, as summarised in a large meta-analysis of 16 prospective cohorts and which included > 60,000 participants [76]. In this pooled analysis, the authors observed inverse associations between T2D risk and both circulating C15:0 (HR = 0.80 [0.73 – 0.87]) and C17:0 (HR = 0.65 [0.59 – 0.72]). These findings were in line with one prospective cohort published since then that was based on dietary intakes of odd-chain SFAs rather than circulating levels, which observed a 12% T2D risk reduction associated with every additional 0.11%TE from odd-chain SFAs [77].

Table 1.2. Prospective human studies investigating the associations between circulating levels of odd-chain or *trans*-fatty acids and incident cardiovascular diseases (CVD), CVD mortality, or incident type 2 diabetes (T2D).

Reference	Fatty acid(s) of interest	Biological fraction measured	Reported study and overall participants characteristics (e.g. n, sex, mean age, mean BMI)	Study design and mean follow-up	Outcomes	No. cases and/or No. of deaths	Confounders included	Summary of observed associations by fatty acid of interest [a]
Incident CVD and CVD mortality								
De Oliveira Otto et al. (2018) [74]	15:0, 17:0, tPA (5^{th} vs. 1^{st} quintile)	plasma phospholipids	Cardiovascular Health Study (USA) n = 2,907 (36% M, 64% F) Age: 74.8 y	prospective cohort study, 22 y	incident CVD, CHD, and stroke total and CVD mortality	1,301 CVDs 876 CHDs 529 strokes 2,428 deaths 614 CVD deaths	age, sex, race, education, enrolment site at baseline, smoking status, alcohol, PA, BMI, drug related hypertension, self-reported general health, circulating total *trans*-FAs, consumption of dairy, dietary fibre, fruits, vegetables, and red meat	**C15:0, C:17:0 tPA** ↔ CVD risk ↔ CHD risk ↔ stroke risk **C15:0, tPA** ↔ total mortality ↔ CVD mortality **C17:0** ↔ total mortality ↓ CVD mortality (HR = 0.77 [0.61-0.98])
Laursen et al. (2018) [75]	15:0, 17:0, tVA (95^{th} percentile vs. 5^{th} percentile)	adipose tissue	Danish Diet, Cancer and Health (Denmark) **Cases (incident stroke)** n = 2,108 (60.5% M, 39.5% F) Age: 60.5 y BMI: 26.2 kg/m^2 **Non-cases** n = 3,186 (54%M, 46% F) Age: 56.3 y, BMI: 25.8 kg/m^2	case-cohort study, 12.8 y	incident stroke and stroke subtypes	2,108 total strokes 1,745 ischemic strokes 249 intracerebral haemorrhages (IH) 102 subarachnoid haemorrhages (SH)	Sex, date of inclusion, education, BMI, waist circumference, PA, smoking status, alcohol intake, baseline hypertension, hypercholesterolemia, diabetes, and myocardial infarction	**C15:0** ↓ total stroke (HR = 0.59 [0.47-0.74]) ↓ ischemic stroke (HR = 0.55 [0.43-0.71]) ↔ IH, SH **C17:0** ↔ total stroke, IH, SH ↓ ischemic stroke (HR = 0.74 [0.58-0.94]) **tVA** ↓total stroke (HR = 0.34 [0.27-0.44]) ↓ ischemic stroke (HR = 0.30 [0.24-0.39]) ↓ IH (HR = 0.45 [0.26-0.78]) ↔ SH
Kleber et al. (2016) [78]	tPA (3^{rd} vs. 1^{st} tertile)	erythrocytes	Ludwigshafen Risk and Cardiovascular Health study (Germany) n = 3,259 (69.7% M, 30.3% F) Age: 62.7 y BMI: 27.5 kg/m^2 Patients hospitalised for coronary angiography	prospective cohort study, 10 y	All-cause and CVD mortality	975 deaths 614 CVD deaths 254 sudden cardiac deaths	Age, sex, BMI, LDL-C, HDL-C, log-transformed TAG, log-transformed fibrinogen, smoking status, hypertension, diabetes, lipid-lowering therapy, glomerular filtration rate, HbA1c, anti-hypertensive medication, alcohol intake	**tPA** ↔ all-cause mortality, CVD mortality ↓ sudden cardiac death (HR = 0.65 [0.47-0.90])

Warensjö et al. (2003) [70]	C15:0, C170, C15:0+C17:0 (continuous)	serum cholesteryl esters, serum phospholipids	Northern Sweden Health and Disease Study n = 1,000 (61.5% M, 38.5% F) Age: 49-64y BMI: 23.2-29.4 kg/m^2	Nested prospective case-control study, 3.1-3.9 y	Incident myocardial infarction	444 cases 556 controls	PA, BMI, smoking status, intakes of fruits and vegetables, education, ApoB/ApoA-I ratio, systolic blood pressure, BMI, prevalence of diabetes	**C15:0, C170, C15:0+C17:0** ↔ myocardial infarction risk
Incident T2D								
Liu et al. (2018) [77]	C15:0, C17:0, C15:0+C17:0 (continuous)	Calculated dietary intakes from food frequency questionnaires	European Prospective Investigation into Cancer and Nutrition-Netherlands n = 37,421 (25.6% M, 74.4% F) Age: 49 y BMI: 25.3-26.0 kg/m^2 across quartiles of dietary SFA	prospective cohort study, 10.1 y	Incident T2D	893 T2D cases	sex, age, sum of other SFA, education, smoking status, PA, BMI, waist circumference, energy-adjusted dietary intakes of: alcohol, animal protein, vegetable protein, *trans*-FAs, vitamin E, fibre, cholesterol	**C15:0** ↔ T2D **C17:0** ↓ T2D (HR = 0.84 [0.73-0.97]) **C15:0+C17:0** ↓ T2D (HR = 0.88 [0.79-0.99])
Liu et al. (2018) [79]	tPA, tVA (5th vs. 1st quintile)	plasma total lipids	National Health and Nutrition Examination Survey (USA) n = 3,801 (48% M, 52% F) Age: 50.1 y (M), 50.0 y (F)	prospective cohort study, 11 y	Incident T2D	505 T2D cases	age, gender, race/ethnicity, education, family income, smoking status, PA, alcohol intake, family history of diabetes, total energy intake, Healthy Eating Index-2010, BMI	**tPA** ↔ T2D (OR = 1.37 [0.90-2.06) **tVA** ↔ T2D (OR = 1.37 [0.95-1.99])

Abbreviations: C15:0, pentadecanoic acid; **C17:0**, heptadecanoic acid; **tPA**, *trans*-palmitoleic acid; **M**, male; **F**, female; **y**, year; **CHD**, coronary heart disease; **CVD**, cardiovascular disease; **T2D**, type 2 diabetes; **IH**, intracerebral haemorrhage; **SH**, subarachnoid haemorrhage; **Apo**, Apolipoprotein; **TAG**, triacylglycerol; **FAs**, fatty acids; **LDL-C**, low-density lipoprotein cholesterol; **HDL-C**, high-density lipoprotein cholesterol; **PA**, physical activity; **HR**, hazard ratio; **OR**, odds ratio; **↑**, direct association; **↓**, inverse association; **↔**, no association.

[a] HRs and ORs presented as *estimate [95% confidence interval]*

Potential physiological role(s) in cardiometabolic health

As previously described, odd-chain SFA metabolism in humans may involve gut microbiota and interactions with other dietary components such as fibre [46]. In addition, it has been suggested that they may contribute to mitochondrial metabolism and to the synthesis of very-long chain SFAs [42]. Thus, the action of odd-chain SFAs might have an indirect effect on CMD risk.

Trans-fatty acids: Trans-palmitoleic (tPA) and vaccenic (tVA) acids

Associations with markers of cardiometabolic health

Cross-sectional studies have observed inverse correlations between circulating levels of tPA and systolic blood pressure [52], fasting glucose [68], and plasma TAG [35]. These studies also suggested inverse correlations between tVA and BMI (in men only) [52], fasting glucose, and C-reactive protein concentrations [35]. In addition, four cohort studies determined cross-sectional associations between baseline levels of tPA and CMD risk biomarkers. Higher circulating tPA levels were associated in three of these studies with elevated LDL-C and high-density lipoprotein cholesterol (HDL-C) concentrations, but lower TAG and biomarkers of T2D risk (i.e. fasting glucose, fasting insulin and insulin resistance index) [49,53,78]. In contrast, a more recent prospective cohort study observed direct cross-sectional relationships between higher circulating tPA and tVA and markers of glycaemic control (i.e. fasting glucose and insulin, HOMA-IR, and HbA1c) [79]. Overall, most of these observations suggest inverse correlations between tPA and TAG together with direct correlations with LDL-C and HDL-C. To date, only one crossover RCT (n = 106) reported that replacing dietary stearic acid with ruminant tVA or industrial *trans*-FAs led to a similar increase in atherogenic lipids such as LDL-C, apolipoprotein B, and lipoprotein (a) [80].

Associations with CMD risk and mortality

A 2018 cross-sectional study (n = 3,504) reported an inverse correlation between CVD risk and circulating tPA, but not tVA [81]. Prior to this, stronger observational evidence from a meta-analysis of prospective studies observed a trend for inverse associations between tPA levels and overall CVD risk (RR = 0.82 [0.67 – 1.02]) and significant inverse associations with T2D risk (RR = 0.58 [0.46 – 0.74], $p < 0.001$) [82]. The latter observation was in line with results from a more recent larger meta-analysis of 16 prospective cohort studies, which found that circulating tPA was associated with an 18% reduced risk of T2D (HR = 0.82 [0.70 – 0.96]) [76].

Since then, two prospective cohort studies observed inverse associations between circulating tVA and risk of strokes [75], or between tPA and sudden cardiac mortality [78], but other studies failed to observe associations between tPA and CVD risk [74] or between tVA and tPA and risk of T2D [79] (*Table 2*).

Potential physiological role(s) in cardiometabolic health

A number of mechanisms have been proposed from *in vitro* and animal studies to explain a potential impact of tPA and tVA on health outcomes. *In vitro* treatment of human endothelial cells with tPA and tVA (50 µmol/L for 24 hours) were reported to significantly decrease TNFα-induced prostaglandin excretion and inflammatory gene expression, thus potentially limiting low-grade inflammation in blood vessels [83]. Secondly, tPA may have similar functions as its *cis*-isomer palmitoleic acid, which has been shown to protect animals from diabetes via the improvement of insulin sensitivity and reduction of hepatic *de novo* lipogenesis [53,84]. Similar improvements in markers of glycaemic control have been observed in obese and diabetic rats supplemented with tVA [85,86].

Phytanic acid (PhA)

To our knowledge, there is no epidemiological data on the associations between dietary or circulating PhA and CMD risk markers or clinical events. A small double blind, parallel trial (n = 5 in control group, n = 9 in intervention group) failed to reveal any significant changes in blood lipids, C-reactive protein, insulin or glucose levels after chronic consumption of PhA-enriched milk, butter and cheese for 4 weeks [64].

Hypotheses regarding PhA in human metabolism and health have been proposed in a number of narrative reviews and mostly derive from *in vitro* and animal studies [61,87]. For example, diets enriched in phytol, a precursor for PhA, led to significant weight loss and reductions in serum total lipids, TAG and cholesterol esters in male mice [88]. Similarly, obese mice fed with high-fat diets enriched in phytol showed reduced plasma TAG, hepatic TAG accumulation and obesity-induced fatty liver via the activation of peroxisome proliferator-activated receptor-α (PPARα) [89]. Recent *in* vitro findings have suggested that the activation of PPARα may also reduce deleterious adiposity by promoting the differentiation of preadipocyte cells into beige adipocytes [90].

Summary of findings

Overall, the evidence linking circulating odd-chain SFAs, tPA and tVA with cardiometabolic health status or CMD events is mostly based on observational studies which reported contrasting results, while only one RCT investigated the effect of PhA on markers of CMD risk. In addition, the physiological role of these FAs and their potential importance in CMD aetiology has not been previously investigated in humans, warranting further research on this topic.

1.2.4 Discussion

Dairy food consumption has often been associated with minimal or decreased CMD risk in epidemiological studies, but current methods of dietary assessment do not provide objective and reliable measures of intake. Thus, the identification of valid and reliable biomarkers of dairy fat intake is an important challenge to best determine the relationship between dairy fat consumption and cardiometabolic health. This review investigated potential biomarker candidates of dairy fat, such as odd-chain, *trans*-, and branched-chain FAs.

According to the available literature, circulating levels of odd-chain SFAs seem to be modestly associated with consumptions of high-fat dairy foods in most populations. Limitations include potential endogenous synthesis and contributions of other dietary sources of these FAs, which crucially need to be further assessed and ideally quantified. Moreover, the reliability of odd-chain SFAs as biomarkers of dietary dairy fat in the context of different dietary patterns (e.g. low-dairy, high-fish, high-meat, high-fibre, or vegan diets) is still unclear. While associations between odd-chain SFAs and CVD risk are still unknown, potential associations are more likely to be driven by circulating C17:0 than C15:0. Meanwhile, the evidence on T2D appears to be more in agreement and suggests an inverse association between circulating odd-chain SFAs and risk of T2D. In general, findings from circulating odd-chain SFAs, which cannot discriminate specific types of dairy foods consumed, are broadly supportive of those from observational studies based on traditional dietary assessment methods [91]. However, these studies highlight that differential associations may exist between specific dairy foods consumed in the diet and CVD risk, and may also be reflective of their overall fat content [94,95]. Importantly, although attempts have been made to quantify dairy fat consumption using traditional dietary assessment methods such as FFQ in prospective cohort studies [92,93], future systematic literature reviews and pooled meta-analyses are warranted to assess the consistency between biomarker and dietary-based approaches to investigate the impact of dairy fat on CMD risk.

Interestingly, prospective cohort studies have reported significant correlations between circulating levels of C15:0, C17:0, and tPA (ranging from r = 0.3 to 0.8), suggesting the utility of tPA and potentially other ruminant *trans*-FAs as novel biomarkers of dairy fat intake [76]. However, with mostly outdated results from observational studies on ruminant *trans*-FAs, there is a striking need for new investigations on the validity of tPA and tVA as biomarkers of dairy fat intakes, especially since circulating levels of tPA are gaining interest in relation to cardiometabolic health [59]. Conversion pathways between the two FAs may also lead to confounding if used as biomarkers of dairy fat consumption separately, although the topic seems to be contentious among experts in the field. In the light of the current evidence, no conclusions can be drawn on the relevance of these FAs to be used as

proxies for dairy fat consumption until more well controlled dose-response research in humans is conducted. Moreover, the observational evidence on circulating ruminant tPA and tVA in relation to cardiometabolic health does not provide a clear picture, with scarcer evidence on tVA than on tPA. Updated meta-analyses are warranted to confirm the inverse associations observed between circulating tPA levels and T2D risk.

Similar to *trans*-FAs, the correlation between circulating concentrations of PhA and consumption of dairy products or dairy fat is still mostly unknown and has not been compared to other potential dietary sources such as fish and meat. While, to our current knowledge, PhA might not represent the most accurate biomarker of dairy fat, its utility might lay in the reflection of different cow feeding regimes (pasture grazing vs. conventional fodder) and ultimately be used as a more refined biomarker of dairy food quality in combination with more robust biomarkers of intake. Besides, the current lack of data on the direct role of PhA in human cardiometabolic health highlights an important need for research in this direction.

Overall, it is still unclear whether these FAs exert a direct effect on cardiometabolic health, or if they only reflect other beneficial components of dairy foods such as bioactive peptides [96]. It may also be difficult to disentangle the potential physiological roles of FAs from those of the rest of the dairy food matrix, which is highly variable among dairy food groups such as cheese and butter [97,98]. In addition, it has been proposed that fermented dairy foods such as yogurts may help prevent obesity via beneficial effects on the gut microbiota, the intestinal barrier function, and the hormonal regulation of appetite [99], and may be associated with lower risks of cerebrovascular diseases [100].

Conclusion

The evidence reviewed here indicates that FAs are often considered as biomarkers of dairy consumption by researchers, rather than biomarkers of dairy fat intakes. This may contribute to an important confounding in the interpretation of the results, since FAs, if validated as reliable biomarkers, may only accurately estimate the consumption of full-fat dairy products. With current public health guidelines often promoting the consumption of low-fat dairy, and prospective cohorts relying on FFQ that may not always distinguish dairy foods according to their fat content, it is crucial to describe FA biomarkers precisely in research papers to avoid confusion within and outside the scientific community. Importantly, the utility of FAs as biomarkers of dairy fat intake needs to be interpreted in the context of overall dietary patterns and potential interactions with other food groups or nutrients.

Overall, well-controlled intervention studies would help strengthen the evidence base on the impact of dairy FAs on traditional biomarkers of CMD risk (such as fasting lipid profiles and markers of glycaemic control), which are easily measured and responsive to dietary changes in a matter of a few weeks. Furthermore, the use of combinations rather than individual FAs, and insights from -omics methods such as lipidomic, proteomic and metabolomic analyses, may be helpful to identify and validate new biomarkers of dairy fat and total dairy intakes [101]. Finally, research in precision nutrition and nutrigenetics have so far showed mixed associations between dairy foods, lactase persistence and cardiometabolic health [102], and further studies which take into account phenotypical traits (e.g. sex, age, adiposity, or physical activity levels) may improve the overall understanding of the relationship between dairy and cardiometabolic health. In the future, validated approaches to identify new functional biomarkers of dairy fat consumption may improve our understanding of the relationship between dairy fat intake and cardiometabolic health and contribute to a better assessment of adherence to public health dietary guidelines.

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1.3 Hypothesis, aims, and objectives

The primary hypothesis of this PhD thesis was that the detrimental impact of dietary SFAs on cardiometabolic health may be modulated by their effect on novel markers of CMD risk and/or the food matrix from which they are consumed.

Therefore, this project aimed to:

1. Investigate the impact of overall and individual dietary SFAs on medium-term CMD risk markers and long-term CMD risk,
2. Assess the utility of dairy foods, and more particularly dairy fat, for CMD prevention at a population level.

To reach these aims, this PhD thesis includes five research studies designed to address specific hypotheses, which are detailed below.

First, I hypothesised that the beneficial impacts on CMD risk observed when replacing overall dietary SFAs with UFAs might be mediated via changes in the plasma lipidome. Therefore, we conducted a joint analysis of the DIVAS randomised controlled trial and the EPIC-Potsdam prospective cohort study to assess the impact of replacing dietary SFAs with UFAs on the plasma lipidome and the associations between plasma lipidome changes and both CMD long-term risk and risk markers (Chapter 2).

Second, I hypothesised that individual dietary SFAs may have differential impacts on CMD risk markers. To address this hypothesis, a systematic literature review and meta-analysis of randomised controlled trials was conducted to investigate the isoenergetic replacement of individual dietary SFAs with another SFA or a mixture of UFAs and its impact on a wide range of traditional and novel biomarkers of CMD risk (Chapter 3).

Third, I hypothesised that dairy foods, as important sources of dietary SFAs in UK adults, may represent an interesting food group to reduce SFA intake at a population level considering the wide availability of lower-fat dairy foods. Thus, we designed a food-based dietary fat exchange model incorporating high-fat or lower-fat dairy foods combined with snacks and cooking fats to replace dietary SFAs with UFAs in UK adult men (the RISSCI-1 study). Plasma phospholipid fatty acids (PL FAs) and dietary pattern analyses were used to assess the efficacy of and compliance to the dietary fat exchange model (Chapter 4).

Fourth, as plasma PL FAs are generally accepted as physiological markers of compliance to dietary fat manipulation to improve traditional dietary assessment methods (e.g. food frequency questionnaires,

food diaries), I hypothesised that profiles of plasma PL FAs could potentially be used as proxies for the consumption of dairy fat. After reviewing the literature on the topic (Chapter 1), a secondary analysis of three intervention studies conducted in the Hugh Sinclair Unit of Human Nutrition (i.e. the DIVAS, RESET, and SATgenε studies) was conducted to identify changes in plasma PL FA abundances associated with the chronic consumption of milk, cheese, yogurt, butter, and total dairy foods, along with their fat fraction (Chapter 5).

Fifth, and finally, I hypothesised that the association between dairy consumption and long-term CVD risk at a population level might be mediated by the specific dairy food matrix consumed. To explore this idea, specific associations between long-term CVD risk (total CVD, coronary heart disease, or cerebrovascular disease) and the consumptions of milk, cheese, yogurt, high-fat or lower-fat dairy, and fermented or non-fermented dairy were investigated in the NutriNet-Santé prospective cohort study (Chapter 6).

Chapter 2: Replacement of dietary saturated with unsaturated fatty acids over sixteen weeks are associated with beneficial effects on cardiometabolic risk related lipidome metabolites in adults at moderate cardiovascular disease risk

Contribution towards PhD thesis: My responsibilities included the preparation of lipidomics analyses and communication with the Metabolon Inc, who performed the sample analyses. After the completion of the sample analyses, I was responsible for developing the statistical analysis protocol and performing the statistical analyses, in close collaboration with Dr Fabian Eichelmann from the German Institute of Human Nutrition. Finally, I prepared the initial draft of the manuscript for submission to the American Journal of Nutrition, and finalised the manuscript presented in this chapter after including the feedback and comments received from co-authors.

This manuscript was approved by all co-authors and is ready for submission to the *American Journal of Clinical Nutrition*.

Replacement of dietary saturated with unsaturated fatty acids over sixteen weeks are associated with beneficial effects on cardiometabolic risk related lipidome metabolites in adults at moderate cardiovascular disease risk

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Running title: Dietary fat, lipidome and cardiometabolic health

Author contributions towards manuscript: LS, KGJ and JAL designed the secondary analysis of the DIVAS study. KGJ and JAL secured the funding and managed the primary analysis of DIVAS study. LS and FE performed the statistical analyses in the DIVAS study and the EPIC-Potsdam cohort study, respectively. LS drafted the manuscript. KGJ and JAL supervised the writing. MBS contributed to data collection in EPIC-Potsdam. FE, KGJ, CW, MBS, and JAL contributed to the data interpretation and revised each draft for important intellectual content. All authors read and approved the final manuscript. KGJ and JAL had primary responsibility for the final content and are the guarantors. The corresponding author (JAL) attests that all listed authors meet authorship criteria and that no other authors meeting criteria have been omitted.

Financial support: LS was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/P028217/1) Fatty Acid Metabolism – Interlinking Diet and Cardiometabolic Health FAME as part of the ERA HDHL: Biomarkers in Nutrition and Health. The DIVAS study was funded by the United Kingdom Food Standards Agency and Department of Health Policy Research Programme (024/0036). Work from the EPIC-Potsdam study was supported by the Federal Ministry of Science, Germany (grant no.01 EA 9401) and the European Union (grant no. SOC 95201408 05 F02) for the recruitment phase of the EPIC-Potsdam Study, by the German Cancer Aid (grant no. 70-2488-Ha I) and the European Community (grant no.SOC9820076905F02) for the follow-up of the EPIC-Potsdam Study by grants from the German Federal Ministry of Education and Research (Bundesministerium fuer Bildung und Forschung) to the German Center for Diabetes Research (DZD grants 82DZD00302,

82DZD03D03) and by a grant from the European Commission and the German Federal Ministry of Education and Research within the Joint Programming Initiative A Healthy Diet for a Healthy Life, as part of the ERA-HDHL cofounded joint call Biomarkers for Nutrition and Health (01EA1704). CW was supported by the SciLifeLab & Wallenberg Data Driven Life Science Program (grant: KAW 2020.0239).

Conflicts of interest: JAL is Deputy Chair of the UK Government Scientific Advisory Committee on Nutrition (SACN) and a previous member of the SACN working group on Saturated Fats and Health. JAL was chair, and KGJ and LS were members of a scientific expert committee for the International Life Sciences Institute (ILSI) on Individual Saturated Fatty acids and Cardiovascular Risk. The other authors have no potential conflicts of interest to disclose.

Acknowledgements: We thank the DIVAS study investigators Katerina Vafeiadou, Michelle Weech, Hana Altowaijri, Susan Todd, Rada Mihaylova, and Parveen Yaqoob. We also thank Cristina Razquin for the helpful discussions regarding the implementation of elastic net regression analyses. We thank the Human Study Centre (HSC) of the German Institute of Human Nutrition Potsdam-Rehbruecke, namely the trustee and the data hub, for data processing, and the biobank for the processing of biological samples. Finally, we thank all participants from the DIVAS and EPIC-Potsdam studies for their contribution.

Abstract

Background: Little is known about the impact of replacing dietary saturated (SFAs) with mono- (MUFAs) and/or polyunsaturated (PUFAs) fatty acids (FAs) on the plasma lipidome in relation to cardiometabolic disease (CMD) risk.

Objective: To assess the impact of replacing dietary SFAs with unsaturated FAs (UFAs) on the plasma lipidome and examine the relationship between lipid metabolites modulated by diet and CMD risk.

Methods: Plasma FA concentrations among 16 lipid classes (within-class FAs) were measured in a subset of participants from the DIVAS parallel randomised controlled trial (n=113), which consisted of three 16-week diets enriched in SFAs (target SFA:MUFA:n-6PUFA ratio=17:11:4% total energy TE), MUFAs (9:19:4%TE), or a mixture of UFAs (9:13:10%TE). Similar lipidomics analyses were conducted in the EPIC-Potsdam prospective cohort study (specific case-cohorts: n=1,707 and n=775 cases for type 2 diabetes [T2D], n=1,886 and n=551 cases for cardiovascular disease [CVD]). Multiple linear regression and multivariable Cox models identified within-class FAs sensitive to the DIVAS interventions and their association with CMD risk in the EPIC-Potsdam study. Elastic-net regression models identified within-class FAs associated with changes in CMD risk markers measured in the DIVAS study.

Results: The DIVAS high-UFA interventions reduced plasma within-class FAs associated with higher CVD risk in the EPIC-Potsdam cohort, especially SFA-containing glycerolipids and sphingolipids, while increasing plasma within-class FAs inversely associated with CVD risk. Results on T2D were less clear. Specific sphingolipids and phospholipids were associated with changes in markers of endothelial function, arterial stiffness, and ambulatory blood pressure, while higher low-density lipoprotein cholesterol concentrations were characterised by greater plasma levels of glycerolipids containing lauric and stearic acids.

Conclusions: These results suggest a mediating role of plasma lipid metabolites in the association between dietary fat and CMD risk. Future research combining interventional and observational findings will further our understanding of the role of dietary fat in CMD aetiology.

Key words: dietary fat, lipidomics, cardiovascular disease, type 2 diabetes, randomised controlled trial, EPIC-Potsdam

2.1 Introduction

Reducing dietary saturated fatty acids (SFAs) has been proposed as a modifiable dietary strategy to help prevent cardiometabolic disease (CMD) risk worldwide [1]. In particular, the replacement of dietary SFAs with unsaturated fatty acids (UFAs) confers a greater benefit than other nutrients (i.e. proteins or carbohydrates) on markers of cardiovascular disease (CVD) risk [2,3]. However, it is unclear whether such cardioprotective effects might stem from UFAs themselves, from reduced intakes of deleterious SFAs, or a combination of both.

Plasma lipids mostly derive from nutrient intakes, along with lipids stored and/or endogenously synthesised in the liver and adipose tissue [4]. In particular, lipids can be solubilised in plasma by binding with proteins, such as free fatty acids with albumins, or as more complex lipids and lipoproteins [5]. More than 600 lipid molecular species have been identified in plasma using high-throughput analytic methods such as lipidome-wide screenings, which differ in their structures and physiological functions [6]. For instance, glycerolipids are characterised by the presence of a glycerol backbone bonding fatty acids (e.g. in mono-, di-, and triacylglycerols), along with a phosphate-containing polar head in glycerophospholipids. Other plasma lipid classes include sphingolipids, such as sphingomyelin and ceramides, and sterols, which are important elements to cellular membrane structure [4]. Complex plasma lipid structures, such as low-density lipoprotein (LDL) or triacylglycerol (TAG), are important in the mechanistic link between diet and CMD risk [7,8]. Further hypotheses on the involvement of plasma lipid molecular species in cardiometabolic health have been generated thanks to the growing popularity of high-throughput lipidomics analyses [9–13]. Nonetheless, little is known on the impact of manipulating dietary fat intakes on the plasma lipidome, and how this may mediate the epidemiological associations observed between dietary fat intakes and CMD risk.

Methodological constraints have been important obstacles to the assessment of the causal relationship between dietary intakes, CMD risk markers, and CMD events. To date, established knowledge on the role of dietary fat on CMD risk derives from interventional studies such as randomised controlled trials (RCTs), which usually investigate the direct effect of dietary fat on short to medium-term changes in CMD risk markers, to observational prospective cohort studies, which allow for the long-term follow-up necessary to observe CMD events in large populations. Therefore, there is a need for intersectional research that would provide novel insights into CMD aetiology. To that end, recent analysis of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam investigated lipidome-wide CMD association analyses across different lipidomics levels and identified direct associations between SFA-containing glycerolipids in plasma and CMD risk (CVD or type 2 diabetes (T2D)), along with direct associations between ceramides and CVD risk [13] (full-text in Appendix 2). In addition, the authors observed that some of the lipid metabolites associated with CMD risk in the EPIC-Potsdam study may be modulated by dietary fat intakes in a subset of participants from the DIVAS RCT. To complement these novel findings, this secondary analysis of the DIVAS RCT and the EPIC-Potsdam prospective

cohort study aimed to assess the impact of a dietary fat exchange of SFAs with UFAs on the plasma lipidome and to identify lipidomic signatures that may be associated with changes in CMD risk markers (e.g. fasting lipid profiles, markers of vascular function and inflammation) measured in the DIVAS RCT. The secondary aim of this analysis was to investigate whether the identified dietary fat-induced lipidome changes were associated with CMD risk in the EPIC-Potsdam prospective cohort study.

2.2 Methods

The DIVAS randomised controlled trial

A subset of 113 participants of the DIVAS randomised controlled trial were broadly matched for dietary intervention, age, sex, and BMI and were selected for inclusion in this secondary analysis (Table 2.1). The protocol of the DIVAS study was previously described in detail [14]. The study was conducted according to the guidelines from the Declaration Helsinki and received ethical approval from the West Berkshire Local Research Ethics Committee (09/H0505/56) along with the University of Reading Research Ethics Committee (09/40). All participants provided their written informed consent before inclusion. Briefly, the DIVAS study was a 16-week, single-blind, parallel RCT conducted between 2009 and 2012 (www.clinicaltrials.gov; NCT01478958), which enrolled non-smoking men and women from the Berkshire (UK) area, aged 21-60 y, and with a moderate risk of CVD identified via a scoring tool developed by Weech *et al*. [15]. Inclusion criteria also included normal fasting lipid profiles, kidney and liver functions, no history of CVD or diabetes in the year preceding inclusion, no medication for hypertension, no pregnancy or lactation, and no excessive physical activity levels (> 3 sessions of 20 min per week) or alcohol consumption (≤ 14 units per week for women and ≤ 21 units for men, corresponding to the recommended alcohol intakes at the time the study was conducted).

Upon inclusion, participants were randomly allocated to one of three 16-week isoenergetic, high-fat (36%TE) dietary intervention arms varying in proportions of SFAs, monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs): a SFA-rich diet (target SFA:MUFA:n-6 PUFA ratio = 17:11:4 %TE), a MUFA-rich diet (target SFA:MUFA:n-6 PUFA ratio = 9:19:4 %TE), or a MUFA/PUFA-rich diet (target SFA:MUFA:n-6 PUFA ratio = 9:13:10 %TE) [15]. The analysis of 4-day weighed diet diaries at baseline and post-intervention confirmed participants' compliance and the successful isoenergetic exchange of dietary fat in each dietary intervention arm [15]. In addition, measurements for a wide range of markers of cardiometabolic risks were measured at baseline and post-intervention (Table 2.1).

EPIC-Potsdam prospective cohort study

The European Prospective Investigation into Cancer and Nutrition-Potsdam (EPIC-Potsdam) is a prospective cohort study which enrolled 27,548 participants from the Potsdam (Germany) area between 1994 and 1998.

The study and recruitment protocols have been previously published elsewhere [13,16,17]. The study received ethical approval from the Medical Society of the State of Brandenburg Ethics Committee, and all participants provided their written informed consent before inclusion. Cases of incident CMD were self-reported by participants during the active follow-up or obtained from death certificates' information. Additional information on T2D incidence were obtained using self-reported T2D medication or T2D-related dietary modifications, along with evidence of T2D detected in clinical files and death certificates originally related to other pathologies. Participants' treating physicians were then requested to validate each case and diagnosis date, following guidelines from the International Statistical classification of Disease and Related Health Problems (ICD-10) [18]. CMD considered for analyses included T2D (ICD-10 code E11), along with fatal or non-fatal cases of primary CVD which included myocardial infarction (ICD-10 code I21) or stroke (ICD-10 codes I63.0 to I63.9 for ischemic stroke, I61.0 to I61.9 for intracerebral and I60.0 to I60.9 for subarachnoid hemorrhage, and I64.0 to I64.9 for unspecified stroke).

To assess the associations between molecular phenotypes and T2D or CVD risks, two nested case-cohorts were constructed among the participants who provided blood samples at baseline (n=26,437). The construction protocol of the nested case-cohorts has been previously described in detail [13]. In summary, a random sub-cohort (n=1,262) was selected as a reference for both T2D and CVD risks, while incident cases of T2D (censoring date 30th November 2006) or CVD (censoring date 31st August 2005) were drawn from the overall EPIC-Potsdam cohort. Participants without follow-up or suffering from prevalent T2D or CVD were excluded from the analysis. The final T2D nested case-cohort included n=1,886 participants (among which n=775 T2D cases), while the CVD nested case-cohort included n=1,671 participants (among which n=551 CVD cases).

Lipidomics Analyses

Fasted blood plasma samples were collected in citrated vacutainer collection tubes at baseline (week 0) and post-intervention (week 16) from participants from the DIVAS study and stored at -80°C until analysis. Citrated plasma samples from the DIVAS study participants (n=113) were analysed by Metabolon Inc. (Morrisville, NC), who performed a Complex Lipids Platform™ (CLP) analysis to measure absolute concentrations of 987 molecular species (in µmol/L). Similar analyses were performed on citrated plasma samples from the baseline assessment in the EPIC-Potsdam study [13]. Briefly, plasma lipids were extracted using a butanol:methanol (BUME) solution as described by Löfgren et al. [19], concentrated under nitrogen and reconstituted in 0.25 mL of 10 mM ammonium acetate dichloromethane:methanol (50:50). Infusion-mass spectrometry analyses were then performed on a Sciex SelexION® -5500 QTRAP mass spectrometer. To determine characteristics fragments and lipid concentrations, analyses were performed in multiple reaction monitoring mode and samples were injected with deuterated internal standards. Metabolon Inc. reported mean relative SD below 5% for all measured lipid species.

Statistical analyses

All statistical analyses were performed in R (version 4.1.2). All statistical tests were two-sided and p-values < 0.05 were considered statistically significant.

Computation of within-class FA concentrations

Missing values in molecular species concentrations were imputed using the "Quantile Regression Imputation of Left-Censored data" method from the *imputeLCMD* R package [20], except for molecular species with ≥ 75% of missing values, which were excluded from analyses.

The CLP analysis measured absolute concentrations of lipid molecular species across lipid classes classified as neutral lipids, sphingolipids, and phospholipids, which all contain between one and three FAs within their molecular structure. Neutral lipids included cholesteryl esters (CE), monoacylglycerols (MAG), diacylglycerols (DAG), and TAG. Sphingolipids included sphingomyelins (SM), ceramides (CER), hexosylceramides (HCER), lactosylceramides (LCER), and dihydroceramides (DCER). Finally, phospholipids included phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylethanolamine ether (PEO), phosphatidylethanolamine plasmalogen (PEP), and lysophosphatidylethanolamine (LPE). Within each lipid class, molecular species containing a specific FA were summed to compute within-class FA concentrations. Thus, for lipid classes containing one FA per molecule (i.e. CE, MAG, CER, DCER, LCER, HCER, SM, SPE, and LPC), within-class FA and molecular species concentrations were equivalent. Lipid classes containing two or three FAs per molecule (i.e. DAG, TAG, PC, PE, PEO, PEP, and PI) were accounted for by weighing their contribution to a specific within-class FA using the amount of FA of interest present in the molecule (e.g. a DAG molecule containing two palmitic acids (16:0) contributed twice to the calculation of the DAG(16:0) within-class FA concentration, while a DAG molecule containing a palmitic acid (16:0) and a myristic acid (14:0) contributed once to the DAG(16:0) and once to the DAG(14:0) within-class FA concentrations). Finally, total lipid class concentrations were calculated by summing all molecular species concentrations from the same lipid class. For conciseness and legibility, within-class FAs will be referred to as their shorthand notations thereafter (e.g. DAG(16:0)). A list of the full names of all FAs investigated in this study is available in Supplementary Table 2.1.

Effects of the DIVAS dietary fat intervention on within-class FA plasma concentrations

The effects of the two UFA-rich diets (MUFA-rich and MUFA/PUFA-rich) from the DIVAS RCT on post-intervention within-class FA concentrations were assessed using multiple linear regression models adjusted for age, BMI, sex, baseline concentration of the within-class FA of interest, along with baseline and post-intervention concentration of the total lipid class of interest. All within-class FA concentrations were log-

transformed and z normalized (mean=0, SD=1) to allow comparison of effect size across within-class FA concentrations and account for potentially skewed distributions. Dietary intervention arms were coded as dummy variables to express linear regression model results as changes in z-score in the MUFA-rich and MUFA/PUFA-rich diets compared to the SFA-rich diet (used as reference). To account for multiple testing, p-values from multiple linear regression models were adjusted using the Bonferroni correction method [21].

All models were checked for linearity using scatter plots, for normality of residuals using QQ-plots, and for homogeneity of variance and independence of residuals using residuals plotted against predicted or observed outcome values, respectively.

Associations between within-class FA plasma concentrations and changes in CMD risk markers in DIVAS

Elastic-net linear regression (ENR) models were conducted to identify and select changes in within-class FA concentrations associated with changes in individual CMD risk markers (dependent variable) measured during the DIVAS RCT. ENR approaches have been successfully implemented in recent nutritional epidemiological research for the identification of simple and predictive models among omics datasets, which often present high dimensionality and risk of collinearity [22–24].

In this analysis, we adapted the method described by Drouin-Chartier et al. [22] to perform ENR analyses using the *glmnet* R package [25]. Briefly, all within-class FA concentrations and CMD risk markers were expressed as change from baseline (week 16 – week 0), and within-class FA concentrations were z normalized (mean=0, SD=1) to ensure comparability of effect sizes. However, due to the presence of negative changes from baseline, variables could not be log-transformed. First, A 10-fold cross-validation (CV) approach (training/testing sets: 80%/20% of the data, respectively) was used to determine the penalty (α) and tuning parameters (λ) which would optimise the complexity of the model (i.e. number of within-class FAs selected) while minimising the mean-squared error between the measured and predicted changes in the dependent variable and mitigating the risk of overfitting. The chosen α and λ parameters were then used to compute ENR models in a separate 10-fold CV procedure similar the one described above, from which we extracted the list of every within-class FAs selected in each of the 10 iterations along with their estimated regression coefficients. To build the final model, we considered within-class FAs consistently selected in the 10-fold CV procedure (i.e. in at least 9 iterations out of 10), and averaged their regression coefficients from each iteration to obtain a final, unique coefficient for each retained within-class FA. Finally, the performance of this approach was evaluated by comparing the predicted dependant variable values (i.e. CMD risk markers) to the actual ones measured during the DIVAS study. To do this, within each 10-fold CV iteration, the model obtained from the training set was fitted to its respective testing set to compute predicted values of the dependant variable. The subsets from

each iteration were then collated into one dataset, and the overall Pearson correlation between the predicted and measured values was used as a proxy for performance.

This method was repeated for each dependent variable of interest, which consisted of 41 CMD risk markers measured in the DIVAS study (Table 2.1), to obtain predictive models based on within-class FA plasma concentrations.

Finally, the associations between within-class FA concentrations and CMD risk markers identified by ENR analyses were further tested in fully adjusted multiple linear regression models, including age (continuous, in years), sex (female/male), BMI (continuous, kg/m^2), baseline CMD risk marker value (continuous, units in Table 2.1), and DIVAS dietary intervention group (SFA-rich, MUFA-rich, or MUFA/PUFA-rich diet).

Associations between within-class FA plasma concentrations and CMD risk in EPIC-Potsdam

Hazard ratios (HR) for the associations between within-class FA concentrations and incident risks of CVD or T2D were computed using multivariable proportional hazards Cox models, adjusted for age (timescale), sex, waist circumference, height, leisure time physical activity, smoking status, alcohol intake, highest achieved education level, fasting status as blood draw, total energy intake, diastolic and systolic blood pressures, circulating total cholesterol, high-density lipoprotein cholesterol (HDL-C), and TAG concentrations, anti-hypertensive medication, lipid-lowering medication, and acetylsalicylic acid medication. In addition, each model was adjusted for the concentration of total lipid class to which the within-class FA concentration of interest belonged (e.g. models on DAG(16:0) were adjusted for total DAG plasma concentration). All within-class FA concentrations were log-transformed and z normalized (mean=0, SD=1) to allow comparison of effect size across within-class FA concentrations and account for potentially skewed distributions. Participants contributed to each model until date of diagnosis, death, loss to follow-up, or censoring date, whichever occurred first. In addition, the case-cohort design and multiple testing were accounted for using assigned weights as proposed by Prentice [26] and the false discovery rate method [27], respectively.

2.3 Results

Baseline characteristics of the DIVAS study participant subset (n=113) and according to their allocated dietary intervention group are presented in Table 2.1. Within the included participants, 60.2% were females, overall mean age was 44 y (SD 10.2), and overall mean BMI 26.8 kg/m^2 (SD 4.3). Baseline characteristics of the participants from the EPIC study have been previously published [13].

Table 2.1 Pre-intervention characteristics and cardiometabolic disease risk markers in the subset of participants from the DIVAS randomised controlled trial (n=113).

		Dietary intervention group		
	Overall	**SFA-rich**	**MUFA-rich**	**MUFA/PUFA-rich**
	n=113	**n=38**	**n=39**	**n=36**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Age, *y*	43.8 (10.2)	46.1 (7.4)	42.2 (12.1)	43.3 (10.2)
Sex, *n (%)*				
Female	68 (60.2%)	21 (55.3%)	23 (59.0%)	24 (66.7%)
Male	45 (39.8%)	17 (44.7%)	16 (41.0%)	12 (33.3%)
BMI, *kg/m²*	26.8 (4.3)	27.7 (4.9)	26.1 (4.0)	26.5 (3.7)
Fasting lipid markers				
Total Cholesterol, *mmol/L*	5.52 (1.12)	5.54 (1.09)	5.35 (0.99)	5.68 (1.28)
LDL-C, *mmol/L*	3.79 (1.02)	3.80 (1.04)	3.65 (0.85)	3.93 (1.16)
HDL-C, *mmol/L*	1.49 (0.37)	1.47 (0.34)	1.47 (0.39)	1.52 (0.38)
Total Cholesterol:HDL-C ratio	3.91 (1.15)	3.99 (1.30)	3.82 (0.99)	3.94 (1.15)
TAG, *mmol/L*	1.24 (0.70)	1.35 (0.89)	1.18 (0.58)	1.19 (0.58)
NEFAs, *µmol/L*	471 (171)	484 (121)	446 (179)	484 (207)
Markers of glycaemic control				
Glucose, *mmol/L*	5.00 (0.44)	5.04 (0.45)	4.95 (0.44)	5.01 (0.44)
Insulin, *pmol/L*	28.0 (15.7)	31.7 (20.2)	27.1 (11.2)	25.2 (13.8)
HOMA-IR	1.05 (0.61)	1.22 (0.81)	0.99 (0.41)	0.94 (0.52)
QUICKI	0.40 (0.04)	0.39 (0.06)	0.39 (0.03)	0.40 (0.04)
rQUICKI	0.46 (0.06)	0.46 (0.08)	0.46 (0.05)	0.47 (0.06)
Markers of endothelial function and arterial stiffness				
FMD, *%*	5.66 (2.87)	5.44 (2.74)	5.27 (2.57)	6.38 (3.29)
Preocclusion artery diameter, *mm*	3.86 (0.64)	3.99 (0.67)	3.80 (0.65)	3.78 (0.59)
LDI Ach, *AU*	1583 (872)	1643(1,072)	1555 (734)	1534 (742)
LDI SNP, *AU*	1491 (683)	1552 (748)	1513 (708)	1383 (563)
Reflection index, *%*	62.7 (12.7)	65.2 (12.4)	61.1 (12.5)	61.6 (13.0)
Pulse wave velocity, *m/s*	6.89 (1.19)	7.12 (1.02)	6.53 (1.20)	7.05 (1.25)
Augmentation index, *%*	23.6 (12.2)	24.8 (10.9)	21.9 (13.5)	24.1 (12.2)
Stiffness index, *m/s*	6.72 (1.81)	6.98 (2.01)	6.35 (1.34)	6.83 (1.99)

	Overall	Dietary intervention group		
		SFA-rich	MUFA-rich	MUFA/PUFA-rich
	n=113	n=38	n=39	n=36
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Markers of ambulatory blood pressure				
24h SBP, *mmHg*	121 (11)	120 (12)	119 (9)	124 (11)
24h DBP, *mmHg*	74 (7)	75 (9)	73 (7)	76 (7)
24h PP, *mmHg*	47 (7)	46 (7)	46 (6)	49 (6)
24h heart rate, *beats/min*	71 (7)	69 (7)	72 (7)	72 (6)
Day SBP, *mmHg*	125 (12)	124 (12)	123 (10)	129 (12)
Day DBP, *mmHg*	78 (8)	77 (8)	76 (8)	79 (9)
Day PP, *mmHg*	48 (7)	47 (7)	47 (7)	50 (7)
Day heart rate, *beats/min*	73 (8)	71 (7)	75 (8)	74 (7)
Night SBP, *mmHg*	106 (12)	106 (14)	105 (10)	109 (11)
Night DBP, *mmHg*	63 (7)	64 (8)	61 (6)	65 (7)
Night PP, *mmHg*	47 (7)	46 (7)	46 (6)	49 (6)
Night heart rate, *beats/min*	62 (6)	62 (7)	62 (6)	63 (6)
Markers of inflammation and endothelial activation				
CRP, *mg/L*	2.15 (3.29)	2.95 (4.40)	1.59 (2.61)	1.90 (2.41)
NOx, *µmol/L*	27.5 (17.8)	32.5 (24.9)	23.6 (9.5)	26.4 (14.7)
VCAM-1, *ng/mL*	670 (171)	662 (142)	670 (199)	676 (172)
ICAM-1, *ng/mL*	218 (53)	221 (58)	216 (42)	217 (60)
IL-6, *pg/mL*	1.44 (1.06)	1.85 (1.29)	1.12 (0.78)	1.38 (0.94)
TNF-α, *pg/mL*	1.17 (0.74)	1.42 (1.01)	1.07 (0.62)	1.00 (0.37)
E-selectin, *ng/mL*	34.6 (14.7)	34.9 (14.5)	33.9 (12.7)	35.1 (17.0)
P-selectin, *ng/mL*	41.7 (12.9)	43.7 (12.9)	43.1 (13.3)	38.0 (12.0)
Von Willebrand factor, *µU/mL*	817 (409)	905 (492)	813 (368)	728 (339)
Urinary microalbumin, *mg/24h*	3.56 (6.47)	2.94 (2.48)	1.99 (1.22)	5.72 (10.63)

Abbreviations: AU, arbitrary units; BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; FMD, flow-mediated dilation; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin 6; LDI Ach, laser doppler imaging microvascular response to acetylcholine; LDI SNP, laser doppler imaging microvascular response to sodium nitroprusside; LDL-C, low-density lipoprotein cholesterol; NEFAs, non-esterified fatty acids; NOx, Nitrogen oxides; PP, pulse pressure; QUICKI, quantitative insulin sensitivity check index; rQUICKI, revised quantitative insulin sensitivity index; SBP, systolic blood pressure; TAG, triacylglycerol; TNF-α, tumour necrosis factor α; VCAM-1, vascular cell adhesion protein 1.

The CLP analysis identified a total of 987 molecular species in plasma samples, of which 101 were excluded from further analyses due to high proportion of missing values (≥ 75%). Among the 886 molecular species retained, 28 different FAs were detected (Supplementary Table 2.1), and a total of 243 within-class plasma FA concentrations across 16 total lipid classes were available for analyses. In plasma samples from the DIVAS participants prior to dietary intervention, palmitic (16:0) and oleic (18:1) acids were the most abundant FAs across all lipid classes (Figure 2.1), while TAG and CE were the most abundant lipid classes (Figure 2.2). As presented in Figure 2.3, 16:0 was the most abundant FA in sphingolipids (LCER, SM) and some phospholipids (LPC and PC), while stearic acid (18:0) accounted for the majority of FAs in most phospholipids (LPE, PE, PEO, PEP, and PI). Other ceramides (CER, DCER, HCER) mostly contained lignoceric acid (24:0). Finally, 18-carbon UFAs were the most abundant FAs in CE (i.e. 18:2), MAG (i.e. 18:4), along with DAG and TAG (i.e. 18:1).

Effects of the DIVAS dietary fat intervention on within-class FA plasma concentrations

The impacts of the 16-week MUFA and MUFA/PUFA-rich diets compared to the SFA-rich diet (as expressed in z-scores) in participants from the DIVAS study are presented in Figure 2.4. Compared to the SFA-rich diet, the MUFA-rich diet resulted in lower concentrations of SFAs across most lipid classes, with the largest reductions observed for DAG(20:0) (z-score = -1.08, standard error SE=0.17, p-value < 10^{-8}) and HCER(14:0) (z-score = -1.08, SE = 0.16, p-value < 10^{-9}) plasma levels. Furthermore, the MUFA-rich diet led to higher concentrations of long-chain MUFAs (18:1, 22:1, and 24:1) in CE, DAG, TAG, PEP, LCER, and SM compared to the SFA-rich diet, with the largest increases observed for SM(24:1) (z-score = 0.55, SE = 0.10, p-value < 10^{-6}) and TAG(22:1) (z-score = 0.53, SE = 0.08, p-value < 10^{-8}) plasma levels. However, some MUFA concentrations were lower following the MUFA-rich diet, such as 22:1 in CER, 14:1 in CE and TAG, along with 18:1, 20:1 and 22:1 in HCER. Similar albeit less diverse results were observed when comparing the effects of the MUFA/PUFA-rich diet to the SFA-rich diet. In particular, the MUFA/PUFA-rich diet led to higher concentrations of 18:2 in DAG and TAG (z-score = 0.29, SE = 0.06, p-value < 10^{-5}, and z-score = 0.30, SE = 0.06, p-value < 10^{-5}, respectively, data not tabulated), but fewer reductions in SFAs compared to the MUFA-rich diet (Figure 2.4).

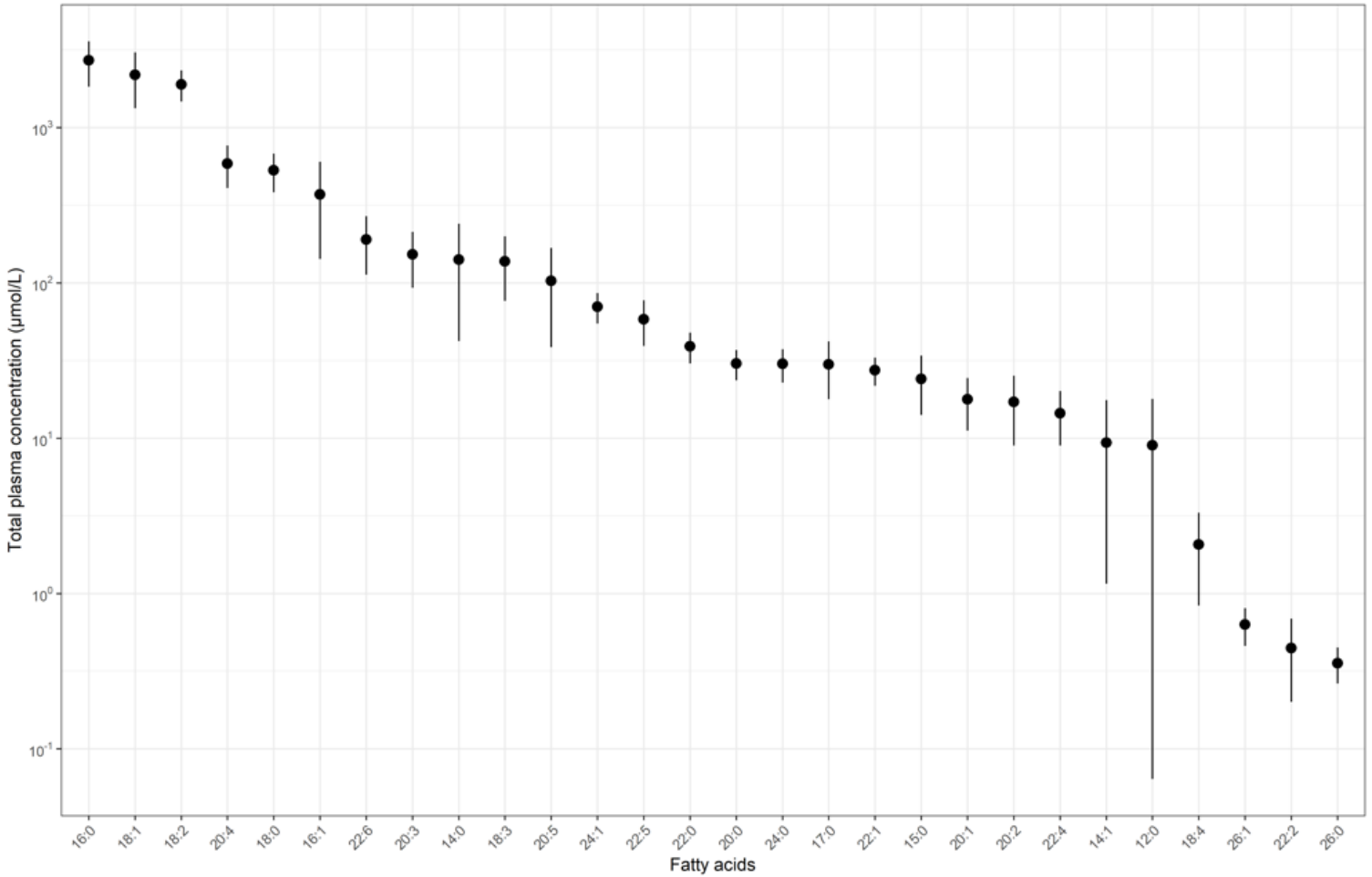

Figure 2.1 Total plasma concentrations of 28 fatty acids identified in lipidome-wide screening among participants from the DIVAS study prior to the start of the dietary intervention (n=113).[a,b]

[a] *Full names of fatty acids are listed in supplementary table 2.1.*

[b] *Data points represented as mean and SD*

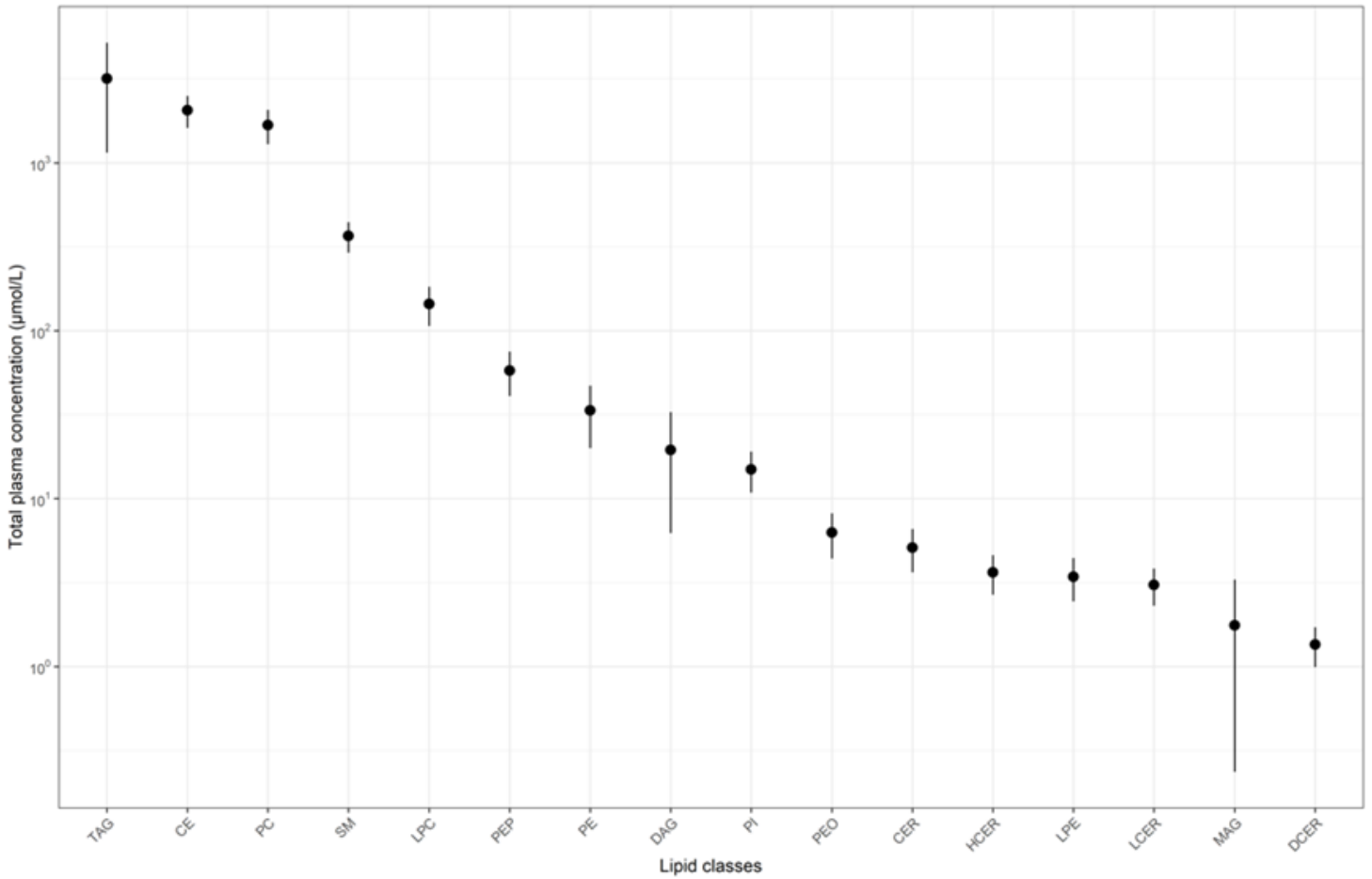

Figure 2.2 Total plasma concentrations of 14 lipid classes identified in lipidome-wide screening among participants from the DIVAS study prior to the start of the dietary intervention (n=113).[a]

[a] Data points represented as mean and SD

Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; DCER, dihydroceramides; HCER, hexosylceramides; LCER, lactosylceramides; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEO, phosphatidylethanolamine ether; PEP, phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; SM, sphingomyelins; TAG, triacylglycerols*

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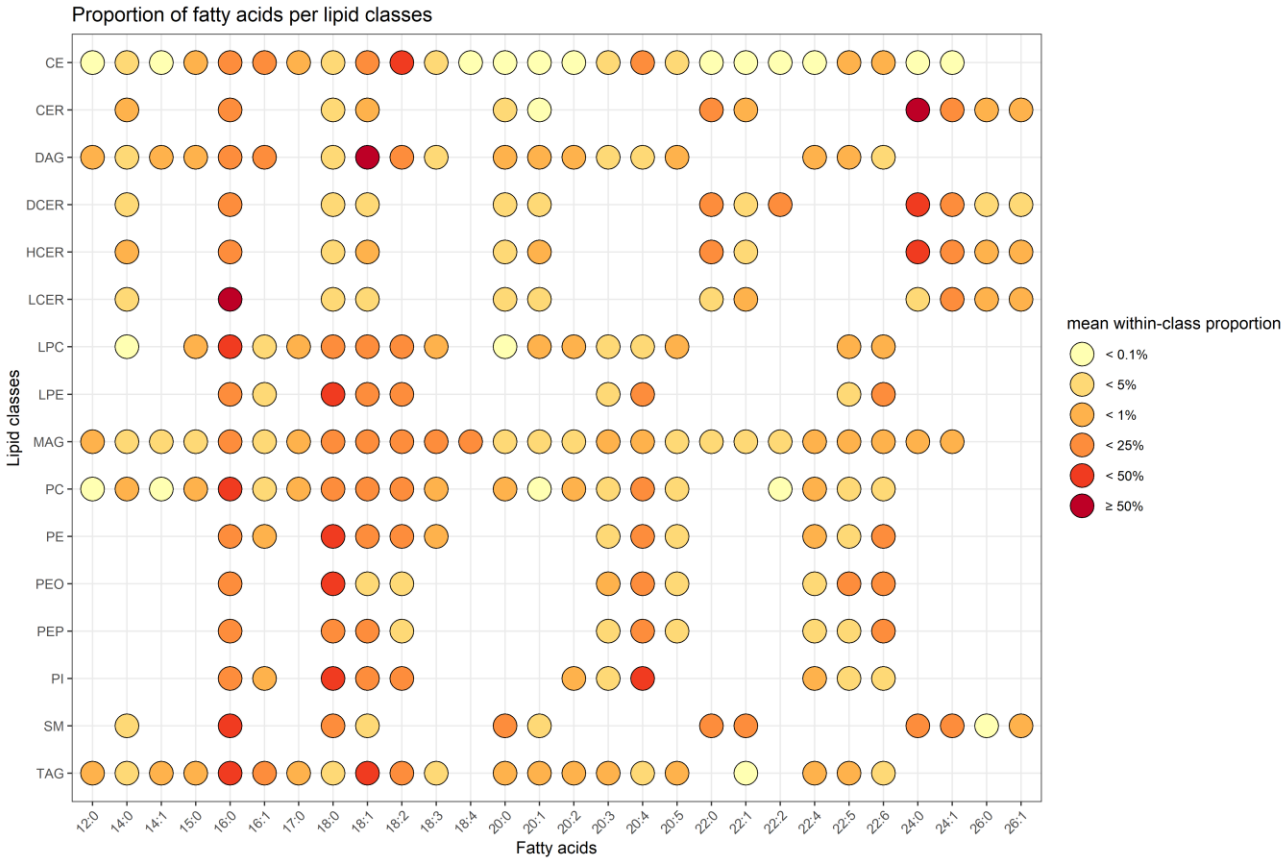


Figure 2.3 Proportion of fatty acids in plasma lipid classes among participants from the DIVAS study prior to the start of the dietary intervention (n=113). [a]

[a] *Full names of fatty acids are listed in supplementary table 2.1.*
Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; DCER, dihydroceramides; HCER, hexosylceramides; LCER, lactosylceramides; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEO, phosphatidylethanolamine ether; PEP, phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; SM, sphingomyelins; TAG, triacylglycerols.*

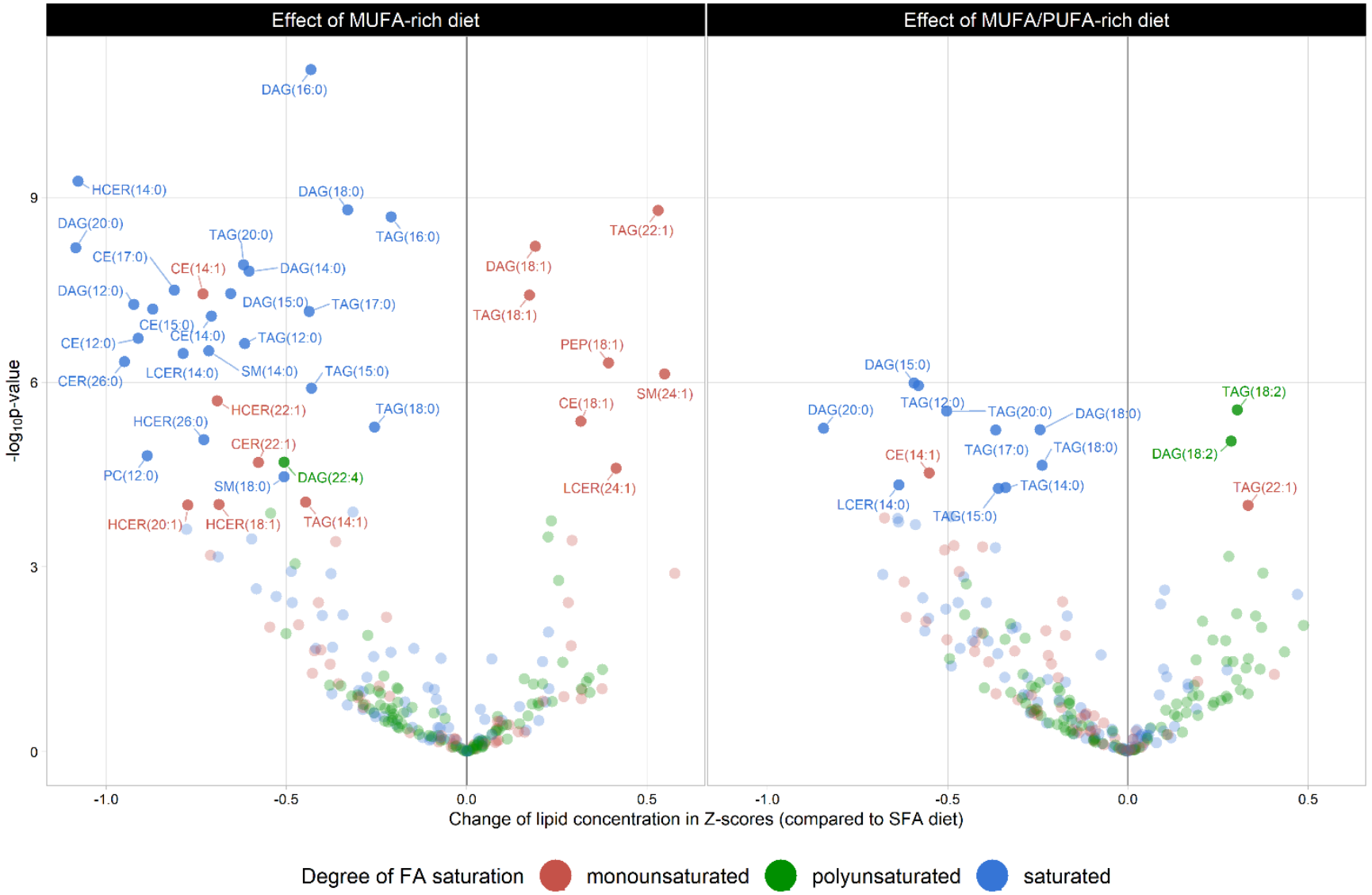


Figure 2.4 Effect of MUFA-rich and MUFA/PUFA rich dietary interventions compared to a SFA-rich diet on plasma lipid metabolites identified in lipidome-wide screening among participants from the DIVAS study (n=113). [a, b]

[a] Assessed using multiple linear regression models adjusted for: age, BMI, sex, baseline concentration of the within-class fatty acid of interest, along with baseline and post-intervention concentration of the total lipid class of interest. P-values were adjusted for multiple testing using the Bonferroni correction method.

[b] Unlabelled data points represent within-class FA concentrations not significantly affected by the DIVAS dietary intervention after Bonferroni correction (p-value ≥ 0.05).

Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; DCER, dihydroceramides; HCER, hexosylceramides; LCER, lactosylceramides; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEO, phosphatidylethanolamine ether; PEP, phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelins; TAG, triacylglycerols.*

Associations between within-class FA plasma levels and changes in CMD risk markers in DIVAS

A total of 14 out of the 41 CMD risk markers measured in the DIVAS study were linked with changes in within-class FAs using ENR models (Figure 2.5, Supplementary Figure 2.1). In particular, increased LDL-C concentrations between post- and pre-intervention measures were associated with increased plasma TAG(12:0), CE(18:0), and PEP(20:3) levels (predictive Pearson correlation = 0.21, Supplementary Table 2.2), while increases in non-esterified FA (NEFA) concentrations were associated with reduced levels of SFAs in plasma LPC (i.e. 15:0, 16:0, 17:0, and 18:0) but higher levels of PE(16:0) (predictive Pearson correlation = 0.26, Supplementary Table 2.2). Furthermore, various changes in within-class FA plasma concentrations were associated with beneficial effects on endothelial function and arterial stiffness estimations, such as laser doppler imaging microvascular response to acetylcholine or sodium nitroprusside (LDI Ach and LDI SNP respectively), reflection and arterial stiffness indexes, and pulse wave velocity. In particular, a higher arterial stiffness index was associated with higher levels of long-chain FAs (i.e. 20:2, 22:2, 24:0, and 22:6) in CE, LPC, or MAG, and with lower levels of 16:0 and 18:1 in HCER, and LPE(22:5) (predictive Pearson correlation = 0.13, Supplementary Table 2.2). Finally, higher tumour necrosis factor α (TNF-α) concentrations were associated with increased levels of 14:1, 16:0 and 18:1 in MAG, and in 12:0 in CE (predictive Pearson correlation = 0.18, Supplementary Table 2.2). Similarly, higher P-selectin concentrations were associated with increased proportions of 12:0 in CE and TAG (predictive Pearson correlation = 0.24, Supplementary Table 2.2).

Results multiple linear regression models are presented in Table 2.2. In fully adjusted models, there was no statistically significant associations between within-class FA concentrations and NEFAs, quantitative insulin sensitivity check index (QUICKI), pulse wave velocity, or TNF-α concentrations. For other CMD risk markers, multiple linear regression models confirmed associations between single within-class FA concentrations and total cholesterol, P-selectin, LDI Ach, LDI SNP, reflection index, night systolic blood pressure (SBP), 24h pulse pressure (PP), and day heart rate levels. Furthermore, changes in LDL-C concentrations remained positively associated with changes in TAG(12:0) and CE(18:0) concentrations (β = 0.15, 95%CI 0.04-0.26, p-value = 0.01, and β = 0.20, 95%CI 0.08-0.32, p-value < 0.01, respectively, Table 2.2). Finally, changes in the arterial stiffness index remained positively associated with changes in plasma concentration of CE(22:2) (β = 0.49, 95%CI 0.10-0.87, p-value = 0.01), LPC(15:0) (β = 0.34, 95%CI 0.01-0.66, p-value = 0.04), and LPC(20:2) (β = 0.45, 95%CI 0.12-0.78, p-value = 0.008, Table 2.2).

Table 2.2 Results from multiple linear regression models on the associations between changes in within-class FA concentrations and CMD risk markers in the DIVAS randomised controlled trial (n=113).[a]

Within-class FA	β estimate [b]	95% CI Lower-bound	95% CI Upper-bound	p-value	R^2	n
Total cholesterol, *mmol/L*						
CE(18:0)	0.26	0.13	0.39	<0.001	0.24	113
LDL-C, mmol/L						
LPC(20:1)	-0.16	-0.26	-0.06	<0.01	0.41	113
PEP(20:3)	0.03	-0.09	0.14	0.64		
TAG(12:0)	0.15	0.04	0.26	0.01		
CE(18:0)	0.20	0.08	0.32	<0.01		
NEFAs, *mmol/L*						
PE(16:0)	19.83	-8.09	47.75	0.16	0.37	113
HCER(20:1)	26.03	-3.24	55.30	0.08		
LPC(15:0)	-5.46	-45.83	34.91	0.79		
LPC(16:0)	-17.24	-74.48	40.01	0.55		
LPC(17:0)	-45.13	-95.46	5.20	0.08		
LPC(18:0)	3.18	-53.19	59.55	0.91		
QUICKI						
PC(22:2)	-0.003	-0.011	0.004	0.34	0.33	97
LDI Ach, *AUC*						
PE(20:3)	230.83	106.96	354.71	<0.001	0.59	89
LDI SNP, *AUC*						
CER(18:0)	172.20	40.13	304.26	0.01	0.56	89
Reflection index, *%*						
PC(22:2)	-3.11	-5.31	-0.91	0.006	0.24	108
Arterial stiffness index, *m/s*						
CE(22:2)	0.49	0.10	0.87	0.01	0.55	109
CE(24:0)	-0.05	-0.47	0.36	0.81		
LPE(22:5)	-0.44	-0.77	-0.10	0.01		
MAG(22:6)	0.12	-0.20	0.43	0.47		
HCER(16:0)	-0.27	-0.66	0.12	0.17		
HCER(18:1)	-0.23	-0.64	0.17	0.26		
LPC(15:0)	0.34	0.01	0.66	0.04		
LPC(20:2)	0.45	0.12	0.78	0.008		
Pulse wave velocity, *m/s*						
HCER(26:1)	-0.18	-0.37	0.01	0.06	0.32	86
Night SBP, *mmHg*						
LPC(22:5)	2.30	0.30	4.31	0.03	0.33	90
24h PP, *mmHg*						
MAG(12:0)	1.92	0.75	3.09	0.002	0.40	81

Day heart rate, *beats/min*						
PC(14:1)	-2.05	-3.67	-0.43	0.01	0.27	84
TNF-α, *pg/mL*						
CE(12:0)	0.04	0.00	0.08	0.08	0.36	110
MAG(14:1)	0.04	-0.01	0.09	0.10		
MAG(16:0)	0.04	-0.01	0.08	0.10		
MAG(18:1)	0.03	-0.02	0.08	0.21		
P-selectin, *ng/mL*						
CE(12:0)	2.41	0.26	4.55	0.03	0.20	112
TAG(12:0)	1.56	-0.49	3.62	0.13		

[a] Multiple linear regression adjusted for age (continuous, in years), sex (female/male), BMI (continuous, kg/m^2), baseline CMD risk marker value (continuous), and dietary intervention group (SFA-rich, MUFA-rich, or MUFA/PUFA-rich diet).

[b] Expressed as change in CMD risk marker per additional SD of plasma within-class FA concentration (μmol/L).

Abbreviations: CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; HCER, hexosylceramides; LDI Ach, laser doppler imaging microvascular response to acetylcholine; LDI SNP, laser doppler imaging microvascular response to sodium nitroprusside; LDL-C, low density lipoprotein cholesterol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; NEFAs, non-esterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine plasmalogen; PP, pulse pressure; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TAG, triacylglycerols; TNF-α, tumour necrosis factor α.

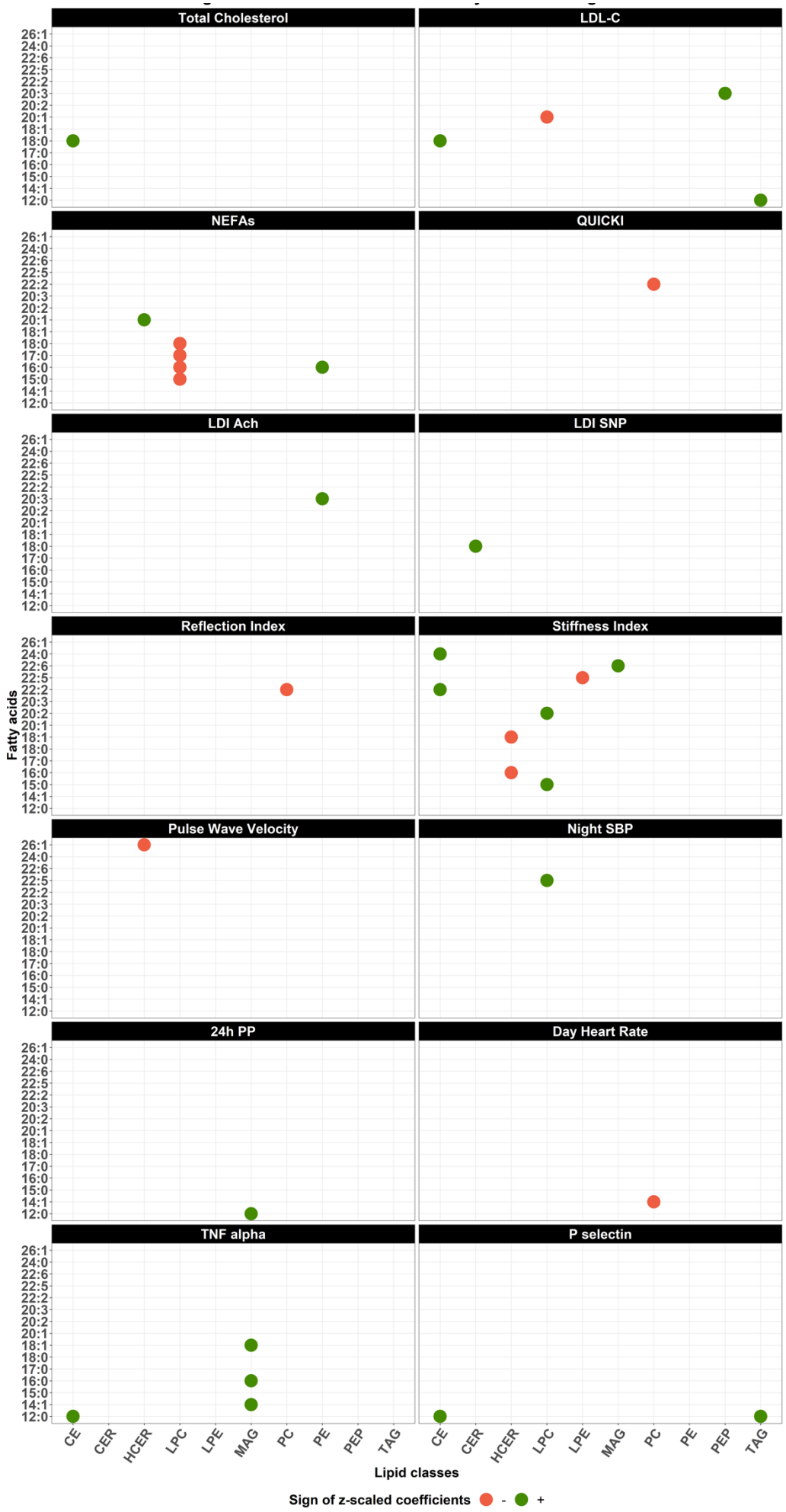

Total Cholesterol
LDL-C
NEFAs
QUICKI
LDI Ach
LDI SNP
Reflection Index
Stiffness Index
Pulse Wave Velocity
Night SBP
24h PP
Day Heart Rate
TNF alpha
P selectin
Fatty acids
26:1
24:0
22:6
22:5
22:2
20:3
20:2
20:1
18:1
18:0
17:0
16:0
15:0
14:1
12:0
CE
CER
HCER
LPC
LPE
MAG
PC
PE
PEP
TAG
Lipid classes
Sign of z-scaled coefficients
-
+

Figure 2.5 Lipid metabolites associated with changes in cardiometabolic risk markers measured among participants from the DIVAS study (n=113). [a]

[a] *Identified using 10-fold cross validated elastic-net regression models.*
Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; HCER, hexosylceramides; LDI Ach, laser doppler imaging microvascular response to acetylcholine; LDI SNP, laser doppler imaging microvascular response to sodium nitroprusside; LDL-C, low density lipoprotein cholesterol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; NEFAs, non-esterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine plasmalogen; PP, pulse pressure; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TAG, triacylglycerols; TNF-α, tumour necrosis factor α.*

Associations between within-class FA plasma concentrations and CMD risk in EPIC-Potsdam

The associations between within-class FAs affected by the DIVAS dietary intervention and long-term CMD risk in the EPIC-Potsdam cohort study are presented in Figure 2.6. Overall, results from Cox proportional hazards models suggest that the DIVAS study MUFA-rich diet significantly decreased the concentrations of some FAs in DAG (i.e. 14:0, 15:0, 16:0, 18:0, 22:4), TAG (i.e. 14:1, 16:0, 17:0, 18:0, 20:0) SM (i.e. 14:0, 18:0), and HCER (i.e. 18:1), and that these lipid metabolites were all associated with higher CVD risk in the EPIC-Potsdam cohort study. Reciprocally, the DIVAS study MUFA-rich diet increased the concentrations of a within-class FA associated with lower CVD risk in the EPIC-Potsdam cohort study (LCER(24:1), HR = 0.73, 95%CI 0.56-0.94, p-value = 0.02). However, the DIVAS MUFA-rich diet increased plasma levels of SM(24:1), a within-class FA associated with a greater CVD risk in the EPIC-Potsdam cohort study (HR = 1.60, 95%CI 1.27-2.02, p-value < 10^{-4}). This beneficial synergy between the DIVAS MUFA-rich diet and long-term CVD risk in the EPIC-Potsdam cohort study was consistent with the effects of the DIVAS MUFA/PUFA-rich diet, although fewer within-class FAs were affected by the latter dietary intervention. Finally, the lipidome-mediated links between the DIVAS dietary intervention and incident T2D risk in the EPIC-Potsdam cohort study were not as clear, but revealed that the DIVAS MUFA-rich diet decreased the concentrations of some of the within-class FAs that were strongly associated with T2D risk, such as TAG(16:0) (HR = 9.80, 95%CI 3.96-24.27, p-value < 10^{-6}), DAG(16:0) (HR = 2.84, 95%CI 1.75-4.61, p-value < 10^{-4}), and DAG(18:0) (HR = 2.22, 95%CI 1.41-3.51, p-value < 10^{-3}).

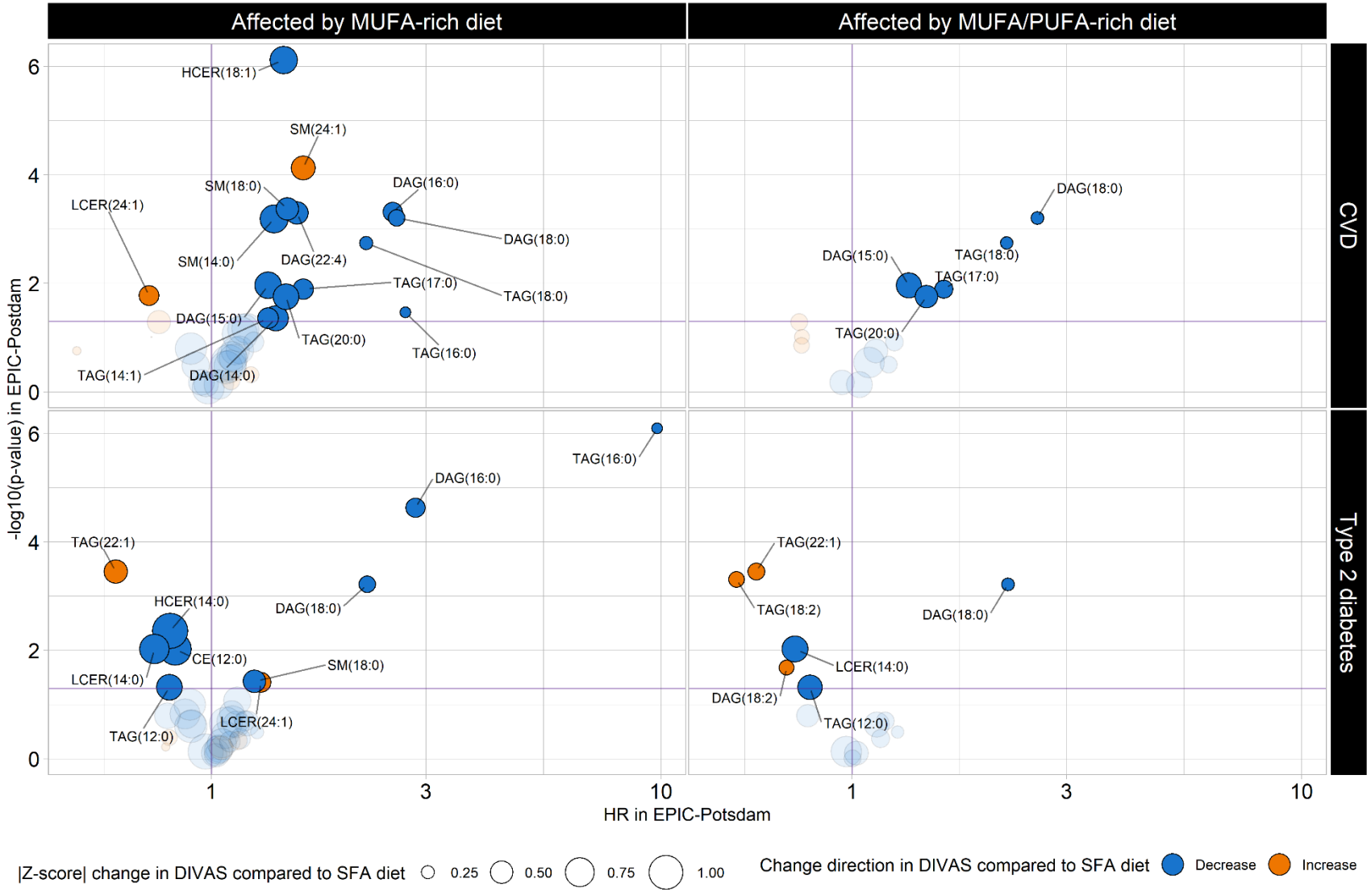


Figure 2.6 Effect of the DIVAS dietary intervention on lipid metabolites identified in lipidome-wide screening and associations with cardiometabolic disease risk in the EPIC-Potsdam cohort study. [a, b]

[a] Assessed using multivariable Cox proportional hazard models adjusted for age (timescale), sex, waist circumference, height, leisure time physical activity, smoking status, alcohol intake, highest achieved education level, fasting status as blood draw, total energy intake, diastolic and systolic blood pressures, circulating total cholesterol, high-density lipoprotein cholesterol, and triacylglycerol concentrations, anti-hypertensive medication, lipid-lowering medication, and acetylsalicylic acid medication. In addition, each model was adjusted for the concentration of total lipid class to which the within-class FA concentration of interest belonged.

[b] Unlabelled data points represent within-class FA concentration not significantly associated with CVD or T2D risk in the EPIC-Potsdam cohort (p-value ≥ 0.05).

Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; DCER, dihydroceramides; HCER, hexosylceramides; HR, hazard ratio; LCER, lactosylceramides; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEO, phosphatidylethanolamine ether; PEP, phosphatidylethanolamine plasmalogen; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SM, sphingomyelins; TAG, triacylglycerols.*

2.4 Discussion

In this secondary analysis of the DIVAS RCT and the EPIC-Potsdam cohort studies, lipidome-wide screening revealed changes in plasma within-class FAs following the exchange of dietary SFAs with UFAs for 16 weeks. In particular, the DIVAS dietary intervention may reduce plasma concentrations of lipid metabolites associated with CVD risk, while increasing the concentrations of those associated with lower CVD risk. Finally, this study revealed correlations between changes in specific lipid metabolites and markers of fasting lipids, microvascular function, arterial stiffness, endothelial activation, and ambulatory blood pressure after the 16-week intervention.

In the DIVAS study, the implementation of diets enriched in UFAs compared to SFAs led to broad reductions in SFA concentrations along with increased concentrations of MUFAs and PUFAs across several plasma lipid classes (i.e. sphingolipids and glycerolipids). These results might be partly explained by the absorption of dietary UFAs throughout the MUFA-rich and PUFA-rich dietary interventions, since glycerolipids are common FA storage structures found within lipoprotein particles such as chylomicrons [28,29]. In addition, this study is the first, to our knowledge, to report direct associations between dietary MUFA intakes and plasma PEP(18:1) levels. In a case cohort study from the PREDIMED RCT (n=787), Wang et al. did not report any effect of a 1-year diet enriched in extra virgin olive oil on the plasmalogen lipid cluster score, which reflected the plasma concentrations of PEP and plasmalogen PC [30]. However, this lack of response in plasmalogen lipids might be partly explained by the high habitual consumption of olive oil within the Spanish cohort, which may have reduced the impact of the PREDIMED dietary intervention in a Spanish population. This highlights the importance of the background FA intakes in response to dietary fat interventions [31,32].

Other consequences of the DIVAS MUFA-rich diet included lower long-chain FA concentrations in plasma sphingolipids such as 18:1, 20:1, and 22:1 in CER and HCER after intervention compared to the SFA-rich diet. While decreased MUFA concentrations in these lipid classes following replacement of dietary SFAs with MUFAs might seem counterintuitive, these results are in line with experimental findings, which suggested that SFAs may be used as precursors for the first step of the endogenous synthesis of 16:1 and 18:1 sphingolipids catalysed by the serine palmitoyltransferase (SPT) enzyme [33]. Therefore, diets enriched in UFAs instead of SFAs might down-regulate SPT activity, leading to lower endogenous synthesis of long-chain sphingolipids [34,35]. Evidence from human studies is sparse, but one cross-sectional study of 2,860 Chinese participants observed similar results and reported inverse associations between dietary total PUFA intakes and plasma long-chain CER and HCER levels, although different lipid metabolite nomenclatures prevent the direct comparison of their results with those from the DIVAS study [36]. Similarly, two overfeeding RCTs reported decreased serum long-chain FAs

(16:0, 18:0, 24:0, and 24:1) in CER and LCER following a PUFA-rich diet [37], and increased plasma long-chain CER levels following a SFA-rich diet [38].

Coordinated CLP analyses between the DIVAS RCT and the EPIC-Potsdam prospective cohort study revealed that both DIVAS study MUFA-rich and MUFA/PUFA-rich diets decreased plasma concentrations of 15:0, 17:0, and 18:0 in glycerolipids (i.e. DAG, TAG), which were associated with higher CVD risk in the EPIC-Potsdam cohort study. Previous epidemiological studies on circulating odd-chain SFAs, often used as biomarkers of dairy consumption [39], have suggested null or inverse associations with CVD risk [40], which contrast with findings from the EPIC-Potsdam cohort study [13]. However, most studies have measured odd-chain SFA concentrations within plasma/serum phospholipids or cholesteryl esters, or in total plasma/serum, while there is no previous epidemiological evidence, to our knowledge, of potential associations between specific DAG or MAG FA compositions and CVD risk. Moreover, analyses from the EPIC-Potsdam study suggested direct associations between CVD risk and some plasma sphingolipids (SM and HCER) containing 14:0, 18:0, and/or 18:1, which were sensitive to the MUFA-rich dietary intervention in the DIVAS study. In line with these findings, in a prospective cohort study of 2,627 Chinese participants (n=152 CVD cases) higher plasma HCER(18:1/16:0), HCER(18:1/18:0), SM(18:1/18:0), and SM(18:2/18:0) levels were associated with higher risks of major cardiovascular events (i.e. non-fatal myocardial infarction, stroke, and cardiovascular death) [41]. Although the exact mechanism underlying these associations have not been elucidated, a cross-sectional analysis of 200 human atherosclerotic carotid plaques reported higher SM concentrations in plaques from symptomatic patients (i.e. patients who reported transient ischemic attacks, strokes, or amaurosis fugax) [42], and further experimental studies have suggested dysfunctional SM synthesis and transport may be associated with atherosclerosis, valvular disease, and cardiomyopathy risks [43].

Overall, the DIVAS dietary interventions seemed less efficient at increasing plasma within-class FAs inversely associated with T2D risk. However, the MUFA-rich dietary intervention seemed to decrease plasma levels of 16:0 in DAG and TAG, along with 18:0 in DAG and SM, which were directly associated with T2D risk in the EPIC-Potsdam prospective cohort [13]. Serum and plasma glycerolipids containing SFAs, especially 16:0 and 18:0, have been previously identified as potential predictors of T2D risk in RCTs and prospective cohort studies [12,44,45], possibly by impairing insulin sensitivity pathways as suggested by animal and in vitro studies [46]. Furthermore, while recent findings from the PREDIMED RCT suggested inverse associations between plasma SM cluster scores and T2D risk [12], other studies reported SFA-containing SM species may be associated with higher T2D risk and impaired insulin sensitivity [47–49]. In particular, a prospective cohort study of 2,302 Chinese participants observed direct

associations between T2D risk and higher plasma levels of two 18:0-containing SM species: SM(16:1/18:0) (HR = 1.45, %95CI 1.18-1.78) and SM(18:1/18:0) (HR = 1.40, 95%CI 1.17-1.68) [47].

In the DIVAS study participants, we identified that higher plasma levels of CE(18:0) and TAG(12:0) were associated with higher LDL-C concentrations, an established CVD risk marker [8], which was independent of the dietary intervention group and other potential confounding factors. These results are in line with previously described general lipid compositions of LDL particles, which contain approximately 42% of CE and 6% of TAG (wt/wt) [50]. In a recent overfeeding RCT (n=36 participants), a 3-week SFA-rich diet led to higher concentrations of SFA-containing TAG in LDL particles, which was associated with higher LDL susceptibility to oxidation and aggregation in the intima [51]. In line with these findings, previous experimental and animal studies suggested dietary SFAs might decrease LDL receptor expression in hepatocytes, leading to increased circulating LDL-C levels [52]. In addition, we identified improved LDI Ach and LDI SNP, two estimates of microvascular reactivity, were associated with higher plasma PE(20:3) and CER(18:0), respectively. The latter association contrasts with findings from a recent case-control study of 90 patients with abnormal coronary endothelial function who displayed higher plasma CER(18:0) levels compared to controls [53], and more generally contrasts with studies suggesting the potential detrimental role of ceramides in atherosclerosis and CVD aetiology [54,55]. Additionally, we observed direct associations between arterial stiffness index measured by digital volume pulse and plasma LPC (i.e. 15:0 and 20:2), and between night SBP and LPC(22:5) levels in DIVAS study participants. Although previous studies reported direct associations between overall LPC and atherosclerosis or arterial stiffness [56,57], other findings suggest inverse associations between CVD risk markers and specific LPC molecular species [58–61]. Finally, this analysis of the DIVAS study revealed potential deleterious associations between MAG(12:0) and 24h PP and between CE(12:0) and P-selectin levels, but inverse associations between PC(14:1) and day heart rate. Besides, two within-class FAs (i.e. TAG(12:0) and CE(12:0)) significantly affected by the DIVAS dietary intervention were also associated with changes in CMD risk markers in fully adjusted linear regression models, which provides promising insights into the beneficial impact of replacing dietary SFAs with UFAs for CVD prevention. Overall, further studies are warranted to assess the strength of these associations and to decipher the potential roles of lipid classes and/or specific FAs in these relationships.

Overall strengths of this study include its extensive panel of investigated lipids, which were then aggregated into within-class FAs to facilitate the interpretability of the findings. Secondly, this coordinated analysis between the DIVAS RCT and the EPIC-Potsdam prospective cohort study provided novel insights bridging the gap between dietary intervention studies, which are often limited to the study of biomarkers of disease risk, and prospective cohort studies, which can investigate long-term disease outcomes but are often unable to implement intervention designs. Finally, this analysis

benefitted from strong methodological approaches to identify lipidomic predictors of CVD risk and CVD risk markers, by adjusting Cox proportional hazard models and linear regression models with a wide range of potential confounders, along with using cross-validation methods and corrections from multiple testing. Nonetheless, some limitations of this study need to be acknowledged. Firstly, although the CLP used for this analysis provided details on the acyl chains within lipid classes, there was no information on the location of the configuration of double bonds in FAs (i.e. cis or trans). Secondly, plasma free fatty acids levels were not available in this analysis. Thirdly, the risk of lipid degradation and oxidation in plasma samples cannot be entirely ruled out, although the DIVAS samples were stored at -80°C since their collection and were not thawed until their analysis [62]. There was no evidence that freeze-thaw cycles in the EPIC-Potsdam study samples had any impact on the observed associations with CMD risk [13]. Fourthly, although previous studies have investigated the relationship between lipidomic patterns and health outcomes [30,63,64], this study focused on the investigation of individual within-class FAs to better understand their specific role in CVD and T2D aetiology. Finally, the diversity of approaches to measure and identify lipidomic species may impede the direct comparison of our findings to those from previous studies, and highlights the need for harmonised approach and nomenclature in future lipidomic investigations.

To conclude, the replacement of dietary SFAs with UFAs in the DIVAS RCT led to modifications of the plasma lipidome metabolites among UK adults at moderate risk of CVD. Furthermore, findings from this study suggested that the associations between lipid metabolites and markers of CMD risk may be specific to certain FAs and specific lipid classes. In particular, some sphingolipids and phospholipids may be associated with novel CVD risk markers including endothelial function, arterial stiffness, and ambulatory blood pressure. Finally, the coordinated lipidome-wide plasma screening performed in the EPIC-Potsdam prospective cohort suggested that the changes in plasma lipid metabolites observed after the replacement of dietary SFAs with UFAs may be beneficially associated with long-term CMD risk. Overall, these results concur with current evidence on the benefits of replacing dietary SFAs with UFAs for CMD risk prevention, and contribute to the evidence base on the role of lipid metabolites in CMD aetiology.

2.5 Supplementary material

Supplementary Table 2.1. List of 28 fatty acids identified in lipidome-wide screening among participants from the DIVAS study, prior to and after the start of the dietary intervention (n=113).

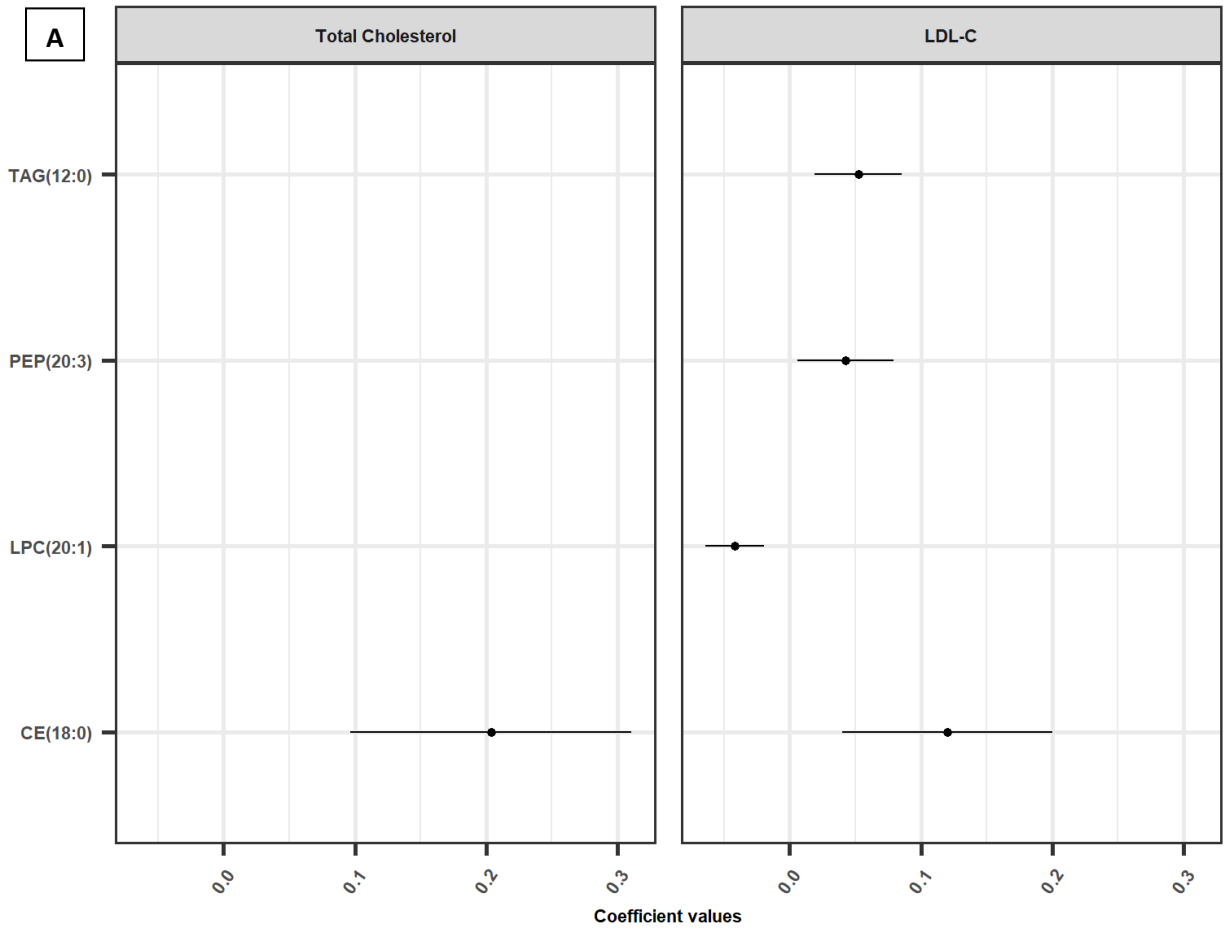
Fatty acid shorthand notation	Fatty acid common name [a]
12:0	Lauric acid
14:0	Myristic acid
14:1	Myristoleic acid
15:0	Pentadecanoic acid
16:0	Palmitic acid
16:1	Palmitoleic acid
17:0	Hexadecenoic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
18:3	α-linolenic acid
18:4	Stearidonic acid
20:0	Arachidic acid
20:1	Paullinic acid
20:2	Dihomo-linoleic acid
20:3	Dihomo-γ-linolenic acid
20:4	Eicosatetraenoic acid
20:5	Eicosapentaenoic acid
22:0	Behenic acid
22:1	Erucic acid
22:2	Docosadienoic acid
22:4	Docosatetraenoic acid
22:5	Docosapentaenoic acid (osbond acid)
22:6	Cervonic acid
24:0	Lignoceric acid
24:1	Nervonic acid
26:0	Cerotic acid
26:1	Ximenic acid

[a] based on the most common double-bond *cis* configurations, as the complex lipid panel performed by Metabolon Inc. in this analysis did not allow for the resolution of double bond position or configuration.

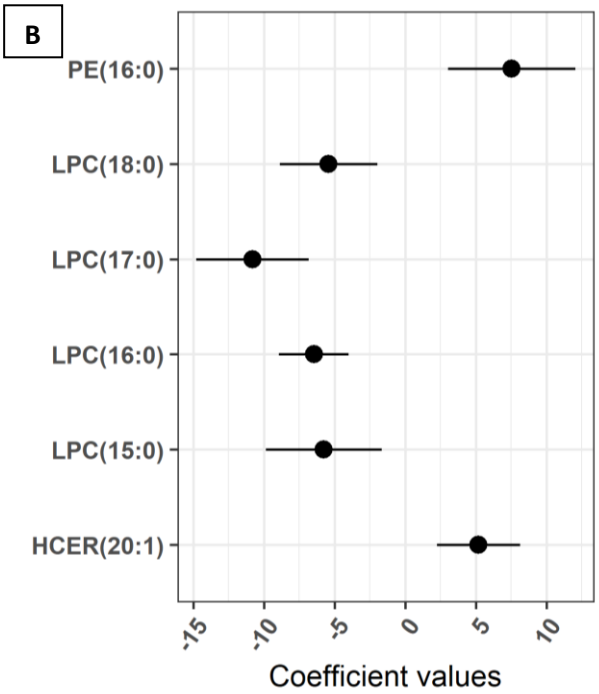
Supplementary Figure 2.1. Final regression coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models conducted among participants from the DIVAS study.

Abbreviations: *CE cholesteryl esters; CER, ceramides; DAG, diacylglycerols; FA, fatty acid; HCER, hexosylceramides; LDI Ach, laser doppler imaging microvascular response to acetylcholine; LDI SNP, laser doppler imaging microvascular response to sodium nitroprusside; LDL-C, low density lipoprotein cholesterol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; NEFAs, non-esterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine plasmalogen; PP, pulse pressure; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TAG, triacylglycerols; TNF-α, tumour necrosis factor α.*

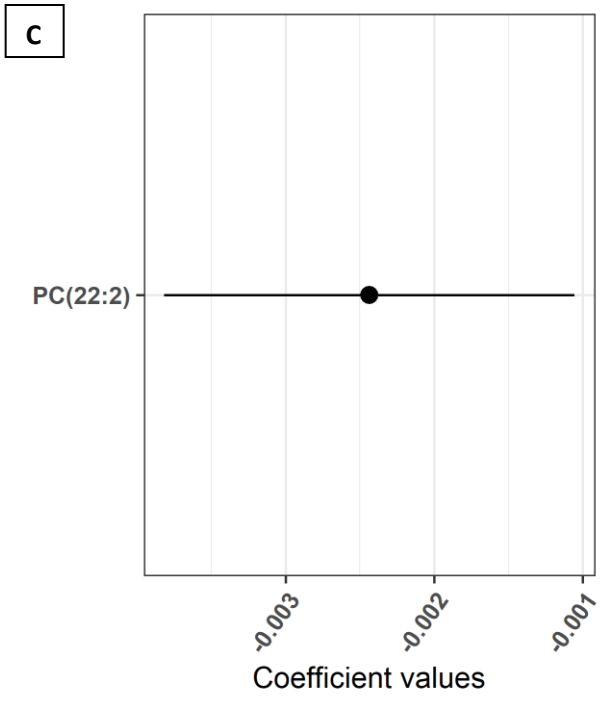
A: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on total cholesterol and low-density lipoprotein cholesterol (LDL-C) among participants from the DIVAS study.



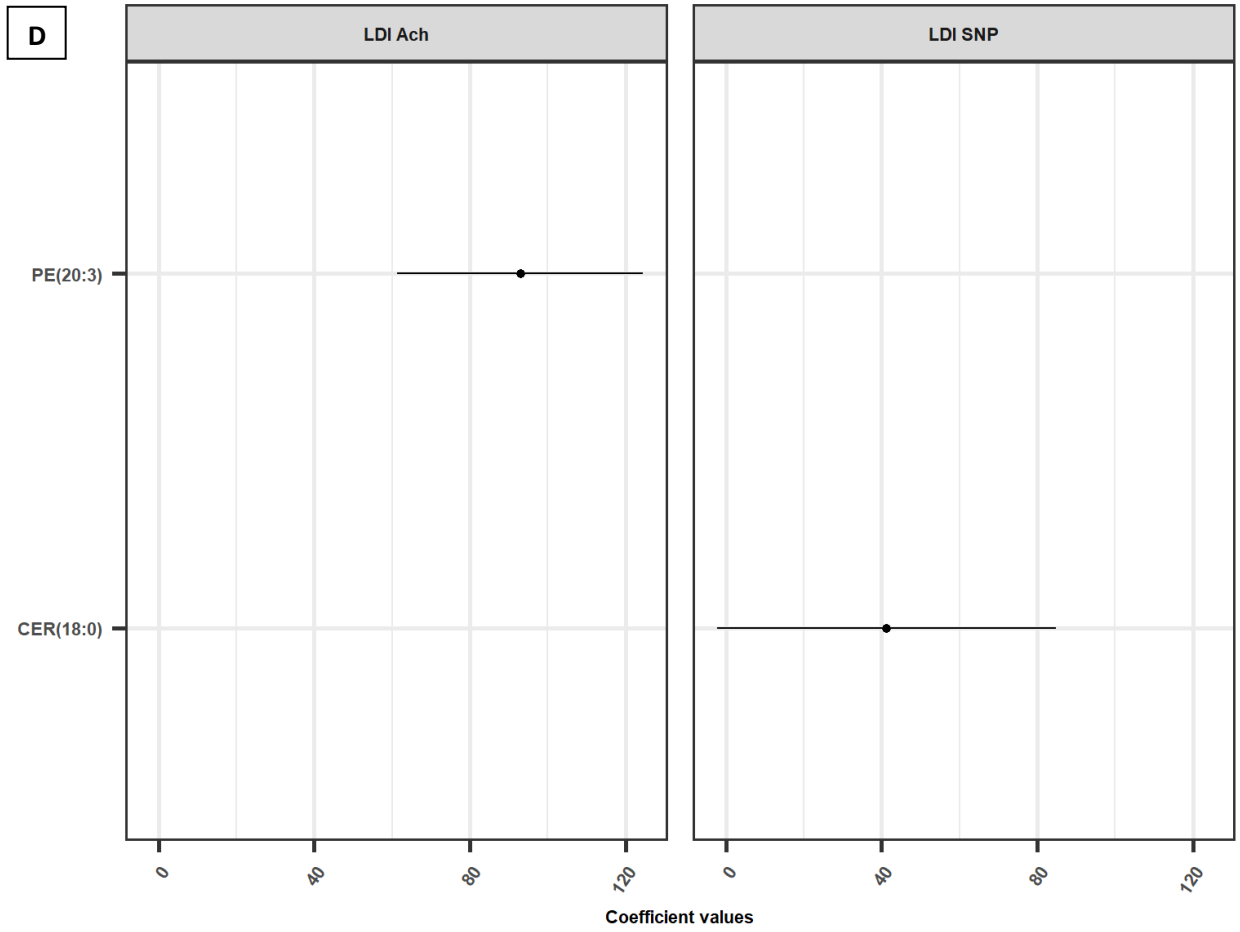
B: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on non-esterified fatty acids (NEFAs) among participants from the DIVAS study.



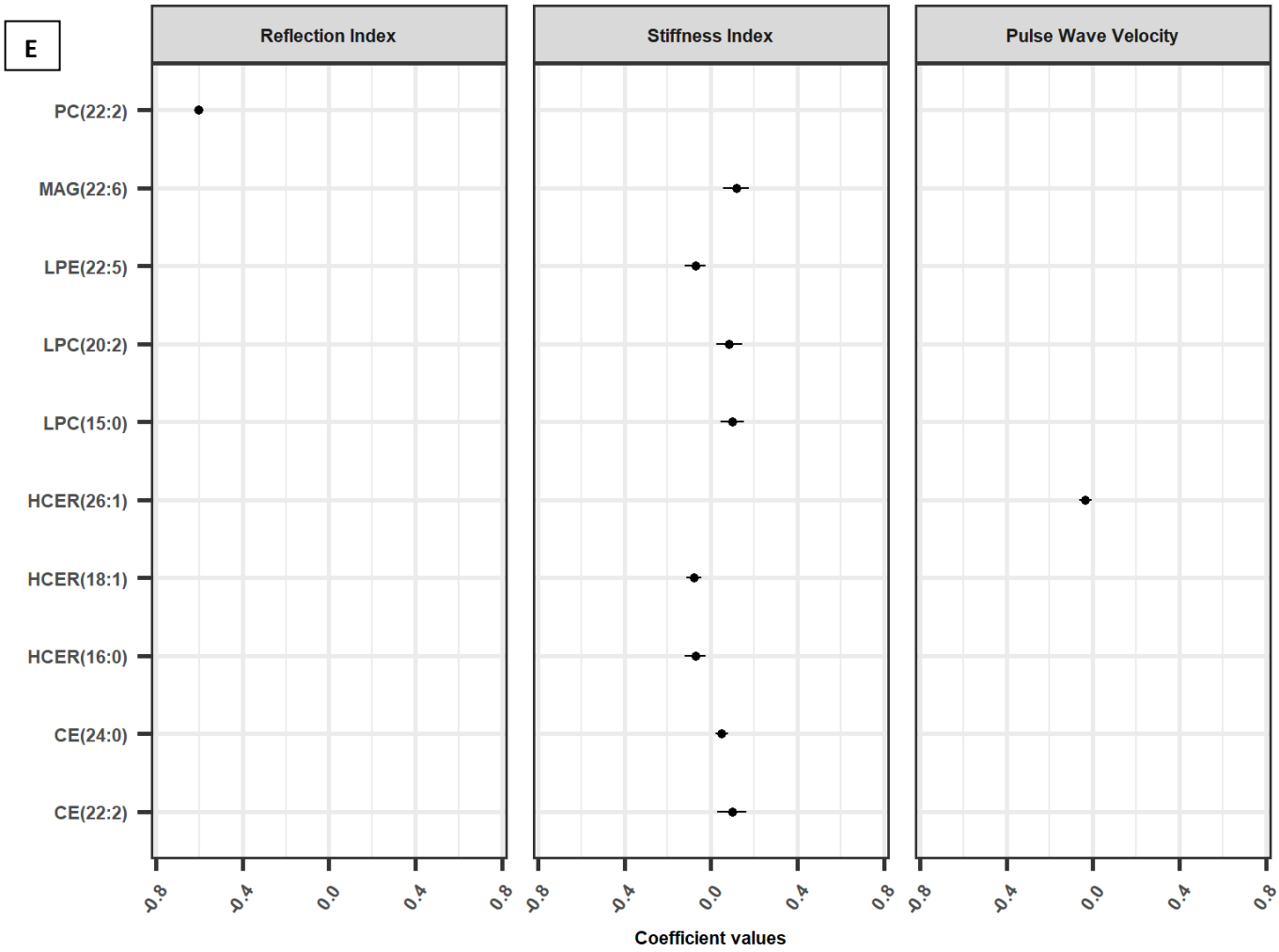
C: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on quantitative insulin sensitivity check index (QUICKI) among participants from the DIVAS study.



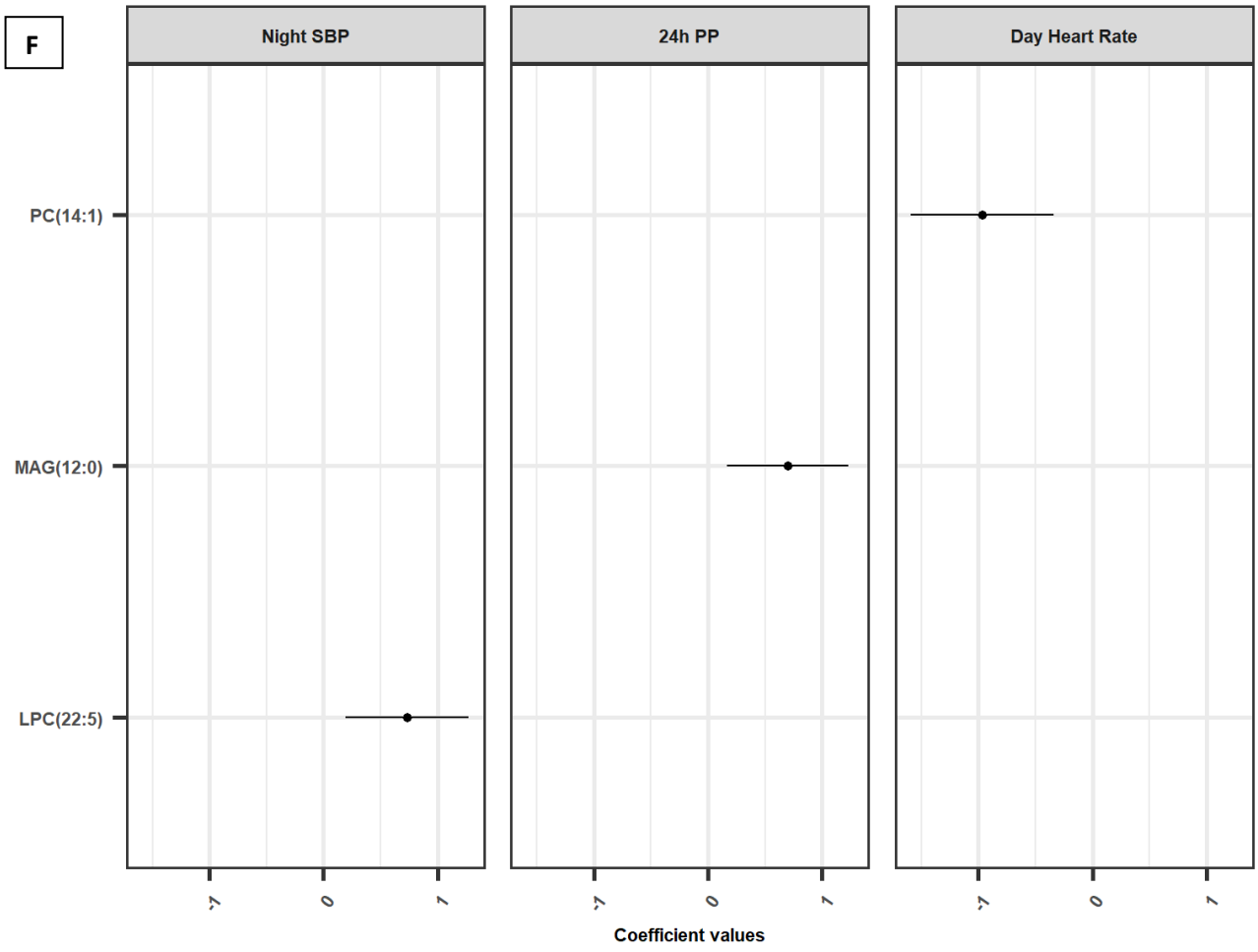
D: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on laser doppler imaging microvascular response to acetylcholine (LDI Ach) and sodium nitroprusside (LDI SNP) among participants from the DIVAS study.



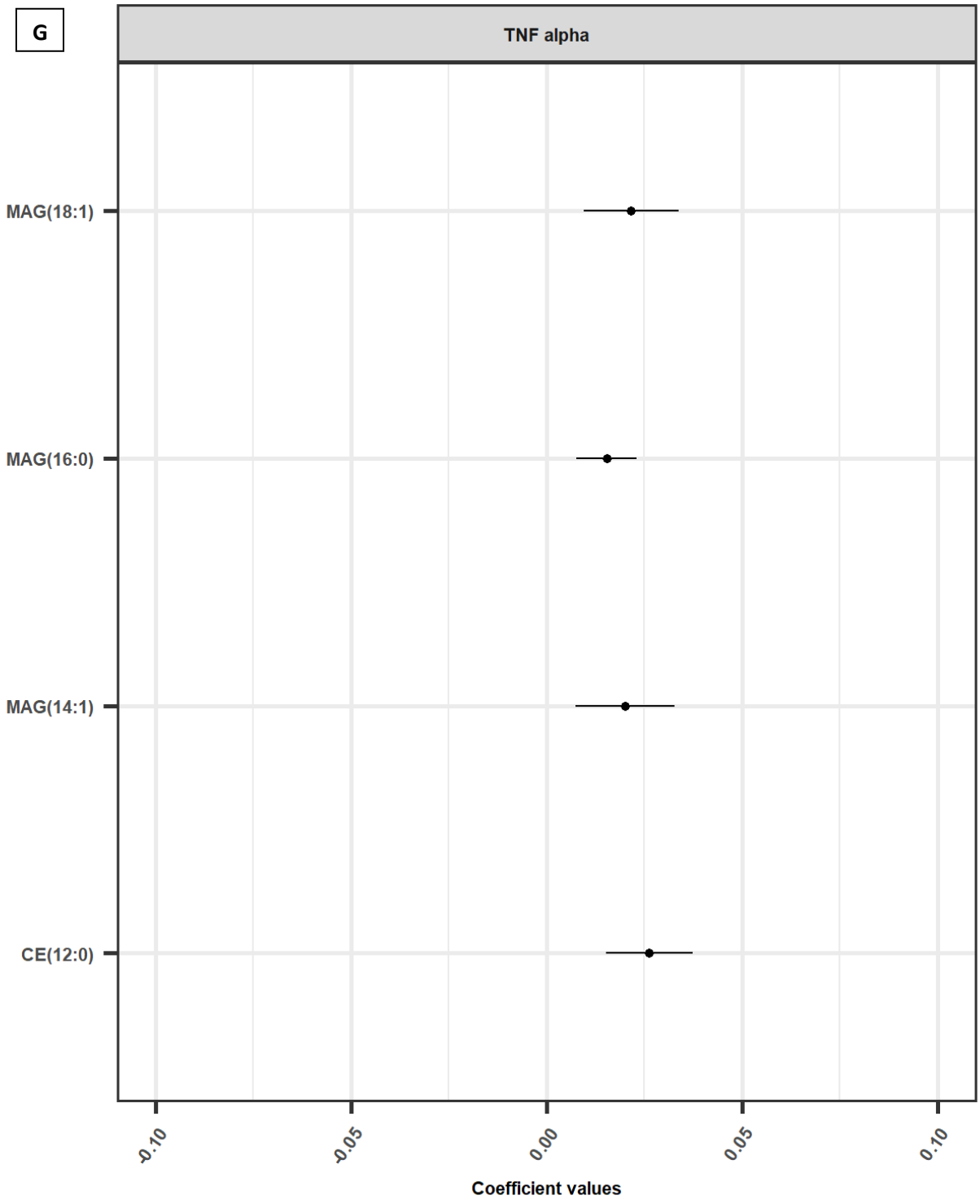
E: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on reflection index, stiffness index, and pulse wave velocity among participants from the DIVAS study.



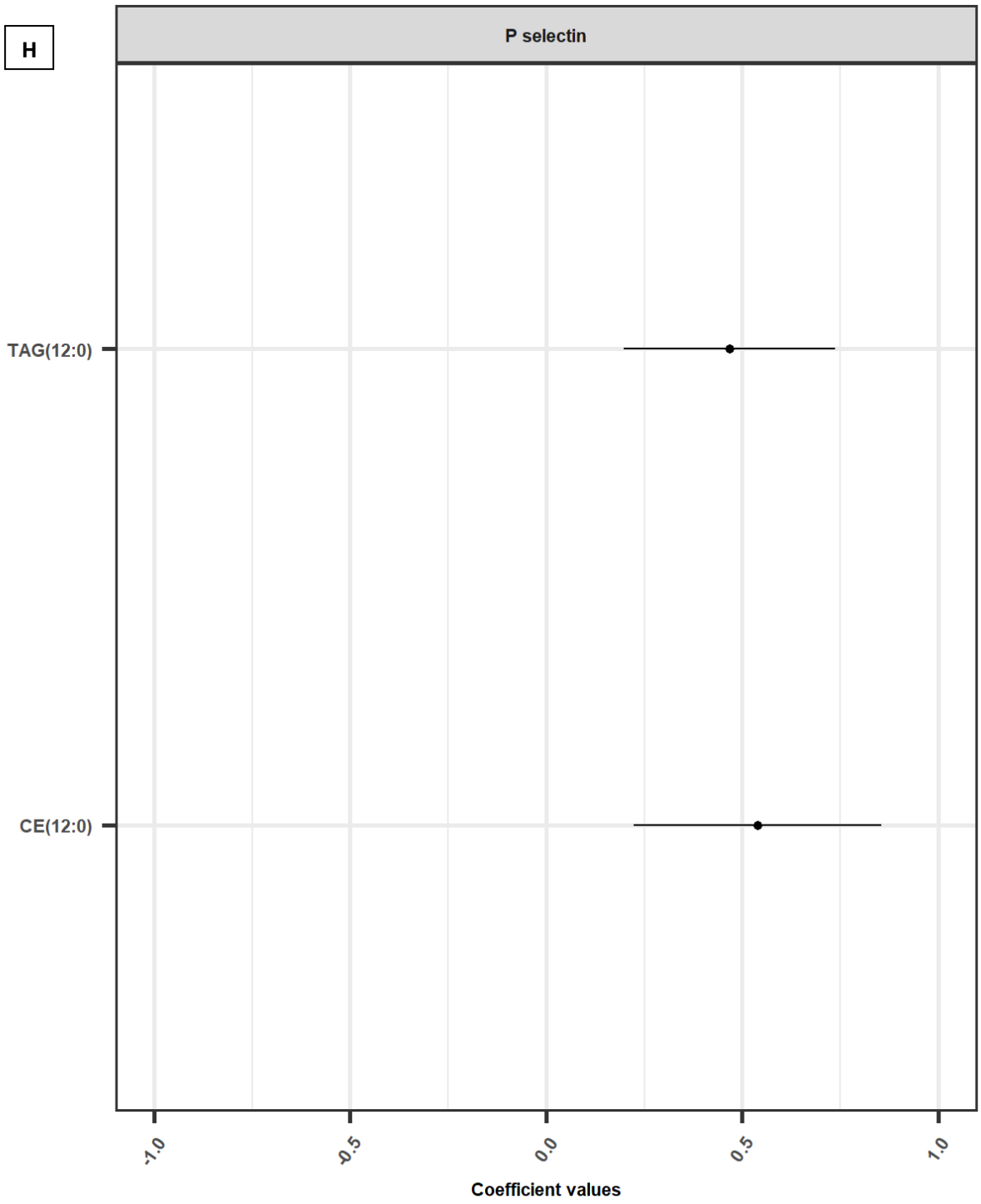
F: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on night systolic blood pressure (SBP), 24h pulse pressure (PP), and day heart rate among participants from the DIVAS study.



G: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on tumour necrosis factor α (TNF-α) among participants from the DIVAS study.



H: Coefficients (mean and SD) for the within-class FAs selected at least 9 times in the elastic-net regression models on P-selectin among participants from the DIVAS study.



Supplementary Table 2.2. Number of within-class FAs correlated with changes in CMD risk markers among participants from the DIVAS study, and Pearson correlation coefficients between multi-metabolite profiles and measured CMD risk markers.

CMD risk marker	Total within-class FAs consistently selected in ENR [a]	Pearson correlation [b]	95%CI
Total cholesterol	1	0.15	0.02 to 0.28
LDL-C	4	0.21	0.08 to 0.33
NEFAs	6	0.26	0.14 to 0.38
QUICKI	1	0.23	0.09 to 0.36
LDI Ach	1	0.63	0.53 to 0.71
LDI SNP	1	0.60	0.50 to 0.69
Reflection Index	1	0.06	-0.07 to 0.19
Stiffness Index	8	0.13	-0.02 to 0.27
Pulse Wave Velocity	1	0.36	0.24 to 0.47
Night SBP	1	0.28	0.14 to 0.41
24h PP	1	0.25	0.10 to 0.39
Day heart rate	1	0.33	0.18 to 0.45
TNF-α	4	0.18	0.05 to 0.31
P-selectin	2	0.24	0.11 to 0.36

[a] Number of within-class FAs selected at least 9 times in the 10-fold cross validation procedure for the elastic net regression approach.

[b] The Pearson correlation coefficients reflect the correlation between the predicted change in CMD risk marker derived from the multi-metabolite models identified by the elastic net regression approach and the actual change in CMD risk marker measured during the DIVAS study (change = post-intervention – pre-intervention value).

Abbreviations: CE cholesteryl esters; CER, ceramides; CMD, cardiometabolic disease; DAG, diacylglycerols; FA, fatty acid; HCER, hexosylceramides; LDI Ach, laser doppler imaging microvascular response to acetylcholine; LDI SNP, laser doppler imaging microvascular response to sodium nitroprusside; LDL-C, low density lipoprotein cholesterol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MAG, monoacylglycerols; NEFAs, non-esterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine plasmalogen; PP, pulse pressure; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TAG, triacylglycerols; TNF-α, tumour necrosis factor α.

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Chapter 3: Impact of individual dietary saturated fatty acid replacement on circulating lipids and other biomarkers of cardiometabolic health: a systematic review and meta-analysis of RCTs in humans.

Contribution towards PhD thesis: This systematic literature project was initially designed by the ILSI task force on "Individual saturated fatty acids and health", which I joined in September 2017 after the task force had performed the initial literature searches. Between 2017 and September 2020, I was involved in the initial screenings of abstracts and full texts. In September 2020, the task force committee provided me with the opportunity to lead the remaining tasks needed for the publication of the systematic literature review in a peer-reviewed journal. Therefore, I was responsible for conducting an updated literature search in January 2021 along with the subsequent screening of abstracts and full-texts with the help of James Lumley, an undergraduate student intern working under the supervision of myself and Julie Lovegrove. I was also responsible for the extraction of the data from selected studies and the development and conduct of the statistical analyses. Finally, I prepared the initial draft of the manuscript for publication, and finalised the published manuscript presented below after including the feedback and comments received from co-authors and journal reviewers.

Manuscript published in *Advances in Nutrition* (November 2021). DOI: 10.1093/advances/nmab143

Impact of individual dietary saturated fatty acid replacement on circulating lipids and other biomarkers of cardiometabolic health: a systematic review and meta-analysis of RCTs in humans.

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Short title: saturated fatty acids and cardiometabolic health

Author contributions towards manuscript: LS, MF, SL, JL, and RPM performed the literature searches. LS, MF, KGJ, PJJ, JL, SL, RPM and JAL contributed to the screening of titles, abstracts, and full-text records. LS, MF, KGJ, PJJ, JL, SL, RPM, SSS-M and JAL contributed to data extraction from full-text records. LS performed the statistical analyses, under the supervision of SSS-M and SL. LS and JL performed the risk of bias assessments. LS drafted the manuscript. MF, KGJ, PJJ, JL, SL, RPM, SS-M, and JAL contributed to the interpretation of the data, and revised each draft of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Financial support: LS was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) UK Joint Programme Initiatives (JPI) 'HDHL Biomarkers: Fatty Acid Metabolism - Interlinking Diet with Cardiometabolic Health (FAME)' (Project Reference: BB/P028217/1). SSS-M has received unrestricted grants from the Global Dairy Platform, Dairy Research Institute, and Dairy Australia for a meta-analysis on cheese and blood lipids (2012) and a meta-analysis of dairy and mortality (2015). She received the Wiebe Visser International Dairy Nutrition Prize and has received recent research funding (2019) for epidemiological studies on dairy products and cardiometabolic diseases from the Dutch Dairy Association and the Danish Dairy Research Foundation.

Conflicts of interest: This work was conducted by an expert group of the European branch of the International Life Sciences Institute (ILSI Europe). The research question addressed in this publication and potential contributing experts in the field were identified by the Qualitative Fat Intake (QFI) Task Force. Industry members of this task force are listed on the ILSI Europe's website http://ilsi.eu/wp-content/uploads/sites/3/2019/11/Qualitative-Fat-Intake_TFonepager_Sept2019.pdf. Experts are not paid for the time spent on this work; however, the non-industry members within the expert group were offered support for travel and accommodation costs from the QFI Task Forces to attend meetings to discuss the manuscript. The expert group carried out the work, i.e. collecting/analyzing data/information and writing the scientific paper separate to other activities of the task forces. The research reported is the result of a scientific evaluation in line with ILSI Europe's framework to provide a precompetitive setting for public-private partnership (PPP). ILSI Europe facilitated scientific meetings and coordinated the overall project management and administrative tasks relating to the completion of this work. For further information about ILSI Europe, please email info@ilsieurope.be or call +32 2 771 00 14. The opinions expressed herein and the conclusions of this publication are those of the authors and do not necessarily represent the views of ILSI Europe nor those of its member companies. JAL is Deputy Chair of the UK Scientific Advisory Committee for Nutrition (SACN) and was an expert on SACN's Saturated Fats working group. MF is an employee of ILSI Europe.

Acknowledgements: Authors would like to thank Dr. Odgerel Baasan, Dr. Wendy Blom, Dr. Marjolijn Bragt, Dr. Nils Billecke, Dr. Mathilde Fleith, and Dr. Agnes Méheust as members of the ILSI Europe expert group who initiated the work and for their early contributions to the literature search.

Statement of significance (1-2 sentence): This is the first systematic review and meta-analysis of randomized controlled trials that assessed the impact of individual saturated fatty acids and their isoenergetic substitution on a wide range of risk markers of cardiometabolic diseases (including lipid profile, markers of glycemic control, markers of inflammation, and metabolic hormones).

Abstract

Little is known of the impact of individual saturated fatty acids (SFAs) and their isoenergetic substitution with other SFAs or unsaturated fatty acids (UFAs) on the prevention of cardiometabolic disease (CMD). This systematic literature review (POSPERO registration: CRD42020084241) assessed the impact of such dietary substitutions on a range of fasting CMD risk markers, including lipid profile, markers of glycemic control and inflammation, and metabolic hormone concentrations. Eligible randomized controlled trials (RCTs) investigated the effect of isoenergetic replacements of individual dietary SFAs for at least 14 days on one or more CMD risk markers in humans. Searches of PubMed, Embase, Scopus and Cochrane CENTRAL databases on 14th February 2021 identified 44 RCTs conducted in participants aged 39.9y (SD 15.2). Studies' risk of bias was assessed using the Cochrane Risk of Bias tool 2.0 for RCTs. Random-effect meta-analyses assessed the effect of at least three similar dietary substitutions on the same CMD risk marker. Other dietary interventions were described in qualitative syntheses. We observed reductions in low-density lipoprotein cholesterol concentrations after the replacement of palmitic acid (C16:0) with UFA (-0.36 mmol/L, 95%CI [-0.50, -0.21], I^2=96.0%, n=18 RCTs) or oleic acid (C18:1) (-0.16 mmol/L, 95% CI [-0.28, -0.03], I^2=89.6%, n=9 RCTs), with a similar impact on total cholesterol and apolipoprotein B concentrations. No effects on other CMD risk markers, including high-density lipoprotein cholesterol, triacylglycerol, glucose, insulin, or C-reactive protein concentrations, were evident. Similarly, we found no evidence of a benefit from replacing dietary stearic acid with UFA on CMD risk markers (n=4 RCTs). In conclusion, the impact of replacing dietary palmitic acid with UFA on lipid biomarkers is aligned with current public health recommendations. However, due to the high heterogeneity and limited studies, relationships between all individual SFAs and biomarkers of cardiometabolic health need further confirmation from RCTs.

Keywords (5-10): palmitic acid, stearic acid, myristic acid, medium-chain fatty acids, saturated fatty acids, unsaturated fatty acids, lipoproteins, fasting lipid profile, glucose, insulin

3.1 Introduction

Cardiovascular diseases (CVD) are the main cause of death worldwide with an estimated 17.9 million deaths from CVD in 2019 [1]. The etiology of CVD is complex and often results from a combination of risk factors, including the presence of other metabolic disorders and cardiometabolic diseases (CMD) such as type 2 diabetes, hypertension, or hyperlipidemia [1]. In particular, the fasting lipid profile and markers of glycemic control are routinely used as clinical biomarkers of risks for CVD and type 2 diabetes (T2D), but more other risk factors such as markers of inflammation or blood hemostasis have been less extensively studied [2,3]. Changes in environmental and behavioral factors, such as dietary habits, tobacco use, and physical activity, have been identified as important strategies to help prevent CMD risk at a population level [4].

Among dietary factors, public health guidelines around the world advocate a reduction of dietary saturated fatty acids (SFAs) in favor of unsaturated fatty acids (UFAs), with a general consensus that dietary SFAs should not exceed approximately 10% total energy (%TE) intakes [5,6]. These recommendations are supported by systematic literature reviews (SLR) and meta-analyses, such as the 2020 updated analysis of 12 randomized controlled trials (RCT) from Hooper *et al.* which reported a 17% decrease in CVD event risk associated with reduced dietary SFAs and showed an inverse linear relationship between the amount of SFA removed from the diet and CVD risk [7]. However, in the context of isoenergetic dietary replacements, a reduction of SFAs can only be achieved with a concomitant increase in another type of dietary fat or another macronutrient. Current epidemiological evidence suggests that replacing dietary SFAs with polyunsaturated fatty acids (PUFAs) might lead to a greater reduction of CMD risk compared to other nutrients which have been less extensively studied (e.g. monounsaturated fatty acids (MUFAs) or other nutrients like carbohydrates) [7,8].

Importantly, dietary guidelines currently consider dietary SFAs as a whole group, but emerging evidence suggest that individual SFAs might have differential impacts on cardiometabolic health [9,10]. In a 2016 World Health Organization SLR and regression analysis on the effect of SFAs on serum lipids and lipoproteins, Mensink [11] predicted that total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations would increase when dietary carbohydrates are replaced with lauric, myristic, or palmitic acids, but not stearic acid. This potential distinction of individual fatty acids (FAs) in the context of cardiometabolic health is reflected in current French dietary guidelines which have recommended since 2011 that the sum of dietary lauric, myristic and palmitic acid should not exceed 8%TE in adults [12], but this is not the case in other countries.

Despite the growing interest in the differential roles of individual SFAs in cardiometabolic health, to our knowledge there are no published SLR or meta-analyses of well-controlled intervention studies investigating this research topic. Therefore, the objective of this SLR and meta-analysis was to address this knowledge gap. The hypothesis of this analysis was that the chronic consumption of individual dietary SFAs will have differential effects on circulating lipids and other markers of CMD risk.

3.2 Methods

This SLR and meta-analysis was conducted according to guidelines from the Cochrane Network and the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [13]. It was registered in the International Prospective Register of Systematic Reviews (PROSPERO) under the registration number CRD42020084241.

Eligibility criteria & Search strategy

This SLR included RCTs which investigated the impact of the dietary replacement of individual SFAs for other individual SFAs or UFAs on markers of cardiometabolic health. Eligible studies included in this SLR were defined as full-text, peer-reviewed, original research reports of RCTs published in English language, which investigated food-based isoenergetic dietary fat interventions implemented for at least 14 days on humans aged over 3 years old, and presented at least one fasting biomarker for circulating lipids, inflammation, glycemic control, hemostasis, or hormones. The groups considered for synthesis were defined as dietary interventions replacing one out of eight groups of dietary SFAs defined by their carbon-chain length (i.e. medium-chain SFAs <C12:0, lauric acid C12:0, myristic acid C14:0, pentadecanoic acid C15:0, palmitic acid C16:0, heptadecanoic acid C17:0, stearic acid C18:0, long-chain SFAs >C18:0) or *trans* FAs for another group of SFAs, UFAs, or *trans* FAs. Only dietary interventions exchanging at least 1.5%TE of palmitic or stearic acid, or 1%TE of other FAs were considered eligible for inclusion. Studies were excluded if any of the above criteria was not met, if more than one group of SFAs was exchanged, if the amount of dietary fat exchanged could not be expressed as %TE, if studies reported results from parenteral/enteral nutrition interventions, or if they included critically ill patients (e.g. cancer).

RCTs published before 14th February 2021 were searched in PubMed, Embase, Scopus and the Cochrane central register for clinical trials using two predefined comprehensive query syntaxes (*supplementary method 1*). The first search focused on serum lipids and lipoproteins, whereas the second search aimed to identify RCTs presenting all other predefined eligible outcomes. Finally, additional references were identified from previous systematic reviews of RCTs on dietary fat and cardiometabolic health.

Selection & data collection process

Results from literature searches were imported in reference manager software packages to remove duplicates (Zotero 5.0, Virginia, USA, and Endnote X9, Pennsylvania, USA). References were then uploaded to the Covidence systematic review software (Veritas Health Innovation, Melbourne, Australia) for further identification of duplicates and screening process. Each title/abstract imported in Covidence was randomly assigned to two independent reviewers for screening, and decision conflicts were addressed by a third reviewer where necessary. Full texts of selected titles/abstracts were retrieved and screened following the same process. Finally, full-text articles deemed eligible were allocated to two independent reviewers who each used a predefined extraction table template to collect all relevant data items. Pairs of extracted data were then compared and combined into a single final version for each eligible study. Numerical data items from figures were extracted using the WebPlotDigitizer web-based tool version 4.4 (available at https://apps.automeris.io/wpd/).

Data items

Outcome data items to be extracted were classified into five categories of biomarkers of CMD risk: circulating lipid profiles, markers of inflammation, markers of glycemic control, markers of hemostasis, or metabolic hormone concentrations (*supplementary table 1*). Results from FA profiles in plasma/serum or other blood fractions were not considered in this SLR, since they are mostly reported in dietary fat replacement RCTs to assess compliance to the intervention diets rather than considered as biomarker of cardiometabolic health. Data from each reported outcome was sought at baseline and at the end of intervention. Since this SLR assessed the effect of chronic dietary fat replacements, only measurements performed on participants in fasting state were extracted.

In addition, study characteristic details were extracted for each eligible RCT and included country, year of publication, industrial funding source (yes/no), participant information (i.e. biological sex, age, body mass index (BMI), body weight, health status, medication, physical activity level, smoking habits, occupation, ethnicity), study design (i.e. crossover or parallel), duration of intervention and any run-in or washout periods (in days), level of participant feeding control (i.e. full control of all foods consumed, control of intervention foods only, or dietary advice only without any food provided), and composition of intervention diets (type of food, macronutrient composition, energy provided, detailed FA composition). No assumption was made in case of missing data from one of the above variables, and data was reported as not specified (NS). However, studies were excluded if detailed FA compositions of the intervention(s) diet(s) were not available or could not be expressed as %TE.

Study risk of bias assessment

Risk of bias of included studies was assessed using the Cochrane Risk of Bias Tool 2.0 for parallel or crossover RCTs where appropriate, with the aim to quantify the effect of adhering to intervention as specified in the study protocol (i.e. "per-protocol") [14]. Briefly, the tool assessed the risk of bias arising from five domains: (i) randomization process, (ii) deviations from the intended interventions, (iii) missing outcome data, (iv) measurement of the outcomes, and (v) selection of the reported results. An additional domain was assessed in crossover RCTs for potential risk of bias arising from period and carry-over effects. Each domain was attributed a risk of bias score (i.e. low risk, some concerns, high-risk), which was used to calculate overall risk of bias scores for each included RCTs. The overall risk of bias score was judged as: "low-risk" if all domains were also judged at low-risk, "some concerns" if one domain was judged as concerning but no domain was judged as high-risk, and "high-risk" if at least one domain was judged at high-risk or if several domains were scored as concerning in a way that may substantially affect the confidence in the reported results. Risk of bias was first assessed by one reviewer and independently validated by a second reviewer, using full-text articles as the main source of information for the assessments along with secondary publications or RCT's registered information (e.g. ClinicalTrials.gov) where necessary.

Eligibility and preparation for synthesis

Outcomes were selected for synthesis in this SLR if presented in at least two of the eligible RCTs. Quantitative meta-analyses were performed separately for every outcome that was reported in at least three independent RCTs which conducted similar dietary fat replacements, and if the outcome of interest could be reasonably assumed to follow a normal distribution. In quantitative syntheses, outcome data items at the end of intervention were expressed as mean and standard deviation (SD) in SI units. Missing data items (missing timepoint, or item not expressed as Mean/SD) were obtained by either contacting the authors of the original full-text articles, or by converting median values, standard error, or interquartile range using the methods proposed by Hozo *et al.* [15]. Since the outcomes of interest were all continuous and measured on similar scales across studies, intervention effects were measured as a weighted mean difference (WMD) between two dietary interventions. To account for within-participant variance in crossover RCTs as opposed to between-participant variance in parallel trials, we calculated effect measures and their standard deviations in crossover RCTs using correlation coefficients from one of the crossover trials included in the meta-analyses [16] (*Supplementary table 2*). Forest plots were generated for each suitable outcome to display results from meta-analyses, grouped by type of dietary fat replacement. Findings ineligible for quantitative meta-analyses were described in qualitative syntheses and tabulated to report details on dietary fat replacements, number of participants, and outcome measurements in each dietary intervention arm.

Statistical analyses

Statistical synthesis

All statistical analyses were conducted on STATA version 16.1 (StataCorp, Texas, USA), and p-values below 0.05 were considered statistically significant. Studies in sufficient number were pooled using an inverse variance random-effect meta-analysis model to account for potential heterogeneity. The restricted maximum likelihood (REML) method was used to estimate heterogeneity variance. This method is recommended for meta-analyses of continuous outcomes containing a small number of studies (approximately $n \leq 10$) as an improvement of the traditional DerSimonian-Laird approach [17,18]. In addition, we used the Knapp-Hartung-Sidik-Jonkman (HKSJ) correction to estimate the 95% Confidence Intervals (CIs) of the summary effects [19,20]. This method provides more conservative 95%CIs compared to the commonly used Wald-type method when pooling a small number of studies [18,21,22]. Statistical heterogeneity was quantified using the τ^2 and I^2 value along with Cochran's Q statistic for heterogeneity.

Sensitivity analyses were performed by repeating the meta-analyses without studies for which the confidence in some of the reported results was particularly low, e.g. when reviewers suspected typing errors in values or units reported. This issue was particularly prevalent in articles published between 1980-2000 and for which original authors could not be contacted.

Methods to explore heterogeneity and publication bias

In meta-analyses including $n \geq 10$ studies, substantial heterogeneity was investigated using meta-regression analyses for the impact of the amount of dietary fat exchanged (per 5%TE) on the observed summary effect, expecting that a larger amount of exchanged dietary fat would lead to greater observed effects. Meta-regression analyses were based on the REML-HKSJ approach and were presented as bubble plots if statistically significant. To comply with Cochrane recommendations on meta-analyses including a small number of studies, no further methods were planned or conducted to explore substantial heterogeneity [23]. In meta-analyses including $n \geq 10$ studies we investigated potential publication bias using funnel plot's visual inspection and Egger's statistical test [24]. Statistically significant Egger's tests were addressed using the trim and fill method based on a linear estimator to correct any funnel plot asymmetry [25].

3.3 Results

Study selection and characteristics

The selection process and the included RCTs are summarized in *Figure 3.1*. We identified 14050 records, of which 7155 were screened after removal of duplicates. After the exclusion of 6472 records at the first stage of screening based on title and abstracts, 683 records were assessed in detail using the full-text articles. A total of 639 records were further excluded for not meeting the predefined inclusion criteria, mainly because they did not report sufficient information about the dietary intervention (n=286), reported ineligible dietary intervention (n=225), outcomes (n=7) or study designs (n=55), or because no English full-text article was available (n=66). Overall, 44 full-text articles met the inclusion criteria and were included in this SLR [16,26–66]. Among those, 35 articles reporting 34 RCTs were included in quantitative meta-analyses, whereas 9 articles reporting 10 RCTs were included solely in the qualitative synthesis.

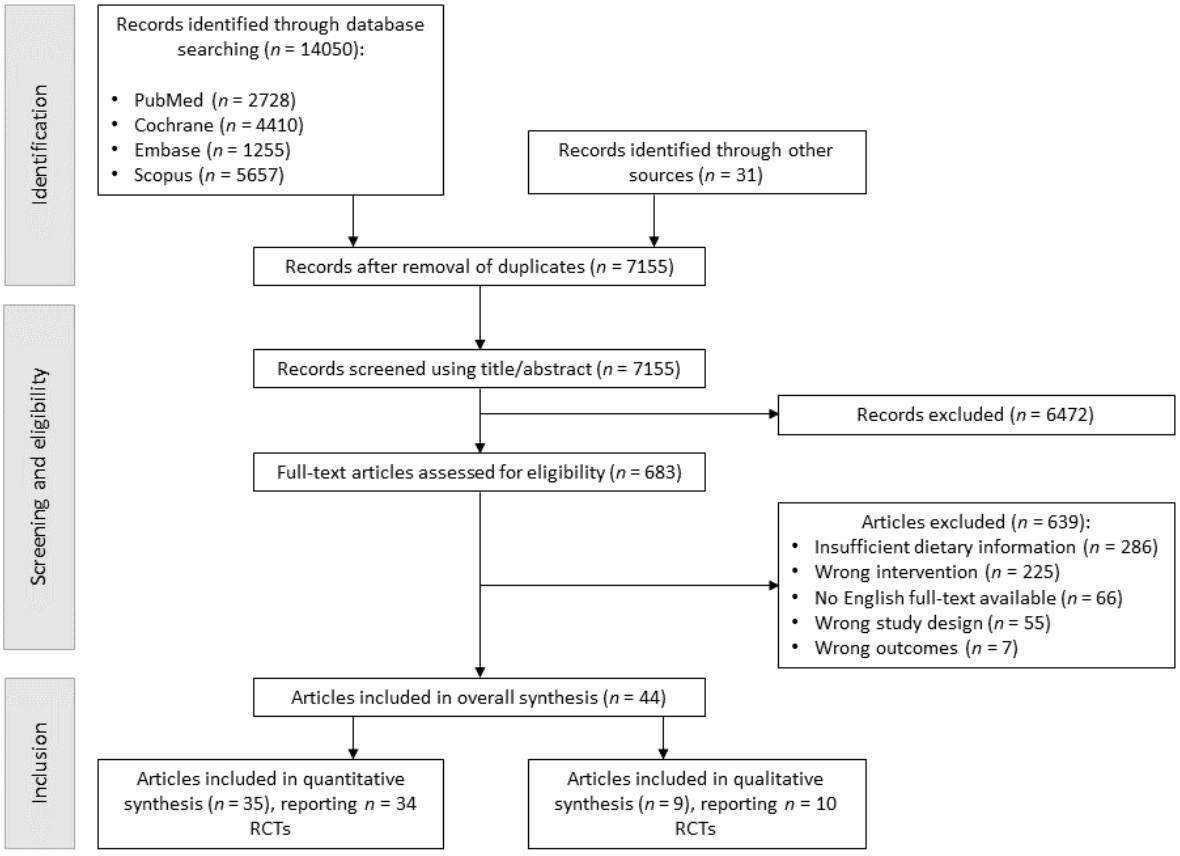


Figure 3.1. PRISMA flow diagram of included randomized controlled trials (RCTs).

Characteristics of the included RCTs along with details of the dietary interventions are presented in *Table 1*. Among the 44 included RCTs, 36 were conducted in a crossover design and 8 in a parallel design. The most common interventions investigated were the dietary substitution of palmitic acid with a mixture of UFA (n=20) [26,27,29–31,34,35,37,40,41,44–46,51,54–58,67], followed by the replacement of palmitic

with oleic acid (n=10) [32,33,36,47,49–53,62], the substitution of palmitic with stearic acid (n=5) [16,28,43,48,56], and the substitution of stearic acid with a mixture of UFA (n=4) [26,38,39,42,56]. The number of participants included in the studies ranged from six to 101 [42,59,60], and intervention duration varied between 14 and 112 days [42,26,67,64]. Most of the RCTs included enrolled both men and women (n=21), apart from 12 RCTs conducted in men only [32,41,42,46,51,52,54,56,57,68,64,65] and five RCTs conducted in women only [35,43,44,48,58]. Finally, most RCTs included healthy participants but 10 trials included participants with moderately to highly elevated fasting serum or plasma lipids, although some authors did not specify the cut-off used for fasting lipids (*Table 1*) [29,35,37,40,41,45,46,57,59,60].

Risk of Bias assessment

Results from the risk of bias assessment of included RCTs are presented in *Table 2*. Thirteen of the 44 RCTs were judged as "low risk of bias" and 18 presented "some concerns". Furthermore, 13 crossover RCTs were judged as "high-risk of bias", mostly due to insufficient washout periods between dietary interventions in comparison to the duration of intervention. Indeed, when assessing the risk of carry-over effects between interventions, washout periods of at least 14 days were deemed acceptable regardless of the duration of interventions, and shorter washout periods were judged acceptable only when combined with interventions of at least 28 days to ensure at least 14 days of dietary intervention with minimal risks of carry-over effects.

Table 3.1. Characteristics of 44 eligible randomized controlled trials (RCTs) on dietary fat exchange and biomarkers of cardiometabolic diseases (CMD).

RCTs included in meta-analyses (n=34)

First Author and year (Country)	No. of participants who completed the study (% men/women)	Mean age (y), mean BMI (kg/m^2)	Participant characteristics	Study design, Type of dietary intervention, duration of run-in, intervention, and washout (days) [a]	No. of dietary intervention arms	Reported dietary fat replacements and amount exchanged (%TE)[1,2]	Outcomes measured	Industrial funding (yes/no)
Van Rooijen 2020 [16] (The Netherlands)	34 (59/41)	61.5y 25.4kg/m^2	Healthy	Crossover, semi-controlled, 28d intervention, ≥ 28d washout	2	C16:0 (6.1) ⟷ C18:0 (6.5)	apoA-I, apoB, apoB/apoA-I ratio, C-peptide, CRP, glucose, HDL-C, HOMA-IR, IL-6, insulin, LDL-C, TC/HDL-C ratio, TNF-α, TAG, TC	Yes
Stonehouse 2020 [26] (Australia)	64 (31/69)	32.6y 22.8kg/m^2	Healthy	Parallel, semi-controlled, 14d run-in, 112d intervention	3	C16:0 (7.3) ⟷ MUFA + PUFA (7.2) C18:0 (10.6) ⟷ MUFA + PUFA (8.3)	apoA-I, apoB, apoB/apoA-I ratio, glucose, HDL-C, LDL-C, leptin, TC/HDL-C ratio, TAG, TC	No
Sun 2019 [27] (China)	100 (47/53)The	40y 22.2kg/m^2	Healthy	Crossover, fully controlled, 35d intervention, 14d washout	2	C16:0 (3.2) ⟷ MUFA + PUFA (3.3)	apoA-I, apoB, glucose, HDL-C, HOMA-IR, insulin, LDL-C, TAG, TC	No
Lv 2018 [67] (China)	88 (47/53)	21.6y 21.0kg/m^2	Healthy	Parallel, semi-controlled, 7d run-in, 112d intervention	3	C16:0 (1.9) ⟷ MUFA + PUFA (1.2)	apoA-I, apoB, apoE, CRP, glucose, HDL-C, HOMA-IR, insulin, LDL-C, leptin, Lp(a), NEFA, TAG, TC	No
Ng 2018 [28] (Malaysia)	85 (25/75)	34.4y 26.1kg/m^2	Healthy	Parallel, fully controlled, 21d run-in, 56d intervention	3	C16:0 (6.0) ⟷ C18:0 (7.0) C16:0 (5.4) ⟷ C18:0 (6.8)	apoA-I, apoB, C-peptide, glucose, HDL-C, HOMA-IR, insulin, LDL-C, leptin, Lp(a), TC/HDL-C ratio, TAG, TC	No
Karupaiah 2016 [29] (Malaysia)	34 (47/53)	23.4y 25.1kg/m^2	Healthy, with normal (n=21) or mildly elevated (n=13) TC levels	Crossover, fully controlled, 28d intervention, 14d washout	2	C16:0 (1.8) ⟷ MUFA + PUFA (1.5)	apoA-I, apoB, CRP, glucose, HDL-C, LDL-C/HDL-c ratio, NEFA, TAG, TC, VLDL-C	Yes
Kien 2014 [30] (USA)	18 (50/50)	29.5y 23.3kg/m^2	Healthy	Crossover, fully controlled, 21d intervention, 7d washout	2	C16:0 (13.7) ⟷ MUFA + PUFA (13.9)	Adiponectin, apoE, HDL-C, LDL-C, LDL-C/HDL-C ratio, TAG, TC	No
Rosqvist 2014 [31] (Sweden)	37 (70.3/29.7)	26.7/27.1y 20.8/19.9 kg/m^2	Healthy	Parallel, semi-controlled, 49d intervention	2	C16:0 (5.2) ⟷ MUFA + PUFA (7.5)	Adiponectin, glucose, insulin	No

Tholstrup 2011 [32] (Denmark)	32 (100/0)	29.6y 22.9kg/m^2	Healthy, n=6 smokers	Crossover, semi-controlled, 21d intervention, no washout	3	C16:0 (4.6) ↔ C18:1 (4.8)	CRP, glucose, HDL-C, insulin, LDL-C, PAI-1 activity, TC/HDL-C ratio, TAG, TC	No
Voon 2011 [33] (Malaysia)	45 (20/80)	30.1y 23.1kg/m^2	Healthy	Crossover, fully controlled, 35d intervention, no washout	3	C16:0 (4.9) ↔ C18:1 (6.8)	apoA-I, apoB, LDL-C, Lp(a), TC/HDL-C ratio, TAG, TC	No
Teng 2010 [34] (Malaysia)	41 (19.5/80.5)	28.8y 21.9kg/m^2	Healthy	Crossover, semi-controlled, 35d intervention, 7d washout	3	C16:0 (5.2) ↔ MUFA + PUFA (9.4) C16:0 (5.8) ↔ C18:1-*trans* (9.9)	apoA-I, apoB, CRP, HDL-C, IL-6, LDL-C, TC/HDL-C ratio, TNF-α, TAG, TC	No
Utarwuthipong 2009 [35] (Thailand)	16 (0/100)	44-67y <25kg/m^2	Hyperlipidemic	Crossover, semi-controlled, 70d intervention, no washout	4	C16:0 (1.8) ↔ MUFA + PUFA (17.3) C16:0 (5.5) ↔ MUFA + PUFA (21.5) C16:0 (2.7) ↔ MUFA + PUFA (18.4) C16:0 (3.8) ↔ C18:2n-6 (4.6) C16:0 (2.8) ↔ C18:2n-6 (3.4)	HDL-C, LDL-C, TAG, TC	No
Mensink 2008 [36] (The Netherlands)	44 (25/75)	41y 23.9kg/m^2	Healthy, normolipidaemic	Crossover, semi-controlled, 21d intervention, 7d washout	2	C16:0 (4.2) ↔ C18:1 (2.9)	CRP, glucose, HDL-C, LDL-C, TC/HDL-C ratio, TC	Yes
Vega-Lopez 2006 [37] (USA)	15 (33/66)	63.9y 26kg/m^2	Elevated LDL-C (> 3.36 mmol/L)	Crossover, fully controlled, 35d intervention, no washout	4	C16:0 (7.5) ↔ MUFA + PUFA (6.2) C16:0 (8.3) ↔ MUFA + PUFA (9.7)	apoA-I, apoA-II, apoB, glucose, HDL_2-C, HDL_3-C, HDL-C, HOMA-IR, insulin, LDL-C, Lp(a), TC/HDL-C ratio, TAG, TC, VLDL-C	No
Thijssen 2005 [38,39] (The Netherlands)	45 (60/40)	51y 24.9kg/m^2	Healthy, normolipidaemic	Crossover, semi-controlled, 35d intervention, 7d washout	3	C18:0 (19.7) ↔ MUFA + PUFA (20.2) C18:0 (19.6) ↔ MUFA + PUFA (20.0)	apoA-I, apoB, fibrinogen, HDL-C, LDL-C, PAI-1 activity, TC/HDL-C ratio, TAG, TC	Yes
Gill 2003 [40] (UK)	35 (49/51)	55y 26.3kg/m^2	Mildly hypercholesterolaemic	Crossover, semi-controlled, 42d intervention, 56d washout	3	C16:0 (3.0) ↔ MUFA + PUFA (3.6) C16:0 (6.1) ↔ MUFA (6.6) C16:0 (3.1) ↔ MUFA (3.0)	apoA-I, apoB, CRP, HDL-C, insulin, LDL-C, Lp(a), NEFA, TAG, TC, VLDL-C	No
Cater 2001 [41] (USA)	7 (100/0)	66y 27kg/m^2	Mildly hypercholesterolaemic, n=3 subjects with history of CHD	Crossover, fully controlled, 21d intervention, 7d washout	3	C16:0 (19.3) ↔ MUFA + PUFA (21.2) >C18:0 (17.0) ↔ MUFA + PUFA (19.0)	HDL-C, LDL-C, TAG, TC, VLDL-C	No
Hunter 2000 [42] (UK)	6 (100/0)	28y 24.7kg/m^2	Healthy	Crossover, fully controlled, 14d intervention, 35d washout	3	C18:0 (10.7) ↔ C18:1 (11.1) C18:0 (10.7) ↔ MUFA + PUFA (10.3)	Fibrinogen, HDL-C, LDL-C, PAI-1 activity, TAG, TC, tPA activity	No

Snook 1999 [43] (USA)	16 (0/100)	28y NS	Healthy	Crossover, fully controlled, 35d intervention, 49d washout	3	C16:0 (10.0) ↔ C18:0 (10.8)	apoA-I, apoB, HDL_2-C, HDL_3-C, HDL-C, LDL-C, TAG, TC	No
Muller 1998 [44] (Norway)	27 (0/100)	27y 26.5kg/m^2	Healthy	Crossover, fully controlled, 17d intervention, 7d washout	3	C16:0 (5.5) ↔ MUFA + PUFA (4.5) Total *trans* (6.8) ↔ MUFA + PUFA (5.6)	apoA-I, apoB, HDL-C, LDL-C, LDL-C/HDL-C ratio, Lp(a), TAG, TC	Yes
Schwab 1998 [45] (Finland)	14 (42.9/57.1)	63y 27.2kg/m^2	Elevated LDL-C (> 3.36 mmol/L)	Crossover, fully controlled, 32d intervention, no washout	5	C16:0 (2.0) ↔ MUFA + PUFA (1.6)	HDL-C, LDL-C, TC, TC/HDL-C, VLDL-C	No
Cater 1997 [46] (USA)	9 (100/0)	66y 27kg/m^2	Mildly hypercholesterolaemic, n=3 subjects with history of CHD	Crossover, fully controlled, 21d intervention, 7d washout	3	C16:0 (19.3) ↔ MUFA + PUFA (21.3)	HDL-C, LDL-C, TAG, TC, VLDL-C	No
Sundram 1997 [47] (Malaysia)	27 (66/33)	29.4y 22.7kg/m^2	Healthy	Crossover, semi-controlled, 28d intervention, no washout	4	C16:0 (4.1) ↔ C18:1 (2.7)	apoA-I, apoB, apoB/apoA-I ratio, HDL-C, LDL-C, LDL-C/HDL-C ratio, Lp(a), TAG, TC, VLDL-C	No
Schwab 1996 [48] (USA)	12 (0/100)	23.5y 22.1kg/m^2	Healthy, n=6 using oral contraceptives	Crossover, semi-controlled, 28d intervention, 14d washout	2	C16:0 (3.3) ↔ C18:0 (4.9)	apoA-I, apoB, HDL-C, HDL-TAG, LDL-C, LDL-TAG, TAG, TC, VLDL-C, VLDL-TAG	No
Temme 1996 [49] (Netherlands)	32 (43.7/56.3)	41y 25kg/m^2	Healthy, n=9 smokers, n=2 using oral contraceptives	Crossover, semi-controlled, 42d intervention, 14 to 21d washout	3	C16:0 (7.5) ↔ 18:1 (8.4)	apoA-I, apoA-I/apoB ratio, apoB, HDL-C, HDL-C/LDL-C ratio, LDL-C, Lp(a), TAG, TC	Yes
Choudhury 1995 [50] (Australia)	21 (48/52)	27.8y 24.1kg/m^2	Healthy	Crossover, semi-controlled, 30d intervention, no washout	2	C16:0 (5.0) ↔ C18:1 (7.3)	HDL-C, LDL-C, TAG, TC	No
Sundram 1995 [51] (Malaysia)	23 (100/0)	21y, 20.1kg/m^2	Healthy	Crossover, fully controlled, 21d intervention, no washout	3	C16:0 (4.3) ↔ C18:1 (5.1) C16:0 (3.0) ↔ MUFA + PUFA (6.8) C16:0 (7.3) ↔ MUFA + PUFA (6.8)	apoA-I, apoB, apoB/apoA-I ratio, HDL_2-C, HDL_3-C, HDL-C, LDL-C, LDL-C/HDL-C ratio, Lp(a), TAG, TC, VLDL-C	NS
Nestel 1994 [52] (Australia)	34 (100/0)	49y 25.7kg/m^2	Healthy	Crossover, semi-controlled, 21d intervention, no washout	3	C16:0 (3.3) ↔ C16:1 (3.8) C16:0 (3.4) ↔ C18:1 (2.7)	HDL-C, LDL-C, TAG, TC	Yes
Zock 1994 [53]	59 (39/61)	Men: 28y	Healthy, n=8 smokers		3	C14:0 (10.2) ↔ C16:0 (10.2)		No

(Netherlands)		22.3kg/m^2 Women: 29y 22.4kg/m^2		Crossover, fully controlled, 21d intervention, no washout		C14:0 (10.5) ↔ C18:1 (10.0) C16:0 (9.9) ↔ C18:1 (9.3)	apoA-I, apoB, HDL-C, HDL-C/LDL-C ratio, LDL-C, TAG, TC	
Denke 1992 [54] (USA)	14 (100/0)	63y 25.5kg/m^2	n=5 subjects with history of CHD, n=7 smokers	Crossover, fully controlled, 21d intervention, 7d washout	3	C12:0 (17.5) ↔ MUFA + PUFA (16.3) C16:0 (15.5) ↔ MUFA + PUFA (16.6)	HDL-C, TAG, TC, VLDL-C	No
Ng 1991 [55] (Malaysia)	27 (74/26)	23.9y 19.5kg/m^2	Healthy, normolipidaemic	Crossover, fully controlled, 35d intervention, no washout	2	C16:0 (4.9) ↔ MUFA + PUFA (4.8)	HDL-C, LDL-C, LDL-C/HDL-C ratio, TAG, TC	No
Bonanome 1988 [56] (USA)	11 (100/0)	64y 24kg/m^2	n=4 subjects with a history of CHD using anti-hypertensives medication and nitroglycerine	Crossover, fully controlled, 21d intervention, no washout	3	C16:0 (14.8) ↔ C18:0 (15.3) C16:0 (15.8) ↔ MUFA + PUFA (17.4) C18:0 (16.3) ↔ MUFA + PUFA (17.7)	HDL-C, LDL-C, LDL-C/HDL-C ratio, TAG, TC, VLDL-C	NS
Mattson 1985 [57] (USA)	20 (100/0)	58.7y BMI: NS	Healthy, with normal or elevated TC and TAG levels	Crossover, fully controlled, 28d intervention, no washout	3	C16:0 (15.3) ↔ MUFA + PUFA (15.2) C16:0 (14.8) ↔ MUFA + PUFA (15.2)	HDL-C, LDL-C, TAG, TC, VLDL-C	No
Baudet 1984 [58] (France)	24 (0/100)	46y NS	Healthy	Crossover, fully controlled, 35d intervention, no washout	4	C16:0 (3.1) ↔ MUFA + PUFA (2.8) C16:0 (2.1) ↔ MUFA + PUFA (0.7)	TAG, TC	Yes
RCTs included in qualitative synthesis only (n=10)								
Reference (Country)	**No. of participants who completed the study (percentage of men/women)**	**Mean age (y) and mean BMI (kg/m^2)**	**Participant characteristics**	**Study design, Type of dietary intervention, duration of intervention and washout (days)**	**No. of dietary intervention arms**	**Eligible dietary fat replacements and amount exchanged (%TE)**	**Outcomes measured**	**Industrial funding (yes/no)**
Liu 2009 [59] (China)	101 (66/34)	53.7y 25.9kg/m^2	Hypertriglyceridaemic	Parallel, semi-controlled, 56d intervention	2	Men: <C12:0 (1.8) ↔ C18:2n-6 (1.7) Women: <C12:0 (1.9) ↔ C18:2n-6 (1.6)	apoA-I, apoB, apoE, glucose, HDL-C, LDL-C, TAG, TC	Yes

Xue 2009 [60] (China)	101 (66/34)	53.7y 25.9kg/m^2	Hypertriglyceridaemic	Parallel, semi-controlled, 56d intervention	2	<C12:0 (1.8) ⟷ MUFA + PUFA (1.3)	Adiponectin, apoA-I, apoA-II, apoB, glucose, HDL-C, LDL-C, TAG, TC	NS
Nosaka 2003 [61] (Japan)	64 (75/25)	37.6y 25kg/m^2	Healthy	Parallel, semi-controlled, 84d intervention	2	<C12:0 (3.0) ⟷ C18:1 (1.6)	glucose, HDL-C, HDL-TAG, LDL-C, LDL-TAG, TAG, TC, VLDL-C, VLDL-TAG	NS
Judd 2002 [68] (USA)	50 (100/0)	42y 26.2kg/m^2	Healthy, smokers and non-smokers	Crossover, fully controlled, 35d intervention, no washout	6	C18:0 (8.0) ⟷ C18:1 (71) C18:0 (4.1) ⟷ Total *trans* (4.1) C18:0 (8.1) ⟷ Total *trans* (8.0) C18:0 (4.0) ⟷ Total *trans* (3.9)	apoA-I, apoB, HDL_2-C, HDL_3-C, HDL-C, TC/HDL-C ratio, TAG, TC	Yes
Temme 1999 [62] (The Netherlands)	32 (43.8/56.2)	Men: 43y 25kg/m^2 Women: 40y 25kg/m^2	Healthy, normolipidaemic, n=9 smokers	Crossover, semi-controlled, 42d intervention, 14 to 21d washout	3	C16:0 (6.1) ⟷ C18:1 (6.8)	fibrinogen, PAI-1 activity	Yes
Temme 1997 [63] (Netherlands)	60 (38/62)	Men: 43y 25kg/m^2 Women: 40y 24kg/m^2	Healthy, n=11 smokers, n=10 using oral contraceptives	Parallel, semi-controlled, 42d intervention	3	<C12:0 (9.9) ⟷ C18:1 (10.1) <C12:0 (9.9) ⟷ C14:0 (9.7) C14:0 (9.6) ⟷ C18:1 (10.4)	apoA-I, apoA-I/apoB ratio, apoB, HDL-C, Lp(a), TC/HDL-C ratio, TAG, TC	Yes
Ghafoorunisa 1995 [64] (India)	12 (100/0)	35y 21kg/m^2	Healthy	Crossover, fully controlled, 56d intervention, 42d washout	2	C16:0 (5.0) ⟷ C18:2n-6 (3.7)	HDL-C, LDL-C, TAG, TC, VLDL-C	No
	24 (50/50)	Men: 43y 23kg/m^2 Women: 38y 24kg/m^2	Healthy	Crossover, semi-controlled, 112d intervention, no washout	2	C16:0 (6.0) ⟷ C18:2n-6 (3.7)	HDL-C, LDL-C, TAG, TC, VLDL-C	No
Tholstrup 1994 [65] (Denmark)	12 (100/0)	23.8y 23.5kg/m^2	Healthy	Crossover, fully controlled, 21d intervention, 35d washout	3	C14:0 (13.4) ⟷ C16:0 (12.8)	apoA-I, apoB, fibrinogen, HDL_2-C, HDL_3-C, HDL-C, LDL-C, LDL-C/HDL-C ratio, TAG, TC, tPA activity, VLDL-C	No
Zock 1992 [66] (Netherlands)	62 (50/50)	24.5y BMI: NS	Healthy, normolipidaemic, n=8 smokers	Crossover, fully controlled, 21d intervention, no washout	3	C18:0 (9.0) ⟷ C18:2n-6 (8.1) Total *trans* (7.6) ⟷ MUFA + PUFA (7.5)	apoA-I, apoA-I/apoB ratio, apoB, HDL-C, HDL-C/LDL-C ratio, LDL-C, TAG, TC	NS

[1] **fully controlled intervention:** all foods consumed were provided to participants, for either home or on-site consumption (e.g. campus, metabolic ward, etc.). **Semi-controlled intervention:** experimental foods were provided to participants along with dietary advice for non-experimental foods.

[2] **fatty acids considered:** C12:0 lauric acid, C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C16:1 palmitoleic acid, C18:1 oleic acid, C18:2n-6 linoleic acid, C18:2 n-3 α-linolenic acid, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

Abbreviations: %TE: % total energy, Apo: apolipoprotein, BMI: body mass index, CHD: coronary heart disease, CMD: cardiometabolic diseases, CRP: C-reactive protein, d: days, HDL: high-density lipoprotein, HOMA-IR: Homeostatic Model Assessment of Insulin Resistance, IL: interleukin, LDL: low-density lipoprotein, Lp(a): lipoprotein (a), NEFA: non-esterified fatty acids, NS: not specified, PAI-1: Plasminogen activator inhibitor-1, RCT: randomized controlled trial, TAG: triacylglycerol, TC: total cholesterol, TNF-α: tumor necrosis factor α, tPA: tissue plasminogen activator, VLDL: very low-density lipoprotein.

Table 3.2. Risk of bias assessment of 44 eligible randomized controlled trials (RCTs) on dietary fat exchange and biomarkers of cardiometabolic diseases (CMD), from the Cochrane RoB 2.0 tool for parallel or crossover RCTs. [14]

RCTs included in meta-analyses (n=34)								
First Author	**Year**	**Journal**	**Randomization Process**	**Deviations from the intended intervention**	**Missing outcome data**	**Measurement of the outcome**	**Selection of the reported result**	**Overall risk of bias**
Van Rooijen [16]	**2020**	Clin Nutr	Low	Low	Low	Low	Low	**Low**
Stonehouse [26]	**2020**	Am J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Sun [27]	**2019**	Asia Pac J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Lv [67]	**2018**	Food Nutr Res	Some concerns	Low	Low	Low	Low	**Some concerns**
Ng [28]	**2018**	Nutrients	Low	Low	Low	Low	Low	**Low**
Karupaiah [29]	**2016**	Lipids Health Dis	Low	Low	Low	Low	Low	**Low**
Kien [30]	**2014**	Am J Clin Nutr	Low	High	Low	Low	Low	**High**
Rosqvist [31]	**2014**	Diabetes	Low	Low	Low	Low	Low	**Low**
Tholstrup [32]	**2011**	Am J Clin Nutr	Low	High	Some concerns	Low	Low	**High**
Voon [33]	**2011**	Am J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Teng [34]	**2010**	Lipids	Low	High	Low	Low	Low	**High**
Utarwuthipong [35]	**2009**	Int J Med Sci	Some concerns	Low	Low	Low	Low	**Some concerns**
Mensink [36]	**2008**	Eur J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Vega-Lopez [37]	**2006**	Am J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Thijssen [38,39]	**2005**	Nutr Metab / Am J Clin Nutr	Some concerns	High	Low	Low	Low	**High**
Gill [40]	**2003**	Am J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Cater [41]	**2001**	Am J Clin Nutr	Low	High	Low	Low	Low	**High**
Hunter [42]	**2000**	J Nutr Biochem	Some concerns	Low	Low	Low	Low	**Some concerns**
Snook [43]	**1999**	Eur J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Müller [44]	**1998**	Lipids	Low	High	Low	Low	Low	**High**
Schwab [45]	**1998**	Nutr Metab	Some concerns	Some concerns	Low	Low	Low	**Some concerns**
Cater [46]	**1997**	Am J Clin Nutr	Low	High	Low	Low	Low	**High**
Sundram [47]	**1997**	J Nutr	Low	Low	Low	Low	Low	**Low**

Schwab [48]	**1996**	Metab Clin Exp	Low	Low	Low	Low	Low	**Low**
Temme [49]	**1996**	Am J Clin Nutr	Some concerns	Some concerns	Low	Low	Low	**Some concerns**
Choudhury [50]	**1995**	Am J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Sundram [51]	**1995**	J Nutr Biochem	Some concerns	High	Low	Low	Low	**High**
Nestel [52]	**1994**	J Lipid Res	Low	High	Low	Low	Low	**High**
Zock [53]	**1994**	J Atheroscler Thromb	Some concerns	High	Low	Low	Low	**High**
Denke [54]	**1992**	Am J Clin Nutr	Some concerns	High	Low	Low	Low	**High**
Ng [55]	**1991**	Am J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Bonanome [56]	**1988**	N Engl J Med	Some concerns	High	Low	Low	Low	**High**
Mattson [57]	**1985**	J Lipid Res	Some concerns	Low	Low	Low	Some concerns	**Some concerns**
Baudet [58]	**1984**	J Lipid Res	Some concerns	Some concerns	Low	Low	Some concerns	**Some concerns**

RCTs included in qualitative synthesis only (n=10)

First author	**Year**	**Journal**	**Randomization Process**	**Deviations from the intended intervention**	**Missing outcome data**	**Measurement of the outcome**	**Selection of the reported result**	**Overall risk of bias**
Liu [59]	**2009**	Asia Pac J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Xue [60]	**2009**	Eur J Clin Nutr	Low	Low	Low	Low	Low	**Low**
Nosaka [61]	**2003**	J Atheroscler Thromb	Some concerns	Low	Low	Low	Some concerns	**Some concerns**
Judd [68]	**2002**	Lipids	Low	Low	Low	Low	Low	**Low**
Temme [62]	**1999**	Thromb Haemost	Some concerns	Some concerns	Low	Low	Low	**Some concerns**
Temme [63]	**1997**	J Lipid Res	Some concerns	Low	Low	Low	Low	**Some concerns**
Ghafoorunisa [64]	**1995**	Lipids	Some concerns	Low	Low	Low	Low	**Some concerns**
			Some concerns	Low	Low	Low	Low	**Some concerns**
Tholstrup [65]	**1994**	Am J Clin Nutr	Some concerns	Low	Low	Low	Low	**Some concerns**
Zock [66]	**1992**	J Lipid Res	Some concerns	High	Low	Low	Low	**High**

Effect of dietary fat replacement on fasting lipid profiles

Total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C)

As illustrated in *Figure 3.2 and Figure 3.3*, quantitative syntheses revealed no statistically significant effects on TC or LDL-C of the dietary replacements of palmitic with stearic acid (WMD -0.34 mmol/L, 95%CI -0.72 to 0.04, I^2=87.2%, n=5 RCTs, and WMD -0.28 mmol/L, 95%CI -0.71 to 0.15, I^2=94.0%, n=5 RCTs, respectively) or stearic acid with a mixture of UFA (WMD 0.03 mmol/L, 95%CI -0.20 to 0.27, I^2=75.0%, n=4 RCTs, and WMD -0.01 mmol/L, 95%CI -0.34 to 0.31, n=4 RCTs, respectively). However, statistically significant decreases in TC and LDL-C concentrations, albeit with evidence of high heterogeneity, were observed when exchanging dietary palmitic acid with either oleic acid (TC: WMD -0.21 mmol/L, 95%CI -0.33 to -0.10, I^2=87.2%, n=9 RCTs, and LDL-C: WMD -0.16 mmol/L, 95%CI -0.28 to -0.03, I^2=89.6%, n=9 RCTs) or a mixture of UFA (TC: WMD -0.41 mmol/L, 95%CI -0.55 to -0.26, I^2=93.1%, n=19 RCTs, and LDL-C: WMD -0.36 mmol/L, 95%CI -0.50 to -0.21, I^2=96%, n=18 RCTs). The latter effect on TC and LDL-C was dependent on the amount of dietary palmitic acid replaced with UFA according to meta-regression analyses, with each additional 5%TE of palmitic acid exchanged associated with a 0.12 and 0.17 mmol/L decrease in fasting TC (p-value=0.03, *Figure 3.4A*) and LDL-C (p-value=0.001, *Figure 3.4B*) concentrations, respectively. Sensitivity analyses on the effect of replacing dietary palmitic with oleic acid, which excluded two RCTs with potential reporting errors in the full text articles, showed similar effects on TC and LDL-C concentrations *supplementary figures 3.1 and 3.2*).

The impact of other dietary fat substitutions on TC and LDL-C concentrations was investigated in 17 [34,35,40–42,44,53,54,59–61,63–66,68] and 18 RCTs [34,35,40–42,44,53,54,59–61,63–66,68], respectively (*Table 3*). In particular, findings from four RCTs suggest that the dietary replacement of medium-chain SFAs with UFA may not have any impact on TC concentrations [59–61,63]. However, decreased TC and LDL-C concentrations were reported after replacing dietary myristic acid with either palmitic [53,65] or oleic acid [53,63]. Furthermore, three RCTs reported decreased or unchanged TC concentrations in response to a replacement of dietary stearic with oleic or linoleic acid [42,66,68]. Finally, two crossover RCTs observed beneficial effects of exchanging 6.8 to 7.6%TE dietary trans-FA with a mixture of UFA on TC and LDL-C concentrations [44,66].

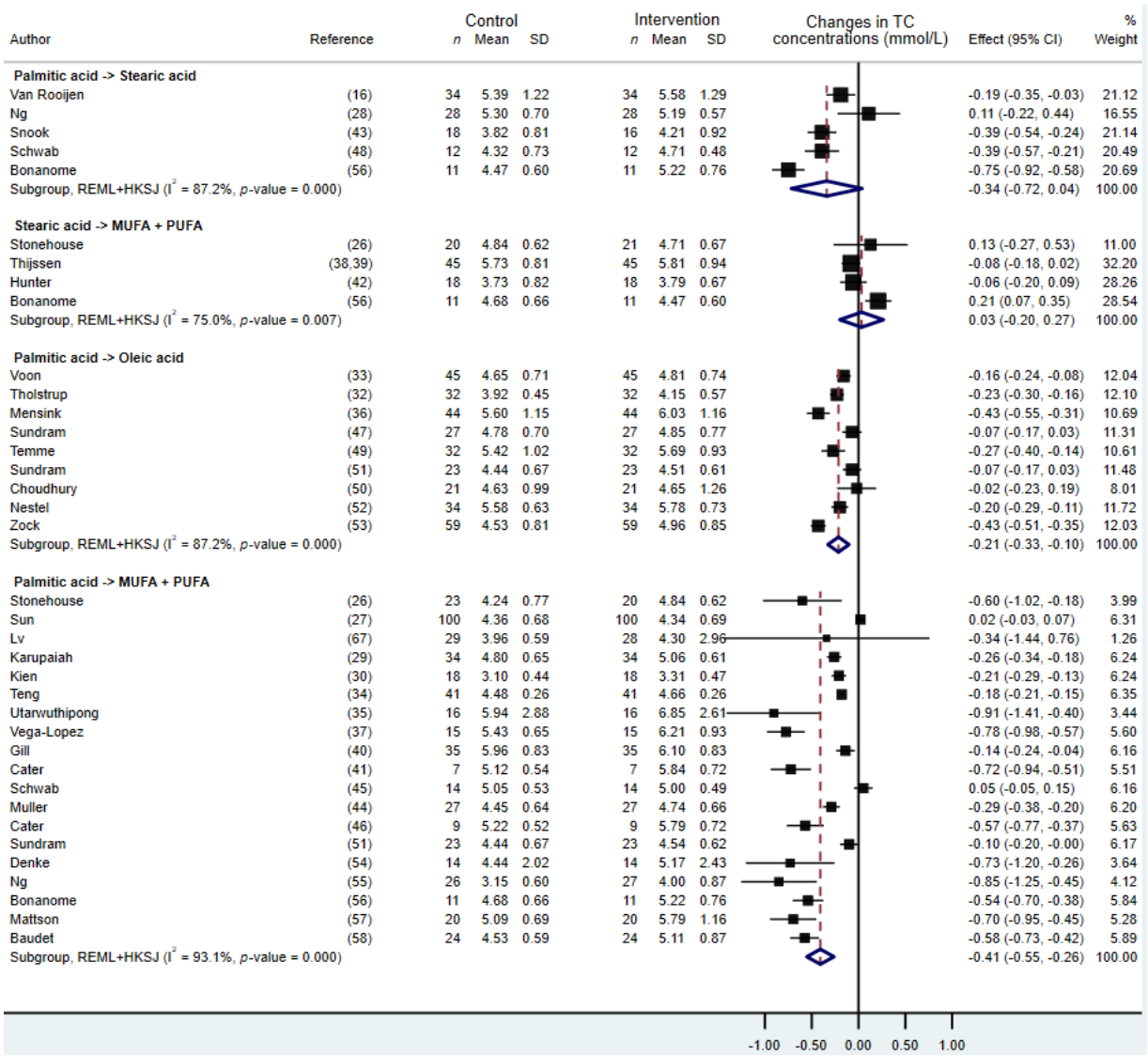


Figure 3.2. Forest plot of the effect of dietary fat substitutions on total cholesterol concentrations in randomized controlled trials. *HKSJ: Hartung-Knapp-Sidik-Jonkman, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood, TC: total cholesterol.*

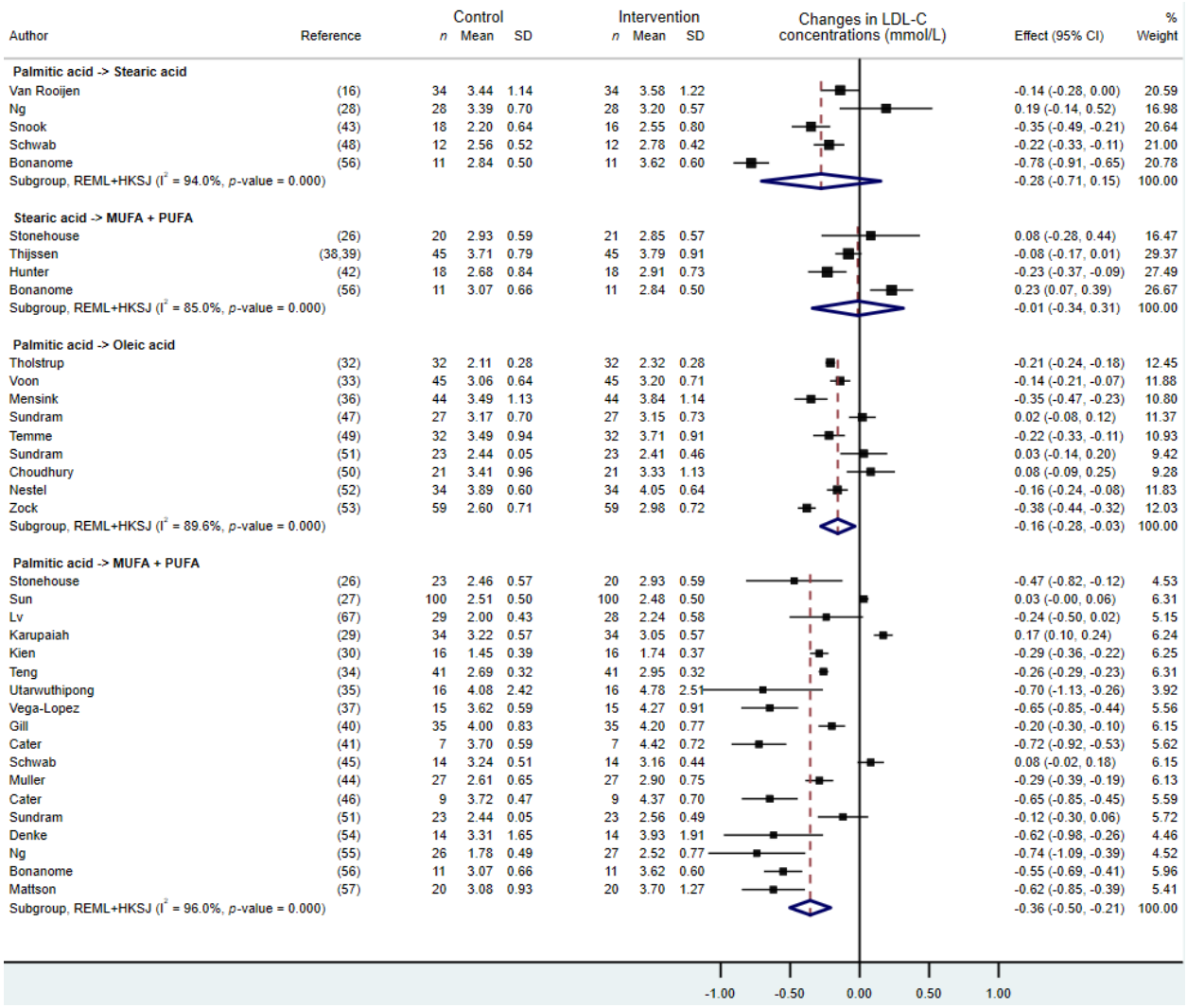


Figure 3.3. Forest plot of the effect of dietary fat substitutions on low-density lipoprotein cholesterol concentrations in randomized controlled trials. *HKSJ: Hartung-Knapp-Sidik-Jonkman, LDL-C, low-density lipoprotein cholesterol, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood.*

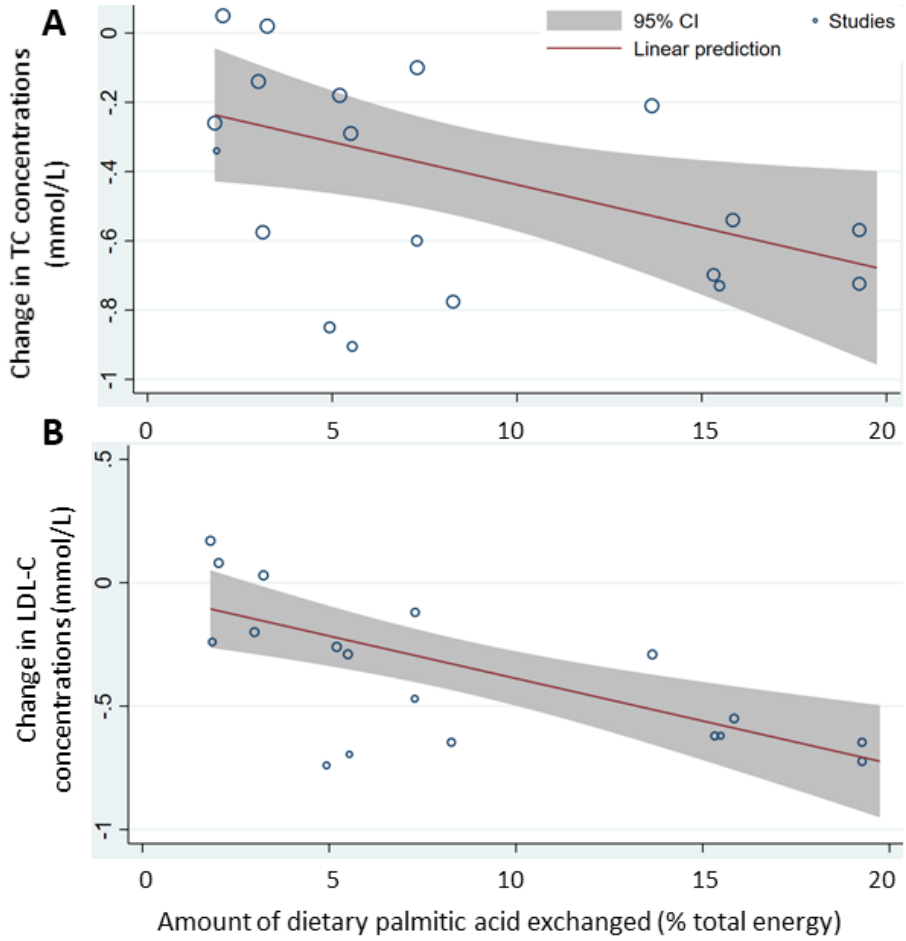


Figure 3.4. Dose-response meta-regression analysis of the change in (A) total cholesterol (TC) or (B) low-density lipoprotein cholesterol (LDL-C) concentrations according to the amount of dietary palmitic acid exchanged with unsaturated fat (MUFA + PUFA).

High-density lipoprotein cholesterol (HDL-C)

Pooled analyses presented in *Figure 3.5* showed no statistically significant effect on HDL-C concentrations of the dietary replacements of palmitic with stearic acid (WMD -0.06 mmol/L, 95%CI -0.14 to 0.03, I^2=75.0%, n=5 RCTs), stearic acid with a mixture of UFA (WMD 0.05 mmol/L, 95%CI -0.02 to 0.12, I^2=32.6%, n=4 RCTs) or palmitic acid with a mixture of UFA (WMD -0.02 mmol/L, 95%CI -0.05 to 0.01, I^2=83.9%, n=18 RCTs). However, there was a statistically significant albeit small decrease in HDL-C concentration, with large heterogeneity, when replacing dietary palmitic with oleic acid based on the pooled analysis of 9 RCTs (WMD -0.05 mmol/L, 95%CI -0.10 to -0.005, I^2=78.6%). Results remained similar when excluding two RCTs with potential reporting errors in the full text articles (*supplementary figure 3.3*) [45,56]. Meta-regression analyses revealed no effect of the amount of dietary palmitic acid exchanged for UFA on the concentrations of HDL-C (p-value=0.48).

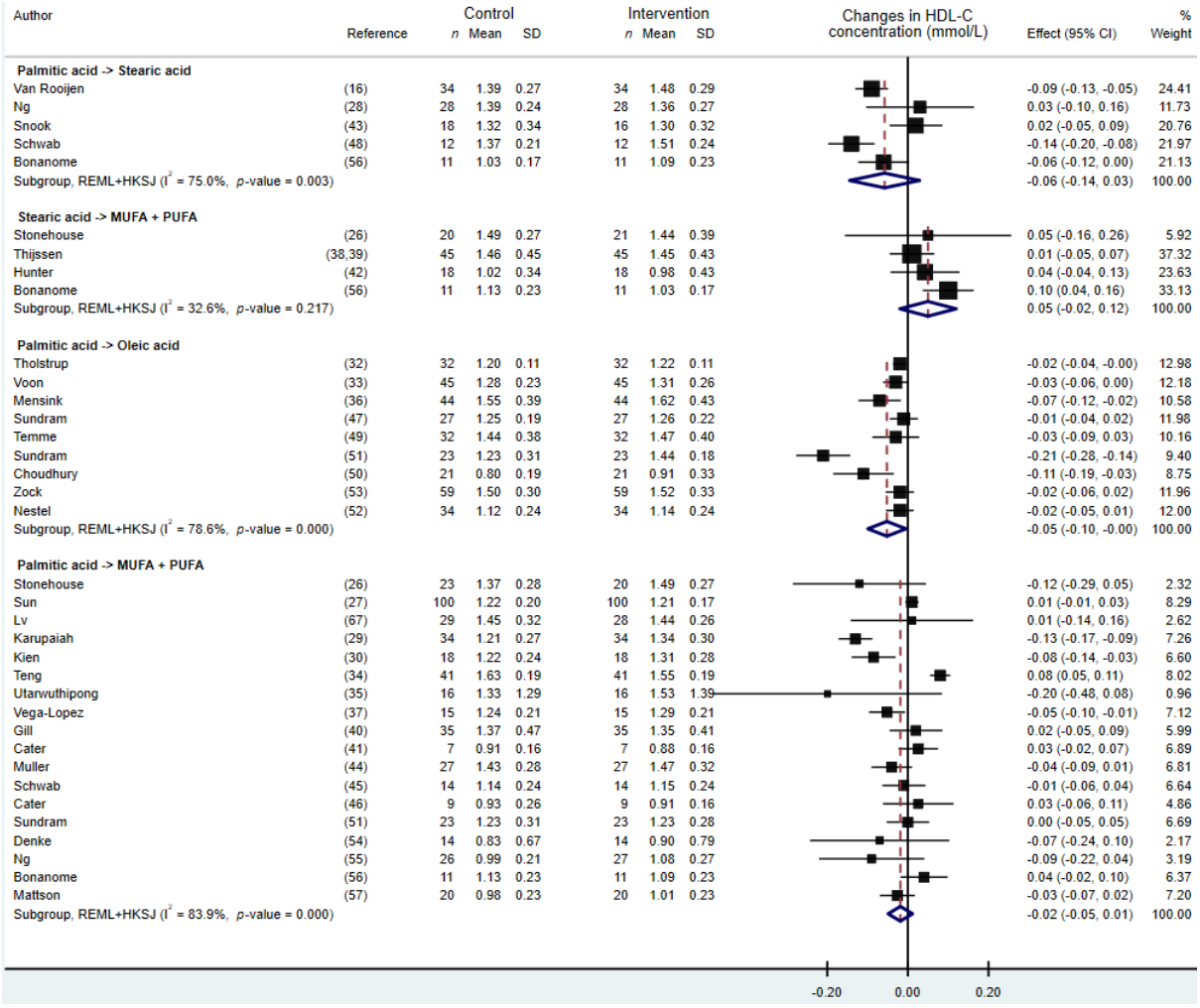


Figure 3.5. Forest plot of the effect of dietary fat substitutions on high-density lipoprotein cholesterol concentrations in randomized controlled trials. *HDL-C, high-density lipoprotein cholesterol, HKSJ: Hartung-Knapp-Sidik-Jonkman, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood.*

The impact of other dietary fatty acid substitutions was further assessed in 14 RCTs conducted between 1992 and 2010 [34,35,42,44,51,59–61,63–66,68], which are described in *Table 3*. Overall, increased HDL-C concentrations were observed when replacing dietary medium-chain SFAs with myristic acid [63], stearic acid with oleic or linoleic acid [66,68], and trans-FA with a mixture of UFA [44,66]. In contrast, the replacement of dietary palmitic acid with UFA [35,51] or *trans* elaidic acid [51], and myristic with palmitic acid [65], decreased concentrations of HDL-C. Finally, the included RCTs did not reveal any significant impact of replacing medium-chain SFAs with UFA on HDL-C concentrations [59–61,63].

Total cholesterol to HDL-C ratio (TC:HDL-C)

Replacing dietary palmitic acid with oleic acid or a mixture of UFA led to a trend for a decrease in the total cholesterol to HDL-C (TC:HDL-C) ratio, but the quantitative syntheses based on three and four RCTs respectively did not reach statistical significance (*supplementary figure 4*).

Other dietary fat replacements were studied in seven RCTs conducted between 1997 and 2020 (*Table 3*) [16,26,28,34,38,63,68]. No effects on the TC:HDL-C ratio were observed when replacing dietary medium-chain SFAs with myristic or oleic acid [63], or when replacing dietary stearic acid with a mixture of UFA [26,38]. Furthermore, the dietary replacement of palmitic with stearic acid was found to either increase or not affect the TC:HDL-C ratio in two recent RCTs [16,28].

LDL-C to HDL-C ratio (LDL-C:HDL-C)

The replacement of dietary palmitic acid with a mixture of UFA and its impact on the LDL-C:HDL-C ratio was investigated in six RCTs and showed a trend for a beneficial effect (i.e. decrease), although the quantitative synthesis did not reach statistical significance (WMD -0.25, 95%CI -0.60 to 0.09, I^2=17.6%, n=6 RCTs, *supplementary figure 3.5*). There was no correlation coefficient value available to correct the effect measures of the LDL-C:HDL-C ratio in crossover trials (*supplementary table 1*).

Other dietary fat replacements and their impact on LDL-C:HDL-C or HDL-C:LDL-C ratios were investigated in eight RCTs which are described in *Table 3* [44,47,49,51,53,56,65,66]. Overall, findings from two RCTs suggest a beneficial effect (i.e. decreased LDL-C:HDL-C ratio) of dietary myristic acid compared to palmitic acid [53,65], and of UFA compared to trans-FAs [44,66]. However, the included RCTs showed no emerging trend of the impact of replacing dietary palmitic with oleic acid [47,49,51,53] or stearic acid with UFA [56,66] on LDL-C:HDL-C ratio.

Very-low density lipoprotein cholesterol (VLDL-C)

We observed no effect of the dietary exchange of palmitic acid for a mixture of UFA on VLDL-C concentrations, and no evidence of inter-study heterogeneity (WMD 0.02 mmol/L, 95%CI -0.09 to 0.13, I^2=0%, n=10 RCTs, supplementary *figure 3.6*). There was no correlation coefficient value available to correct the effect measures of VLDL-C concentrations in crossover trials (*supplementary table 1*).

The impact of other dietary fat replacements on VLDL-C fasting concentrations was investigated in 11 additional RCTs [40,41,47,48,51,54,56,61,64,65] conducted between 1988 and 2003. As described in *Table 3*, the included RCTs showed no changes in VLDL-C concentrations after replacing dietary palmitic with stearic [48,56] or linoleic acid [64]. In particular, one crossover RCT conducted in 27 participants over 28 days observed reductions in VLDL-C concentrations when replacing dietary palmitic (4.1%TE) with oleic acid (2.7%TE) [47]. In contrast, a 2003 parallel RCT conducted in 64 participants over 84 days found

that dietary oleic acid might increase VLDL-C concentrations compared to dietary medium-chain SFAs [61].

Triacylglycerol (TAG)

There was no statistically significant effect on TAG concentrations of the dietary substitution of palmitic with stearic acid, stearic acid with a mixture of UFA, palmitic with oleic acid, or palmitic acid with a mixture of UFA (*Figure 3.6*). In the latter, we found no effect of the amount of dietary palmitic acid exchanged for UFA (p-value=0.36). These results remained unchanged when excluding two RCTs with potential reporting errors in the full text articles (*supplementary figure 3.7*) [32,51].

Other dietary fat substitutions and their impact on TAG concentrations were described in 17 RCTs conducted between 1992 and 2010 (*Table 3*) [34,35,40–42,44,53,54,59–61,63–66,68]. Four RCTs focused on medium-chain SFAs and suggested their potential beneficial effect (i.e. decreased TAG concentrations) compared to diets enriched in linoleic acid or a mixture of UFA [59,60], but not oleic acid [61,63]. Furthermore, dietary myristic acid does not seem to impact TAG concentrations compared to palmitic or oleic acids according to two trials [53,63]. However, one crossover RCT which included 12 participants over 21 days observed beneficial effects of dietary myristic acid compared to palmitic acid on TAG concentrations [65]. Finally, there was no evidence of an impact of replacing dietary trans-FA with a mixture of UFA on TAG concentrations, according to two trials [44,66].

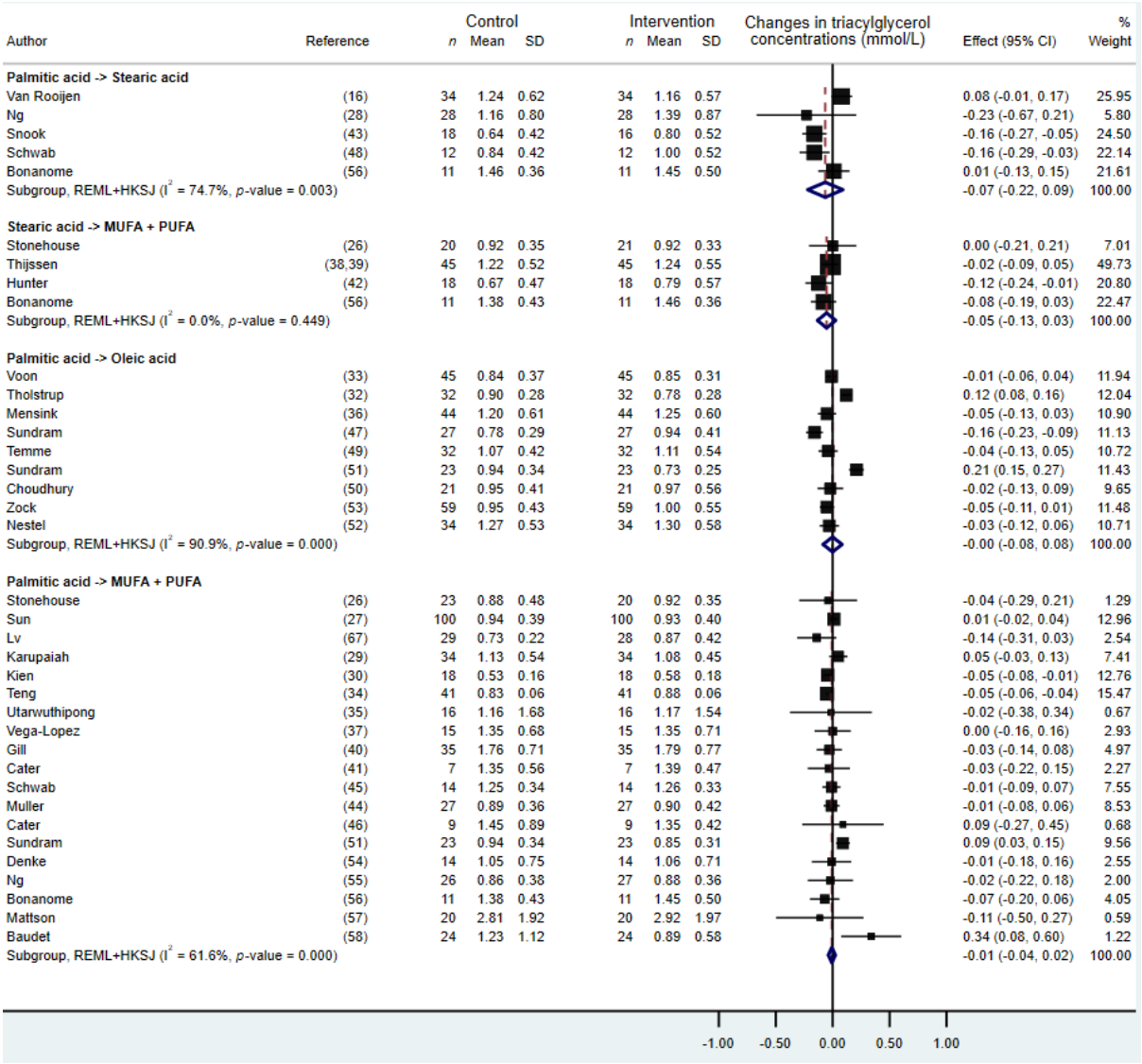


Figure 3.6. Forest plot of the effect of dietary fat substitutions on triacylglycerol concentrations in randomized controlled trials. *HKSJ: Hartung-Knapp-Sidik-Jonkman, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood.*

Apolipoprotein A-I (apoA-I)

In quantitative syntheses presented in *Figure 3.7*, we observed no statistically significant effect on apoA-I concentrations after the dietary replacement of palmitic with stearic acid, oleic acid, or a mixture of UFA. These results remained unchanged after the exclusion of two RCTs containing potential reporting errors in the full text articles (*supplementary figure 3.8*) [28,29]. The impact of other dietary fat replacements on apoA-I concentrations were investigated in 12 RCTs conducted between 1992 and 2020 (*Table 3*) [26,34,38,40,44,53,59,60,63,65,66,68]. Among those, three RCTs reported significant increases in apoA-I when replacing dietary medium-chain SFAs with myristic acid [63], stearic with oleic

acid [68], or trans-FA with UFA [66] and one RCT reported reductions in apoA-I concentrations when replacing dietary myristic with palmitic or oleic acid [53].

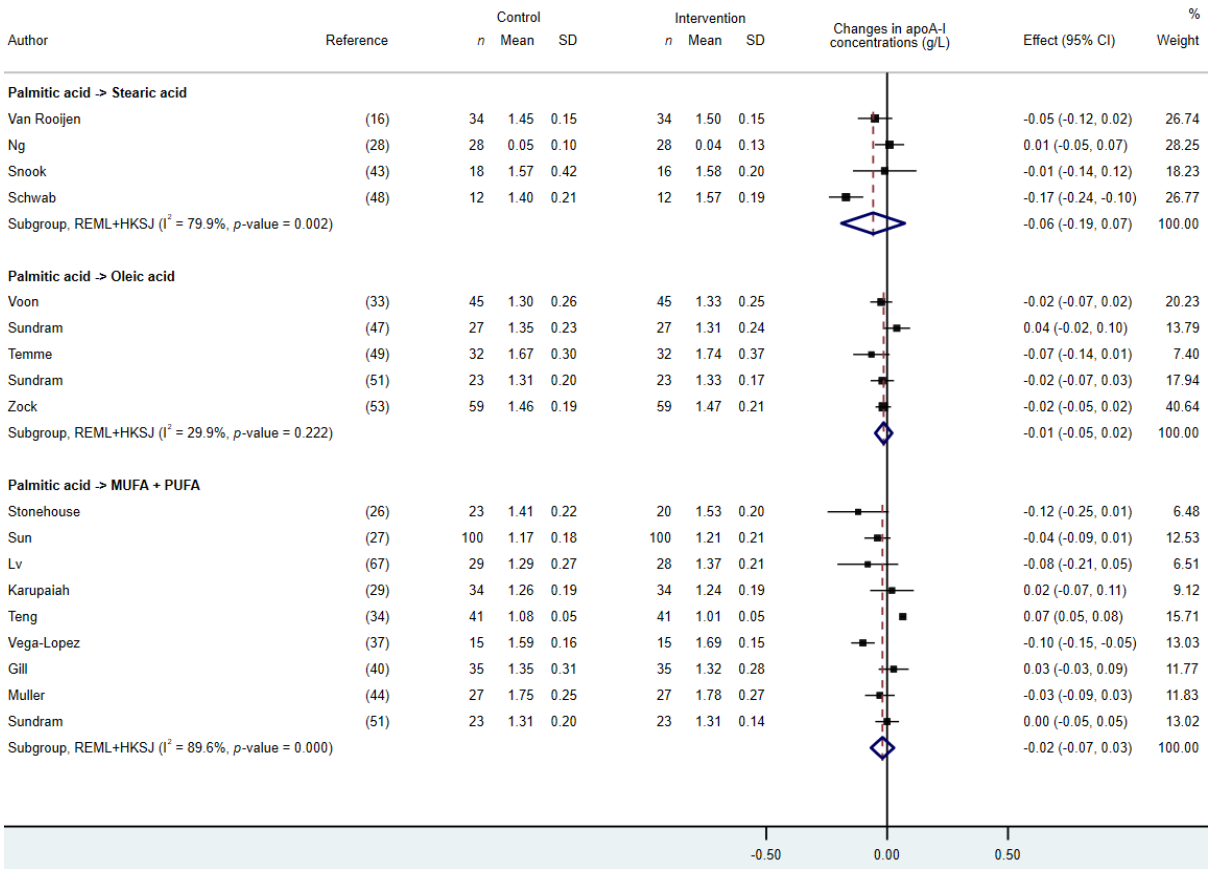


Figure 3.7. Forest plot of the effect of dietary fat substitutions on apolipoprotein A-I concentrations in randomized controlled trials. *ApoA-I, apolipoprotein A-I, HKSJ: Hartung-Knapp-Sidik-Jonkman, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood.*

Apolipoprotein B (apoB)

In pooled meta-analyses, we observed no statistically significant effect on apoB concentrations of the dietary replacement of palmitic with stearic acid (*Figure 3.8*). However, small reductions in apoB concentration were observed in response to the dietary replacement of palmitic acid with oleic acid (WMD -0.05 g/L, 95%CI -0.07 to -0.04, I^2=0.0%, n=5 RCTs) or a mixture of UFA (WMD -0.06 g/L, 95%CI -0.10 to -0.01, I^2=98.8%, n=9 RCTs), with the latter showing evidence of high statistical heterogeneity.

Other dietary fat replacements and their impact on apoB concentrations were investigated in 12 RCTs and are described in *Table 3* [26,34,38,40,44,53,59,60,63,65,66,68]. The effects of replacing medium-chain SFAs with UFA was investigated in three RCTs which reported no effect of oleic acid [63], a beneficial effect (decreased apoB concentrations) of linoleic acid [59], and a deleterious effect of a mixture of UFA [60].

Furthermore, there was no evidence for a significant effect of myristic acid compared to palmitic acid [53,65], however one crossover RCT which included 59 participants over 21 days reported decreased apoB concentrations when myristic acid was replaced with oleic acid [53]. Finally, two RCTs observed reductions in apoB concentrations when replacing dietary trans-FA with a mixture of UFA [44,66].

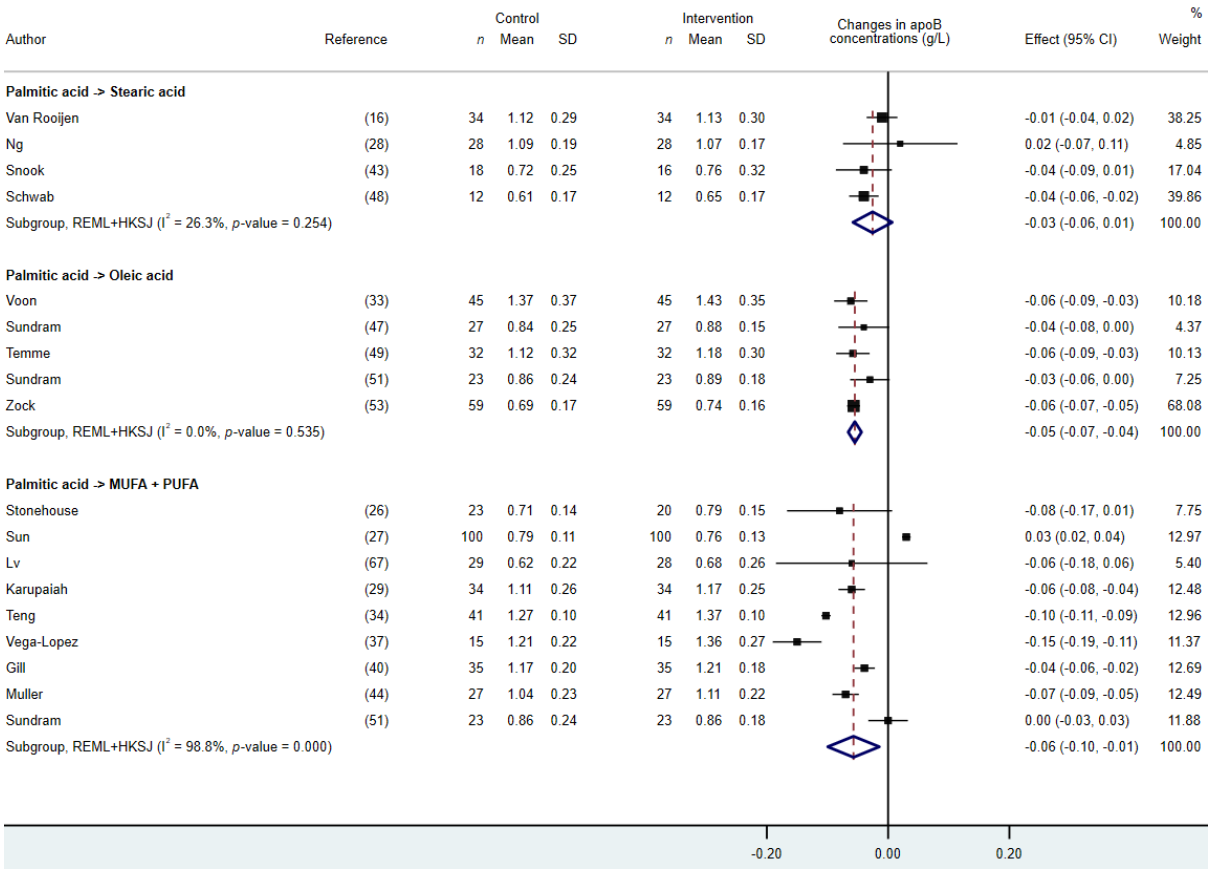


Figure 3.8. Forest plot of the effect of dietary fat substitutions on apolipoprotein B concentrations in randomized controlled trials. *ApoB, apolipoprotein B, HKSJ: Hartung-Knapp-Sidik-Jonkman, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, REML: restricted maximum likelihood.*

Other lipid related outcomes

Three RCTs reported replacing dietary palmitic acid with a mixture of UFA did not affect the concentrations of non-esterified fatty acids (NEFA) (*supplementary figure 3.9*). Findings on other lipid outcomes, including apoA-I:apoB ratio, apoA-II, apoE, HDL_2-C, HDL_3-C, TAG content in lipoproteins, and lipoprotein (a) (Lp(a)), are described in *Table 3*. The following qualitative synthesis section will highlight the main findings on these outcomes reported in eligible RCTs.

Additional lipoprotein concentrations, such as apoA-I:apoB ratio, apoA-II, or apoE, were measured in 11 RCTs [16,26,37,47,49,51,59,60,63,66,67]. In particular, three RCTs found beneficial effects (i.e. increase) on the

apoA-I:apoB ratio following the dietary replacement of medium-chain SFAs with myristic or oleic acid [63], stearic with linoleic acid [66], or trans-FA with a mixture of UFA [66]. In contrast, one recent crossover RCT which included 35 participants over 21 days observed deleterious effects of dietary stearic acid compared to palmitic acid on the apoA-I:apoB ratio [16]. Moreover, one RCT observed significant increases in apoA-II concentrations with substitution of dietary medium-chain SFAs with a mixture of UFA [60]. One trial further reported increased apoE concentrations, in men only, when replacing dietary medium-chain SFAs with linoleic acid [59]. Based on findings from two RCTs, apoA-II and apoE concentrations were not affected by the dietary replacement of palmitic acid with a mixture of UFA [37,67].

Five RCTs measured the concentrations of HDL_2-C and HDL_3-C in plasma or serum in response to dietary replacements of myristic with palmitic acid [65], palmitic acid with stearic acid [43], oleic acid [51], or a mixture of UFA [37,51], and stearic acid with oleic acid or *trans* FA [68]. While no effects were observed on HDL_2-C, one study reported decreased HDL_3-C concentrations in response to the replacement of dietary palmitic acid with a mixture of UFA [37], and another reported a greater HDL_3-C concentration after replacing dietary stearic with oleic acid [68]. Furthermore, two RCTs measured the TAG content of HDL, LDL and VLDL fractions, and reported no changes after replacing dietary medium-chain SFAs with oleic acid [61] or palmitic with stearic acid [48].

Finally, 10 RCTs assessed the impact of various dietary fat exchanges on concentrations of Lp(a) [28,33,37,40,44,47,49,51,63,67], including the replacement of palmitic acid with a mixture of UFA (n=5 RCTs) [37,40,44,51,67] or oleic acid (n=4 RCTs) [33,47,49,51]. None of the included RCTs reported significant changes in Lp(a) concentrations after the dietary interventions.

Effect of dietary fat replacement on markers of glycemic control

Glucose

Six RCTs investigated the impact of replacing dietary palmitic acid with a mixture of UFA on fasting glucose concentrations, without showing any statistically significant overall effect (WMD -0.04 mmol/L, 95%CI -0.10 to 0.01, I^2=35.5%, n=6 RCTs, *supplementary figure 3.10A*). In addition, eight RCTs measured glucose concentrations in response to other dietary fat exchanges [16,26,28,32,36,59–61], but none reported statistically significant changes (*Table 3*).

Insulin

Similar results were observed in the pooled analysis of five RCTs which showed no overall effect of replacing dietary palmitic acid with a mixture of UFA on fasting insulin concentrations (WMD -2.60 pmol/L, 95%CI -9.66 to 4.47, I^2=68.9%, n=5 RCTs, *supplementary figure 3.10B*). Four RCTs further

assessed the effects of replacing dietary palmitic acid with stearic acid [16,28], oleic acid [32], or MUFA [40] on insulin concentrations. Results from these trials, as described in *Table 3*, suggest a tendency for increased fasting insulin concentrations when replacing dietary palmitic acid with stearic acid or MUFA [16,40].

Other markers of glycemic control and insulin resistance

As detailed in *Table 3*, two RCTs investigated the impact of replacing dietary palmitic with stearic acid on Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and C-peptide concentrations [16,28], one of which observed decreased C-peptide concentrations and increased HOMA-IR after a diet rich in stearic acid compared to a palmitic acid-rich diet [16]. Furthermore, three trials investigated the effects of replacing dietary palmitic acid with a mixture of UFA, without showing any significant impact on HOMA-IR [27,37,67].

Effect of dietary fat replacement on markers of inflammation

Three RCTs investigated the effects of replacing dietary palmitic acid with a mixture of UFA on C-reactive protein (CRP) or high-sensitivity CRP concentrations and showed no overall effect (WMD -0.02 mg/L, 95%CI -0.04 to 0.01, I^2=0.0%, n=3 RCTs, *supplementary figure 3.11*). In addition, CRP concentrations in response to the dietary replacement of palmitic acid with stearic acid, oleic acid, *trans* elaidic acid or MUFA was assessed in five trials (*Table 3*) [16,32,34,36,40]. Only one crossover study, which included 41 participants over 35 days, observed a detrimental impact on CRP concentrations (i.e. increase) after a diet rich in trans-FAs compared to a palmitic acid-rich diet [34]. Other markers of inflammation, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) were investigated in two RCTs [16,34], one of which showed detrimental changes in these two markers (i.e. increased concentrations) after replacing dietary palmitic with stearic acid [16].

Effect of dietary fat replacement on metabolic hormones concentrations and markers of hemostasis

Six RCTs measured concentrations of adiponectin [30,31,60] or leptin [26,28,67] in response to dietary fat replacements (*Table 3*). The authors did not report any effects on these two hormones of replacing dietary palmitic acid with stearic acid or a mixture of UFA, medium-chain SFAs with a mixture of UFA, or stearic acid with a mixture of UFA.

Similarly, five RCTs investigated the response of markers of hemostasis, such as fibrinogen concentration, tissue-type plasminogen activator (tPA) activity, and plasminogen activator inhibitor type 1 (PAI-1) activity to dietary fat exchanges (*Table 3*) [32,39,42,62]. The dietary interventions assessed in these trials included the replacement of myristic with palmitic acid, of palmitic or stearic acids with

oleic acid, and of stearic acid with a mixture of UFA. Only one of these RCTs observed a beneficial effect (i.e. decrease) of replacing palmitic with oleic acid on PAI-1 activity [62].

Reporting biases

As per our predefined protocol, the assessment of reporting bias was conducted for quantitative syntheses that included at least 10 RCTs, which applied to those assessing the impact of replacing dietary palmitic acid with a mixture of UFA on concentrations of TC, LDL-C, HDL-C, and TAG. The results from Egger's tests did not indicate any signs of publication bias or small study effects in the quantitative synthesis of HDL-C (p-value=0.12). However, potential publication bias was found in the syntheses of TC (p-value=0.006), LDL-C (p-value=0.04), and TAG (p-value=0.04). For these outcomes, contour-enhanced funnel plots and corrections using the trim-and-fill method yielded similar summary effects to the ones observed without corrections (WMD for TC -0.30, 95%CI -0.44 to -0.15, *supplementary figure 3.12A*, WMD for LDL-C -0.34 mmol/L, 95%CI -0.47 to -0.20, *supplementary figure 3.12B*, and WMD for TAG 0.00 mmol/L, 95%CI -0.03 to 0.03, *supplementary figure 3.12C*).

Table 3.3. Qualitative synthesis of dietary fatty acid exchanges and measured outcomes from eligible randomized controlled trials (RCTs) which were not eligible for quantitative meta-analyses.

First Author and year	Type and amount of dietary FA exchanged (%TE) [1]		Outcomes measured and not pooled	Changes in outcomes in the intervention diet compared to control [2]
	Control Diet	Intervention Diet		
Van Rooijen 2020 [16]	C16:0 (6.1)	C18:0 (6.5)	apoB/apoA-I ratio, C-peptide, CRP, glucose, HOMA-IR, IL-6, insulin, TC/HDL-C ratio, TNF-α	↑ apoB/apoA-I ratio, HOMA-IR (women only), IL-6, insulin (women only), TC/HDL-C ratio, TNF-α ↓ C-peptide (men only)
Stonehouse 2020 [26]	C16:0 (7.3)	MUFA + PUFA (7.2)	apoB/apoA-I ratio, leptin	NS
	C18:0 (10.6)	MUFA + PUFA (8.3)	apoA-I, apoB, apoB/apoA-I ratio, glucose, leptin, TC/HDL-C ratio	NS
Sun 2019 [27]	C16:0 (3.2)	MUFA + PUFA (3.3)	HOMA-IR	NS
Lv 2018 [67]	C16:0 (1.9)	MUFA + PUFA (1.2)	apoE, HOMA-IR, Leptin, Lp(a)	NS
Ng 2018 [28]	C16:0 (6.0)	C18:0 (7.0)	C-peptide, glucose, leptin, Lp(a), TC/HDL-C ratio, HOMA-IR, insulin	NS
	C16:0 (5.4)	C18:0 (6.8)	C-peptide, glucose, leptin, Lp(a), TC/HDL-C ratio, HOMA-IR, insulin	NS
Karupaiah 2016 [29]	C16:0 (1.8)	MUFA + PUFA (1.5)	N/A	NS
Kien 2014 [30]	C16:0 (13.7)	MUFA + PUFA (13.9)	adiponectin	NS
Rosqvist 2014 [31]	C16:0 (5.2)	MUFA + PUFA (7.5)	adiponectin	NS
Tholstrup 2011 [32]	C16:0 (4.6)	C18:1 (4.8)	CRP, glucose, insulin, PAI-1 activity	NS
Voon 2011 [33]	C16:0 (4.9)	C18:1 (6.8)	Lp(a)	NS
Teng 2010 [34]	C16:0 (5.2)	MUFA + PUFA (9.4)	IL-6, TNF-α	NS
	C16:0 (5.8)	C18:1-*trans* (9.9)	apoA-I, apoB, CRP, IL-6, TC/HDL-C ratio, TNF-alpha, TC, TAG, LDL-C, HDL-C	↑ CRP, TC/HDL-C ratio ↓ HDL-C
Utarwuthipong 2009 [35]	C16:0 (1.8)	MUFA + PUFA (17.3)	HDL-C, LDL-C, TAG, TC	↓ HDL-C
	C16:0 (5.5)	MUFA + PUFA (21.5)	N/A	NS
	C16:0 (2.7)	MUFA + PUFA (18.4)	HDL-C, LDL-C, TAG, TC	↓ HDL-C
	C16:0 (3.8)	C18:2n-6 (4.6)	HDL-C, LDL-C, TAG, TC	↓ LDL-C, TC

	C16:0 (2.8)	C18:2n-6 (3.4)	HDL-C, LDL-C, TAG, TC	↓ LDL-C, TC
Mensink 2008 [36]	C16:0 (4.2)	C18:1 (2.9)	CRP, glucose	NS
Vega-Lopez 2006 [37]	C16:0 (7.5)	MUFA + PUFA (6.2)	apoA-II, HDL_2-C, HDL_3-C, HOMA-IR, Lp(a)	↓ HDL_3-C
	C16:0 (8.3)	MUFA + PUFA (9.7)		NS
Thijssen 2005 [38,39]	C18:0 (19.7)	MUFA + PUFA (20.2)	apoA-I, apoB, fibrinogen, PAI-1 activity, TC/HDL-C ratio	NS
	C18:0 (19.7)	MUFA + PUFA (20.0)		NS
Gill 2003 [40]	C16:0 (3.0)	MUFA + PUFA (3.6)	Lp(a)	NS
	C16:0 (6.1)	MUFA (6.6)	apoA-I, apoB, CRP, HDL-C, insulin, LDL-C, Lp(a), NEFA, TAG, TC, VLDL-C	↑ insulin ↓ apoB, LDL-C, TC
	C16:0 (3.1)	MUFA (3.0)		↑ insulin ↓ apoB, LDL-C, TC
Cater 2001 [41]	C16:0 (19.3)	MUFA + PUFA (21.2)	N/A	NS
	>C18:0 (17.0)	MUFA + PUFA (19.0)	HDL-C, LDL-C, TAG, TC, VLDL-C	↓ LDL-C, TC
Hunter 2000 [42]	C18:0 (10.7)	C18:1 (11.1)	Fibrinogen, HDL-C, LDL-C, PAI-1 activity, TAG, TC, tPA activity	NS
	C18:0 (10.7)	MUFA + PUFA (10.3)	Fibrinogen, PAI-1 activity, tPA activity	NS
Snook 1999 [43]	C16:0 (10.0)	C18:0 (10.8)	HDL_2-C, HDL_3-C	NS
Müller 1998 [44]	C16:0 (5.5)	MUFA + PUFA (4.5)	Lp(a)	NS
	Total *trans* (6.8)	MUFA + PUFA (5.6)	apoA-I, apoB, HDL-C, LDL-C, LDL-C/HDL-C ratio, Lp(a), TAG, TC	↑ HDL-C ↓ apoB, LDL-C, LDL-C/HDL-C ratio, TC
Schwab 1998 [45]	C16:0 (2.0)	MUFA + PUFA (1.6)	N/A	NS
Cater 1997 [46]	C16:0 (19.3)	MUFA + PUFA (21.3)	N/A	NS
Sundram 1997 [47]	C16:0 (4.1)	C18:1 (2.7)	apoB/apoA-I ratio, LDL-C/HDL-C ratio, Lp(a), VLDL-C	↓ VLDL-C
Schwab 1996 [48]	C16:0 (3.3)	C18:0 (4.9)	HDL-TAG, LDL-TAG, VLDL-C, VLDL-TAG	NS

Temme 1996 [49]	C16:0 (7.5)	18:1 (8.4)	apoA-I/apoB ratio, HDL-C/LDL-C ratio, Lp(a)	NS
Choudhury 1995 [50]	C16:0 (5.0)	C18:1 (7.3)	N/A	NS
Sundram 1995 [51]	C16:0 (4.3)	C18:1 (5.1)	apoB/apoA-I ratio, HDL_2-C, HDL_3-C, LDL-C/HDL-C ratio, Lp(a), VLDL-C	↑ LDL-C/HDL-C ratio ↓ HDL-C
	C16:0 (3.0)	MUFA + PUFA (6.8)	apoA-I, apoB, apoB/apoA-I ratio, HDL-C, HDL_2-C, HDL_3-C, LDL-C, LDL-C/HDL-C ratio, Lp(a), TAG, TC, VLDL-C	↓ LDL-C/HDL-C ratio
	C16:0 (7.3)	MUFA + PUFA (6.8)	apoB/apoA-I ratio, HDL_2-C, HDL_3-C, Lp(a)	NS
Nestel 1994 [52]	C16:0 (3.3)	C16:1 (3.8)	HDL-C, LDL-C, TAG, TC	↓ HDL-C
	C16:0 (3.4)	C18:1 (2.7)	N/A	NS
Zock 1994 [53]	C14:0 (10.2)	C16:0 (10.2)	apoA-I, apoB, HDL-C, HDL-C/LDL-C ratio, LDL-C, TAG, TC	↓ apoA-I, HDL-C, HDL-C/LDL-C ratio, LDL-C, TC
	C14:0 (10.5)	C18:1 (10.0)		↑ HDL-C/LDL-C ratio ↓ apoA-I, apoB, HDL-C, LDL-C, TC
	C16:0 (9.9)	C18:1 (9.3)	HDL-C/LDL-C ratio	↑ HDL-C/LDL-C ratio
Denke 1992 [54]	C12:0 (17.5)	MUFA + PUFA (16.3)	HDL-C, LDL-C, TAG, TC, VLDL-C	↓ LDL-C, TC
	C16:0 (15.5)	MUFA + PUFA (16.6)	N/A	NS
Ng 1991 [55]	C16:0 (4.9)	MUFA + PUFA (4.8)	N/A	NS
Bonanome 1988 [56]	C16:0 (14.8)	C18:0 (15.3)	LDL-C/HDL-C ratio, VLDL-C	↓ LDL-C/HDL-C ratio
	C16:0 (15.8)	MUFA + PUFA (17.4)	N/A	NS
	C18:0 (16.3)	MUFA + PUFA (17.7)	LDL-C/HDL-C ratio, VLDL-C	NS
Mattson 1985 [57]	C16:0 (15.3)	MUFA + PUFA (15.2)	N/A	NS
	C16:0 (14.8)	MUFA + PUFA (15.2)	N/A	NS

Baudet 1984 [58]	C16:0 (3.1)	MUFA + PUFA (2.8)	N/A	NS
	C16:0 (2.1)	MUFA + PUFA (0.7)	N/A	NS
Liu 2009 [59]	Men: <C12:0 (1.81) Women: <C12:0 (1.87)	Men: C18:2n-6 (1.69) Women: C18:2n-6 (1.59)	apoA-I, apoB, apoE, glucose, HDL-C, LDL-C, TAG, TC	Observed in men only: ↑ TAG, apoE ↓ apoB, LDL-C
Xue 2009 [60]	<C12:0 (1.8)	MUFA + PUFA (1.3)	Adiponectin, apoA-I, apoA-II, apoB, apoE, glucose, HDL-C, LDL-C, TAG, TC	↑ apoA-II, apoB, LDL-C, TAG
Nosaka 2003 [61]	<C12:0 (3.0)	C18:1 (1.6)	glucose, HDL-C, TDL-TAG, LDL-C, LDL-TAG, TAG, TC, VLDL-C, VLDL-TAG	↑ VLDL-C
Judd 2002 [68]	C18:0 (8.0)	C18:1 (71)	apoA-I, apoB, HDL_2-C, HDL_3-C, HDL-C, LDL-C, TAG, TC, TC/HDL-C ratio	↑ apoA-I, HDL-C, HDL_3-C ↓ LDL-C, TAG, TC, TC/HDL-C ratio
	C18:0 (4.1)	Total *trans* (4.1)		NS
	C18:0 (8.1)	Total *trans* (8.0)		↑ LDL-C, TC, TC/HDL-C ratio ↓ apoB
	C18:0 (4.0)	Total *trans* (3.9)		↑ apoB, LDL-C, TC
Temme 1999 [62]	C16:0 (6.1)	C18:1 (6.8)	Fibrinogen, PAI-1 activity	↓ PAI-1 activity
Temme 1997 [63]	<C12:0 (9.9)	C18:1 (10.1)	apoA-I, apoA-I/apoB ratio, apoB, HDL-C, LDL-C, Lp(a), TC/HDL-C ratio, TAG, TC	↑ apoA-I/apoB ratio
	<C12:0 (9.9)	C14:0 (9.7)		↑ apoA-I, apoA-I/apoB ratio, HDL-C
	C14:0 (9.6)	C18:1 (10.4)		↓ LDL-C, TC
Ghafoorunisa 1995 [64]	C16:0 (5.0)	C18:2n-6 (3.7)	HDL-C, LDL-C, TAG, TC, VLDL-C	NS
	C16:0 (6.0)	C18:2n-6 (3.7)		NS
Tholstrup 1994 [65]	C14:0 (13.4)	C16:0 (12.8)	apoA-I, apoB, fibrinogen, HDL_2-C, HDL_3-C, HDL-C, LDL-C, LDL-C/HDL-C ratio, TAG, TC, tPA activity, VLDL-C	↑ LDL-C, LDL-C/HDL-C ratio, TAG ↓ HDL-C, TC

Zock 1992 [66]	C18:0 (9.0)	C18:2n-6 (8.1)	apoA-I, apoA-I/apoB ratio, apoB, HDL-C, HDL-C/LDL-C ratio, LDL-C, TAG, TC	↑ apoA-I/apoB ratio, HDL-C, HDL-C/LDL-C ratio ↓ apoB, LDL-C, TAG, TC
	Total (7.6)	*trans* MUFA + PUFA (7.5)		↑ apoA-I, apoA-I/apoB ratio, HDL-C, HDL-C/LDL-C ratio ↓ apoB, LDL-C, TC

[1] **fatty acids considered:** C12:0 lauric acid, C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C16:1 palmitoleic acid, C18:1 oleic acid, C18:2n-6 linoleic acid, C18:2 n-3 α-linolenic acid, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

[2] Comparisons reported as statistically significant in the original full-text articles.

Abbreviations: ↑: increase, ↓ decrease, %TE: % total energy, Apo: apolipoprotein, CHD: coronary heart disease, CRP: C-reactive protein, FAs: fatty acids; HDL: high-density lipoprotein, HOMA-IR: Homeostatic Model Assessment of Insulin Resistance, IL: interleukin, LDL: low-density lipoprotein, Lp(a): lipoprotein (a), MUFA: monounsaturated fatty acids, NEFA: non-esterified fatty acids, NS: no significant difference, PAI-1: Plasminogen activator inhibitor-1, PUFA: polyunsaturated fatty acids, TAG: triacylglycerol, TC: total cholesterol, TNF-α: tumor necrosis factor α, tPA: tissue plasminogen activator, VLDL: very low-density lipoprotein.

3.4 Discussion

This systematic review of RCTs is the first, to our knowledge, to investigate the role of individual dietary SFA replacement on biomarkers of CMD risk. In our meta-analyses, we found the isoenergetic dietary replacement of at least 1.5%TE of palmitic acid with oleic acid or UFA for a duration of at least 14 days had significant beneficial impacts on lipid CMD risk markers, including TC, LDL-C, and apoB concentrations in adults, albeit with high heterogeneity. In particular, there was a significant linear relationship between the amount of dietary palmitic acid exchanged with UFA and the decreases observed in fasting LDL-C and TC concentrations. There was, however, no significant effect of the latter dietary substitution on other lipid CMD risk markers such as circulating HDL-C, VLDL-C, TAG, apoA-I, NEFA concentrations, TC:HDL-C or LDL-C:HDL-C ratios. Our qualitative synthesis highlighted the abundance of RCTs investigating the effects of dietary palmitic acid, and to a lesser extent stearic acid, whereas trials on other SFAs such as myristic or lauric acids were much scarcer. Most of the trials included focused on traditional biomarkers of CMD risk such as fasting lipid profiles, with little evidence on other risk factors such as markers of inflammation, hemostasis, glycemic control, or metabolic hormones.

Our findings are in line with results from the 2019 UK Scientific Advisory Committee on Nutrition (SACN) on SFA and health, which reported adequate evidence from RCTs supporting the hypothesis that a replacement of overall dietary SFA with MUFA or PUFA might lead to lower concentrations of TC and LDL-C [8]. In addition, the report did not suggest any effect on HDL-C concentrations (moderate evidence quality), TAG concentrations (adequate evidence quality) or TC:HDL-C ratio (limited evidence quality) [8]. Furthermore, our SLR adds novel evidence regarding the apolipoprotein responses to individual dietary SFA, which was not investigated in the 2019 SACN report. In quantitative meta-analyses, we observed similar effects of the replacements of dietary palmitic acid with UFA or oleic acid on apoB and LDL-C, and on apoA-I and HDL-C concentrations, which reflects the main apoproteins associated with these lipoproteins.

Whilst there is no previous SLR and meta-analysis of RCTs on the effects of individual dietary SFA available in the literature to our knowledge, in 2014 Fattore *et al*. systematically reviewed RCTs on the effects of palm olein-rich diets compared to other dietary fats on fasting lipids [69]. Their meta-analyses included some RCTs also reviewed in our SLR, and authors observed beneficial effects on LDL-C concentrations of diets rich in stearic acid (n=8) or MUFA (n=20), but not PUFA (n=14), in comparison to palm olein-rich diets. They further reported that PUFA-rich diets might decrease concentrations of TC (n=16), HDL-C (n=16), apoA-I (n=7), and apoB (n=7) [69]. These results suggested contrasting effects of palm olein on different CVD risk markers and might be confounded by the composition of the palm

olein used in the included RCTs, which contained palmitic acid as the main source of SFA but was also a small dietary source of oleic and linoleic acids. Overall, our findings are supported by experimental evidence suggesting that as opposed to dietary UFA, SFA and particularly C16:0 are associated with a down-regulation of the expression of LDL receptors on the surface of hepatocytes. This can result in higher circulating levels of LDL-C and a potential increased risk of developing and/or exacerbating atherosclerosis [70,71]. In addition, previous RCTs have suggested that dietary SFA may be associated with higher concentrations of E-selectin [72,73], a biomarker of atherosclerosis and endothelial activation, although this SLR did not identify RCTs investigating the impact of individual SFAs on this cell adhesion molecule.

The limited number of RCTs may have contributed to the lack of identified statistically significant effects of replacing dietary palmitic acid with UFA on markers of glycemic control (i.e. fasting glucose and insulin concentrations) or inflammation (i.e. CRP concentrations) in our meta-analyses. However, when looking at overall dietary SFA, authors from the 2019 SACN report observed beneficial effects of SFA substitution with PUFA but not MUFA on fasting glucose levels, potential deleterious effect of dietary MUFA but not PUFA on fasting insulin concentrations, and beneficial effects of both MUFA and PUFA on hemoglobin A1c (a long-term biomarker of glycemic control) based on adequate evidence quality from RCTs [8]. There is, to our knowledge, no SLR or meta-analysis looking at the effect of such dietary replacements on CRP concentrations.

Furthermore, replacing dietary palmitic acid with stearic acid may have little to no effect on lipid CMD risk markers, such as LDL-C, TC, and apoB, but the evidence based on 4 to 5 RCTs with low number of participants is very uncertain. Our findings on the dietary substitution of stearic acid with UFA, which did not impact concentrations of LDL-C, HDL-C, TC, or TAG in meta-analyses based on 4 RCTs, contrast with meta-analyses on dietary palmitic acid substituted with UFA. This supports the hypothesis that dietary stearic acid might be less detrimental than other SFAs such as palmitic acid on lipid CMD risk markers. However, these findings were based on only four RCTs, two of which were classified as high risk of bias because of insufficient washout periods between the intervention diets. Predictive studies based on linear regression equations previously suggested the potential lack of detrimental effects of dietary stearic acid compared to other SFAs on fasting lipids [11,74]. The underlying mechanisms to support this proposal are not yet elucidated, and some studies suggested stearic acid might be poorly absorbed compared to other SFAs [56,75] or could be directly converted into oleic acid, although this metabolic pathway seems to be minimal in humans [76].

Finally, this SLR led to the identification of important gaps in the literature regarding individual dietary SFAs. In particular, there is a lack of RCTs investigating the impact of medium-chain SFAs, lauric acid,

and myristic acid in comparison to other SFAs and/or UFA. Overall, our qualitative synthesis suggested dietary myristic acid might have more deleterious effects than palmitic acid, oleic acid, or medium-chain SFAs on fasting lipid profiles, but no other emerging trends were evident from the other included RCTs. The potential atherogenic effect of dietary myristic acid has been previously investigated in predictive regression studies [74,77], but a consensus on the true effect of myristic acid on cardiometabolic health has not yet been reached [78–80]. Moreover, our qualitative synthesis highlighted that very few RCTs assessed the impact of individual SFAs on other biomarkers of cardiometabolic health status, such as metabolic hormone concentrations or markers of hemostasis and inflammation.

Overall, strengths of this SLR and meta-analysis pertain to its broad yet specific focus on individual dietary SFAs substitutions. Our findings are the first and the most up-to-date, to our knowledge, to provide an exhaustive overview of the currently known causal effects of single dietary SFAs on a wide range of CMD risk biomarkers. This "single SFA" approach, which was ensured by strict *a priori* defined inclusion criteria, allowed the investigation of the causal effect of specific isoenergetic dietary substitution on cardiometabolic health while minimizing potential confounding from other dietary fatty acids or macronutrients. This SLR also benefitted from a strong methodology based on the Cochrane and PRISMA guidelines. Nevertheless, some limitations also need to be acknowledged. Firstly, some of the included crossover trials with insufficient washout periods might present a high risk of bias due to potential carry-over effects. This may have led to unprecise estimations of the beneficial effects of UFA-rich diets compared to palmitic acid-rich diets on some of the lipid outcomes reviewed in this SLR. In addition, we observed high statistical heterogeneity in meta-analyses on TC, LDL-C, HDL-C, and apolipoprotein concentrations, which might have prevented the detection of statistically significant effect sizes, particularly in meta-analyses with few RCTs reporting effects distributed around the null. However, some of the observed high heterogeneity might be explained by correlation coefficients used to estimate corrected intervention effects which account for the intra-participant variation specific for this type of study design. While this approach is recommended by the Cochrane handbook for systematic reviews [23], this may have led to underestimated confidence intervals of the effects from individual RCTs. Furthermore, our analyses focused on individual dietary SFAs and might not account for potential food matrix effects from dietary sources of SFAs, such as red meat [81], dairy foods [82], or coconut, palm, and to a lesser extent, other plant-based oils [83]. For instance, dairy food intakes may be associated with lower CMD risks, despite being important dietary sources of C16:0 and C18:0 [84,85]. Finally, the small number of studies included in quantitative syntheses precluded the detailed investigation of potential dose-response relationships, high inter-study heterogeneity, and publication bias.

To conclude, our findings on the replacement of dietary palmitic acid with oleic acid or UFA are overall in line with current public health recommendations which suggest reducing dietary SFA in favor of UFA to help prevent CMD. This further supports the hypothesis that dietary SFA should not be considered as a homogeneous nutrient group, and that individual SFAs might have differential impacts on cardiometabolic health. However, our quantitative findings need to be interpreted with caution due to the presence of high statistical heterogeneity and low number of RCTs. With most of the available evidence focusing on dietary palmitic, and to a lesser extent stearic acid, and their impact on lipid profiles, further RCTs designed to investigate different SFAs such as lauric and myristic acids and their impact on other clinical biomarkers of CMD risk such as markers of inflammation, endothelial activation, and glycemic control are warranted. Overall, a more complete picture of the impact of dietary SFAs on metabolic health status would greatly contribute to the improvement of public health guidelines for the prevention of CMD.

3.5 Supplementary material

Supplementary method 1. Detailed search queries used to identify records in PubMed, Embase, Scopus and the Cochrane central register for clinical trials.

Search 1: Update of the 2016 WHO systematic review report on dietary saturated fat, serum lipids and lipoproteins. [11]

(("saturated" AND ("fat" OR “fats” OR "fatty acid" OR “fatty acids”)) OR “SFA” OR "Butyric" OR "butanoic" OR "butyrates" OR "butyrate" OR "butanoates" OR "butanoate" OR "BTA" OR "C4:0" OR "caproic" OR "hexaenoic" OR "hexanoic" OR "hexanoate" OR "hexanoates" OR "caproates" OR "caproate" OR "C6:0" OR "caprylic" OR "octanoic" OR "C8:0" OR "capric" OR "decanoic" OR "decanoate" OR "decanoates" OR "caprate" OR “caprates" OR "C10:0" OR "lauric" OR "dodecanoic" OR "laurate" OR "laurates" OR "C12:0" OR "tetradecanoic" OR "myristic" OR "myristate" OR "myristates" OR "C14:0" OR "pentadecylic" OR "pentadecanoic" OR "C15:0" OR "palmitic" OR "palmitate" OR "palmitates" OR "hexadecanoic" OR "margaric" OR "heptadecanoic" OR "heptadecanoate" OR "C17:0" OR "stearic" OR "octadecanoic" OR "stearate" OR "stearates" OR "C18:0" OR "arachidic" OR "arachic" OR "eicosanoic" OR "icosanoic" OR "C20:0" OR "behenic" OR "docosanoic" OR "C22:0" OR "lignoceric" OR "tetracosanoic" OR "C24:0")

AND

“cholesterol” OR “high density lipoprotein” OR “HDL” OR “low density lipoprotein” OR “LDL” OR “triacylglycerol” OR “TC” OR “triglyceride” OR “TG” OR “ApoA-I” OR “Apolipoprotein A-I” OR “apoB” OR “apolipoprotein B” OR “Lipoproteins” OR “serum lipids”

AND

(randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR clinical trials as topic[mesh:noexp] OR randomly[tiab] OR trial[ti] NOT (animals[mh] NOT humans [mh]))

AND

(“diet” OR “diets” OR “dietary” OR “intake” OR “intakes” OR “consumption” OR “consume” OR “substitution” OR “replacement” OR “change” OR “replace” OR “nutrition” OR “nutritional” OR “eat” OR “food” OR “foods” OR “source” OR “sources”

NOT

(“acute” OR “postprandial”))

<u>Search 2:</u> Search for RCTs investigating all other biomarkers of cardiometabolic disease risk.

(("saturated" AND ("fat" OR “fats” OR "fatty acid" OR “fatty acids”)) OR “SFA” OR "Butyric" OR "butanoic" OR "butyrates" OR "butyrate" OR "butanoates" OR "butanoate" OR "BTA" OR "C4:0" OR "caproic" OR "hexaenoic" OR "hexanoic" OR "hexanoate" OR "hexanoates" OR "caproates" OR "caproate" OR "C6:0" OR "caprylic" OR "octanoic" OR "C8:0" OR "capric" OR "decanoic" OR "decanoate" OR "decanoates" OR "caprate" OR “caprates" OR "C10:0" OR "lauric" OR "dodecanoic" OR "laurate" OR "laurates" OR "C12:0" OR "tetradecanoic" OR "myristic" OR "myristate" OR "myristates" OR "C14:0" OR "pentadecylic" OR "pentadecanoic" OR "C15:0" OR "palmitic" OR "palmitate" OR "palmitates" OR "hexadecanoic" OR "margaric" OR "heptadecanoic" OR "heptadecanoate" OR "C17:0" OR "stearic" OR "octadecanoic" OR "stearate" OR "stearates" OR "C18:0" OR "arachidic" OR "arachic" OR "eicosanoic" OR "icosanoic" OR "C20:0" OR "behenic" OR "docosanoic" OR "C22:0" OR "lignoceric" OR "tetracosanoic" OR "C24:0")

AND

("free fatty acid" OR "free fatty acids" OR "nonesterified fatty acid" OR "nonesterified fatty acids" OR “NEFA” OR “lipoprotein a” OR “lipoprotein (a)” OR “Lp(a)” OR “apoC2” OR “apolipoprotein C2” OR “apoC3” OR “apolipoprotein C3” OR “apolipoprotein CIII” OR “blood lipid” OR “dyslipidemia” OR

“hyperlipidemia” OR “hyperlipoproteinemia” OR “hypercholesterolemia” OR “postprandial lipemia” OR “postprandial lipaemia” OR “serum lipids” OR "TNF-a" OR “TNF alpha” OR “TNFA” OR "IL-6" OR "IL6" OR "IL-10" OR "IL10" OR “CRP” OR “C-reactive protein” OR “reactive protein” OR “hsCRP” OR “high sensitivity CRP” OR “tumor necrosis factor” OR “inflammatory” OR “glucose” OR “insulin” OR "C-peptide" OR “homocysteine” OR “CGM” OR "dicarbonyl stress" OR "1,5-anhydroglucitol" OR “fructosamine” OR "glycated albumin" OR "glycated proteins" OR "advanced glycated end products" OR “HbA1c” OR “glycosylated haemoglobin” OR "hyperglycemic clamp" OR “IVGTT” OR "minimal model" OR “homeostatic model assessment” OR “HOMA” OR “QUICKI” OR “OGTT” OR “clamp” OR “glycemia” OR “glycaemia” OR “BMI” OR “body mass index” OR "waist-hip ratio" OR "waist-to-hip ratio" OR "waist circumference” OR “Body mass” OR “body fat” OR “fat mass” OR “Quetelet index” OR “Ponderal index” OR “Abdominal fat” OR “Visceral fat” OR “subcutaneous fat” OR “Overweight” OR “Over weight” OR “Obesity” OR “Obese” OR “Fatness” OR “glucagon” OR “GPL1” OR “GPL 1” OR “GIP” OR “CCK” OR “PPAR alpha” OR “gluco insulinotropic” OR “Cholecystokinin” OR “peroxisome receptor” OR “anthropometric” OR “fibrinogen” OR "platelet aggregation" OR “fibrinolysis” OR "red blood cell size" OR “factor VII” OR “FVII” OR “ischemia” OR “ischemic” OR “leptin” OR “adiponectin” OR "blood pressure" OR "vascular function" OR “vascular reactivity” OR "endothelial dysfunction" OR "flow-mediated dilation" OR “FMD” OR "intima media thickness" OR “IMT” OR "digital volume pulse" OR “DVP” OR "pulse wave analysis" OR "pulse wave velocity" OR “PWV” OR “ICAM” OR “VCAM” OR “*-selectin” OR “MCP1” OR “endothelial function” OR “arterial stiffness” OR “ankle-brachial index” OR “ABI” OR “ankle-brachial pressure index” OR “ABPI” OR “laser Doppler imaging” OR “endoPAT” OR “coagulation factors” OR “coagulation factor”)

AND

(randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR clinical trials as topic[mesh:noexp] OR randomly[tiab] OR trial[ti] NOT (animals[mh] NOT humans [mh]))

AND

(“diet” OR “diets” OR “dietary” OR “intake” OR “intakes” OR “consumption” OR “consume” OR “substitution” OR “replacement” OR “change” OR “replace” OR “nutrition” OR “nutritional” OR “eat” OR “food” OR “foods” OR “source” OR “sources”

NOT

(“acute” OR “postprandial”))

Supplementary table 3.1. Outcomes reported in eligible randomized controlled trials for inclusion in the systematic literature review.

Outcome category	Outcomes included in SLR
Fasting lipid concentrations	ApoA-I, apoA-II, apoB, apoB:apoA-I ratio, apoE, HDL-C, HDL_2-C, HDL_3-C, LDL-C, LDL-C:HDL-C ratio, Lp(a), NEFA, TAG, TAG content of lipoproteins, TC, TC:HDL-C ratio, VLDL-C
Blood pressure and vascular function	N/A
Markers of inflammation	CRP, IL-6, TNF-α
Markers of glycaemic control	C-peptide, glucose, HOMA-IR, insulin
Markers of haemostasis	Fibrinogen, PAI-1 activity, tPA activity
Metabolic hormones concentrations	Adiponectin, leptin

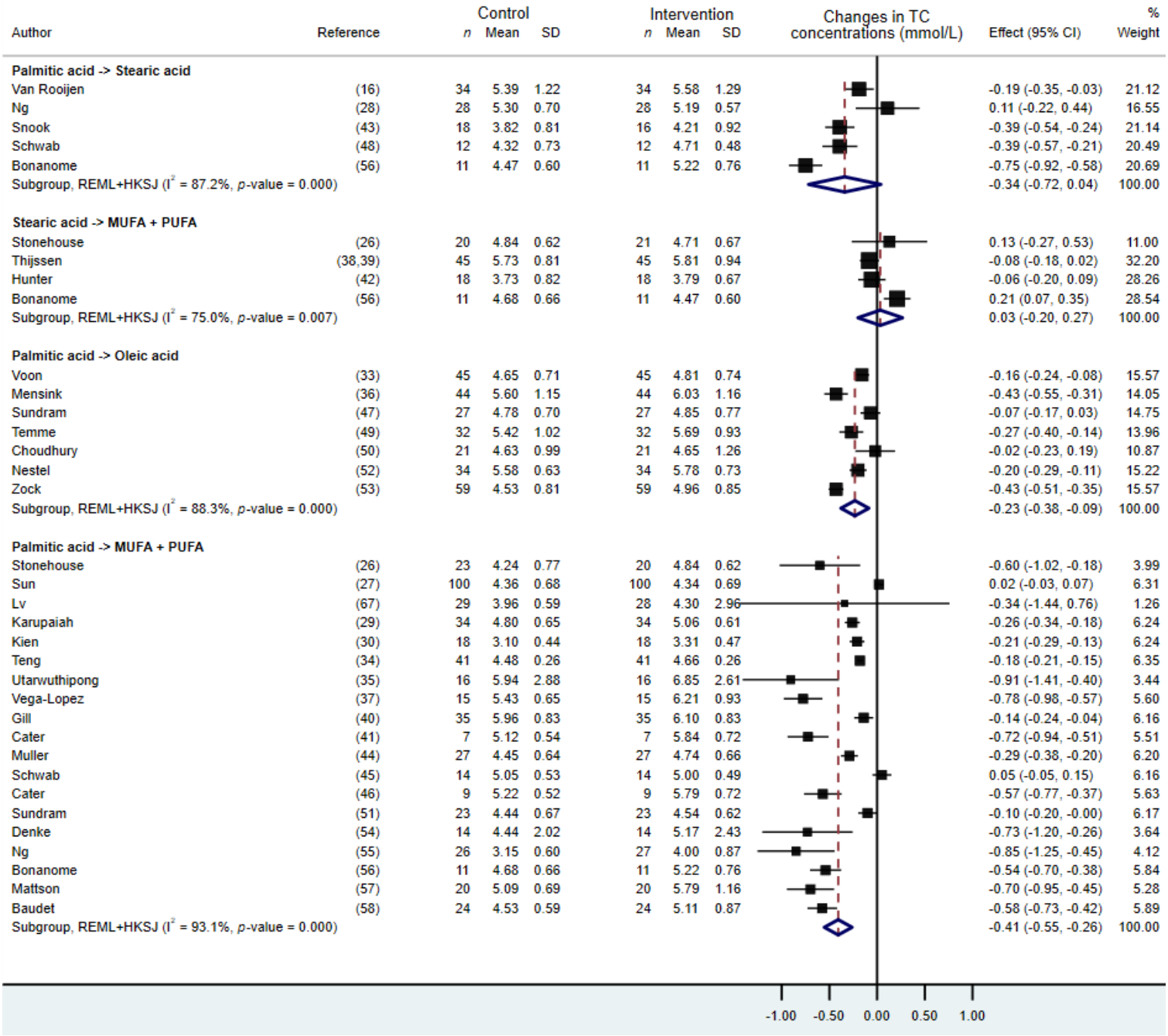
Abbreviations: Apo: apolipoprotein, CRP: C-reactive protein, HDL: high-density lipoprotein, HOMA-IR: Homeostatic Model Assessment of Insulin Resistance, IL: interleukin, LDL: low-density lipoprotein, Lp(a): lipoprotein (a), N/A: not applicable, NEFA: non-esterified fatty acids, PAI-1: Plasminogen activator inhibitor-1, SLR: systematic literature review, TNF-α: tumor necrosis factor α, tPA: tissue plasminogen activator, VLDL: very low-density lipoprotein.

Supplementary table 3.2. Within-participant correlation coefficients used for the statistical syntheses of crossover randomized controlled trials.

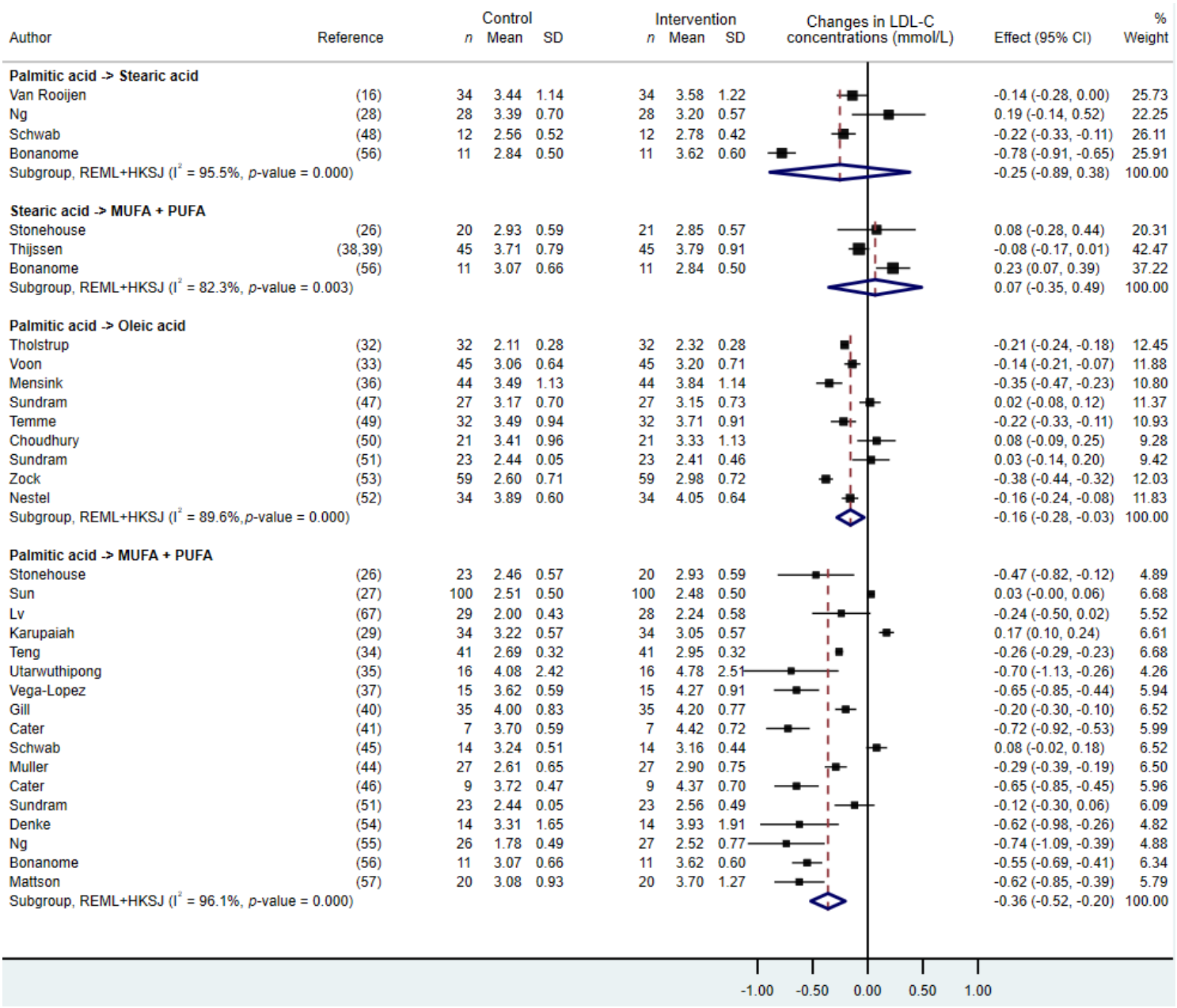
Outcome	Correlation coefficient [1]
ApoA-I	0.807
ApoB	0.968
NEFA	0.693
TAG	0.899
HDL-C	0.909
LDL-C	0.937
LDL-C to HDL-C ratio	N/A
VLDL-C	N/A
Total cholesterol	0.934
Total cholesterol to HDL-C ratio	0.959
CRP	0.472
Glucose	0.895
Insulin	0.818

[1] derived from Van Rooijen et al. (2020) [16]

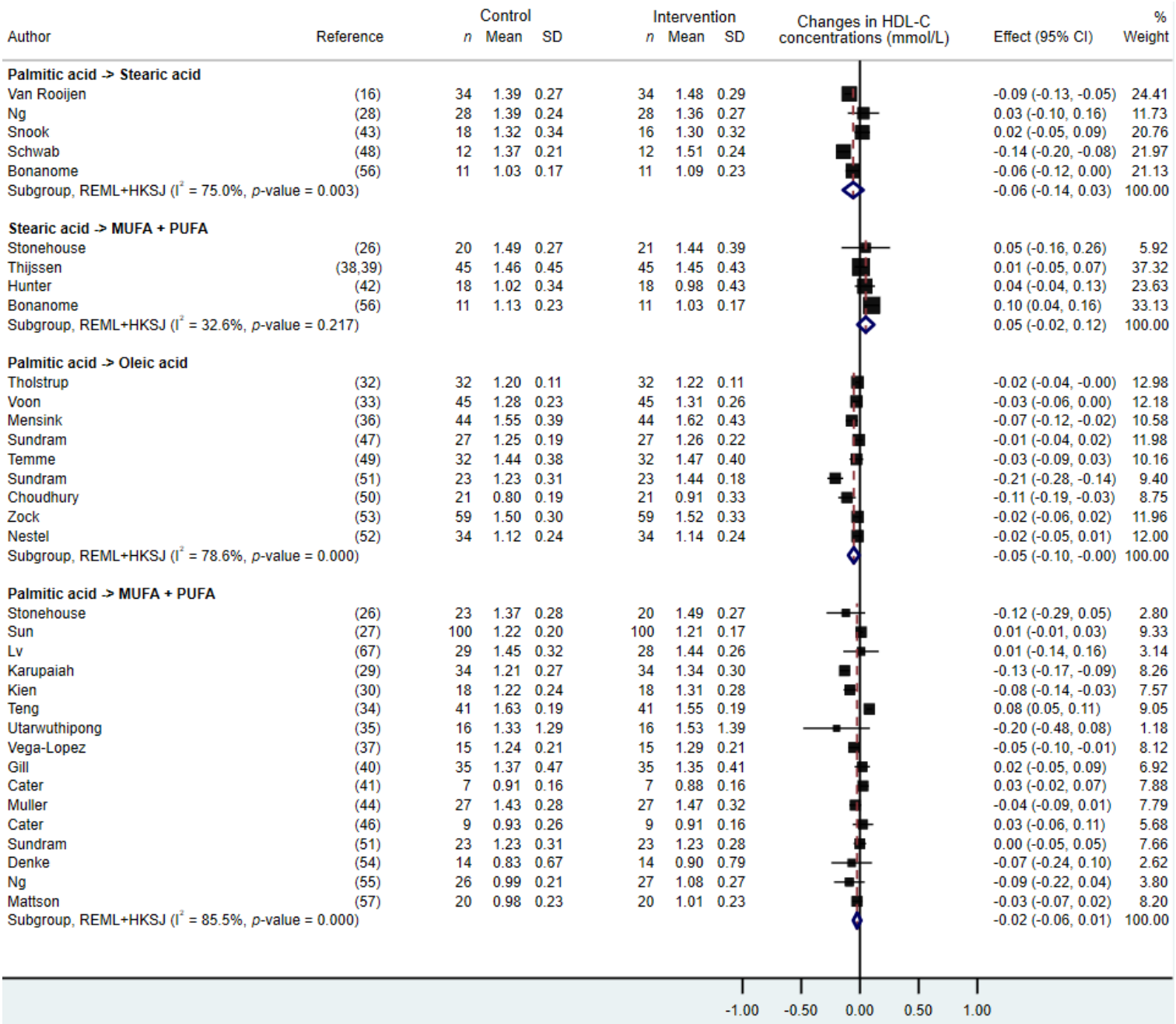
Abbreviations: Apo: apolipoprotein, CRP: C-reactive protein, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, N/A: not available, NEFA: non-esterified fatty acids, TAG: triacylglycerol, VLDL-C: very low-density lipoprotein cholesterol.



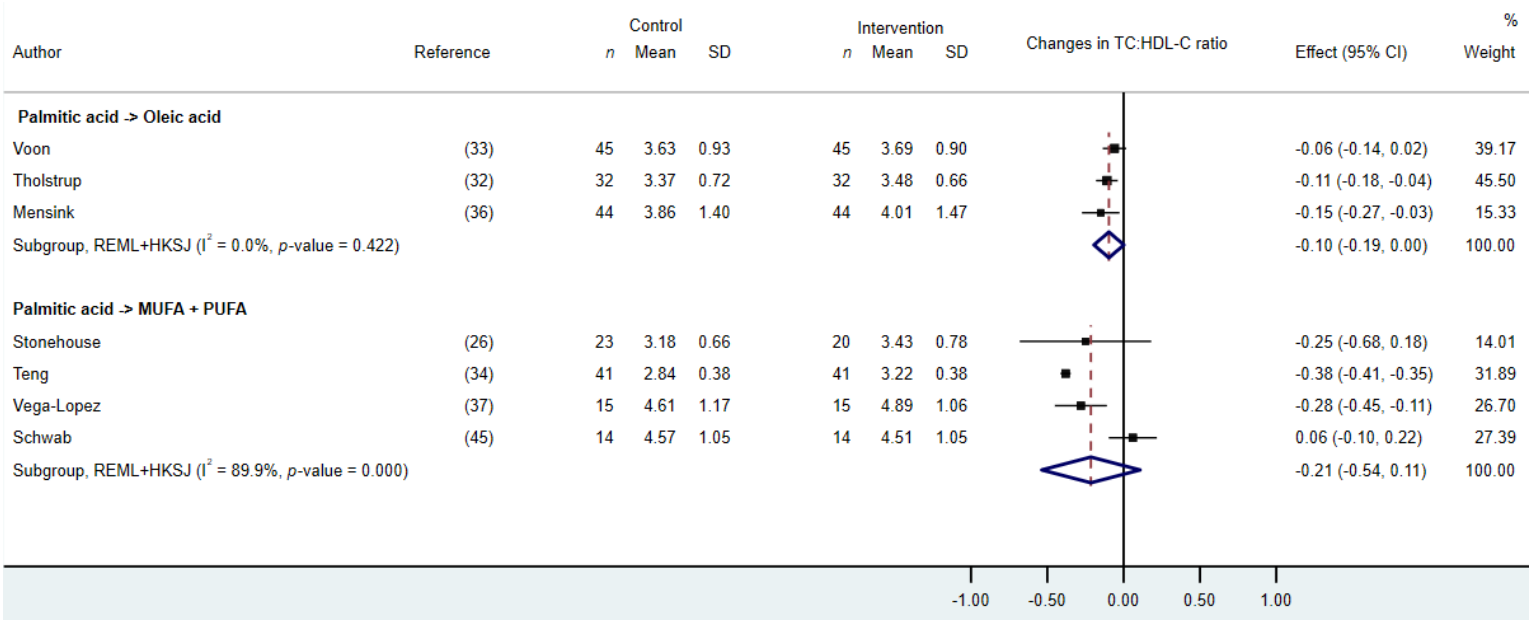
Supplementary figure 3.1. Sensitivity analyses of the impact of dietary fat replacements on total cholesterol (TC) concentrations, excluding trials with potential reporting errors in the full-text articles. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*



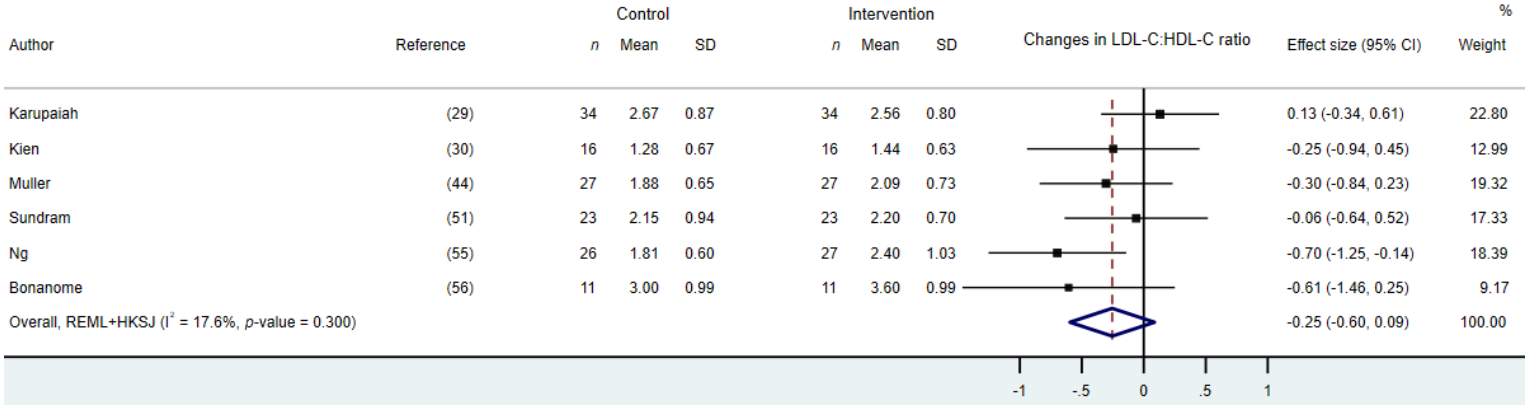
***Supplementary figure 3.2. Sensitivity analyses of the impact of dietary fat replacements on low-density lipoprotein cholesterol (LDL-C) concentrations, excluding trials with potential reporting errors in the full-text articles. Abbreviations**: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*



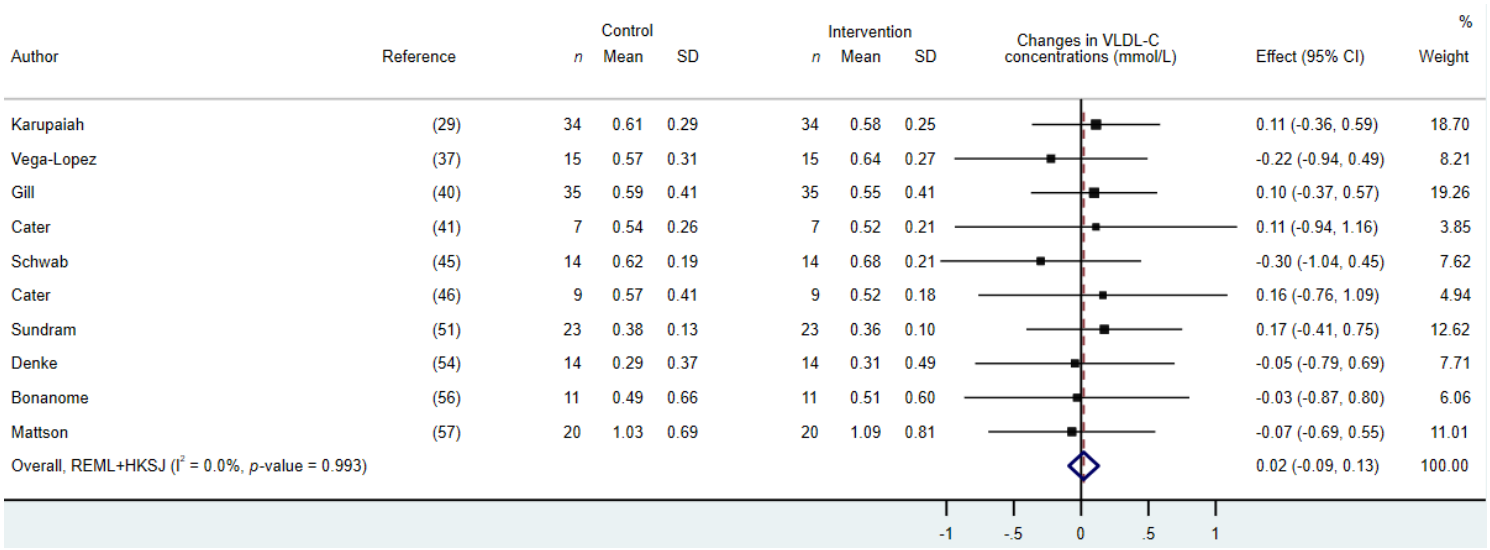
***Supplementary figure 3.3. Sensitivity analyses of the impact of dietary fat replacements on high-density lipoprotein cholesterol (HDL-C) concentrations, excluding trials with potential reporting errors in the full-text articles. Abbreviations**: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*



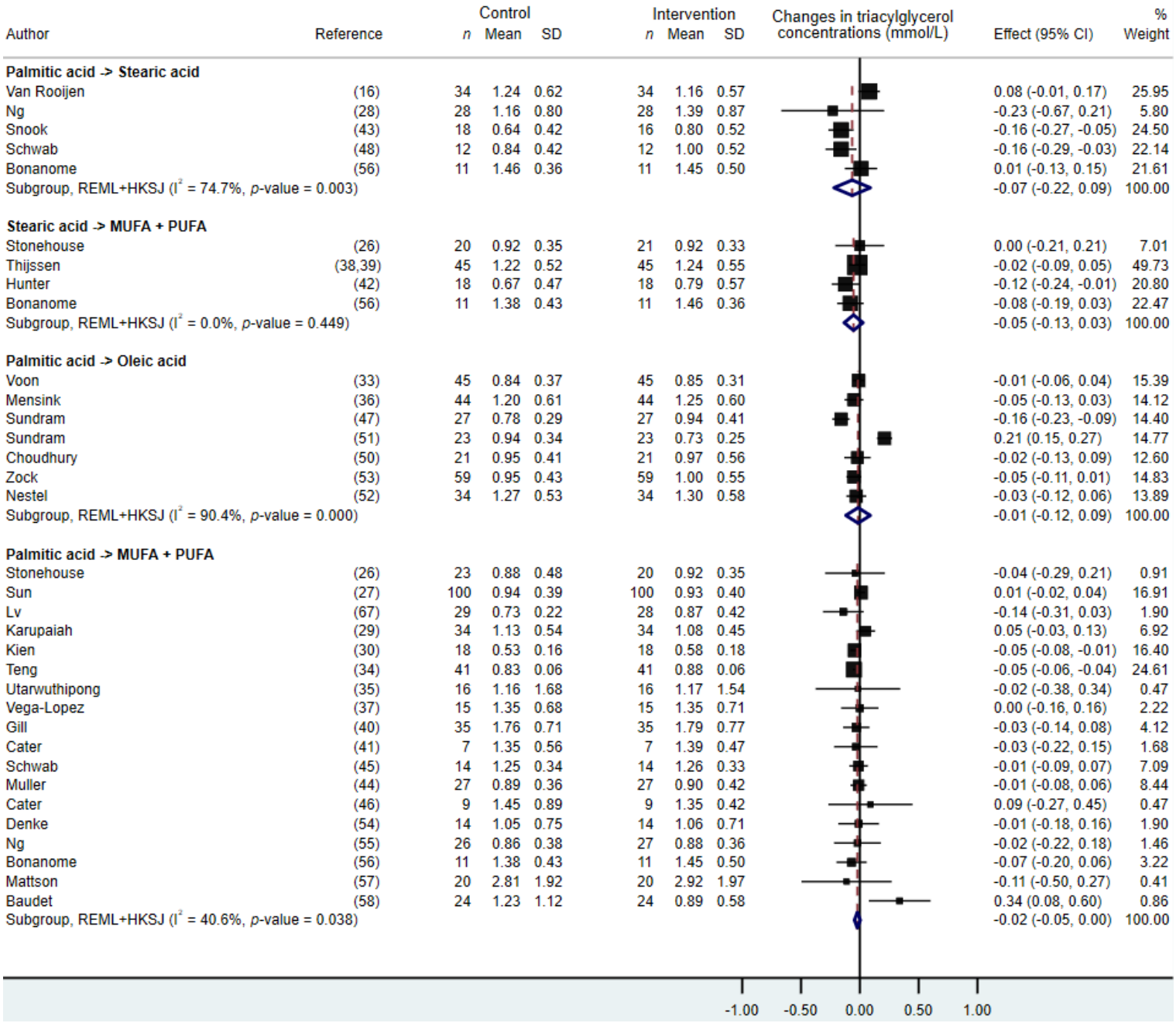
***Supplementary figure 3.4. Forest plot of the effect of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on total cholesterol to high-density lipoprotein cholesterol (TC:HDL-C) ratio in randomized controlled trials. Abbreviations**: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.*



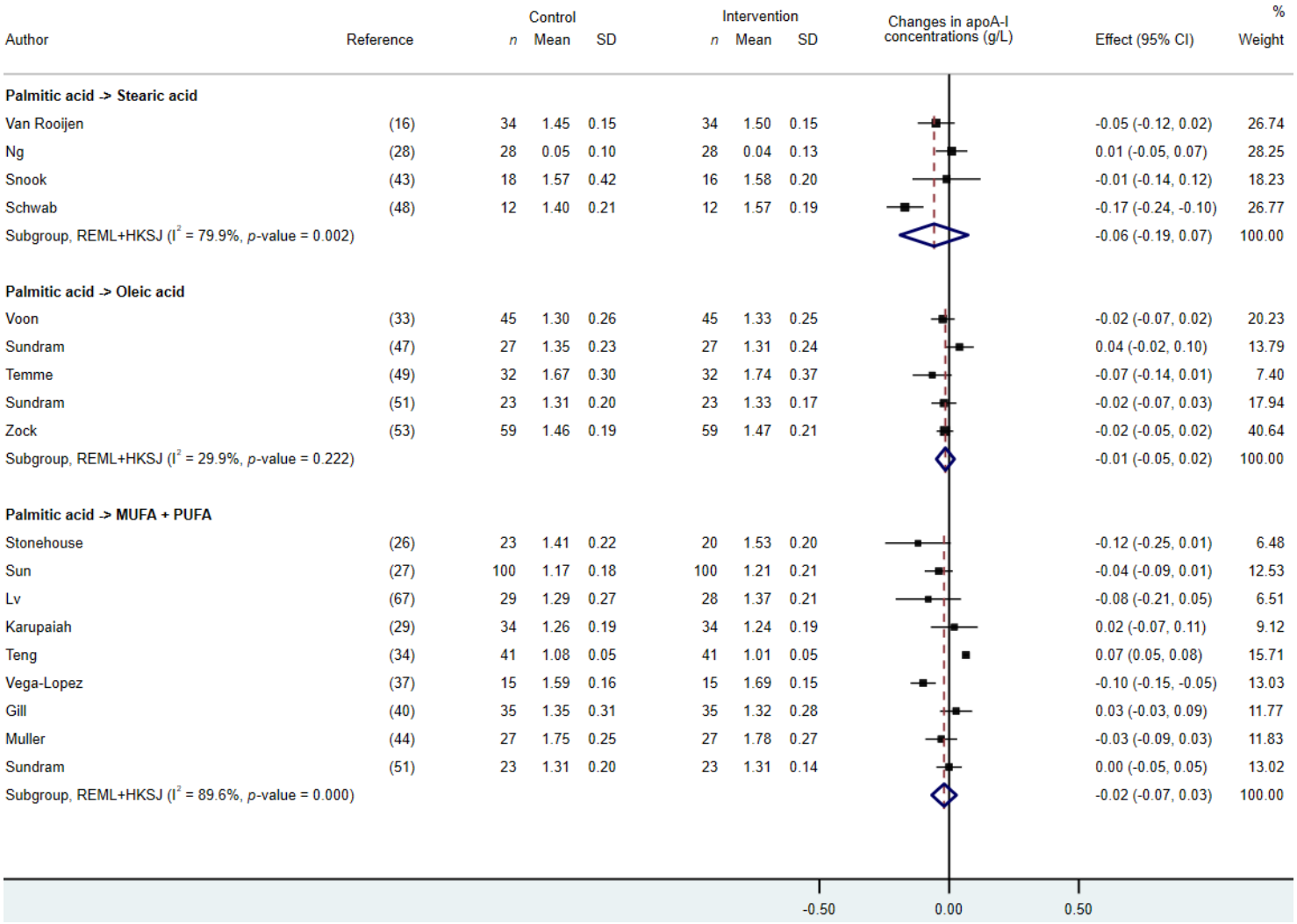
***Supplementary figure 3.5. Forest plot of the effect of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on low-density lipoprotein cholesterol to high-density lipoprotein cholesterol (LDL-C:HDL-C) ratio in randomized controlled trials. Abbreviations**: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.*



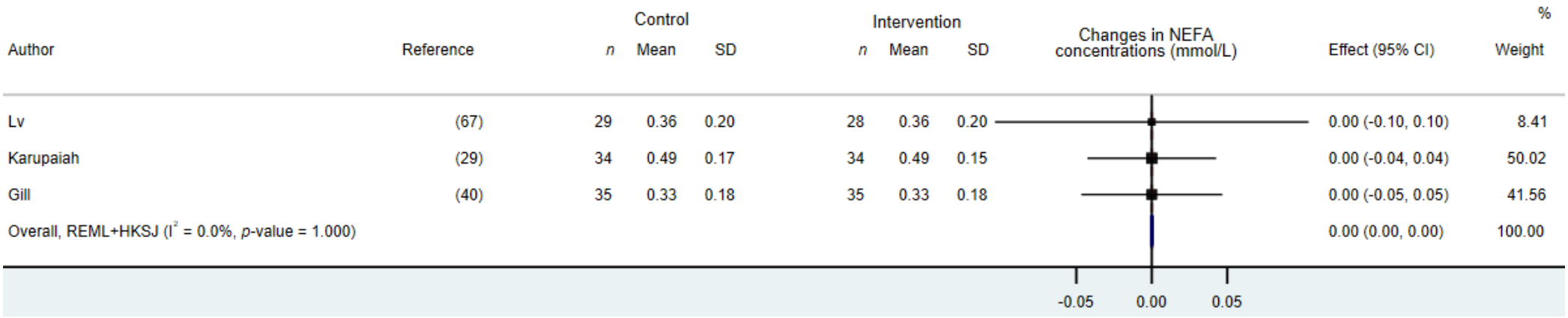
Supplementary figure 3.6. Forest plot of the effect of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on very low-density lipoprotein cholesterol (VLDL-C) in randomized controlled trials. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.*



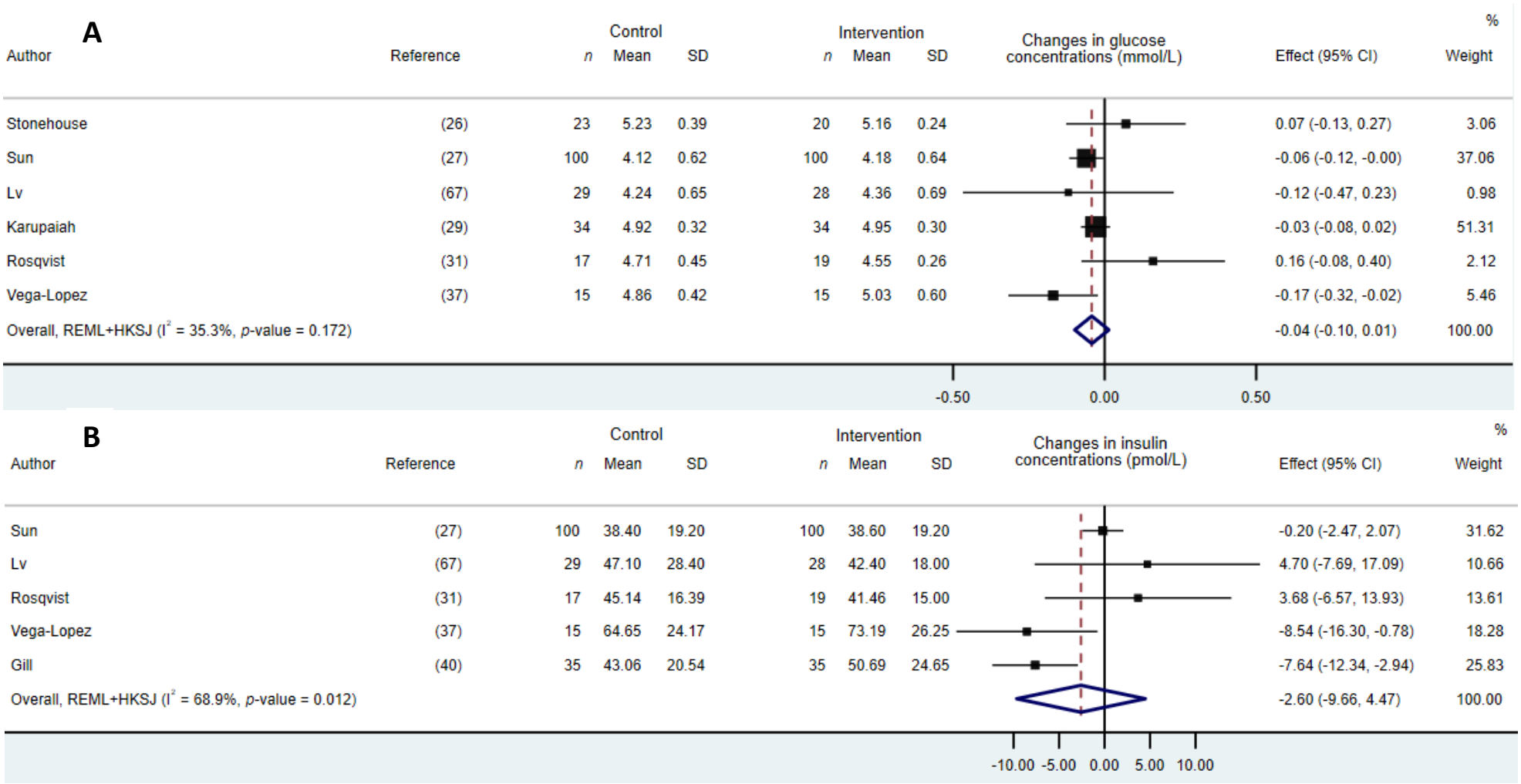
Supplementary figure 3.7. Sensitivity analyses of the impact of dietary fat replacements on triacylglycerol concentrations, excluding trials with potential reporting errors in the full-text articles.
***Abbreviations**: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*



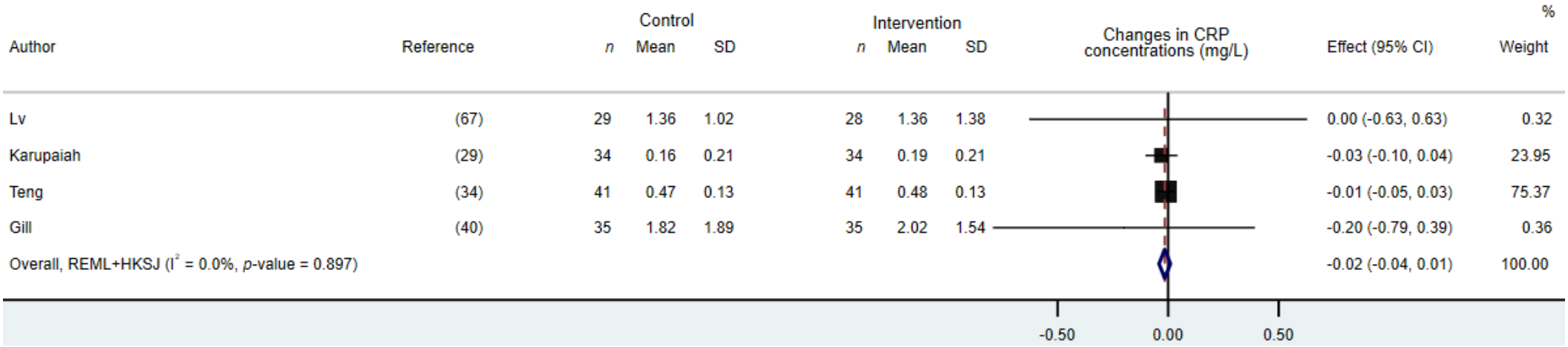
Supplementary figure 3.8. Sensitivity analyses of the impact of dietary fat replacements on apolipoprotein A-I (apoA-I) concentrations, excluding trials with potential reporting errors in the full-text articles. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*



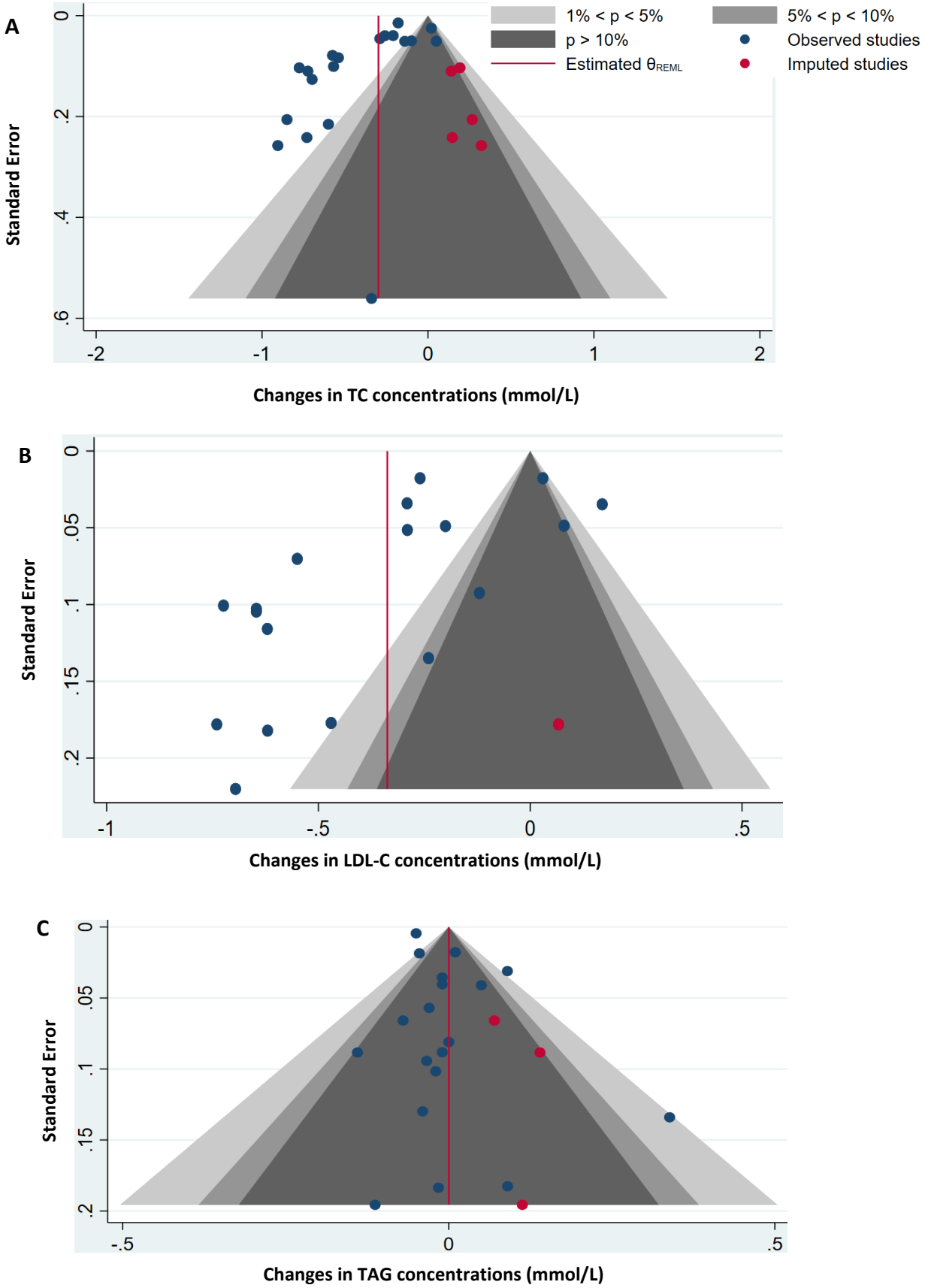
Supplementary figure 3.9. Forest plot of the effect of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on non-esterified fatty acid (NEFA) concentrations in randomized controlled trials. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.*



Supplementary figure 3.10. Forest plots of the effect of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on (A) fasting glucose and (B) insulin concentrations in randomized controlled trials. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.*



Supplementary figure 3.11. Forest plot of the effect of the dietary substitution of dietary palmitic acid with unsaturated fat (MUFA+ PUFA) on C-reactive protein (CRP) concentrations in randomized controlled trials. Abbreviations*: MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids*.



Supplementary figure 3.12. Contour-enhanced funnel plots of RCTs investigating the impact of the dietary substitution of palmitic acid with unsaturated fat (MUFA + PUFA) on (A) total cholesterol (TC), (B) low-density lipoprotein cholesterol (LDL-C), and (C) triacylglycerol (TAG) concentrations, corrected with a trim and fill method and showing regions of statistical significance. ***Abbreviations****: LDL-C: low-density lipoprotein cholesterol, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TAG: triacylglycerol, TC: total cholesterol.*

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Chapter 4: Impact of a food-based dietary fat exchange model for replacing dietary saturated with unsaturated fatty acids in healthy men on plasma phospholipids fatty acid profiles and dietary patterns.

Contribution towards PhD thesis: The RISSCI-1 study was conducted between 2017 and 2020. During that time, I was involved in the recruitment of participants in collaboration with Dr Athanasios Koutsos, Gloria Wong, and Ezgi Ozen. Throughout the study, I was responsible for the daily communication with participants, the monitoring of their compliance and the qualitative assessment of their food diaries. Moreover, I participated in the management of screening visits, main study visits, shorter follow-up visits, along with the processing of the biological samples collected from participants (i.e. blood, urine, and stools). Furthermore, I analysed the food diaries from participants enrolled in Reading, while Dr Rona Antoni analysed those from the University of Surrey. I conducted the statistical analyses presented in this manuscript which included all of dietary data collected from participants at the universities of Reading and Surrey. Finally, I prepared the initial draft of the manuscript for publication, and finalised the published manuscript presented below after including the feedback and comments received from co-authors and journal reviewers.

Manuscript published in the *European Journal of Nutrition* (June 2022). DOI: 10.1007/s00394-022-02910-2

Impact of a food-based dietary fat exchange model for replacing dietary saturated with unsaturated fatty acids in healthy men on plasma phospholipids fatty acid profiles and dietary patterns.

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Author contributions towards manuscript: BAG, JAL, KGJ, BF and MDR obtained the funding and designed the study. AK, BAG, JAL, KGJ, MW, and RA developed the food exchange model and methodology for data collection. LS, RA, AK, EO, GW, and HA collected the data. LS and RA performed the dietary analyses in University of Reading and University of Surrey, respectively. MS, AW performed the fatty acid analysis. LS performed statistical analyses. LS prepared the original draft of the manuscript and revised each version of the manuscript, under the supervision of JAL and KGJ. LS, RA, AK, EO, GW, HA, MW, BF, MDR, KGJ, BAG, and JAL contributed to the interpretation of the data, read, and approved the final manuscript.

Financial support: The RISSCI study was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) project 'Mechanisms to Explain Variation in Serum Low Density Lipoprotein Cholesterol Response to Dietary Saturated Fat' (Project references: BB/P010245/1 and BB/P009891/1).

Conflicts of interest: JAL is a member of the UK Government's Scientific Advisory committee on Nutrition (SACN). JAL (Chair), LS and KGJ are part of the International Life Science Institute (ILSI) Europe expert group on "Update on health effects of different dietary saturated fats". The other authors have no conflicts of interest or competing interests to declare.

Acknowledgements: The authors thank the RISSCI-1 participants for their time and diligence throughout the study, along with Unilever (Wageningen, The Netherlands), who provided, in kind, the fat spreads used in the dietary intervention.

Abstract

Purpose: UK guidelines recommend dietary saturated fatty acids (SFAs) should not exceed 10% total energy (%TE) for cardiovascular disease prevention, with benefits observed when SFAs are replaced with unsaturated fatty acids (UFAs). This study aimed to assess the efficacy of a dietary exchange model using commercially available foods to replace SFAs with UFAs.

Methods: Healthy men (n=109, age 48, SD 11y) recruited to the Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1 (RISSCI-1) study (ClinicalTrials.Gov n°NCT03270527) followed two sequential 4-week isoenergetic moderate-fat (34%TE) diets: high-SFA (18%TE SFAs, 16%TE UFAs) and low-SFA (10%TE SFAs, 24%TE UFAs). Dietary intakes were assessed using 4-day weighed diet diaries. Nutrient intakes were analysed using paired *t*-tests, fasting plasma phospholipid fatty acid (PL-FA) profiles and dietary patterns were analysed using orthogonal partial least square discriminant analyses.

Results: Participants exchanged 10.2%TE (SD 4.1) SFAs for 9.7%TE (SD 3.9) UFAs between the high and low-SFA diets, reaching target intakes with minimal effect on other nutrients or energy intakes. Analyses of dietary patterns confirmed successful incorporation of recommended foods from commercially available sources (e.g. dairy products, snacks, oils, and fats), without affecting participants' overall dietary intakes. Analyses of plasma PL-FAs indicated good compliance to the dietary intervention and foods of varying SFA content.

Conclusions: RISSCI-1 dietary exchange model successfully replaced dietary SFAs with UFAs in free-living healthy men using commercially available foods, and without altering their dietary patterns. Further intervention studies are required to confirm utility and feasibility of such food-based dietary fat replacement models at a population level.

Keywords (4-6): dietary fat composition, food-exchange model, dietary compliance, dairy biomarkers, dietary fat replacement.

4.1 Introduction

A quarter of all deaths in the UK are attributed to cardiovascular diseases (CVD), which represent a major burden on public health worldwide [1]. While the aetiology of CVD is multifactorial, elevated circulating low-density lipoprotein cholesterol (LDL-C) has been established as a causal risk factor for the development of atherosclerosis [2]. Evidence from epidemiological prospective cohort studies, strictly controlled metabolic ward studies, and randomised controlled trials supports consistent associations between a high consumption of dietary saturated fatty acids (SFAs) and elevated serum LDL-C [3–6]. This evidence has formed the basis of public health guidelines in the UK, which since 1983, have recommended dietary SFAs should not exceed 10% of total energy (%TE) intake in adults [7,8].

To study the impact of reducing dietary SFAs on health, many previous dietary interventions replaced SFAs with unsaturated fatty acids (UFAs) i.e. mono- (MUFAs) or polyunsaturated fatty acids (PUFAs) [9]. However, these studies often used dietary fats manufactured specifically for the purpose of the intervention, which limited the translation and applicability of the findings to non-experimental, free-living people settings [10–13]. This limitation raises the importance of developing interventions based on commercially available whole-foods to improve the practicability of reducing dietary SFAs and adherence to dietary guidelines, while minimising the impact on other dietary components. In particular, since about a third of dietary SFAs is consumed from dairy foods and fat spreads in UK adults aged 19-64 y [14], the replacement of full-fat dairy and butter for lower fat or plant-based alternatives has been proposed as a food-based strategy to help reduce dietary SFAs in this group [15].

In parallel with developing food-based interventions, the assessment of dietary compliance beyond traditional approaches using diet diaries, or food-frequency questionnaires linked with food composition databases, would increase understanding of the impact and feasibility of dietary intervention studies in free-living individuals. Plasma phospholipid fatty acids (PL-FAs) correlate with the short to medium-term intake of dietary fatty acid (FA) [16,17], and as such, PL odd-chain SFAs (e.g. pentadecanoic or heptadecanoic acids) have been used as biomarkers of dairy fat consumption [18,19]. The use of plasma PL-FA as an objective tool to assess dietary compliance may thus be particularly effective in the context of interventions that manipulate dietary fat using full-fat dairy foods. Furthermore, the analysis of dietary patterns can identify residual confounding from changes in dietary habits, which are not routinely assessed in dietary intervention studies.

The Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1 ('RISSCI'-1) study was based on a tailored, dietary fat-exchange model, matched to the average diet of UK adult men. The study aimed to replace dietary SFAs with UFAs using common, commercially available foods, while

minimising impacts on dietary habits, and improving dietary compliance and reproducibility, with the primary outcome of measuring variability in LDL-C responses to saturated fat [20]. The present paper assessed the efficacy of a food-based dietary fat exchange model, that replaced dietary SFAs with MUFAs and PUFAs in free-living UK men, with endpoint measures of nutrient intakes, overall dietary patterns and plasma PL-FAs.

4.2 Methods

Study design

The RISSCI-1 study was a single-blind sequential dietary intervention study (ClinicalTrials.Gov registration No. NCT03270527). The study was given a favourable ethical opinion for conduct by the University of Reading Research Health Ethics Committee (17/29) and the University of Surrey Ethics Committee (UEC/2017/41/FHMS) and was conducted in accordance with the Declaration of Helsinki guidelines. Written informed consent was collected from all participants before inclusion in the study.

Participants

The RISSCI-1 study included healthy men aged 30 to 65 y, which were recruited from the Reading, Berkshire and Guildford, Surrey areas between 2017 and 2019. Eligible participants were required to meet the following inclusion criteria: body mass index (BMI) between 19-32 kg/m^2; fasting serum total cholesterol < 7.5 mmol/L and triacylglycerol < 2.3 mmol/L; blood pressure < 140/90 mmHg; fasting glucose < 7.0 mmol/L; haemoglobin > 130 g/L; no history of myocardial infarction, stroke, diabetes, or any other endocrine disorder in the past 12 months; no history of kidney, liver, or gastrointestinal disorder, or history of cancer; not taking any medication for hyperlipidaemia, hypertension, inflammation, or prescribed antibiotics in the last three months; not smoking; drinking ≤ 14 units of alcohol per week; participating in vigorous exercise ≤ 3 times per week; not participating or planning to participate in a weight-loss diet; not taking any dietary supplements known to influence circulating lipids or gut microbiota (e.g. plant stanols, fish oil, phytochemicals, natural laxatives, probiotics and prebiotics); not being involved in another dietary intervention study and willing to regularly consume study intervention products (butter/spreads, oils, dairy foods, snacks). Upon inclusion, participants were advised to maintain their usual physical activity levels, and to inform the researchers of any important changes to their health or medication use.

Dietary intervention and food exchange model

The replacement of dietary SFAs with MUFAs/PUFAs was based on a food exchange model which was successfully implemented in previous intervention studies at the University of Reading [11–13]. The food exchange model aimed to identify dietary sources of exchangeable fat that would not impact total energy or other macronutrient intakes. Estimated amounts of dietary exchangeable fat from oil, butter and fat spreads, dairy foods, and snacks were calculated using data from the National Diet and Nutrition Survey (NDNS) (y 1 to 4) in UK adult men aged 19-64 y [21], and the Dietary Intervention and Vascular function (DIVAS) randomised controlled trial (RCT) [12] (*Table 1)*. These estimates were then converted into servings of common commercially available cooking oils and fat spreads, dairy foods, and sweet and savoury snacks that participants were required to consume daily to achieve the nutrient targets in each dietary intervention period (*Table 2*).

To achieve the exchange of dietary fat, the RISSCI-1 sequential dietary intervention consisted of two, 4-week, isoenergetic, moderate-fat diets (34% TE from fat). The first intervention period was a high-SFA diet (target%TE SFA:MUFA:PUFA = 18:12:4), and the second intervention period was a low-SFA, high-MUFA/PUFA diet (target%TE SFA:MUFA:PUFA = 10:14:10). Both 4-week diets were otherwise broadly matched for other macronutrients, and aimed to comply with the COMA 1991 recommendations which stated n-6 PUFA should not exceed 10%TE [7]. To reproduce a transition from a high intake of SFA to the lower intake representative of the UK public health guideline for SFA intake of no more than 10%TE with recommendations to replace with unsaturated fats, all participants received the high-SFA diet for the first 4-week period, followed by the low-SFA, high-MUFA/PUFA diet for the second 4-week period without a washout period.

Implementation of intervention diets

Participants were invited to attend three study visits: at baseline upon inclusion (week 0), after completing the high-SFA diet (week 4), and low-SFA diet (week 8). At the first two study visits, participants were provided with a detailed information booklet containing instructions on how to comply with the high-SFA or low-SFA dietary guidelines, along with tailored recommendations to suit their lifestyle (e.g. meals out of the home, cooking for the family meal ideas and recipes). To improve compliance, participants also received free-of-charge study food items to incorporate into their baseline diets. Supplied food items included fat spreads, cooking oils, and an assortment of sweet and savoury snacks in sufficient quantity for each 4-week dietary intervention period. Due to their shorter shelf-life, dairy foods such as milk and cheese were not supplied, and participants were instructed to purchase these foods. All the intervention foods were commercially available from major UK supermarkets.

Table *4.*1. Identified sources of dietary exchangeable fat in the RISSCI-1 food exchange model [a]

	Total Energy	Total Fat		SFAs		MUFAs		PUFAs	
	MJ/d	g/d	%TE	g/d	%TE	g/d	%TE	g/d	%TE
Total baseline intake (including alcohol) [b]	8.80	77.7	32.8	28.4	11.9	28.5	12.0	13.4	5.7
Sources of exchangeable fat									
Added oils [c]	0.38	8.7	3.7	0.8	0.3	3.2	1.4	1.5	0.6
Added fats (butter and spreads)	0.29	7.8	3.3	2.8	1.2	2.9	1.2	1.4	0.6
Milk	0.44	4.3	1.8	2.7	1.2	1.1	0.5	0.1	<0.1
Cheese	0.26	5.0	2.1	3.0	1.3	1.3	0.6	0.2	<0.1
Sweet and savoury snacks[d]	0.86	9.9	4.2	3.8	1.6	3.4	1.5	1.6	0.7
Total exchangeable fat intake	2.15	35.8	15.3	13.1	5.6	12.0	5.1	4.8	2.1
Non-exchangeable fat intake	6.65	41.9	17.9	15.3	6.5	16.5	7.1	8.6	3.7

Abbreviations:%TE, % total energy; **MJ/d**, megajoules/day; **SFAs**, saturated fatty acids; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **RISSCI-1**, Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1.

[a] Adapted from Weech *et al.* [12]

[b] Calculation based on the National Diet and Nutrition Survey (y 1 to 4) in men aged 19-64y [21].

[c] Calculation based on the Dietary Intervention and Vascular function (DIVAS) randomised controlled trial [12]

[d] Included biscuits, buns, cakes, pastries, fruit pies, savoury snacks, and chocolate.

Table *4*.2. Recommended daily servings of intervention food items for the achievement of the RISSCI-1 dietary fat exchange.

Intervention food item	High-SFA diet		Low-SFA diet	
	Description	*Recommended amount (g/d)*	*Description*	*Recommended amount (g/d)*
Fat spreads	Salted butter [a]	14	Vegetable fat spread [a,b]	17
Cooking fats	Salted butter [a]	6	Sunflower oil [a]	11
Cheese or yogurt	Cheese with ≥ 25% fat, or full-fat yogurt	25 (cheese) or 100 (yogurt)	Cheese with < 25% fat, or virtually fat free yogurt	25 (cheese) or 100 (yogurt)
Milk	Full fat or semi-skimmed	200	< 1% fat	200
Snacks	Chocolates, biscuits, and crackers [a]	50	Crisps and nuts [a]	50

Abbreviations: **SFA**, saturated fatty acid; **MUFA**, monounsaturated fatty acid; **PUFA**, polyunsaturated fatty acid; **RISSCI-1**, Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1.

[a] Food items provided by researchers. Items provided for the high-SFA diet included: Wyke Farms "Salted Butter", Whitworths "Banana Chips", McVitie's "Gold Bar", Mrs Crimble's "Big Choc Macaroon", McVitie's "Trio Toffee Biscuit bar", Sainsbury's "Belgian Chocolate Chunk Shortbread", Tunnock's "Caramel Wafer", Sainsbury's "Cheddar Cheese Crispies", Arden's "Cream Cheese and Spring Onion Melts", and Jacob's "Savours Sweet Chilli Thins Crackers". Items provided for the low-SFA diet included: Flora "Buttery Spread", KTC "100% Sunflower Seed Oil", Tesco "Crispy Seedy Nutty Bites", Sainsbury's "Unsalted Mixed Nuts and Raisins", Tesco "Sweet Chilli Coated Peanuts", Sesame Snaps ®, Tesco "Bombay Mix", Nik Naks "Nice & Spicy Corn Snacks", Tesco "Ready Salted Crisps", Walkers "Max Paprika Crisps", and Pringles "Original Crisps".

[b] 79% vegetable fat spread with 5% sunflower oil and 24% rapeseed oil.

To ensure compliance to dietary guidelines, each dietary intervention period was scheduled outside of major holiday periods (e.g. Christmas and Easter), and participants were required to avoid any extended periods away from their home. Participants were also asked to return any leftover study items from the high-SFA diet before starting the low-SFA dietary intervention period. To help incorporate the study foods into their usual diet, and to assess compliance, participants were provided with daily tick sheets to be completed throughout each intervention period. Participants were free to consume the provided food items either as part of their main meals or at any other time of day. Participants were also permitted to consume more than the minimum required daily servings of any study food items, if their habitual intake exceeded the recommended amount for the intervention and if they were maintaining a stable body weight (± 1 kg from week 0). The importance of the latter was emphasised to the participants at follow-up visits at the mid-point of each dietary intervention (weeks 2 and 6). During these short visits, daily tick sheets were reviewed, and participants were supplied with any additional study food items required to complete the remainder of the intervention period. If body weight varied by greater than 1 kg from baseline or the previous study visit, participants were advised to reduce or increase their consumption of the provided snacks or other food items as appropriate.

Collection of dietary data

Participants were instructed to complete a 4-day weighed diet diary, a week before each study visit, to assess their baseline, habitual dietary intake (week -1), and during each dietary period to assess compliance to the interventions (weeks 3-4 and 7-8). Each diet diary included 3 weekdays and 1 weekend day during which participants were provided with digital scales to record the amount and description of all food items and beverages consumed. To improve the accuracy of the diet diaries, participants received additional diary templates to record all individual ingredients used in homemade recipes, along with published food portion tables to record foods consumed outside of the home [22]. Researchers assessed the completion and accuracy of the diet diary during each study visit, and requested any additional information necessary to improve data entry precision.

Paper diet diaries were analysed using Nutritics Research Edition v5.64 (Dublin, 2019) to assess foods consumed and nutrient intakes. Every item consumed was matched to its closest equivalent in the McCance and Widdowson's Composition of Foods Integrated Dataset (CoFID)[23], which was used to calculate daily dietary consumptions of total energy, and selected macro- and micro-nutrients: protein, carbohydrate, free sugars, Association of Analytical Chemists (AOAC) fibre, alcohol, total fat, SFAs, MUFAs, PUFAs, n-3 PUFAs, n-6 PUFAs, *trans* fatty acids (TFAs), cholesterol, and sodium. In addition, researchers used the NDNS Rolling Programme nutrient databank to impute missing values

of n-3/n-6 PUFAs in food items contributing to at least 1 g of PUFAs in each diet diary [21]. Food items consumed (in g/d) were classified into 40 food categories (*supplementary table 1*), which were used to assess dietary patterns.

Assessment of underestimation of energy consumption

Underestimation of dietary TE at baseline and during each dietary intervention periods was checked by the method proposed by Black [24]. Researchers estimated the basal metabolic rate of each participant using the Henry equations for men, based on age and body weight [25]. On the basis of a sedentary lifestyle (physical activity level score of 1.2 [12]), the lower 95% confidence limit of the Goldberg cut-off to identify under-reporters of dietary TE was estimated to lie between 1.13 and 1.16.

Phospholipid fatty acid analyses

Blood was collected into EDTA vacutainers after an overnight fast (12 hours) at baseline (week 0) and at the end of each dietary intervention period (weeks 4 and 8). After collection, vacutainers were chilled on ice for 20 min before centrifugation at 1750 x g (3000 rpm) for 15 min at 4°C for the collection of plasma, which was stored at -80°C before subsequent analysis.

The extraction of fatty acids methyl esters (FAME) from plasma PL was performed using a 3-step protocol (i.e. lipid extraction, solid phase extraction and transmethylation) based on methods from Metges *et al.* [26], Kaluzny *et al*. [27], and Baylin *et al.* [28]. Briefly, plasma lipids were extracted using a tert-butyl methyl ether (MTBE)/methanol solution and PL were eluted in methanol using solid phase extraction on aminopropyl-silica columns (Chromabond, MachereyNagel GmbH & Co. KG, Düren, Germany). Dried PL were then suspended in 200 µL of toluene and 15 µL of trimethyl sulfonium hydroxide solution (TMSH, 0.2 mol/L in methanol, Macherey-Nagel, 701 520.101) to obtain fatty acid methyl esters. FAMEs were separated using a gas chromatograph (GC) (Agilent 7890A, Agilent Technologies, Waldbronn, Germany) and flame ionization detector (FID) equipped with a 100m capillary column (HP-88, 100 m x 0.25 mm I.D., 0.2 µm film thickness, Agilent). Finally, FAMEs were identified against a standard mixture of 37 FAMEs (Supelco™) containing FAMEs of chain-length between C4-C24. In subsequent analyses, fatty acid concentrations were calculated as weight percentage of total fatty acids detected (wt%). Inter-assay coefficients of variation (n=10) were all below 6.4% (range 0.5% to 6.4%).

Measurement of anthropometrics and physical activity levels

The evening before each study visit (weeks 0, 4, and 8), participants were asked to consume a supplied, low-fat meal (< 1.5 MJ and < 7 g total fat content) with low-nitrate water (Buxton Mineral Water, Nestlé Waters, Buxton, UK) and to fast overnight for at least 12 hours consuming only the low-nitrate

water provided. On the morning of the study visit, researchers recorded height (to the nearest 0.1 cm), body weight (to the nearest 0.1 kg), and calculated the BMI of each participant using a wall-mounted stadiometer and a Tanita BC-418 (Reading) or Tanita BC-420MA (Surrey) digital scale (Tanita Europe). An allowance of 1 kg was included for light clothing when assessing body weight, and the digital scale was operated under the "standard body type" setting. Physical activity habits were assessed through the participants' completion of the International long version of the Physical Activity Questionnaire (IPAQ), and physical activity levels were classified into three categories (i.e. "Low", "Moderate", and "High") using the IPAQ guidelines for categorisation [29].

Power calculations and statistical analyses

A required sample size of 92 participants was estimated for the detection of a 0.16 mmol/L (SD 0.54) difference in fasting LDL-C concentrations (primary outcome in the main RISSCI-1 study) between the high- and low-SFA diets, as observed in the DIVAS parallel RCT [30], with an 80% statistical power and a 5% significance level. After accounting for a 15% dropout rate, this increased to a total of 106 participants. A sample size of 106 participants was also adequate for the investigation of PL-FA responses to the interventions. In this study, the successful replacement of dietary SFAs with MUFAs/PUFAs was expected to decrease the abundance of total SFAs in plasma PL-FAs by an estimated 0.46% of area of total PL-FAs (SD 0.8) [12], leading to a required sample size of 30 participants (i.e. n=26 participants for a detection with an 80% statistical power and a 5% significance levels, and n=4 participants to allow for a 15% dropout).

Since the RISSCI-1 dietary intervention was isoenergetic, the stability of BMI throughout the intervention was assessed using a linear mixed model which included age (continuous, y), study visit (week 0, week 4, or week 8), and study centre (University of Reading, University of Surrey) as fixed effects, and participants as a random effect. Daily average nutrient intakes from 4-day diet diaries and plasma PL-FA concentrations were compared between the high-SFA diet (week 4) and the low-SFA diet (week 8) using paired *t*-tests. All variables were checked for normality and log-transformed if necessary. In the case of alcohol consumption, *t*-tests were performed on alcohol consumers only and non-consumers were excluded from statistical analyses.

Furthermore, food categories and plasma PL-FA concentrations during the high-SFA and low-SFA diet were analysed using orthogonal partial least square discriminant analyses (OPLS-DA) to identify dietary patterns and circulating FA profiles in response to the RISSCI-1 dietary intervention [31,32]. All variables were mean-centred and divided by their standard deviation (SD). Statistical significance of the OPLS-DA models was tested using internal cross-validation permutation tests (n=1000 permutations), and goodness of fit and predictive accuracy were assessed using the R^2Y and Q^2 values,

respectively. For the interpretation of the models, variable loadings scaled as correlations towards the predictive model (p(corr)) were used to identify the variables that contributed the most to the discrimination of dietary patterns or plasma PL-FA profiles between the high-SFA and the low-SFA diets.

In exploratory analyses, a constraint-based feature selection algorithm was used to identify plasma PL-FAs associated with dairy fat consumption [33]. This method is based on a forward-backward feature selection approach and aims to reduce the dimension of a given dataset by providing multiple statistically equivalent subsets of features with maximised predictive accuracy. In prospective analyses, plasma PL-FA concentrations were calculated as changes between the high-SFA diet (week 4), which was enriched in full-fat dairy foods, and baseline (week 0). In addition, cross-sectional analyses aimed to identify predictors of baseline dairy fat consumption among baseline concentrations of plasma PL-FAs. In both approaches, selected predictors among plasma PL-FAs were fitted in multiple linear regression models with adjustments for age (y), BMI (kg/m^2), baseline dairy fat consumption (g/d, in prospective models only), and energy intakes at baseline (kcal/d). Predictive R^2 coefficients were used to assess the predictive accuracy of multiple linear regression models. All statistical analyses were conducted in R (version 4.0.4), except from OPLS-DA models which were fitted in MetaboAnalyst version 5.0 [34].

4.3 Results

The flowchart of participants included in the RISSCI-1 study is presented in *Figure 4.1*. A total of 118 participants were enrolled to follow the first dietary intervention period (i.e. high-SFA diet), including 9 participants who withdrew from the study at the end of the first diet (n=6 due to time or work commitments, n=2 due to loss of interest in the study, n=1 due to newly prescribed medication). The remaining 109 participants completed both the first (high-SFA) and second dietary intervention period (low-SFA diet), giving an overall dropout rate of 7.6%.

Baseline characteristics of participants are presented in *Table 3*. Participants mean age was 48 y (SD 11), with a BMI of 25.1 kg/m^2 (SD 3.3). Participants were of Asian or UK Asian (7.3%), Black or UK Black (2.8%), Chinese (1.8%), Mixed Ethnic (1.8%), or White (86.2%) self-reported ethnic backgrounds. Finally, most participants had moderate or high, self-reported physical activity levels (31.2% and 47.7%, respectively).

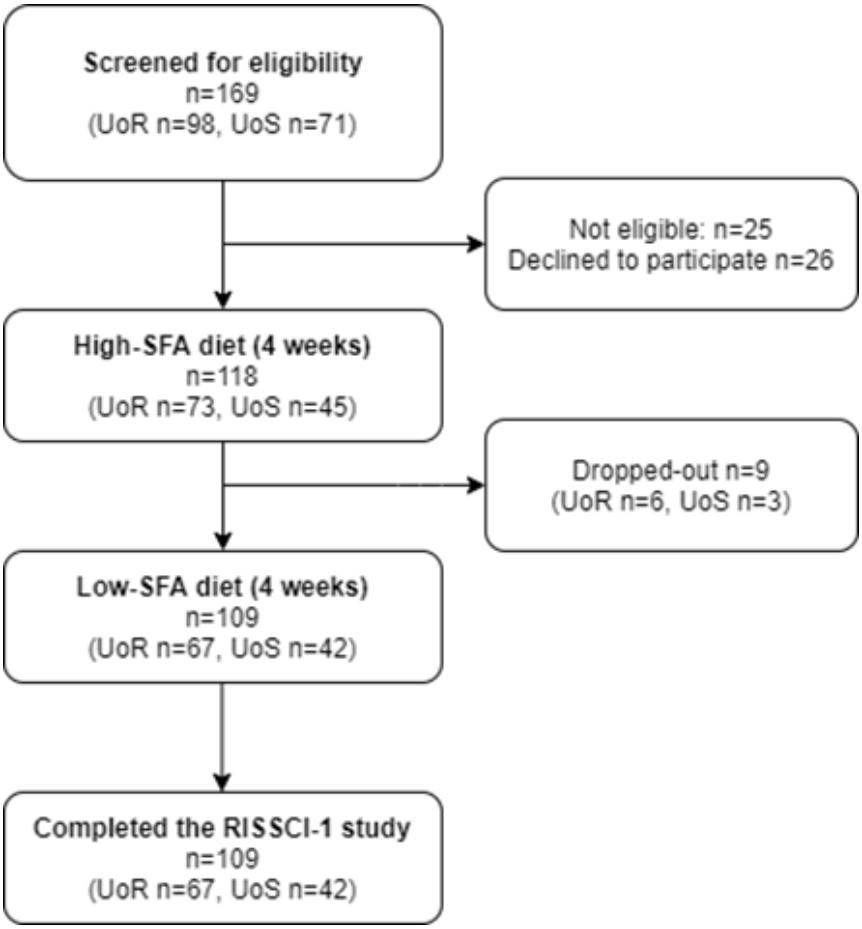

Figure 4.1. Flow-chart of participants from the RISSCI-1 study. *UoR, University of Reading; UoS, University of Surrey.*

Dietary consumption

Nutrient intakes during each dietary intervention period are shown in *Table 4*. Out of the 109 participants who completed the RISSCI-1 study, nine were excluded from the dietary analyses due to insufficient or incomplete dietary data. There were no significant differences between the dietary energy, macronutrients (total fat, carbohydrates, and proteins), AOAC dietary fibre or alcohol consumption during the high-SFA and low-SFA diets. Data on average daily nutrient consumption indicated a successful exchange of dietary SFAs for MUFAs and PUFAs during the second dietary intervention period, with dietary SFA consumption decreasing from 19.1%TE (SD 3.5) during the high-SFA diet to 8.9%TE (SD 2.1) during the low-SFA diet ($p < 10^{-3}$). The observed decrease in SFA intake was compensated for by a rise in MUFA and PUFA consumptions from 11.1%TE (SD 2.8) and 3.7%TE (SD 1.3), respectively during the high-SFA diet to 13.4%TE (SD 2.9), and 11.1%TE (SD 3.6) during the low-SFA diet (both $p < 10^{-3}$). In addition, participants consumed less TFAs ($p < 10^{-3}$), dietary cholesterol ($p < 10^{-3}$), and sodium ($p=0.04$) during the low-SFA diet compared to the high-SFA diet.

Table 4.3. Baseline characteristics of adult men from the RISSCI-1 study (n=109).

	Mean	SD
Age, *y*	48.4	10.8
Self-reported ethnicity, n (*%*)		
Asian or UK Asian	8 (7.3)	
Black or UK Black	3 (2.8)	
Chinese	2 (1.8)	
Mixed Ethnic Background (not specified)	2 (1.8)	
White	94 (86.2)	
BMI, *kg/m²*	25.1	3.3
Physical activity level, n (*%*) [a]		
Low	6 (5.5)	
Moderate	34 (31.2)	
High	52 (47.7)	
Missing	17 (15.6)	
Total energy		
kcal/d	2320	635
MJ/d	9.7	2.7
Total fat,%TE	36.2	7.8
SFAs,%TE	12.7	3.8
MUFAs,%TE	13.3	3.5
***n-3* PUFAs,%TE**	0.8	0.4
***n-6* PUFAs,%TE**	4.6	1.8
Total PUFAs,%TE	5.8	2.1
TFAs, %TE	0.5	0.3
Cholesterol, *mg/d*	235	116
Protein,%TE	16.3	3.3
Carbohydrates,%TE	44.3	9.4
Free sugars,%TE	7.6	4.8
Dietary fibre (AOAC), *g/d*	25.8	9.5
Alcohol,%TE [b]	4.0	(1.4-7.7)
Sodium, *g/d*	2.6	1.0

Abbreviations: **AOAC**, Association of Analytical Chemists; **BMI**, body mass index; **d**, day; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **RISSCI-1**, Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1; **SD**, standard deviation; **SFAs**, saturated fatty acids; **TFAs**, trans fatty acids;%TE, % total energy

[a] Categories derived from the International Physical Activity Questionnaire (IPAQ) [29].

[b] Values presented as *median (interquartile range)* and based on n=45 participants who consumed alcohol (n=55 non-consumers).

Table 4.4. Recorded and target daily nutrient intakes following each dietary intervention period (high-SFA and low-SFA diets) in adult men from the RISSCI-1 study (n=100).

	High-SFA Diet			Low-SFA Diet			
	Target	**Mean**	**SD**	**Target**	**Mean**	**SD**	**p [a]**
Total energy							
kcal/d		2354	546		2282	558	0.13
MJ/d		9.9	2.3		9.6	2.3	0.14
Total fat,%TE	34.0	38.4	6.5	34.0	38.2	6.6	0.79
SFAs,%TE	18.0	19.1	3.5	10.0	8.9	2.1	$< 10^{-3}$
MUFAs,%TE	12.0	11.1	2.8	14.0	13.4	2.9	$< 10^{-3}$
***n-3* PUFAs,%TE**		0.6	0.4		1.2	0.5	$< 10^{-3}$
***n-6* PUFAs,%TE**		2.5	1.0		9.5	3.5	$< 10^{-3}$
Total PUFAs,%TE	4.0	3.7	1.3	10.0	11.1	3.6	$< 10^{-3}$
TFAs,%TE		0.8	0.3		0.2	0.2	$< 10^{-3}$
Cholesterol, *mg/d*		273	112		201	166	$< 10^{-3}$
Protein,%TE		16.0	3.0		16.3	3.1	0.28
Carbohydrates,%TE		42.6	7.9		42.9	8.0	0.61
Free sugars,%TE		5.0	3.9		4.7	3.2	0.35
Dietary fibre (AOAC), *g/d*		24.4	10.3		25.9	11.9	0.06
Alcohol,%TE [b]		4.5	(2.2-6.2)		3.6	(2.0-5.6)	0.83 [c]
Sodium, *g/d*		2.67	0.88		2.45	0.91	0.04

Abbreviations: **AOAC**, Association of Analytical Chemists; **d**, day; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **RISSCI-1**, Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1; **SD**, standard deviation; **SFAs**, saturated fatty acids; **TFAs**, trans fatty acids;%TE, % total energy

Means and SD based on n = 100 participants, unless specified otherwise.

[a] From paired *t*-tests.

[b] Values presented as *median* (interquartile range) and based on n=45 participants who consumed alcohol (n=55 non consumers).

[c] From paired T-test on log-transformed values between the high-SFA and low-SFA diets.

Energy balance

There was no statistically significant impact of the dietary interventions on participants' BMI (p=0.7 for the high-SFA diet, and 0.1 for the low-SFA diet, compared to baseline). Estimated marginal means for BMI at baseline, following the high-SFA diet, and following the low-SFA diet were 25.1 kg/m^2 (95%CI 24.4-25.7), 25.1 kg/m^2 (95%CI 24.4-25.7), and 25.0 kg/m^2 (95%CI 24.4-25.7), respectively. The proportions of under-reporters of energy intake at baseline, following the high-SFA diet and following the low-SFA diet were estimated at 28%, 17%, and 27%, respectively, based on the assumption that participants remained in energy balance throughout the study.

Analysis of plasma PL-FAs

Relative concentrations of plasma PL-FAs after each 4-week dietary intervention period are shown in *Table 5*. All plasma PL-FA concentrations were significantly different between the high-SFA and low-SFA diets apart from those of elaidic acid (18:1 n-9 *trans*), γ-linolenic acid (18:3 n-6), and α-linolenic acid (18:3 n-3). Overall, plasma PL samples after the low-SFA diet had lower abundances of 16 individual and classes of plasma PL-FAs which included palmitic acid (16:0, difference between high-SFA and low-SFA diet (Δ) =-1.23 wt%, p <10^{-4}), total SFAs (Δ=-0.84 wt%, p <10^{-4}), n-3 PUFAs (Δ=-0.52% total FA, p <10^{-4}), dihomo-γ-linolenic acid (20:3 n-6, Δ=-0.41 wt%, p <10^{-4}), and total MUFAs (Δ=-0.31 wt%, p <10^{-2}), but higher abundances of 10 individual and classes of plasma PL-FAs, which included linoleic acid (18:2 n-6, Δ=1.87 wt%, p <10^{-4}), n-6 PUFAs (Δ=1.69 wt%, p <10^{-4}), total PUFAs (Δ=1.15 wt%, p <10^{-4}), stearic acid (18:0, Δ=0.53 wt%, p <10^{-4}), and arachidonic acid (20:4 n-6, Δ=0.31 wt%, p <10^{-2}).

In OPLS-DA of the plasma PL-FA abundances during the high-SFA and low-SFA diets, the first component of the model, which explained 13.6% of the total variation, was retained for interpretation (*Figure 4.2A*). The OPLS-DA, which aimed to discriminate plasma PL-FA profiles specific to each dietary intervention period, revealed moderate fitness (R^2Y=0.66, empirical permutation p < 0.01 (0/1000)) and predictive accuracy (Q^2=0.57, empirical permutation p < 0.01 (0/1000)). As shown in *Figure 4.2B*, discriminating plasma PL-FAs during the high-SFA diet included pentadecanoic acid (15:0, p(corr)=0.72), *trans* vaccenic acid (18:1 n-7 *trans*, p(corr)=0.69), palmitic acid (16:0, p(corr)=0.58), myristic acid (14:0, p(corr)=0.46), and n-6 docosapentaenoic acid (22:5 n-6, p(corr)=0.38). In contrast, the low-SFA plasma PL-FA profile showed higher abundances of eicosenoic acid (20:1 n-9, p(corr)= -0.63), arachidic acid (20:0, p(corr)= -0.60), behenic acid (22:0, p(corr)= -0.48), linoleic acid (18:2 n-6, p(corr)= -0.41), and stearic acid (18:0, p(corr)= -0.36).

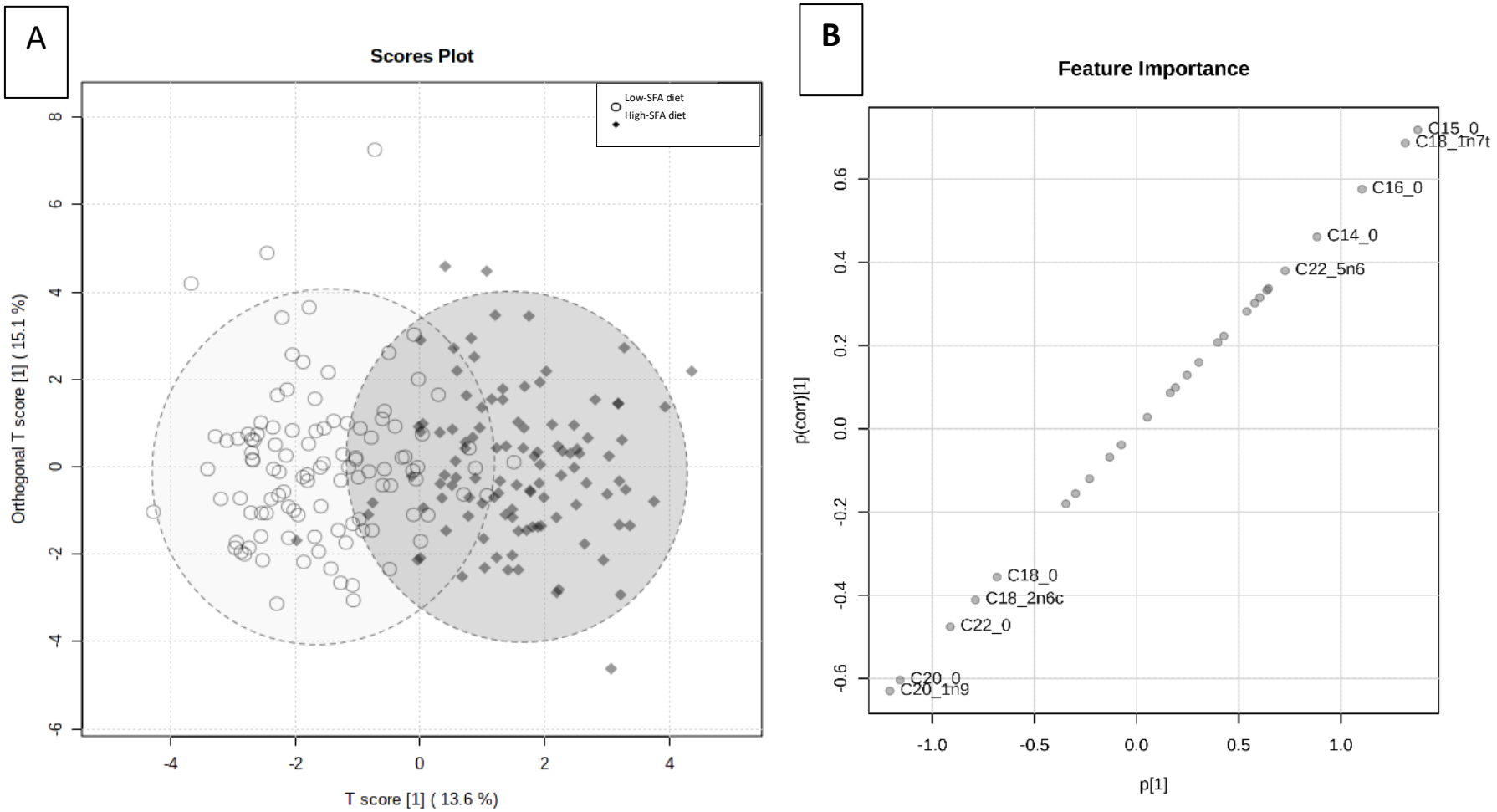

Figure 4.2. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) based on plasma phospholipid fatty acids (PL-FAs) in adult men from the RISSCI-1 study between the high-SFA and the low-SFA diets (n=108). A: *Scores plot showing a moderate discrimination between two PL-FA profiles during the high-SFA and low-SFA diets.* ***B:*** *Feature loadings scaled as correlation coefficients (p(corr)[1]) towards the OPLS-DA predictive component (p[1]), showing the individual PL-FAs contributing to each discriminated FA profile.*

Table 4.5. Fasting abundances of plasma phospholipid fatty acids following the low-SFA and high-SFA diets in adult men from the RISSCI-1 study (n=108).

Fatty acid abundances	High-SFA Diet		Low-SFA Diet		Δ [a]		
(wt%)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p [b]**
Total SFAs	46.0	0.9	45.1	1.1	-0.84	0.90	$< 10^{-4}$
14:0	0.55	0.12	0.46	0.11	-0.09	0.12	$< 10^{-4}$
15:0	0.28	0.05	0.21	0.04	-0.06	0.04	$< 10^{-4}$
16:0	30.3	1.2	29.0	1.3	-1.23	1.20	$< 10^{-4}$
17:0	0.44	0.06	0.42	0.06	-0.02	0.04	$< 10^{-4}$
18:0	14.3	1.0	14.9	1.0	0.53	0.74	$< 10^{-4}$
20:0	0.09	0.01	0.11	0.02	0.02	0.02	$< 10^{-4}$
22:0	0.03	0.01	0.03	0.01	0.01	0.01	$< 10^{-4}$
Total MUFAs	12.6	1.3	12.3	1.3	-0.31	1.11	$< 10^{-2}$
16:1 n-7 *cis*	0.52	0.21	0.42	0.18	-0.09	0.13	$< 10^{-4}$
18:1 n-9 *cis*	10.2	1.2	9.9	1.2	-0.26	1.02	$< 10^{-2}$
18:1 n-7 *cis*	1.43	0.20	1.49	0.22	0.06	0.17	$< 10^{-4}$
20:1 n-9	0.18	0.04	0.23	0.05	0.05	0.04	$< 10^{-4}$
16:1 n-7 *trans*	0.01	0.00	0.01	0.00	-0.003	0.004	$< 10^{-4}$
18:1 n-9 *trans*	0.15	0.04	0.15	0.04	0.004	0.040	0.37
18:1 n-7 *trans*	0.18	0.06	0.11	0.04	-0.07	0.06	$< 10^{-4}$
Total PUFAs	41.4	1.6	42.5	1.6	1.15	1.34	$< 10^{-4}$
20:3 n-9	0.15	0.04	0.13	0.04	-0.02	0.05	$< 10^{-3}$
Total PUFAs n-6	35.5	2.1	37.1	2.0	1.69	1.73	$< 10^{-4}$
18:2 n-6 *cis*	21.4	2.5	23.2	2.4	1.87	1.75	$< 10^{-4}$
18:3 n-6	0.09	0.05	0.09	0.05	-0.005	0.041	0.26
20:2 n-6	0.33	0.05	0.34	0.06	0.01	0.05	0.01
20:3 n-6	3.38	0.83	2.97	0.74	-0.41	0.52	$< 10^{-4}$
20:4 n-6	9.70	1.71	9.99	1.86	0.30	0.97	$< 10^{-2}$
22:4 n-6	0.35	0.08	0.32	0.09	-0.03	0.04	$< 10^{-4}$
22:5 n-6	0.20	0.06	0.16	0.06	-0.04	0.03	$< 10^{-4}$
18:2 n-6 *trans*	0.06	0.01	0.06	0.01	-0.002	0.007	$< 10^{-2}$
Total PUFAs n-3	5.76	1.49	5.23	1.19	-0.52	0.92	$< 10^{-4}$
18:3 n-3	0.22	0.07	0.22	0.08	0.00	0.07	0.53
20:5 n-3	1.25	0.69	0.99	0.53	-0.26	0.48	$< 10^{-4}$
22:5 n-3	1.08	0.20	0.95	0.20	-0.12	0.14	$< 10^{-4}$
22:6 n-3	3.21	0.90	3.07	0.79	-0.14	0.50	$< 10^{-2}$

Abbreviation: **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **RISSCI-1**, Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1; **SD**, standard deviation; **SFAs**, saturated fatty acids; **wt%**, weight percentage of total fatty acids.

[a] Δ = low-SFA – high-SFA values.
[b] from paired *t*-tests.

Analysis of dietary patterns

For the recorded consumption of 40 food categories during the high-SFA and low-SFA diets, the first component of the model (OPLS-DA) was retained for the discrimination of dietary patterns during the two diets, and explained 7.5% of the overall variation (*Figure 4.3A*). The retained model showed adequate fitness (R^2Y=0.82, empirical permutation p < 0.01 (0/1000)) and predictive accuracy (Q^2=0.68, empirical permutation p < 0.01 (0/1000)). As shown in *Figure 4.3B*, the high-SFA dietary pattern was characterised by higher intakes of SFA-rich fat (correlation scaled loading p(corr)=0.89), full-fat dairy foods (p(corr)=0.57), and biscuits and cakes (p(corr)=0.27). In contrast, the low-SFA dietary pattern was characterised by higher consumptions of MUFA-rich fat (p(corr)= -0.80), PUFA-rich fat (p(corr)= -0.71), nuts (p(corr)= -0.63), savoury snacks (p(corr)= -0.31), and low-fat dairy (p(corr)= -0.23). Other food categories, such as cereals and grains, meats, fish, or fruits and vegetables, did not contribute significantly to the dietary pattern discrimination between the low-SFA and high-SFA diets.

Associations between dairy consumption and plasma PL-FAs

In accordance with the dietary fat exchange model developed for the RISSCI-1 study (*Table 1*), dietary intakes from the 4-day weighed diet diaries showed that total dairy foods were important contributors of total fat (39.6%, SD 11.5) and SFA consumption (50.1%, SD 12.6) during the high-SFA diet compared to baseline (16.6% SD 11.4 for total fat, and 28.5% SD 17.5 for SFAs) (*supplementary table 2*).

Prospective constraint-based feature selection analyses identified two independent predictors of changes in dairy fat consumption among plasma PL-FAs after the high-SFA diet compared to baseline: pentadecanoic acid (15:0) and *trans* vaccenic acid (18:1 n-7 *trans*). In prospective multiple linear regression models between the end of the high-SFA diet and baseline (n=104 participants), each additional 1% (%wt total FA) of pentadecanoic acid abundance in PL-FAs was associated with a 158 g/d increase in the reported intake of dairy fat (95% CI 81-235, $p < 10^{-3}$). In a separate linear regression model, each additional unit of circulating *trans* vaccenic acid was associated with an increase of 84 g/d of reported dairy fat intake (95%CI 26-142, p=0.005). In addition, the linear regression model based on pentadecanoic acid abundance had a slightly better predictive accuracy (predictive R^2=0.27) than the model based on *trans* vaccenic acid (predictive R^2=0.21).

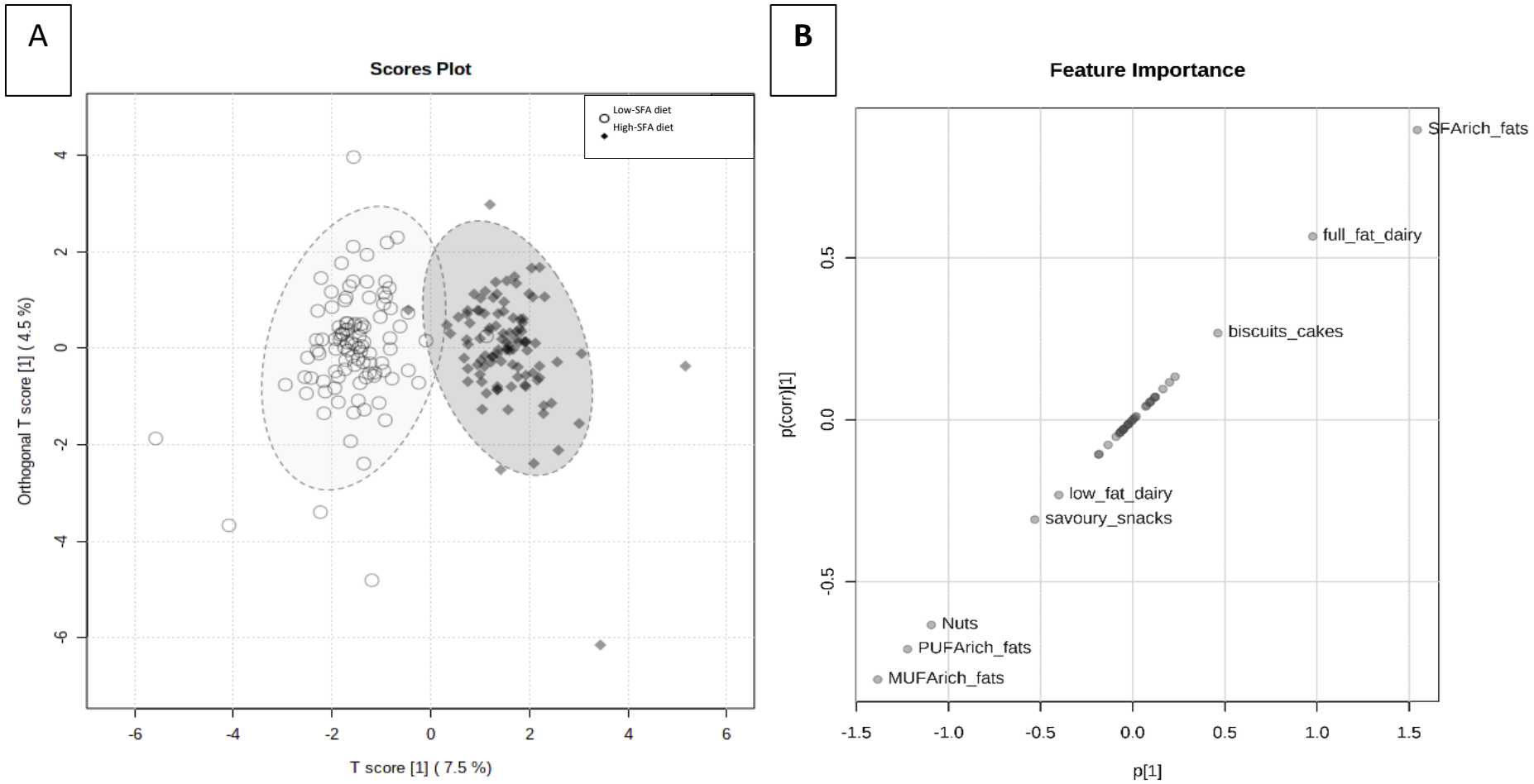

Figure 4.3. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) based on dietary intakes in adult men from the RISSCI-1 study between the high-SFA and the low-SFA diets (n=100). A*: Scores plot showing the discrimination between two dietary patterns during the high-SFA and low-SFA diets.* ***B:*** *Feature loadings scaled as correlation coefficients (p(corr)[1]) towards the OPLS-DA predictive component (p[1]), showing the food groups contributing to each discriminated dietary pattern.*

In cross-sectional analyses of baseline data (n=106), pentadecanoic acid and trans vaccenic acid were also identified as two independent predictors of dairy fat consumption. However, linear regression models for both pentadecanoic acid (β=92 g/d of reported dairy fat, 95%CI 42-142, p <10-3) and trans vaccenic acid (β=100 g/d of reported dairy fat, 95%CI 50-150, p <10-3) showed weaker prediction accuracy, compared to prospective models (predictive R2=0.10 for pentadecanoic acid, and 0.12 for trans vaccenic acid).

4.4 Discussion

The analyses of 4-day weighed diet diaries and plasma PL-FA profiles confirmed that the participants reached the nutritional targets set in our model, by reducing their consumption of dietary SFAs by 10.2%TE from the high-SFA diet to the low-SFA diet. This decrease in SFAs was mostly compensated by an increase in dietary MUFAs and PUFAs by 2.3%TE and 7.4%TE, respectively, while maintaining other macronutrient intakes. The exchange of dietary SFAs for UFAs was achieved without affecting total energy intake or BMI, which confirmed that participants remained in energy balance throughout the study. In addition, discriminant analyses of dietary patterns constituted a novel method of confirming compliance to the RISSCI-1 dietary guidelines, by showing that participants integrated the recommended and supplied study foods into their diets to exchange dietary SFAs for UFAs, without modifying their overall dietary patterns (e.g. via changes in intakes of meat, fish, cereals and grains, fruits, and vegetables).

The analysis of plasma PL-FAs during the two dietary intervention periods provides further evidence in support of the successful implementation of the RISSCI-1 dietary fat exchange, by revealing a 0.84 wt% decrease in total SFAs, 0.31 wt% decrease in total MUFAs, and 1.15 wt% increase in total PUFAs during the low-SFA compared to the high-SFA diet. The rise in plasma PL PUFAs during the low-SFA diet was driven by n-6 PUFAs (1.70 wt% increase), whereas circulating n-3 PUFAs decreased by 0.53 wt%. These results reflect the type of dietary fat consumed during the two diets, albeit on a much smaller scale, and with the caveat that even-chain SFAs and UFAs are subject to endogenous synthesis and oxidation in humans, limiting their reliability and utility as biomarkers of fat consumption [35]. In this respect, it is noteworthy that while total circulating palmitic acid has been reported to be associated with dietary intakes of carbohydrates and alcohol [36,37], intakes of these macronutrients in the current study were not significantly different between the diets.

Furthermore, dietary analyses revealed small but significantly higher intakes of dietary TFAs and cholesterol during the high- compared to the low-SFA diet (decreases in 0.6%TE and 72mg during the low-SFA diet, respectively). Since the abundance of elaidic acid (a *trans* FA mostly found in industrially

processed food) in plasma PL did not differ between the high- and low-SFA diets, these differences may be explained by the guidelines to consume full-fat dairy foods and butter during the high-SFA diet, which contain naturally occurring ruminant *trans* FAs and cholesterol [38,39]. However, participants remained well below the dietary reference value for TFAs of 2%TE [7], and small variations in dietary cholesterol (i.e. equivalent to less than that from a single egg yolk [23]) are unlikely to impact on plasma LDL-C. Moreover, current epidemiological evidence suggests that TFAs from dairy may not be associated with deleterious cardiometabolic outcomes as opposed to industrial TFAs [40,41]. Similarly, higher sodium intakes were observed during the high-SFA diet compared to the low-SFA diet. This may reflect the dietary guidelines for this diet, which recommended daily servings of salted butter and cheese with higher salt content (e.g. Cheddar and Red Leicester) than those recommended during the low-SFA diet (e.g. cottage cheese and spreadable cream cheese). On average, study participants exceeded UK dietary recommendations for sodium of 2.4 g/d (6 g/d salt) at baseline and throughout the RISSCI-1 dietary intervention, but remained below the national average for men aged 19-64y which was estimated at 3.7 g/d (SD 1.7) in 2020 [42].

The dietary fat exchange model developed for this study used dairy as a key food group for the exchange of dietary SFA. Indeed, dairy foods represent an important entry point for SFA in the food chain as on average, they contribute 21% of dietary SFA intake in UK adults [14]. Nonetheless, and despite their SFA content, epidemiological evidence suggests an inverse or neutral association between dairy food consumption and cardiometabolic disease risk [43,44]. This may stem from beneficial components and food matrix effects specific to some types of dairy food, such as bioactive peptides, fermentation process, or calcium-dependent fat sequestration [45]. These effects have not been demonstrated with butter, which may explain the detrimental associations observed between its consumption and cardiometabolic health outcomes [46,47]. Apart from butter, other sources of dietary SFA, such as red and processed meat products, may have detrimental effects on cardiovascular health [48,49] and were considered for the development of the previously implemented dietary fat exchange models [11]. However, a meat-based exchange of SFA was not achievable without compromising isoenergetic and equivalent macronutrient target intakes. In this context, the use of low-fat dairy products to reduce dietary SFA intakes in this study presents several advantages, as it helped avoid the exchange of dietary fat impacting on the intake of other nutrients (e.g. bioactive peptides, calcium and iodine) and potentially beneficial dairy components.

The plasma PL-FA profile associated with the high-SFA diet was characterised by higher proportions of pentadecanoic acid (C15:0) and vaccenic acid (C18:1 n-7 *trans*). These two FAs have been previously used as biomarkers of dairy fat consumption, as odd-chain SFAs and ruminant TFAs are synthesised in the rumen of cows before being integrated into the fat fraction of dairy foods [19,38]. As plasma PL-FAs

are thought to reflect short to medium-term dietary FA consumption [12,16,17], the importance of these two FAs in the high-SFA diet plasma PL-FA profile may be explained by a higher consumption of full-fat dairy products, which contributed to 39.6% of dietary total fat and 50.1% of dietary SFAs during the high-SFA diet. The strong association between dairy fat consumption and pentadecanoic acid or vaccenic acid in plasma PL from the RISSCI-1 study participants was further confirmed in prospective and cross-sectional multiple linear regression models, which identified these FAs as two independent predictors of dairy fat consumption among the 25 other FAs measured in plasma PL. In particular, the large effect estimates observed in linear regression models suggested that a large amount of dairy fat would need to be consumed in order to observe a 1% increase (%wt total FA) in the abundance of pentadecanoic or *trans*-vaccenic acid in plasma phospholipids. These findings from plasma PL-FAs are consistent with those from previous RCTs, which reported moderate but consistent associations between total dairy consumption and circulating levels of pentadecanoic acid in serum or plasma total lipids [50–52]. However, these findings from the RISSCI-1 study provide novel evidence for the utility of vaccenic acid as a biomarker for dairy fat consumption, a ruminant TFA that has been previously under studied in intervention studies [19]. The predictive accuracy of circulating pentadecanoic or vaccenic acids as biomarkers of dairy fat consumption, reflected by the predictive R^2 value, was significantly improved when using prospective multiple regression models (i.e. changes between baseline and high-SFA diet) compared to cross-sectional models. This might provide an important area of future research for the use of these FAs in observational epidemiology studies, which often rely on a single measurement of dairy-specific FAs (e.g. pentadecanoic, heptadecanoic, or vaccenic acids) to investigate associations with mortality or incidence of cardiometabolic diseases [53–55].

In contrast to the high-SFA diet, the low-SFA diet was associated with higher abundances of long-chain MUFAs and n-6 PUFAs, such as eicosenoic and linoleic acids in plasma PL, which may reflect the increased dietary consumption of MUFAs and PUFAs from sunflower oil and vegetable spread during the low-SFA diet [16,17]. Moreover, the low-SFA plasma PL-FA profile was also characterised by higher concentrations of long-chain SFAs (i.e. ranging from 18 to 22 carbons). These results might be partly explained by the endogenous synthesis of long-chain SFAs in humans together with the fat composition of sunflower oil, vegetable spreads, and nut-based snacks recommended during the low-SFA diet, which contain very small amounts of long-chain SFAs [56,57]. In line with this hypothesis, a prospective study of changes in plasma PL-FA concentrations over 13 y among participants of the EPIC-Norfolk study reported that each additional 100 g/d of nut and seeds intake was associated with a 2.33% increase in plasma PL long-chain SFAs (20 to 24 carbons, 95%CI: 0.15-4.55) [58]. In addition, the low-SFA diet resulted in lower abundances of long-chain n-3 FAs in plasma PL. Since plasma PL-FA are expressed in relative (%wt) rather than absolute concentrations, the lower abundances of long-chain

n-3 PUFAs in plasma PL after the low-SFA diet might represent higher abundances of other FAs. In particular, this may reflect the exchange of dietary SFA with mostly n-6 PUFA, in line with the consistent evidence on replacing dietary SFA with n-6 PUFA for cardiovascular disease (CVD) risk prevention [8].

A major strength of the RISSCI-1 dietary intervention was its success in replacing dietary SFAs with UFAs from commonly available commercial foods in healthy, free-living men living in the UK. The reduction of dietary SFAs achieved in the dietary intervention exceeded public health guidelines by reducing dietary SFA consumption to below 10%TE [8]. The dietary intervention was also reported to be well received by the participants, on the basis of self-reports and low attrition rate. This may be explained, in part, by the wide range of commercially available food products recommended and supplied during each dietary intervention period, which facilitated compliance, and minimised disruption to the participants' habitual dietary habits.

Limitations of the dietary intervention included the use of self-reported dietary records, which may have influenced the eating behaviour of participants, and introduced bias towards healthier dietary patterns and under-reporting of energy intakes [59,60]. Such self-reporting bias may partly account for the moderate proportion of under-reporting of energy intakes among participants at baseline (28%) and during the low-SFA diet (27%), which were similar to that observed in previous dietary intervention studies in free-living participants [11–13]. Interestingly, under-reporting of dietary energy was much less prevalent during the high-SFA diet (17%), which might, in part, be explained by increased awareness of the importance of accurate dietary records after being enrolled in the study. However, this been attenuated throughout the course of the 8-week intervention, as reflected in the higher degree of under-reporting observed at the end of the study, which may reflect participants' fatigue. Moreover, dietary intakes were calculated using food composition databases, which could have introduced measurement errors through missing values and lack of diversity in food items. PUFAs (n-3 and n-6) were the main nutrients affected by this limitation, and their consumptions were estimated more accurately by using the NDNS nutrient databank [21] to complement missing data from the CoFID database [23]. In addition, since food composition databases did not allow for the reliable estimation of the intake of specific FAs, dietary SFAs were considered as a whole. Although specific SFAs are known to exert different effects on markers of CVD risk, such as serum LDL-cholesterol [61], this was not of immediate relevance to the outcomes reported here. Another possible limitation of this study included the 4-week duration of each dietary intervention, which may not have been sufficient for plasma PL-FA concentrations to stabilise and potentially led to carry-over effects from the high- to the low-SFA diet. In particular, such carry-over effects may have underestimated the changes in plasma PL-FA abundances between the two diets. However, the observed changes in

abundances of individual PL-FAs (*Table 5*) and patterns of PL-FAs (*Figure 4.2*) both align with the dietary guidelines provided and were sufficient to reveal differences between the two dietary interventions. Moreover, participants were healthy men, many with optimal BMI (between 18.5 and 24.9 kg/m^2, n=56, 52.8%), high self-reported physical activity levels (n=52, 47.7%), and white ethnic background (n=94, 86.2%), which may limit the generalisability of the study findings to a wider population. However, self-reported ethnicity from the RISSCI-1 closely match data from the 2011 Census in England and Wales [62]. Finally, the application of this food-exchange model in non-interventional 'real-life' settings may be affected by factors influencing food purchases, such as personal preference, financial and familial situations, as well as cultural background.

In conclusion, the RISSCI-1 dietary fat exchange model was successful in exchanging dietary SFAs for UFAs in healthy UK men, in accordance with current UK public health guidelines for adults. The replacement of dietary SFAs with UFAs, was based on commercially available foods and relied mostly on dairy foods, snacks, and cooking oil, and did not interfere with the overall dietary patterns of participants. Confirmation of the feasibility and efficacy of this food-based dietary exchange model will require its use in larger populations and intervention studies of longer duration.

4.5 Supplementary material

Supplementary table 4.1. Definition of food categories used to assess dietary patterns in the RISSCI-1 study.

Food category	Example items	Calculation details
Fruits	Banana, apple, berries, etc.	Canned, stewed, and dried fruit as equivalent weight of whole fruit, including fruit within composite dishes
Whole vegetables	Cucumber, tomatoes, spinach, etc.	All cooked or raw vegetables, including tomato puree as equivalent weight of whole vegetable.
Pulses	Lentils, beans, chickpeas, etc.	Equivalent cooked weight
Vegetarian processed foods and ready meals	Potato dishes, pizza, salads, egg dishes,	Weight as consumed
Soups	any vegetable soup, including meat or fish soups	Weight as consumed
Cooking sauces	Tomato sauce, creamy sauces, pesto, etc.	Weight as consumed
Sauces and stock	Gravy, chicken stock, etc.	Weight as consumed
Nut butters	Peanut butter, Tahini paste, etc.	Weight as consumed
Nuts	Walnuts, hazelnuts, etc.	Weight as consumed

Seeds	Sesame seeds, etc.	Weight as consumed
Red and processed meats, offals	Beef, lamb, cured meats, sausages, etc.	Equivalent cooked weight, excluding waste (e.g. bones)
Poultry	Turkey, chicken, etc.	Equivalent cooked weight, excluding wastage (e.g. bones)
Meat alternatives	Quorn, tofu, etc.	Weight as consumed
Red and processed meat dishes	Meat pies, meat curry dishes, etc.	Weight as consumed
White fish	Cod, plaice, etc.	Equivalent cooked weight
Oily fish	Salmon, mackerel, etc.	Equivalent cooked weight
Shellfish	Mussels, clams, crab, etc.	Excluding wastage (e.g. shells)
Fish dishes	Fish pies, breaded fish, etc.	Oily and white fish, and shellfish included
Full-fat dairy foods	Whole milk, medium and full-fat cheese, full-fat yogurts, dairy desserts.	Weight as consumed
Reduced-fat dairy foods	Semi-skimmed and skimmed milk, low-fat and fat free yogurts, low-fat cheese	Weight as consumed
Dairy alternatives	Plant-based milks, plant-based yogurts, etc.	Included fortified and non-fortified dairy alternatives
Eggs	All types of eggs	Weight as consumed
Refined grains	Pasta, rice, etc.	Equivalent cooked weight
Refined grain foods	Bread, flour, crackers, etc.	Weight as consumed
Whole grains	Pasta, rice, etc.	Equivalent cooked weight
Whole grain foods	Bread, flour, crackers, etc.	Weight as consumed
Oats	Porridge and rolled oats	Equivalent dry weight
Condiments	Vinegar, mustard, salad dressing, herbs, spices, etc.	Weight as consumed
MUFA-rich fat	Olive oil, vegetable fat spread	Weight as consumed
PUFA-rich fat	Sunflower oil, vegetable fat spread	Weight as consumed
SFA-rich fat	Butter, animal fat, coconut fat	Weight as consumed
Biscuits and cakes	Sweet bakery products, biscuits, etc.	Weight as consumed
Savoury snacks	Crisps, crackers, corn/maize based snacks, etc.	Weight as consumed
Sugary products	Marmalades, jams, syrups, sugar, etc.	Weight as consumed
Sugar alternatives	Stevia, aspartame, etc.	Weight as consumed
Coffee	All coffee drinks	Weight as consumed
Tea	Green, black, herbal tea drinks	Weight as consumed
Sweetened drinks	Sodas, tonics, squashes, etc.	Equivalent ready to drink weight
Sugar free drinks	Sodas, tonics, squashes, etc.	Equivalent ready to drink weight
Alcoholic drinks	Beers, liqueurs, spirits, cocktails, etc.	Weight as consumed

Supplementary table 4.2. Contribution of total dairy foods to nutrient intakes (%) in the RISSCI-1 study participants. [a]

Nutrients	Baseline [b]		High-SFA diet [c]		Low-SFA diet [d]	
	Mean, %	SD	Mean, %	SD	Mean, %	SD
Energy	10.6	6.4	20.8	6.3	6.3	3.0
Protein	14.0	8.5	20.9	7.8	15.3	6.9
Carbohydrates	5.4	4.2	5.9	3.5	6.6	3.6
Sugars	2.6	5.3	0.2	1.0	0.5	0.8
AOAC Fibre	0.6	1.7	0.1	0.7	0.4	1.1
Total fat	16.6	11.4	39.6	11.5	3.3	4.0
SFAs	28.5	17.5	50.1	12.6	8.1	8.4
MUFAs	12.5	9.6	35.8	12.5	2.5	3.3
PUFAs	4.2	4.9	13.7	7.4	0.4	0.9
n-3 PUFAs	5.7	7.9	19.7	13.8	0.4	1.5
n-6 PUFAs	2.9	3.7	10.6	7.3	0.2	0.7
TFAs	45.2	26.0	74.3	18.9	22.5	25.7
Cholesterol	21.7	16.5	44.0	19.4	13.0	16.4
Sodium	10.6	7.8	21.9	9.2	9.8	5.6
Iodine	50.5	22.4	66.1	18.5	61.7	20.9
Calcium	37.5	17.1	53.4	14.0	42.5	15.1

Abbreviations: AOAC, American Association of Analytical Chemists; **MUFAs**, monounsaturated fatty acids; **PUFAs,** polyunsaturated fatty acids; **SD** standard deviation; **SFAs**, saturated fatty acids; **TFAs**, trans fatty acids.

[a] total dairy foods included milk, cheese, yogurt, dairy cream, butter, and dairy from milky drinks (e.g. milkshakes and cappuccino).

[b] based on n=106 participants.

[c] based on n=104 participants.

[d] based on n=100 participants.

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Chapter 5: Identification of plasma phospholipid fatty acids associated with chronic dairy consumption in UK adults: a secondary analysis of controlled dietary intervention studies

Contribution towards PhD thesis: My responsibilities included the preparation of all plasma samples, which consisted of locating all samples, extracting the lipid fraction, isolating the phospholipid fraction, and isolating the purified fatty acid methyl esters. Their subsequent analysis by gas chromatography was conducted by Dr Kirsty Kliem. After the acquisition of the data, I was responsible for the integration of chromatograms for the identification and quantification of plasma phospholipid fatty acids. I was also responsible for the preparation of the dietary data with the help of Dr Michelle Weech and Arife Yilmaz, along with the development and conduct of the statistical analyses. Finally, I prepared the initial draft of the manuscript for submission to the *American Journal of Nutrition*, and finalised the manuscript presented in this chapter after including the feedback and comments received from Julie Lovegrove and Kim Jackson.

This manuscript was approved by my supervisors (Julie Lovegrove and Kim Jackson) and will be prepared for submission to the *American Journal of Clinical Nutrition* after feedback from co-authors.

Identification of plasma phospholipid fatty acids associated with chronic dairy consumption in UK adults: a secondary analysis of controlled dietary intervention studies

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Short title: plasma phospholipid fatty acid and dairy intakes

Author contributions towards manuscript: LS, AMM, KGJ and JAL designed the secondary analysis of the DIVAS, RESET, and SATgenε studies. KGJ, and JAL designed and secured the funding for the primary analysis of the DIVAS and RESET studies. KGJ, JAL, and AMM designed and secured the funding for the primary analysis of the SATgenε study. MW developed the method for the analysis of reported dietary intakes, along with the food categorization method which was implemented by LS and AY. LS conducted the laboratory extractions of plasma phospholipid fatty acids in preparation for GC analyses, under the supervision of KGJ. KEK developed the method for GC analyses and performed the sample analysis. LS performed the statistical analyses. LS drafted the manuscript and KGJ and JAL supervised the writing. KK, MW, AY, AMM, KGJ, and JAL contributed to the data interpretation and revised each draft for important intellectual content. All authors read and approved the final manuscript. KGJ and JAL had primary responsibility for the final content and are the guarantors. The corresponding author (JAL) attests that all listed authors meet authorship criteria and that no other authors meeting criteria have been omitted.

Financial Support: LS and this secondary analysis were funded by the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/P028217/1) Fatty Acid Metabolism – Interlinking Diet and Cardiometabolic Health FAME as part of the ERA HDHL: Biomarkers in Nutrition and Health.

Conflicts of Interest: JAL is Deputy Chair of the UK Government Scientific Advisory Committee on Nutrition (SACN) and a previous member of the SACN working group on Saturated Fats and Health. JAL was chair, and KGJ and LS were members of a scientific expert committee for the International Life Sciences Institute (ILSI) on Individual Saturated Fatty acids and Cardiovascular Risk. The other authors have no potential conflicts of interest to disclose.

Acknowledgements: The authors would like to thank the research team members involved in the primary data collection from the DIVAS, RESET, and SATgenε studies: Katerina Vafeiadou, Hana Altowaijri, Susan Todd, Parveen Yaqoob, Oonagh Markey, Dafni Vasilopoulou, Colette Fagan, Alistair Grandison, David Humphries, Ian Givens, Andrew Carvalho-Wells, and Stacey Lockyer. The authors also thank Cristina Razquin from the University of Navarra for the helpful conversations about the implementation of elastic-net regression procedures as part of the FAME Consortium.

Abstract

Background: Intervention and epidemiological studies often rely on circulating pentadecanoic and heptadecanoic acids to investigate the relationship between dairy consumptions and cardiometabolic disease risk. However, these fatty acids (FAs) only modestly correlate with reported dairy consumptions and few studies have investigated a wider range of FAs.

Objective: To identify changes in plasma phospholipid (PL) FAs associated with increased overall and specific dairy and dairy fat intakes in UK adults.

Methods: This secondary analysis of three dietary intervention studies included 138 adults who followed a high-saturated fat (SFA) diet (17 to 19% total energy from SFA) for 8 to 16 weeks, which mostly relied on daily consumption of butter, full-fat cheese, and milk. Fasting plasma PL FAs (in %wt) were measured using gas chromatography before and after intervention, and dietary intakes (in g/d) were monitored using 3 to 4-day diet-diaries. Prospective associations between plasma PL FAs and dairy intakes were assessed using 10-fold cross-validated elastic-net regression followed by multiple linear regression models.

Results: Increased total dairy fat intakes were associated with increased plasma PL myristic acid abundance (14:0, β=16.9, 95%CI 2.4;31.5, p-value=0.02), while higher consumptions of cheese and cheese fat were related to higher undecanoic acid abundance (11:0, β=208.3, 95%CI 78.9;337.7, p-value=0.002, and β=46.7, 95%CI 8.9;84.5, p-value=0.02, respectively). Increased butter intakes were associated with decreased *cis*-10-heptedecenoic acid abundance (17:1*cis*-10, β=-21.7, 95%CI -40.0;-3.5, p-value=0.02). There were no associations between PL FA abundances and intakes of milk, yogurt, cream, or fat from these foods.

Conclusions: This secondary analysis of dietary intervention studies identified novel potential proxies of chronic consumptions of total dairy fat, cheese, and cheese fat among plasma PL FAs in UK adults. Further dose-response intervention studies are warranted to replicate these results, which may improve the monitoring of dairy consumption in population studies and provide more objective markers of dietary intakes.

Keywords: dairy biomarkers, phospholipid fatty acids, myristic acid, undecanoic acid.

5.1 Introduction

Worldwide dietary guidelines recommend that dietary saturated fatty acids (SFAs) and *trans* fatty acids (FAs) remain below 10% and 2% of total energy (%TE), respectively, for the prevention of cardiometabolic diseases (CMD) [1–3]. From a food perspective, dairy products are one of the main sources of dietary SFAs in European and US adult diets, which raised the question of their role in CMD aetiology [4,5]. In this respect, epidemiological studies have mostly reported null or inverse associations between dairy consumption and CMD risk [6–8]. These studies have traditionally relied on self-declared dietary intakes from food frequency questionnaires, dietary recalls, or food diaries, the latter being considered more precise but practically more difficult to implement in large cohorts [9]. More recently, baseline measurements of circulating fatty acids in various blood fractions have emerged as biomarkers of dairy food consumption in cross-sectional and prospective cohort studies, with a particular interest in odd-chain SFAs (e.g. pentadecanoic acid 15:0 and heptadecanoic acid 17:0) and ruminant *trans*-FAs (e.g. trans palmitoleic acid 16:1 *trans*-9) [10–12]. However, their circulating levels only modestly correlate with reported dairy consumptions and dose-response intervention studies validating their utility as objective biomarkers of dairy intakes are lacking. In a recent narrative review, we identified other FAs such as trans vaccenic (18:1 *trans*-11) and phytanic acids which have been understudied so far and show potential to improve the assessment of dairy consumption [13]. In addition, we observed a lack of dietary intervention studies investigating (i) a wide range of potential biomarkers of dairy intakes among circulating FAs and (ii) their associations with the consumption of specific dairy food groups along with their fat fraction. Thus, the present study aimed to address these two knowledge gaps, by conducting a secondary analysis of three dietary intervention studies which relied on full-fat dairy foods to implement high-SFA diets in UK adults [14–16]. In particular, the objective of this analysis was to identify changes in plasma phospholipid (PL) FAs associated with increased overall and specific dairy consumptions (e.g. cheese, milk, yogurt, cream, and butter) throughout high-SFA dietary interventions enriched in full-fat dairy foods.

5.2 Methods

Study design and population

This secondary analysis was based on a subset of participants from three dietary intervention studies conducted in the Hugh Sinclair Unit of Human Nutrition (Reading, UK): the "Dietary Intervention and Vascular Function" (DIVAS) parallel randomised controlled trial (RCT), the "Replacement of Saturated fat in dairy on Total cholesterol" (RESET) crossover RCT, and the "*APOLIPOPROTEIN* E genotype as a

determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation" (SATgenε) sequential study, which have been previously described in detail [17–19].

An overview of the DIVAS, RESET, and SATgenε study designs, target population, and dietary interventions is presented in Table 5.1. Briefly, these three intervention studies investigated the impact of replacing dietary SFAs with monounsaturated fatty acids (MUFAs) and/or polyunsaturated fatty acids (PUFAs) over 8 to 16 weeks on CMD risk markers (including fasting lipid profiles, vascular function, and markers of inflammation and endothelial activation) in UK adults [14–16]. To achieve the exchange of dietary SFAs for MUFAs and PUFAs, the studies relied on food-based dietary fat exchange models based on the 2000/2001 National Diet and Nutrition Survey (NDNS) in UK adults (aged 19-64 y) [20]. In addition, the RESET and DIVAS studies included the baseline macronutrient and energy intakes among adults with higher cardiovascular disease (CVD) risk using reported dietary data from the RISCK study [21]. Although each trial had its own specific design, they all included one dietary intervention arm consisting of a high-fat diet (36 to 38 %TE from total fat) enriched in SFAs (17 to 19 %TE), with the remaining dietary fat consumed as MUFAs and PUFAs. Thus, the present study included a subset of participants who followed a SFA-rich dietary intervention within the DIVAS, RESET, and SATgenε trials.

Implementation of SFA-rich dietary interventions

The DIVAS, RESET, and SATgenε high-SFA diets all required participants to replace their habitual dairy foods, cooking fats, and/or snacks, by those provided and/or advised by researchers [17–19]. Firstly, participants from the DIVAS study who were allocated to the high-SFA diet received butter and high-SFA snacks (e.g. crackers, cookies, chocolate) to be consumed daily (25.5 g/d of butter and 2 snack portions per day), and were advised to consume commercially available semi-skimmed milk (i.e. at least 215 g/d) and full-fat cheese (i.e. at least 21 g/d) for 16 weeks [17]. Secondly, participants from the RESET study were provided with butter, cheese, and milk with a FA profile representative of commercially available products. They were advised to consume the provided foods daily (i.e. 340 g/d of milk, 45 g/d of cheese, and 21.5 g/d of butter) throughout the duration of the dietary intervention (i.e. 12 weeks) [18]. Finally, dietary guidelines for the SATgenε study participants were provided separately for males and females (i.e. 20 g/d or 10 g/d of provided butter, 25 g/d or 15 g/d of full-fat cheese, 230 g/d or 190 g/d of full-fat milk, respectively). In addition, all participants were required to consume two daily portions of provided high-SFA snacks (e.g. chocolate bars, biscuits, crisps), along with 2 g/d of oil capsules providing a FA profile representative of a typical UK diet (i.e. 44% SFAs, 39% MUFAs, and 17% *n-6* PUFAs) [19].

Table 5.1. Overview of the three dietary intervention studies included for the analysis of plasma PL FAs.

Study name	Recruitment dates	Population recruited	sample size (n)	Study design	Intervention duration	Isoenergetic diets and dietary fatty acid targets
SATgenε [19]	2009-2011	Men and women aged 35-70 y, APOE3/E3 and APOE4 carrier groups matched for age, sex, and BMI	88	Single-blind, sequential trial	3 x 8-week diets, with no washout period	*1 low-fat diet followed by 2 high-fat diets:* *Low-fat + palm oil/soybean oil capsule* (%TE total fat:SFA:MUFA:PUFA=24:8:8:6) *High-SFA + palm oil/soybean oil capsule* (%TE total fat:SFA:MUFA:PUFA=38:18:12:6) *High-SFA + DHA-enriched capsules* (%TE total fat:SFA:MUFA:PUFA=38:18:12:6 + 3g/d DHA)
DIVAS [17]	2009-2012	Men and women aged 21-69 y with moderate risk of CVD	195	Single-blind, randomised, parallel controlled trial	16-week diets	high-fat (36%TE) diets: *SFA-rich* (%TE SFA:MUFA:*n-6* PUFA =17:11:4) *MUFA-rich* (%TE SFA:MUFA:*n-6* PUFA =9:19:4) *MUFA/PUFA-rich* (%TE SFA:MUFA:*n-6* PUFA =9:13:10)
RESET [18]	2014-2016	Men and women aged 25-70 y with moderate risk of CVD	54	Double-blind, randomised, cross-over placebo trial	2 x 12-week diets, separated by a 4-week washout period	high-fat (38%TE) diets: *Conventional dairy* (%TE SFA:MUFA:PUFA =19:11:8) *Modified dairy* (%TE SFA:MUFA:PUFA =16:14:8)

Abbreviations: APOE, apolipoprotein E; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DIVAS, Dietary Intervention and Vascular Function; FAs, fatty acids; MUFAs, monounsaturated fatty acids; PL, phospholipids; PUFAs, polyunsaturated fatty acids; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation; SFAs, saturated fatty acids; y, years; %TE, % total energy.

Assessment of nutrient and dairy intakes

Participants from the DIVAS, RESET, and SATgenε completed food diaries during the last week of the high-SFA dietary intervention, in which they entered the description and weight of all food items and beverages consumed over 3 to 4 days. Food diaries were analysed for daily nutrient intakes using the nutrient analysis software DietPlan version 6.6 and 7 (Forestfield Software Ltd.) based on the McCance and Widdowson's composition of Foods 2015 Integrated Dataset [22] and/or the NDNS Rolling Programme nutrient databank [23]. Furthermore, dairy foods and total fat from dairy foods consumed over the duration of the diet diaries were extracted and averaged to reflect daily intakes (in g/d) of: butter, cream, milk, cheese, and yogurt. In this analysis, dairy consumptions were defined as those directly reported by participants in their diet diaries, i.e. dairy foods consumed on their own or within homemade recipes. However, dairy foods included as ingredients in ready-meals or processed food items (e.g. milk powder, butter in pastries, cheese in pizzas) were not included in this analysis. Total dairy intake was calculated as the sum of all dairy food consumed (i.e. milk, cheese, and yogurt), excluding butter and cream which are not considered as recommended dairy foods in UK dietary guidelines [24]. For the same reason, dairy-based desserts (such as ice cream and cheese cakes) were not considered as outcomes of interest in this analysis.

Blood sample collection and analysis of plasma phospholipid fatty acids

Fasted blood samples were collected into lithium heparin vacutainers before and after each high-SFA dietary intervention period. After collection, vacutainers were chilled on ice for up to 30 min and were centrifuged at 3,000 rpm for 15 min at 4°C to isolate plasma. DIVAS and RESET plasma samples were stored at – 80°C and SATgenε plasma samples were stored at -20°C without being thawed until subsequent analysis.

Fatty acid methyl esters (FAME) were extracted from plasma PL using a 3-step protocol derived from methods developed by Folch et al. [25] and Burdge et al. [26]. Firstly, thawed plasma samples were mixed with 0.1 mL of internal standard (1,2-ditridecanoyl-sn-glycero-3-phosphocholine, 0.4 mg/mL in choloroform). Plasma total lipids were extracted using a chloroform:methanol (2:1 volume ratio) solution containing butylated hydroxytoluene (50 mg/L) and a sodium chloride solution (0.1 mL at 0.9%, and 1 mL at 1 mol/L). Secondly, plasma PL were isolated from total lipids using solid phase extraction on Bond Elut NH2 cartridges (Agilent, Santa Clara, CA) and chloroform for elution. Finally, plasma PL were suspended in 0.4 mL of toluene and incubated with 0.8 mL of 1.5% sulphuric acid in a 70°C water bath for one hour to catalyse the transmethylation of FAMEs.

Purified plasma PL FAMEs were analysed using a gas chromatography (GC) protocol developed by Kliem et al. to allow optimal separation of dairy-specific fatty acids [27]. Briefly, FAMEs were quantified using a Bruker 450 GC system (Bruker Corporation, Billerica, MA) equipped with a flame ionisation detector and a high-resolution 100 m fused silica capillary column (CP-Sil 88 for FAME, Agilent, Santa Clara, CA). FAMEs were identified against a mixture of 52 FAMEs (GLC #463, Nu-Chek-Prep Inc., Elysian, MN) and those not available as authentic standards, such as dairy specific isomers of palmitoleic and oleic acids, were identified based on retention times previously observed using GC coupled with mass spectrometry analyses [27,28]. Finally, correction factors accounting for carbon deficiency in the flame ionisation detector response to FAMEs with short carbon chains (4 to 10 carbon atoms) were applied for the calculation of relative abundances of FAMEs in plasma PL (%wt) as proposed by Ulberth et al.[29].

Statistical analyses

Plasma PL FA abundances with ≥ 75% of missing values due to non-detection by the GC system were excluded from further analyses. Changes in dairy consumption and plasma PL FA abundances throughout the high-SFA dietary intervention periods were expressed as the difference between post-intervention and pre-intervention measurements and were z normalised (mean=0, SD=1) to ensure comparability of effect sizes. For the DIVAS and RESET studies, pre-intervention values corresponded to habitual, baseline participant diets and fasted plasma samples. For the SATgenε study, pre-intervention values corresponded to the end of an 8-week run-in low-fat diet.

We conducted elastic-net linear regression (ENR) models for the identification of changes in plasma PL FA abundances associated with changes in dairy food group consumption (dependent variables) measured during high-SFA dietary intervention within the DIVAS, RESET, and SATgenε studies. ENR methods have been successfully implemented in nutrition research to identify biomarkers of dietary intakes among large molecular datasets with high dimensionality and risk of collinearity [30–32]. This analysis adapted the method proposed by Drouin-Chartier [30] to perform ENR analyses using the *glmnet* R package [33]. First, the penalty (α) and tuning parameters (λ) used to optimise the number of plasma PL FAs selected were determined using a 10-fold cross-validation (CV) approach (training/testing sets: 80%/20% of the data, respectively), which aimed to minimise the mean-squared error between the measured and predicted changes in the dependent variable. Then, the selected α and λ parameters were used in a second 10-fold CV procedure, similar to the first one, from which we extracted the list of all selected plasma PL FAs along with their regression coefficient within each CV iteration. To determine the final predictive model, we retained the plasma PL FAs consistently selected in the 10-fold CV procedure (i.e. at least in 8 iterations out of 10), and averaged their coefficients from the individual iterations to obtain one final, unique predictive coefficient for each plasma PL FA

retained in the final predictive model. This method was repeated for each dairy food group of interest (i.e. cheese, milk, yogurt, butter, and total dairy), along with total fat intakes from these dairy food groups, to obtain predictive models based on changes in plasma PL FA abundances.

To assess the predictive performance of the 10-fold CV ENR approach, we compared the dairy food consumptions predicted by each ENR iteration to the actual consumptions reported in the DIVAS, RESET, and SATgenε participants' food diaries. To do this, we fitted the model obtained from a training set to its respective testing set in each 10-fold CV iteration to obtain 10 subsets of predicted dependent variable values. The overall Pearson correlation between the predicted and reported values was then used as an indicator of predictive performance.

Finally, the strength of the associations between changes in plasma PL FA abundances and changes in dairy food consumptions was further tested in multiple linear regression models adjusted for age (continuous, in years), sex (male/female), baseline BMI (continuous, in kg/m^2), intervention duration (continuous, in weeks), baseline energy intakes (continuous, in kcal/d), and baseline consumption of the dairy food group or total fat of interest (e.g. the model on changes in total dairy consumption was adjusted for baseline total dairy consumption).

All statistical analyses were conducted using R (version 4.1.2). All statistical tests were two-sided and p-values < 0.05 considered statistically significant.

5.3 Results

This analysis included 138 participants who followed a high-SFA dietary intervention during the DIVAS, RESET, or SATgenε studies (Table 5.2). Overall, participants were aged 49 years (SD 10), had a mean BMI of 25.9 kg/m^2 (SD 4.0), and 55% of them were female. Participants from the DIVAS study were overall younger than those from the RESET and SATgenε (mean age 45, 53, and 51 years respectively, p-value < 0.001, Table 5.2). However, there was no statistically significant difference between studies for other baseline characteristics and dairy food consumptions before a high-SFA diet. Of the 74 individual plasma PL FAs identified in GC analyses, 23 were excluded due to high proportions of non-detection (≥ 75%) and so 51 plasma PL FAs were included in the statistical analyses (Table 5.3). In ENR analyses, nine plasma PL FAs abundances were associated with at least one dairy food consumption outcome (Figure 5.1). However, only 17.4% of participants consumed cream (Table 5.2), which prevented the analysis of this dairy food group in ENR models. There was no association between changes in plasma PL FA abundances and consumptions of milk, yogurt, or fat from these dairy food groups. Furthermore, final 10-fold CV ENR models on dairy fat intakes displayed more PL FAs associations and better prediction accuracy than models on dairy food group consumptions (Table

5.4). Indeed, higher total dairy consumption was associated with a greater abundance of *trans*-11-octodecenoic acid (18:1 *trans*-15) in plasma PL (mean coefficient β=295.8 [SD 126.1], Pearson correlation=0.15), while higher total dairy fat intakes were associated with higher PL abundances of myristic acid (14:0, β=8.3 [SD 2.8]), 18:1 *trans*-15 (β=41.8 [SD 23.0]), and linoleic acid (18:2 *n-6*, β=0.4 [SD 0.2]) with a Pearson correlation of 0.46 between predicted and reported total fat intake values. Similarly, higher cheese consumption was positively associated with the abundance of undecanoic acid (11:0) in plasma PL. However, cheese fat intakes were positively associated with higher PL abundances of 11:0, 14:0, *trans*-10-hexedecenoic acid (16:1 *trans*-10), and dihomo-γ-linolenic acid (20:3 *n-6*), but inversely associated with nonadecanoic acid (19:0) and nervonic acid (24:1 *cis*-15) abundances in plasma PL, with minimal differences in prediction accuracy. Finally, there was no positive associations between butter or butter fat intakes and plasma PL FAs abundances, but we observed inverse associations between these outcomes and *cis*-10-heptadecenoic acid (17:1 *cis*-10) abundance in plasma PL (β= -12.1 [SD 7.4] for butter, and β= -12.0 [SD 4.6] for butter fat, Table 5.4).

In fully adjusted multiple linear regression models, changes in 14:0 abundance in plasma PL remained the only FA associated with total dairy fat intakes (β=16.9, 95%CI 2.4 to 31.5, p-value=0.02, Table 5.5), while there was no statistically significant association between plasma PL FA and total dairy consumption. In addition, each additional SD of change in plasma PL 11:0 abundance (%wt) was associated with an additional consumption of 208 g/d cheese (95%CI 79 to 338, p-value=0.002) and 477 g/d cheese fat (95%CI 9 to 85, p-value=0.02). Finally, inverse associations between 17:1 *cis*-10 abundance in plasma PL and butter or butter fat intakes in fully adjusted multiple linear regression models had similar effect sizes to those observed after the initial ENR procedure.

Table 5.2. Pre-intervention characteristics of participants from the DIVAS, RESET, and SATgenε studies included in the secondary analysis (n = 138).

	Overall n=138	DIVAS n=50	RESET n=39	SATgenε n=49 [a]	p-value [b]
Age, y [c]	49 (10)	45 (9)	53 (14)	51 (9)	<0.001
BMI, kg/m² [c]	25.9 (4.0)	26.7 (4.3)	25.8 (3.4)	25.3 (4.1)	0.18
Sex, n (%)					0.63
Female	76 (55.1)	29 (58.0)	19 (48.7)	28 (57.1)	
Male	62 (44.9)	21 (42.0)	20 (51.3)	21 (42.9)	
Energy, kcal/d [c]	2,099 (672)	2,143 (803)	1,992 (511)	2,142 (640)	0.56
Energy, MJ/d [c]	8.8 (2.8)	9.0 (3.4)	8.3 (2.1)	9.0 (2.7)	0.56
Total dairy consumption, g/d [d]	204 (116–334)	195 (146–321)	219 (114–377)	203 (100–337)	0.64
Of which total fat, g/d [d]	8.0 (4.1–15.0)	8.1 (4.5–15.4)	7.7 (4.5–14.0)	7.4 (3.6–15.0)	0.60
Consumers, n (%)	130 (94.2)	50 (100.0)	39 (100.0)	41 (83.7)	
Cheese consumption, g/d [d]	16.7 (4.6–29.4)	15.8 (6.5–39.4)	17.5 (6.3–25.5)	16.7 (1.7–28.3)	0.76
Of which total fat, g/d [d]	4.8 (1.0–9.6)	4.0 (1.8–11.5)	4.9 (1.1–8.0)	4.8 (0.6–9.4)	0.75
Consumers, n (%)	106 (76.8)	41 (82.0)	31 (79.5)	34 (69.4)	
Milk consumption, g/d [d]	150 (75–254)	148 (92–241)	188 (81–289)	125 (50–237)	0.39
Of which total fat, g/d [d]	2.0 (0.7–4.0)	2.3 (1.2–4.0)	2.4 (0.7–5.1)	1.7 (0.2–3.6)	0.23
Consumers, n (%)	120 (87.0)	47 (94.0)	35 (89.7)	38 (77.6)	
Yogurt consumption, g/d [d]	0.0 (0.0–52.7)	6.2 (0.0– 43.4)	17.7 (0.0–62.5)	0.0 (0.0–41.7)	0.63
Of which total fat, g/d [d]	0.0 (0.0–0.7)	0.0 (0.0–0.5)	0.1 (0.0– 0.7)	0.0 (0.0–1.0)	0.78
Consumers, n (%)	65 (47.1)	25 (50.0)	21 (53.8)	19 (38.8)	
Cream consumption, g/d [d]	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.37
Of which total fat, g/d [d]	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.40
Consumers, n (%)	24 (17.4)	11 (22.0)	4 (10.3)	9 (18.4)	
Butter consumption, g/d [d]	0.0 (0.0–7.0)	0.0 (0.0–2.4)	3.5 (0.0–9.3)	0.0 (0.0–7.2)	0.05
Of which total fat, g/d [d]	0.0 (0.0–5.8)	0.0 (0.0–2.2)	2.9 (0.0–7.1)	0.0 (0.0–5.9)	0.07
Consumers, n (%)	58 (42.0)	16 (32.0)	24 (61.5)	18 (36.7)	

[a] Including n=4 participants with missing dietary data before intervention.
[b] From Fisher tests and Pearson's χ^2 tests, as appropriate.
[c] Values presented as mean (SD).
[d] Values presented as median (interquartile range).
Abbreviations: BMI, body mass index; DIVAS, Dietary Intervention and Vascular Function; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation; SD, standard deviation; y, years.

Table 5.3. Plasma phospholipid fatty acid abundances (%wt, mean ± SD) among a subset of participants from the DIVAS, RESET, and SATgenε studies prior to and after a high-SFA dietary intervention (n=138). [a]

Fatty acid name	Overall n=138		DIVAS n=50		RESET n=39		SATgenε n=49	
	Pre-	Δ [b]	Pre-	Δ [b]	Pre-	Δ [b]	Pre-	Δ [b]
11:0	0.02 ± 0.03	0.00 ± 0.00	0.04 ± 0.05	-0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00
12:0	0.03 ± 0.03	0.00 ± 0.00	0.03 ± 0.03	0.00 ± 0.01	0.02 ± 0.02	0.00 ± 0.01	0.05 ± 0.04	0.00 ± 0.01
12:1 *cis*-9	0.09 ± 0.08	0.00 ± 0.01	0.06 ± 0.08	0.00 ± 0.02	0.13 ± 0.09	0.00 ± 0.02	0.09 ± 0.06	0.00 ± 0.01
14:0	0.39 ± 0.13	0.05 ± 0.02	0.4 ± 0.18	0.04 ± 0.04	0.36 ± 0.09	0.08 ± 0.03	0.42 ± 0.1	0.04 ± 0.02
14:1 *cis*-9	0.02 ± 0.03	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.00	0.05 ± 0.04	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
15:0	0.22 ± 0.06	0.02 ± 0.01	0.21 ± 0.08	0.02 ± 0.02	0.21 ± 0.04	0.04 ± 0.01	0.23 ± 0.05	0.02 ± 0.01
16:0	32.65 ± 6.38	-0.13 ± 0.76	31.99 ± 9.23	0.31 ± 1.86	29.2 ± 1.32	0.37 ± 0.29	36.07 ± 2.37	-0.99 ± 0.46
16:1 *trans*-6/7/8	0.01 ± 0.02	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
16:1 *trans*-9	0.18 ± 0.07	0.04 ± 0.01	0.18 ± 0.08	0.04 ± 0.02	0.18 ± 0.06	0.04 ± 0.01	0.19 ± 0.05	0.03 ± 0.01
16:1 *cis*-9	0.75 ± 0.32	0.01 ± 0.04	0.67 ± 0.38	0.00 ± 0.07	0.67 ± 0.21	0.06 ± 0.06	0.9 ± 0.28	-0.03 ± 0.07
16:1 *trans*-10	0.01 ± 0.02	0.00 ± 0.00	0.01 ± 0.03	0.00 ± 0.01	0.01 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
16:1 *trans*-11/12/13	0.16 ± 0.05	-0.01 ± 0.01	0.14 ± 0.06	-0.02 ± 0.01	0.14 ± 0.03	-0.01 ± 0.01	0.2 ± 0.03	-0.02 ± 0.01
17:0	0.34 ± 0.09	0.00 ± 0.01	0.34 ± 0.12	-0.02 ± 0.02	0.3 ± 0.06	0.03 ± 0.01	0.37 ± 0.08	0.00 ± 0.02
17:1 *cis*-10	0.24 ± 0.16	-0.01 ± 0.02	0.3 ± 0.2	-0.02 ± 0.04	0.25 ± 0.15	-0.01 ± 0.03	0.17 ± 0.06	0.01 ± 0.01
18:0	13.56 ± 3.19	0.06 ± 0.38	13.58 ± 4.4	-0.33 ± 0.9	12.45 ± 1.02	0.03 ± 0.25	14.43 ± 2.57	0.48 ± 0.43
18:1 *trans*-6/7/8	0.06 ± 0.05	0.01 ± 0.01	0.08 ± 0.07	0.00 ± 0.01	0.06 ± 0.04	0.01 ± 0.01	0.04 ± 0.04	0.02 ± 0.01
18:1 *cis*-6/*trans*-12	0.09 ± 0.06	0.02 ± 0.01	0.11 ± 0.08	0.01 ± 0.01	0.09 ± 0.04	0.01 ± 0.01	0.06 ± 0.05	0.03 ± 0.01
18:1 *trans*-9	0.04 ± 0.04	0.01 ± 0	0.05 ± 0.04	0.01 ± 0.01	0.04 ± 0.03	0.01 ± 0.01	0.02 ± 0.03	0.01 ± 0.01
18:1 *cis*-9	11.1 ± 3.07	0.09 ± 0.37	9.32 ± 3.59	0.09 ± 0.7	10.64 ± 1.67	-0.2 ± 0.37	13.29 ± 1.79	0.32 ± 0.39
18:1 *trans*-10	0.01 ± 0.05	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.00	0.02 ± 0.08	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.00
18:1 *trans*-11	0.36 ± 0.2	0.02 ± 0.02	0.4 ± 0.27	0.01 ± 0.05	0.31 ± 0.13	0.00 ± 0.03	0.36 ± 0.12	0.05 ± 0.03
18:1 *cis*-11	1.42 ± 0.42	-0.03 ± 0.13	1.23 ± 0.5	0.19 ± 0.34	1.32 ± 0.2	-0.1 ± 0.04	1.71 ± 0.28	-0.21 ± 0.06
18:1 *cis*-12	0.06 ± 0.08	0.01 ± 0.01	0.08 ± 0.09	0.00 ± 0.02	0.03 ± 0.04	0.00 ± 0.01	0.06 ± 0.08	0.02 ± 0.02
18:1 *cis*-13	0.04 ± 0.06	0.01 ± 0.01	0.04 ± 0.06	0.02 ± 0.01	0.03 ± 0.04	0.00 ± 0.01	0.03 ± 0.06	0.00 ± 0.01
18:1 *trans*-15	0.05 ± 0.03	0.01 ± 0	0.03 ± 0.03	0.01 ± 0.01	0.05 ± 0.03	0.01 ± 0.01	0.07 ± 0.03	0.02 ± 0.01
18:1 *trans*-16/*cis*-14	0.45 ± 0.37	-0.01 ± 0.04	0.53 ± 0.52	-0.02 ± 0.1	0.31 ± 0.17	0.00 ± 0.04	0.48 ± 0.26	-0.01 ± 0.06
18:2 *trans*-9, *cis*-12	0.03 ± 0.04	0.00 ± 0.01	0.04 ± 0.06	0.01 ± 0.01	0.04 ± 0.03	0.00 ± 0.01	0.01 ± 0.02	0.01 ± 0.01
18:2 *n-6*	18.44 ± 5.12	0.28 ± 0.63	17.05 ± 7.17	-0.29 ± 1.46	21.39 ± 2.89	-0.13 ± 0.62	17.5 ± 2.43	1.19 ± 0.53
18:2 *trans*-11,15	0.03 ± 0.07	0.00 ± 0.01	0.07 ± 0.1	-0.01 ± 0.02	0.03 ± 0.02	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00

CLA	2.43 ± 2.01	-0.14 ± 0.24	2.34 ± 2.5	-0.22 ± 0.46	1.78 ± 1.08	-0.19 ± 0.27	3.05 ± 1.85	-0.04 ± 0.41
18:3 *n-6*	0.45 ± 0.29	-0.02 ± 0.03	0.53 ± 0.4	-0.05 ± 0.07	0.34 ± 0.15	0.00 ± 0.04	0.46 ± 0.22	-0.01 ± 0.05
18:3 *n-3*	0.39 ± 0.21	-0.01 ± 0.02	0.47 ± 0.26	-0.01 ± 0.05	0.4 ± 0.16	-0.02 ± 0.04	0.29 ± 0.13	0.01 ± 0.03
19:0 [c]	0.07 ± 0.13	0.00 ± 0.01	0.12 ± 0.19	-0.01 ± 0.04	0.03 ± 0.02	0.00 ± 0.01	0.05 ± 0.05	0.00 ± 0.01
19:1 *cis*-7	0.16 ± 1.56	0.01 ± 0.2	0.42 ± 2.59	0.02 ± 0.54	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
20:0	0.15 ± 0.08	0.00 ± 0.01	0.18 ± 0.12	-0.01 ± 0.02	0.11 ± 0.03	0.00 ± 0.01	0.15 ± 0.04	0.00 ± 0.01
20:1 *cis*-8	0.05 ± 0.07	0.00 ± 0.01	0.1 ± 0.1	0.00 ± 0.02	0.04 ± 0.04	0.00 ± 0.01	0.02 ± 0.04	0.01 ± 0.01
20:1 *cis*-11	0.16 ± 0.13	-0.03 ± 0.01	0.16 ± 0.18	-0.05 ± 0.03	0.11 ± 0.12	-0.01 ± 0.03	0.2 ± 0.07	-0.02 ± 0.01
20:2 *n-6*	2.36 ± 1.03	0.13 ± 0.14	2.81 ± 1.22	0.11 ± 0.27	2.8 ± 0.6	0.33 ± 0.16	1.55 ± 0.43	-0.02 ± 0.09
20:3 *n-6*	0.27 ± 0.07	0.00 ± 0.01	0.28 ± 0.11	0.01 ± 0.02	0.28 ± 0.04	0.01 ± 0.01	0.27 ± 0.05	-0.01 ± 0.01
20:3 *n-3*	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
20:4 *n-6*	6.67 ± 3.44	-0.33 ± 0.41	8.05 ± 3.26	-0.28 ± 0.62	9.14 ± 1.86	-0.32 ± 0.4	3.28 ± 1.26	-0.4 ± 0.24
20:5 *n-3*	0.22 ± 0.19	0.01 ± 0.03	0.2 ± 0.28	0.01 ± 0.07	0.14 ± 0.05	0.00 ± 0.01	0.3 ± 0.09	0.03 ± 0.02
22:0	0.31 ± 0.09	0.01 ± 0.01	0.35 ± 0.1	-0.01 ± 0.02	0.32 ± 0.06	0.02 ± 0.01	0.26 ± 0.09	0.01 ± 0.02
22:1 *cis*-13	0.06 ± 0.14	-0.01 ± 0.01	0.1 ± 0.22	-0.04 ± 0.03	0.03 ± 0.03	0.01 ± 0.01	0.06 ± 0.07	-0.01 ± 0.01
22:2 *n-6*	0.01 ± 0.02	0.00 ± 0.00	0.02 ± 0.03	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
22:3 *n-3*	0.16 ± 0.17	0.00 ± 0.02	0.16 ± 0.19	0.01 ± 0.04	0.03 ± 0.06	0.03 ± 0.03	0.26 ± 0.14	-0.04 ± 0.03
22:4 *n-6*	0.2 ± 0.12	0.02 ± 0.02	0.25 ± 0.13	0.04 ± 0.03	0.27 ± 0.07	0.02 ± 0.02	0.09 ± 0.06	-0.01 ± 0.01
22:5 *n-3*	0.67 ± 0.36	-0.01 ± 0.04	0.86 ± 0.34	-0.01 ± 0.07	0.89 ± 0.15	0.04 ± 0.03	0.31 ± 0.13	-0.04 ± 0.02
22:6 *n-3*	2.49 ± 1.59	-0.2 ± 0.19	3.21 ± 1.49	-0.11 ± 0.3	3.31 ± 1.36	-0.19 ± 0.27	1.1 ± 0.65	-0.3 ± 0.11
24:0	1 ± 0.95	-0.08 ± 0.1	1.19 ± 1.02	-0.01 ± 0.18	1.19 ± 0.64	-0.01 ± 0.12	0.64 ± 1	-0.22 ± 0.15
24:1 *cis*-15	0.44 ± 0.39	0.01 ± 0.05	0.17 ± 0.1	-0.01 ± 0.02	0.23 ± 0.1	0.00 ± 0.02	0.89 ± 0.32	0.04 ± 0.06
Total SFAs	81.05 ± 15.1	-0.21 ± 1.79	80.08 ± 22.68	0.32 ± 4.5	73.1 ± 2.54	0.9 ± 0.59	88.37 ± 3.65	-1.65 ± 0.82
Total *cis* MUFAs	15.2 ± 3.81	0.06 ± 0.46	13.43 ± 4.66	0.19 ± 0.91	13.96 ± 1.84	-0.24 ± 0.43	17.99 ± 2.03	0.15 ± 0.46
Total *trans* MUFAs	1.42 ± 0.62	0.09 ± 0.07	1.56 ± 0.88	0.04 ± 0.16	1.22 ± 0.38	0.09 ± 0.09	1.43 ± 0.41	0.13 ± 0.09
Total PUFAs	34.83 ± 8.79	-0.28 ± 1.04	36.35 ± 11.68	-0.8 ± 2.29	40.85 ± 1.85	-0.43 ± 0.47	28.48 ± 2.31	0.38 ± 0.54
Total *n-3* PUFAs	3.97 ± 1.83	-0.21 ± 0.22	4.98 ± 1.69	-0.13 ± 0.34	4.8 ± 1.36	-0.14 ± 0.27	2.27 ± 0.77	-0.34 ± 0.12
Total *n-6* PUFAs	28.43 ± 8.05	0.07 ± 0.95	29.03 ± 10.77	-0.46 ± 2.13	34.27 ± 2.2	-0.1 ± 0.49	23.16 ± 2.54	0.76 ± 0.55

[a] High-SFA dietary interventions in each study are detailed in Table 5.1.[b] Change calculated as *Δ = post-intervention – pre-intervention value*.[c] Co-eluted with 18:1 *cis*-15 in gas chromatography analyses. **Abbreviations:** DIVAS, Dietary Intervention and Vascular Function; MUFAs, monounsaturated fatty acids; PL FA, phospholipid fatty acid; PUFAs, polyunsaturated fatty acids; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation; SD, standard deviation; SFAs, saturated fatty acids

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Table 5.4. Final coefficients for the plasma phospholipid fatty acids selected during elastic-net regression procedures in relation to the amount and type of dairy consumptions among participants included in the secondary analysis (n=138).

Type of dairy consumption	Plasma PL FA	Final 10-fold CV ENR model		Prediction performance	
		Mean coefficient [a, b]	SD	Pearson correlation [c]	95%CI
Total dairy	18:1 *trans*-15	296	126	0.15	0.03 – 0.26
Total dairy fat	14:0	8.3	2.8	0.46	0.36 – 0.55
	18:1 *trans*-15	41.8	23.0		
	18:2 *n-6*	0.4	0.2		
Cheese	11:0	123	80	0.68	0.61 – 0.74
Cheese fat	11:0	26.3	5.9	0.61	0.53 – 0.68
	14:0	4.0	1.8		
	16:1 *trans*-10	13.7	5.9		
	19:0	-15.5	7.1		
	20:3 *n-6*	1.0	0.7		
	24:1 *cis*-15	-2.5	1.3		
Butter	17:1 *cis*-10	-12.1	7.4	0.14	0.02 – 0.25
Butter fat	17:1 *cis*-10	-12.0	4.6	0.13	0.01 – 0.24

[a] Mean coefficients were calculated using the z-scaled regression coefficients of each iteration of the 10-fold cross validated ENR models. PL FAs imputed in the ENR procedure needed to be selected in at least 8 iterations out of 10 to be selected as consistent markers of dairy consumption.

[b] Expressed as change in dairy consumption (g/d) per additional SD of change in plasma PL FA abundance (%wt).

[c] The Pearson correlation coefficients reflect the correlation between the predicted change in dairy consumption derived from the multi-metabolite models identified by the elastic net regression approach and the actual change in dairy consumption measured during the combined studies (change = post-intervention – pre-intervention value).

Abbreviations: DIVAS, Dietary Intervention and Vascular Function; PL FA, phospholipid fatty acid; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation; SD, standard deviation; 95%CI, 95% confidence interval.

Table 5.5. Results from multiple linear regression models on the associations between plasma phospholipid fatty acid abundances and the amount and type of dairy consumptions among participants included in the secondary analysis (n=138). [a]

Type of dairy consumption	Plasma PL FA	β estimate [b]	95%CI	p-value	R^2
Total dairy	18:1 *trans*-15	1012	-318 – 2341	0.13	0.18
Total dairy fat	14:0	16.9	2.4 – 31.5	0.02	0.39
	18:1 *trans*-15	42.8	-19.8 – 105.4	0.18	
	18:2 *n-6*	0.5	-0.2 – 1.3	0.15	
Cheese	11:0	208	79 – 338	0.002	0.55
Cheese fat	11:0	46.7	8.9 – 84.5	0.02	0.58
	14:0	8.2	-0.3 – 16.8	0.06	
	16:1 *trans*-10	32.2	-16.2 – 80.6	0.19	
	19:0	-18.9	-38.0 – 0.2	0.05	
	20:3 *n-6*	2.2	0.2 – 4.2	0.03	
	24:1 *cis*-15	-4.7	-11.7 – 2.3	0.19	
Butter	17:1 *cis*-10	-21.7	-40.0 – -3.5	0.02	0.31
Butter fat	17:1 *cis*-10	-18.3	-33.2 – -3.5	0.02	0.30

[a] Multiple linear regression adjusted for age (continuous, in years), sex (female/male), BMI (continuous, in kg/m^2), pre-intervention consumption of the dairy consumption of interest (continuous, in g/d), baseline total energy intake (continuous, in kcal/d), and intervention duration (continuous, in weeks).

[b] Expressed as change in dairy consumption (g/d) per additional SD of change in plasma PL FA abundance (%wt).

Abbreviations: DIVAS, Dietary Intervention and Vascular Function; PL FA, phospholipid fatty acid; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgenε, *APOLIPOPROTEIN* E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation; SD, standard deviation; 95%CI, 95% confidence interval.

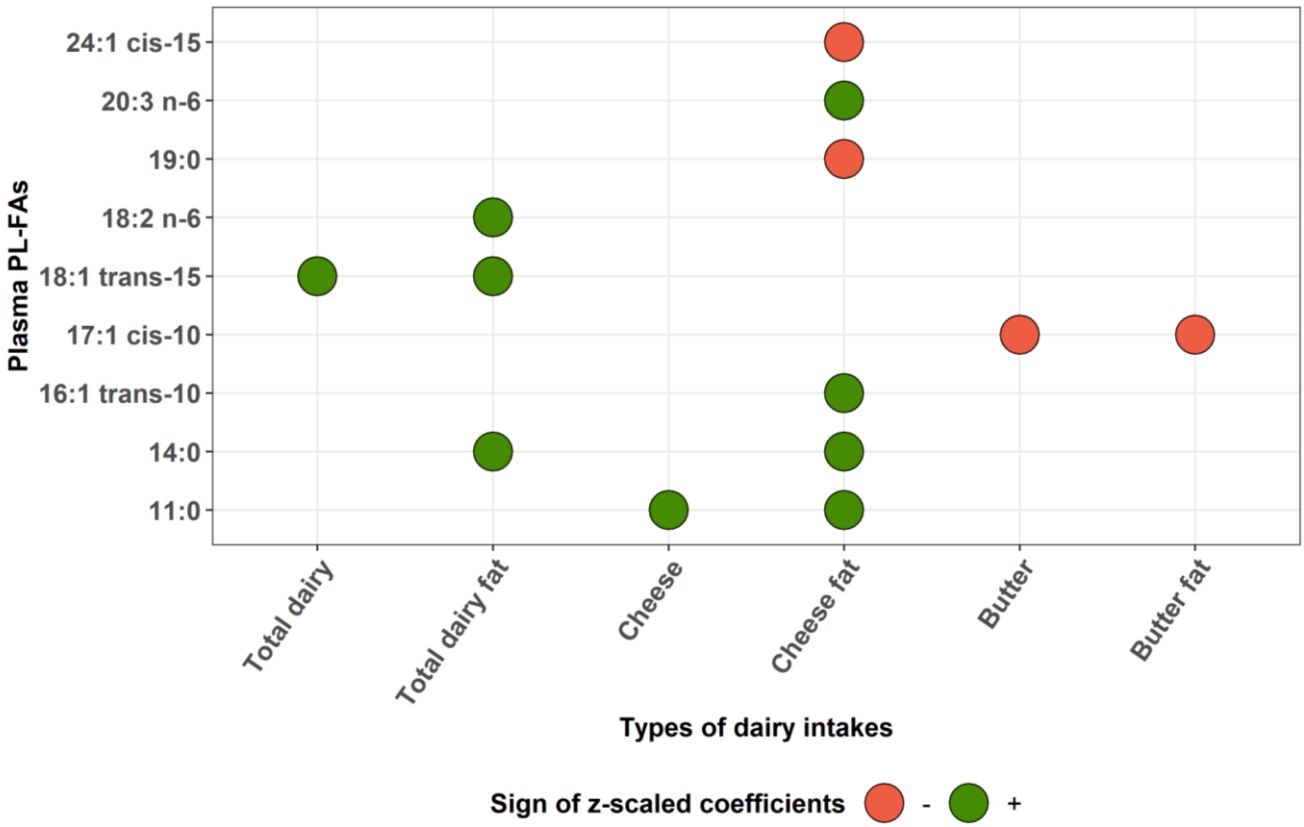

Figure 5.1. Plasma phospholipid fatty acids associated with changes in the amount and type of dairy consumptions among participants included in the secondary analysis (n=138). [a]

[a] *Identified using 10-fold cross validated elastic-net regression models.*
Abbreviations*: DIVAS, Dietary Intervention and Vascular Function; PL FA, phospholipid fatty acid; RESET, Replacement of Saturated fat in dairy on Total cholesterol; SATgen, APOLIPOPROTEIN E genotype as a determinant of the low-density lipoprotein cholesterol response to dietary fat manipulation.*

5.4 Discussion

In this secondary analysis of three high-SFA dietary intervention studies incorporating full-fat dairy foods, we identified individual plasma PL FA abundances specifically associated with increased total dairy, cheese, and butter consumptions, along with the fat intakes from these food groups. However, there was no consistent associations between plasma PL FA abundances and intakes of milk, yogurt, or fat from these dairy foods.

Previous RCTs have mostly focused on odd-chain SFAs (e.g. 15:0 and 17:0) and ruminant *trans* FAs such as 16:1 *trans*-9 as biomarkers of dairy consumption [11,12,34,35], but these plasma PL FAs were not significantly associated with total or specific dairy intakes in the present analysis. However, we identified that increased plasma PL abundance of 14:0 was the strongest predictor of higher total dairy fat intake in fully adjusted models. These results align with recent findings from a meta-analysis of

eight observational studies which reported a significant, direct, cross-sectional association between 14:0 abundances in various blood fractions (expressed as %wt total FAs) and dairy fat intakes in g/d (total effect size = 0.16, 95%CI 0.10 to 0.22, n=5,209 participants) [10]. Moreover, these results may partly reflect the high content of 14:0 in dairy foods, as this FA is the second most abundant SFA in dairy fat (i.e. 10 to 11%wt total milk FAs) after palmitic acid 16:0 (i.e. 30 to 33%wt total milk FAs) [27,36,37]. Nonetheless, 14:0 is also endogenously synthesised by classic FA elongation metabolic pathways along with shortening of 16:0 by peroxisomal β-oxidation [38,39]. Therefore, the higher abundance of 14:0 in plasma PL observed in the present analysis after a chronic high-SFA dietary intervention might derive from increased total dairy intakes, increased endogenous synthesis of this FA, or a combination of both.

In addition, we observed positive associations between the abundance of 11:0 in plasma PL and cheese and cheese fat intakes in fully adjusted linear regression models. In particular, our results suggest that a large increase in cheese intake (208 g/d) is required to increase 11:0 abundance in plasma PL by one SD (SD <0.01%wt total FAs, Table 5.3). Like the longer odd-chain SFAs 15:0 and 17:0, 11:0 is produced by bacteria in ruminant animals by *de novo* FA synthesis using propionic acid (3:0) as a substrate, or by α-oxidation of even-chain FAs [40–42]. In dairy cows, 11:0 is then utilised in the mammary gland as a minor contributor to the milk lipid fraction (0.03 to 0.06%wt total FAs) [27]. As expected, 11:0 is also found in cheese fat and represents 0.03 to 0.04%wt of triacylglycerol FAs in UK cheddar cheese fat, depending on the cows' feeding regimen [43]. Although odd-chain SFAs were thought to exclusively derive from dietary intakes, their utility as biomarkers of dietary consumptions of dairy has been challenged in recent *in vitro* studies which suggested their potential synthesis by the human gut microbiota from propionyl-CoA or from the decarboxylation of even-chain FAs through α-oxidation [44–46]. However, these metabolic pathways have been proposed for the synthesis 15:0 and 17:0, and there is, to our knowledge, no quantitative estimate of the potential endogenous synthesis of 11:0 in humans.

The inverse association between 17:1 *cis*-10 in plasma PL and butter consumption observed in this analysis somewhat contrasts with previous findings, although studies which have quantified this FA are scarce. In a cross-sectional study including 205 Norwegian adults, butter intakes (in g/d) assessed by a food frequency questionnaire were significantly correlated with 17:1 in serum cholesteryl esters (Spearman's rank correlation coefficient r=0.19), but not in adipose tissue, serum free FAs, serum triacylglycerols, or serum phospholipids [47]. However, the absence of details on the position or configuration of the double bond investigated limits the comparability of their findings to those observed in the present study. In particular, an analytical study from Alves et al. showed that 17:1 *cis*-9 are more abundant than 17:1 *cis*-10 isomers in milk [48]. Despite using a similar GC system than the

authors in the present study, isomers of 17:1 could not be identified among FAMEs from human plasma PL. Combined with the lack of 17:1 isomer detail in other human studies, our findings suggest a potential suboptimal resolution of 17:1 isomers in human plasma. This adds to the limited commercial availability of authentic standards for 17:1 *cis*-9 and *cis*-8, which may altogether contribute to the lack of available evidence on the potential of 17:1 as a proxy of dairy fat consumption.

Importantly, previous RCTs often investigated circulating FAs as proxies for dairy consumption, rather than dairy fat consumption [11,34,35]. In this study, we showed that the associations between individual plasma PL FAs were stronger and provided better predictive accuracy when considering reported dairy fat intakes rather than the consumption of whole dairy foods. Indeed, FA biomarkers are likely to inaccurately capture the consumption of low-fat dairy foods, which would introduce confounding in their associations with overall dairy intakes. This hypothesis aligns with the lack of associations between plasma PL FA abundances and consumptions of lower-fat dairy food items (i.e. milk and yogurt) in the present analysis, and with previous observations of null or weak associations observed between circulating odd-chain SFAs and intake of low-fat dairy foods [49,50].

Strengths of this analysis included its prospective design, which allowed for the measurement of dairy intake and plasma PL FA profiles before and after the chronic consumption of higher fat dairy foods as part of a high-SFA diet. As illustrated in the present analysis and in a previous intervention study, this prospective approach may improve the precision of the estimated associations between plasma PL FA abundances and dairy intakes compared to cross-sectional study designs [51]. Furthermore, the combined analysis of three RCTs conducted within the same research groups and using the same dietary assessment methods allowed for a large sample size (n=138), which surpassed most previous RCTs which investigated similar research questions. Therefore, the associations observed in this analysis may provide novel insights into the response to increased full-fat dairy food consumptions, and may improve the current evidence base on FAs as biomarkers of dairy consumption. Moreover, all participants were free-living and consumed commercially available dairy foods or manufactured dairy foods with FA profiles typical of commercially available items, which improves the applicability of our findings to real-life settings. Finally, this analysis relied on strong methodological approaches to maximise the accuracy of outcome measurements, using comprehensive food diaries to analyse dietary intakes and GC methods specifically developed for the detection of a wide range of dairy-derived FAs and their isomers. Nevertheless, some limitations of this analysis need to be acknowledged. First, this study was a secondary analysis of RCTs, which were not initially powered or designed to detect associations between dairy consumption and plasma PL FA abundances. In particular, the high-SFA dietary intervention, while relying mostly on full-fat dairy foods, also

contained high-SFA snacks which might have contributed to the changes observed in plasma PL FA abundances. In addition, only 17.4% of participants consumed dairy cream as the high-SFA diets did not specify guidelines for this particular food, which prevented the detection of associations between dairy cream intakes and plasma PL FAs. Second, this analysis investigated the changes in plasma PL FA profiles in relation to the consumption of dairy foods consumed as whole foods or within homemade recipes, but did not account for the presence of dairy in processed foods or ready-to-eat meals due to the absence of decomposed data for these food items in the McCance and Widdowson's composition of Foods 2015 Integrated Dataset used for the analysis of dietary intakes [22]. While this approach may have underestimated total dairy consumptions, it was aligned with our initial aim to identify proxies of dairy products consumed as whole foods, as recommended in UK dietary guidelines [24]. Finally, pre-intervention dairy consumptions in participants from the DIVAS and RESET studies corresponded to their habitual dietary intakes, which broadly aligned with those of a typical UK adult diet of about 9%TE from total dairy products [23]. This baseline level of consumption of dairy might have affected the effect size of the high-SFA dietary intervention on dairy intakes, and therefore underestimated the associations between changes in plasma PL FA abundances and changes in dairy intakes in the present analysis.

To conclude, this secondary analysis of dietary intervention studies revealed that dietary intakes of dairy foods and dairy fat may in part modulate plasma PL FA profiles in UK adults consuming a high-SFA diet enriched in full-fat dairy foods. Provided that these results are replicated in confirmatory cohorts, they may improve the assessment of dairy consumption at a population level and estimate adherence to dietary guidelines.

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Chapter 6: Consumption of dairy products and cardiovascular disease risk: results from the French prospective cohort NutriNet-Santé

Contribution towards PhD thesis: Thanks to my previous work within the Nutritional Epidemiology Research Team (EREN) in Paris, I initiated this collaborative project between the Hugh Sinclair Unit of Human Nutrition and Dr Mathilde Touvier's research team and contributed to the definition of the research question for this study. I visited the EREN research facilities on two occasions (January 2019 and October 2019) to perform the data management and statistical analyses presented in this manuscript. Finally, I prepared the initial draft of the manuscript for publication, and finalised the published manuscript presented in this chapter after including the feedback and comments received from co-authors and journal reviewers.

Manuscript published in the *British Journal of Nutrition* (April 2021).DOI:10.1017/S0007114521001422

Consumption of dairy products and cardiovascular disease risk: results from the French prospective cohort NutriNet-Santé

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Short title: dairy consumption and cardiovascular disease risk

Author contributions towards manuscript: LS, MT and JL designed the research. SH, PG, MT, CJ, LKF and EK-G conducted the research. LS performed the statistical analyses. BS supervised the statistical analyses. LS drafted the manuscript. MS, JL and KGJ supervised the writing. BS, KGJ, SH, PG, EK-G, CJ, LKF, MD, JL and MT contributed to the data interpretation and revised each draft for important intellectual content. All authors read and approved the final manuscript. MT and JL had primary responsibility for the final content and are the guarantors. The corresponding author (BS) attests that all listed authors meet authorship criteria and that no other authors meeting criteria have been omitted.

Financial support: The NutriNet-Santé study was supported by the Ministère de la Santé, Santé Publique France, Institut National de la Santé et de la Recherche Médicale (INSERM), Institut National de la Recherche Agronomique (INRA), Conservatoire National des Arts et Métiers (CNAM), and Université Paris 13. Laury Sellem was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Joint Programme Initiatives (JPI) "HDHL Biomarkers: Fatty Acid Metabolism - Interlinking Diet with Cardiometabolic Health (FAME)" (Project Reference: BB/P028217/1). Researchers were independent from funders. Funders had no role in the study design, the collection, analysis, and interpretation of data, the writing of the report, and the decision to submit the article for publication.

Conflicts of interest: Julie Lovegrove is Deputy Chair of the UK Scientific Advisory Committee for Nutrition (SACN) and was an expert on SACN's Saturated Fats working group. All others have no conflict of interest to declare.

Acknowledgements: The authors warmly thank all the volunteers of the NutriNet-Santé cohort. We also thank Younes Esseddik (IT manager), Thi Hong Van Duong, Régis Gatibelza, and Jagatjit Mohinder (computer scientists), Fabien Szabo de Edelenyi, PhD (data management supervisor), Julien Allègre, Nathalie Arnault, Laurent Bourhis (data-managers/biostatisticians), Sandrine Kamdem, MD (physician), and Nathalie Druesne-Pecollo (operational coordinator) for their technical contribution to the NutriNet-Santé study.

Abstract

In France, dairy products contribute to dietary saturated fat intake, of which reduced consumption is often recommended for cardiovascular disease (CVD) prevention. Epidemiological evidence on the association between dairy consumption and CVD risk remains unclear, suggesting either null or inverse associations. This study aimed to investigate the associations between dairy consumption (overall and specific foods) and CVD risk in a large cohort of French adults. This prospective analysis included participants aged ≥ 18 years from the NutriNet-Santé cohort (2009–2019). Daily dietary intakes were collected using 24h-dietary records. Total dairy, milk, cheese, yogurts, fermented and reduced-fat dairy intakes were investigated. CVD cases (n=1,952) included cerebrovascular (n=878 cases) and coronary heart diseases (CHD, n=1,219 cases). Multivariable Cox models were performed to investigate associations. This analysis included n=104,805 French adults (mean age at baseline 42.8 years (SD 14.6), mean follow-up 5.5 years (SD 3.0, i.e. 579,155 persons years). There were no significant associations between dairy intakes and total CVD or CHD risks. However, the consumption of at least 160 g/d of fermented dairy (e.g. cheese and yogurts) was associated with a reduced risk of cerebrovascular diseases compared to intakes below 57 g/d (HR=0.81 [0.66-0.98], p-trend=0.01). Despite being a major dietary source of saturated fats, dairy consumption was not associated with CVD or CHD risks in this study. However, fermented dairy was associated with a lower cerebrovascular disease risk. Robust randomized controlled trials are needed to further assess the impact of consuming different dairy foods on CVD risk and potential underlying mechanisms.

Keywords: dairy, cardiovascular disease, fermented dairy, cerebrovascular disease, cheese, yogurt

6.1 Introduction

Cardiovascular diseases (CVD), including coronary heart diseases (CHD) and cerebrovascular diseases such as strokes are still a leading cause of mortality worldwide, causing 17.9 million deaths every year [1,2]. In France, CVDs are the primary cause of death in women and the second most common cause in men [3]. Beside smoking, lifestyle factors such as nutritional status and dietary habits, have been identified as one of the main modifiable risk factors of CVD [4].Thus, public health guidelines around the world target the consumption of specific nutrients and food groups as a strategy for reducing CVD risk at a population level. In particular, reducing the dietary consumption of saturated fat (SFA) is often recommended to help lower circulating levels of low-density lipoprotein cholesterol, a well-established risk factor for CVD [5,6]. In France, public health guidelines suggest a consumption of SFA below 12% of dietary energy (without alcohol), with an emphasis on three fatty acids that should remain below 8% of dietary energy (lauric C12:0, myristic C14:0 and palmitic C16:0 acids) [7]. In parallel, the French National Health and Nutrition Programme (PNNS) focuses on recommendations related to food groups, and suggests a daily consumption of two servings of dairy products per day for adults, to be chosen among milk, cheese and yogurts, but not including butter, cream or dairy desserts (e.g. custard, ice cream and cheese cake) [8].

Dairy products are nutrient-rich foods containing various minerals and vitamins, such as calcium, potassium, phosphorus, and vitamins B, K and D which are associated with health benefits. However, full-fat dairy foods can also be energy-dense and may contain high levels of sodium and SFA. In French adults, dairy contribute to 24% of total dietary SFA intake [9]. Despite this high contribution to dietary SFA, epidemiological evidence on the association between dairy product consumption and CVD risk remains unclear. Recent meta-analyses of prospective cohort studies suggest either null or slightly inverse associations between total dairy foods and incident CVDs [10–13]. Similar trends were also observed in more recent prospective cohort studies, such as the PURE study, which included data from 21 countries across five continents and observed a reduced risk of CVD associated with total dairy consumption [14]. In addition, epidemiological evidence on specific types of dairy remains inconsistent, although a recent meta-analysis of nine prospective cohort studies suggested a reduced CVD risk associated with the consumption of fermented dairy foods only, which included cheese, yogurt and fermented milk, as opposed to intakes of total dairy or non-fermented milks [11]. These results raise the question of the importance of dairy food types and potential fermentation in relation to cardiovascular health.

This study aimed to investigate the associations between the consumption of dairy foods (overall and specific types) and CVD risk in a large cohort of French adults.

6.2 Methods

The NutriNet-Santé cohort

The NutriNet-Santé study is an ongoing French web-based cohort, launched in 2009 with the aim of investigating the associations between nutrition, dietary behaviours, determinants of nutrition status, and health. Detail of this study has been published previously [15]. Briefly, since May 2009 participants aged 18 years and over with access to the Internet have been continuously recruited among the French population using vast multimedia campaigns. All questionnaires are completed online using a dedicated website (www.etude-nutrinet-sante.fr).

Ethical approval

The NutriNet-Santé study is conducted according to the Declaration of Helsinki guidelines. It was approved by the Institutional Review Board of the French Institute for Health and Medical Research (IRB Inserm n°0000388FWA00005831) and the "Commission Nationale de l'Informatique et des Libertés" (CNIL n°908450/n°909216). Each participant provides their informed consent electronically. The study is registered at clinicaltrials.gov as NCT03335644.

Data collection

Upon inclusion, participants completed a set of five questionnaires related to socio-demographic and lifestyle characteristics, such as sex, occupation, educational level (< high school degree, < 2 years after high school, ≥ 2 years after high school), smoking status (never smoked, former smoker, current smoker), alcohol consumption (g/d), number of children [16], anthropometrics [17,18] (e.g. height and weight, which were validated against a random sample of participants), dietary intakes [19], physical activity levels (as low, moderate of high, from validated seven day International Physical Activity Questionnaire (IPAQ)) [20,21], and health status (e.g. personal and family history of diseases, prescribed medication).

Dietary data

Usual dietary intakes were assessed at inclusion and then every six months, using a series of three non-consecutive web-based 24h-dietary records, randomly assigned over a 2-week period (2 weekdays and 1 weekend day). The web-based questionnaires used in the study have been tested and validated against both in-person interviews by trained dietitians, and urinary and blood markers [17,22].

In this analysis, we calculated the usual baseline dietary intakes as the average of all 24h-dietary records completed during the first two years of each participant's follow-up, with a mandatory requirement of at least two 24h-dietary records during this period to be included in the analysis.

At all times throughout their assigned dietary record period, participants had access to a dedicated interface of the study website to declare all foods and beverages consumed during a 24h-period: three main meals (breakfast, lunch, dinner) and any other eating occasion. When participants could not provide weights for the food items consumed, they were invited to estimate portion sizes using validated photographs or usual containers [23]. A French food composition database (>3,500 items) [24] was used to estimate mean daily energy, alcohol, macro- and micro-nutrient intakes. These estimates included contributions from composite dishes using French recipes validated by food and nutrition professionals. Individual dietary data were not communicated to participants to avoid any changes in dietary behaviours. Finally, those that under-reported total energy intake were identified and excluded based on the method proposed by Black [25], using the basal metabolic rate, Goldberg cut-off, and a physical activity level (PAL) cut-off of 1.55 which corresponds to the WHO value for "light" activity. For this calculation, intra-individual coefficients of variations for BMR and PAL were fixed using the values recommended by Black, i.e. 8.5 % and 15%, respectively. About 20.0% of participants of the cohort were considered as under-reporters of energy intake and were excluded from the analyses. There was no sign of over reporters as the highest energy intakes ranged within plausible values (99^{th} percentile = 3,289 kcal/d).

Dairy products classification

Trained dietitians categorised all dairy foods of the NutriNet-Santé composition table into one of the five dairy groups defined in the PNNS: milk, cheese, yogurts, curd cheese and "petit-suisses". As per the PNNS guidelines, milk-based products containing more than 12% of sugars were classified in a separate "dairy desserts" category, while creams and butter which were considered as fat sources. Thus, these three food groups were not included in this analysis. We calculated a larger "yogurt-like dairy products" category which included the consumption of yogurt along with curd cheese and petit-suisses, as those were not consumed frequently enough to be analysed separately. Total dairy intakes were calculated by combining the consumption of each of the five dairy groups (milk, cheese, yogurt, curd cheese and "petit-suisses"). In addition, we defined fermented dairy foods as cheese, curd cheese, petit-suisses, yogurts and fermented milk, whereas non-fermented dairy included all milks (UHT, pasteurised, concentrated and flavoured) except fermented ones. Finally, reduced-fat dairy products included skimmed and semi-skimmed milk, low-fat yogurts, curd cheese, petit-suisses and cheese containing less than 20g of fat per 100g final product.

Case ascertainment

Participants were invited to declare any major health event on a dedicated interface on the study website, either through the yearly health status questionnaire, through a specific health check-up questionnaire sent out every three months, or spontaneously. We asked participants to send their medical records (e.g. complementary examinations for diagnosis, hospitalisations and electrocardiograms) to support any health event declaration. A physician expert committee validated every major health event after reviewing the participants' medical records and collecting additional information from the participants' treating physicians or medical practices. In the absence of any response to the study website for more than one year, the physician expert committee contacted the participants' family or physicians. In addition to this process, which constituted the main source of case ascertainment, cohort data from participants was linked to medico-administrative databases from the National Health Insurance (SNIIRAM, authorisation by the Council of State No 2013-175). Finally, deaths and potentially missed CVD events in deceased participants were identified using data from the French national cause-specific mortality registry (CépiDC).

The International Classification of Diseases-Clinical Modification codes (ICD-CM, 10th revision) was used to classify CVD cases. This study focused on first incident cases of myocardial infarction (I21), angioplasty (Z95.8), acute coronary syndrome (I20.0 and I21.4), angina pectoris (I20.1, I20.8 and I20.9), stroke (I64) and transient ischemic attack (TIA, G45.8 and G45.9) occurring between inclusion and January 2019. CHD included all cases of myocardial infarction, angioplasty, acute coronary syndrome and angina pectoris, and cerebrovascular diseases included all cases of stroke and TIA.

Statistical analyses

As of the 1st of January 2019, participants with no history of CVD who had completed at least two valid 24-h dietary records were included in the analyses. Mean daily dairy intakes (overall and by type of dairy food) were coded as sex-specific quartiles, as potential distinctions in dietary patterns of French adult men and women have been previously reported [26,27]. Missing values represented less than 5% for all covariates, except for physical activity, and were imputed with the modal or median value for categorical and continuous variables, respectively. Physical activity scores were only calculated when all the answers from the IPAQ were provided by the participants, which resulted in a higher percentage of missing value for this variable (13.9%). Therefore, we introduced a missing class for this variable in the main analysis. Nonetheless, we performed additional analyses including complete cases and multiple imputation for missing values. We used the MICE method [28] to create 10 imputed datasets with fully conditional specification for the outcome [29] and the following covariates: physical activity level, education level, smoking status and BMI. We used the SAS PROC MIANALYZE procedure [30] to

combine the results from the imputed datasets, based on the combination rules proposed by Rubin [31,32].

Two dietary patterns were identified using a principal component analysis based on 20 food categories derived from the 58 food groups defined in the French PNNS (Supplementary Table 1). The analysis was conducted with the SAS PROC FACTOR procedure (SAS Institute Inc, Cary, NC). For easier interpretation, we used the SAS "varimax" option to rotate the principal components orthogonally and maximise the independence of the retained principal components. The first two principal components explained 10.6% and 7.0% of the variance, respectively, which was consistent with proportion of variance observed in other nutritional epidemiology studies [33]. The first principal component was characterised by higher intakes of fruits, vegetables, soups and broths, unsweetened soft drinks, and wholegrains, along with lower intakes of sweetened soft drinks, which we defined as a "Healthy" dietary pattern. In contrast, the second principal component was characterised by higher consumptions of fats and sauces (which included butter and dairy cream), alcohol, meat, and starchy foods, which we defined as a "Western" dietary pattern. We calculated an adherence score to each dietary pattern and for each participant, using the food categories factor loadings to weigh the sum of all food categories observed consumption. Thus, the adherence score measures a participant's diet conformity to the identified dietary pattern.

Multivariable Cox proportional hazard models with age as the primary time variable were used to characterise the associations between each type of dairy consumption and incidence of CVD, CHD and cerebrovascular diseases, and to calculate cause-specific hazard ratios and 95% confidence intervals. In the CHD model, cerebrovascular disease cases were censored at the date of diagnosis but were considered as non-cases for CHD, and reciprocally for the cerebrovascular disease model. The Schoenfeld residuals were used to confirm risk proportionality assumptions [34]. P-values for linear trends were obtained by coding quartiles of dairy consumption as an ordinal variable. Participants contributed person-time to the Cox model until the date of CVD diagnosis, the date of the last completed questionnaire, the date of death or 1st January 2019, whichever occurred first. Models were adjusted for age (time-scale), sex, physical activity (low, moderate, high, missing, computed following IPAQ recommendations), BMI (kg/m^2, continuous), education level (<high-school degree, <2 years after high-school degree, ≥2 years after high-school degree), without alcohol energy intake (kcal/d, continuous), alcohol intake (g/d, continuous), smoking status (never smoked, former smoker, current smoker), number of dietary records (continuous), family history of CVD (yes/no) (model 1).

In exploratory analyses, we performed an additional model to account for the potential influence of the nutritional quality of the diet. This included adjusting model 1 for a healthy dietary pattern derived

by principal component analysis (model 2, Supplementary table 1). Finally, further adjustments were added to model 1 to include the influence of baseline prevalence and treatment of self-reported type 2 diabetes, hypercholesterolemia, hypertension, and hypertriglyceridemia (model 3).

When Cox models revealed significant associations, sensitivity analyses were performed based on model 1 by adding further adjustments for a Western dietary pattern derived from principal component analysis (Supplementary Table 1). Finally, CVD cases diagnosed in the first two years of each participant's follow-up were excluded to account for reverse causation bias in statistically significant associations. All tests were two-sided and p-values ≤ 0.05 were considered statistically significant. All analyses were carried out with SAS software (version 9.4; SAS Institute Inc., Cary, NC).

6.3 Results

This analysis included 104,805 participants (see Figure 6.1) with a mean age of 42.8 years at baseline (SD 14.6), among which 22,291 were men (21.3%) and 82,517 were women (78.7%). Participants included in this analysis had completed on average 5.7 (SD 3.1) 24h-dietary records, with 8.1% of the participants having only completed the minimum two dietary records for inclusion in the analyses. The participants' baseline characteristics according to sex-specific quartiles of dairy intake are shown in Table 1. Overall, there was no significant difference in baseline characteristics between low consumers (1st quartile) and high consumers of dairy foods (4th quartile). In addition, 65% of our participants had moderate to high physical activity scores from the IPAQ, 65.4% had ≥ 2 years of education after high school and 82.8% did not smoke.

Participants consumed on average 222g/d of dairy foods (SD 151), including 110g/d of milk (SD 127), 37.7g/d of cheese (SD 28.3) and 79.1g/d of yogurt (SD 84.9), which was similar to the consumption levels observed in the general French population [9]. In addition, dairy foods contributed to 18.3% of total fat intakes (SD 13.7) and 28.9% of SFA intakes (SD 24.4) (Supplementary Table 2).

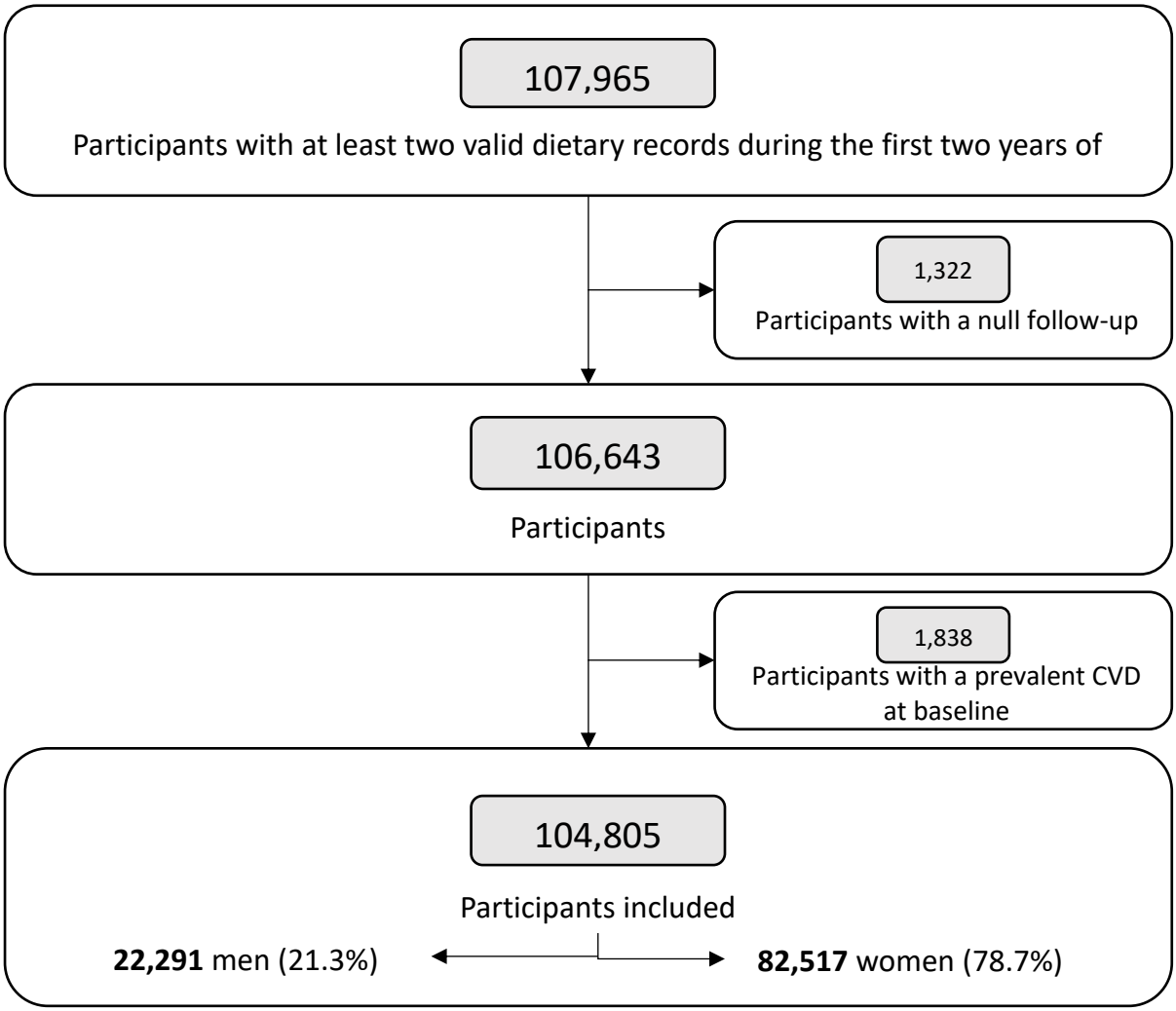

Figure 6.1. Flow chart of participants included in the study, NutriNet-Santé cohort, France, 2009-2019.

Table 6.1. Baseline characteristics of study population according to sex-specific quartiles of dairy consumption, NutriNet-Santé cohort, France, 2009-2019 (n=104,805).

Characteristics	All participants (n=104,805)		Quartiles of total dairy intake [a]								P-value [b]
			First (n=26,201) (lowest intake)		Second (n=26,202)		Third (n=26,203)		Fourth (n=26,202) (highest intake)		
Age, *years*	42.8	14.6	42.7	14.6	42.8	14.6	42.8	14.6	42.8	14.6	0.89
Sex											1.00
Female, *n*	82,517	78.7	20,629	78.7	20,629	78.7	20,630	78.7	20,629	78.7	
Male, *n*	22,291	21.3	5,572	21.3	5,573	21.3	5,573	21.3	5,573	21.3	
BMI, *kg/m²*	23.7	4.5	23.7	4.5	23.7	4.5	23.7	4.5	23.7	4.4	0.11
Physical Activity [c]											0.97
Low	2,2049	21.0	5,439	20.8	5,513	21.0	5,538	21.1	5,559	21.2	
Moderate	3,8712	36.9	9,710	37.1	9,676	36.9	9,633	36.8	9,693	37.0	
High	2,9447	28.1	7,366	28.1	7,376	28.2	7,370	28.1	7,335	28.0	
Education level											0.55
< High school degree	18,323	17.5	4,545	17.4	4,543	17.3	4,617	17.6	4,618	17.6	
< 2 years after high school	17,969	17.1	4,571	17.5	4,474	17.1	4,516	17.2	4,408	16.8	
≥ 2 years after high school	68,516	65.4	17,085	65.2	17,185	65.6	17,070	65.2	17,176	65.6	
Smoking status											0.66
Never	52,325	49.9	13,093	50.0	13,139	50.2	13,033	49.7	13,060	49.8	
Former	34,479	32.9	8,607	32.8	8,567	32.6	8,595	32.8	8,710	33.3	
Current	18,004	17.2	4,501	17.2	4,496	17.2	4,575	17.5	4,432	16.9	
Family history of CVD [d], *yes*	32,760	31.3	8,267	31.6	8,138	31.1	8,121	31.0	8,234	31.4	0.43
Prevalent morbidity, *yes*											
Type 2 diabetes	1,498	1.4	357	1.4	348	1.3	425	1.6	368	1.4	0.02
Hypertension	8,691	8.3	2,100	8.0	2,224	8.5	2,170	8.3	2,197	8.4	0.23
Hypercholesterolemia	8,396	8.0	2,073	8.0	2,135	8.0	2,097	8.0	2,091	8.0	0.79
Hypertriglyceridemia	1,536	1.5	383	1.5	378	1.4	390	1.5	385	1.5	0.98
Intakes of: [e]											
Energy, *kcal/d*	1,847	452	1,850	456	1,845	450	1,845	452	1,847	451	0.47
Alcohol, *g/d*	7.8	11.9	7.7	11.9	7.7	11.8	7.9	12.0	7.8	11.9	0.33
Total lipids, *g/d*	81.5	25.3	81.6	25.6	81.3	25.2	81.4	25.3	81.6	25.2	0.39
Carbohydrates, *g/d*	198.1	57.6	198.7	57.8	197.8	57.2	198.0	57.6	197.9	57.7	0.30
Proteins, *g/d*	78.8	21.5	78.9	21.6	79.0	21.5	78.7	21.4	78.8	21.5	0.58
Sodium, *g/d*	2.7	0.9	2.7	0.9	2.7	0.9	2.7	0.9	2.7	0.9	0.70
Total dietary fibre, *g/d*	19.5	7.2	19.5	7.2	19.5	7.1	19.5	7.3	19.4	7.2	0.54
Dietary Calcium, *mg/d*	921	299	922	299	922	299	919	299	921	299	0.61
Fruits and Vegetables, *g/d*	465	233	467	231	465	231	467	237	463	231	0.14
Total dairy, *g/d*	222	151	65	32	150	22	241	32	431	123	<0.001
Milk, *serving/d*	0.55	0.81	0.56	0.82	0.55	0.81	0.55	0.81	0.55	0.82	0.56
Cheese, *serving/d*	1.23	0.93	1.23	0.93	1.23	0.94	1.22	0.92	1.23	0.94	0.74
Yogurt, *serving/d*	0.47	0.55	0.09	0.16	0.38	0.33	0.64	0.51	0.77	0.77	<0.001

All values are presented as means ± SDs or *n* (%).**Abbreviations**: **CVD**, cardiovascular disease. **d**, day.

[a] Sex-specific quartiles of total dairy consumption. Cut-offs were 112, 190 and 303g/d for males and 112, 191 and 301g/d for females.

[b] P-value comparing quartiles of total dairy consumption, using two-sided χ^2 tests or Fisher tests as appropriate.

[c] Physical activity categories according to the International Physical Activity Questionnaire (IPAQ) [20] IPAQ data was missing for 14,600 participants (13.9%).

[d] Amongst first-degree relatives.

[e] Standard French serving sizes defined as 150ml for milk, 30g for cheese and 125g for yogurt

Associations between dairy consumption and CVD risk

Between 2009 and 2019 and a mean follow-up of 5.5 years (SD 3.0, 579,155 person years), 2,098 cases of CVD were diagnosed, among which there were 1,220 cases of CHD (82 acute coronary syndromes, 318 angina pectoris, 148 myocardial infarctions and 672 angioplasties) and 878 cases of cerebrovascular diseases (118 strokes and 760 TIAs).

Schoenfeld residuals were not significantly associated with time, which supported the proportional hazard assumption (Supplementary Table 3). The associations between the consumption of dairy foods and the risks of overall cardiovascular, coronary heart, and cerebrovascular diseases are presented in Table 2. The basic multivariable Cox proportional hazard (model 1) did not reveal any statistically significant association between the consumption of any dairy type and overall or coronary heart diseases. These associations remained statistically non-significant in models 2, 2b, and 3 (data not shown). However, high consumption (≥ 161.6g/d for males and ≥ 160.9g/d for females) of fermented dairy foods (yogurt, cheese and fermented milk) compared to low-consumption (< 57.3 g/d for males, < 54.3g/d for females) was associated with a 19% decreased risk of cerebrovascular disease (HR = 0.81, 95%CI = 0.66-0.98, p-trend=0.01). This association was borderline significant when considering the continuous intake of fermented dairy with an increment of 100g/d (HR = 0.98, 95%CI = 0.97-1.00, p-value=0.05). In addition, the restricted cubic spline presented in Figure 6.2 verified the linearity assumption between the consumption of fermented dairy foods and the risk of cerebrovascular disease (p-value for non-linear association=0.23) [35].

Exploratory and Sensitivity analyses

The association between fermented dairy consumption and cerebrovascular risk remained statistically significant when comparing the highest intake (4th quartile) to the lowest intake (1st quartile), after further adjustments in models 2 and 3 (Table 3). Similarly, this association remained stable in further exploratory analyses adjusting for a Western dietary pattern derived from principal component analysis (HR = 0.81, 95%CI = 0.66-0.98, p-trend=0.01). Furthermore, the use of multiple imputation with the MICE method to manage missing values strengthened the inverse association between continuous consumption of fermented foods and cerebrovascular disease risk (HR = 0.91, 95%CI = 0.84-0.99, p-value=0.02), but did not significantly impact other associations between continuous dairy consumption and overall, coronary heart or cerebrovascular disease risk. When excluding CVD cases that required more extensive documentation to ascertain diagnosis (i.e. TIAs and angina), all associations between dairy consumption and disease risk where non-significant, likely due to a loss of statistical power.

Finally, the exclusion of cerebrovascular disease cases diagnosed during the first two years of follow-up (n=144 cases excluded) suggested a 9% decreased risk of cerebrovascular disease associated with each additional 100g of fermented dairy food consumed daily (n=734 cases / 103,927 non cases, HR = 0.91, 95%CI = 0.83-0.99, p-trend=0.03), and a 21% decreased risk when comparing the highest (4th quartile) to the lowest (1st quartile) consumption of fermented dairy (HR = 0.79, 95%CI = 0.64-0.98, p-trend=0.001).

6.4 Discussion

In this prospective cohort study of French adults, we did not observe a statistically significant association between the consumption of dairy food and the risk of CVD or CHD. However, our results suggest a possible lower risk of cerebrovascular disease (i.e. stroke and TIA) associated with a higher consumption of fermented dairy foods, such as cheese and yogurts.

It is recommended to limit dietary SFA intakes for CVD prevention; however, this study did not reveal any direct association between the consumption of dairy products and total CVD or CHD risk, despite contributing to 28.9% of dietary SFA (Supplementary Table 2b). This supports the existing epidemiological evidence on the topic, especially from meta-analyses of prospective studies which consistently reported null or weak inverse associations between the consumption of total dairy and CVD risk [10–13,36]. In a 2018 meta-analysis, Soedamah-Muthu and de Goede reported a non-statistically significant association between total dairy and CHD risk when pooling the results from 15 prospective cohort studies, and reported an 8% reduced risk of stroke associated with an increment of 200g of milk consumption per day (Risk Ratio (RR) = 0.92, 95% confidence interval (CI) = 0.88-0.97, I^2 = 85%) [13]. Since then, the PURE prospective cohort study observed 5,855 CVD events over 9.1 years of follow-up from both urban and rural populations in 21 countries [14]. In this large prospective study, authors reported that a total dairy consumption of >2 servings per day, compared to no dairy consumption, was associated with a 22% risk reduction of major CVD (i.e. MI, stroke, or heart failure) (Hazard Ratio (HR) =0.78, 95%CI = 0.67-0.90, p-trend=0.0001) and a 34% reduced risk of incident stroke (HR = 0.66, 95%CI = 0.53-0.82, p-trend=0.0003). More recently, a small prospective cohort study from Greece, which included 2,020 participants followed-up for 10 years, observed an inverse association between total dairy intake and total CVD risk (HR = 0.48, 95%CI = 0.23-0.90) in women only. This inverse association in women was stronger when the authors looked at yogurt consumption, with a 14% CVD risk reduction associated with a 200g/d increment in yogurt intake (HR = 0.86, 95%CI = 0.49-0.98) [37].

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Table 6.2. Associations between dairy consumption and cardiovascular disease risk from multivariable Cox proportional hazard models [a], NutriNet-Santé cohort, France, 2009-2019 (n=104,805).

				Quartiles of dairy food intakes [b]				
		Continuous [c]	p-value	First (low intake)	Second	Third	Fourth (high intake)	p-trend
Total CVD								
Milk	Cases/non-cases	1,952/102,856		484/25,717	514/25,688	468/25,735	486/25,716	
	HR (95% CI)	0.99 (0.93-1.04)	0.57	1	1.05 (0.92-1.19)	0.96 (0.84-1.09)	1.00 (0.89-1.14)	0.70
Cheese	Cases/non-cases	1,952/102,856		468/25,732	507/25,691	489/25,731	488/25,702	
	HR (95% CI)	1.00 (0.95-1.04)	0.86	1	1.09 (0.96-1.24)	1.05 (0.93-1.20)	1.06 (0.93-1.20)	0.54
Yogurts	Cases/non-cases	1,952/102,856		530/30,180	405/21,173	544/26,781	473/24,722	
	HR (95% CI)	0.99 (0.94-1.04)	0.72	1	1.09 (0.96-1.24)	1.16 (1.03-1.30)	1.09 (0.96-1.23)	0.10
High-fat	Cases/non-cases	1,952/102,856		502/25,699	505/25,724	493/25,681	452/25,752	
	HR (95% CI)	0.92 (0.85-0.99)	0.04	1	1.01 (0.89-1.14)	0.99 (0.87-1.12)	0.91 (0.80-1.03)	0.12
Reduced-fat	Cases/non-cases	1,952/102,856		461/25,740	502/25,700	513/25,690	476/25,726	
	HR (95% CI)	1.00 (0.99-1.01)	0.93	1	1.09 (0.96-1.24)	1.11 (0.98-1.26)	1.04 (0.91-1.18)	0.57
Fermented	Cases/non-cases	1,952/102,856		487/25,714	515/25,687	463/25,740	487/25,715	
	HR (95% CI)	1.00 (0.99-1.01)	0.72	1	1.04 (0.92-1.18)	0.94 (0.83-1.07)	1.00 (0.88-1.13)	0.60
Non-fermented	Cases/non-cases	1,952/102,856		686/35,249	401/22,536	435/22,543	430/22,528	
	HR (95% CI)	1.00 (0.99-1.01)	0.55	1	0.91 (0.81-1.03)	0.99 (0.88-1.12)	0.99 (0.87-1.11)	0.97
Total dairy	Cases/non-cases	1,952/102,856		486/25,715	513/25,689	473/25,730	480/25,722	
	HR (95% CI)	0.99 (0.96-1.02)	0.48	1	1.06 (0.94-1.20)	0.98 (0.87-1.12)	0.99 (0.88-1.13)	0.62
Coronary Heart Disease [d]								
Milk	Cases/non-cases	1,219/103,586		296/25,905	347/25,853	301/25,902	275/25,926	
	HR (95% CI)	1.01 (0.94-1.08)	0.83	1	1.12 (0.96-1.31)	1.07 (0.91-1.26)	1.10 (0.93-1.30)	0.40
Cheese	Cases/non-cases	1,219/103,586		270/25,927	339/25,870	320/25,878	290/25,911	
	HR (95% CI)	0.99 (0.93-1.06)	0.85	1	1.03 (0.87-1.21)	0.93 (0.79-1.09)	0.96 (0.81-1.15)	0.42
Yogurts	Cases/non-cases	1,219/103,586		297/30,135	280/21,690	321/26,162	321/25,599	
	HR (95% CI)	0.96 (0.89-1.03)	0.21	1	0.95 (0.80-1.13)	0.97 (0.83-1.14)	0.87 (0.74-1.02)	0.12
High-fat	Cases/non-cases	1,219/103,586		288/25,911	316/25,888	299/25,902	316/25,885	
	HR (95% CI)	0.94 (0.85-1.04)	0.23	1	0.89 (0.76-1.04)	0.83 (0.70-0.98)	0.86 (0.73-1.02)	0.07
Reduced-fat	Cases/non-cases	1,219/103,586		287/25,915	339/25,861	305/25,897	288/25,913	
	HR (95% CI)	1.00 (0.96-1.04)	0.93	1	1.08 (0.92-1.27)	0.95 (0.81-1.12)	1.01 (0.85-1.19)	0.63
Fermented	Cases/non-cases	1,219/103,586		252/25,949	320/25,881	305/25,897	342/25,859	
	HR (95% CI)	0.99 (0.98-1.01)	0.21	1	1.00 (0.84-1.18)	0.84 (0.71-0.99)	0.89 (0.75-1.05)	0.05
Non-fermented	Cases/non-cases	1,219/103,586		298/25,903	349/25,852	293/25,909	279/25,922	
	HR (95% CI)	1.00 (0.99-1.01)	0.84	1	1.11 (0.95-1.30)	1.05 (0.89-1.23)	1.11 (0.94-1.31)	0.39
Total dairy	Cases/non-cases	1,219/103,586		287/25,914	335/25,866	304/25,898	293/25,908	
	HR (95% CI)	0.99 (0.95-1.03)	0.56	1	0.98 (0.84-1.15)	0.88 (0.75-1.04)	0.95 (0.80-1.12)	0.30
Cerebrovascular Disease [e]								
Milk	Cases/non-cases	878/103,927		207/25,994	248/25,952	227/25,976	196/26,005	
	HR (95% CI)	1.02 (0.94-1.11)	0.65	1	1.11 (0.92-1.34)	1.17 (0.96-1.41)	1.13 (0.92-1.38)	0.19
Cheese	Cases/non-cases	878/103,927		185/26,012	272/25,937	217/25,981	204/25,997	

	HR (95% CI)	0.96 (0.88-1.04)	0.33	1	1.19 (0.99-1.44)	0.91(0.74-1.11)	0.99 (0.80-1.22)	0.26
Yogurts	Cases/non-cases	878/103,927		187/30,245	221/21,749	235/26,248	235/25,685	
	HR (95% CI)	0.93 (0.85-1.01)	0.08	1	1.08 (0.88-1.32)	1.04 (0.86-1.27)	0.92 (0.76-1.12)	0.30
High-fat	Cases/non-cases	878/103,927		174/26,025	251/25,953	223/25,978	230/25,971	
	HR (95% CI)	0.96 (0.85-1.08)	0.45	1	1.16 (0.96-1.42)	1.01 (0.83-1.42)	1.00 (0.81-1.23)	0.54
Reduced-fat	Cases/non-cases	878/103,927		219/25,983	245/25,955	207/25,995	207/25,994	
	HR (95% CI)	0.99 (0.94-1.04)	0.62	1	1.02 (0.85-1.22)	0.85 (0.70-1.03)	0.94 (0.78-1.15)	0.23
Fermented	Cases/non-cases	878/103,927		180/26,021	229/25,972	232/25,970	237/25,964	
	HR (95% CI)	0.98 (0.97-1.00)	0.05	1	0.97 (0.79-1.18)	0.84 (0.69-1.02)	0.81 (0.66-0.98)	0.01
Non-fermented	Cases/non-cases	878/103,927		211/25,990	246/25,955	224/25,978	197/26,004	
	HR (95% CI)	1.00 (0.99-1.01)	0.67	1	1.08 (0.90-1.30)	1.14 (0.94-1.38)	1.12 (0.91-1.36)	0.23
Total dairy	Cases/non-cases	878/103,927		212/25,989	244/25,957	205/25,997	217/25,984	
	HR (95% CI)	0.98 (0.94-1.03)	0.42	1	0.95 (0.79-1.14)	0.79 (0.65-0.96)	0.93 (0.76-1.13)	0.19

Abbreviations: CVD, cardiovascular disease. **HR**, hazard ratio. **CI**, confidence interval.

[a] Cox models were adjusted for age (time-scale), sex, physical activity (low, moderate, high, computed following IPAQ recommendations), BMI (kg/m^2, continuous), education level (<high-school degree, <2 years after high-school degree, ≥2 years after high-school degree), without alcohol energy intake (kcal/d, continuous), alcohol intake (g/d, continuous), smoking status (never smoked, former smoker, current smoker), number of dietary records (continuous), family history of CVD (yes/no) (model 1).

[b] Sex specific cut-offs for milk were 16.1, 51.6 and 153.8 g/d in males and 16.3, 50.8 and 153.7 g/d in females. Cut-offs for cheese were 17.7, 32.9 and 51.4 g/d in males and 17.8, 33.0 and 51.6 g/d in females. Cut-offs for yogurt-like dairy were 0.04, 44.7 and 109.8 g/d in males and 11.9, 60.2 and 125.0 g/d in females. Cut-offs for high-fat dairy were 26.2, 50.5 and 84.6 g/d in males and 26.3, 50.0 and 83.9 g/d in females. Cut-offs for reduced-fat dairy were 46.7, 117.7 and 232.9 g/d for males and 47.1, 117.4 and 232.3 g/d in females. Cut-offs for fermented dairy were 57.3, 102.8, 161.6 g/d for males and 54.3, 100.6 and 160.9 g/d in females. Cut-offs for non-fermented dairy were 16.8, 54.3 and 168.6 g/d in males and 15.9, 48.8 and 147.5 g/d in females. Cut-offs for total dairy were 112.0, 186.8 and 303.0 g/d in males and 111.7, 190.8 and 301.5 g/d in females.

[c] Hazard Ratios for an absolute increment of 150 g/d of milk, 30 g/d of cheese, and 100 g/d of yogurts, high-fat, reduced-fat, fermented, non-fermented and total dairy.

[d] Includes myocardial infarction, angioplasty, acute coronary syndrome, and angina pectoris.

[e] Includes stroke and transient ischemic attack.

Table 6.3. Associations between fermented dairy foods and cerebrovascular disease risk from multivariable Cox proportional hazard models, NutriNet-Santé cohort, France, 2009-2019 (n=104,805). [a]

Proportional hazard Cox models [b,c]		**Quartiles of fermented dairy food intakes [d]**				
		First (low intake)	**Second**	**Third**	**Fourth (high intake)**	**p-trend**
	Cases/non-cases	180/26,021	229/25,972	232/25,970	237/25,964	
Model 1	**HR (95% CI)**	1	0.97 (0.79-1.18)	0.84 (0.69-1.02)	0.81 (0.66-0.98)	0.01
Model 2	**HR (95% CI)**	1	0.97 (0.79-1.18)	0.84 (0.69-1.02)	0.81 (0.66-0.98)	0.01
Model 2b	**HR (95% CI)**	1	0.97 (0.79-1.18)	0.84 (0.69-1.02)	0.81 (0.66-0.98)	0.01
Model 3	**HR (95% CI)**	1	0.96 (0.79-1.17)	0.83 (0.68-1.02)	0.80 (0.66-0.98)	0.01

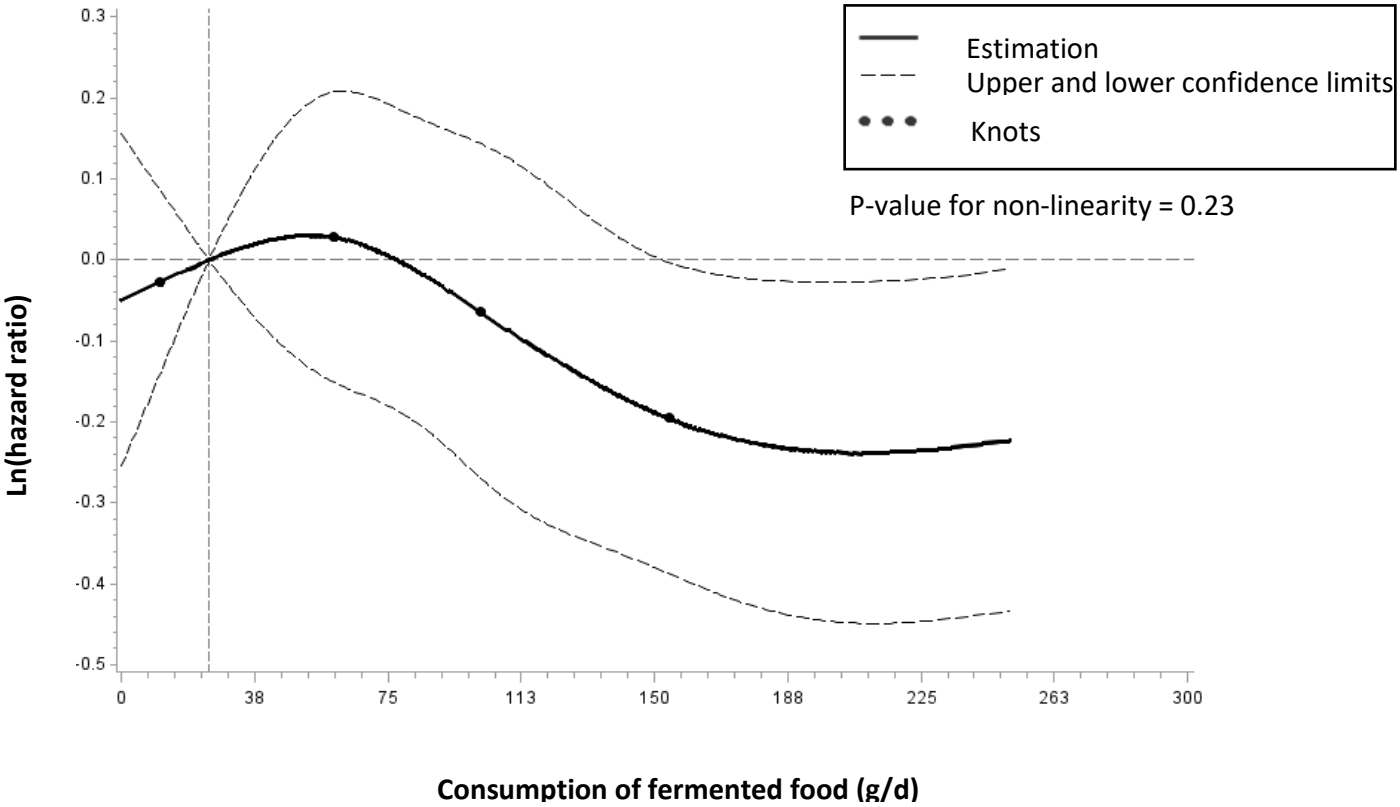
[a] Cerebrovascular disease included incident events of strokes and transient ischemic attacks.

[b] Cox models were adjusted for age (time-scale), sex, physical activity (low, moderate, high, computed following IPAQ recommendations), BMI (kg/m^2, continuous), education level (<high-school degree, <2 years after high-school degree, ≥2 years after high-school degree), without alcohol energy intake (kcal/d, continuous), alcohol intake (g/d, continuous), smoking status (never smoked, former smoker, current smoker), number of dietary records (continuous), family history of CVD (yes/no) (**model 1**). **Model 2**=Model 1 + healthy dietary pattern (derived by principal component analysis). **Model 2b**=Model 1 + Western dietary pattern derived by principal component analysis. **Model 3**=Model 1 + prevalence and treatment of type 2 diabetes, dyslipidaemia, hypertension and hypertriglyceridemia.

[c] Hazard Ratios for an absolute increment of 100 g/d of fermented dairy foods

[d] Sex specific cut-offs for fermented dairy were 57.3, 102.8, 161.6 g/d for males and 54.3, 100.6 and 160.9 g/d in females. Fermented dairy foods included yogurt, cheese and fermented milk.

a) Consumption of fermented dairy and risk of cerebrovascular diseases



b) Consumption of total dairy and risk of total CVD

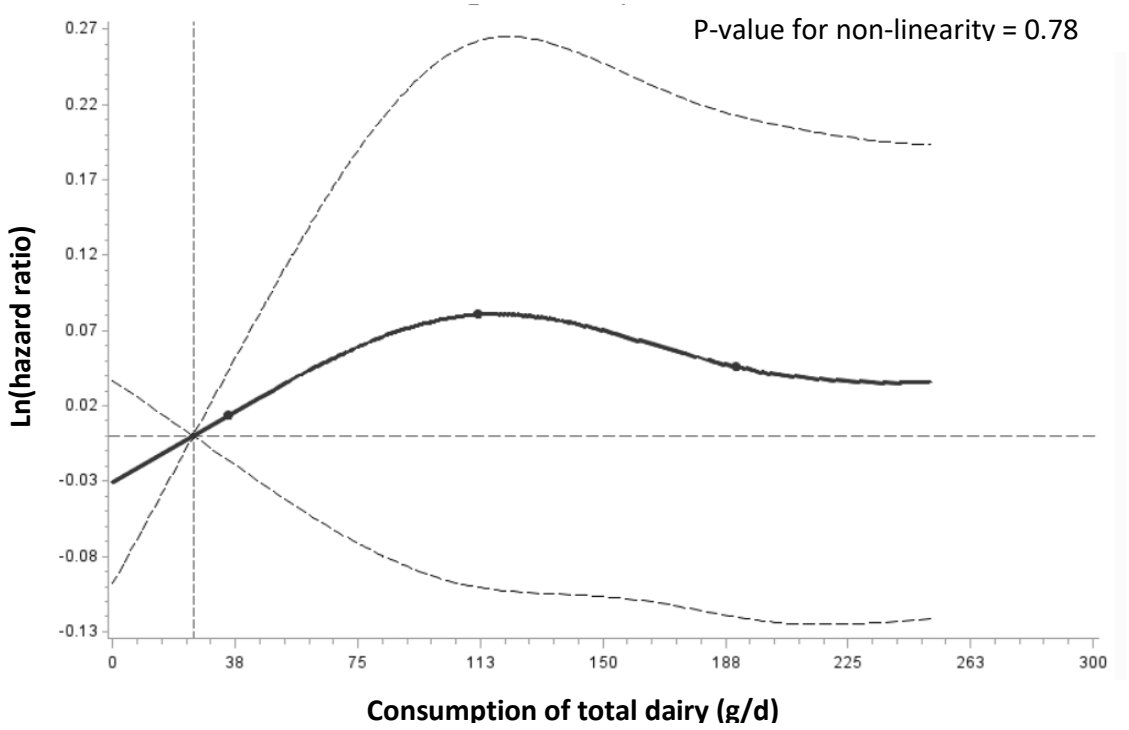


Figure 6.2. Spline plot for the linearity assumption of the association between the consumption of dairy foods and risk of cardiovascular diseases, NutriNet-Santé cohort, France, 2009-2019 (n=104,805). *Restricted cubic spline SAS macro developed by Desquilbet and Mariotti [35].*

The inverse association between fermented dairy and cerebrovascular risk observed in this study may suggest a differential effect of these types of dairy foods on cardiovascular health. In a 2019 meta-analysis of randomised controlled trials and prospective studies, Companys et al. observed that the consumption of fermented milk was associated with a reduced risk of stroke and ischemic heart disease (RR = 0.96, 95% CI 0.94 to 0.98, high heterogeneity I^2=95.9%) [38]. This finding was in line with those reported in an extensive review of meta-analyses conducted by Fontecha et al. in 2019, which observed a reduced risk of stroke and stroke mortality associated with the consumption of fermented dairy, including fermented milk [39] and cheese [12,39–42], but not yogurt [10].

Another potential source of variation in the health effects of dairy consumption relates to the nutrient content of specific types of dairy foods. In the large European EPIC-Netherlands study, Laursen et al. observed 884 stroke cases over a 15.2-year follow-up. They reported that the consumption of each additional daily serving of whole-fat yogurt as a substitution for any other dairy group (low-fat yogurt, cheese, butter, buttermilk or milk) was associated with a lower risk of ischemic stroke (HR between 0.33 and 0.36) [43]. These findings were in line with previous results observed by the same authors in another European prospective study, the Danish Diet, Cancer and Health cohort [44]. However, emerging evidence suggest that the nutrient content of dairy should be considered in relation to the dairy food matrix, which refers to the physical structure of food and may have an impact on nutrient absorption and biomarkers of CVD risk, such as blood pressure and cholesterol metabolism. In particular, one hypothesis suggests that cheese, despite being high in fat, possesses similar features to milk and yogurt, rather than to butter, due to its high calcium, protein and milk fat globule membrane content [45]. In addition, the fermentation process involved in the production of cheese and yogurt often results in the presence of bacteria within the food matrix, which may produce short-chain fatty acids and bioactive peptides [45]. All these components of dairy, particularly present in cheese and yogurt, may interfere with the intestinal absorption and digestibility of fat (which is mostly SFA in dairy foods) and therefore attenuate the effect of SFA on cholesterol metabolism and potentially provide a protective effect on cardiovascular health [46–49]. Although more well-controlled intervention studies in humans are necessary to further investigate these potential mechanisms, this would be in line with observational evidence from meta-analyses of prospective studies, which suggest that fermented dairy consumption may be associated with lower total and low-density lipoprotein cholesterol levels [50–53]. Finally, a potential hypotensive effect of bioactive peptides found in fermented dairy foods may reduce cerebrovascular diseases risk, which would be in line with observational epidemiological studies, but still needs further research to be fully elucidated [54].

The prospective design of this study contributed to its strengths, allowing the assessment of mid-term associations of dairy consumption with CVD risks. In addition, this study used repeated 24h-dietary

records to provide detailed and up-to-date dietary intake assessment, as opposed to food frequency questionnaires which are often used in nutritional epidemiology. In this study, we identified two Healthy and Western dietary patterns using a principal component analysis (Supplementary Table 1), and these patterns did not influence the associations observed between fermented dairy consumption and cerebrovascular risk. Finally, the use of the SNIIRAM national register allowed the maximisation of CVD case ascertainment, limiting the omission of cases when participants did not report their disease to the study investigators. However, some limitations of this study also pertain to its observational design. Indeed, residual confounding cannot be ruled out and a causal link between the consumption of fermented dairy and a decreased risk of cerebrovascular disease cannot be established from this prospective cohort study alone, although the inclusion of many potential confounders in our main analyses suggest a robust inverse association. Furthermore, a relatively limited statistical power precluded the investigation of specific types of CVDs. The NutriNet-Santé cohort is volunteered-based, and as highlighted in Table 1, the participants included in this study were generally more health-conscious, younger, more highly educated, more often women, consumed more fruits and vegetables [9] and were less likely to smoke or be overweight [55], compared to the general French population.

In conclusion, in this large prospective cohort study we found that the consumption of dairy foods may not be associated with overall CVD or CHD risks in French adults. However, we observed a higher consumption of fermented dairy products (e.g. cheese and yogurt) to be associated with a lower risk of stroke and TIA. Overall, these observational findings provide insight on the potential role of specific dairy foods in cardiometabolic health. However, future RCTs are warranted to confirm these associations.

6.5 Supplementary material

Supplementary table 6.1. Factor loadings from principal component analysis used to derive dietary patterns.

The principal component analysis creates linear combinations (called principal components) of the 20 food categories, with the aim to group together food categories that are correlated while explaining as much variation from the dataset as possible.

Food categories used for this principal component analysis were derived from the 58 foods groups defined in the French PNNS. Notably, the “Dairy products” category included milk, cheese, yogurt, cheese, curd-cheese, and “petit-suisses”, whereas butter and dairy cream were included in the “Fats and sauces” categories [(8)].

The coefficients derived from the selected principal components are called factor loadings. A positive factor loading indicates a positive contribution of the food category to the principal component, whereas a negative factor loading indicates a negative contribution to the principal component. For the interpretation of the two principal components selected, we considered the food categories contributing the most to the component, i.e. with loading coefficients under -0.25 or over 0.25. We then label the principal components descriptively, based on the most contributing food categories. The healthy pattern (explaining 10.6% of the variance) was characterised by higher intakes of fruits, vegetables, soups and broths, unsweetened soft drinks, and whole grains, and lower sweetened soft drinks intake. The Western pattern (explaining 7.0% of the variance) was characterised by higher intakes of fat and sauces, alcohol, meat, and starchy foods.

Food categories	**Factor loadings**	
	Healthy Pattern	**Western Pattern**
Alcoholic drinks	-.09	**0.28**
Breakfast cereals	0.07	-.18
Cakes and biscuits	-.19	0.00
Dairy products	0.06	-.01
Eggs	0.07	0.04
Fats and sauces	0.01	**0.54**
Fish and seafood	0.20	0.10
Fruit	**0.35**	0.05
Meat	-.18	**0.31**
Pasta and rice	-.21	**0.34**
Potatoes and tubers	-.02	**0.40**
Poultry	-.03	0.06
Processed meat	-.22	0.20
Pulses	0.19	0.02
Soups and broths	**0.26**	0.22
Sugar and confectionery	-.08	0.12
Sweetened soft drinks	**-.28**	-.00
Unsweetened soft drinks	**0.25**	0.15
Vegetables	**0.47**	0.23
Whole grains	**0.38**	-.04

Supplementary table 6.2a. Consumption of dairy food in the NutriNet-Santé cohort, France, 2009-2019 (n=104,805).

Dairy food	Consumers (%)	Consumption (g/d)		Consumption in a representative French population (g/d) [a]	
		Mean	SD	Mean	SD
Milk	95.6	110	126.9	172.3	176.3
Cheese	94.8	37.7	28.3	38.5	30.4
Yogurts	79.2	79.1	84.9	76.7	78.7
High-fat	96.4	63.7	58	*NA*	
Reduced-fat	98.1	158.3	145.3	*NA*	
Fermented	97.9	117.5	87.3	*NA*	
Non-fermented	95.6	104.4	125.9	*NA*	
Total dairy	99.5	221.9	151.1	*NA*	

[a] As reported in the Third Individual and National Survey on Food consumption (INCA3) [(9).]

Supplementary table 6.2b. Contribution of dairy foods to key nutrient intakes in the NutriNet-Santé cohort, France, 2009-2019 (n=104,805).

Nutrient	Contribution from total dairy foods[a]				
	g/d		*% total nutrient*		*% total nutrient in the French population[b]*
	Mean	SD	Mean	SD	
Total fats	13.5	8.3	18.3	13.7	15.0
SFA	8.3	5.2	28.9	24.4	24.0
Protein	16.7	8.9	22.8	14.4	15.0
Sugars	9.9	7.7	12.4	12.3	10.0
Calcium	0.46	0.25	55.9	0.39	38.0
Iodine	36.4	22.0	29.0	25.1	20.0

[a] Values are presented as mean ± SD.

[b] As reported in the Third Individual and National Survey on Food consumption (INCA3) [(9).]

Supplementary table 6.3. Assessment of the proportional hazard assumption using the Schoenfeld residual method, NutriNet-Santé cohort, France, 2009-2019 (n=104,805).

The Schoenfeld residual method was used to test the proportional hazard assumption when performing Cox proportional hazard model [34]. The assumption is supported if there is no statistically significant correlation between the Schoenfeld residuals and time. P-values from Person correlations between the Schoenfeld residuals of each dairy food consumption in g/d and timescale (age, in years) are reported in the table below, and confirm that the proportional hazard assumption is verified.

Dairy food	**p-value**
Milk	0.33
Cheese	0.88
Yogurts	0.41
High-fat	0.53
Reduced-fat	0.12
Fermented	0.40
Non-fermented	0.35
Total dairy	0.21

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Chapter 7: General discussion and perspectives for future work

7.1 General discussion

The detrimental impact of high dietary saturated fatty acid (SFA) intakes on cardiometabolic disease risk (CMD) risk has been established for decades and is reflected in current public dietary guidelines worldwide, with a general consensus that SFAs should not exceed 10% total energy (TE) in adults [1,2]. While most of the previous research focused on the impact of overall dietary SFAs on traditional biomarkers of CMD risk, such as fasting lipid profiles, or long-term CMD risk, their overall and/or specific effect on other risk factors (e.g. markers of inflammation, haemostasis, glycaemic control) or novel omics markers such as the plasma lipidome have not been fully elucidated yet [1,2]. In addition, specific food sources of dietary SFAs may exert differential effects on cardiometabolic health status, as illustrated by the seemingly paradoxical lack of deleterious associations between dairy foods and CMD risk observed in epidemiological studies despite their high SFA content [3–5]. Overall, this PhD thesis aimed to explore these two knowledge gaps, with the overarching hypothesis that the detrimental impact of dietary SFAs on cardiometabolic health may be modulated by their effect on novel markers of CMD risk and/or the food matrix from which SFAs are consumed.

The impacts of specific and overall dietary SFAs on traditional and more novel CMD risk markers were investigated in Chapters 2 and 3. In a joint lipidomics analysis of the DIVAS randomised controll ed trial (RCT) and the EPIC-Potsdam prospective cohort study, we observed that the replacement of dietary SFAs with unsaturated fatty acids (UFAs) in the DIVAS RCT reduced the plasma concentrations of SFA-containing lipid metabolites such as glycerolipids and sphingolipids, which were associated with higher CMD risk in the EPIC-Potsdam cohort (Chapter 2). These novel results complemented those previously published from the EPIC-Potsdam study group, which revealed glycerolipids containing palmitic (16:0) and stearic acids (18:0) had the strongest associations with CVD and T2D risk among the 282 within-class FA plasma concentrations investigated [6]. In addition, prospective analyses in the DIVAS RCT revealed that increased low-density lipoprotein cholesterol (LDL-C) serum concentrations over 16 weeks were associated with higher plasma concentrations of glycerolipids containing lauric (12:0) and stearic acids (18:0). Similar prospective associations were also observed between changes in specific plasma sphingolipids and/or phospholipids and other markers of CMD risk, such as markers of endothelial function, arterial stiffness, and ambulatory blood pressure. Altogether, these results suggest that the benefits of replacing dietary SFAs with UFAs for CMD prevention may be mediated by specific changes in the plasma lipidome involving sphingolipids (i.e. ceramides and sphingomyelin) and glycerolipids (i.e. mono-, di-, and triacylglycerol) containing specific fatty acids (FAs).

To further explore the hypothesis that individual FAs may differentially impact CMD risk, we conducted a systematic literature review of RCTs, with a focus on dietary SFAs rather than circulating ones (Chapter 3). In particular, our meta-analyses revealed that the isoenergetic replacement of at least 1.5% total energy from palmitic acid (16:0) with oleic acid (18:1 *cis*-9) or total UFAs for at least 14 days may decrease total cholesterol, LDL-C, and apolipoprotein B concentrations in adults, which broadly aligned with current public dietary recommendations for CMD prevention. However, our quantitative analyses suggested high heterogeneity between studies and therefore need to be interpreted with caution. Furthermore, the systematic literature review process highlighted important research gaps on the isoenergetic substitutions of short-chain SFAs, lauric acid (12:0), or myristic acid (14:0), and only few studies reported impacts on less traditional markers of CMD risk such as markers of inflammation or endothelial activation. Overall, this work contributes to the evidence-base advocating for dietary SFA reduction for CMD risk prevention by investigating the potential physiological effects of SFA replacement on the plasma lipidome and assessing the impact of individual dietary SFAs on cardiometabolic health status. Nonetheless, several research gaps still need to be addressed to reach a consensus on the recent controversies surround dietary SFAs [7]. In particular, the benefits of SFA reduction to lower CVD risk have been questioned after recent studies observed null or inverse associations between SFA intakes and disease risk [8–10]. Among those, the Prospective Urban Rural Epidemiology (PURE) observed inverse associations between SFA intakes and stroke risk among 135,335 adults from 21 countries, although the interpretation of this result needs to account for the 8.0% TE median SFA intake in this cohort, which is below most current public health recommendations [10]. In addition, the upper limit set at 10% TE for SFAs in adults has been challenged by experts in the field, on the basis that the impact of dietary SFAs on disease risk may be modulated by the food sources of dietary SFAs (e.g. dairy, meat, dark chocolate) and the inter-individual responses to high SFA intakes [11].

From a food perspective, the analyses performed in the NutriNet-Santé prospective cohort study (Chapter 6) broadly aligned with previous epidemiological studies, which often failed to report statistically significant associations between most dairy consumptions and cardiovascular disease (CVD) risk. Nonetheless, we observed that high fermented dairy food intakes (i.e. at least 160 g/d of combined intakes of cheese, yogurt, and fermented milk) were associated with a 19% reduction in cerebrovascular risk compared to study participants who consumed less than 57 g/d of these foods. These results might be partly explained by possible beneficial properties of the fermented dairy food matrix, as suggested in previous studies [12]. In addition, calcium and proteins in dairy may interfere with the intestinal absorption of dairy fat and possibly mitigate the deleterious effects of the latter on cholesterol metabolism and blood pressure regulation, thus generally improving cardiometabolic

health status [13–15]. These potentially beneficial health effects of the dairy food matrix influenced the design of the SATgenε, DIVAS, and RISSCI-1 food-based dietary fat exchange models, which incorporated lower-fat dairy foods to successfully reduce dietary SFAs in UK adults without losing the potential health benefits of dairy food components [16–18]. In compliance analyses on plasma phospholipid fatty acids (PL FAs) among the RISSCI-1 study participants (Chapter 4), we showed that dietary guidelines on dietary SFAs could be achieved in free-living UK adult men mostly by replacing commercially available high-fat dairy foods (e.g. butter, whole milk, high-fat cheese like Cheddar) with lower-fat alternatives (e.g. skimmed or semi-skimmed milk, cottage or spreadable cheese). Importantly, we showed that dietary SFAs could be isoenergetically replaced with UFAs in this population to reach dietary recommendation levels without impacting other macronutrient intakes (i.e. carbohydrates and proteins), and without disrupting participants habitual consumptions of non-dairy food groups (e.g. meat, fish, fruits and vegetable, etc.). Overall, our findings support the hypothesis that dairy foods can be consumed as part of healthy dietary patterns, although previous research has mostly focused on low-fat dairy intakes. For instance, while Mediterranean dietary patterns, which are traditionally low in dairy foods [19], have been previously associated with lower CMD risk in large prospective studies such as the PREDIMED RCT [20] and the EPIC-Potsdam cohort [21], a recent modelling study suggested that increasing dairy food consumption within a Mediterranean diet may improve adult's adherence dietary reference intakes for calcium, vitamin D, and potassium in US adults [22]. Furthermore, a crossover RCT (n = 37 adults) observed improved CMD risk marker profiles (i.e. reduced blood pressure, total to high-density lipoprotein cholesterol ratio, and triacylglycerol) after following a Mediterranean diet enriched with 3 to 4 daily servings of low to medium-fat dairy for eight weeks compared to a low-fat control Mediterranean diet [23]. Other dietary patterns such as the Nordic diet or the Dietary Approach to Stop Hypertension (DASH), which incorporate low-fat dairy food consumption in modest amounts, have also been associated with beneficial cardiometabolic health status [24,25].

One common aspect that emerged from our work on dietary intervention trials and larger-scale prospective cohort studies was the importance of an accurate and reliable method for the assessment of dairy intakes. The RISSCI-1 study (Chapter 4), similarly to the SATgenε, DIVAS, and RESET trials (Chapter 5), relied on paper diet diaries in which participants were required to record the weight and description all food items and beverages consumed over three to four days. In the NutriNet-Santé cohort study (Chapter 6), a dedicated web-based platform was developed for study participants to record all foods and drinks consumed throughout the day either within a database of > 3,500 items or by inputting their consumptions manually where necessary, providing important cost and logistic advantages over paper-based diet diaries while maintaining high data quality [26]. Finally, other large

prospective cohort studies, such as the EPIC-Potsdam cohort (Chapter 2), rely on food frequency questionnaires to collect dietary data. This method, although routinely used in epidemiological research as it captures medium-term habitual dietary intakes, may introduce recall bias and a lack of precision in dietary assessments. In addition, all these methods require nutrient information from data food composition tables which highly vary in quality and comprehensiveness, potentially introducing an additional source of measurement error.

In the case of dairy intake assessment, our narrative literature review (Chapter 1) identified the need for novel approaches relying on objective biomarkers of dairy consumption that would complement more traditional dietary assessment methods. An important interest in using circulating odd-chain SFAs (e.g. pentadecanoic 15:0 and heptadecanoic acids 17:0) along with *trans*-palmitoleic acid (16:1 *trans*-9) as biomarkers of dairy intake has emerged in epidemiological research. However, our review highlighted that they only moderately correlate with reported dairy consumptions in randomised controlled trials, that they are commonly used as proxies for total dairy intakes despite their lack of correlation with low-fat dairy, and that odd-chain fatty acids in particular may be endogenously synthesised in humans in small amounts. Other circulating fatty acids such as *trans* vaccenic (18:1 *trans*-11) and phytanic acids also presented interesting characteristics as potential biomarkers of intakes, but have only been scarcely studied in human RCTs in relation to dairy consumptions. Interestingly, we found that plasma PL pentadecanoic acid (15:0) and *trans* vaccenic acids (18:1 *trans*-11) were independently associated with increased dairy fat consumptions in the RISSCI-1 study (Chapter 4), but these results were not replicated in the pooled secondary analysis of the SATgenε, DIVAS, and RESET intervention studies (Chapter 5). In fact, the latter analysis only identified plasma PL myristic acid (14:0) and undecanoic acid (11:0) as possible proxies for increased consumptions of total dairy fat, and cheese or cheese fat, respectively, but failed to identify plasma PL FAs associated with increased milk, yogurt, cream, or butter intakes. Such discrepancies highlight the important need for large-scale, long-term RCTs specifically designed for the identification and validation of individual circulating FAs as biomarkers of dairy fat consumption.

The primary strength of this collection of research projects pertained to the variety of the study designs and research approaches included, which allowed for the interpretation of the role of dairy foods as contributors to dietary SFAs in relation to CMD risk at different levels. First, experimental approaches using gas chromatography (Chapter 5) and high-throughput lipidomics (Chapter 2) allowed for the identification and quantification of up to n=74 plasma PL FAs and n=987 individual plasma lipid metabolites, respectively. This wide range of human plasma metabolites measured before and after the exchange of dietary fat in two RCTs (i.e. the DIVAS, RESET studies) and two sequential

intervention studies (i.e. the SATgenε and RISSCI-1 studies) allowed for the investigation of prospective associations in controlled conditions, which strengthened the design of our analyses. The secondary analysis of previously conducted intervention studies (Chapter 5), and the pooled analysis 44 RCTs in Chapter 3, also contributed to the overall strength of this PhD thesis by allowing more confident causal inference compared to observational studies. Nonetheless, our epidemiological analyses within the EPIC-Potsdam (Chapter 2) and NutriNet-Santé (Chapter 6) prospective cohort studies, despite being observational, allowed for the assessment of long-term CMD risk in relation to dietary intakes at a large scale thanks to the detection of validated CVD and T2D diagnosis events. More specific strengths of our individual research projects included the combined analysis of the DIVAS RCT and the EPIC-Potsdam cohort study, which allowed for the prospective investigation of both medium-term changes in CMD risk markers and long-term incident CMD risk (Chapter 2). This work contributed to strengthening the evidence base for a link between dietary SFAs and CMD risk, and generated new hypotheses about the mediating role of the plasma lipidome in these associations. In addition, we conducted the first, to our knowledge, systematic literature review to provide a comprehensive overview of the current knowledge on individual SFAs and their impact on CMD risk markers (Chapter 3). Finally, the development of a food-based approach based on dairy foods in the RISSCI-1 study explored the possible practical applications to achieve public dietary guidelines on SFAs in free living UK adult men following a typical UK diet without disrupting their dietary habits (Chapter 4).

Nonetheless, some limitations need to be acknowledged. First, the joint analysis of the DIVAS RCT and the EPIC-Potsdam cohort (Chapter 2) provided an interesting perspective on bridging the gap between interventional and observational research, but the two studies were based on different populations, which limited the interpretation of our findings in a causal context. Second, the high variation in reporting methods in RCTs limited the inclusion of some studies in our systematic review and meta-analysis (Chapter 3), particularly when assessing older RCTs which had different reporting standards at the time of their publication. Third, the dietary intervention studies analysed for the identification of plasma PL FAs associated with chronic dairy consumption (Chapter 5) were not specifically designed for the detection of biomarkers of dairy intakes. In particular, the consumption of high-SFA snacks to achieve the nutritional targets of high-SFA dietary intervention, along with the lack of quantification of dairy fat within processed foods and ready-to-eat meals, may have impaired the precision of the observed associations between plasma PL FA abundances and dairy consumptions. In addition, the identification of specific plasma PL FAs would have been improved by a coupled gas chromatography – mass spectrometry analysis, rather than relying on gas chromatography retention times alone. Finally, observational associations in prospective studies (Chapters 2 and 6) cannot entirely rule out

potential residual confounding, although a wide range of potential confounders have been included in the statistical models to help mitigate this risk.

7.2 Conclusion

To conclude, my results on the role of dietary SFAs in cardiometabolic health concur with current public dietary guidelines which recommend their reduction to less than 10% total energy. In particular, I observed favourable effects of replacing dietary palmitic acid (16:0) with UFAs on fasting lipid profiles in a meta-analysis of 44 RCTs, and showed that the long-term reduction of CMD risk observed when lowering overall dietary SFAs may be mediated by beneficial modulations of the plasma lipidome. In addition, my results broadly align with current evidence that dairy foods, despite being important contributors of dietary SFAs in European adult diets, are not associated with increased CVD risk. On the contrary, our findings suggest that some elements of the dairy food matrix, such as the fermentation process, may contribute to the beneficial properties of dairy foods in the context of cardiometabolic health.

Nonetheless, my work highlighted important challenges for future research, including the need for better dietary assessment methods which may combine traditional methods with novel validated biomarkers of intakes. In this respect, our search for plasma PL FAs associated with dairy consumptions yielded contrasting results, which may be partly explained by the design of the included intervention studies which did not primarily aim to identify biomarkers of dairy intakes. Finally, the research studies presented in this thesis provide novel insights into the potential of combining interventional and observational study designs to identify causal pathways between dietary fat intakes and long-term CMD risk.

7.3 Future work

The current PhD thesis focused on the investigation of the detrimental impact of dietary SFAs on cardiometabolic health and how it may be modulated by their effect on the plasma lipidome and/or the food matrix from which SFAs are consumed, with the example of dairy foods. While this work provided novel evidence addressing this research question, other aspects still need to be investigated.

First, emerging work in prospective cohort studies have identified lipidomics and/or metabolomics biomarkers of dairy consumption in large populations, such as the recent work by Drouin-Chartier et al. among the PREDIMED intervention study, the Nurses' Health Study, the Nurses' Health Study II, and the Health Professionals Follow-Up Study [27]. Their analysis identified 38 plasma metabolites associated with higher reported dairy consumptions, among which myristic acid (14:0) in

sphingomyelin and C34:0 phosphatidylethanolamine. While these findings were observational, applying such omics methods to well-controlled dietary intervention studies would provide important and novel insights into the metabolomic signatures associated with dairy consumption, which may significantly improve the way dietary intakes are assessed.

Second, recent research has suggested that the strength of statistical associations between dietary SFAs and CVD risk may be affected by important variations in LDL-C response to dietary SFAs between individuals, potentially due to differential cholesterol synthesis and reabsorption pathways, bile acid metabolism, and/or genetic factors [28,29]. The RISSCI-1 study described in Chapter 4 was designed to identify high- and low-responders to a reduction in dietary SFAs among UK adult men based on changes in fasting LDL-C concentration. While results from this study have not been published yet, the research team successfully identified non-responder and responder groups to the dietary replacement of SFAs with UFAs, which were invited to participate in an acute-within-chronic follow-up study based on the same food-based dietary fat exchange model (RISSCI-2). Results from this follow-up study are still being analysed, and will hopefully further elucidate the mechanisms regulating the individual LDL-C response to dietary SFAs.

Finally, an increasing body of evidence suggests the dairy food matrix contains beneficial components for cardiometabolic health, with a particular interest in the fermentation process and its impact on yogurt, cheese, and fermented milk food matrices. Indeed, these foods contain bacteria producing short-chain FAs and bioactive peptides, which have been suggested to improve the gut barrier integrity, lipid metabolism, and blood pressure [12,30,31]. Nonetheless, little is known about the impact of different types of dairy products, strains of bacteria involved in the fermentation process, or other foods consumed with dairy on these beneficial health effects, and future research in this direction is warranted to better understand the place of dairy foods within a healthy diet.

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Appendix 1: Full-text abstracts published in conference proceedings as a first author

The impact of dietary saturated fat replacement with unsaturated fat on the plasma lipidome and cardiometabolic disease risk

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Winter Conference 2021, 7–8 December 2021, Obesity and the brain. **Published in Proceedings of the Nutrition Society. Cambridge University Press; 2022;81(OCE1):E51.**

Evidence from epidemiological studies and randomised controlled trials (RCTs) suggests that replacing dietary saturated (SFAs) with unsaturated fatty acids (UFA) may have beneficial impacts on cardiometabolic disease (CMD) risk [1]. However, interdisciplinary research narrowing the gap between interventional and observational evidence is lacking. Recent findings have suggested the utility of high-throughput lipidomics to identify potential CMD risk markers and provide novel aetiological insights into the relationship between dietary fat composition and CMD risk [2]. Thus, this study aimed to assess the lipidome-mediated impact of replacing dietary SFAs with UFAs on CMD risk. Plasma fatty acid (FA) concentrations among 14 lipid classes were measured using high-throughput lipidomics analyses (Metabolon, USA) in samples from the DIVAS parallel RCT (n = 113), which investigated the effects of three 16-week diets enriched in SFAs (target SFA:monounsaturated fatty acids MUFA:n-6 polyunsaturated fatty acids PUFA ratio = 17:11:4% total energy TE), MUFAs (target SFA:MUFA:n-6PUFA ratio = 9:19:4%TE), or a mixture of UFAs (target SFA:MUFA:n-6PUFA ratio = 9:13:10% TE) on CMD risk markers such as fasting lipid profiles, and markers of inflammation, endothelial function, and arterial stiffness(3) . Similar lipidomics analyses were conducted on samples from two case-cohorts from the EPIC-Potsdam prospective cohort study [n = 1,707 for type 2 diabetes (T2D) and n = 1,886 for cardiovascular diseases (CVD)][2]. Within-class FAs sensitive to the DIVAS dietary intervention were identified using multiple linear regression models and related to CMD risks in each EPIC-Potsdam case-cohort using multivariable Cox proportional hazard models. Finally, within- class FAs associated with changes in CMD risk markers assessed in the DIVAS study were identified using constraint-based feature selection algorithms and multiple linear regression models. Analysis of within-class plasma FA

concentrations revealed high-UFA intervention diets from the DIVAS study broadly reduced the concentrations of FAs associated with higher CVD risk, and to a lesser extent T2D risk, in the EPIC-Potsdam cohort, such as palmitic (16:0) and stearic (18:0) acids in di- and triacylglycerol, and myristic acid (14:0) in hexosylceramides, with clearer effects of the high- MUFA diet compared to the mixed-UFA. Reciprocally, the high-UFA diets increased the concentrations of FAs associated with lower CMD risk, such as erucic acid (22:1) in triacylglycerol and nervonic acid (24:1) in lactosylceramides. Furthermore, increased low-density lipoprotein cholesterol and total cholesterol concentrations were associated with a higher abundance of arachidic acid (20:0) in cholesteryl esters and diacylglycerol ($p < 10-3$ and $p = 0.001$, respectively), whilst increased interleukin-6 and P-selectin concentrations were associated with higher proportions of arachidic acid (20:0) in mono- ($p = 0.008$) and triacylglycerol ($p = 0.02$). Overall, these findings suggest a potential mediating role of plasma lipid metabolites in the association between dietary fat and CMD risk. Future research combining interventional and observational findings and investigating the identified within-class FAs is warranted to improve our understanding of dietary fat composition in CMD aetiology.

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Plasma phospholipid fatty acid profiles confirm compliance to the dietary exchange of saturated with unsaturated fat in healthy men using full-fat or lower-fat dairy foods: results from the Reading, Imperial, Surrey, Saturated fat, Cholesterol Intervention (RISSCI) study

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Summer Conference, 6–8 July 2021, Nutrition in a changing world. **Published in Proceedings of the Nutrition Society (2021), 80 (OCE5), E177.**

Dairy foods contribute to 21% of dietary saturated fatty acids (SFA) in British adults and may represent a key food group to help reduce dietary SFA to under 10% of total energy for the prevention of cardiovascular disease [1]. The dietary exchange of SFA for monounsaturated (MUFA) and polyunsaturated fat (PUFA) was successfully implemented in 100 healthy men from the Reading, Imperial, Surrey, Saturated fat, Cholesterol Intervention (RISSCI) study. Participants broadly achieved the required nutritional targets while consuming two sequential 4-week isoenergetic diets with high (18% total energy) or low (≤10% total energy) SFA, with MUFA/PUFA replacing SFA in the latter [2]. The modulation of dietary SFA intake was primarily achieved by advising participants to purchase and consume either full-fat dairy foods (butter, high-fat cheese, and whole milk) in the high-SFA diet or reduced-fat dairy foods (fat-free yogurt, low-fat cheese, and skimmed milk) in the low-SFA diet. Snacks and vegetable spreads/oils rich in MUFA/ PUFA were also provided to all participants for daily consumption. Thus, the current analysis aimed to evaluate the compliance to dietary advice using plasma phospholipid fatty acids profiles as a biomarker of dairy fat and/or high MUFA/PUFA consumptions. Plasma samples collected at the end of each 4-week dietary intervention period were analysed using gas chromatography and flame ionisation detection to assess the relative concentrations of 27 fatty acids in phospholipids. Differences in fatty acid profiles of the intervention diets were assessed by orthogonal partial least square discriminant analysis (OPLS-DA). Goodness of fit and predictability of the model were assessed by the R2Y and Q2 values, respectively, and permutation tests (n = 1,000 permutations) were used to assess the statistical significance of the model. The OPLS-DA revealed a statistically significant discrimination of plasma phospholipid fatty acid

profiles between the two diets with moderate goodness of fit (R2Y = 0.66, ppermutation < 0.001) and predictive accuracy (Q2 = 0.57, ppermutation < 0.001). Predictive loading values (pcorr) of individual fatty acids indicated that the observed separation was mostly driven by higher relative concentrations of C15:0 (pcorr = 0.72), C18:1 trans-11 (pcorr = 0.69), and C16:0 (pcorr = 0.58) in plasma phospholipids during the high-SFA diet, and of C20:1 cis-11 (pcorr =−0.63), C20:0 (pcorr =−0.60) and C22:0 (pcorr=−0.48) during the low-SFA, high-MUFA/PUFA diet. These results indicate that higher relative concentrations of fatty acids typically found in dairy fat (C15:0 and C18:1 trans-11) [3] were incorporated into plasma phospholipids when participants were advised to consume high-fat dairy foods as part of a high-SFA diet. Overall, these findings complement previous analyses from the RISSCI study to confirm the successful exchange of dietary SFA for MUFA/PUFA in healthy men [2,4] and provide evidence to support the use of dairy foods as a key food group to achieve a reduction in dietary SFA at a population level.

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Dietary pattern analysis reveals key food groups contributing to the successful exchange of saturated with unsaturated fatty acids in healthy men

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Nutrition Society Live 2020, 14–15th July 2020. **Published in Proceedings of the Nutrition Society (2020), 79 (OCE3), E772.**

Reducing dietary saturated fatty acids (SFA) to under 10% of total energy is a key strategy for cardiovascular disease (CVD) prevention in the UK. Recent evidence suggests replacing SFA with monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids could lead to a greater CVD risk reduction compared to a replacement with carbohydrates.[1] To assess the effects of replacing dietary SFA with unsaturated fatty acids on variability in fasting serum low-density lipoprotein cholesterol, 100 healthy men (30–65 y; 19–30 kg/m^2) participated in a sequential dietary intervention study (Reading, Imperial, Surrey Saturated fat Cholesterol Intervention (RISSCI) study), following two 4-week isoenergetic diets with high (18% total energy) and lower (≤10% total energy) SFA, with MUFA/PUFA replacing SFA in the latter. The two diets were designed using data from the NDNS years 1–4 rolling programme among men aged 19–64 years (2014) and a previous dietary intervention study "DIVAS" [2], identifying the following food groups as sources of exchangeable fats: butter/spreads, oils, full-fat and low-fat dairy foods, and snacks. Analysing daily nutrient intakes in a subsample from the same cohort of participants confirmed the two diets both broadly achieved their nutritional targets.[3] Thus, the current analysis aimed to investigate the impact of implementing the exchange of SFA for MUFA/PUFA to overall dietary patterns. Dietary intake from 4-day weighed diet-diaries was categorised into 135 food groups. Differences in the overall dietary patterns of the intervention diets and intake of these specific food groups were assessed by orthogonal partial least square discriminant analysis (OPLS-DA) and Fisher tests respectively. Goodness of fit and predictability of the model were assessed by the R2 Y and Q2 values, respectively, and permutation tests (n = 1,000 permutations) were used to assess the statistical significance of the model. The OPLS-DA revealed a clear difference in dietary patterns between the two diets (R2 Y = 0.899 and Q2 =0.743, empirical p-values R2 Y: $p < 0.001$ (0/1000) and Q2: $p < 0.001$ (0/1000)). As indicated by the corresponding predictive loading values (p(corr)), this separation was driven by the foods supplied to the participants to facilitate the dietary

fat exchange including solid animal fats (e.g. butter), high-fat cheese, full-fat yogurts and biscuits in the high-SFA diet (p(corr)= -0.87, -0.78, -0.29 and -0.24, respectively), and plant-based spreads, low-fat cheese, PUFA-rich oils, nuts, low-fat yogurt and savoury snacks (e.g. crisps) in the lower-SFA diet (p (corr)=0.78, 0.72, 0.67, 0.62, 0.33 and 0.30, respectively). Furthermore, there were significant differences in the level of consumption of the food groups (listed above) between the two diets ($p < 0.05$). These findings provide evidence that the foods containing the exchangeable fat were effective in achieving the dietary fat exchange. They also support compliance to the dietary advice, without significantly changing other dietary components such as meats, fish, fruits and vegetables, or carbohydrate sources.

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Consumption of dairy products and cardiovascular disease risk: results from the French prospective cohort NutriNet-Santé

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The 13th European Nutrition Conference, FENS 2019, Dublin Convention Centre, 15–18 October 2019.

Published in Proceedings of the Nutrition Society (2020), 79 (OCE2), E152.

Current French National Health and Nutrition Plan (PNNS) recommends 2 servings of dairy products per day for adults. However, dairy contributes to dietary saturated fat intake, of which reduced consumption is often recommended for cardiovascular disease (CVD) prevention. Epidemiological evidence on the association between dairy product consumption and CVD risk remains unclear, with findings from recent prospective cohorts suggesting either null or inverse associations between dairy intake and CVD risk.[1,2] This study aimed to investigate the associations between intakes of dairy products (overall and specific types) and CVD risk in a large cohort of French adults. This prospective study included self-selected participants aged ≥ 18 years from the NutriNet-Santé cohort (2009–2019). Dietary data were collected every 6 months using 24 h-dietary records, averaged in daily intakes and coded as sex-specific quartiles. Dairy foods were classified according the PNNS dairy groups: milk, cheese, and yogurts (i.e. yogurts, curd cheese and petit-suisses). Total, fermented and low-fat dairy intakes were also investigated. CVD cases (n = 1,952) included cerebrovascular (i.e. stroke and transient ischemic attack, n = 878 cases) and coronary heart diseases (i.e. myocardial infarction, angina, acute coronary syndrome and angioplasty, n = 1,219 cases). Multivariable Cox models were performed to characterize associations and were adjusted for age, gender, without-alcohol energy intake, number of 24h-dietary records, smoking status, educational level, physical activity, BMI, alcohol intake and family history of CVD. This analysis included n = 104,805 French adults with a mean age 42.8 (SD 14.6) years and the mean number of dietary records per subject was 5.7 (SD 3.1). There was no association between total or specific dairy intakes and total CVD or coronary heart disease risks. However, consumption of fermented dairy, such as cheese and yogurts, was associated with a 19% reduction in the risk of cerebrovascular disease (HRQ4 vs. Q1= 0.81 [0.66–0.98], p trend = 0.01). Despite being important dietary sources of saturated fat, dairy product consumption was not associated with total CVD or coronary heart disease risks in a large cohort of French adults. However,

fermented dairy products may be associated with a lower risk of cerebrovascular diseases. Further observational and interventional studies may be needed to further assess the impact of dairy on CVD risk and to identify potential mechanisms underlying the beneficial effects of fermented dairy products on cerebrovascular disease risk.

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Appendix 2: Deep lipidomics in human plasma – cardiometabolic disease risk and effect of dietary fat modulation

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Manuscript published in *Circulation* (April 2022). DOI: 10.1161/CIRCULATIONAHA.121.056805.

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Short title: Plasma lipidomics and cardiometabolic diseases

Total word count (including Title Page, Abstract, Text, References, Tables and Figures Legends): 7932

Acknowledgements

We thank the Human Study Centre (HSC) of the German Institute of Human Nutrition Potsdam-Rehbruecke, namely the trustee and the data hub, for data processing, and the biobank for the processing of biological samples, and Manuela Bergmann, the head of the HSC, for the contribution to the study design and leading the underlying processes of data generation. We thank the DIVAS study investigators Katerina Vafeiadou, Michelle Weech, Hana Altowaijri, Susan Todd, and Parveen Yaqoob. Finally, we thank all EPIC-Potsdam and DIVAS participants for their invaluable contribution to the respective studies.

Sources of Funding

The work was supported by the Federal Ministry of Science, Germany (grant no.01 EA 9401) and the European Union (grant no. SOC 95201408 05 F02) for the recruitment phase of the EPIC-Potsdam Study, by the German Cancer Aid (grant no. 70-2488-Ha I) and the European Community (grant no.SOC9820076905F02) for the follow-up of the EPIC-Potsdam Study, by a grant from the German

Federal Ministry of Education and Research (Bundesministerium fuer Bildung und Forschung) to the German Center for Diabetes Research (DZD grant 82DZD00302) and by a grant from the European Commission and the German Federal Ministry of Education and Research within the Joint Programming Initiative A Healthy Diet for a Healthy Life, as part of the ERA-HDHL cofounded joint call Biomarkers for Nutrition and Health (01EA1704). DIVAS was funded by the United Kingdom Food Standards Agency and Department of Health Policy Research Programme (024/0036) and LS was funded by the Biotechnology and BiologicalSciences Research Council (BBSRC) (BB/P028217/1) Fatty Acid Metabolism – Interlinking Diet and Cardiometabolic Health FAME as part of the ERA-HDHL: Biomarkers in Nutrition and Health

Disclosures

JAL is Deputy Chair of the UK Government Scientific Advisory Committee on Nutrition (SACN) and a previous member of the SACN working group on Saturated Fats and Health. JAL was chair, and KGJ and LS were members of a scientific expert committee for the International Life Sciences Institute (ILSI) on Individual Saturated Fatty acids and Cardiovascular Risk. The other authors have no potential conflicts of interest to disclose.

Abstract

Background: In blood and tissues, dietary and endogenously generated fatty acids (FAs) occur in free form or as part of complex lipid molecules that collectively represent the lipidome of the respective tissue. We assessed associations of plasma lipids derived from high-resolution lipidomics with incident cardiometabolic diseases and subsequently tested if the identified risk-associated lipids were sensitive to dietary fat modification.

Methods: The European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam cohort study comprises 27,548 participants recruited within an age-range of 35–65 years from the general population around Potsdam, Germany. We generated two disease-specific case-cohorts based on a fixed random subsample (n=1,262) and all respective cohort-wide identified incident primary cardiovascular disease (CVD, composite of fatal and non-fatal myocardial infarction and stroke) (n=551) and type 2 diabetes (T2D) (n=775) cases. We estimated the associations of baseline plasma concentrations of 282 class-specific FA abundances (calculated from 940 distinct molecular species across 15 lipid classes) with the outcomes in multivariable-adjusted Cox models. We tested the effect of an isoenergetic dietary fat modification on risk-associated lipids in The Dietary Intervention and VAScular function randomized controlled trial (DIVAS) (n=113). Participants consumed either a diet rich in saturated FAs (control), monounsaturated FAs, or a mixture of monounsaturated and n–6 polyunsaturated FAs for 16 weeks.

Results: 69 lipids associated (false discovery rate (FDR)<0.05) with at least one outcome (both=8, only CVD=49, only T2D=12). In brief, several monoacylglycerols and FA16:0 and FA18:0 in diacylglycerols were associated with both outcomes, cholesteryl esters, free fatty acids, and sphingolipids were largely CVD-specific, and several (glycero)phospholipids T2D-specific. In addition, nineteen risk-associated lipids were affected (FDR<0.05) by the diets rich in unsaturated dietary FAs compared to the saturated fat diet (17 in a direction consistent with a potential beneficial effect on long-term cardiometabolic risk). For example, the monounsaturated FA-rich diet decreased DG(FA16:0) by 0.4 (95%-CI:0.5,0.3) SD-units and increased TG(FA22:1) by 0.5 (95%-CI:0.4,0.7) SD-units.

Conclusions: We identified several lipids associated with cardiometabolic disease risk. A subset was beneficially altered by a dietary fat intervention, which supports substitution of dietary saturated FAs with unsaturated FAs as a potential tool for primary disease prevention.

Clinical Perspective

What is new?

High-resolution lipidomics uncovered several cardiometabolic risk biomarkers across a range of lipid classes that associate with incident CVD and T2D independent of standard clinical biomarkers

Several identified risk-associated lipid markers were beneficially altered by a controlled 16-weeks intervention when comparing a diet rich in saturated fatty acids with diets rich in mono-unsaturated fatty acids or a mixture of mono- and n-6 polyunsaturated fatty acids

What are the clinical implications?

Identified risk-associated lipids could serve as risk biomarkers and implicate underlying disease-specific pathways

Lipids sensitive to dietary fat modification could serve as biomarkers of intervention effects in dietary intervention trials

Observed intervention effects on risk-associated lipids provide further evidence for beneficial effects of exchanging dietary saturated with unsaturated fatty acids

Introduction

Plasma concentrations of total triacylglycerol (TG), high-density (HDL-C) and low-density lipoprotein (LDL-C) cholesterol are important predictors and potential causal factors of future cardiometabolic disease risk, including myocardial infarction, stroke, and type 2 diabetes (T2D)[1–3]. Accordingly, these biomarkers are routinely used in clinical decision-making and underlying molecular pathways are among the targets of first-line drugs for primary and secondary cardiometabolic disease prevention[4]. Preceding a drug prescription, adopting a healthier diet is considered a cornerstone of prevention[5,6]. Particularly, the dietary fatty acid (FA) profile poses a plausible link to lipid metabolism and subsequent health effects[7].

In blood and tissues, dietary and endogenously generated FAs occur in a free form or as part of complex lipid molecules that collectively represent the lipidome of the respective tissue (Figure 1A). Major sources of plasma lipids are adipose tissue, liver, and dietary lipids (Figure 1B). Lipid classes differ in terms of molecular structure of the headgroups, which can be largely classified into non-glycerides (e.g. cholesteryl esters and sphingolipids) and glycerides (e.g. phospholipids and glycerolipids) (Figure 1C, Supplemental Figure 1). Diversity within the lipid classes is furthermore increased by the attached FAs, that exhibit different structural features (Figure 1D).

High-throughput lipid profiling technologies (lipidomics) generate detailed information on the (plasma) lipidome's composition, including identification of single FAs attached to a lipid molecule[8]. This enables researchers to precisely dissect lipid-disease associations and diet intervention effects. Recent studies assessed lipidomics in relation to cardiometabolic disease outcomes and elucidated connections between lipid metabolism and cardiometabolic diseases; however, low to intermediate level resolution of the lipidome (not determining lipid class-specific FA abundance, see Table 1), analyses targeted on few or single lipid classes (e.g., only phospholipids or ceramides), or using patient cohorts rather than a general population sample represent shortcomings[9–19]. Furthermore, even though T2D and cardiovascular diseases (CVD) are both intricately related to lipid metabolism and ectopic lipid deposition, direct comparison of the relationships with plasma lipid profiles are currently sparse and could add valuable etiological insights.

Within the population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study we therefore conducted lipidome-wide cardiometabolic disease association analyses across different lipidomics levels and tested the effect of modified dietary fat intake on identified risk-associated lipids in a separate dietary randomized controlled parallel intervention trial, the Dietary Intervention and VAScular function (DIVAS) study. Throughout the analyses, we present commonalities

and differences between the investigated disease outcomes - primary composite CVD (stroke or myocardial infarction) and T2D.

Methods

EPIC-Potsdam data supporting the findings of this study are available from the corresponding author upon reasonable request. Requests to access the dataset from the DIVAS study may be sent to Prof Julie Lovegrove, j.a.lovegrove@reading.ac.uk. Code used to generate the results, figures, and tables are available upon request.

Study designs & study populations

EPIC-Potsdam

The EPIC-Potsdam study is a prospective cohort study that recruited 27,548 participants (16,644 women and 10,904 men, age-range: 35-65 years) from the general population of Potsdam, Germany, and the surrounding geographical area from 1994 to 1998. Participants were then actively followed-up every 2-3 years, by mailed questionnaires and, if necessary, by telephone. Response rates ranged between 90% and 96% per follow-up round[20]. The study protocol was approved by the ethics committee of the Medical Society of the State of Brandenburg, Germany, and all participants provided a statement of written informed consent prior to enrollment.

Incident CVD was defined as incidence of primary non-fatal and fatal myocardial infarction (MI) and stroke (International Statistical Classification of Diseases and Related Health Problems (ICD)-10 code: I21 for acute MI, I63.0 to I63.9 for ischemic stroke, I61.0 to I61.9 for intracerebral and I60.0 to I60.9 for subarachnoid hemorrhage and I64.0 to I64.9 for unspecified stroke). Incidence of CVD was captured by participants' self-reports or based on information from the death certificates, which were validated by contacting the treating physicians. Inquired information included ICD-10 code, date of occurrence and further information on symptoms and diagnosis criteria. For myocardial infarction, diagnostic criteria included clinical symptoms, electrocardiograms, cardiac enzymes and known coronary heart disease. For stroke, diagnosis was based on anamnesis, clinical symptoms, CT/MRT, angiogram, lumbar puncture, echocardiogram, Doppler and ECG, plus imaging techniques if available. Participants with silent cardiovascular events that had not been documented within 28 days after occurrence were excluded as non-verifiable cases from all analyses.

Information on incidence of T2D was systematically acquired through self-report of a diagnosis, of T2D-relevant medication, or of dietary treatment due to T2D diagnosis during follow-up. Additionally, death

certificates and information from tumor centers, physicians, or clinics that provided assessments for other diagnoses were screened for indication of incident T2D. For participants that were classified as potential cases based on that information, a standard inquiry form was sent to the treating physician. Only physician-verified cases with a diagnosis of T2D (ICD-10 code: E11) and a diagnosis date after the baseline examination were considered confirmed incident cases of T2D.

Nested case-cohorts were constructed for efficient study of molecular phenotypes. From all participants who provided blood at baseline (n=26,437), a random sample (subcohort, n=1,262) was drawn, which served as a common reference population for both endpoints. For each endpoint, all incident cases that occurred in the full cohort until a specified censoring date were included in the analysis. After excluding prevalent cases of the respective outcomes, the analytical sample for T2D comprised 1,886 participants including 775 incident cases (26 cases in the subcohort) and for CVD 1,671 participants, including 551 incident cases (28 cases in the subcohort). Follow-up was defined as the time between enrollment and study exit which was determined by diagnosis of the respective disease, death, drop out, or final censoring date, whichever came first. Endpoint-specific censoring dates were 30th of November 2006 for stroke and MI and 31st of August 2005 for T2D.

Anthropometric and blood pressure measurements were conducted according to a standardized protocol [21,22]. Blood plasma was obtained at baseline and stored in liquid nitrogen tanks at -196°C or in deep freezers at -80°C until time of analysis. Baseline plasma concentrations of standard blood lipids (total cholesterol (TC), HDL-C and TG) were measured in 2007. Plasma samples, from which aliquots were drawn for the lipidomics measurements in 2016, were never or only once thawed and refrozen during storage (93 samples defrosted and refrozen once for aliquoting for unrelated analysis).

Detailed information on measurements of non-lipidomics biomarkers, anthropometric measures, and socioeconomic factors are provided in the Supplemental material.

DIVAS

Lipidomics analysis was performed in a subset of participants (n=113 of 195) from DIVAS, a 16-week randomized controlled trial. This study recruited men and women, aged between 21-60 years and with estimated moderate CVD risk which were randomized to either one of three isoenergetic diets: rich in saturated FAs (SFA), rich in monounsaturated FAs (MUFA), or rich in mixed unsaturated fatty acids (UFA) including both MUFA and n–6 polyunsaturated FAs (PUFA). The target compositions (%total energy (TE) of total fat:SFA:MUFA:PUFA) were 36:17:11:4 for the SFA-rich diet (n=38), 36:9:19:4 for the MUFA-rich diet (n=39), and 36:9:13:10 for the mixed UFA-rich diet (n=36). All participants provided written informed consent, were non-smokers, not pregnant or lactating, had normal blood

biochemistry, liver and kidney function, did not take dietary supplements, medication for hypertension, raised lipids, or inflammatory disorders, had no prior diagnosis of a myocardial infarction, stroke, or diabetes, did not consume excessive amounts of alcohol (males:<21units/week; females:<14units/week), and performed <3*30min of aerobic exercise/week. The trial was single-blinded and randomization was conducted by a study researcher using minimization stratified for sex, age, BMI, and estimated CVD risk[23]. Blood samples were taken at baseline and after 16 weeks at a similar time of day in a fasted state. Additional information on the intervention diets is provided in the Supplemental material.

Lipidomics profiling in EPIC-Potsdam and DIVAS

Lipidomics analysis was performed with Metabolon's Complex Lipid Panel™ for EPIC-Potsdam and the DIVAS trial separately. Details on this platform are provided in the Supplemental material.

The Complex Lipid Panel™ produced measurements for 15 lipid classes (free fatty acids, FFA; cholesteryl esters, CE; monoacylglycerols, MG; ceramides, Cer; dihydroceramides, dhCer; lactosylceramides, LacCer; hexosylceramides, HexCer; sphingomeylins, SM, lysophosphatidylethanolamines, LPE; lysophosphatidylcholines, LPC; diacylglycerols, DG; triacylglycerols, TG; phosphatidylcholines, PC; phosphatidylethanolamines, PE; phosphatidylinositol, PI). In case of PE, the species from the two subclasses phosphatidylethanolamine ether (PEO) and phosphatidylethanolamine plasmalogen (PEP) were detected and hence presented separately from PE where necessary. Measured concentrations of molecular species were used to calculate lipid class sums (by summing all molecular species from one class), total FA sums (by summing concentrations of all molecular species containing a specific FA), and within-class FA sums (summing all concentrations of molecular species containing a specific FA within a lipid class). Within-class FA sums are synonymous with molecular species level in lipid classes containing only one reported variable FA per molecule (one-FA-containing classes: FFA, CE, MG, Cer, dhCer, LacCer, HexCer, SM, LPE, LPC). For comparability with other studies we further calculated the species level for those classes with more than one FA per molecule (i.e. DG, TG, PC, PE, PEO, PEP, PI), by summing all species with the same total atomic mass and degree of saturation of the contained FAs (i.e. isobaric species) (Table 1). In total, 940 distinct lipid molecular species, 28 total FA sums, 282 within-class FA sums, and 249 species were finally available for analysis. We used the updated shorthand notations from the LIPIDMAPS initiative where applicable[24]. We only refer to the shorthand notations of FAs for brevity. Respective common names of FAs are presented in Supplemental Table 1.

Statistical analysis

All lipidomics variables were log-transformed and z-scaled (mean=0, SD=1) to allow comparison of association strength across lipids and to stabilize skewed distributions.

Lipidome-wide association analysis in EPIC-Potsdam

We conducted a lipidome-wide screen estimating hazard ratios (HR-s) for the associations between the lipid variables (class sums, total FA sums, within-class FA sums, species, molecular species) and incident CVD and T2D with Cox proportional hazards models. The case-cohort design was accounted for by assigning weights as proposed by Prentice[25]. These weights are realized by counting survival time of participants of the random subcohort fully (cases and non-cases) and survival time of incident cases outside of the subcohort only at the date of diagnosis. Age was the underlying time variable, with entry time as age at baseline and exit time as age at event or censoring. The fully adjusted model included age, sex, waist circumference, height, leisure-time physical activity, highest achieved education level, fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), standard clinical blood lipid markers (TC, HDL-C and TG), anti-hypertensive medication, lipid-lowering medication, and acetylsalicylic acid medication as covariates. Models for incident CVD were additionally adjusted for drug treatment for prevalent T2D (insulin or other) and proportion glycated hemoglobin. Models for total FA sums were adjusted for the total sums of all lipid classes, to disentangle FA abundances from within-class abundances. Models on within-class FA sums and (molecular) species levels were adjusted for the respective class sum to separate the association from the class sum. We accounted for multiple hypothesis testing by controlling the false discovery rate (FDR) at 5% separately for each outcome and lipidomics level[26]. To check if presentation of unstratified results was warranted, we tested the potential for effect measure modification by sex by including lipid*sex interaction terms into the respective most adjusted model.

Effect of modified fat intake intervention in DIVAS

We considered all identified risk associated (FDR<0.05) within-class FA sums from EPIC-Potsdam as readouts in the DIVAS trial. We assessed the difference in post-intervention within-class FA sum concentrations among the trial arms via linear regression models with trial arm coded as indicator variable (SFA-rich diet as reference) and adjusted for respective baseline concentrations in addition to age, BMI, and sex. Similarly, to the disease outcome analyses in EPIC-Potsdam, the models were further adjusted for baseline and post-intervention concentrations of the respective class sums.

Software

All analyses were performed using R (version 4.1.0, package versions reported in supplemental material).

Results

EPIC-Potsdam cohort characteristics

Compared to the respective subcohort participants, incident cases tended to be older, more likely to be male, current or former smokers, and on medication. On average, incident cases were characterized by higher BMI and waist circumference, elevated total cholesterol, TG, and blood pressure, and lower HDL-C (Table 2). The median accrued follow-up time in the T2D analysis was 6.5 years (interquartile range 6.0 to 8.7 years) and 8.4 years (interquartile range 7.6 to 9.2 years) for the CVD analysis.

Lipid abundances and correlation analyses in a general population sample

Overall, CE, FFA, TG, PC and SM were the most abundant classes in plasma with average concentrations in the range of 10-100 µM (Supplemental Figure 2). The least abundant classes were Cer, dhCer, LacCer, HexCer, PEO, and LPE with close to, or below 0.1 µM average concentrations. Most abundant FAs in total were FA16:0, FA18:0, FA18:1, FA18:2, FA20:4 with >10 µM concentrations, while the least abundant FAs were FA18:4, FA22:2, FA26:0, and FA26:1 with average concentrations well below 0.1 µM (Supplemental Figure 3). CE and MG had the widest diversity of detected FAs followed by FFA, TG, DG, and PC (Supplemental Figure 3). Certain FAs were prominent in within-class profiles, e.g., FA16:0 was among the most abundant within each lipid class (e.g., median relative proportion in LacCer 61%, Supplemental Figure 4, detailed distributions in Supplemental Figure 5).

On class level, strong positive correlations were observed between structurally close classes such as for example TG-DG ($r=0.87, p<0.001$), PEP-PEO ($r=0.82, p<0.001$), LPC-LPE ($r=0.79, p<0.001$), and PI-PC ($r=0.75, p<0.001$) (Supplemental Figure 6). Among the total FA sums, most of the stronger correlations were in line with FA elongation (e.g., FA20:0-FA22:0 ($r=0.63, p<0.001$)) and FA desaturation steps (e.g., FA22:4-FA22:5 ($r=0.59, p<0.001$)) (Supplemental Figure 7). These correlations were also present on a within-class FA sum level (Supplemental Figures 8-24).

Lipidome-wide cardiometabolic risk association analyses

Results of the lipidome-wide screen across all levels, classes, and adjustment models are included in Supplemental Table 4. For both CVD and T2D, we did not detect statistically significant (FDR<0.05) effect measure modification for the association between lipids and cardiometabolic disease risk by sex and therefore present unstratified results.

With the exception of FFA and DG, class sums of all classes were associated with at least one disease outcome (nominal $p<0.05$) (Figure 2A, Supplemental Table 4). All classes associated with incident CVD, were positively associated. For T2D, only PE was statistically significantly positively associated and

LacCer, HexCer, LPC, LPE, and SM inversely associated. Of note, the associations of LacCer, HexCer, LPC, and LPE were opposite for T2D and CVD (i.e. higher risk observed for CVD and lower risk for T2D). However, no association remained after controlling for multiple testing. Associations of total FA sums were characterized by relatively low precision (i.e. wide confidence intervals). After accounting for multiple testing, FA22:2 and FA22:4 were significantly positively associated with CVD and FA22:5 inversely with T2D. (Figure 2B, Supplemental Table 4).

Lipidome-wide screening of all molecular species and within-class FA sums indicated that within-class FA sums largely showed similar or stronger (and more precise) risk associations compared to molecular species in two-FA-containing classes (DG, PC, PE, PEP, PEO, PI) and TGs (Supplemental Figures 25&26). Within-class FA sums of two or more FA-containing classes are therefore presented together with molecular species of one-FA-containing classes to address the associations of FA class-specific abundances across all classes.

Analyses of within-class FA sums, species, and molecular species were specifically geared towards associations of FA composition within lipid classes by adjusting for the corresponding class sum. Taken together, this analysis comprised 282 distinct variables, of which in total 69 were significantly associated (FDR<0.05) with at least one outcome. When contrasting the disease associations, we observed lipids associated with both outcomes (n=8) and outcome-specific associations (CVD: n=49, T2D: n=12) (Figure 3A/B). Among lipids associated with both outcomes, only MG(15:0) was inversely associated, while CE(20:3), MG(14:0), MG(18:1), MG(18:2) DG(FA16:0), DG(FA18:0), and PC(FA20:2) were positively associated (Figures 3 and 4). We found CEs, FFAs, and SMs nearly exclusively associated with CVD. Observed associations of CEs were all positive, whereas FFAs and SMs exhibited associations in both directions (Figures 3 and 4). Several LacCers and single other ceramides were associated. Further associations, aside from the ones associated with both outcomes, were detected among MGs and other glycero(phospho)lipid classes (Figures 3 and 4). In contrast to CVD, fewer lipids were specifically associated with T2D among which glycero(phospho)lipids represented the majority. Particularly FA16:0 was associated with higher T2D-risk as part of MG, DG, TG, and PEP. Among sphingolipids only two positive associations (LacCer(20:0) and LacCer(22:0)) were detected.

Risk associations according to FA carbon chain length and or number of double bonds

Most species associated with higher risk contained shorter carbon chains, were saturated or had only few double bonds. (Supplemental Figure 27). This observation was more pronounced for T2D than CVD. The species level represented a mixture of different isobaric molecular species. In most cases, one specific molecular species was statistically significantly associated with the outcome, whereas the remaining isobaric molecular species were not. For example, PEP(36_3) was positively associated with

T2D risk and the only similarly associated molecular species that represents this species was PE(P-16:0/20:3) (Supplemental Figures 28&29).On the molecular species level, lipids containing specific FAs (i.e. FA16:0 and FA18:0) were often associated with higher risk (Supplemental Figures 28&298). Lipids that were associated with lower risk did not contain common FAs, but rather a wider range of (unsaturated) FAs and an overall absence of FA16:0 and FA18:0.

Impact of dietary fat modification on risk-associated lipids

From the identified 69 statistically significantly disease-associated lipids in EPIC-Potsdam, 55 were available for analysis in the DIVAS study. Among those, we found plasma concentrations of 19 significantly increased or decreased (FDR <0.05) by an UFA-rich diet relative to the SFA-rich diet (Figure 5A, Supplemental Table 3). The MUFA-rich diet increased concentrations of TG(FA22:1), SM(24:1), and TG(FA18:2) and decreased DG(FA16:0), DG(FA18:0) TG(FA16:0), TG(FA18:0), DG(FA22:4), SM(18:0), SM(14:0), PEP(FA22:5), PE(FA16:1), HexCer(18:1), LPC(14:0), LacCer(20:1), and MG(20:0). The mixed UFA-rich diet decreased concentrations of DG(FA16:0), DG(FA18:0), TG(FA18:0), HexCer(18:1), PE(FA16:1), SM(14:0), PEP(FA22:5), PE(FA20:3), and LPC(14:0) and increased TG(FA22:1), TG(FA18:2), LacCer(16:0), and CE(24:0). High cardiometabolic disease risk-associated lipids were decreased and low risk-associated lipids increased by the MUFA-rich and mixed UFA-rich intervention diets (Figure 5A/B). Only SM(24:1) for the MUFA-rich and CE(24:0) for the mixed UFA-rich diet as high-risk associated lipids did not follow the above-mentioned pattern and were increased instead of decreased. The effects with the lowest p value (baseline concentration-adjusted difference between both UFA-rich and SFA-rich intervention arms in z-scores, all $p < 0.001$) were for the MUFA-rich diet DG(FA16:0) (-0.40, 95%-CI:-0.51,-0.30) and TG(FA22:1) (0.53, 95%-CI:0.37,0.69) and for mixed UFA-rich DG(FA18:0) (-0.24, 95%-CI:0.34,-0.14) and TG(FA18:2) (0.30, 95%-CI:0.18,0.43).

Discussion

In the population-based EPIC-Potsdam cohort study, we screened the lipidome on different levels (class sums, species, molecular species, and within-class FA sums) to identify risk biomarkers, which we subsequently assessed for sensitivity to a dietary intervention aimed to modify dietary FA intakes. Class-specific FA abundances and molecular species showed strong associations that were independent of the respective class sum level associations and standard clinical blood lipid markers. From 282 distinct within-class FA sums, 69 were associated with at least one outcome, from which 8 were associated with both CVD and T2D, 49 to only CVD, and 12 to only T2D. We showed that 19 disease-associated lipids (12 CVD risk-specific, 5 T2D risk-specific and 2 associated with both) were changed by substituting dietary SFA with UFA in the DIVAS trial. In general, high-risk-associated lipids were lowered, whereas low-risk-associated lipids were increased by the UFA interventions.

We identified several risk-associated lipids, particularly contained in DG and TG, but also in other classes, that were sensitive to the modification of the dietary FA intake. Most prominently, the high-risk associated TG(FA16:0), TG(FA18:0), DG(FA16:0), and DG(FA18:0) were decreased, while low-risk associated lipids (e.g. TG(FA22:1) and TG(FA18:2)) were increased with both UFA-rich diets. The achieved effects were of a magnitude that could translate into long-term cardiometabolic disease risk reduction, when considering the respective observed risk estimate sizes from EPIC-Potsdam. For example, baseline-adjusted post-intervention concentrations of DG(FA16:0) were reduced by 0.4 SD units with the MUFA-rich diet vs. the SFA-rich diet, while the respective HR for T2D was 2.8 per SD. Therefore, our results suggest substitution of dietary SFA with UFA could improve cardiometabolic disease risk[27].

Our modeling approach in the lipidome-wide screen allowed us to distinguish between the disease risk association of lipid classes (class sum) and specific FA residues-containing lipid metabolites within a particular class (by class sum-adjusting molecular species and within-class FA sums). Others recently showed this approach uncovers intricate disease associations[11,16].

Our analyses make apparent that direction, association strength and precision of class-specific FA proportions (within-class FA sums) and FA combinations (molecular species) can diverge substantially from those of total FA sums and can vary considerably among classes. For example, our analyses showed inverse associations of TGs containing FA18:2 and FA18:3 with T2D and LPC(18:2) with CVD, but these same FAs as part of MGs were associated with higher cardiometabolic disease risk. Our results, therefore, add nuance to findings from large pooled analyses on total FA concentrations largely indicating no or inverse associations of FA18:2 and FA18:3 with CVD and T2D risk across varying lipid compartments[28–31]. In a similar fashion, the association of arachidonic acid (FA20:4) with CVD was inconclusive in recent reports[29,31]. In our data, FFA(20:4) and MG(20:4) were positively associated with CVD. As another example, total dihomo-gamma-linoleic acid (FA20:3) in phospholipids were previously reported as strongly positively associated with incident T2D in our [32] and other studies[33], whereas the associations with CVD outcomes were inconsistent[7]. We observed statistically significant positive associations with T2D in phospholipids (PE(FA20:3) and PC(FA20:3)), but also in CE(20:3). For CVD this was the case for CE(20:3), FFA(20:3), and MG(20:3). However, in contrast to risk-associated lipids containing linoleic acid (FA18:2), lipids containing dihomo-gamma-linolenic acid (FA20:3) were not increased through higher dietary UFA and lower SFA intakes, which is in line with other reports[34]. This might indicate that for those lipids, endogenous FA metabolism plays a greater role as determinant of plasma concentrations (and hence risk associations) compared to dietary fat composition.

MGs were the class with the most lipids significantly associated with both outcomes. To our knowledge, only one report from the PREDIMED study assessed the association of MGs with incident coronary heart disease, but the molecular species that were significantly associated in our analysis, were not identified in their lipidomics panel[12]. Surprisingly, most observed significant associations in our data were positive, including molecular species containing UFAs generally considered to be beneficial or not harmful (e.g., FA18:2 = linoleic acid)[29,31]. Our finding on MG(15:0) and FFA(15:0) is in line with recent reports that found FA15:0, probably as a biomarker of dairy intake, inversely associated with T2D risk[35]; however, the current evidence for a relationship with CVD is inconclusive[36]. We recently investigated odd-chain FAs in relation to T2D in a targeted approach and found the association of MG(15:0) the only consistent species between men and women in terms of direction and precision of the observed association[40]. In the exploratory setting of the current analyses, we did not identify sufficient evidence for effect measure modification by sex ($p>0.05$ after accounting for multiple testing) to warrant deeper investigation of sex differences here.

Lipids that associated more specifically with CVD were CEs, FFAs, ceramides (especially LacCers) and SMs, whereas lipids from glycerophospholipid classes were largely T2D-specific. The observed positive associations of several CEs with CVD are in line with enrichment of CEs in atherosclerotic plaques[38]. However, results from PREDIMED indicated inverse associations[11,12], whereas Stegemann et al. reported positive associations with incident CVD in the Bruneck study[9]. A potential explanation could be differences in study populations, as PREDIMED recruited persons at high CVD risk whereas the Bruneck study and EPIC-Potsdam are population-based with lower baseline cardiometabolic disease risk profiles among the participants.

Sphingolipid molecular species (Cer, dhCer, HexCer, LacCer, and SM) exhibited stronger associations with CVD than with T2D, although the less precise associations from the T2D analyses were overall directionally consistent with CVD. The weaker findings in relation to incident T2D in our data seem to be in line with overall rather heterogeneous reports from other studies[17,19,39,40]. A recent meta-analysis pooling longitudinal studies on various adverse cardiovascular outcomes found positive associations of Cer(d18:1/16:0), Cer(d18:1/18:0), and Cer(d18:1/24:1) with CVD risk, but reported substantial heterogeneity among the included studies[41].

The possibility to determine FA abundances within lipid classes is a major advantage over previous work in similarly sized population-based cohort studies. Our findings suggest that FA carbon chain length and saturation level are not universally determinant of the direction or strength of the risk associations. Our data further allowed us to refine previously reported associations generated from lower resolution lipidomics. For example, Rhee et al. reported TG(52_1) as their strongest association

with incident T2D with an OR per SD of 1.9 (95%-CI 1.2, 3.2)[10]. We replicated the high T2D risk association of TG(52_1) and further attributed this association to the isobaric species TG(52_1-FA16:0) and TG(52_1-FA18:0). Furthermore, our data suggest that the purported higher risk from shorter and less saturated species can be attributed to contained FA16:0 in most of the investigated glycerolipids. This is in line with evidence that shows total FA16:0 concentration, irrespective of lipid compartment, is associated with T2D[28]. However, the inverse association of LacCer(16:0) and FFA(16:0) with CVD suggests that the role of FA16:0 is also lipid class-specific and warrants deeper investigation into class-specific FA proportions.

The plasma lipid pool is derived from different tissues and integrates modifiable (e.g. habitual diet, physical activity, and fasting state) and non-modifiable factors (e.g. genetics, and pathological disturbances)[8]. Dietary lipids from the intestine occur alongside de-novo generated lipids and lipid derivatives originating from different tissues (e.g. liver, adipose tissue, muscle). Plasma lipids, therefore, reflect disease-relevant metabolic disturbances (e.g. hepatic glucose metabolism), habitual intake levels (e.g. dietary fat quality), or specific organ damage (e.g. inflammatory processes in atherosclerosis). For example, observed associations of specific CE and SM molecular species might be representative of specific lipoprotein subclasses that exhibit greater cardiovascular risk than is captured by traditional lipoprotein measurements (i.e. HDLC, LDLC)[42,43]. FA16:0, as another example, is the major product of hepatic de-novo lipogenesis, a hallmark of non-alcoholic fatty liver disease and was recently shown to be associated with incident T2D[28,43]. In vivo and in vitro studies found FA16:0 impaired intracellular insulin signaling in a range of tissues, including hepatocytes and endothelial cells, and thereby drive endothelial dysfunction, inflammation, and ectopic fat storage[44].Our data extend these results by highlighting FA16:0 in TG and DG as strongly associated with incident T2D and CVD. Our intervention analysis in DIVAS showed TG and DG FA16:0 were sensitive to dietary fat quality, but further work is needed to determine whether the reductions of FA16:0 achieved in our study represented the different dietary FA compositions alone or reflected improved metabolic status. Plasma lipids might furthermore be reflective of cell membrane compositions and fluidity, which impact intercellular signaling and substrate flux[45]. However, connecting our findings on plasma lipids to membrane compositions is not straight forward. For example, saturated phospholipids are suggested to be detrimental to membrane functionality, but we found mostly PUFA phospholipids associated with higher risk.

When interpreting the results of our study, certain caveats apply. One, we cannot rule out that some molecular species were missing from our analysis even though Metabolon's Complex Lipid Panel™ is, to our knowledge, the high-throughput platform with the largest coverage with this level of resolution. Of note, Metabolon recently removed FFAs from the Complex Lipid Panel™, whichmade FFAs

unavailable in DIVAS. Two, some associations might have been attenuated by the low reliability of specific lipids. However, in a pilot study comprising 35 EPIC-Potsdam participants, we found 80% of the covered lipids had at least a fair, often good, or excellent reliability score over 4 months (data not shown). Three, plasma samples were stored at -80 °C at all times after sampling and processing with no or only one additional freeze-thaw cycle to minimize sample deterioration (e.g. FA oxidation)[46]. In a sensitivity analysis we did not find evidence that the additional freeze-thaw cycle of some samples (n = 93) was the cause for the observed associations. Four, the platform does not resolve the n-configuration i.e., position of a double bond within a FA, not allowing exact attribution to the specific molecular isomers of FAs. Five, we modelled disease associations in the lipidome-wide screen relative to class sums. This enabled the etiologically relevant distinction between the cardiometabolic disease risk associations of the total lipid class plasma level versus specific FA residues within that class. However, this came at the expense of introducing higher imprecision in the associations of some lipids, which could have led to missed associations. To facilitate comparisons with prior and future reports, we provide all risk estimates from differently adjusted models, including class-unadjusted models, in the supplement. Six, lipidome-wide screening was not geared towards assessing the cardiometabolic disease risk associated with multi-metabolite patterns or ratios. However, we and others have previously shown that the relationship between lipids may reflect etiologically critical processes in lipid metabolism[32,47,48]. Seven, the lipidome-wide screen was of an exploratory nature. Further studies are, therefore, needed to judge the generalizability of our findings and to increase (combined) sample sizes to detect smaller associations, which did not withstand multiple testing adjustment in our study. Last, identifying cases by self-report could lead to cases remaining undetected. However, case verification ensured no false positives and the false negatives do not bias risk associations if this misclassification is nondifferential to the exposure of interest[49].

In conclusion, lipid class-specific FA compositions available through deep lipidomics allow detailed investigation of the links between lipid metabolism and cardiometabolic diseases. We found different risk-associated lipids for CVD and T2D with minor overlap, emphasizing the differing etiologies, but also highlighting potential unifying pathways. Our data furthermoreshow sensitivity of several risk-associated lipids to a dietary fat modulation, by comparing the effect of a SFA-rich diet with UFA-rich diets.

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Table 1 Lipidomics levels with examples

Level	**Example**	
Lipid class sums (n = 17)	TG, PE, Cer, PEO, MG	
Total FA sums (n = 28)	FA16:0, FA22:2, FA18:3	
	1 FA-containing class	**2 or 3 FA-containing class**
Species (n = 249)	Cer(20:0)	TG(50:1)
	FFA(20:4)	PE(34:1)
	MG(18:1)	PEO(34:1)
Molecular species (n = 940)	Cer(20:0)	TG(50_1-FA18:1)*
	MG(18:1)	PE(16:0_18:1)
	FFA(20:4)	PE(O-16:1/18:0)**
Within-class FA sums*** (n = 282)	Cer(20:0)	TG(FA18:1)
	MG(18:1)	PE(FA16:0)
	FFA(20:4)	PEO(FA16:1)

*Termed "molecular species" for convenience. The level for TG with the highest resolution detected by the lipidomics platform was the "FA abundance per isobaric species".

**Compared to other molecular species, PEO and PEP could be determined to exact sn-position, therefore FAs are separated by "/" instead of "_" in accordance with LIPIDMAPS[24].

*** Within-class FA sums is the focus of the presented analyses

Table 2 Baseline characteristics of EPIC-Potsdam participants by outcome-specific subcohort membership and incident cases status

Variable*	Subcohort (CVD analysis)	Incident CVD	Subcohort (T2D analysis)	Incident T2D
N	1,148	551	1,137	775
N incident cases of respective outcome	28	551	26	775
Women	710 (61.8%)	188 (34.1%)	689 (60.6%)	325 (41.9%)
Age [years]	49.4 (42.1-57.7)	57.9 (52.3-62.2)	49.4 (42.1-57.6)	56.5 (49.5-60.9)
BMI [kg/m²]	25.5 (23.0-28.2)	27.0 (24.7-29.8)	25.4 (23.0-28.0)	29.8 (27.3-32.8)
Waist circumference [cm]	85.0 (75.0-94.0)	93.0 (85.0-101.0)	85.0 (75.0-93.5)	100.0 (92.0-107.5)
Prevalent T2D	47 (4.1%)	4 (0.7%)	0 (0%)	0 (0%)
Prevalent hypertension	564 (49.1%)	398 (72.2%)	563 (49.5%)	600 (77.4%)
Prevalent cancer	73 (6.4%)	23 (4.2%)	72 (6.3%)	45 (5.8%)
Prevalent CVD	0 (0%)	0 (0%)	36 (3.2%)	47 (6.1%)
Leisure time physical activity [h/week]	4.5 (2.0-8.0)	5.0 (2.0-10.0)	5.0 (2.0-8.0)	4.5 (1.5-8.5)
Highest level of education				
Primary school	438 (38.2%)	222 (40.3%)	439 (38.6%)	355 (45.8%)
Sec./high school	273 (23.8%)	137 (24.9%)	271 (23.8%)	182 (23.5%)
College/higher	437 (38.1%)	192 (34.8%)	427 (37.6%)	238 (30.7%)
Smoker				
Never	557 (48.5%)	176 (31.9%)	552 (48.5%)	266 (34.3%)
Former	358 (31.2%)	180 (32.7%)	358 (31.5%)	344 (44.4%)
Current smoker (<20 U/day)	173 (15.1%)	116 (21.1%)	168 (14.8%)	92 (11.9%)
Current smoker (≥20 U/day)	60 (5.2%)	79 (14.3%)	59 (5.2%)	73 (9.4%)
Antihypertensive medication	217 (18.9%)	190 (34.5%)	227 (20%)	304 (39.2%)
Lipid-lowering medication	52 (4.5%)	38 (6.9%)	58 (5.1%)	81 (10.5%)
Acetylsalicylic acid medication	101 (8.8%)	52 (9.4%)	114 (10%)	100 (12.9%)
Alcohol intake				
None	30 (2.6%)	33 (6%)	32 (2.8%)	28 (3.6%)

Low	458 (39.9%)	188 (34.1%)	447 (39.3%)	288 (37.2%)
Moderately low	223 (19.4%)	97 (17.6%)	221 (19.4%)	156 (20.1%)
Moderately high	221 (19.3%)	100 (18.1%)	221 (19.4%)	139 (17.9%)
High	190 (16.6%)	110 (20%)	191 (16.8%)	134 (17.3%)
Very high	26 (2.3%)	23 (4.2%)	25 (2.2%)	30 (3.9%)
Total energy intake [kJ/day]	8,424 (6,804-10,292)	8,893 (7,277-10,514)	8,446 (6,790-10,300)	8,844 (7,169-10,710)
Systolic blood pressure [mmHg]	127.5 (116.5-139.5)	137.5 (126.5-152.5)	127.5 (116.5-140.0)	138.0 (127.5-151.0)
Diastolic blood pressure [mmHg]	82.5 (76.0-90.5)	87.5 (81.5-95.5)	83.0 (76.0-90.5)	89.5 (82.5-95.5)
Total cholesterol [mg/dL]	204.3 (177.9-229.8)	216.0 (189.5-243.6)	204.3 (177.9-230.2)	212.5 (186.6-239.5)
Triglycerides [mg/dL]	106.8 (75.5-162.8)	140.9 (94.6-209.1)	105.8 (75.1-162.8)	170.2 (127.9-239.7)
High-density lipoprotein cholesterol [mg/dL]	54.8 (46.1-64.7)	47.7 (40.7-59.2)	54.9 (46.3-64.6)	45.8 (38.9-52.8)
Glycated hemoglobin (HbA1c) [%]	5.4 (5.1-5.7)	5.7 (5.4-6.2)	5.4 (5.1-5.7)	6.1 (5.7-6.7)

* n (%) or median (interquartile range)

BMI, body mass index; T2D, Type 2 diabetes mellitus; CVD, cardiovascular diseases including fatal and non-fatal myocardial infarction and stroke

Figure 1 Lipid class occurrence in cell and plasma compartments (panel A), major sources of plasma lipids (panel B), lipid classes (panel C), fatty acid features (panel D), generated lipid diversity through lipid class and fatty acid differences (panel E)

Legend: (A) Lipids have several functions in the organism, which determine their location in cells and tissues. They make-up membranes and thereby determine membrane fluidity and function, serve as energy storage, exert intracellular signaling propeties, and are precursors of hormones (steroids and eicosanoids). In plasma, FFAs are mostly transported bound to albumin, while complex lipids are transported as part of lipoproteins. Lipids are continuously exhanged between plasma and tissues (B); The plasma lipidome largly comprises lipids ingested from the diet, released from adipose tissue, or produced by the liver. Particularly the liver is the central hub of lipid metabolism though lipoprotein production and de-novo lipogenesis. (C) The lipid classes depicted here are covered by Metabolon's Complex Lipid Panel™ and represent most of the major lipid classes found in the human plasma. More detailed information on the molecular differences between the lipid classes is shown in Supplemental Figure 1. (E) Metabolon's Complex Lipid Panel™ allows investigation of lipids on different aggregated levels. The main results of this publication refer to "Within-class FA sum". More details on the different naming conventions used throughout are in Table 1.

The figure was produced using **smart.servier.com.**

Figure 2 Disease associations of class sums and total FA sums

Legend: Hazard ratios from models adjusted for age (as underlying time variable), sex, waist circumference, height, leisure time physical activity, smoking status, average alcohol intake, highest achieved education level, fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG), anti-hypertensive medication, lipid-lowering medication, and acetylsalicylic acid medication. Models for CVD further adjusted antidiabetic medication and proportion glycated hemoglobin; **(A)** class sums; **(B)** total FA sums, additionally adjusted for all class sums.

Figure 3 Disease associations of lipid class-specific FA abundances

Legend: P values and hazard ratios from models adjusted for age (as underlying time variable), sex, waist circumference, height, leisure time physical activity, smoking status, average alcohol intake, highest achieved education level, fasting status at blood draw, total energy intake, blood pressure

(systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG), anti-hypertensive medication, lipid-lowering medication, acetylsalicylic acid medication, and respective class sum. Models for CVD further adjusted antidiabetic medication and proportion glycated hemoglobin; **(A)** Scatter plot of p values for T2D vs CVD, all labeled points were statistically significant after accounting for multiple testing**; (B)** Hazard ratios (95%.CI) of all significant associations after controlling for multiple testing in (A).

Figure 4 Disease associations of lipid class-specific FA abundances by FA carbon chain length and number of unsaturated bonds

Legend: P values from models adjusted for age (as underlying time variable), sex, waist circumference, height, leisure time physical activity, smoking status, average alcohol intake, highest achieved education level, fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG), anti-hypertensive medication, lipid-lowering medication, acetylsalicylic acid medication, and respective class sum. Models for CVD further adjusted for antidiabetic medication and proportion glycated hemoglobin.

Figure 5 Effect of MUFA and mixed UFA-rich diets versus a SFA-rich diet on risk-associated lipids in the DIVAS trial

Legend: (A) Volcano plot of effect of diet intervention on within class-FA sum concentrations from a linear regression model with post intervention concentration as dependent variable and diet type (indicator variable for MUFA-rich (yes/no), mixed UFA-rich (yes/no), SFA-rich as reference), respective lipid baseline concentration, respective baseline and post intervention class sum concentration, age, sex, and BMI as independent variables. Direction of associations in EPIC-Potsdam was consistent between T2D and CVD for all risk-associated lipids. All labeled points were statistically significant after accounting for multiple testing. **(B)** Word clouds for overlap between change through diet type and observed risk association in EPIC-Potsdam by disease.

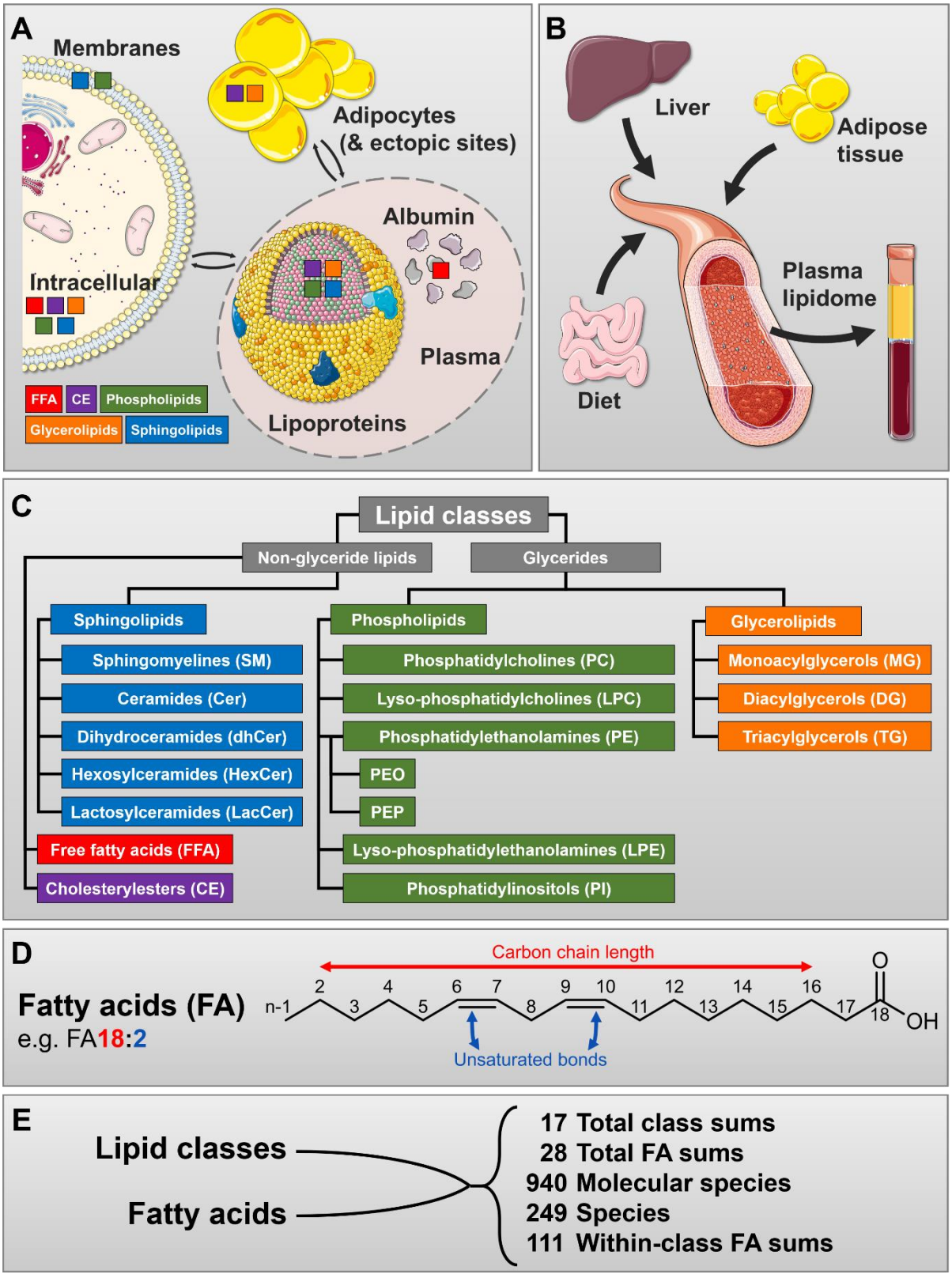
A
Membranes
Adipocytes
(& ectopic sites)
Albumin
Intracellular
Plasma
Lipoproteins
FFA
CE
Phospholipids
Glycerolipids
Sphingolipids
B
Liver
Adipose
tissue
Plasma
lipidome
Diet
C
Lipid classes
Non-glyceride lipids
Glycerides
Sphingolipids
Sphingomyelines (SM)
Ceramides (Cer)
Dihydroceramides (dhCer)
Hexosylceramides (HexCer)
Lactosylceramides (LacCer)
Free fatty acids (FFA)
Cholesterylesters (CE)
Phospholipids
Phosphatidylcholines (PC)
Lyso-phosphatidylcholines (LPC)
Phosphatidylethanolamines (PE)
PEO
PEP
Lyso-phosphatidylethanolamines (LPE)
Phosphatidylinositols (PI)
Glycerolipids
Monoacylglycerols (MG)
Diacylglycerols (DG)
Triacylglycerols (TG)
D
Fatty acids (FA)
e.g. FA18:2
Carbon chain length
n-1
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
O
OH
Unsaturated bonds
E
Lipid classes
Fatty acids
17 Total class sums
28 Total FA sums
940 Molecular species
249 Species
111 Within-class FA sums

Figure 1

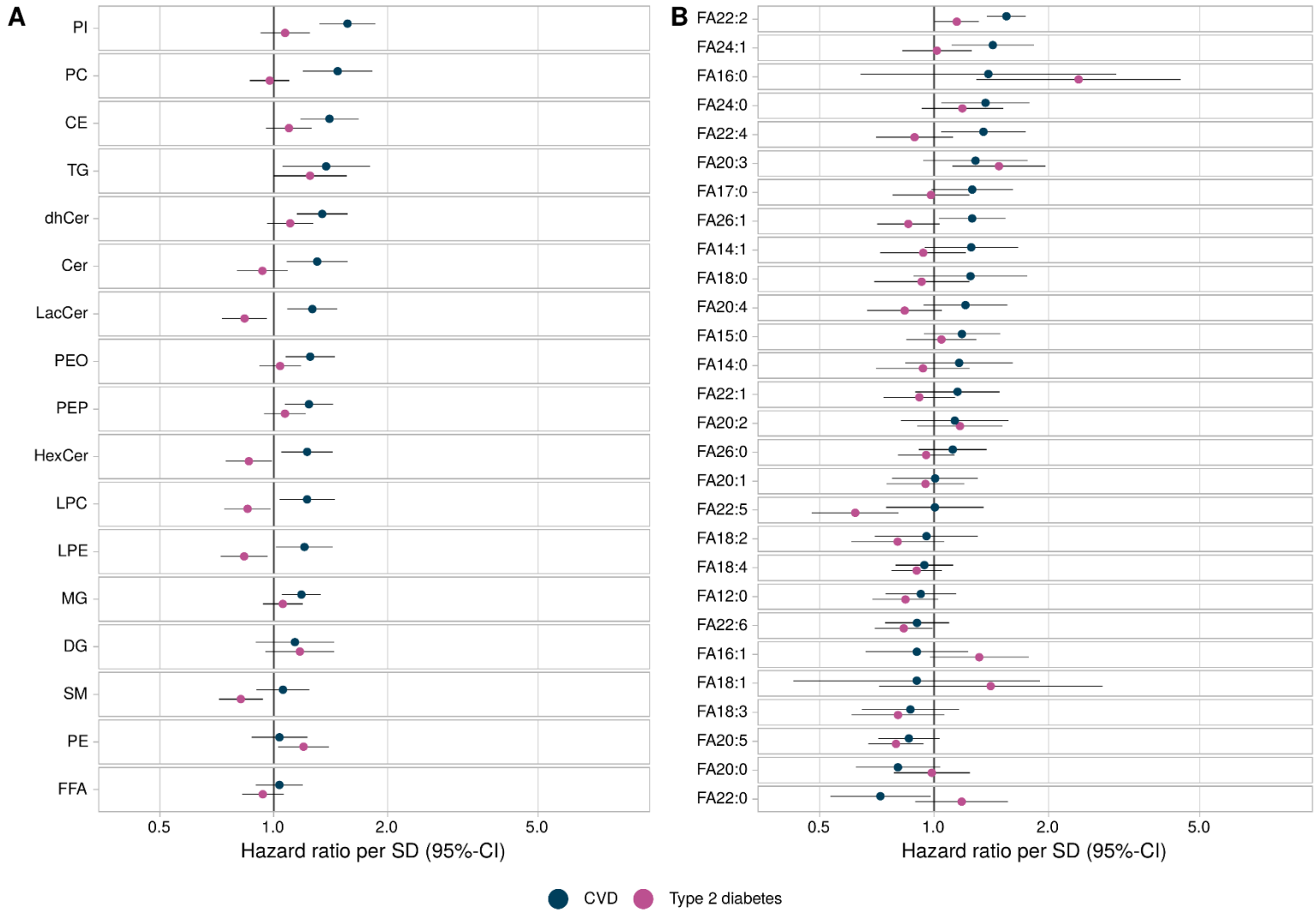
A
PI
PC
CE
TG
dhCer
Cer
LacCer
PEO
PEP
HexCer
LPC
LPE
MG
DG
SM
PE
FFA
0.5
1.0
2.0
5.0
Hazard ratio per SD (95%-CI)
B
FA22:2
FA24:1
FA16:0
FA24:0
FA22:4
FA20:3
FA17:0
FA26:1
FA14:1
FA18:0
FA20:4
FA15:0
FA14:0
FA22:1
FA20:2
FA26:0
FA20:1
FA22:5
FA18:2
FA18:4
FA12:0
FA22:6
FA16:1
FA18:1
FA18:3
FA20:5
FA20:0
FA22:0
0.5
1.0
2.0
5.0
Hazard ratio per SD (95%-CI)
CVD
Type 2 diabetes

Figure 2

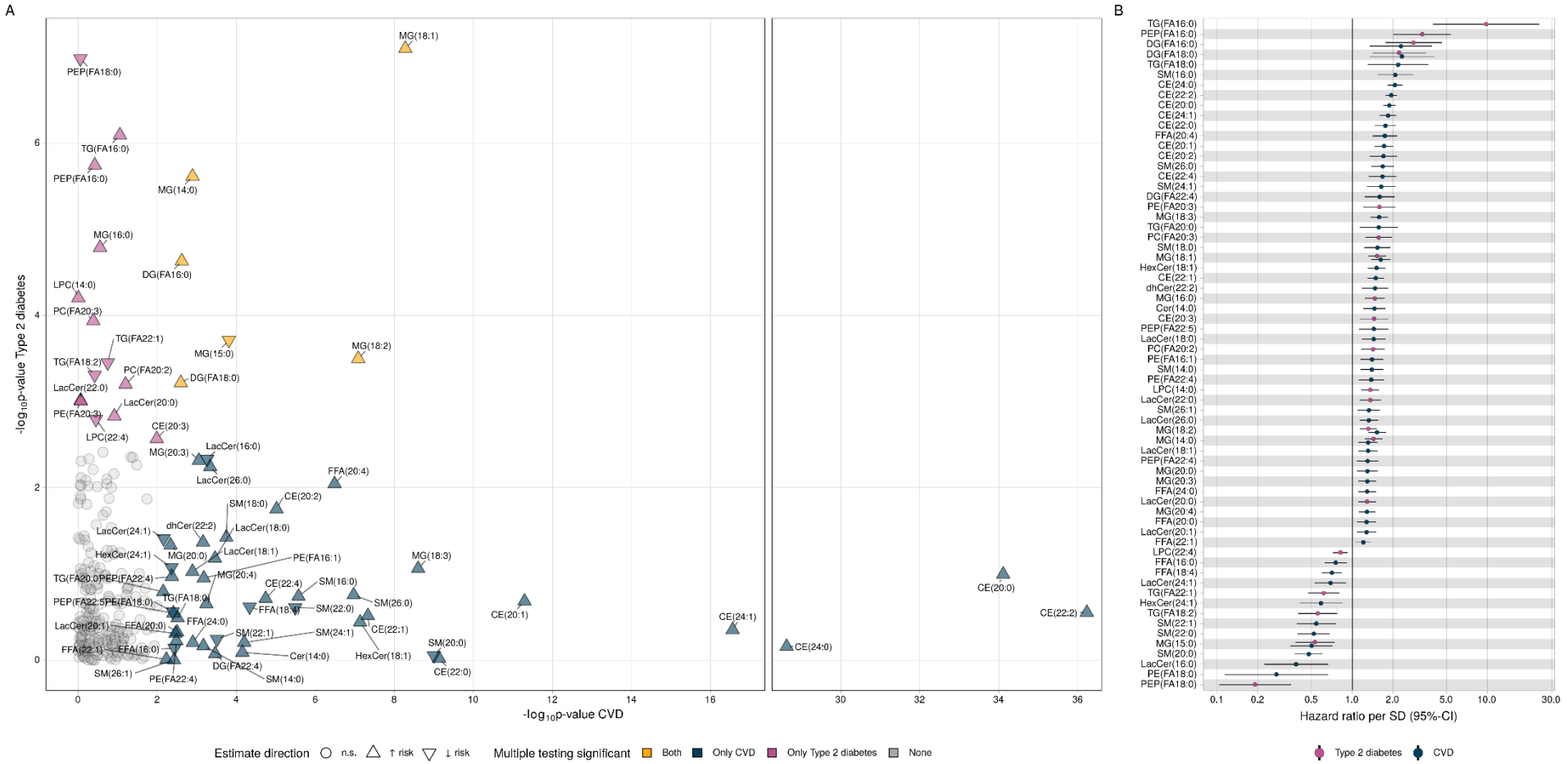
A
B
-log10p-value Type 2 diabetes
-log10p-value CVD
Hazard ratio per SD (95%-CI)
Estimate direction
n.s.
↑ risk
↓ risk
Multiple testing significant
Both
Only CVD
Only Type 2 diabetes
None
Type 2 diabetes
CVD

Figure 3

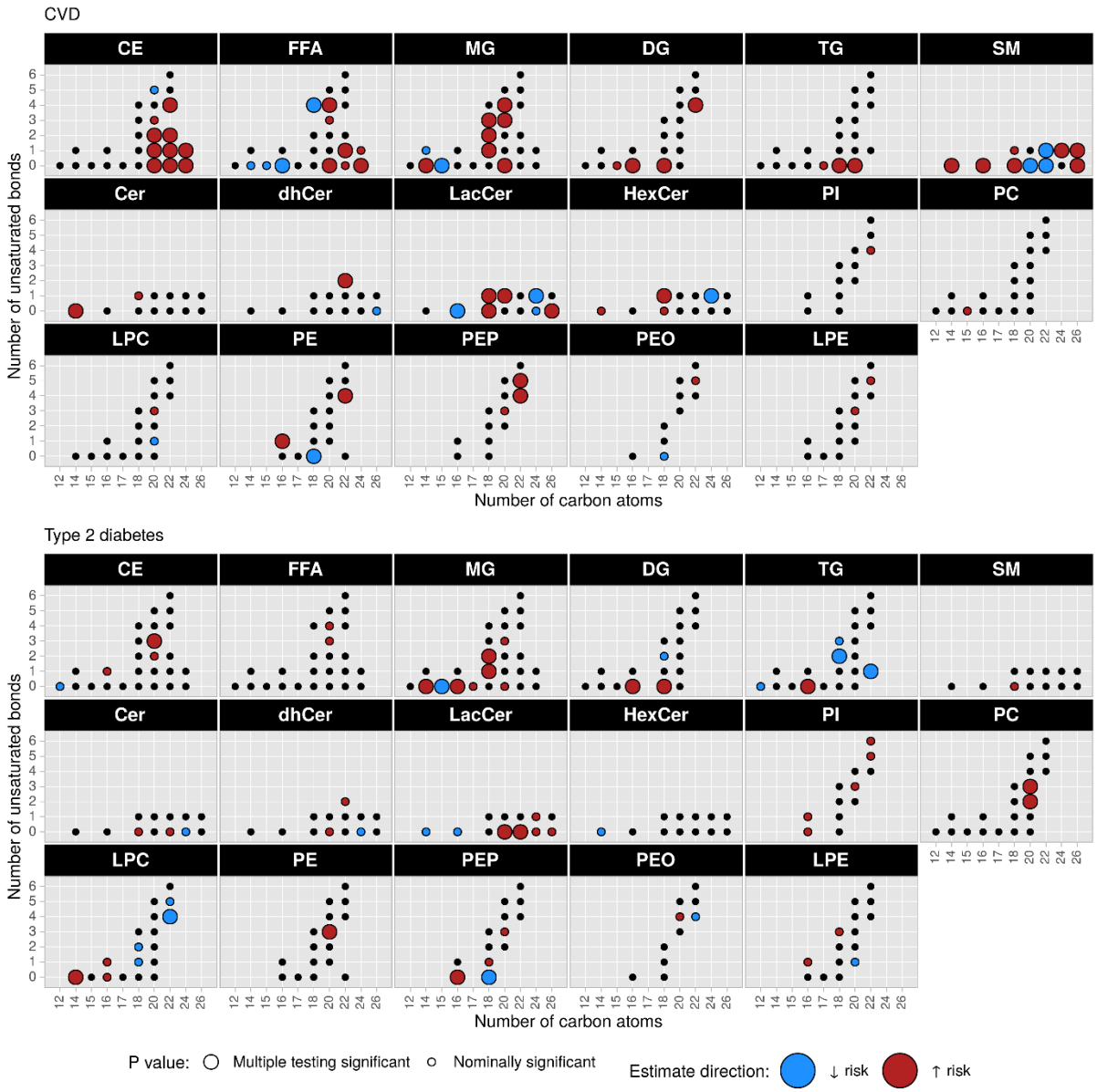
CVD
CE
FFA
MG
DG
TG
SM
Cer
dhCer
LacCer
HexCer
PI
PC
LPC
PE
PEP
PEO
LPE
Number of unsaturated bonds
Number of carbon atoms
Type 2 diabetes
P value:
Multiple testing significant
Nominally significant
Estimate direction:
↓ risk
↑ risk

Figure 4

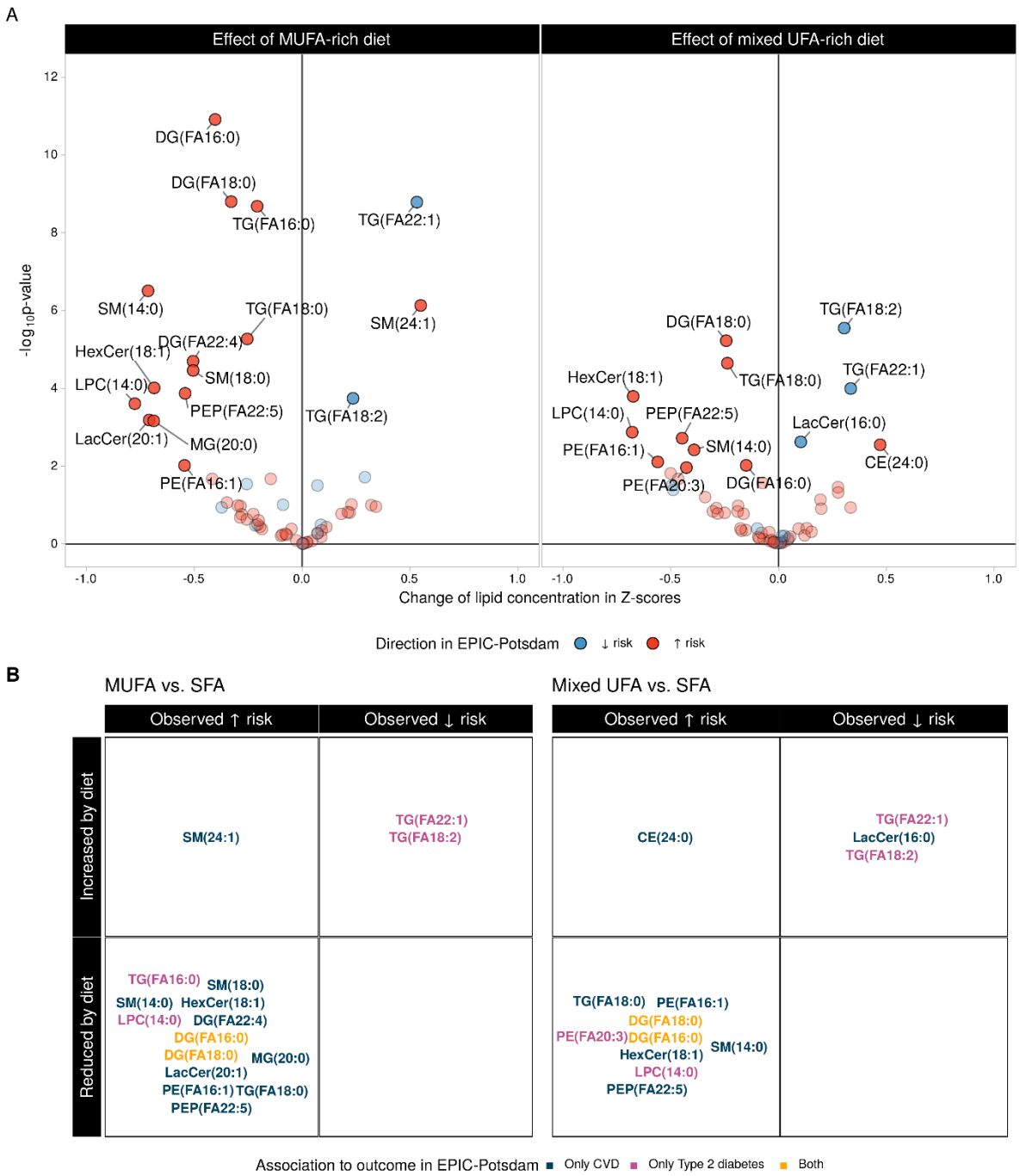
A
Effect of MUFA-rich diet
Effect of mixed UFA-rich diet
-log10p-value
DG(FA16:0)
DG(FA18:0)
TG(FA16:0)
TG(FA22:1)
SM(14:0)
TG(FA18:0)
SM(24:1)
DG(FA22:4)
HexCer(18:1)
SM(18:0)
LPC(14:0)
PEP(FA22:5)
TG(FA18:2)
LacCer(20:1)
MG(20:0)
PE(FA16:1)
TG(FA18:2)
DG(FA18:0)
HexCer(18:1)
TG(FA18:0)
TG(FA22:1)
LPC(14:0)
PEP(FA22:5)
LacCer(16:0)
PE(FA16:1)
SM(14:0)
CE(24:0)
PE(FA20:3)
DG(FA16:0)
Change of lipid concentration in Z-scores
Direction in EPIC-Potsdam
↓ risk
↑ risk
B
MUFA vs. SFA
Mixed UFA vs. SFA
Observed ↑ risk
Observed ↓ risk
Increased by diet
Reduced by diet
SM(24:1)
TG(FA22:1)
TG(FA18:2)
CE(24:0)
TG(FA22:1)
LacCer(16:0)
TG(FA18:2)
TG(FA16:0)
SM(18:0)
SM(14:0)
HexCer(18:1)
LPC(14:0)
DG(FA22:4)
DG(FA16:0)
DG(FA18:0)
MG(20:0)
LacCer(20:1)
PE(FA16:1)
TG(FA18:0)
PEP(FA22:5)
TG(FA18:0)
PE(FA16:1)
DG(FA18:0)
PE(FA20:3)
DG(FA16:0)
HexCer(18:1)
SM(14:0)
LPC(14:0)
PEP(FA22:5)
Association to outcome in EPIC-Potsdam
Only CVD
Only Type 2 diabetes
Both

Figure 5