Fatty acid biomarkers of intake and metabolism and their association with type 2 diabetes

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1. Summary

Background: The role of fatty acid (FA) intake and metabolism in type 2 diabetes (T2D) incidence is controversial. Some FAs are not synthesised endogenously and, therefore, these circulating FAs reflect dietary intake, for example, the trans fatty acids (TFAs), saturated odd chain fatty acids (OCFAs), and linoleic acid, an n-6 polyunsaturated fatty acids (PUFA). It remains unclear if intake of TFA influence T2D risk and whether industrial TFAs (iTFAs) and ruminant TFAs (rTFAs) exert the same effect. Unlike even chain saturated FAs, the OCFAs have been inversely associated with T2D risk, but this association is poorly understood. Furthermore, the associations of n-6 PUFAs intake with T2D risk are still debated, while delta-5 desaturase (D5D), a key enzyme in the metabolism of PUFAs, has been consistently related to T2D risk. To better understand these relationships, the FA composition in circulating lipid fractions can be used as biomarkers of dietary intake and metabolism. The exploration of TFAs subtypes in plasma phospholipids and OCFAs and n-6 PUFAs within a wide range of lipid classes may give insights into the pathophysiology of T2D.

Aim: This thesis aimed mainly to analyse the association of TFAs, OCFAs and n-6 PUFAs with self-reported dietary intake and prospective T2D risk, using seven types of TFAs in plasma phospholipids and deep lipidomics profiling data from fifteen lipid classes.

Methods: A prospective case-cohort study was designed within the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study, including all the participants who developed T2D (median follow-up 6.5 years) and a random subsample of the full cohort (subcohort: n=1248; T2D cases: n=820). The main analyses included two lipid profiles. The first was an assessment of seven TFA in plasma phospholipids, with a modified method for analysis of FA with very low abundances. The second lipid profile was derived from a high-throughout lipid profiling technology, which identified 940 distinct molecular species and allowed to quantify OCFAs and PUFAs composition across 15 lipid classes. Delta-5 desaturase (D5D) activity was estimated as 20:4/20:3-ratio. Using multivariable Cox regression models, we examined the associations of TFA subtypes with incident T2D and class-specific associations of OCFA and n-6 PUFAs with T2D risk.

Results: 16:1n-7*t*, 18:1n-7*t*, and *c*9*t*11-CLA were positively correlated with the intake of fat-rich dairy foods. iTFA 18:1 isomers were positively correlated with margarine. After adjustment for confounders and other TFAs, higher plasma phospholipid concentrations of two rTFAs were associated with a lower incidence of T2D: 18:1n-7*t* and *t*10*c*12-CLA. In

contrast, the rTFA *c*9*t*11-CLA was associated with a higher incidence of T2D. rTFA 16:1n-7*t* and iTFAs (18:1n-6*t*, 18:1n-9*t*, 18:2n-6,9*t*) were not statistically significantly associated with T2D risk.

We observed heterogeneous integration of OCFA in different lipid classes, and the contribution of 15:0 versus 17:0 to the total OCFA abundance differed across lipid classes. Consumption of fat-rich dairy and fiber-rich foods were positively and red meat inversely correlated to OCFA abundance in plasma phospholipid classes. In women only, higher abundances of 15:0 in phosphatidylcholines (PC) and diacylglycerols (DG), and 17:0 in PC, lysophosphatidylcholines (LPC), and cholesterol esters (CE) were inversely associated with T2D risk. In men and women, a higher abundance of 15:0 in monoacylglycerols (MG) was also inversely associated with T2D. Conversely, a higher 15:0 concentration in LPC and triacylglycerols (TG) was associated with higher T2D risk in men. Women with a higher concentration of 17:0 as free fatty acids (FFA) also had higher T2D incidence.

The integration of n-6 PUFAs in lipid classes was also heterogeneous. 18:2 was highly abundant in phospholipids (particularly PC), CE, and TG; 20:3 represented a small fraction of FA in most lipid classes, and 20:4 accounted for a large proportion of circulating phosphatidylinositol (PI) and phosphatidylethanolamines (PE). Higher concentrations of 18:2 were inversely associated with T2D risk, especially within DG, TG, and LPC. However, 18:2 as part of MG was positively associated with T2D risk. Higher concentrations of 20:3 in phospholipids (PC, PE, PI), FFA, CE, and MG were linked to higher T2D incidence. 20:4 was unrelated to risk in most lipid classes, except positive associations were observed for 20:4 enriched in FFA and PE. The estimated D5D activities in PC, PE, PI, LPC, and CE were inversely associated with T2D and explained variance of estimated D5D activity by genomic variation in the FADS locus was only substantial in those lipid classes.

Conclusion: The TFAs' conformation is essential in their relationship to diabetes risk, as indicated by plasma rTFA subtypes concentrations having opposite directions of associations with diabetes risk. Plasma OCFA concentration is linked to T2D risk in a lipid class and sex-specific manner. Plasma n-6 PUFA concentrations are associated differently with T2D incidence depending on the specific FA and the lipid class. Overall, these results highlight the complexity of circulating FAs and their heterogeneous association with T2D risk depending on the specific FA structure, lipid class, and sex. My results extend the evidence of the relationship between diet, lipid metabolism, and subsequent T2D risk. In addition, my work generated several potential new biomarkers of dietary intake and prospective T2D risk.

2. List of Abbreviations

AA	Arachidonic acid (20:4n-6)
CE	Cholesteryl esters
Cer	Ceramides
D5D	Delta-5 desaturase
DG	Diacylglycerols
DGLA	Dihomo-gamma-linolenic acid (20:3n-6)
dhCer	Dihydroceramides
EPIC	European Prospective Investigation into Cancer and Nutrition
FA	Fatty acid
FFA	Free fatty acids
FFQ	Food frequency questionnaire
GLA	Gamma-linolenic acid (18:3n-6)
HexCer	Hexosylceramides
LA	Linoleic acid
LacCer	lactosylceramides
LPC	Lysophosphatidylcholines
LPE	Lysophosphatidylethanolamines
MG	Monoacylglycerols
OCFA	Odd chain fatty acid
PC	Phosphatidylcholines
PE	Phosphatidylethanolamines
PI	Phosphatidyl inositol
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid

SM	Sphingomyelins
T2D	Type 2 diabetes
TFA	Trans fatty acids
TG	Triacylglycerols

3. Introduction

Type 2 diabetes

Five hundred thirty-seven million adults worldwide were estimated to have diabetes in 2021, and approximately 90-95% of them have type 2 diabetes (T2D), the most common form of diabetes (1, 2). T2D is a chronic condition derived from insufficient insulin secretion by the pancreas and peripheral insulin resistance. These two interrelated mechanisms lead to dysregulation of blood glucose uptake and, therefore, high glucose concentration in the blood. Chronic hyperglycemia can induce damage to the circulatory, nervous, and immune systems, apart from organs like the eyes and kidneys (1). Therefore, prevention, early detection, and management of T2D are public health priorities (3).

The specific causes of T2D are not fully understood; still, there is a strong link with some risk factors, for example, overweight and obesity, increasing age, lack of physical activity, and ethnicity (4). While some risk factors are not modifiable, modifying certain lifestyle aspects may constitute an effective strategy for preventing diabetes-related morbidity, complications, and mortality. The recommendations suggest achieving and maintaining a healthy body weight and a healthy diet (2). Regarding diet, randomised controlled trials have demonstrated that dietary modifications can largely prevent T2D (5-8), in line with meta-analyses of prospective cohort studies (9, 10). There is consensus that interventions that modify diets to achieve weight loss can prevent the development of T2D in individuals at risk, however, uncertainty exists over which dietary composition is best to reduce T2D risk, independent of the weight loss (11).

Dietary and circulating fatty acids

One aspect that has been under debate for decades is the role of dietary fat as a risk factor for T2D. Nutritional guidelines have recommended limiting total and saturated fat to reduce the risk of chronic diseases like T2D (12). However, accumulating evidence has pointed out that the type of dietary fat is more important than just quantity. For example, a systematic review of prospective cohort studies indicated that total fat intake was not associated with T2D risk, but intake of polyunsaturated fatty acids (PUFAs) in exchange for carbohydrates or saturated fatty acids (SFAs) was associated with a reduced risk of T2D (13). Overall, investigations suggest that the role of dietary fats depends on the specific fatty acid (FA) and the food source. Advances in nutritional research have highlighted a complex relationship between

dietary fat and cardiometabolic diseases, with pathophysiological pathways yet to be discovered.

A major difficulty in the investigations into the role of dietary fat in the development of T2D is documenting the intake. In epidemiological research, the assessment of fat intake is commonly carried out with validated methods such as food frequency questionnaires (FFQ), food diaries, and 24-hour recalls. These self-reporting tools, which are subjective in nature, have many advantages but are limited by measurement errors. Errors arise from under- or over-reporting (frequency and portion size), which could be intentional (e.g., underreporting food perceived as unhealthy, changing food intake to make recording simpler) or unintentional (inability to estimate portion size). The accuracy of self-reported methods could also be influenced by many other reasons; for example, day/seasonal variations in foods consumed, the ability of the interviewer to assist in recalling and estimating portion sizes, the imprecise estimation of FA intake due to variations in composition tables or variations in FA food content (e.g., among different types of cheese), miscoding, among others (14). Furthermore, compared with other diet components, the amount and type of dietary fat are particularly challenging to measure with traditional self-report assessments. This is because fat is added in food preparation (frying, cooking) and is hidden in dressing, sauces, or composite foods that are difficult to quantify and recognise. Besides, high-fat foods are underreported by people with overweight or obesity (14).

Self-reported methods have been validated by comparing results with another method that is presumed to be more accurate. However, the exact value is unknown because even weighted records depend on reports by the participants. Given these limitations, there has been a growing interest in using circulating FAs as biomarkers of intake, which allows an objective measurement of dietary intake. Intake biomarkers are an alternative to the traditional assessment by self-report in nutritional studies or a promising addition to calibrate or validate the accuracy of self-reported methods (15).

Controlled feeding and supplementation studies have confirmed that a higher intake of dietary FAs is reflected in higher proportions in the blood (16). FAs not synthesised endogenously tend to have stronger associations between dietary intake and circulating levels, potentially reflecting dietary intake. Examples are trans fatty acids (TFAs), saturated odd chain fatty acids (OCFAs), and linoleic acid, an n-6 PUFA. These FAs are found in blood and other tissues as part of lipid molecules or non-esterified (free) (17). However, in addition to reflecting intake, circulating biomarkers might also reflect nutrient status and metabolism. As

part of lipid metabolism, for example, enzymatic processes modify the length and saturation of the FA carbon chain or affect the composition of lipid tissues (16). Thus, the determinants of FA biomarkers include dietary intake and endogenous FA modification or relocation, providing a more integrated measure of exposure.

FAs in adipose tissue are considered a good choice as biomarkers for long-term intake (years) because adipose tissue has a slow turnover rate (18). However, the sampling of adipose tissue is very invasive and not easily available in large cohort studies. Investigators, especially in studies with a large number of participants, prefer to measure FAs in plasma or other blood compartments. FAs in total plasma or total plasma phospholipids are commonly used in many epidemiological studies. Furthermore, FAs measured from triglycerides or triacylglycerols (TG), erythrocytes phospholipids, cholesteryl esters (CE), and total plasma lipids are also often presented in the literature (16). Plasma, moreover, is a complex tissue with many more lipid classes available (**Figure 1**).

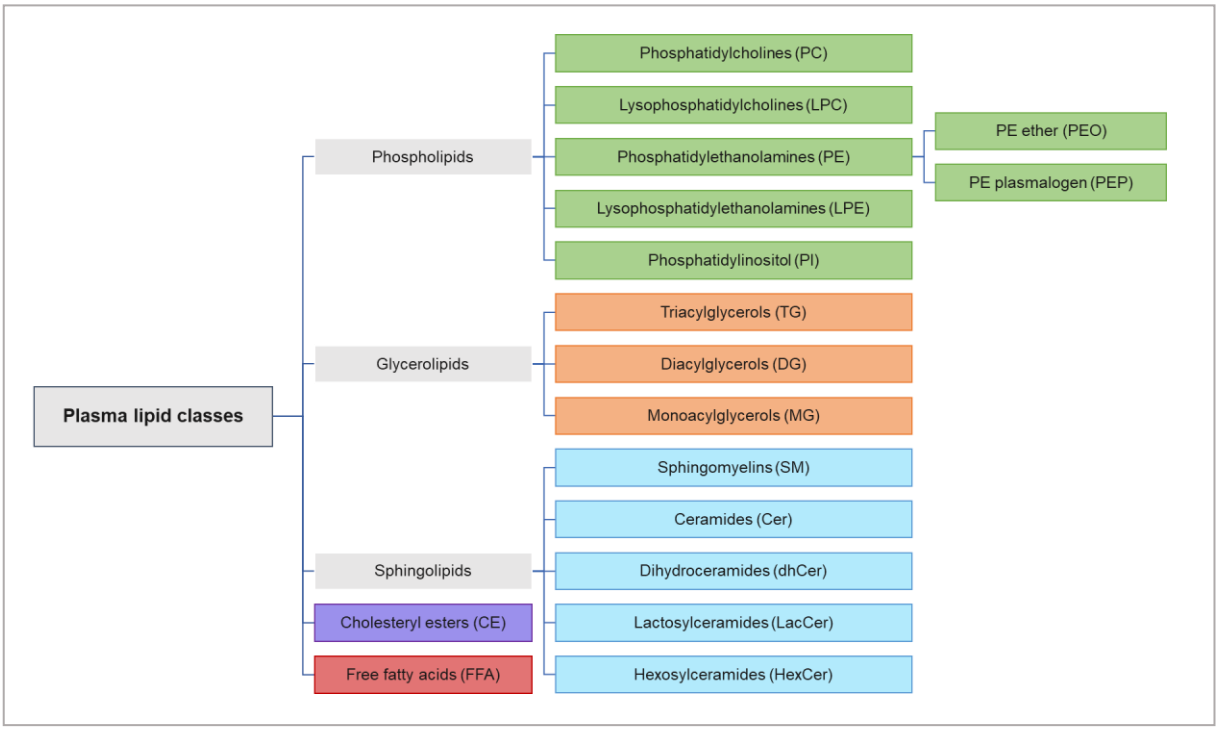

Figure 1. Plasma lipid classes in lipidome profiles

Regardless of the interest in FA biomarkers in clinical and epidemiological trials, the selective incorporation of FAs into the different plasma lipid classes is poorly researched. Compared with earlier investigations, nutrition science has benefited from advanced methodologies like

metabolomic platforms that allow the characterisation of diverse lipid molecules across many lipid classes. The lipid classes differ in headgroup structure and FAs attached (17).

Phospholipids can be subdivided into different classes, depending on the headgroups: phosphatidylcholines (PC), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (LPE), lysophosphatidylcholines (LPC), and phosphatidylinositol (PI). The glycerolipids are a group that includes TG, diacylglycerols (DG), and monoacylglycerols (MG) and are formed by one glycerol with three, two, or one FA, respectively. The sphingolipids involve sphingomyelins (SM), ceramides (Cer), dihydroceramides (dhCer), lactosylceramides (LacCer), and hexosylceramides (HexCer). CE are cholesterol backbones with FAs linked to the hydroxyl group. The free fatty acids (FFA) are FAs that circulate non-esterified.

The lipid class may influence the associations between specific FAs and T2D incidence analysed, given the differences in origin (liver, adipose tissue, diet) and functions (16). For example, phospholipids are part of the lipid bilayer of cell membranes, secreted mainly by the liver and released into circulation in lipoproteins (19). Phospholipids influence cell properties, like their permeability and fluidity, and are involved in metabolism and signalling (insulin signalling, receptor binding affinities, glucose transporters) (20). CE are formed from free cholesterol esterified to a FA for transport in circulating lipoproteins and storage in lipid droplets within cells. In circulation, CE are transported from digestive organs to tissues by very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) and from tissues to the liver by high-density lipoprotein (HDL) (21). Phospholipids and CE reflect recent intake (in terms of weeks or months) (16).

Lipoproteins also carry TG derived from dietary fat intake or endogenous synthesis, mainly in the liver and adipose tissue. TG are a major form of energy storage, especially in adipose tissue, but also in various cells and represent very recent intake (days). Another group is the plasma FFA, derived from adipose tissue during fasting and from lipoproteins after absorption from dietary fat intake (16), and thus are used as biomarkers of adipose tissue composition in fasted individuals.

Irrespective of the lipid fraction, the FAs measured therein are conventionally grouped into broad categories of FAs, such as total SFAs, total TFAs, and total n-6 PUFAs. This conventional classification ignores structural and biological differences. Within each category, there is diversity in carbon chain length, number and location of double bonds, and cis or trans conformation. This thesis focused on these three categories, commonly used as

biomarkers of intake and part of the current scientific debate. But instead of a broad classification into these main groups, specific FAs were investigated across total phospholipids (TFAs) or several lipid fractions (OCFAs and n-6 PUFAs).

Trans fatty acids

TFAs are unsaturated FAs with at least one double bond in *trans* configuration. TFAs are formed during the industrial partial hardening of vegetable oils. Therefore, they are found in margarine, frying fats, baked goods, confectionery, and convenience foods (22). Industrial TFAs are used in food products because they improve some aspects of the industrial fat: texture, structure, thermal and oxidative stability, and provide longer shelf life. Ruminant TFAs, on the other hand, are formed in the digestive tract of ruminants and are a natural component of milk and butter along with beef, goat, and sheep meat. Both industrial and ruminant sources of TFAs contain a diverse distribution of TFA subtypes (22).

TFA subtypes vary by the carbon chain length and the number and location of the double bonds (**Figure 2**). A commonly used nomenclature indicates the number of carbons (first number), the number of double bonds (second number), and the position of the double bond from the methyl end (omega end or n-carbon). For example, 18:1n-7*t* (*trans*-vaccenic acid) has 18 carbons and 1 double bond in the location n-7 in *trans* configuration. The conjugated linoleic acids (CLA) are a particular type of TFA with only one carbon between two double bonds, one in *trans* and one in *cis* configuration. By convention, their nomenclature differs from other TFAs. The position of the double bonds is indicated by the carbon atom counting from the carboxylic end of the carbon chain. *cis*-9,*trans*-11 CLA (*c*9*t*11-CLA or rumenic acid), with 18 carbons and 2 double bonds, has a *cis* double bond in carbon 9 and a *trans* double bond in carbon 11, counting from the carboxylic end.

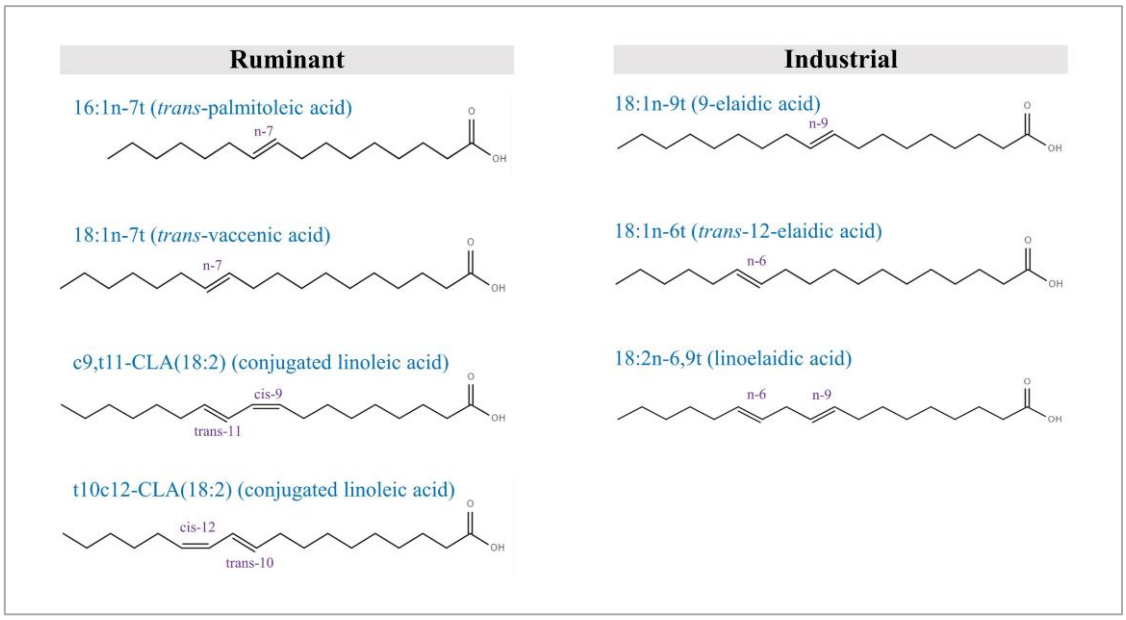

Figure 2. Ruminant and industrial trans fatty acid structures

Investigating TFA intake is challenging. Apart from the mentioned measurement errors (e.g., imprecision in estimating food intakes by participants, selective underreporting of fat-rich foods), TFAs are present in very small quantities in foods, and there is limited availability of detailed and accurate food composition databases for specific TFA isomers. Furthermore, TFA food composition has changed over time due to reformulation (23), which might not be reflected in food composition databases. That is why the use of TFAs biomarkers in blood for studying TFA intake is of high interest, and, as mentioned, it is assumed that circulating TFAs come mostly from dietary intake.

Consensus exists on the strong association between high TFA intake and increased risk of coronary heart disease and mortality (24, 25). However, in prospective cohort studies, total TFA intake was not linked to a higher risk of T2D (26) and, instead, higher circulating ruminant-derived *trans*-palmitoleic acid (16:1n-7*t*) was associated with lower T2D risk (26, 27). Evidence for *trans*-vaccenic acid (18:1n-7*t*), a major ruminant-derived TFA, is scarce and has not been studied compared to the other dairy TFAs. Other ruminant-derived TFAs are the *c*9*t*11-CLA and *trans*-10,*cis*-12 CLA (*t*10*c*12-CLA), common CLA isomers found in dairy fat (28). The relation between CLA and T2D has not been well established, and prospective cohort studies investigating circulating levels of specific CLA isomers with type 2 diabetes are lacking.

Because of this lack of evidence about specific TFA subtypes and their association with T2D incidence, the work presented in the first manuscript of this thesis aimed to examine the

relation of seven TFAs (from industrial and ruminant sources) measured in plasma phospholipids with T2D risk and dietary sources.

Saturated fatty acids

Dietary SFAs have traditionally been associated with an increased risk of T2D and its complications (29, 30). In prospective cohort studies, while higher concentrations of plasma SFAs with an even number carbon chain were associated with higher T2D risk (31, 32), plasma SFAs with an odd chain were associated with a lower T2D risk (27, 31, 32). Pentadecanoic acid (15:0) and heptadecanoic acid (17:0) are the main OCFAs found in human plasma.

Ruminants, such as cows, sheep, and goats, produce OCFAs by bacterial fermentation, and thus OCFAs are found in dairy fat. Despite being present in small amounts of total dairy fat, 15:0 and 17:0 have been extensively used as biomarkers of dairy fat intake, as they were assumed to be consumed mostly through dairy products. These OCFAs correlated with dairy fat consumption in many observational (33) and interventional studies (34). Furthermore, higher intakes of total dairy are linked with a reduced T2D risk (35), but this association appears to be driven primarily by some dairy subgroups, such as low-fat dairy and yoghurt (35). Still, no conclusive pathway has been determined to confirm the involvement of OCFAs (or ruminant TFAs) as mediators of this association between dairy and T2D.

It should be noted that researchers have also proposed an endogenous synthesis of OCFAs by intestinal bacterial fermentation of dietary fibre (36) and by branched-chain amino acids (37). And some criticism has emerged for OCFAs as valid biomarkers of dairy intake because low amounts are present in fish, beef, veal, lamb, and other foods (38). Thus, examining plasma OCFAs determinants is relevant to validating them as reliable biomarkers.

Although results from OCFAs in plasma phospholipids and T2D risk are available (27), results from other lipid classes or types of phospholipids are scarce. The selective incorporation of OCFAs into different plasma lipid classes is poorly understood. Moreover, it remains unclear whether the associations of plasma OCFAs with dairy intake and T2D risk are similar across plasma lipid classes. With the help of high-resolution lipidomics, the analyses presented in the second manuscript involved the distribution of OCFAs in different lipid classes, their class-specific associations with T2D incidence, and their associations with food groups as potential determinants.

Polyunsaturated Fatty Acids

A meta-analysis of randomised controlled feeding trials provided evidence that replacing SFA or carbohydrate intake with PUFAs improved glycemia, insulin resistance, and insulin secretion (39). Three main PUFAs groups are commonly found in human tissues: n-3, n-6, and n-9. The n nomenclature reveals the position of the first double bond counting from the methyl end, also known as the omega end. The most abundant PUFA in plasma is linoleic acid (LA, 18:2n-6).

Circulating 18:2n-6 was inversely associated with T2D incidence in a meta-analysis of 27 prospective cohorts (40). 18:2n-6 cannot be endogenously synthesised and, thus, is consumed by dietary intake. This FA is metabolised to n-6 PUFAs with longer chains and more double bonds (**Figure 3**). Therefore, the linoleic acid concentration in tissues depends on the dietary intake and the activity of enzymes in the elongation and desaturation pathway. The addition of carbons (elongation) and double bonds (desaturation) leads to the formation of gamma-linolenic acid (GLA, 18:3n-6), dihomogamma-linolenic acid (DGLA, 20:3n-6), and arachidonic acid (AA, 20:4n-6). Prospective cohort studies have revealed different associations of these FAs with T2D risk. For example, 20:3n-6 is related to an increased risk of T2D (30, 32, 41-48), while 20:4n-6 was not associated with T2D risk (30, 32, 41-44, 48-53).

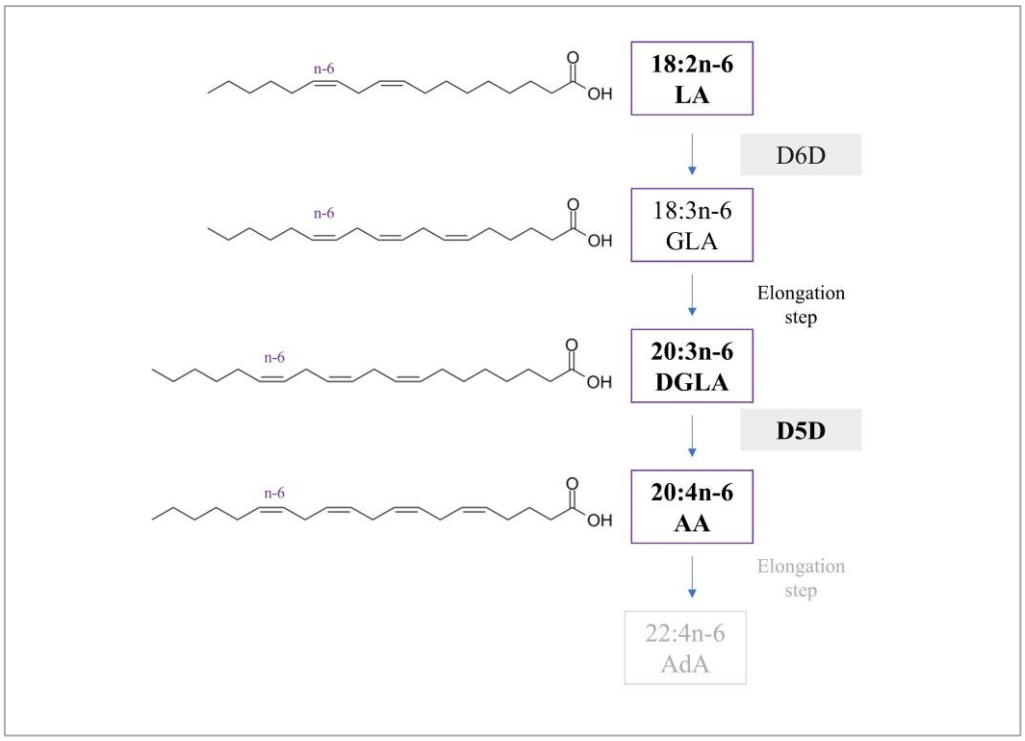

Figure 3. N-6 polyunsaturated fatty acids synthesis pathway

Most studies that have measured circulating n-6 PUFAs have reported these FAs in total plasma or phospholipids, with only a few other lipid compartments evaluated for PUFA concentrations. A pooled analysis from 20 prospective cohort studies from ten countries presented associations of 18:2n-6 and 20:4n-6 with T2D by lipid compartment. Only four lipid pools were included: total plasma, total phospholipids, CE, and one study with adipose tissue (49). The inverse associations between 18:2n-6 biomarkers and T2D were generally similar in these lipid compartments, and 20:4n-6 levels were generally not associated with T2D. However, other lipid classes have not been assessed, and thus, the potential differences in associations of n-6 PUFAs measured in other lipid classes with T2D risk are unknown.

In the PUFAs desaturation pathway, one of the desaturase enzymes well characterised is the delta-5 desaturase (D5D). The delta nomenclature indicates that the double bond is added in the 5th position from the carboxyl end. D5D then produces 20:4n-6 (delta 5,8,11,14) from the desaturation of 20:3n-6 (delta 8,11,14). Because higher concentrations of 20:4n-6 may result from increased enzymatic conversion from 20:3n-6, an increase in the ratio of 20:4n-6 to 20:3n-6 has been used to reflect higher D5D activity. Individuals with higher estimated D5D activity had a lower risk of T2D in prospective studies (41, 42, 45, 52, 54), supported by the indication of a causal effect from desaturase genotype information in a mendelian randomisation study (55).

The higher or lower D5D enzymatic activity is partly influenced by genetic factors (56). Within the FA desaturase (*FADS*) gene cluster, single-nucleotide polymorphisms (SNPs) in the genes encoding for D5D have been associated with the estimated desaturase activity using the ratio of 20:4n-6/20:3n-6 in total plasma/serum or red blood cell phospholipids (55, 57, 58), individual phospholipids (PC, PE, and PI) (56, 59), CE and adipose tissue (60). Still, evidence of associations of genetic variants in the *FADS* region with estimated D5D activity in these and other lipid classes is little explored. Distinguishing between classes enables us to identify which compartments are more representative of genetic factors.

Because the essential n-6 PUFA 18:2n-6 cannot be synthesised by humans and seem to play a role in T2D prevention, biomarkers of n-6 PUFAs status are of research interest. There is limited evidence on how n-6 PUFAs are distributed among lipid classes and if the n-6 PUFAs class-specific abundance is relevant in terms of their association with T2D risk. With the analyses presented in the third manuscript, this thesis adds evidence to those points to contribute to understanding n-6 PUFAs biomarkers. Moreover, gene variants were evaluated regarding their association with estimated D5D activity across the lipid classes.

Detailed fatty acid profiles in EPIC Potsdam case-cohort study

To characterise FA biomarkers (different TFA subtypes, and OCFAs and n-6 PUFAs in a range of lipid classes), data from participants of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort study was used in this thesis. The main analyses included two lipid profiles. The first was an assessment of TFAs in plasma phospholipids. With a modified method for analysis of FAs with very low abundances, seven TFAs subtypes were measured in total plasma phospholipids. The second lipid profile was derived from a high-throughout lipid profiling technology, which generated detailed information to allow the identification of 940 distinct molecular species concentrations and FA composition across 15 lipid classes (FFA, CE, MG, Cer, dhCer, LacCer, HexCer, SM, LPE, LPC, PI, DG, TG, PC, PE). The associations with T2D risk were assessed with a prospective nested case-cohort study design.

Overall, with high-resolution lipidomics and deep characterisation of TFA subtypes, novel biomarkers of health and risk were identified to understand further the relationships between diet, metabolism, and disease. The investigation of the plasma FA biomarkers may contribute to recognising potential biological pathways connecting FA intake with T2D incidence and could provide further evidence for a causal role of FAs or enzymes in T2D incidence.

3.1 Objectives and Outline

Main Objective

The main objective of this thesis was to investigate TFAs, OCFAs, and n-6 PUFAs and their link to T2D incidence by profiting from technological advances that allow better definition of these three categories. The work presented aimed to examine TFAs according to their specific conformation, and OCFAs and n-6 PUFAs within diverse lipid classes.

Specific Objectives

- To examine seven individual TFAs from industrial and ruminant sources (including two CLAs) measured in plasma phospholipids in relation to food sources and T2D risk

In the first manuscript, a prospective study was conducted to analyse the associations of seven subtypes of TFAs with T2D risk. This manuscript also presents mediation analysis by cardiometabolic biomarkers and different levels of model adjustments. Correlations between TFAs and food group intake derived from an FFQ were also reported.

- To characterise the distribution of OCFAs (15:0 and 17:0) among lipid classes and investigate the class-specific association of the lipids containing OCFAs with T2D incidence and their dietary determinants

The second manuscript, using lipidomic profiling, served the purpose of identifying the abundance of OCFAs in a wide range of 15 plasma lipid classes, the OCFA class-specific prospective associations with T2D, and the differences in associations between men and women. Also, we evaluated the correlations between OCFAs in phospholipid classes and dietary determinants based on the self-reported dietary assessment by FFQ.

- To investigate the prospective associations of PUFA abundance across lipid classes and class-specific estimated desaturase activity with T2D risk

In the third manuscript, the n-6 PUFAs concentrations across 15 plasma lipid classes were investigated. With a prospective case-cohort study, we investigated the associations of n-6 PUFAs abundance in lipid classes with T2D risk. This manuscript also presents estimated desaturase activities in different lipid classes and their associations with T2D risk and genomic variants.

4. Manuscripts

4.1 List of manuscripts and contributions

Prada M, Wittenbecher C, Eichelmann F, Wernitz A, Kuxhaus O, Kröger J, et al. **Plasma Industrial and Ruminant Trans Fatty Acids and Incident Type 2 Diabetes in the EPIC-Potsdam Cohort.** Diabetes Care. 2022; 45(4):845-853 (Impact Factor 19.1)

Contribution: Literature search, research questions formulation, data extraction, data management, statistical analysis, risk of bias assessment, illustration and interpretation of the results, and writing of the manuscript.

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Manuscript 1: Plasma Industrial and Ruminant Trans Fatty Acids and Incident Type 2 Diabetes in the EPIC-Potsdam Cohort

Plasma Industrial and Ruminant *Trans* Fatty Acids and Incident Type 2 Diabetes in the EPIC-Potsdam Cohort

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OBJECTIVE

Although dietary intake of *trans* fatty acid (TFA) is a major public health concern because of the associated increase in the risk of cardiovascular events, it remains unclear whether TFAs also influence risk of type 2 diabetes (T2D) and whether industrial TFAs (iTFAs) and ruminant TFAs (rTFAs) exert the same effect on health.

RESEARCH DESIGN AND METHODS

To investigate the relationship of 7 rTFAs and iTFAs, including 2 conjugated linoleic acids (CLAs), plasma phospholipid TFAs were measured in a case-cohort study nested within the European Prospective Investigation Into Cancer and Nutrition–Potsdam cohort. The analytical sample was a random subsample (*n* = 1,248) and incident cases of T2D (*n* = 801) over a median follow-up of 6.5 years. Using multivariable Cox regression models, we examined associations of TFAs with incident T2D.

RESULTS

The TFA subtypes were intercorrelated with each other, with other fatty acids, and with different food sources. After controlling for other TFAs, the iTFAs (18:1n-6*t*, 18:1n-9*t*, 18:2n-6,9*t*) were not associated with diabetes risk. Some rTFA subtypes were inversely associated with diabetes risk: vaccenic acid (18:1n-7*t*; hazard ratio [HR] per SD 0.72; 95% CI 0.58–0.89) and *t*10*c*12-CLA (HR per SD 0.81; 95% CI 0.70–0.94), whereas *c*9*t*11-CLA was positively associated (HR per SD 1.39; 95% CI 1.19–1.62). *Trans*-palmitoleic acid (16:1n-7*t*) was not associated with diabetes risk when adjusting for the other TFAs (HR per SD 1.08; 95% CI 0.88–1.31).

CONCLUSIONS

The TFAs' conformation plays an essential role in their relationship to diabetes risk. rTFA subtypes may have opposing relationships to diabetes risk. Previous observations for reduced diabetes risk with higher levels of circulating *trans*-palmitoleic acid are likely due to confounding.

Trans fatty acids (TFAs), which are unsaturated fatty acids (FAs) with at least 1 double bond in *trans* configuration, have been a focus of public health initiatives (1) because their intake has been related to coronary heart disease and all-cause

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mortality (2). Although initiatives to reduce TFA intake have focused on TFAs derived industrially by partial hydrogenation of vegetable oils, TFAs can also be found naturally in dairy foods.

Industrial TFAs (iTFAs) and ruminant TFAs (rTFAs) exist in a range of different FA molecules, differing in carbon-chain length and position of the double bond. The rTFAs and iTFAs share common isomers; however, the proportions are different (3). For example, *trans*-vaccenic acid (18:1n-7*t*) is predominant in rTFAs, whereas elaidic acid (18:1n-9*t*) is predominant in iTFAs (3). The conjugated linoleic acids (CLAs) are TFAs with only 1 carbon between 2 double bonds, 1 in *trans* configuration and 1 in *cis* configuration. Two common CLA isomers found in dairy fat are *cis*-9,*trans*-11 CLA (*c*9*t*11-CLA) and *trans*-10,*cis*-12 CLA (*t*10*c*12-CLA) (4).

Whether iTFA and rTFA exert the same effect on health is an ongoing debate. iTFAs and rTFAs have similar effects on blood lipoprotein concentrations (5), but the epidemiological evidence predominantly links iTFA intake with coronary heart disease risk (2). A higher intake of total TFAs is not clearly linked to higher risk of type 2 diabetes (T2D) in prospective cohort studies (2). However, in cohort studies, researchers have observed lower diabetes risk with higher blood concentrations of the ruminant-derived *trans*-palmitoleic acid (16:1n-7*t*) (2,6). Still, evidence for the role of iTFAs and rTFAs in diabetes is limited given that most studies examined only single TFA isomers (6–9) or did not distinguish between specific isomers from industrial and ruminant sources (7,10–13).

Furthermore, the role of CLA for T2D remains largely unknown. Short-term randomized controlled trials usually used a mix of CLA isomers and results suggest an influence on insulin sensitivity (14), which may depend on *PPARG* polymorphism (15). However, we are not aware of prospective studies investigating blood levels of specific CLA isomers with T2D risk. Therefore, in our study, we aimed to examine the relation of individual TFAs from industrial and ruminant sources, including 2 CLAs, measured in plasma phospholipids as a marker of intake, with T2D risk in a large, population-based cohort study.

RESEARCH DESIGN AND METHODS

Study Design and Population

The European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study is part of the multicenter prospective cohort study EPIC. In Potsdam, Germany, 27,548 participants (n = 16,644 women, most aged between 35 and 65 years; n = 10,904 men, most aged between 40 and 65 years) from the general population were recruited between 1994 and 1998 (16). The baseline examination included collecting blood samples, anthropometry, a self-administered validated food frequency questionnaire (FFQ), an interview including questions on prevalent diseases, and a questionnaire on sociodemographic and lifestyle characteristics (17,18). Follow-up questionnaires were administered every 2–3 years to identify incident cases of chronic diseases, including diabetes. Response rates for follow-up rounds 1, 2, and 3 were 96%, 95%, and 91%, respectively. We also considered questionnaires within the fourth follow-up round sent out until 31 January 2005, of which 90% were returned by 31 August 2005.

For efficient molecular phenotyping, a case-cohort study within the EPIC-Potsdam study was designed (19). From all participants with blood samples (n = 26,437), we randomly selected a subsample (n = 1,248) and all case patients with incident diabetes (n = 801) (Supplementary Figure 1). By randomly selecting a subcohort, the results are expected to be generalizable to the entire cohort without measuring biomarkers in the whole cohort (19). We excluded participants who had missing TFA analyses (n = 5), prevalent diabetes (n = 58), and missing follow-up information (n = 31). The analytical sample thus included 1,927 participants: 1,159 from the subcohort and 796 incident diabetes case patients, with an overlap of 28 participants. The median follow-up was 6.5 years. Consent was obtained from all participants, and approval was given by the Ethical Committee of the State Brandenburg, Germany.

Ascertainment of Diabetes Cases

Incident diabetes case patients were identified during follow-up via self-reports of diabetes diagnosis, diabetes-relevant medication, or dietary treatment due to diabetes. Diabetes information was also obtained from death certificates or other sources such as tumor centers, physicians, or clinics. All incident cases were verified by questionnaires mailed to the diagnosing physician asking about the date and type of diagnosis, diagnostic tests, and treatment of diabetes. Only cases with a physician diagnosis of T2D (ICD10 code E11) and a diagnosis date after the baseline examination were considered confirmed incident cases of T2D.

Dietary Assessment

All participants were asked to complete a validated semiquantitative FFQ (20–22), which was used to assess the average intake frequency and portion size of 148 foods consumed during the 12 months before the examination. Additional questions were asked about fat products used for food preparation. Moderate to high correlations were found with repeated administration of the FFQ at a 6-month interval: milk and milk products (r = 0.55), cheese (r = 0.61), bread (r = 0.49), cereals (r = 0.73), fruits (r = 0.61), vegetables (r = 54), meat (r = 0.77), and processed meat (r = 0.73) (22).

We analyzed dairy foods in 2 main groups based on their fat content: Low-fat dairy and full-fat dairy (Supplementary Table 1). In addition, the following dairy subgroups were considered separately: low-fat cheese, full-fat cheese, and butter. Apart from dairy foods, we included other potential dietary sources of TFAs or food that might correlate with TFA intake and thus potentially confound associations: margarine, other vegetable fat (vegetable fat or oil for cooking or salads), other fat (animal cooking fat, various fats and oils), eggs, poultry, meat, processed meat, fish, nuts, fried potatoes, confectionery, cakes, cookies, and desserts, fresh fruit, raw vegetables, cooked vegetables, grains, pasta and rice, bread (whole grain and other), and alcohol from alcoholic beverages.

Plasma Phospholipid TFAs

Venous blood samples were collected at baseline following standardized procedures. Phospholipid TFAs were measured from plasma samples that were stored at −80°C (Supplementary Material). In brief, we measured TFAs using total lipid extraction with *tert*-butyl methyl ether/methanol, solid-phase separation, hydrolysis,

and methylation with trimethyl sulfonium hydroxide, and subsequently analyzed samples by gas chromatography–tandem mass spectrometry. The method was modified for biomarker-relevant FA profile analysis with low abundances in plasma phospholipids. The area count values of multiple reaction monitoring were calibrated against area count values of detection by flame ionization detector (FID) using appropriate standard FAs. This enables relativization between low abundance TFA data with high abundance FA data based on FID detection. A full FA profile, including the odd-chain FAs 15:0 and 17:0, was assessed simultaneously by FID detection, splitting the eluting gas flow for FID and mass spectrometry detection.

We defined 16:1n-7*t*, 18:1n-7*t*, *c*9*t*11-CLA, and *t*10*c*12-CLA as rTFAs. All other TFAs, 18:1n-6*t*, 18:1n-9*t*, and 18:2n-6,9*t*, were defined as iTFAs (Supplementary Table 2).

Other Measurements

Measurements of plasma HDL cholesterol, triglycerides, γ-glutamyltransferase (GGT), hs-CRP, adiponectin, fetuin-A, and red blood cell concentrations of hemoglobin A_{1c} (HbA_{1c}) were performed as described in previous publications (23,24). Participants were genotyped for the Pro-12Ala polymorphism in *PPARG* using the TaqMan technology (Applied Biosystems, Foster City, CA). The reproducibility of the genotyping method was ≥99.5%.

Statistical Analysis

All TFA variables were log-transformed to stabilize skewed distributions (Supplementary Figure 2) and standardized (mean = 0, SD = 1). We explored intercorrelations of the TFAs and food groups, using Pearson correlation coefficients with adjustment for age, sex, and total energy intake in the subcohort; their 95% CIs were obtained by Fisher *z*-transformation.

We used Cox proportional hazard regression models stratified by age to study the relationship between FAs and diabetes risk in the case cohort, accounting for the oversampling of cases by Prentice weighting (19). We estimated hazard ratios (HRs) and 95% CIs per 1-SD increase in log-transformed TFA concentrations. The following variables were considered covariables in the models: waist circumference (continuous), BMI (continuous), smoking status (never, past, current <20 cigarettes/day, current ≥20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, ≥5 h/week), sports activity (0, 0.1–4.0, >4.0 h/week), occupational activity (light, moderate, heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/day), red meat intake (energy adjusted), coffee intake (energy adjusted), fiber intake (energy adjusted), fasting status, and total energy intake. For energy adjustment, the residual method was used (25). To evaluate the independent associations of each TFA subtype, we further mutually adjusted all TFAs in a separate model. To further explore intercorrelations, a principal component (PC) analysis (PCA) based on the individual TFA was performed on the subcohort's data. We then calculated a PC score for all participants and constructed Cox models adjusting for this score. We also assessed the nonlinear association of TFAs with diabetes, using restricted cubic splines with 3 knots placed at the 5th, 50th, and 95th percentiles.

In sensitivity analyses, we included TFA food sources as covariates in the models to confirm that TFAs associated with T2D risk are not just markers of intake of these food groups. We also considered other FAs for adjustment: the dairy fat–derived 15:0 and 17:0 (expected to correlate with rTFAs), FAs in the de novo lipogenesis pathway as suggested in a previous study (10), and other FAs as potential confounders derived from our correlation analyses. We then examined whether associations between TFAs and diabetes were modified by sex, alcohol intake, BMI, and *PPARG* gene polymorphism (wild type vs. Ala carriers), because it has been reported that CLA isomers influence insulin sensitivity depending on the *PPARG* polymorphism (15). Tests for statistical interactions were conducted by including cross-product terms in the models. Last, we excluded participants with baseline HbA_{1c} >6.5%, thus potentially those with undiagnosed or nonreported diabetes.

We used Pearson correlation analysis adjusted for age and sex to examine the relationship of the TFAs to cardiometabolic risk biomarkers (namely, triglycerides, non-HDL cholesterol, HDL cholesterol, HbA_{1c}, GGT, adiponectin, hs-CRP, and fetuin-A) and the fatty liver index (FLI) (26) in the subcohort. Then, we conducted mediation analyses to assess the extent to which significantly TFA-related biomarkers potentially explained the associations between TFAs and diabetes risk. Cox regression models with and without adjustment for the potential mediator were compared. Differences between the TFA β-coefficients of the reference model and the TFA β-coefficients of the biomarker-fitted model, their stability as well as the corresponding HRs, were estimated as median and dispersion from a bootstrapping procedure (n = 500 bootstrap replicates). All statistical analyses were conducted with SAS Software Enterprise Guide 7.1 (SAS Institute, Cary, NC).

RESULTS

Baseline characteristics of the random subcohort were similar to those in the full EPIC-Potsdam cohort (Supplementary Table 3). The characteristics of the 1,159 subcohort participants (n = 454 men and 705 women) were considered according to the distribution of ruminant TFAs in plasma phospholipids (Supplementary Table 4). The most abundant of the rTFAs was *c*9*t*11-CLA (0.26%), followed by 18:1n-7*t* (0.19%). Only small portions were 16:1n-7*t* (0.04%) and *t*10*c*12-CLA (0.01%). The most abundant iTFA was 18:1n-9*t* (0.16%), followed by its isomer 18:1n-6*t* (0.09%). Study participants with higher rTFA concentrations were more likely to be women and nonsmokers and to have lower waist circumference and less alcohol consumption than participants with lower rTFA concentrations. Age, BMI, and other characteristics were similar across quintiles.

The rTFAs were positively intercorrelated, with the strongest correlation observed between 16:1n-7*t* and 18:1n-7*t* (r = 0.70) (Supplementary Table 5). Of note is that the rTFAs 16:1n-7*t*, 18:1n-7*t*, and *t*10*c*12-CLA were moderately positively correlated with some of the iTFAs (r = 0.29–0.66). Among the iTFAs, the most abundant, 18:1n-9*t*, was strongly correlated with its isomer, 18:1n-6*t* (r = 0.75); and moderately correlated with the ruminant isomer, 18:1n-7*t* (r = 0.50), and some of the 18:2 isomers (18:2n-6,9*t*, r = 0.32; *t*10*c*12-CLA, r = 0.34). All rTFAs were positively correlated to 15:0 and 17:0

(r = 0.16–0.62), whereas iTFAs were mainly correlated with 17:0 (r = 0.22–0.41). TFAs were also correlated with polyunsaturated fatty acid (PUFA) concentrations, particularly n-6 PUFAs.

The rTFAs 16:1n-7*t*, 18:1n-7*t*, and *c*9*t*11-CLA were positively correlated with intake of fat-rich dairy foods, particularly with butter (strongest for *c*9*t*11-CLA; r = 0.34), whereas low-fat dairy foods were not appreciably correlated (Fig. 1). In contrast, these rTFAs were negatively correlated with margarine consumption, especially *c*9*t*11-CLA (r = −0.29). Noteworthy, *c*9*t*11-CLA correlated with dietary variables differently from the other 18:2 isomers. The iTFA 18:1 isomers showed correlations contrasting with those observed for rTFAs: positive for margarine (18:1n-9*t*; r = 0.17) and negative for butter (18:1n-9*t*; r = −0.16). Still, both rTFAs and iTFAs were positively correlated to a similar degree with confectionery, cakes, cookies, and desserts. Correlations with alcohol intake were inverse and relatively strong compared with most other foods for iTFAs and rTFAs.

In models adjusted for age and sex, inverse associations with diabetes risk were observed for all TFAs except *c*9*t*11-CLA (Fig. 2). Among the iTFAs, adjustment for demographic, lifestyle, and anthropometric risk factors rendered the associations of 18:1n-6*t* and 18:1n-9*t* with diabetes risk nonsignificant, whereas 18:2n-6,9*t* remained inversely associated (HR per SD 0.87; 95% CI 0.77–0.98). This association was largely attenuated when adjusted for other TFAs (HR per SD 0.95; 95% CI 0.81–1.12). Among the rTFAs, 18:1n-7*t* and *t*10*c*12-CLA were inversely associated with diabetes risk, and these associations were unaffected or strengthened by further adjustment for other TFAs (for 18:1n-7*t* and *t*10*c*12-CLA, respectively: HR per SD 0.72 [95% CI 0.58–0.89]; and 0.81 [95% CI 0.70–0.94]). In contrast, *c*9*t*11-CLA was positively associated with diabetes risk (in the fully adjusted model, HR 1.39; 95% CI 1.19–1.62). The inverse association of 16:1n-7*t* with diabetes risk in the age- and sex-adjusted model was attenuated with further adjustment, particularly for other TFAs. Multivariable-adjusted restricted cubic splines did not indicate relevant deviation from linearity (P for linearity > 0.05 for each rTFA) (Supplementary Figure 3).

The first PC from PCA accounted for a large variance proportion and was positively correlated with all TFAs (Supplementary Table 6). Subsequently, we used the PC1 score for adjustment in Cox models, which did not substantially affect our results (Supplementary Figure 4).

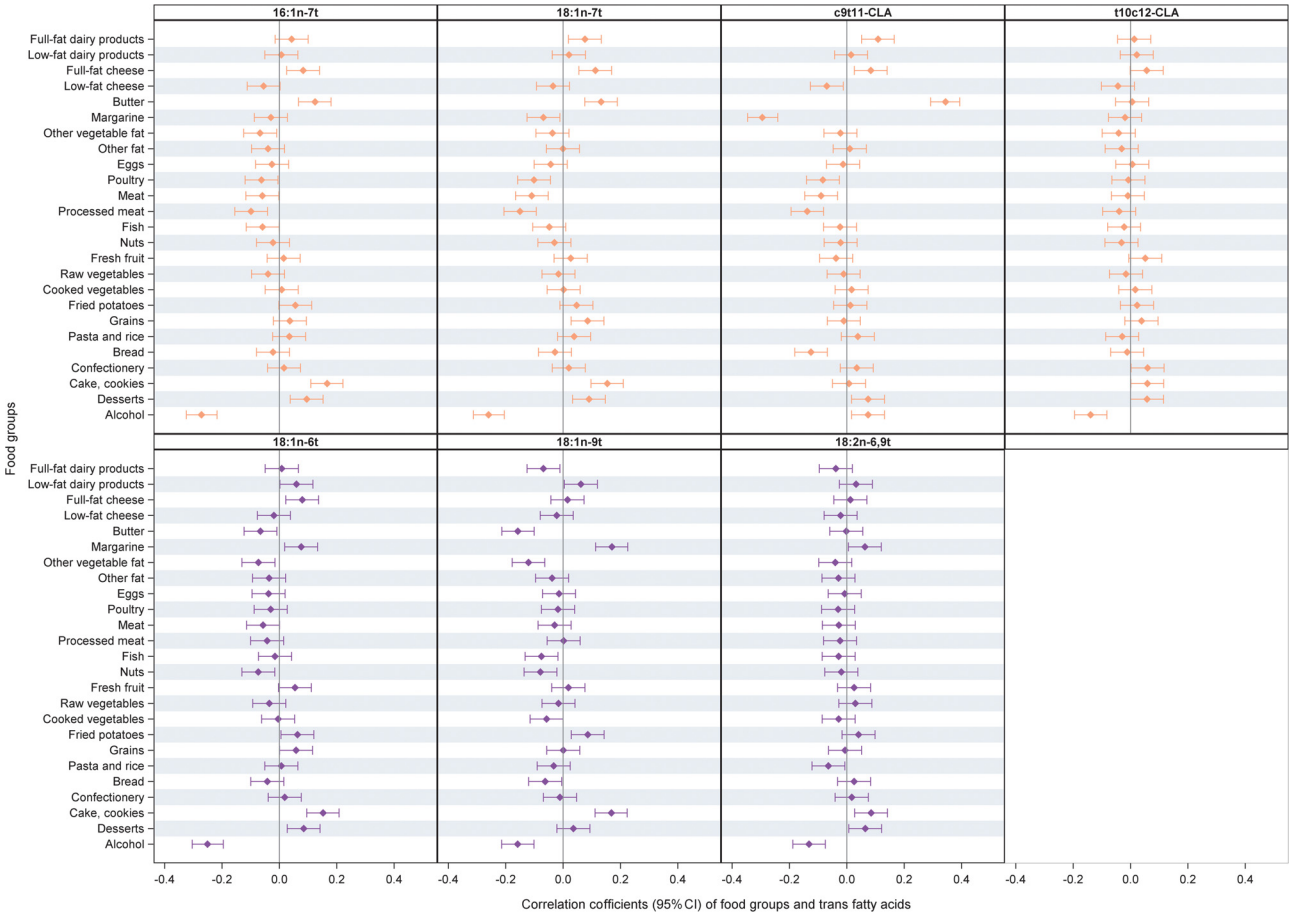

Figure 1—Partial correlations between rTFAs (orange) or iTFAs (purple) and food groups, adjusted for age, sex, and total energy intake, in the EPIC-Potsdam subcohort (n = 1,159). Full-fat dairy included full-fat milk and milk beverages (>1.5% fat), full-fat yogurt (>1.5% fat), curd cheese (>5% fat), heavy cream, and full-fat cheese (namely, full-fat cream cheese, Gouda, Emmental, Tilsiter, Camembert, Brie, Gorgonzola, processed cheese). Low-fat dairy included low-fat milk and milk beverages (≤1.5% fat), low-fat yogurt (≤1.5% fat), kefir (≤1.5% fat), curd cheese (≤5% fat), and low-fat cheese (reduced fat or lean: cream cheese, Gouda, Emmental, Tilsiter, Camembert, Brie, Gorgonzola).

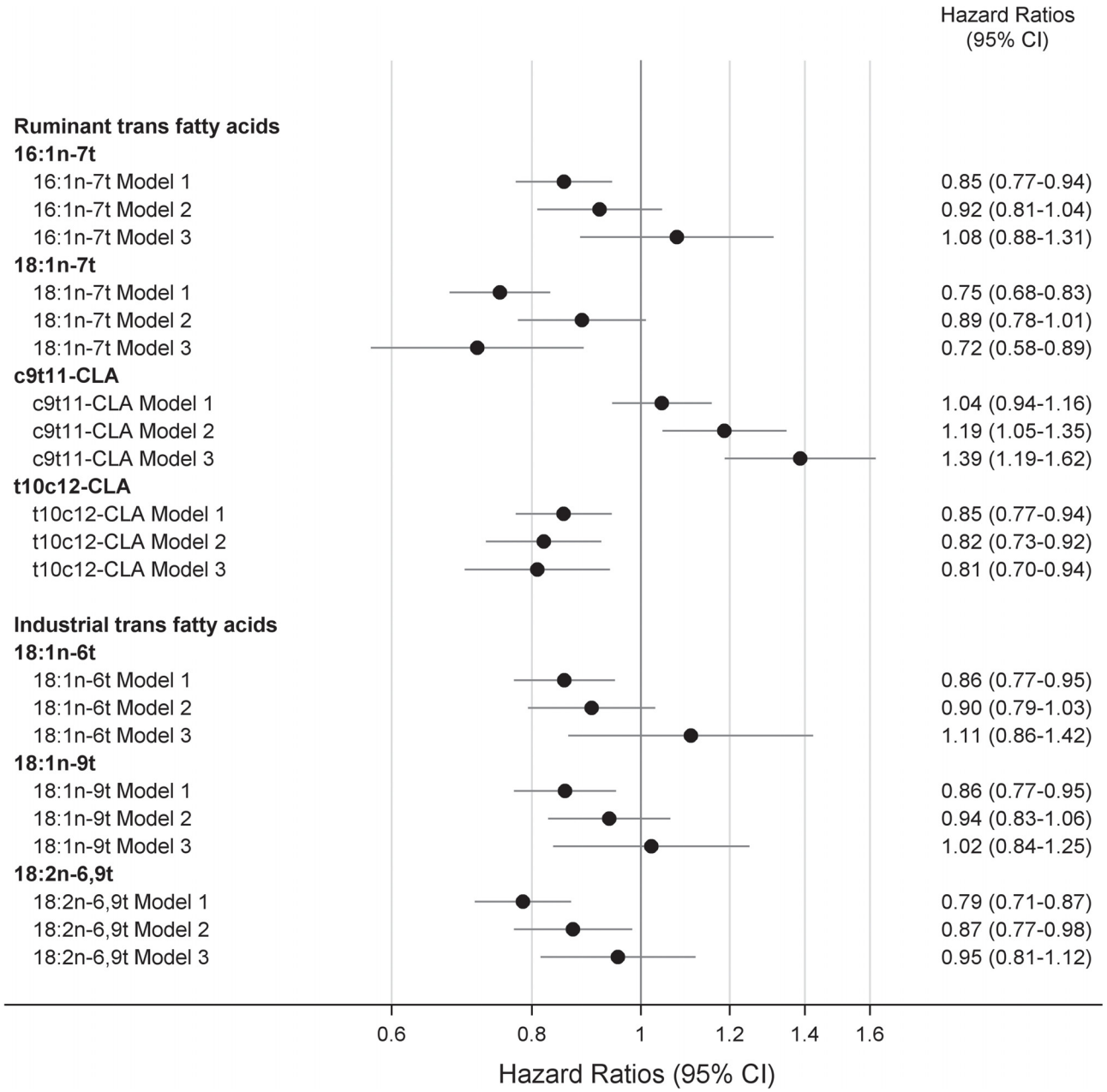

Figure 2—Prospective associations of plasma TFAs with risk of T2D in EPIC-Potsdam (*N* = 1,927; cases, *n* = 796). HR and 95% CI per 1 SD increase of each TFA. Model 1 was adjusted for age (stratum variable) and sex. Model 2 was further adjusted for waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or ≥5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/day), red meat intake (energy adjusted), coffee intake (energy adjusted), fiber intake (energy adjusted), fasting status, and total energy intake. Model 3 was further adjusted for all other TFA subtypes: 16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, and *t*10*c*12-CLA.

In additional sensitivity analyses, we adjusted the models of the 3 significantly diabetes risk–associated rTFAs for other potential confounders (Table 1). Adjustment variables included the strongest food correlates (butter and margarine), dairy-fat biomarkers (15:0 and 17:0), and FAs in the de novo lipogenesis pathway (16:0, 16:1n-7*c*, 18:0, and 18:1n-9*c*). All adjustments had little impact on the rTFA–diabetes risk associations. When adjusting for n-6 PUFA concentrations (18:2n-6*c*, 20:3n-6*c*, and 20:4n-6*c*), the inverse association of *t*10*c*12-CLA with diabetes risk remained unchanged. However, the associations for 18:1n-7*t* and *c*9*t*11-CLA were somewhat attenuated, although directions remained unchanged (for 18:1n-7*t* and *c*9*t*11-CLA, respectively, HR per SD: 0.86 [0.67–1.11] and 1.21 [0.99–1.48]). We did not observe significant interactions of TFAs with alcohol intake, sex, BMI, or the *PPARG* Pro12Ala polymorphism on

Table 1—Prospective association between TFAs and T2D in the EPIC-Potsdam study (*N* = 1,927; cases, *n* = 796), with additional models*

	HR per 1 SD (95% CI)			
	18:1n-7*t*	*c*9*t*11-CLA	*t*10*c*12-CLA	AIC
Reference model†	0.72 (0.58–0.89)	1.39 (1.19–1.62)	0.81 (0.70–0.94)	4,408
+ 15:0 and 17:0	0.78 (0.61–0.99)	1.41 (1.20–1.66)	0.82 (0.70–0.95)	4,407
+ Butter	0.72 (0.58–0.89)	1.39 (1.18–1.63)	0.81 (0.70–0.94)	4,411
+ Margarine	0.72 (0.58–0.89)	1.41 (1.20–1.65)	0.81 (0.70–0.94)	4,409
+ Total dairy	0.72 (0.58–0.89)	1.38 (1.18–1.61)	0.81 (0.70–0.94)	5,271
+ FAs in the de novo lipogenesis pathway‡	0.68 (0.51–0.90)	1.44 (1.16–1.79)	0.78 (0.67–0.91)	4,374
+ n-6 PUFAs§	0.86 (0.67–1.11)	1.21 (0.99–1.48)	0.81 (0.69–0.94)	4,332

*Adjusted for dairy fat–derived FAs pentadecanoic acid (15:0) and heptadecanoic acid (17:0), butter, margarine, total dairy, FAs in the de novo lipogenesis pathway, and n-6 PUFAs. AIC, Akaike information criterion. †Model adjusted for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or ≥5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, vocational training, technical school, or technical college or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/day), red meat intake (energy adjusted), coffee intake (energy adjusted), fiber intake (energy adjusted), fasting status, total energy intake, and all other TFA subtypes: 16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA. ‡16:0, 16:1n-7*c*, 18:0, and 18:1n-9*c*. §18:2n-6*c*, 20:3n-6*c*, and 20:4n-6*c*.

diabetes risk (Supplementary Tables 7–10). Associations remained unaffected when excluding participants with baseline HbA_{1c} >6.5% (Supplementary Figure 5).

The different cardiometabolic biomarkers generally had comparable correlations with all TFA isomers (Fig. 3) but *c*9*t*11-CLA. Most TFA subtypes were negatively correlated with blood lipids (with the strongest correlations observed for triglycerides), negatively with GGT, and positively with adiponectin. The correlations of *c*9*t*11-CLA with biomarkers appeared different in comparison: triglyceride levels (fasted) were positively correlated, whereas other lipids and nonlipid biomarkers showed little correlation overall.

We next evaluated whether adjustment for the correlated biomarkers attenuated the observed associations between rTFAs and diabetes risk (Supplementary Table 11). No appreciable attenuation was observed for the inverse association of 18:1n-7*t* with diabetes risk. The associations of *c*9*t*11-CLA and *t*10*c*12-CLA with T2D risk were attenuated when adjusting for triglycerides (−20% and −35%, respectively) and FLI (−26% and −38%, respectively). Other metabolic markers had no significant impact on the associations.

CONCLUSIONS

In this prospective cohort study, we found opposite diabetes risk associations

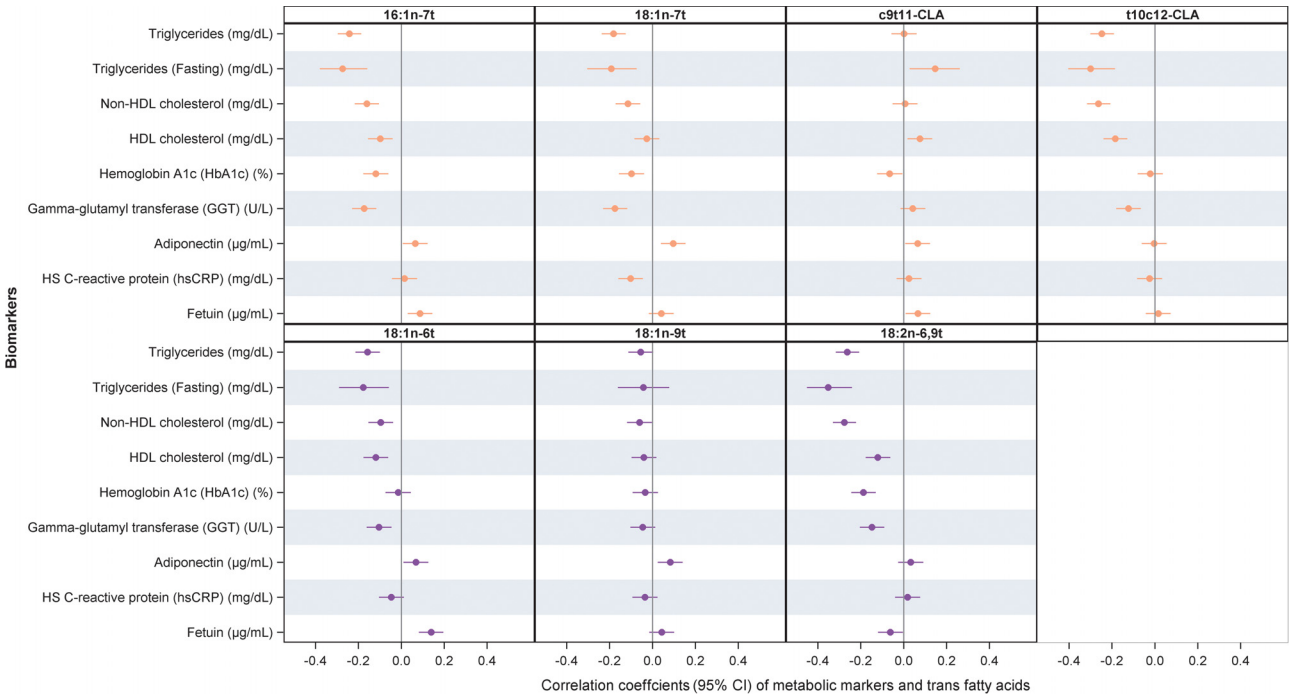

Figure 3—Partial correlations between rTFAs (orange) or iTFAs (purple) and biomarkers, adjusted for age and sex, in the EPIC-Potsdam subcohort. Biomarkers: triglycerides (*N* = 1,138; fasting, *n* = 271), non-HDL cholesterol (*n* = 1,144), HDL cholesterol (*n* = 1,144), HbA_{1c} (*n* = 1,109), GGT (*n* = 1,138), adiponectin (*n* = 1,134), hs-CRP (*n* = 1,141), and fetuin (*n* = 1,146).

among 7 different iTFAs and rTFAs measured in plasma phospholipids. The iTFA subtypes were not associated with diabetes risk independently of other TFAs, and 3 rTFA subtypes were associated with diabetes risk. An inverse association was found for 18:1n-7*t* and *t*10*c*12-CLA, whereas *c*9*t*11-CLA was associated with higher diabetes risk. The inverse associations of 18:1n-7*t* and the positive association of *c*9*t*11-CLA were essentially unchanged when models were adjusted for correlated foods, FA biomarkers of dairy intake (i.e., 15:0 and 17:0), or FA biomarkers of de novo lipogenesis. However, adjustment for n-6 PUFA concentration attenuated these associations. In contrast, *t*10*c*12-CLA remained inversely associated with diabetes risk irrespective of such adjustments. The rTFA 16:1n-7*t* was not associated with diabetes risk when controlling for other correlated TFAs.

We observed that individual TFAs had markedly different associations with diabetes risk, other FAs, cardiometabolic risk markers, and dietary intake. Individual TFAs reflect different food sources (industrial vs. ruminant) and distinct chemical structures, for example, total *trans*-18:1 (7,10,11,13,27), total *trans*-18:2 (10,11,13), and total CLA (12). Our results highlight that collapsing individual TFAs into 1 broad category may obscure potential etiological roles and health relevance. For example, in the Cardiovascular Health Study (CHS), all *trans*-18:1 isomers were summed because of shared food correlations and high intercorrelations (10,28), suggesting that assessing total *trans*-18:1 is sufficient to investigate health outcomes (28). However, although all *trans*-18:1 isomers were similarly associated with confectionery, cake, cookies, desserts, and alcohol in our study, only *trans*-vaccenic acid (18:1n-7*t*) was positively associated with full-fat dairy, full-fat cheese, and butter. Consistently, previous studies reported that 18:1n-7*t* was more abundant in ruminant fats, and 18:1n-6*t* and 18:1n-9*t* were more present in industrial products (3). In addition, 18:1n-7*t* was not positively associated with margarine in our study, in contrast with the CHS study (28). We found 18:1n-7*t* more strongly correlated with 16:1n-7*t* than the other *trans*-18:1 isomers, consistent with their similar dietary sources. The difference in the observed diabetes risk associations (null for the iTFAs 18:1n-6*t* and 18:1n-9*t*; inverse for 18:1n-7*t*) indicates that analyzing the *trans*-18:1 isomers as a whole group appears oversimplified. Our detailed analyses might also explain the mixed results reported from prior prospective cohort studies on total *trans*-18:1 and diabetes risk (10,13,27).

Considering 18:1n-7*t* is the predominant TFA in ruminant fat (3), we further adjusted for total dairy intake and odd-chain FAs as markers of dairy intake. The inverse association of 18:1n-7*t* with T2D risk was largely unaffected. Therefore, 18:1n-7*t* does not appear as just a biomarker of dairy intake. Potential explanations for a possible antidiabetic role include GLUT2- and PPAR-γ–mediated increase of insulin secretion and enhanced β-cell proliferation through 18:1n-7*t*, shown in animal models and human islets (29). Other studies suggest that 18:1n-7*t* may activate insulin signaling genes, including *PPARA* and *PPARG* in adipose tissue (30,31), and 18:1n-7*t* may improve energy use and fat distribution (30). However, in double-blind, randomized, controlled clinical trials, diets enriched in 18:1n-7*t* for 4–5 weeks did not modify fasting insulinemia and glycemia (32,33). The supplementation of 18:1n-7*t* decreased liver fat accumulation in rats (30), but FLI did not explain the 18:1n-7*t* diabetes risk association in our study.

In several other cohort studies, researchers have reported inverse associations of *trans*-palmitoleic acid (16:1n-7*t*) with diabetes risk (6,8,9,34). Importantly, our observation that the initial inverse association of 16:1n-7*t* was completely attenuated when adjusting for other TFAs indicates that previous observations may reflect confounding. In our study, 16:1n-7*t* was associated with dairy products, as previously reported (28), and was highly correlated with 18:1n-7*t*, consistent with their common food sources. A small randomized controlled trial revealed that 16:1n-7*t* increased after supplementation with 18:1n-7*t*, suggesting that 16:1n-7*t* may be endogenously synthesized (8). To our knowledge, no previous study has analyzed the independence of associations with diabetes risk of these 2 rTFA subtypes.

We also analyzed several *trans*-18:2 isomers, including 2 CLAs naturally found in dairy products (4). The weak intercorrelations, variations in the correlations with food groups, and opposing associations with diabetes risk indicate that CLA isomers should be evaluated individually. We are unaware of other epidemiological studies on individual circulating CLA isomers and diabetes risk. Possible heterogeneous effects are particularly relevant because several randomized controlled trials tested the effects of a mix of CLA isomers on diabetes-related traits (14). Some of the few intervention studies focusing on *c*9*t*11-CLA reported neutral effects on plasma insulin, glucose, and insulin resistance (measured by HOMA for insulin resistance) (14). However, authors of 1 study reported reduced insulin sensitivity (measured by hyperinsulinemic clamp) (35), which supports our observation of a positive association of this CLA with diabetes risk. Still, *c*9*t*11-CLA supplementation did not affect concentrations of circulating triglycerides (15,35). Thus, our observation that triglycerides and hepatic steatosis attenuate the association of *c*9*t*11-CLA with diabetes risk must be interpreted with caution. This notion also applies to the isomer *t*10*c*12-CLA, because supplementation did not affect concentrations of circulating triglycerides in human intervention studies (15,36). Furthermore, in contrast to the reduced diabetes risk we observed for higher circulating concentrations of this CLA, supplementation of *t*10*c*12-CLA had either neutral or adverse effects on insulin, glucose, and insulin sensitivity (14).

It is worth noting that 18:1n-7*t* and *t*10*c*12-CLA (inversely associated with diabetes risk) were inversely associated with alcohol intake, whereas *c*9*t*11-CLA (positively associated with diabetes risk) was positively associated with alcohol intake. Associations of TFAs with alcohol intake have been found previously (10). Alcohol intake activates hepatic de novo lipogenesis, an important pathway leading to the development of diabetes (37). However, it is unlikely that alcohol intake and its effect on lipogenesis explain the associations observed for TFAs, because moderate alcohol intake is associated with reduced diabetes risk (38); thus, the correlation of alcohol intake with TFAs instead would result in an underestimation of association. Nevertheless, our models were comprehensively adjusted for alcohol intake.

Consistent with reports from the CHS cohort (10), adjusting for de novo lipogenesis FAs did not appreciably attenuate the associations of 18:1n-7*t*, *c*9*t*11-CLA, and *t*10*c*12-CLA with diabetes risk in our study.

Another finding is that the associations for 18:1n-7*t* and *c*9*t*11-CLA were somewhat attenuated when we adjusted for n-6 FA concentrations. Similar to what we found in our study, phospholipid FA levels have shown strong intercorrelation patterns (39). This intercorrelation may introduce confounding when evaluating individual FAs (40). Thus, low TFA blood levels may partly reflect a replacement by other FAs, with n-6 PUFAs being well established to relate to diabetes risk (41). Although we have controlled for intercorrelated FA in our analysis, this has not been the case in most previous studies of TFA biomarkers (7–9,11,12).

This study has important strengths. We have quantified 7 different TFA subtypes, including 2 CLAs—a large panel compared with most previous studies on TFA subtypes, which included 1 to 3 TFAs in their analyses (7–9,11). Individual analyses of several TFAs, without combining in broader groups, uncovered strikingly different associations. Also, to our knowledge, this is the first prospective study of blood concentrations of specific CLA isomers and diabetes risk. Our study includes a comprehensive investigation of intercorrelations and evaluation of potential pathways. Although our study is observational and, therefore, associations may be prone to confounding, we comprehensively adjusted for various diabetes risk factors, including demographic, lifestyle, and dietary variables.

This study also had limitations. First, we did not measure the stability of the TFAs during storage time from the plasma sample collection to the analysis several years after. However, previous evidence suggested sufficient long-term stability of plasma FA concentration when stored at −80°C (42). Second, the association with food groups was investigated using an FFQ that included a limited number of food groups, which probably underestimated TFA sources. Third, TFAs were assessed in single plasma samples at study baseline. Food intake may have changed over time, and iTFA content in foods has been reduced by food reformulation, which is not reflected in the single baseline measurements. Fourth, we cannot rule out that the TFA concentrations correlate with other unmeasured FAs. Fifth, although several TFAs were significantly associated with diabetes, we might have had limited statistical power to detect weaker associations. 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 (43).

In conclusion, our study findings suggest that TFA conformation plays an essential role in TFAs' relationship to diabetes risk. Although iTFAs were not appreciably associated with risk, rTFA isomers may have different relations to diabetes risk, with 18:1n-7*t* and *t*10*c*12-CLA being inversely associated and *c*9*t*11-CLA positively associated. Previous observations of reduced diabetes risk with higher circulating *trans*-palmitoleic acid are likely due to confounding.

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SUPPLEMENTARY MATERIAL

Plasma industrial and ruminant *trans* fatty acids and incident type 2 diabetes in the EPIC-Potsdam cohort

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Supplementary Table 1. Dairy food groups

Dairy groups	Foods included
Low-fat dairy	low-fat milk and milk beverages (≤1.5% fat), low-fat yogurt (≤1.5% fat), kefir (≤1.5% fat), curd cheese (≤5% fat), and low-fat cheese (reduced fat or lean: cream cheese, gouda, emmental, tilsiter, camembert, brie, gorgonzola).
Full-fat dairy	full-fat milk and milk beverages (>1.5% fat), full-fat yogurt (>1.5% fat), curd cheese (>5% fat), heavy cream, and full-fat cheese (full-fat cream cheese, gouda, emmental, tilsiter, camembert, brie, gorgonzola, processed cheese).

Supplementary Table 2. Trans fatty acids profile

Class	Lipid name	Common name	Systematic Name
Ruminant Trans Fatty Acids	16:1n-7*t*	*trans*-palmitoleic acid	9E-hexadecenoic acid
	18:1n-7*t*	*trans*-vaccenic acid	11E-octadecenoic acid
	*c*9*t*11-CLA	Rumenic acid	9Z,11E-octadecadienoic acid
	*t*10*c*12-CLA	10E,12Z-octadecadienoic acid	10E,12Z-octadecadienoic acid
Industrial Trans Fatty Acids	18:1n-9*t*	9-elaidic acid	9E-octadecenoic acid
	18:1n-6*t*	*trans*-12-elaidic acid	12E-octadecenoic acid
	18:2n-6,9*t*	Linoelaidic acid	9E,12E-octadecadienoic acid

Supplementary Table 3. Comparison between full cohort with blood samples and random subcohort before exclusions

Characteristics	Full cohort	Subcohort before exclusions	Subcohort after exclusions
N	26437*	1248	1159
Sex (% women)	59.8	60.1	60.8
Age (median, IQR)	50.7 (42.6-58.4)	50.7 (42.4-58.0)	49.4 (42.1-57.7)
BMI (median, IQR)	25.7 (23.2-28.6)	25.6 (23.2-28.2)	25.4 (23.0-28.0)
Education (%)			
Currently in training/no certificate	3.5	3.8	3.5
Skilled worker	35.0	34.6	35.1
Professional school	24.8	24.0	23.8
High education/university	36.7	37.5	37.5
Smoking status (%)			
Never smoker	46.8	47.5	48.5
Ex-smoker	32.5	31.6	31.4
Smoker <20 units/day	14.8	15.3	14.8
Smoker >=20 units/day	5.8	5.6	5.3

*There were missing values for BMI (n=198), education (n=17), smoking (n=16)

Supplementary Table 4. Baseline characteristics according to quintiles of total dairy trans fatty acids (sum of 16:1n-7*t*, 18:1n-7*t*, *c*9*t*11-CLA and *t*10*c*12-CLA), EPIC-Potsdam subcohort (n=1159)

	Fifths of plasma phospholipid total dairy trans-fatty acid concentration					
	First	Second	Third	Fourth	Fifth	Total
Ruminant trans fatty acids (%) *, †	**0.37 (0.34-0.39)**	**0.44 (0.43-0.46)**	**0.50 (0.49-0.52)**	**0.58 (0.56-0.60)**	**0.71 (0.66-0.78)**	**0.50 (0.43-0.60)**
16:1n-7*t* (%) †	0.03 (0.02-0.03)	0.04 (0.03-0.04)	0.04 (0.03-0.04)	0.04 (0.04-0.05)	0.05 (0.04-0.06)	0.04 (0.03-0.05)
18:1n-7*t* (%) †	0.13 (0.11-0.15)	0.17 (0.15-0.18)	0.19 (0.17-0.21)	0.23 (0.20-0.26)	0.28 (0.25-0.32)	0.19 (0.15-0.24)
*c*9*t*11-CLA (%) †	0.20 (0.17-0.22)	0.23 (0.22-0.25)	0.27 (0.24-0.29)	0.30 (0.27-0.33)	0.37 (0.32-0.43)	0.26 (0.22-0.31)
*t*10*c*12-CLA (%) †	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.01)
Industrial TFA						
18:1n-6*t* (%) †	0.08 (0.06-0.09)	0.09 (0.08-0.11)	0.10 (0.08-0.11)	0.11 (0.09-0.13)	0.11 (0.09-0.16)	0.09 (0.08-0.12)
18:1n-9*t* (%) †	0.14 (0.12-0.16)	0.16 (0.14-0.19)	0.16 (0.14-0.19)	0.18 (0.14-0.23)	0.19 (0.14-0.27)	0.16 (0.13-0.20)
18:2n-6,9*t* (%) †	0.02 (0.02-0.02)	0.02 (0.02-0.03)	0.02 (0.02-0.03)	0.02 (0.02-0.03)	0.02 (0.02-0.03)	0.02 (0.02-0.03)
Age (years) †	48.8 (42.2-56.2)	49.5 (41.7-57.4)	52.7 (42.3-58.5)	46.7 (41.4-57.5)	50.9 (43.0-58.5)	49.4 (42.1-57.7)
Sex (% women)	42.7	56.0	64.5	69.4	71.6	60.8
Body mass index (kg/m²) †	26.4 (24.0-29.5)	26.0 (23.3-29.3)	25.2 (23.3-28.1)	24.5 (22.2-27.4)	24.5 (22.4-26.8)	25.4 (23.0-28.0)
Waist (cm) †	89.3 (82.0-97.0)	87.0 (76.0-96.0)	83.0 (75.2-93.0)	80.8 (73.0-90.3)	80.5 (72.0-90.0)	85.0 (75.0-93.8)
Leisure time physical activity (h/week) †	0 (0-2)	0 (0-1)	0 (0-1)	0 (0-1.5)	0 (0-1.8)	0 (0-1.5)
Alcohol from alcoholic drinks (g/day) †	15.6 (3.4-29.3)	8.6 (3.1-19.3)	7.9 (2.9-16.3)	6.8 (2.4-18.8)	6.1 (2.0-14.9)	8.4 (2.9-20.1)
Type of work. (%)						
(Heavy) manual work	6.9	7.3	4.3	6.5	4.7	6.0
Sedentary occupation	52.6	59.9	61.0	63.4	60.3	59.4
Standing occupation	40.5	32.8	34.6	30.2	34.9	34.6
Hypertension (%)	35.3	33.2	36.4	28.9	33.2	33.4
Education (%)						
High education/university	34.5	36.6	41.6	37.9	37.1	37.5
Currently in training/no certificate	5.2	1.3	4.3	3.0	3.9	3.5
Professional school	19.1	25.4	19.0	28.0	27.6	23.8
Skilled worker	41.4	36.6	35.1	31.0	31.5	35.1

Smoking status (%)						
Never smoker	35.8	43.1	54.5	51.3	57.8	48.5
Ex-smoker	37.9	36.2	29.0	28.0	25.9	31.4
Smoker < 20 units/day	18.1	15.5	12.6	15.9	12.1	14.8
Smoker ≥ 20 units/day	8.2	5.2	3.9	4.7	4.3	5.3
HbA_{1c} (%) †	5.4 (5.2-5.8)	5.4 (5.1-5.8)	5.4 (5.2-5.8)	5.3 (5.1-5.6)	5.4 (5.1-5.6)	5.4 (5.1-5.7)
Triglycerides (mg/dL) †	126 (82-184)	108 (74-170)	102 (77-148)	94 (70-136)	99 (72-143)	106 (74-161)
Non-HDL cholesterol (mg/dL) †	154 (124-178)	149 (123-180)	146 (122-172)	145 (118-166)	141 (115-173)	147 (119-173)
HDL cholesterol (mg/dL) †	52 (45-63)	55 (46-63)	55 (47-64)	57 (48-67)	57 (47-66)	55 (46-65)
Adiponectin (µg/ml) †	6.7 (4.8-9.1)	7.3 (5.6-9.8)	8.3 (5.5-11.6)	8.5 (6.2-11.9)	8.4 (6.0-11.6)	7.8 (5.5-10.8)
Fetuin (µg/ml) †	257 (224-299)	268 (222-302)	265 (224-312)	271 (230-316)	270 (233-312)	265 (227-308)
hsCRP (mg/dL) †	0.07 (0.02-0.27)	0.10 (0.04-0.25)	0.06 (0.02-0.18)	0.07 (0.02-0.15)	0.07 (0.02-0.16)	0.07 (0.02-0.20)
GGT (units/L) †	25 (14-45)	19 (12-35)	16 (10-28)	14 (10-27)	15 (10-27)	17 (12-32)

* Ruminant trans fatty acids are the sum of the relative concentrations of the fatty acids 16:1n-7*t*, 18:1n-7*t*, *c*9*t*11-CLA, *t*10*c*12-CLA
† Values are presented as median (interquartile range)
HbA_{1c}, hemoglobin A_{1c}; hsCRP, high-sensitivity C-reactive protein; GGT, γ-glutamyl transferase

Supplementary Table 5. Pearson correlation coefficients between specific plasma phospholipid trans fatty acids adjusted for age and sex, EPIC-Potsdam subcohort (n=1159)

		Ruminant TFA				Industrial TFA			Cis FA or SFA												
		16:1n-7t	18:1n-7t	c9t11-CLA	t10c12-CLA	18:1n-6t	18:1n-9t	18:2n-6,9t	15:0	16:0	16:1n-7c	17:0	18:0	18:1n-9c	18:1n-7c	18:2n-6c	20:3n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
	16:1n-7*t*	1																			
	18:1n-7*t*	0.70	1																		
	*c*9*t*11-CLA	0.34	0.47	1																	
	*t*10*c*12-CLA	0.49	0.39	0.12	1																
	18:1n-6*t*	0.61	0.66	0.16	0.54	1															
	18:1n-9*t*	0.39	0.50	0.05	0.34	0.75	1														
	18:2n-6,9*t*	0.46	0.29	-0.04	0.53	0.41	0.32	1													
	15:0	0.46	0.49	0.50	0.16	0.17	-0.12	0.06	1												
	16:0	0.00	-0.23	0.28	-0.11	-0.22	-0.20	-0.07	0.09	1											
	16:1n-7*c*	-0.27	-0.32	0.36	-0.18	-0.16	-0.03	-0.21	-0.17	0.40	1										
	17:0	0.52	0.62	0.28	0.44	0.40	0.22	0.41	0.51	-0.22	-0.42	1									
	18:0	-0.05	0.11	-0.22	0.12	0.10	0.10	0.07	-0.13	-0.79	-0.29	0.18	1								
	18:1n-9c	-0.20	-0.18	0.41	-0.10	-0.13	0.03	-0.20	-0.09	0.23	0.70	-0.27	-0.21	1							
	18:1n-7*c*	-0.06	-0.16	0.02	-0.03	-0.07	0.06	0.10	-0.26	0.14	0.26	-0.07	-0.28	0.27	1						
	18:2n-6*c*	0.23	0.33	-0.24	0.10	0.18	0.15	0.17	0.11	-0.32	-0.47	0.18	0.04	-0.37	-0.21	1					
	20:3n-6	-0.16	-0.21	0.12	-0.10	-0.10	-0.11	-0.23	0.03	0.16	0.26	-0.17	0.02	0.07	-0.14	-0.31	1				
	20:4n-6	-0.14	-0.22	-0.24	-0.09	-0.13	-0.09	-0.06	-0.17	-0.01	-0.08	-0.08	0.00	-0.22	0.15	-0.51	0.03	1			
	20:5n-3	-0.05	0.03	0.16	0.00	0.04	-0.05	-0.04	0.06	0.02	0.02	0.07	0.06	-0.06	-0.09	-0.44	-0.12	0.05	1		
	22:5n-3	-0.04	0.06	0.17	0.08	0.06	0.00	-0.03	0.14	-0.28	0.04	0.19	0.31	0.12	-0.01	-0.37	-0.04	0.11	0.36	1	
	22:6n-3	0.10	0.02	0.04	0.07	0.10	-0.09	0.08	0.08	0.02	-0.14	0.15	0.04	-0.27	0.04	-0.39	-0.15	0.07	0.56	0.29	1

Supplementary Table 6. Factor loadings of the seven trans fatty acids on the retained principal component

TFA	Principal Component 1 (PC1)*
16:1n-7*t*	0.83
18:1n-7*t*	0.83
*c*9*t*11-CLA	0.38
*t*10*c*12-CLA	0.69
18:1n-6*t*	0.88
18:1n-9*t*	0.73
18:2n-6,9*t*	0.60

*53% explained total variance

Supplementary Table 7. Prospective associations of TFA and T2D risk, with and without adjusting for alcohol intake and according to quartiles of alcohol intake

Fatty acid	Hazard Ratio per 1-SD (95%CI)* by Quartiles of Alcohol Intake				p-interaction ‡
	First: 1.0 (0.5-1.9) † (n=501)	Second: 5.2 (4.0-6.7) † (n=472)	Third: 12.8 (10.0-16.1) † (n=468)	Fourth: 33.9 (24.5-46.4) † (n=486)	
18:1n-7*t*	0.44 (0.27-0.72)	0.72 (0.47-1.10)	0.62 (0.39-0.98)	0.67 (0.45-0.99)	0.05
***c*9*t*11-CLA**	1.70 (1.25-2.30)	1.47 (1.09-1.99)	1.20 (0.88-1.62)	1.42 (1.08-1.87)	0.74
***t*10*c*12-CLA**	0.76 (0.55-1.04)	0.75 (0.57-0.98)	1.12 (0.80-1.57)	0.78 (0.59-1.02)	0.35

* Hazard Ratios (HR) and 95% confidence intervals (95% CI) derived from a multivariable Cox regression model adjusted for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake and all the other trans fatty acids (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA)

† Values are median (interquartile range) of grams of alcohol intake per day

‡ Derived from tests for statistical interactions of trans fatty acids concentrations with quartiles of alcohol intake by including cross-product terms in the fully-adjusted models

Supplementary Table 8. Prospective associations of plasma trans fatty acids with risk of type 2 diabetes in men (total n=904, cases n=462) and women (total n=1023, cases n=334) from EPIC-Potsdam

Fatty acid	Men	Women	p- interaction *
16:1n-7*t*			
Median concentration (IQR) (%) †	0.04 (0.03-0.04)	0.04 (0.03-0.05)	
Hazard Ratio per 1 SD (95% CI) ‡	1.05 (0.80-1.38)	1.32 (1.00-1.72)	0.73
18:1n-7*t*			
Median concentration (IQR) (%) †	0.17 (0.14-0.22)	0.20 (0.16-0.25)	
Hazard Ratio per 1 SD (95% CI) ‡	0.74 (0.56-0.99)	0.55 (0.40-0.75)	0.82
***c*9*t*11-CLA**			
Median concentration (IQR) (%) †	0.25 (0.21-0.29)	0.27 (0.23-0.32)	
Hazard Ratio per 1 SD (95% CI) ‡	1.39 (1.13-1.72)	1.45 (1.18-1.79)	0.80
***t*10*c*12-CLA**			
Median concentration (IQR) (%) †	0.01 (0.01-0.01)	0.01 (0.01-0.01)	
Hazard Ratio per 1 SD (95% CI) ‡	1.03 (0.85-1.25)	0.59 (0.46-0.74)	0.06
18:1n-6*t*			
Median concentration (IQR) (%) †	0.09 (0.07-0.11)	0.10 (0.08-0.12)	
Hazard Ratio per 1 SD (95% CI) ‡	0.91 (0.65-1.25)	1.23 (0.87-1.74)	0.59
18:1n-9*t*			
Median concentration (IQR) (%) †	0.16 (0.13-0.19)	0.16 (0.13-0.21)	
Hazard Ratio per 1 SD (95% CI) ‡	1.08 (0.82-1.43)	1.15 (0.87-1.52)	0.15
18:2n-6,9*t*			
Median concentration (IQR) (%) †	0.02 (0.02-0.03)	0.02 (0.02-0.03)	
Hazard Ratio per 1 SD (95% CI) ‡	0.82 (0.67-1.00)	1.13 (0.89-1.43)	0.13

* Derived from tests for statistical interactions of trans fatty acids concentrations with sex by including cross-product terms in the continuous fully-adjusted models

† Plasma TFA concentrations based on the subcohort sample (men n=454, women n=705)

‡ The standard deviation (SD) was derived from full cohort. Hazard ratio (HR) and 95% confidence interval (CI) per-1SD increase of each trans fatty acid, after accounting for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake and all other TFA subtypes (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA)

Supplementary Table 9. Prospective associations of plasma trans fatty acids with risk of type 2 diabetes according to BMI (above and below the median) in EPIC-Potsdam

Fatty acid	Hazard Ratio per 1-SD (95%CI) * BMI ≤27 (n = 955)	BMI > 27 (n = 972)	p- interaction BMI †
Ruminant TFA			
16:1n-7*t*	1.35 (0.98-1.86)	1.00 (0.79-1.27)	0.42
18:1n-7*t*	0.57 (0.41-0.79)	0.76 (0.58-1.01)	0.47
***c*9*t*11-CLA**	1.55 (1.21-2.00)	1.31 (1.07-1.59)	0.38
***t*10*c*12-CLA**	0.76 (0.59-0.98)	0.81 (0.68-0.98)	0.90
Industrial TFA			
18:1n-6*t*	1.34 (0.90-2.01)	1.04 (0.76-1.43)	0.72
18:1n-9*t*	0.80 (0.53-1.18)	1.06 (0.84-1.35)	0.56
18:2n-6,9*t*	1.05 (0.76-1.45)	0.99 (0.83-1.19)	0.96

* Hazard ratio (HR) and 95% confidence interval (CI) per-1SD increase of each trans fatty acid in the EPIC-Potsdam subcohort (total n= 1927, cases n=796), after accounting for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake and all other TFA subtypes (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA)

† Derived from tests for statistical interactions of trans fatty acids concentrations with BMI≤27 and BMI>27 by including cross-product terms in the continuous fully-adjusted models

Supplementary Table 10. Prospective associations of plasma trans fatty acids with risk of type 2 diabetes according to polymorphisms in the *PPARG* gene, in EPIC-Potsdam

	Hazard Ratio per 1-SD (95%CI) *		
Fatty acid	***PPARG* Pro12Pro (Wild type) (n = 1362)**	***PPARG* Pro12Ala (Ala carriers) (n = 485)**	**p-interaction †**
***c*9*t*11-CLA**	1.37 (1.13-1.65)	1.48 (1.12-1.96)	0.92
***t*10*c*12-CLA**	0.78 (0.66-0.92)	0.82 (0.59-1.14)	0.50

* Hazard ratio (HR) and 95% confidence interval (CI) per-1SD increase of each trans fatty acid in the EPIC-Potsdam subcohort (total n= 1927, cases n=796), after accounting for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake and other TFA subtypes (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA)

† Derived from tests for statistical interactions of conjugated linoleic acid concentrations with *PPARG* polymorphisms (wild type vs ala carriers) by including cross-product dichotomous terms in the continuous fully-adjusted models

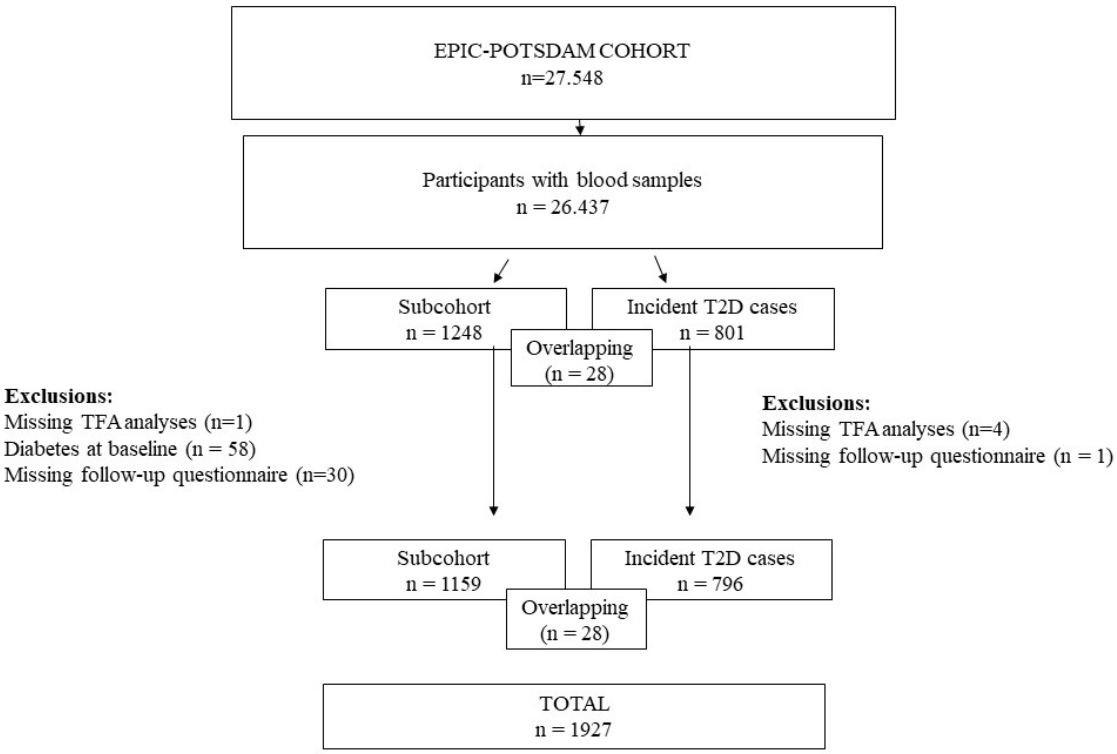
Supplementary Table 11. Mediation analysis of associations of trans fatty acids with type 2 diabetes by metabolic markers and liver fat in EPIC-Potsdam (total n= 1927, cases n=796)

	18:1n-7t			*c*9*t*11-CLA			*t*10*c*12-CLA		
	Hazard ratio per 1 SD (95%-CI)		% change (95%-CI) *	Hazard ratio per 1 SD (95%-CI)		% change (95%-CI) *	Hazard ratio per 1 SD (95%-CI)		% change (95%-CI) *
Reference Model †	**0.61**	**(0.47-0.79)**	-	**1.53**	**(1.29; 1.87)**	-	**0.78**	**(0.60-0.98)**	-
+ Triglycerides	0.60	(0.45-0.79)	5 (-16; 24)	1.40	(1.15-1.73)	-20 (-47; -5)	0.85	(0.63; 1.10)	-35 (-189; -6)
+ Non-HDL cholesterol	0.61	(0.47-0.81)	-0.8 (-9; 4)	-	-	-	0.79	(0.6; 1.01)	-7 (-77; 19)
+ HDL-cholesterol	-	-	-	-	-	-	0.78	(0.58; 1.00)	1 (-45; 30)
+ HbA_{1c}	0.54	(0.37-0.75)	27 (-24; 116)	1.42	(1.12-1.88)	-16 (-65; 28)	-		-
+ GGT	0.57	(0.43-0.75)	16 (-13; 49)	-	-	-	0.81	(0.61; 1.04)	-12 (-107; 21)
+ Adiponectin	0.59	(0.45-0.79)	6 (-9; 24)	-	-	-	-		-
+ hsCRP	0.53	(0.39-0.74)	29 (1; 68)	-	-	-	0.78	(0.59; 0.98)	0 (-38; 53)
+ Fetuin	-	-	-	-	-	-	-		-
+ FLI ‡	0.60	(0.45; 0.78)	6 (-23; 39)	1.37	(1.12-1.71)	-26 (-58; -6)	0.86	(0.64; 1.10)	-38 (-205; -6)

* The change (%) reflects the change of the estimate after additional adjustment for each biomarkers and fatty liver index, relative to the estimate from the reference model. Its stability as well as the corresponding hazard ratio were estimated as median and dispersion from a bootstrapping procedure (500 bootstrap replicates)

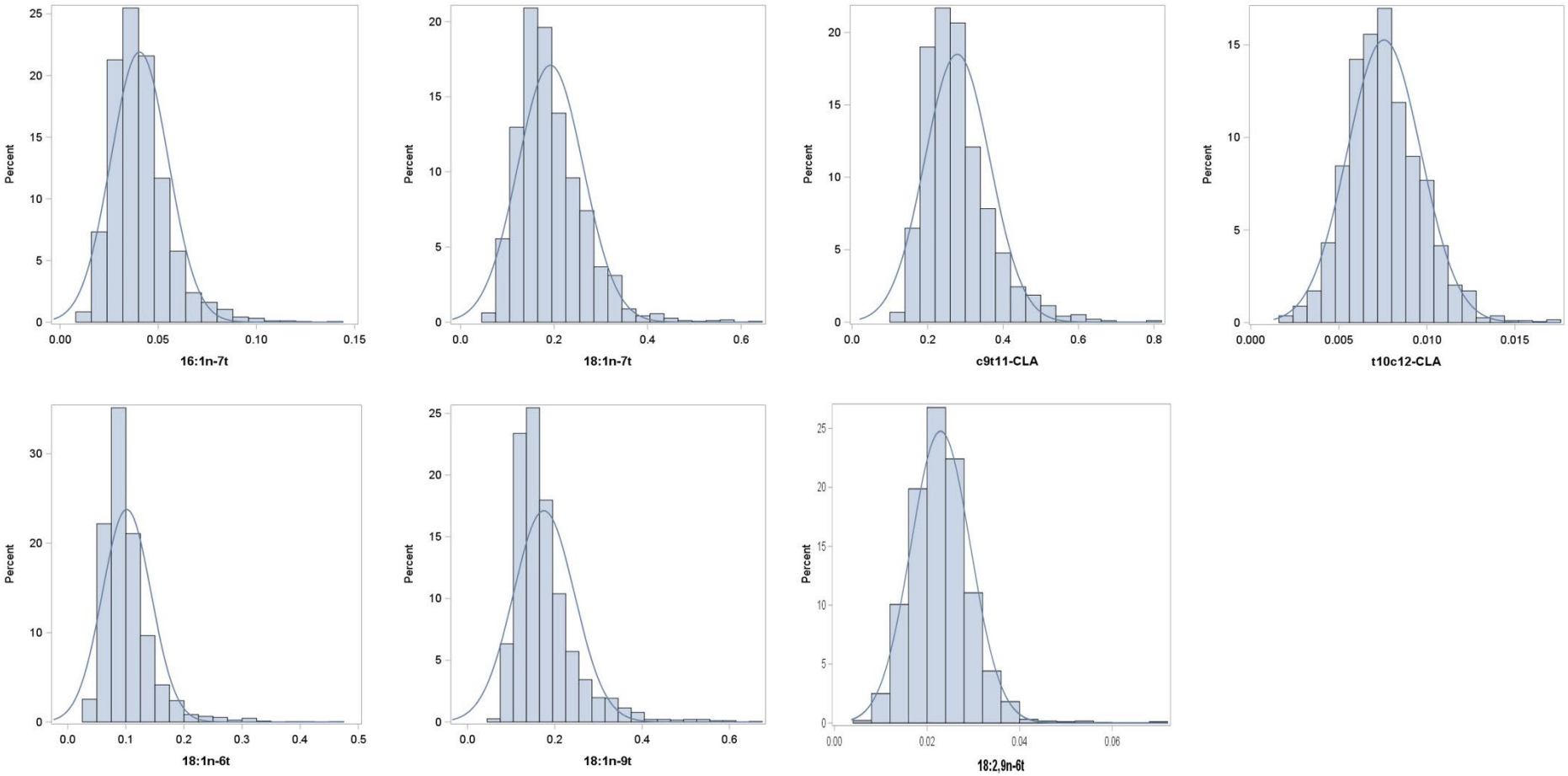
† Hazard ratio (HR) and 95% confidence interval (CI) per-1SD increase of each trans fatty acid, estimated as median and dispersion from a bootstrapping procedure (500 bootstrap replicates). Models adjusted for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake, all other TFA subtypes (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA)

‡ Fatty liver index (FLI) was calculated with the following formula: $FLI = (e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(ggt) + 0.053*\text{waist circumference} - 15.745}) * 100$

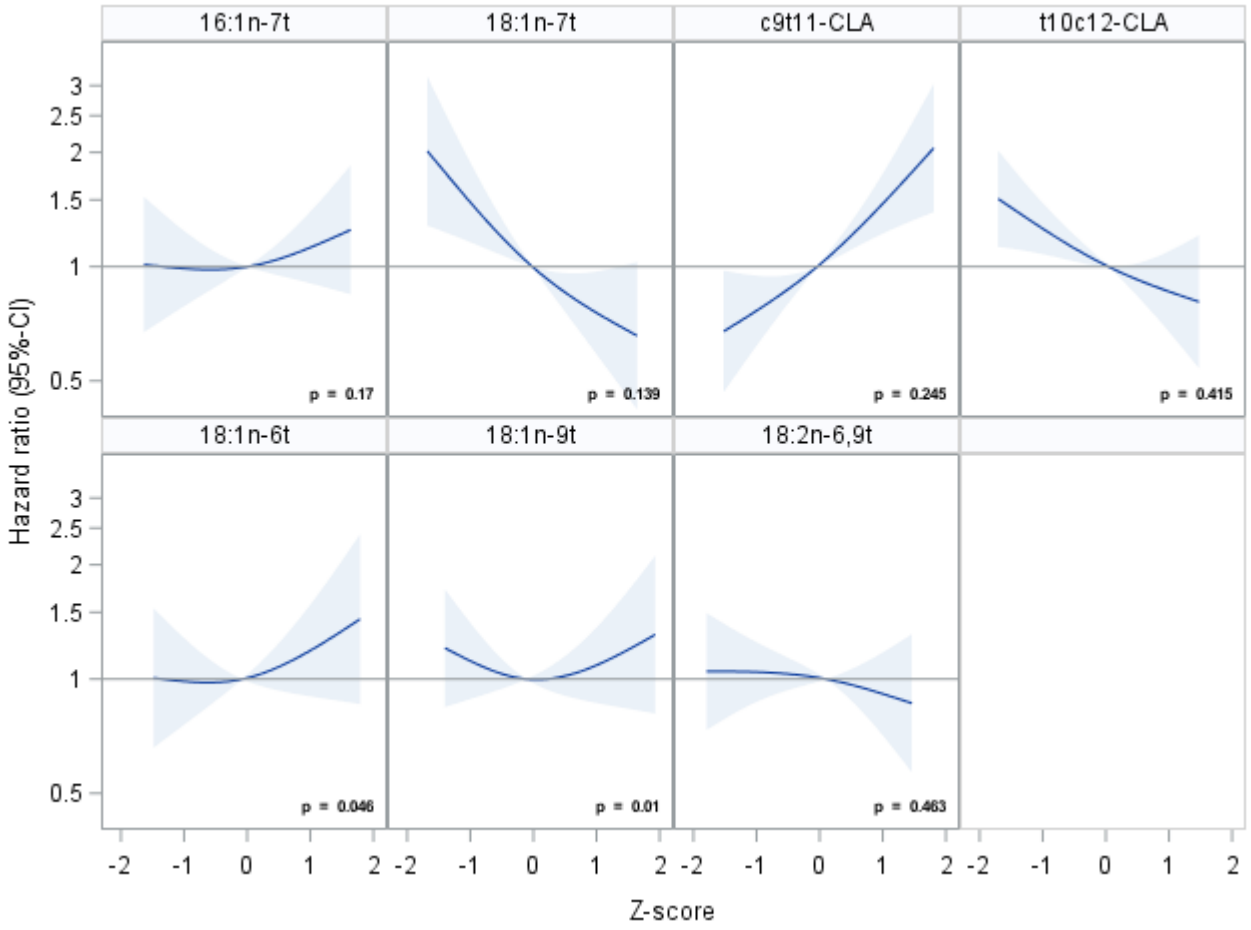


Supplementary Figure 1. Flow chart describing study sample

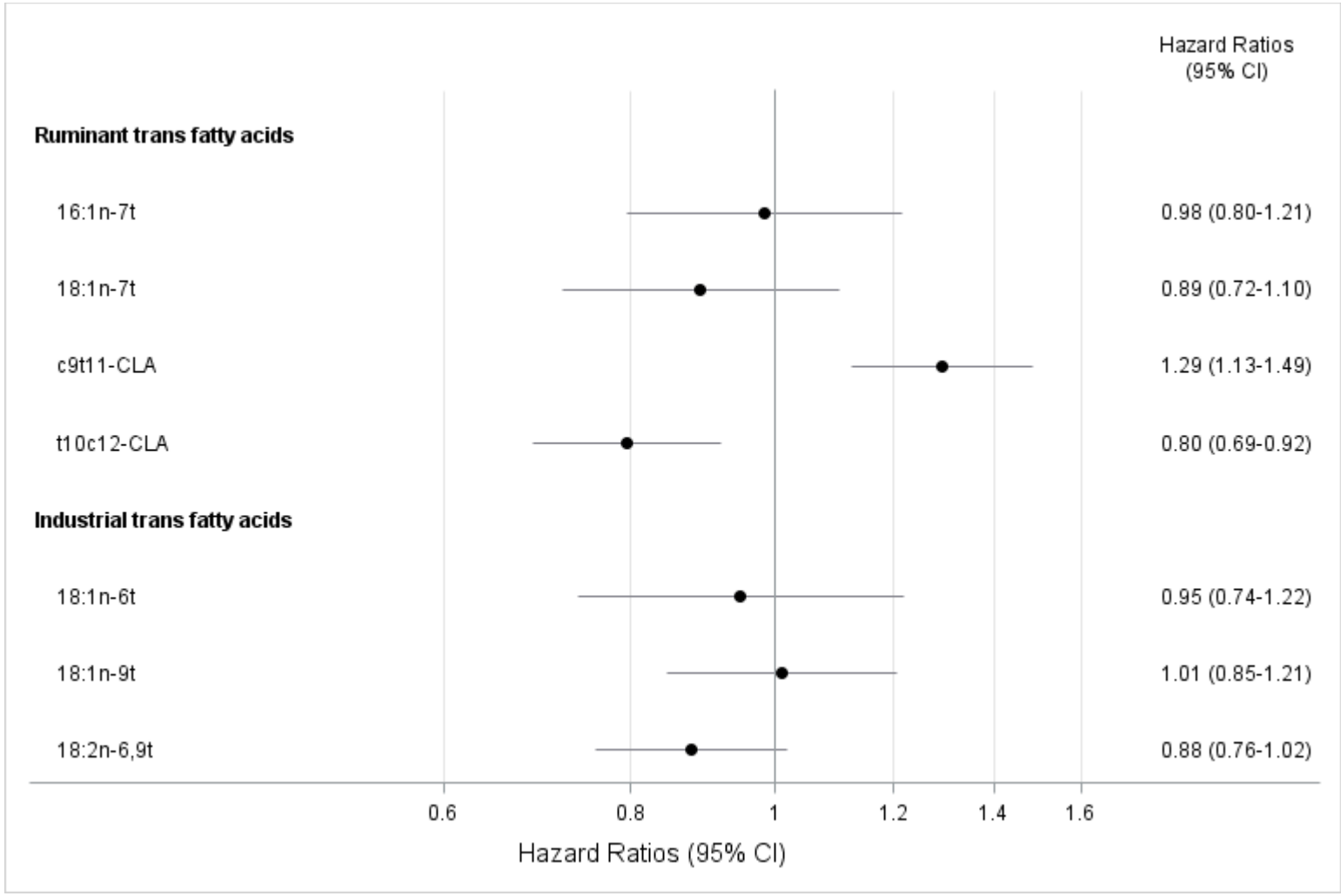
T2D: type 2 diabetes



Supplementary Figure 2. Trans fatty acids histograms

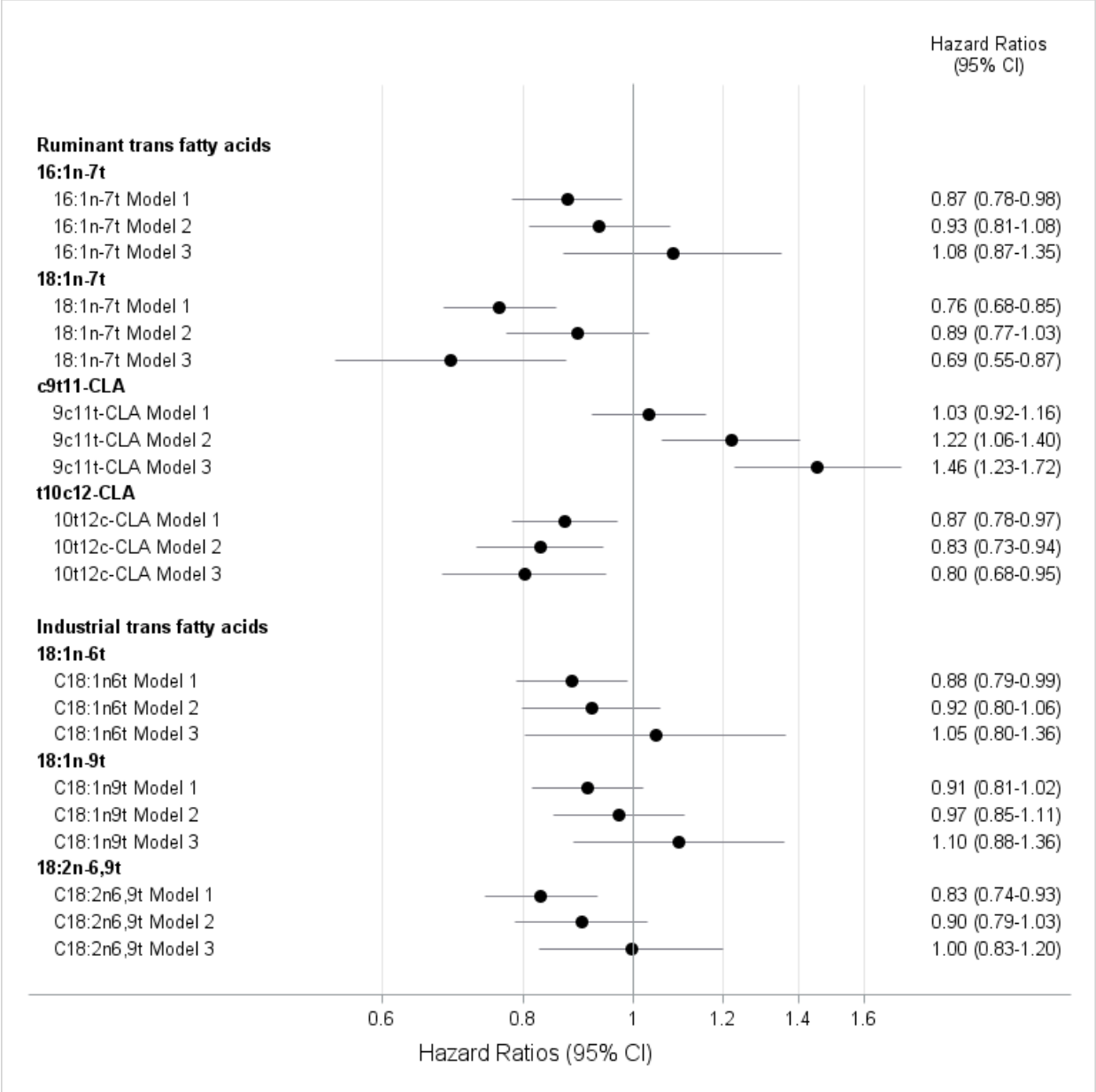


Supplementary Figure 3. Prospective associations between trans fatty acids and incidence of T2D modelled using a restricted cubic spline function with 3 knots placed at the 10th, 50th and 90th percentiles in the EPIC Potsdam (total n= 1927, cases n=796). Shaded areas are 95% confidence intervals. Numbers in the bottom right of each panel are p-values based on the likelihood ratio test



Supplementary Figure 4. Prospective associations of plasma trans fatty acids with risk of type 2 diabetes in EPIC-Potsdam (total n= 1927, cases n=796), with a model further adjusted for PC score. Hazard ratio (HR) and 95% confidence interval (CI) per-1SD increase of each trans fatty acid

Model adjusted for age (stratum variable), sex, waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake and PC score



Supplementary Figure 5. Sensitivity Analysis, excluding participants with HbA1c>6.5 (Total n=1651, cases n=564)

Model 1: adjusted for age (stratum variable) and sex
Model 2: further adjusted for waist circumference, BMI, smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, skilled worker, technical school, or university degree), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), red meat intake (energy-adjusted), coffee intake (energy-adjusted), fiber intake (energy-adjusted), fasting status, total energy intake
Model 3: further adjusted for all other TFA subtypes (16:1n-7*t*, 18:1n-6*t*, 18:1n-7*t*, 18:1n-9*t*, 18:2n-6,9*t*, *c*9*t*11-CLA, *t*10*c*12-CLA

Fatty Acid Measurements

In detail, 50 µL of plasma was transferred into 16 x 100 mm teflon coated screw capped vials. After addition of 1 mL deionized water total lipids were extracted with 3 mL *tert*-butyl methyl ether (MTBE)/methanol solution (2/1, v/v) (MTBE containing 0.01% butyl hydroxytoluene (BHT)). The mixture was vortexed for 15 min. After centrifugation, the upper layer containing the lipid fractions was transferred into another vial to evaporate to dryness under a stream of N_2 at 40°C.
For SPE the dried lipids were redissolved into 500 µL chloroform. The mixture was applied to conditioned SPE columns (1 mL) containing 100 mg of aminopropyl-modified silica. The columns are placed on a vacuum elution apparatus equipped with vents and manometer. Conditioning of columns was performed by washing with 2 x 1 mL *n*-hexane and 1 x 1 mL chloroform/*i*-propanol (2/1, v/v). The vacuum (~10 kPa) was released in time to prevent columns from becoming completely dry. Firstly, neutral lipids and free FA were eluted with 4 x 1 mL chloroform/methanol/acetic acid (100/2/2, v/v). After changing vials the PL were eluted with 2 x 1 mL methanol. Solvents were evaporated under a stream of N_2 at 40°C.
For hydrolysis and methylation of FA the dried PL were redissolved into 230 µL toluene and transferred into GC-vials. 20 µL of trimethyl sulfonium hydroxide solution (TMSH, 0.25 mol/L in methanol, were added to form FA methyl esters (FAME) from FA of PL (except of sphingolipids). Vials were vortexed (30 min, 750 min^{-1}, 40°C). Samples containing the FAMEs were analyzed by GC.
Analysis of FAMEs was performed using an Agilent GC system 7890A equipped with Agilent 7000 GC/MS Triple Quad (Agilent Technologies, Waldbronn, Germany) and a FID. 1 µL of sample was injected using a CIS 4C PTV-type GC-inlet (Gerstel) in splitless mode at 30°C, 30-260°C, ramp 12°C/s, held 2 min, 260-320°C, ramp 12°C/s.
The FAMEs were separated on a GC capillary column (HP-88, 100 m x 0.25 mm I.D., 0.2 µm film thickness, Agilent) using a constant He carrier gas flow of 1.3 mL/min. The eluting gas flow was splitted for FID and MS detection (1:1) using a 2-way splitter.
FID conditions were as follows: heater at 250°C, H_2 flow at 40 mL/min, air flow at 400 mL/min, and makeup flow at 30 mL/min. GC-MS/MS conditions were as follows: EI ion source at 230°C, He quench gas at 2.25 mL/min, and N_2 collision gas at 1.5 mL/min.
The following FAME standards of odd chain fatty acids (OCFA) and trans fatty acids (TFA) were used for calibration: 15:0 and 17:0 (Sigma-Aldrich, Taufkirchen, Germany), 16:1n-7*t*, 18:1n-9*t*, 18:1n-7*t*, 18:1n-6*t*, 18:2n-6*t*, *c*9*t*11-CLA, and *t*10*c*12-CLA (NuChek-Prep, Elysian, USA). The MRM counts were related to corresponding FID counts using calibration standards resulting in individual linear regression curves and conversion factors. The MRM counts of OCFA and TFA of each sample were transformed into FID analogous counts using corresponding conversion factors of each FA. The results were expressed in area percentage of each FA relative to total area of all 9 FA. Simultaneously, a basic FA spectrum of 13 FA can be evaluated by FID detection and expressed in area percentage of each FA relative to total area of all 13 FA. The spectrum consists of the following FA: 15:0, 16:0, 16:1n-7*c*, 17:0, 18:0, 18:1n-9*c*, 18:1n-7*c*, 18:2n-6*c*, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3.
We did not use lower limits of detection (LOD) because we worked with area sum%. Instead, we used a signal-to-noise ratio better than 3 (S/N=3) for all analytes. This means that all peaks that were at least three times larger than the noise were evaluated. This was true for all fatty acids indicated.

The reproducibility of the analytes was determined by a comparison of intra- and interassay coefficients of variation (CV%, n=10) of the OCFA and TFA detected by MRM (two extractions injected twice) calculated according to Rodbard (1974) were as follows (see table):

Fatty acid	Systematic name	Mean ± SD (area sum%)	Intra-assay CV (%)	Inter-assay CV (%)
15:0	pentadecanoic acid	17.10 ± 3.18	1.57	1.97
16:1n-7*t*	9E-hexadecenoic acid	3.04 ± 0.44	1.67	3.65
17:0	heptadecanoic acid	26.90 ± 2.87	0.69	0.90
18:1n-9*t*	trans-9-octadecenoic acid	16.89 ± 3.99	2.00	2.71
18:1n-7*t*	trans-11-octadecenoic acid	15.94 ± 1.93	1.08	1.11
18:1n-6*t*	trans-12-octadecenoic acid	6.45 ± 1.86	1.21	1.98
18:2n-6,9*t*	9E,12E-octadecadienoic acid	0.55 ± 0.12	4.17	2.50
*c*9*t*11-CLA	9Z,11E-octadecadienoic acid	12.90 ± 5.91	1.59	1.96
*t*10*c*12-CLA	10E,12Z-octadecadienoic acid	0.23 ± 0.06	9.51	4.66

Reference:

Rodbard D (1974) Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clinical chemistry 20 (10):1255-1270

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Original article

Association of the odd-chain fatty acid content in lipid groups with type 2 diabetes risk: A targeted analysis of lipidomics data in the EPIC-Potsdam cohort

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SUMMARY

Background: Plasma odd-chain saturated fatty acids (OCFA) are inversely associated with type 2 diabetes (T2D) risk and may serve as biomarkers for dairy fat intake. Their distribution across different lipid classes and consequences for diabetes risk remain unknown.
Aim: To investigate the prospective associations of OCFA-containing lipid species with T2D risk and their dietary determinants.
Methods: Within the European Prospective Investigation into Cancer and Nutrition–Potsdam study (n = 27,548), we applied a nested case-cohort design (subcohort: n = 1,248; T2D cases: n = 820; median follow-up 6.5 years). OCFA-containing lipids included triacylglycerols, free fatty acids (FFA), cholesteryl esters (CE), phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines, lysophosphatidylethanolamines, monoacylglycerols, and diacylglycerols. We estimated lipid class-specific associations between OCFA-containing lipids and T2D in sex-stratified Cox proportional-hazards models. We investigated correlations between lipids and dietary intakes derived from food-frequency questionnaires.
Results: We observed heterogeneous integration of OCFA in different lipid classes: triacylglycerols, FFA, CE, and phosphatidylcholines contributed most to the total OCFA-plasma abundance. The relative concentration of OCFA was particularly high in monoacylglycerols, and the contribution of C15:0 versus C17:0 to the total OCFA-abundance differed across lipid classes. In women, several OCFA-containing phospholipids were inversely associated with T2D risk [phosphatidylcholine(C15:0), HR Q5 vs Q1: 0.56, 95% CI 0.32–0.97; phosphatidylcholine(C17:0), HR per SD: 0.59, 95% CI 0.48–0.71; lysophosphatidylcholine(C17:0), HR Q5 vs Q1: 0.42, 95% CI 0.23–0.76]. In men, we did not detect statistically significant inverse associations in phospholipids, and lysophosphatidylcholine(C15:0) was associated with higher T2D risk (HR Q5 vs. Q1: 1.96, 95% CI 1.06–3.63). Besides, CE(C17:0), monoacylglycerols(C15:0), and diacylglycerols(C15:0) were inversely associated with T2D risk; FFA(C17:0) was positively associated with T2D risk in women. Consumption of fat-rich dairy and fiber-rich foods were positively and red meat inversely correlated to OCFA-containing lipid plasma levels.
Conclusions: OCFA-containing lipids are linked to T2D risk in a lipid class and sex-specific manner, and they are correlated with several foods.

1. Introduction

Dietary saturated fatty acids (SFA) have been traditionally related to higher type 2 diabetes (T2D) risk and complications [1,2]. However, recent research shows that different lengths of SFA have

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Abbreviations

C15:0	pentadecanoic acid
C17:0	heptadecanoic acid
CE	cholesteryl esters
DAG	diacylglycerols
FA	fatty acids
FFA	free fatty acids
FFQ	food frequency questionnaire
L-C15:0	lipid species containing C15:0
L-C17:0	lipid species containing C17:0
LPC	lysophosphatidylcholines
LPE	lysophosphatidylethanolamines
MAG	monoacylglycerols
OCFA	odd-chain saturated fatty acids
PC	phosphatidylcholines
PE	phosphatidylethanolamines
PL	phospholipids
PL-C15:0	phospholipid species containing C15:0
PL-C17:0	phospholipid species containing C17:0
PL-OCFA	phospholipid species containing odd-chain fatty acids
SFA	saturated fatty acids
T2D	type 2 diabetes
TAG	triacylglycerols

opposing effects on cardiometabolic risk. In several prospective cohort studies, including EPIC-InterAct [3] and EPIC-Postdam [4], the quantitatively most abundant even-chain SFA were associated with higher T2D risk. In contrast, higher levels of odd-chain SFA (OCFA) – pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) – were inversely associated [3–5]. Interestingly, these inverse associations were stronger for women compared to men [5].

In observational studies, OCFA are proposed biomarkers of dairy fat intake [5], while total dairy intake is also inversely associated with T2D risk [6]. Still, it remains unclear whether OCFA are involved in dairy's protective effects, considering OCFA constitute a very small fraction of dairy fat, plus the correlations between OCFA and dairy fat intake in previous studies are relatively low [7]. Besides, the association between total dairy and T2D seems to be driven by dairy subgroups, such as low-fat dairy and yogurt [6]. In addition, fiber-rich foods may also be linked to circulating OCFA-concentrations through intestinal production of short-chain fatty acids and their hepatic elongation [8]. Recent studies point out that OCFA may influence metabolism, inflammation, mitochondrial function, and antifibrotic activities [9], which might explain their inverse association with diabetes risk. Clarifying the dietary determinants that affect OCFA concentrations could provide insight into understanding their mechanistic role in T2D.

Novel lipidomic biomarkers may help to elucidate the links between dairy and fiber-rich food consumption, circulating OCFA, and T2D risk in more detail. Previous studies on OCFA biomarkers were restricted to the level of total serum lipids [10–12], plasma phospholipids [3,13–16], erythrocyte membranes phospholipids [4,16–18], and, less frequently, total plasma [17] and cholesteryl esters (CE) [5]. Lipid class-specific analysis allows the investigation of the OCFA abundance in diverse lipid subclasses, e.g., different phospholipid classes, instead of the sum of a blood fraction or subclasses. The question of whether the lipid class containing the OCFA is relevant has to be addressed, considering that some lipid classes have been associated in a different way with diabetes risk. For instance, among phospholipids, several lysophosphatidylcholines (LPC) showed an inverse relationship with T2D [19–22], while predominantly positive associations were found for phosphatidylcholines (PC) [19,23] and phosphatidylethanolamines (PE) [22]. Currently, the distribution of the OCFA across different lipid classes is unknown, and evidence on the associations of major OCFA-containing lipid metabolites with incident T2D is lacking.

We aimed to characterize the diverse lipid class-specific distribution of C15:0 and C17:0, using the novel lipidomic profiling involving 15 lipid classes. We investigated the sex-specific association of the lipids containing OCFA with T2D incidence and their dietary determinants based on comprehensive dietary assessment. Besides, to assess the comparability with previous studies, we also measured and analyzed total C15:0 and C17:0 in plasma phospholipids by gas chromatography.

2. Material & methods

2.1. Study population and baseline measurements

The European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort study included participants mainly within the age range of 35–65 years that were recruited in Potsdam and surrounding communities between 1994 and 1998 (n = 27,548) [24,25]. At baseline, the participants were asked to complete self-administered questionnaires and a guided interview that evaluated information about prevalent diseases, lifestyle (smoking, occupation, physical activity), and education. Also, we measured anthropometry following standard protocols [25]. The procedures and measures of quality control were previously described in detail [26,27]. Participants were contacted every 2–3 years to identify incident cases of chronic diseases such as diabetes until the censoring date in August 2005. The response rates ranged between 90% and 96% per follow-up round. The median follow-up time was 6.5 years (interquartile range 6.0–8.7).

For molecular phenotyping, we designed a case-cohort among the participants who provided blood samples (n = 26,437). According to this study design [28], a random subsample was selected (subcohort, n = 1,248) to be representative of the cohort population at baseline, and we included all cases with incident T2D (n = 820) recorded until 31st of August 2005 (Supplemental Fig. 1). We excluded participants due to insufficient plasma samples (n = 75), prevalent or non-verifiable T2D cases (n = 56), and missing follow-up information (n = 25). The analytical sample thus included 1886 participants: 1137 from the subcohort sample and 775 incident cases with T2D, with an overlap of 26 subjects who belonged both to the random subcohort and the case group. All participants gave informed consent to use their data for research, and the Ethics Committee of the State Brandenburg, Germany, approved the study.

2.2. Type 2 diabetes diagnosis

Participants with potential incident diabetes were detected based on self-report of the diagnosis, antidiabetic medication use, or diabetes dietary treatment. Additionally, T2D information from death certificates or sources as the tumor centers, physicians, or clinics that provided assessments from other diagnoses was obtained. When a participant was identified as a potential case, we inquired the treating physician to verify the diagnosis of type 2

diabetes (International Classification of Diseases, 10th revision code: E11; http://apps.who.int/classifications/icd10/browse/2016/en). Only cases verified by a physician and having a date of diagnosis posterior to the baseline examination date were included as incident cases.

2.3. Dietary assessment

The habitual diet was assessed by a self-administered, validated semiquantitative food frequency questionnaire (FFQ) [29–31]. The FFQ inquired about the average consumption frequency and portion size of 148 foods consumed during the previous 12 months. The participants had to select frequencies from never to five times per day or more, portions relative to standard portion sizes, and the usual fat content of dairy foods consumed. To assist with the portion size estimation, pictures illustrated the standard portion sizes of butter, cheese, yogurt, kefir, curd cheese, and heavy cream, whereas milk portion size was in cups. Additional questions asked about fat products used for food preparation, including the use of butter and cream. Milk and milk products showed moderate correlations when compared to a FFQ administered six months later ($r = 0.55$) and when compared with twelve 24-h recalls ($r = 0.58$) [30]. Other food groups showed moderate to high correlations with repeated administration of the FFQ at a 6-month interval: cheese ($r = 0.61$), bread ($r = 0.49$), cereals ($r = 0.73$), fruits ($r = 0.61$), vegetables ($r = 54$), meat ($r = 0.77$), processed meat ($r = 0.73$).

We analyzed dairy foods in two main groups based on their fat content and FFQ questions. “Low-fat dairy” included low-fat milk and milk beverages (1.5% fat or less), low-fat yogurt (1.5% fat or less), kefir (1.5% fat or less), curd cheese (5% fat or less), and low-fat cheese (reduced fat or lean: cream cheese, gouda, emmental, tilsiter, camembert, brie, gorgonzola). “Full-fat dairy” included full-fat milk and milk beverages (>1.5% fat), full-fat yogurt (>1.5% fat), curd cheese (>5% fat), heavy cream, and full-fat cheese (full-fat cream cheese, gouda, emmental, tilsiter, camembert, brie, gorgonzola, processed cheese). In addition to these two main groups, total dairy fat intake and specific dairy subgroups were considered separately: low-fat cheese, full-fat cheese, milk 3.5% fat, milk ≤1.5% fat, low-fat yogurt (1.5% fat), full-fat yogurt (≥3.5% fat), and butter. Apart from dairy foods, we considered other food groups based on previously reported correlations with OCFA concentrations [3]. Those groups involved: whole grains (whole grain bread, grain flakes, grains, muesli) and fruits and vegetables, due to their fiber content, which can result in propionate, a possible precursor of endogenous OCFA production [8]; bread, pasta, and rice to compare with the fiber-rich foods; red meat and processed meat because they contain OCFA [32].

2.4. Lipidomic profiling

Venous blood samples were collected at baseline by qualified medical personnel following standardized procedures. Samples were fractioned, and plasma samples were stored in tanks of liquid nitrogen (approximately −196 °C) or deep freezers (−80 °C) [25]. Lipidomics analysis was performed by Metabolon (USA) using the Metabolon® Complex Lipid Panel. This platform identified 1044 molecular species concentrations and complete fatty acid composition across 15 lipid classes. In short, lipids were extracted from plasma samples in the presence of deuterated internal standards using an automated BUME extraction [33]. Extracts were infused, and MS analyzed with a Shimadzu LC with nano PEEK tubing and a Sciex SelexION® −5500 QTRAP mass spectrometer. Upon ionization, the lipids passed through a SelexIon differential mobility spectrometry (DMS), which separates lipids by class. After the DMS filtering, lipids enter the multiple reaction monitoring (MRM), in which both the mass of lipid and the mass of its characteristic fragments are measured [34]. Individual lipid species were quantified by taking the ratio of the signal intensity of each target compound to that of its assigned internal standard, then multiplying by the concentration of internal standard added to the sample. The coefficients of variation (CVs) of lipid class concentrations were all below 10%. The median CV of species at a 1uM concentration in serum or plasma was approximately 5% [35]. Fasting blood samples ($n = 35$) collected at two time points four months apart indicate moderate to good reliability for most OCFA-containing lipids, with the exception of individual PC and TAG species (Supplemental Table 1).

2.5. Measurements of fatty acid composition in plasma phospholipids

We measured total C15:0 and C17:0 in total plasma phospholipids to compare with concentrations of individual phospholipid species derived from the lipidomics analyses by mass spectrometry. We measured plasma phospholipids fatty acids using total lipid extraction with tert-butyl methyl ether/methanol. The mixture was vortexed using a rotatory shaker. After centrifugation, the upper layer containing the lipid fractions was transferred into another vial to evaporate to dryness. For solid-phase separation, the dried lipids were dissolved into chloroform. The mixture was applied to conditioned solid-phase separation columns containing aminopropyl-modified silica and placed on a vacuum elution apparatus. Firstly, neutral lipids and free fatty acids (FFA) were eluted with chloroform/methanol/acetic acid, and, after changing vials, the phospholipids were eluted with methanol. Solvents were evaporated under a stream of N_2 at 40 °C. The dried phospholipids were redissolved into toluene for hydrolysis and methylation of fatty acids. A trimethyl sulfonium hydroxide solution was added to form fatty acids methyl esters. Vials were vortexed at 40 °C using a thermomixer equipped with a block thermostat. Subsequently, samples were analyzed by gas chromatography with a flame ionization detector. The proportions of the fatty acids are expressed as a percentage of the total fatty acids present in the chromatogram. The spectrum consists of the following fatty acids: C15:0, C16:0, C16:1n-7c, C17:0, C18:0, C18:1n-9c, C18:1n-7c, C18:2n-6c, C20:3n-6, C20:4n-6, C20:5n-3, C22:5n-3, C22:6n-3.

2.6. Statistical analyses

In data preparation, we imputed missing information on covariates by sex-specific median imputation (height: $n = 1$) and model-based single imputation (body mass index: $n = 4$, blood pressure: $n = 116$). No dietary variables were missing. The missing concentrations of lipids species were imputed with half of the minimum detected value for that lipid, as it is very likely that missing values were below the limit of detection. The amount of missing values were 9% of lysophosphatidylethanolamine (C17:0) (lysophosphatidylethanolamines, LPE), 31% PE(C17:0), 0.05% LPC(C15:0), 0.05% LPC(C17:0), 0.3% PC(C15:0). However, LPE(C15:0) and PE(C15:0) were excluded from analyses due to the high percentage of missing values, 84% and 99%, respectively. There were no missing values of total C15:0 and C17:0 concentrations in plasma phospholipids derived from gas chromatography.

For primary analyses, we selected all lipids containing OCFA ($n = 69$) (Supplemental Table 2). For lipid classes that contain two or three fatty acids (PC, PE, diacylglycerol [DAG], triacylglycerol [TAG]), the sum within a class was used for calculations, i.e., all lipids in a class containing C15:0 were summed, and all lipids containing C17:0. OCFA-containing phospholipids were also evaluated in broader groups: PL-OCFA reflects the sum of all phospholipid

species containing OCFA: LPE(C17:0), PE(C17:0), PC(C17:0), PC(C15:0), LPC(C17:0), and LPC(C15:0); PL-C15:0 reflects the sum of all phospholipids containing C15:0 (PC(C15:0) and LPC(C15:0)) and PL-C17:0 the sum of phospholipids containing C17:0 (LPE(C17:0), PE(C17:0), PC(C17:0), and LPC(C17:0)). Subsequently, we log-transformed the lipid species concentrations to approximate a normal distribution. Values being more than five standard deviations (SD) above or below the mean were considered as outliers and excluded [FFA(C15:0) n = 3, monoacylglycerols (MAG) (C15:0) n = 10, total(C15:0) n = 9, total(C17:0) n = 1].

All descriptive analyses were based on the random subcohort, a representative sample of the full EPIC-Potsdam cohort. To describe the relationship between OCFA measured in total plasma phospholipids by gas chromatography and those from lipidomics analysis, we assessed correlations between lipid variables by Spearman's rank correlation coefficient. Similarly, we assessed pairwise correlations between all phospholipid species containing OCFA. To estimate the link between dietary exposures and circulating lipid species, we calculated Spearman's rank correlation coefficients.

The longitudinal associations with diabetes risk were evaluated in Cox proportional hazards models. The models were weighted with the Prentice method to account for the case-cohort design [36], with age as underlying timescale and including age (years) as strata-variable. We estimated multivariable-adjusted hazard ratios (HR) and 95% confidence intervals (CI) considering lipid concentrations as continuous variables standardized to standard deviation (SD) increments. Cox models were adjusted for age, waist circumference, body mass index (BMI), cycling (0, 0.1–2.4, 2.5–4.9, or ≥ 5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), occupational activity (light, moderate, or heavy), education (in or no training, vocational training, technical school, or technical college or university degree), smoking status (never, past, current < 20 cigarettes/d, or current > 20 cigarettes/d), prevalent hypertension, fasting status, total energy intake (kJ/day), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), coffee intake (energy-adjusted), fibre intake (energy-adjusted), and red meat intake (energy-adjusted). For energy adjustment, the residual method was used [37]. Additionally, we conducted tests for statistical interactions of lipids concentrations with sex by including cross-product terms in the continuous fully-adjusted models. We produced sex-stratified models to assess how associations of lipids and T2D differ by sex. To investigate the shape of the associations, we used restricted cubic splines with three knots (located at 5th, 50th, and 95th percentiles) to model the relation between lipids and T2D incidence after adjusting for the same covariables previously mentioned. The likelihood ratio test was used to compare the fit between the linear and restricted cubic spline models. When there was evidence ($p < 0.05$ in likelihood ratio test) for a non-linear association, we estimated the HR and CI for quintiles of concentration of lipid species containing OCFA, taking the lowest quintile as reference. The quintiles were calculated based on the subcohort distributions.

All analyses were performed with the statistical software SAS Enterprise Guide 7.1.

3. Results

3.1. Baseline characteristics

The distribution of baseline characteristics across quintiles of PL-OCFA concentration in the subcohort is presented in Table 1. Among the participants with higher concentrations of PL-OCFA, more were women and never smokers, the median age was higher, and, in men, the median alcohol consumption was lower. BMI, waist circumference, hypertension, and other variables were similar across the categories.

3.2. OCFA distribution in lipid classes in human plasma

We investigated how OCFA-containing lipids were distributed across the different lipid classes. From the total absolute median concentration of OCFA-containing lipids (1.77 μM, Interquartile range [IQR]: 1.44–2.29, Fig. 1A), the TAG accounted for a median proportion of 37% (IQR: 29–47%), FFA contributed 19% (IQR: 15–24%), and CE 19% (IQR: 15–22%) (Fig. 1B). The phospholipid species (PC, LPC, PE, LPE) contributed 16% (IQR: 13–20%) of the total lipids containing OCFA. The largest proportion of PL-OCFA was attributable to phosphatidylcholines (84%, IQR: 81–86%), followed by lysophosphatidylcholines (14%, IQR: 12–16%) (Fig. 1C). For comparison, the lipid classes with the highest total class concentrations were CE, followed by PC and FFA (Supplemental Table 2). Within distinct lipid classes, the proportion of lipids containing OCFA varied from 38% (IQR: 31–45%) in MAG to less than 0.1% in PE (IQR: 0.01–0.16%), but it was below 2% in most lipid classes (Fig. 2). The relative contribution of L-C15:0 versus L-C17:0 to the total of OCFA-containing lipids was heterogeneous across lipid classes. DAG and MAG with OCFA almost exclusively contained C15:0, while the OCFA content in PE and LPE was predominantly attributable to C17:0. In the other lipid classes, both L-C17:0 and L-C15:0 were present, but the proportion of lipids containing C15:0 (compared to C17:0) in PC, LPC, and FFA were lower than in CE and TG. Women had higher concentrations than men in all OCFA-containing lipids (Supplemental Table 3).

We evaluated the correlations between lipidomics assessment of relative OCFA-content with traditional gas chromatography-based measurement of relative OCFA abundance (Supplemental Table 4). The relative total plasma phospholipid C15:0 assessed by gas chromatography and the relative PL-C15:0 concentrations based on mass spectrometry were very strongly correlated ($r = 0.90$), while this correlation was lower for C17:0 ($r = 0.62$). Among individual phospholipid classes, relative OCFA-content in PC and LPC correlated well with the relative OCFA-content in total plasma phospholipids determined by gas chromatography, while correlations for PE and LPE were weaker.

3.3. Mutual relationship of OCFA-containing lipids

To assess intercorrelations among the PL-OCFA, we calculated partial correlations (Supplemental Table 5). Controlling for age and sex only, we observed moderate to strong correlations between all OCFA-containing phospholipids, with the strongest positive correlations between LPC(C17:0) and LPC(C15:0), between PC(C17:0) and PC(C15:0), and between LPC(C17:0) and LPE(C17:0). After further adjustment for all other PL-OCFA concentrations, only LPC(C17:0) and LPC(C15:0) remained strongly correlated ($r = 0.71$); PC(C15:0) and PC(C17:0) were negatively correlated with LPC(C17:0) and LPC(C15:0), respectively.

3.4. Associations between OCFA-containing lipids and T2D risk

We next investigated the longitudinal association between PL-OCFA and T2D risk, adjusting for a variety of confounding factors - including sociodemographic characteristics, anthropometry, lifestyle, and dietary variables (Table 2), apart from the minimally adjusted model (Supplemental Table 6). A multiplicative interaction test indicated that the risk association differed between men and women for PL-OCFA (p-interaction = 0.023), PL-C15:0 (p-interaction = 0.090) and PL-C17:0 (p-interaction = 0.016). Higher concentrations of total PL-OCFA were associated with lower

Table 1
Baseline characteristics of subcohort participants by quintiles of odd chain fatty acids in phospholipid species (PL-OCFA), derived from lipidomics analysis (n = 1137).

		Quintiles of PL-OCFA					Total subcohort
		1	2	3	4	5	
PL-OCFA (μM)	Men	0.18 (0.07–0.22)[a]	0.25 (0.22–0.27)[a]	0.30 (0.27–0.33)[a]	0.36 (0.33–0.39)[a]	0.44 (0.40–0.75)[a]	0.26 (0.20–0.33)[b]
	Women	0.19 (0.12–0.22)[a]	0.25 (0.22–0.27)[a]	0.30 (0.27–0.33)[a]	0.36 (0.33–0.40)[a]	0.45 (0.40–0.76)[a]	0.33 (0.26–0.40)[b]
Sex (%)	Men	64	43	39	30	21	39
	Women	36	57	61	70	79	61
Age at recruitment (years) [b]	Men	51 (44–58)	54 (46–59)	54 (44–60)	50 (44–58)	56 (48–62)	53 (44–59)
	Women	46 (39–54)	43 (39–54)	46 (40–56)	50 (41–58)	53 (45–59)	47 (40–57)
Body Mass Index [b]	Men	26 (24–29)	27 (25–29)	26 (25–28)	26 (24–28)	27 (24–29)	26 (24–29)
	Women	25 (22–29)	24 (22–28)	24 (22–28)	24 (22–28)	24 (22–27)	24 (22–28)
Height (cm) [b]	Men	175 (171–178)	176 (173–179)	174 (170–178)	175 (171–179)	173 (169–177)	175 (171–179)
	Women	163 (159–168)	163 (160–167)	163 (159–168)	162 (158–167)	162 (158–166)	163 (159–167)
Waist circumference (cm) [b]	Men	93 (88–100)	93 (88–100)	93 (87–98)	91 (86–96)	95 (88–99)	93 (87–100)
	Women	79 (71–88)	77 (70–87)	78 (71–87)	78 (71–87)	78 (71–85)	78 (71–87)
Sport, biking, gardening (h/week)	Men	6 (2–10)	5 (3–9)	6 (3–12)	5 (3–9)	6 (3–10)	6 (3–10)
	Women	3 (2–6)	5 (3–8)	4 (2–7)	5 (2–7)	5 (2–8)	5 (2–8)
Education (%)							
In training/no certificate/part skilled worker	Men	3	2	2	3	0	2
	Women	5	5	3	6	3	4
Skilled worker	Men	38	31	34	38	33	35
	Women	35	37	33	34	37	35
Professional school	Men	14	15	13	18	10	14
	Women	30	34	35	27	26	30
Higher education, university	Men	46	52	51	41	56	48
	Women	30	24	29	33	34	30
Alcohol from alcoholic beverages (g/day) [b]	Men	24 (11–43)	15 (8–25)	19 (9–30)	14 (6–26)	14 (7–26)	18 (8–33)
	Women	6 (2–12)	5 (2–9)	6 (3–13)	5 (2–10)	4 (1–9)	5 (2–10)
Type of work (%)							
Sedentary occupation	Men	51	56	61	46	58	54
	Women	54	65	63	62	68	63
Standing occupation	Men	37	34	31	41	31	35
	Women	46	34	32	36	29	34
Manual/Heavy work	Men	12	10	8	13	10	11
	Women	0	2	5	2	3	3
Smoking (%)							
Never smoker	Men	23	32	28	34	42	29
	Women	55	55	57	63	69	61
Ex-smoker	Men	46	40	55	47	40	46
	Women	20	23	27	22	18	22
Smoker <20 u/d	Men	17	19	9	10	15	15
	Women	22	20	13	14	11	15
Current smoker ≥ 20 u/d	Men	14	9	8	9	4	10
	Women	2	2	3	1	2	2
Prevalent hypertension (%)	Men	61	66	61	47	63	60
	Women	35	39	42	49	43	43
Dairy fat intake (g/day)	Men	9 (6–14)	10 (7–15)	10 (6–16)	12 (8–17)	13 (9–19)	10 (6–15)
	Women	11 (7–15)	10 (7–16)	10 (7–15)	12 (7–16)	11 (8–17)	11 (7–16)

[a] Median (Range).
[b] Median (IQR).

diabetes risk in women (HR per SD: 0.63, 95% CI 0.51–0.78), while no association was observable in men. A similar picture emerged when evaluating individual OCFA: higher concentrations of both PL-C15:0 and PL-C17:0 were inversely associated with diabetes risk among women, but not men. Noteworthy, even though the association of PL-C17:0 with T2D risk was non-linear in women (p for non-linearity = 0.047, Supplemental Fig. 2), women showed lower T2D risk in higher categories (for example, HR Q5 vs. Q1: 0.21, 95% CI 0.11–0.39). In contrast, associations for both PL-C15:0 and PL-C17:0 appeared to be non-linear in men, and categorical analyses showed no clear association pattern. Among the phospholipid subgroups, we observed statistically significant inverse associations of PC(C15:0) with T2D risk in women (p non-linearity = 0.049, HR Q5 vs Q1: 0.56, 95% CI 0.32–0.97), but not in men (p non-linearity = 0.039, HR Q5 vs Q1: 0.98, 95% CI 0.54–1.77). Similarly, PC(C17:0) was inversely associated with T2D risk in women (HR per SD: 0.59, 95% CI 0.48–0.71), but not in men (p non-linearity <0.001, HR Q5 vs. Q1: 0.97; 95% CI 0.53–1.78). Tests for statistical interaction suggested effect measure modification by sex for the risk associations with PC(C17:0) (p-interaction = 0.003) and, to a lesser extent, PC(C15:0) (p-interaction = 0.134).

Noteworthy, LPC(C15:0) was not associated with T2D incidence in women (HR per SD: 0.90, 95% CI 0.74–1.09), but in men, this lipid was positively associated with T2D incidence (p non-linearity = 0.47, HR Q5 vs Q1: 1.96, 95% CI 1.06–3.63) (p-interaction = 0.017). For LPC(C17:0), the restricted cubic spline analysis indicated significant deviation from linearity (women p = 0.002, men p < 0.001), but still, we observed a lower T2D risk with higher relative LPC(C17:0) content in women (HR Q5 vs. Q1: 0.42, 95% CI 0.23–0.76), while this association was weaker and not statistically significant in men (HR Q5 vs. Q1: 0.71, 95% CI 0.39–1.29). The lipids PE(C17:0) and LPE(C17:0) were not appreciably associated with T2D incidence in women or men.

We analyzed other lipids apart from the phospholipid classes. CE(C15:0) was not significantly associated with T2D risk. In women, CE(C17:0) was inversely associated with T2D risk (HR per SD: 0.73, 95% CI 0.60–0.89), while the associations were weaker and non-

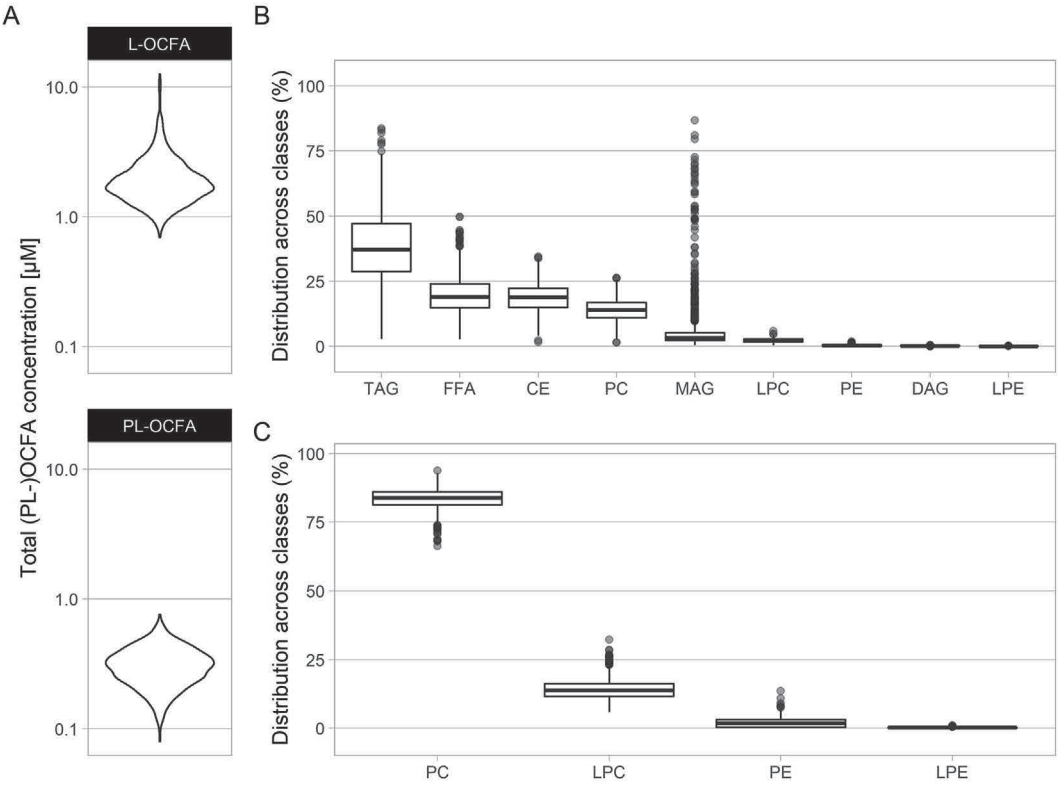

Fig. 1. (A) Total concentration of lipids containing odd-chain fatty acids C15:0 and C17:0 (L-OCFA) and total concentration of phospholipid species containing odd-chain fatty acids C15:0 and C17:0 (PL-OCFA) (B) proportion of total L-OCFA from each class (C) proportion of total PL-OCFA from each phospholipid class; based on the subcohort population (n = 1137). **CE**, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **L-OCFA**, lipids containing odd-chain fatty acids; **LPC**, lysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG**, monoacylglycerols; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **PL-OCFA**, phospholipid species containing odd-chain fatty acids (sum of PC, LPC, PE, LPE); **TAG**, triacylglycerols.

significant for men (HR per SD: 0.93, 95% CI 0.78–1.11). Higher concentrations of MAG(C15:0) were associated with a lower risk of T2D in women (HR Q5 vs. Q1: 0.50, 95% CI 0.30–0.82) and men (HR Q5 vs. Q1: 0.53, 95% CI 0.31–0.91). The association of MAG(C17:0) with T2D risk was not statistically significant in both sexes. Women with higher concentrations of DAG(C15:0) were at lower risk of T2D (HR per SD: 0.78, 95% CI 0.64–0.94), whereas in men, this association was not statistically significant (HR per SD: 0.91, 95% CI 0.77–1.08). We did not observe statistically significant associations between higher concentrations of TAG(C15:0) or TAG(C17:0) and T2D risk for women. In men, we found a positive association between TAG(15:0) and T2D risk (HR per SD: 1.20, 95% CI 1.02–1.41), as well as a similar association between TAG(17:0) and T2D risk (1.17, 95% CI 0.99–1.37).

With regard to FFA, FFA(C15:0) was not appreciably associated; however, FFA(C17:0) was positively associated with T2D risk in women (HR per SD: 1.24, 95% CI 1.04–1.48) and men, although not statistically significant in the latter (HR per SD: 1.13, 95% CI 0.96–1.34). These associations remained largely unchanged when models were further adjusted for total FFA (Supplemental Table 7). When these analyses were stratified by fasting status, FFA(C17:0) was positively associated with T2D risk both in fasted and non-fasted individuals, although the association was stronger among fasted. FFA(C15:0) was positively associated with T2D in fasted individuals but inversely among non-fasted.

3.5. Associations of OCFA-containing lipids and dietary variables

Moreover, we analyzed the correlations between lipids and dietary variables, including total dairy fat intake, dairy foods, grains, fruits and vegetables, and red meat (Fig. 3). Habitual dairy fat intake was weakly positively correlated with PL-C15:0 (men: r = 0.25, women: r = 0.13) and PL-C17:0 (men: r = 0.23, women: r = 0.10). PL-C15:0 and PL-C17:0 were positively correlated with the habitual consumption of several dairy fat-containing food groups, including total full-fat dairy, butter, full-fat milk, and full-fat yogurt. Butter was the source of dairy fat most strongly correlated with PL-OCFA (men: r = 0.36, women: r = 0.43). In contrast, low-fat dairy consumption (i.e., total low-fat dairy, low-fat cheese, milk, and yogurt) was not correlated with lipids containing OCFA. Remarkably, fiber-containing foods (whole grains) were comparably correlated with PL-OCFA (PL-C15:0 men r = 0.19, women r = 0.12; PL-C17:0 men r = 0.17, women r = 0.15), and meat consumption was inversely correlated with PL-OCFA (PL-C15:0 men r = −0.25, women r = −0.15; PL-C17 men r = −0.21, women r = −0.14). The correlations between lipids containing OCFA and whole grains became stronger after adjusting for butter intake (Supplemental Table 8). When analyzing phospholipid subgroups (Fig. 4), the correlations were consistently stronger with full-fat dairy foods compared to low-fat dairy products. The stronger correlation of the relative abundance of PL-C15:0 compared to PL-C17:0 with dairy fat consumption was also consistent in phospholipid subgroups, specifically LPC and PC.

Although men and women had similar total fat intake from dairy products, women consumed more yogurt (Supplemental Table 9). We divided men into high yogurt intake and low yogurt intake, based on women's median as cutpoint (53 g/day), to evaluate men with intake as high as women with high intake. In the analyses restricted to men with high yogurt intake, PL-OCFA and T2D risk tended to be inversely associated (HR per SD 0.77, 95% CI 0.57–1.04, n = 325). This was not the case when analyses were restricted to

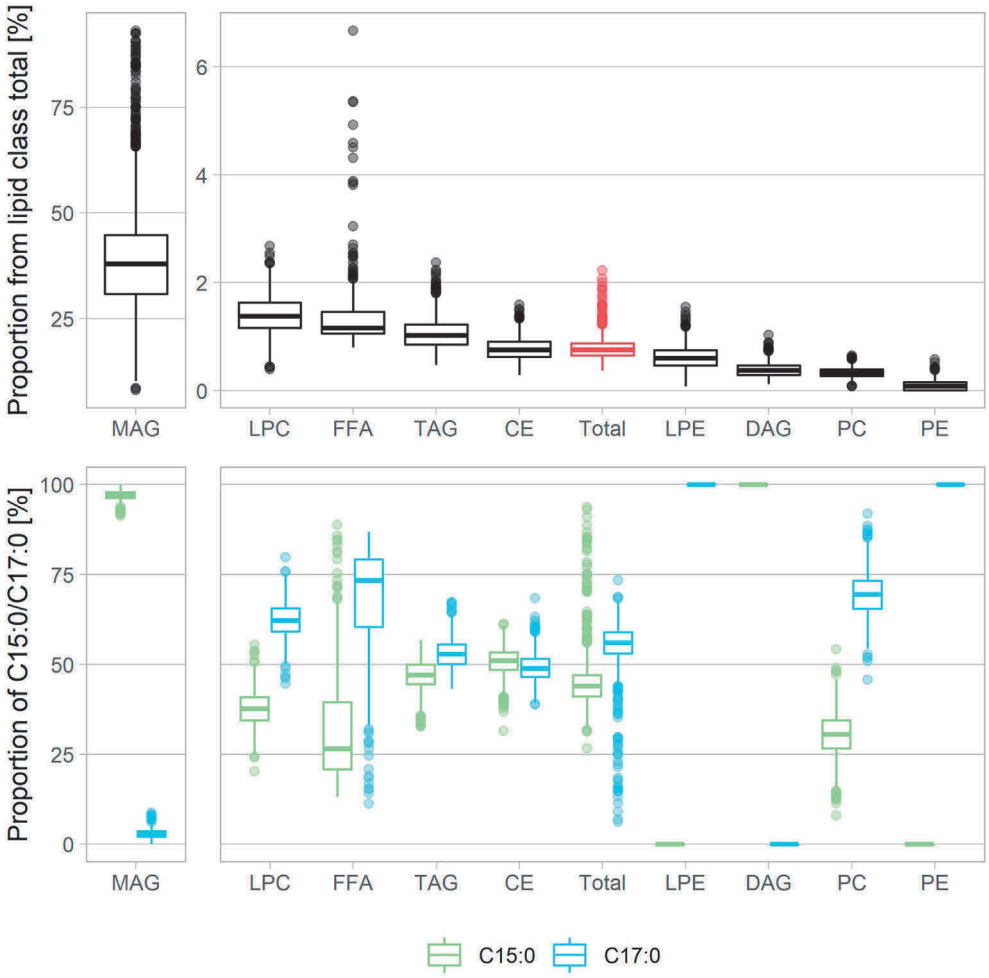

Fig. 2. Proportion of lipid species containing odd chain fatty acids (OCFA) within each lipid class (upper panel) and relative proportion of lipid species containing C15:0 versus lipids containing C17:0 in each lipid class (lower panel), based on the subcohort population (n = 1137). **C15:0**, pentadecanoic acid; **C17:0**, heptadecanoic acid; **CE**, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **LPC**, lysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG**, monoacylglycerols; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **TAG**, triacylglycerols.

men below the cutpoint (HR per SD 1.11, 95% CI 0.89–1.40, n = 561). In women with yogurt intake above the median, the inverse association of PL-OCFA and T2D risk was stronger (HR per SD 0.51, 95% CI 0.38–0.68, n = 576) than when restricted to women below the median (HR per SD 0.81, 95% CI 0.61–1.09, n = 424).

Further exploratory analyses revealed a similar picture when dividing men and women into groups based on women's median fiber intake as cutpoint (21 g/day). Among men consuming higher amounts of fiber (more than 21 g/day), we observed an inverse association of PL-OCFA with T2D risk (HR per SD 0.77, 95% CI 0.62–0.96, n = 523); while it was not the case when analyses were restricted to men consuming less than 21 g/day of fiber (HR per SD 1.20, 95% CI 0.93–1.55, n = 363). Women consuming more fiber than the median had a stronger inverse association of PL-OCFA with T2D risk (HR per SD 0.58, 95% CI 0.42–0.81, n = 464) than women with less fiber intake (HR per SD 0.72, 95% CI 0.53–0.98, n = 536).

Finally, the main models, already adjusted for fiber intake, were further adjusted for butter and full-fat dairy intake in a sensitivity analysis. The adjustment for these foods had a minor impact on the associations (data not shown).

4. Discussion

We investigated OCFA-containing lipids in a large prospective cohort study, using a comprehensive lipidomics panel with 69 OCFA-containing lipids among 15 classes. The TAG, FFA, CE, and PC involving OCFA contributed the most to the total OCFA-plasma abundance. A large fraction of circulating MAG contained OCFA, while their relative concentration in other classes was low. The relative contribution to the total OCFA content of C15:0 versus C17:0 differed across lipid classes. Moreover, higher baseline levels of total PL-C15:0, PL-C17:0, PC(C15:0), PC(C17:0), LPC(C17:0), MAG(15:0), CE(C17:0), DAG(C15:0) were inversely associated with T2D incidence; with most associations being stronger for women. Conversely, positive associations between LPC(C15:0) (in men) and FFA(C17:0) (in women) and T2D risk were observed. In addition, we found that fat-rich dairy products but also fiber-rich foods were correlated to all OCFA-containing lipids. Our results suggest that part of the divergent associations between women and men may be explainable by gender differences in the intake levels of diabetes-related dietary OCFA-determinants.

Table 2
Sex-stratified associations between lipids[a] containing odd-chain fatty acids (OCFA) and incident diabetes among men (n = 886, cases = 450) and women (n = 1000, cases = 325) in the EPIC-Potsdam case-cohort study[b].

	PL-OCFA (C15:0 +C17:0)					
	Men		Women		p-interaction[d]	
Continuous analysis[c]	Non-linear		0.63 (0.51–0.78)		0.023	
Q2 vs. Q1	1.61 (1.01–2.57)					
Q3 vs. Q1	0.67 (0.37–1.20)					
Q4 vs. Q1	0.65 (0.36–1.16)					
Q5 vs. Q1	1.11 (0.61–2.03)					
	C15:0			C17:0		
	Men	Women	p-interaction[d]	Men	Women	p-interaction[d]
Total PL species						
Continuous analysis[c]	Non-linear	0.76 (0.61–0.93)	0.090	Non-linear	Non-linear	0.016
Q2 vs. Q1	1.37 (0.84–2.25)			1.57 (0.98–2.51)	0.56 (0.35–0.90)	
Q3 vs. Q1	1.58 (0.92–2.73)			0.76 (0.44–1.30)	0.54 (0.33–0.87)	
Q4 vs. Q1	1.00 (0.58–1.75)			0.67 (0.37–1.21)	0.46 (0.26–0.80)	
Q5 vs. Q1	1.11 (0.61–2.01)			0.88 (0.48–1.61)	0.21 (0.11–0.39)	
PC						
Continuous analysis[c]	Non-linear	Non-linear	0.134	Non-linear	0.59 (0.48–0.71)	0.003
Q2 vs. Q1	1.10 (0.67–1.83)	0.68 (0.42–1.09)		1.76 (1.10–2.83)		
Q3 vs. Q1	1.60 (0.95–2.71)	0.69 (0.42–1.12)		0.93 (0.54–1.60)		
Q4 vs. Q1	0.97 (0.56–1.70)	0.82 (0.50–1.35)		0.77 (0.42–1.39)		
Q5 vs. Q1	0.98 (0.54–1.77)	0.56 (0.32–0.97)		0.97 (0.53–1.78)		
LPC						
Continuous analysis[c]	Non-linear	0.90 (0.74–1.09)	0.017	Non-linear	Non-linear	0.435
Q2 vs. Q1	1.43 (0.82–2.49)			1.20 (0.74–1.95)	0.91 (0.57–1.46)	
Q3 vs. Q1	2.37 (1.33–4.23)			0.88 (0.52–1.49)	1.23 (0.74–2.03)	
Q4 vs. Q1	1.71 (0.96–3.04)			0.52 (0.28–0.96)	0.52 (0.31–0.89)	
Q5 vs. Q1	1.96 (1.06–3.63)			0.71 (0.39–1.29)	0.42 (0.23–0.76)	
PE						
Continuous analysis[c]	–	–		1.15 (0.98–1.36)	1.16 (0.97–1.38)	0.467
LPE						
Continuous analysis[c]	–	–		0.95 (0.82–1.11)	0.89 (0.75–1.05)	0.594
OTHER CLASSES						
CE						
Continuous analysis[c]	1.10 (0.92–1.31)	0.85 (0.70–1.04)	0.119	0.93 (0.78–1.11)	0.73 (0.60–0.89)	0.208
MAG						
Continuous analysis[c]	Non-linear	Non-linear	0.463	Non-linear	Non-linear	0.051
Q2 vs. Q1	1.01 (0.62–1.63)	0.40 (0.25–0.66)		0.99 (0.63–1.57)	1.46 (0.88–2.43)	
Q3 vs. Q1	0.43 (0.26–0.71)	0.32 (0.19–0.53)		0.80 (0.48–1.32)	1.07 (0.66–1.73)	
Q4 vs. Q1	0.61 (0.37–1.00)	0.30 (0.18–0.51)		0.72 (0.43–1.21)	1.10 (0.67–1.80)	
Q5 vs. Q1	0.53 (0.31–0.91)	0.50 (0.30–0.82)		0.75 (0.45–1.26)	0.63 (0.36–1.12)	
DAG						
Continuous analysis[c]	0.91 (0.77–1.08)	0.78 (0.64–0.94)	0.324	–	–	
TAG						
Continuous analysis[c]	1.20 (1.02–1.41)	Non-linear	0.396	1.17 (0.99–1.37)	Non-linear	0.355
Q2 vs. Q1		0.96 (0.57–1.64)			0.51 (0.30–0.85)	
Q3 vs. Q1		0.45 (0.25–0.80)			0.43 (0.25–0.72)	
Q4 vs. Q1		1.05 (0.64–1.71)			0.52 (0.31–0.86)	
Q5 vs. Q1		1.20 (0.72–2.02)			0.87 (0.54–1.42)	
FFA						
Continuous analysis[c]	0.89 (0.74–1.07)	0.96 (0.81–1.15)	0.927	1.13 (0.96–1.34)	1.24 (1.04–1.48)	0.552

C15:0, pentadecanoic acid; **C17:0**, heptadecanoic acid; **CE**, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **LPC, l**ysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG**, monoacylglycerols; **OCFA**, odd-chain saturated fatty acids; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **PL**, phospholipids; **PL-OCFA**, phospholipid species containing odd-chain fatty acids; **Q**, quintile; **TAG**, triacylglycerols.

[a] Lipids derived from lipidomics mass spectrometry analysis.

[b] Values are Hazard Ratios (HR) and 95% confidence intervals derived from a multivariable Cox regression model adjusted for: age (strata), waist circumference, BMI, cycling (0, 0.1–2.4, 2.5–4.9, or ≥ 5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), education (in or no training, vocational training, technical school, or technical college or university degree), smoking status (never, past, current, <20 cigarettes/day, or current > 20 cigarettes/day), occupational activity (light, moderate, or heavy), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or 0.40.0 g/d), coffee intake (energy-adjusted), fiber intake (energy-adjusted), red meat intake (energy-adjusted), total energy intake (kJ/day), fasting status and prevalent hypertension.

[c] Continuos analysis modeled per 1 SD in Cox regression. If the cubic splines regression analysis showed a non-linear association ($p < 0.05$) the results for continuous analysis are not presented; instead, the table shows HR calculated for quintiles.

[d] p-interaction with sex obtained by including cross-product terms in the continuous fully-adjusted models.

4.1. OCFA distribution across lipid classes

PC contributed the most to the total PL-OCFA abundance, which was largely explained by high absolute PC concentrations relative to other phospholipids. We also observed that C15:0 versus C17:0 were selectively enriched in specific lipid classes. While MAG represents only 0.1% concentration of all lipids, their relative OCFA-content was about a magnitude higher than in other classes and almost exclusively was C15:0. Contrarily, C17:0 seemed to be enriched relative to C15:0 in lipids with a phosphoethanolamine headgroup. Heterogeneous integration of OCFA in different lipids may be due to mechanisms related to the

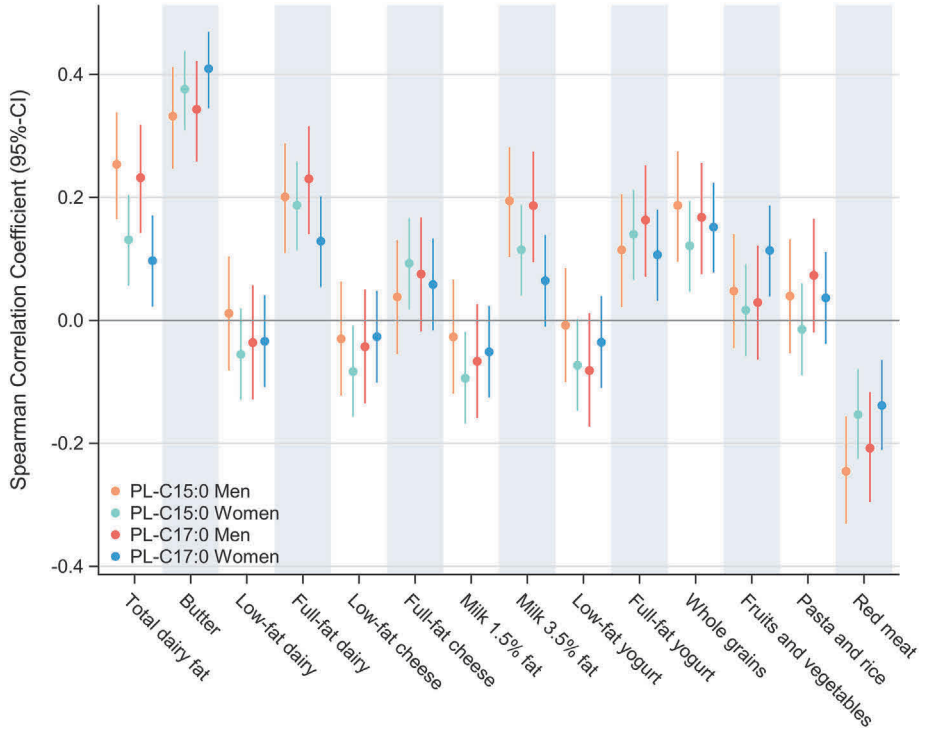

Fig. 3. Correlations of phospholipid species containing C15:0 (PL-C15:0) and phospholipids containing C17:0 (PL-C17:0) with total dairy fat and food groups intake at baseline in the subcohort population (n = 1137, men = 448 women = 689), adjusted for age and total daily energy intake. **C15:0**, pentadecanoic acid; **C17:0**, heptadecanoic acid.

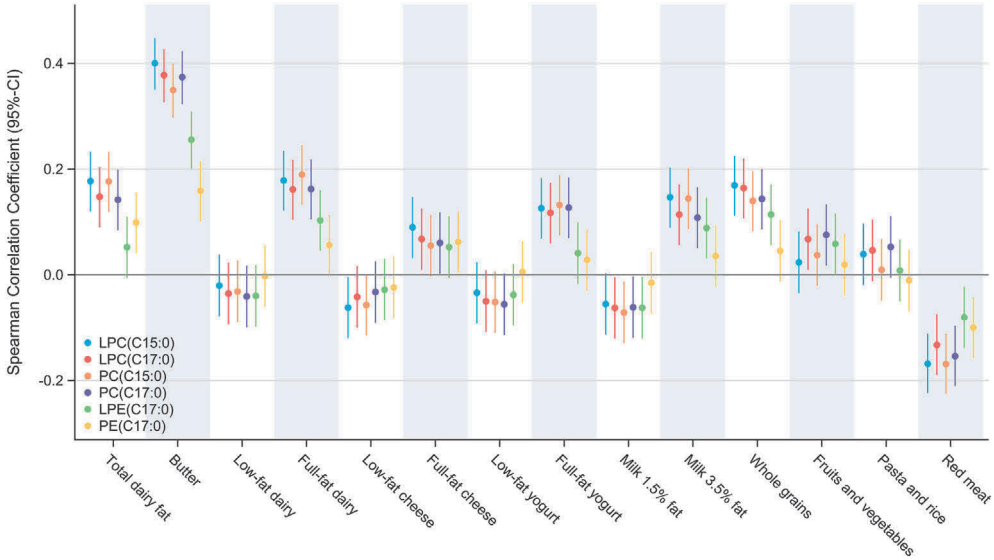

Fig. 4. Correlations of lipid species containing OCFA with total dairy fat and food groups consumption at baseline in the subcohort population (n = 1137), adjusted for age, sex, and total daily energy intake. **15:0**, pentadecanoic acid; **17:0**, heptadecanoic acid; **LPC**, lysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines.

different turnover rates of the lipid classes or fatty acid selectivity of the synthesizing enzymes, which, for example, has been described for the hydrolysis of MAG [38,39]. Regardless of the precise mechanisms, our observations indicate that the distribution of OCFA across lipids underlies a complex metabolic regulation, resulting in heterogenous enrichment patterns of OCFA across distinct lipid classes.

4.2. OCFA-containing lipids and T2D risk

Higher plasma concentrations of phospholipids PC(C15:0), PC(C17:0), and LPC(17:0) were associated with lower T2D risk in our study. Most of the studies which linked OCFA in plasma lipids to lower T2D risk detected the OCFA in total phospholipids [3–5]. Our results extend these findings by identifying PC as the main

responsible phospholipid group, followed by LPC. We furthermore observed stronger inverse associations between T2D risk and lipids with C17:0, i.e., PC(C17:0), LPC(C17:0), in comparison to the ones involving C15:0. These results are in line with the previous prospective studies on OCFA in total plasma phospholipids [3,5]. Likewise, a previous study reported CE(C17:0) more strongly associated with a lower risk of T2D than CE(C15:0) [5], similar to our observation.

Our analyses revealed that the inverse links of OCFA-containing lipids with T2D risk were almost exclusively driven by the strong inverse association in women. In a meta-analysis of sixteen prospective cohorts, inverse associations of C15:0 and C17:0 with T2D risk were stronger in women than in men [5]. A potential explanation for the observed sex differences is that OCFA biomarkers reflect different dietary exposures. In our study, women consumed more yogurt than men. Inverse associations of dairy foods and diabetes risk seem particularly present for yogurt [6,40], and stronger inverse associations of yogurt consumption and T2D risk were observed in women compared with men [40]. Our analyses restricted to high consumers of yogurt suggest that differences in food sources of OCFA may at least in part explain observed sex differences.

Apart from reflecting dietary intake, OCFA may be involved in pathways related to diabetes pathogenesis. One hypothesis is that OCFA oxidation provides intermediates to the tricarboxylic acid cycle, which improves mitochondrial function [41]. Other pathways might be related to anti-inflammatory activities [9], or hepatocyte protection from palmitate-induced insulin resistance and dysfunction — by increasing glycogen and monounsaturated fatty acids concentration and reducing the output of glucose and tumor necrosis factor alpha (TNFα) [42]. However, it remains unclear if and how OCFA-containing lipids are mechanistically involved in T2D development.

Interestingly, FFA(C17:0) was associated with higher T2D risk in our study. Previous prospective studies linked elevated total FFA concentrations with increased T2D risk [43]. However, as the association of FFA(C17:0) was independent of total FFA concentrations in our study, it does not reflect the detrimental effects of FFA per se. Fasting plasma FFA(C15:0) and FFA(C17:0) are strongly correlated with C15:0 and C17:0 in adipose tissue [44]. At the same time, C15:0 in adipose tissue is strongly correlated with total dairy consumption, while C17:0 is not [45,46]. FFA(C17:0) might rather reflect metabolic processes in the adipose tissue than acute dietary exposure. The hypothesis that FFA(C17:0) is a marker for endogenous determinants rather than acute dietary intake is supported by its excellent reliability over several weeks, in contrast to all other FFA with greater intraindividual variability. Still, it is unclear why non-esterified OCFA have a contrasting association with T2D in comparison with other lipids. These results require further replication and mechanistic clarification.

4.3. Food determinants of OCFA-containing lipids

We observed stronger correlations of OCFA-lipids with full-fat dairy foods in comparison to the low-fat versions. Other cross-sectional studies also observed that specific phospholipids (PC, LPC) with OCFA are associated with full-fat dairy intake [47,48]. Our findings, however, indicate that none of the OCFA-containing lipids was specific for dairy, but they were also positively correlated with fiber-rich foods, which are inversely related to T2D risk [49]. Plasma C17:0 levels increase after dietary supplementation with inulin and propionate [8], and endogenous production (through elongation of propionyl-CoA or α-oxidation of C18:0) seems an important source of circulating C17:0 [50]. Indeed, in some studies, C17:0 in phospholipids was more strongly correlated with fiber intake than with dairy intake [51,52], while C15:0 had stronger correlations with dairy intake [7]. In our data, however, lipids with C17:0 correlated with dietary variables similarly as the lipids with C15:0. Additionally, we found inverse correlations between OCFA-lipids and red meat, which has been positively associated with incident type 2 diabetes [53]. Overall, our results indicate several OCFA-lipid metabolites linked to dairy fat consumption; however, these lipids are not specific biomarkers of dairy intake because they are also sensitive to other foods.

4.4. Strengths and limitations

One strength is the availability of a comprehensive lipidomics analysis in a large sample size within a prospective cohort study. To our knowledge, our analyses are the first focusing on a large variety of lipids containing OCFA, their detailed characterization, and their association with T2D risk. However, for lipids with more than one fatty acid (PC, PE, DAG, TAG), we only considered the OCFA for our exposure classification without taking into account diversity related to other fatty acids present in these lipid molecules. Also, we have not considered interconnections between OCFA containing lipid species and other lipids within a class. While most lipids demonstrated reasonable reliability in a pilot study, differences in intra-individual variability may partly explain differences in risk associations observed. Although we used FFQ with reasonable reliability and validity to assess dietary intake, total dairy consumption may be challenging to assess by self-report. Moreover, whether OCFA-containing lipids are mediators linking specific foods to diabetes risk or whether they are indicators of their intake (and other food constituents are the causal factors) cannot be fully answered from our observational study. However, the inverse associations with risk were unaffected by the adjustment for fiber, butter, and full-fat dairy intake, which presents an argument against OCFA being simply an indicator of intake. Finally, because of the observational study design, the possibility of residual confounding cannot be ruled out.

5. Conclusion

Our findings suggest that lipids containing OCFA have heterogeneous distribution across lipid classes, indicating that OCFA are subject to complex metabolic regulation. They are differently linked to T2D risk, depending on lipid class and sex, with positive associations observed for non-esterified OCFA and inverse associations observed in women for OCFA in several other lipid classes (PC, LPC, CE, MAG, DAG). Our results furthermore suggest that OCFA-containing lipids are biomarkers for the metabolic response to several T2D-related foods.

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Author contributions

The authors' responsibilities were as follows, MP: conceptualization, data curation, formal analysis, writing - original draft; CW: conceptualization, data curation, supervision, writing - original draft; FE: methodology, visualization, validation, writing - review & editing; AW: investigation, writing - review & editing; JD: conceptualization, writing - review & editing; MS: conceptualization, funding acquisition, project administration, supervision, writing - review & editing; and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.06.006.

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Association of the odd-chain fatty acid content in lipid groups with type 2 diabetes risk: a targeted analysis of lipidomics data in the EPIC-Potsdam cohort.

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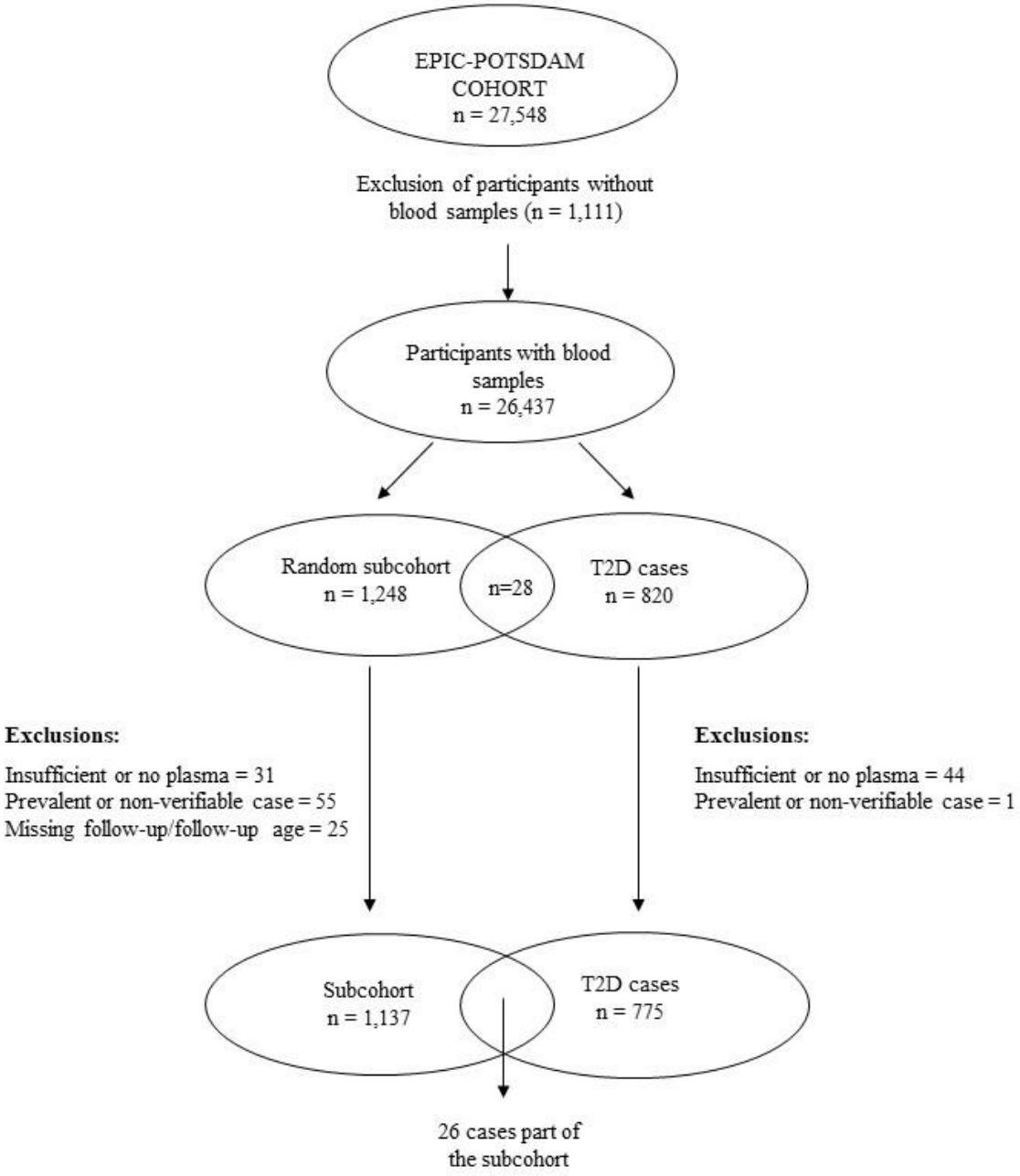
Supplementary Material



Supplemental Figure 1. Participant flow chart

T2D, Type 2 diabetes

Supplemental Table 1. Intraclass Correlation Coefficients (ICC) and 95% Confidence Interval for lipids containing odd-chain fatty acids (C15:0 and C17:0), derived from fasting blood samples collected at two time points (n=35)

	Median	95% Confidence Interval	
Lipid	**ICC**	**Lower limit**	**Upper limit**
CE(15:0)	0.691	0.273	0.869
CE(17:0)	0.661	0.301	0.851
DAG(15:0/18:1)	0.564	0.270	0.775
DAG(15:0/18:2)	0.595	0.309	0.758
FFA(15:0)	0.438	0.181	0.669
FFA(17:0)	0.974	0.258	0.989
LPC(15:0)	0.732	0.501	0.839
LPC(17:0)	0.676	0.421	0.827
LPE(17:0)	0.601	0.321	0.827
PC(17:0/16:1)	0.295	0.000	0.665
PC(17:0/18:1)	0.620	0.398	0.793
PC(17:0/18:2)	0.648	0.389	0.816
PC(17:0/18:3)	NA	NA	NA
PC(17:0/20:3)	0.386	0.057	0.654
PC(17:0/20:4)	0.723	0.491	0.848
PC(17:0/20:5)	0.114	0.000	0.673
PC(17:0/22:5)	0.000	0.000	0.000
PC(17:0/22:6)	0.561	0.079	0.843
PC(15:0/16:1)	0.271	0.000	0.590
PC(15:0/18:1)	0.714	0.494	0.832
PC(15:0/18:2)	0.765	0.470	0.916
PC(15:0/20:3)	0.178	0.000	0.685
PC(15:0/20:4)	0.751	0.597	0.888
PC(15:0/20:5)	NA	NA	NA
PC(15:0/22:5)	NA	NA	NA

PC(15:0/22:6)	0.116	0.000	0.543
PE(17:0/18:1)	NA	NA	NA
PE(17:0/18:2)	NA	NA	NA
PE(17:0/20:4)	NA	NA	NA
PE(17:0/22:6)	NA	NA	NA
TAG55:7-FA15:0	0.691	0.440	0.870
TAG53:4-FA17:0	0.557	0.279	0.751
TAG53:1-FA17:0	0.596	0.192	0.776
TAG53:2-FA17:0	0.544	0.320	0.757
TAG53:3-FA17:0	0.644	0.331	0.812
TAG51:0-FA17:0	0.627	0.206	0.832
TAG51:1-FA15:0	0.647	0.246	0.825
TAG51:1-FA17:0	0.585	0.211	0.819
TAG51:2-FA15:0	0.621	0.286	0.818
TAG51:2-FA17:0	0.524	0.193	0.797
TAG51:3-FA15:0	0.741	0.514	0.868
TAG51:3-FA17:0	0.431	0.144	0.651
TAG51:4-FA15:0	0.690	0.457	0.842
TAG49:0-FA15:0	0.598	0.183	0.841
TAG49:0-FA17:0	0.566	0.162	0.826
TAG49:1-FA15:0	0.606	0.277	0.842
TAG49:1-FA17:0	0.476	0.146	0.760
TAG49:2-FA15:0	0.568	0.249	0.831
TAG49:2-FA17:0	0.415	0.104	0.723
TAG49:3-FA15:0	0.545	0.260	0.759
TAG47:0-FA15:0	0.570	0.184	0.820
TAG47:0-FA17:0	0.441	0.059	0.720
TAG47:1-FA15:0	0.453	0.130	0.778
TAG47:1-FA17:0	0.363	0.009	0.657
TAG47:2-FA15:0	0.334	0.056	0.730

TAG45:0-FA15:0	0.451	0.073	0.753
TAG45:1-FA15:0	0.422	0.068	0.707
Total TAG	0.666	0.400	0.837
Total DAG	0.601	0.314	0.777
Total CE	0.716	0.350	0.893
Total FFA	0.154	0.000	0.424
Total PE	0.628	0.429	0.757
Total LPC	0.695	0.338	0.859
Total LPE	0.643	0.211	0.848
Total PC	0.722	0.533	0.830

Supplemental Table 2. Total lipid class concentration in the subcohort participants (n=1137) and lipids involving C15:0 and C17:0 within each class

	Total class concentration [2]		Lipids with C15:0 or C17:0	
Lipid class [1]	**Absolute (µM)**	**Relative [3] (Mol %)**		
Cholesteryl esters	44.19 (38.32-50.61)	29.37 (27.18-31.54)	CE(15:0)	CE(17:0)
Phosphatidylcholines	38.17 (33.58-42.86)	25.18 (23.13-27.22)	PC(15:0/16:1) PC(15:0/18:1) PC(15:0/18:2) PC(15:0/18:3) PC(15:0/20:3) PC(15:0/20:4) PC(15:0/20:5) PC(15:0/22:5) PC(15:0/22:6)	PC(17:0/16:1) PC(17:0/18:1) PC(17:0/18:2) PC(17:0/18:3) PC(17:0/20:3) PC(17:0/20:4) PC(17:0/20:5) PC(17:0/22:5) PC(17:0/22:6)
Free fatty acids	7.63 (23.77-32.31)	18.4 (15.69-21.47)	FFA(15:0)	FFA(17:0)
Triacylglycerols	20.71 (14.65-30.48)	14.06 (10.73-18.97)	TAG45:0-FA15:0 TAG45:1-FA15:0 TAG47:0-FA15:0 TAG47:1-FA15:0 TAG47:2-FA15:0 TAG49:0-FA15:0 TAG49:1-FA15:0 TAG49:2-FA15:0 TAG49:3-FA15:0 TAG51:1-FA15:0 TAG51:2-FA15:0 TAG51:3-FA15:0 TAG51:4-FA15:0 TAG55:7-FA15:0	TAG47:0-FA17:0 TAG47:1-FA17:0 TAG49:0-FA17:0 TAG49:1-FA17:0 TAG49:2-FA17:0 TAG51:0-FA17:0 TAG51:1-FA17:0 TAG51:2-FA17:0 TAG51:3-FA17:0 TAG53:1-FA17:0 TAG53:2-FA17:0 TAG53:3-FA17:0 TAG53:4-FA17:0
Phosphatidylethanolamines	3.10 (2.56-3.72)	2.04 (1.77-2.34)	PE(15:0/20:2) PE(15:0/22:6) PE(18:0/15:0)	PE(17:0/18:1) PE(17:0/18:2) PE(17:0/20:2) PE(17:0/20:3)

				PE(17:0/20:4) PE(17:0/22:4) PE(17:0/22:5) PE(17:0/22:6) PE(18:0/17:0)
Lysophosphatidylcholines	3.05 (2.53-3.55)	2.01 (1.64-2.38)	LPC(15:0)	LPC(17:0)
Diacylglycerols	0.45 (0.31-0.67)	0.30 (0.23-0.41)	DAG(15:0/18:1)	DAG(15:0/18:1)
Monoacylglycerols	0.17 (0.11-0.24)	0.10 (0.07-0.16)	MAG(15:0)	MAG(17:0)
Lysophosphatidylethanolamines	0.14 (0.11-0.18)	0.09 (0.08-0.12)	LPE(15:0)	LPE(17:0)

[1] Only classes that contain lipids with odd-chain fatty acids are presented
[2] Values are median (Interquartile range) concentration of the sum of all lipid species in each class
[3] Relative to total lipid concentration

Supplemental Table 3. Concentrations of lipids containing OCFA according to quintiles, among 886 men (450 cases) and 1000 women (325 cases) in the EPIC-Potsdam case-cohort study

			Quintiles of lipids containing OCFA					Total subcohort
			1	2	3	4	5	
PL-OCFA	Men	Median (Range)[1]	0.37 (0.16-0.43)	0.50 (0.44-0.57)	0.62 (0.58-0.66)	0.72 (0.67-0.77)	0.86 (0.77-1.26)	0.59 (0.16-1.26)
		Cases	103	140	75	66	65	449
	Women	Median (Range) [1]	0.51 (0.20-0.59)	0.63 (0.59-0.68)	0.72 (0.68-0.77)	0.82 (0.77-0.88)	0.99 (0.88-1.30)	0.70 (0.20-1.30)
		Cases	119	64	61	52	29	325
PL-C15:0	Men	Median (Range)[1]	0.09 (0.02-0.12)	0.14 (0.12-0.16)	0.18 (0.16-0.20)	0.22 (0.20-0.25)	0.28 (0.25-0.80)	0.17 (0.02-0.80)
		Cases	97	101	106	79	67	450
	Women	Median (Range) [1]	0.14 (0.05-0.17)	0.19 (0.17-0.21)	0.23 (0.21-0.25)	0.27 (0.25-0.29)	0.34 (0.29-0.47)	0.22 (0.05-0.47)
		Cases	93	67	79	49	37	325
PL- C17:0	Men	Median (Range) [1]	0.26 (0.13-0.31)	0.35 (0.31-0.39)	0.43 (0.40-0.46)	0.50 (0.46-0.54)	0.59 (0.54-0.89)	0.41 (0.13-0.89)
		Cases	108	134	82	73	52	449
	Women	Median (Range) [1]	0.35 (0.14-0.40)	0.43 (0.40-0.47)	0.50 (0.47-0.53)	0.57 (0.53-0.60)	0.67 (0.60-0.95)	0.48 (0.14-0.95)
		Cases	119	75	57	49	25	325
	Phospholipid species							
LPC(15:0)	Men	Median (Range) [1]	0.26 (0.14-0.32)	0.37 (0.32-0.42)	0.46 (0.42-0.50)	0.54 (0.50-0.59)	0.69 (0.59-1.02)	0.46 (0.14-1.02)
		Cases	67	101	104	83	94	449
	Women	Median (Range) [1]	0.38 (0.19-0.45)	0.49 (0.45-0.52)	0.56 (0.52-0.60)	0.65 (0.60-0.70)	0.80 (0.70-1.17)	0.55 (0.19-1.17)
		Cases	77	52	76	73	47	325
LPC(17:0)	Men	Median (Range) [1]	0.51 (0.22-0.60)	0.67 (0.60-0.73)	0.77 (0.73-0.84)	0.89 (0.84-0.96)	1.03 (0.96-1.49)	0.75 (0.22-1.49)
		Cases	107	122	83	76	61	449

	Women	Median (Range) [1]	0.65 (0.26-0.74)	0.79 (0.74-0.84)	0.90 (0.84-0.96)	1.02 (0.96-1.09)	1.18 (1.09-1.71)	0.88 (0.26-1.71)
		Cases	106	61	77	49	32	325
PC(15:0)	Men	Median (Range) [1]	0.04 (0.01-0.05)	0.06 (0.05-0.08)	0.09 (0.08-0.10)	0.11 (0.10-0.12)	0.14 (0.12-0.24)	0.08 (0.01-0.24)
		Cases	101	88	112	81	66	448
	Women	Median (Range) [1]	0.07 (0.02-0.08)	0.09 (0.08-0.10)	0.11 (0.10-0.12)	0.13 (0.12-0.15)	0.17 (0.15-0.23)	0.11 (0.02-0.23)
		Cases	91	68	75	54	37	325
PC(17:0)	Men	Median (Range) [1]	0.13 (0.05-0.15)	0.17 (0.15-0.19)	0.21 (0.19-0.23)	0.24 (0.23-0.26)	0.29 (0.26-0.45)	0.20 (0.05-0.45)
		Cases	99	135	97	64	54	449
	Women	Median (Range) [1]	0.17 (0.07-0.2)	0.21 (0.20-0.23)	0.24 (0.23-0.26)	0.28 (0.26-0.30)	0.33 (0.30-0.44)	0.24 (0.07-0.44)
		Cases	120	66	62	49	28	325
LPE(17:0)	Men	Median (Range) [1]	0.08 (0.08-0.37)	0.45 (0.37-0.50)	0.55 (0.50-0.60)	0.66 (0.60-0.72)	0.82 (0.73-1.55)	0.53 (0.08-1.55)
		Cases	103	98	103	71	75	450
	Women	Median (Range) [1]	0.40 (0.08-0.48)	0.54 (0.48-0.59)	0.64 (0.59-0.69)	0.76 (0.70-0.81)	0.90 (0.81-1.55)	0.63 (0.08-1.55)
		Cases	98	67	69	37	54	325
PE(17:0)	Men	Median (Range) [1]	0.01 (0.01-0.04) [2]		0.07 (0.04-0.09)	0.12 (0.09-0.14)	0.19 (0.14-0.52)	0.07 (0.01-0.52)
		Cases	169 [2]		94	94	93	450
	Women	Median (Range) [1]	0.01 (0.01-0.01)	0.06 (0.02-0.08)	0.11 (0.09-0.13)	0.16 (0.13-0.20)	0.25 (0.20-0.58)	0.11 (0.01-0.58)
		Cases	81	48	66	68	62	325
	Other lipid species							
CE(15:0)	Men	Median (Range) [1]	0.21 (0.12-0.25)	0.28 (0.25-0.32)	0.35 (0.32-0.38)	0.41 (0.38-0.45)	0.53 (0.45-0.90)	0.35 (0.12-0.90)
		Cases	85	113	94	78	80	450
	Women	Median (Range) [1]	0.28 (0.14-0.32)	0.35 (0.32-0.38)	0.41 (0.38-0.44)	0.48 (0.44-0.52)	0.59 (0.52-0.81)	0.40 (0.14-0.81)
		Cases	83	69	83	46	44	325

CE(17:0)	Men	Median (Range) [1]	0.25 (0.16-0.28)	0.31 (0.28-0.33)	0.35 (0.33-0.38)	0.40 (0.38-0.44)	0.49 (0.44-0.69)	0.34 (0.16-0.69)
		Cases	106	112	77	90	65	450
	Women	Median (Range) [1]	0.28 (0.18-0.31)	0.34 (0.31-0.35)	0.37 (0.35-0.40)	0.43 (0.40-0.46)	0.51 (0.46-0.79)	0.37 (0.18-0.79)
		Cases	95	75	74	38	43	325
DAG(15:0)	Men	Median (Range) [1]	0.21 (0.08-0.25)	0.27 (0.25-0.31)	0.33 (0.31-0.37)	0.41 (0.37-0.46)	0.53 (0.46-1.03)	0.32 (0.08-1.03)
		Cases	122	104	97	58	69	450
	Women	Median (Range) [1]	0.25 (0.13-0.30)	0.33 (0.30-0.37)	0.40 (0.37-0.43)	0.47 (0.43-0.51)	0.59 (0.51-0.88)	0.38 (0.13-0.88)
		Cases	110	101	51	39	24	325
MAG(15:0)	Men	Median (Range) [1]	21.90 (7.64-26.91)	30.29 (26.94-33.24)	35.76 (33.24-38.49)	41.44 (38.52-44.90)	64.72 (45.49-95.58)	34.43 (7.64-95.58)
		Cases	143	91	68	82	66	450
	Women	Median (Range) [1]	25.54 (5.24-29.50)	32.52 (29.50-35.15)	37.65 (35.16-40.24)	42.97 (40.25-47.08)	65.48 (47.13-95.75)	37.05 (5.24-95.75)
		Cases	101	66	48	49	61	325
MAG(17:0)	Men	Median (Range) [1]	0.48 (0.07-0.72)	0.85 (0.72-0.95)	1.01 (0.95-1.08)	1.14 (1.08-1.22)	1.33 (1.22-1.77)	0.99 (0.07-1.77)
		Cases	96	116	86	78	68	444
	Women	Median (Range) [1]	0.36 (0.05-0.73)	0.89 (0.74-0.98)	1.06 (0.99-1.13)	1.19 (1.13-1.28)	1.39 (1.28-1.78)	1.04 (0.05-1.78)
		Cases	67	77	72	66	35	317
TAG(15:0)	Men	Median (Range) [1]	0.28 (0.17-0.33)	0.37 (0.33-0.41)	0.45 (0.41-0.49)	0.55 (0.49-0.62)	0.74 (0.62-1.35)	0.45 (0.17-1.35)
		Cases	75	90	98	94	93	450
	Women	Median (Range) [1]	0.34 (0.20-0.39)	0.43 (0.39-0.47)	0.50 (0.47-0.54)	0.59 (0.54-0.66)	0.76 (0.66-1.19)	0.50 (0.20-1.19)
		Cases	68	79	52	69	57	325
TAG(17:0)	Men	Median (Range) [1]	0.37 (0.26-0.42)	0.46 (0.42-0.49)	0.53 (0.49-0.56)	0.60 (0.56-0.64)	0.72 (0.64-1.04)	0.53 (0.26-1.04)
		Cases	68	87	105	89	101	450
	Women	Median (Range) [1]	0.43 (0.30-0.47)	0.50 (0.47-0.53)	0.56 (0.53-0.59)	0.63 (0.59-0.66)	0.73 (0.66-1.06)	0.56 (0.30-1.06)
		Cases	71	59	70	54	71	325
FFA(15:0)	Men	Median (Range) [1]	0.17 (0.12-0.20)	0.22 (0.20-0.26)	0.30 (0.26-0.35)	0.49 (0.35-0.61)	0.72 (0.61-5.99)	0.29 (0.12-5.99)
		Cases	88	92	106	96	68	450

	Women	Median (Range) [1]	0.19 (0.13-0.22)	0.25 (0.22-0.28)	0.31 (0.28-0.36)	0.55 (0.36-0.65)	0.75 (0.65-5.92)	0.31 (0.13-5.92)
		Cases	59	74	67	65	60	325
FFA(17:0)	Men	Median (Range) [1]	0.75 (0.62-0.78)	0.80 (0.78-0.82)	0.84 (0.82-0.87)	0.89 (0.87-0.92)	0.98 (0.92-1.40)	0.85 (0.62-1.40)
		Cases	91	81	82	93	103	450
	Women	Median (Range) [1]	0.76 (0.64-0.79)	0.82 (0.79-0.83)	0.86 (0.84-0.88)	0.91 (0.88-0.95)	1.00 (0.95-1.28)	0.86 (0.64-1.28)
		Cases	53	63	78	68	63	325

[1] Values expressed as percentages in the group or class.
[2] Due to the very low concentrations of PE(15:0), the analysis was based on Quintile 1 and 2 together as a reference

C15:0, pentadecanoic acid; **C17:0**, heptadecanoic acid; **CE**, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **LPC, l**ysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG,** monoacylglycerols; **OCFA**, odd-chain saturated fatty acids; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **PL,** phospholipids; **PL-C15:0**, phospholipid species containing C15:0; **PL-C17:0**, phospholipid species containing C17:0; **PL-OCFA**, phospholipid species containing odd-chain fatty acids; **TAG**, triacylglycerols.

Supplemental Table 4. Correlation between the odd chain fatty acids (OCFA) concentrations in plasma phospholipids measured by gas chromatography[1] with concentrations of phospholipids containing OCFA measured by mass spectrometry (Metabolon Complex Lipid Panel)[2], based on the subcohort population (n=1137)

OCFA	**Lipidomics analysis (mass spectrometry, Metabolon)**				
	Total Phospholipids	**PC**	**LPC**	**LPE**	**PE**
C15:0	0.90	0.84	0.87	N/A	N/A
C17:0	0.62	0.64	0.66	0.46	0.23

[1] Relative proportion of C15:0 and C17:0 in total plasma phospholipids
[2] Relative proportion of lipids containing C15:0 and C17:0 within individual plasma phospholipid classes and in the total of the phospholipids classes
All p-value <0.0001

LPC, lysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines

Supplemental Table 5. Pairwise correlations between phospholipid species containing odd chain fatty acids, based on the subcohort population (n=1137) [1]

	LPC(17:0)	LPC(15:0)	LPE(17:0)	PC(17:0)	PC(15:0)	PE(17:0)
LPC(17:0)	-	0.71*	0.28*	0.28*	-0.19*	-0.08*
LPC(15:0)	0.83*	-	0.16*	-0.17*	0.25*	0.05
LPE(17:0)	0.69*	0.66*	-	0.05	-0.08*	0.12*
PC(17:0)	0.57*	0.57*	0.45*	-	0.52*	0.21*
PC(15:0)	0.48*	0.64*	0.38*	0.74*	-	0.05
PE(17:0)	0.31*	0.35*	0.33*	0.51*	0.43*	-

[1] Values below the diagonal are adjusted for age and sex, and above the diagonal are additionally mutually adjusted for all other OCFA-containing lipids levels.
*p-value <0.01

Supplemental Table 6. Associations between lipids [1] containing odd-chain fatty acids (OCFA) and incident diabetes among men (n=886, cases=450) and women (n=1000, cases=325) in the EPIC-Potsdam case-cohort study [2]

	PL-OCFA (C15:0 +C17:0)	
	Men	**Women**
Continuous analysis	0.83 (0.74-0.94)	0.52 (0.43-0.62)
Q2 vs Q1	1.24 (0.84-1.83)	0.56 (0.37-0.85)
Q3 vs Q1	0.72 (0.47-1.11)	0.54 (0.36-0.82)
Q4 vs Q1	0.40 (0.25-0.64)	0.36 (0.23-0.56)
Q5 vs Q1	0.54 (0.35-0.84)	0.17 (0.10-0.28)

	C15:0		C17:0	
	Men	**Women**	**Men**	**Women**
Total PL species				
Continuous analysis	0.89 (79-0.99)	0.62 (0.52-0.74)	0.82 (0.73-0.93)	0.51 (0.42-0.61)
Q2 vs Q1	1.07 (0.71-1.62)	0.65 (0.43-0.99)	1.29 (0.87-1.91)	0.46 (0.31-0.70)
Q3 vs Q1	1.04 (0.69-1.57)	0.84 (0.56-1.26)	0.74 (0.49-1.12)	0.40 (0.26-0.62)
Q4 vs Q1	0.69 (0.45-1.08)	0.46 (0.29-0.72)	0.50 (0.32-0.79)	0.31 (0.20-0.48)
Q5 vs Q1	0.64 (0.41-0.99)	0.33 (0.20-0.53)	0.43 (0.27-0.68)	0.13 (0.08-0.22)
PC				
Continuous analysis				0.50 (0.41-0.60)
	0.87 (0.77-0.98)	0.64 (0.54-0.76)	0.83 (0.73-0.93)	
Q2 vs Q1	0.86 (0.57-1.29)	0.65 (0.43-0.99)	1.45 (0.98-2.14)	0.41 (0.27-0.63)

Q3 vs Q1	1.06 (0.70-1.59)	0.83 (0.55-1.25)	0.83 (0.55-1.26)	0.39 (0.26-0.60)
Q4 vs Q1	0.75 (0.49-1.15)	0.56 (0.36-0.86)	0.50 (0.32-0.79)	0.29 (0.19-0.46)
Q5 vs Q1	0.56 (0.36-0.88)	0.33 (0.21-0.54)	0.51 (0.32-0.80)	0.14 (0.08-0.23)
LPC				
Continuous analysis	1.04 (0.92-1.17)	0.78 (0.66-0.92)	0.82 (0.73-0.93)	0.59 (0.50-0.69)
Q2 vs Q1	1.27 (0.81-1.97)	0.56 (0.36-0.87)	1.12 (0.75-1.67)	0.58 (0.38-0.89)
Q3 vs Q1	1.35 (0.87-2.10)	0.82 (0.53-1.26)	0.68 (0.45-1.05)	0.69 (0.45-1.05)
Q4 vs Q1	1.11 (0.70-1.74)	0.78 (0.52-1.18)	0.61 (0.40-0.95)	0.37 (0.23-0.57)
Q5 vs Q1	1.14 (0.73-1.77)	0.49 (0.31-0.77)	0.49 (0.31-0.76)	0.20 (0.12-0.32)
PE				
Continuous analysis	-	-	1.01 (0.88-1.16)	0.96 (0.84-1.11)
Q2 vs Q1			0.65 (0.16-2.68)	1.01 (0.64-1.59)
Q3 vs Q1			0.99 (0.68-1.44)	0.86 (0.58-1.28)
Q4 vs Q1			1.02 (0.70-1.47)	0.95 (0.63-1.43)
Q5 vs Q1			1.05 (0.72-1.52)	0.85 (0.56-1.30)
LPE				
Continuous analysis	-	-	0.85 (0.75-0.96)	0.74 (0.65-0.85)
Q2 vs Q1			1.01 (0.66-1.55)	0.66 (0.44-0.98)
Q3 vs Q1			1.01 (0.66-1.53)	0.65 (0.43-0.98)
Q4 vs Q1			0.74 (0.48-1.15)	0.29 (0.18-0.47)
Q5 vs Q1			0.60 (0.38-0.95)	0.41 (0.26-0.63)

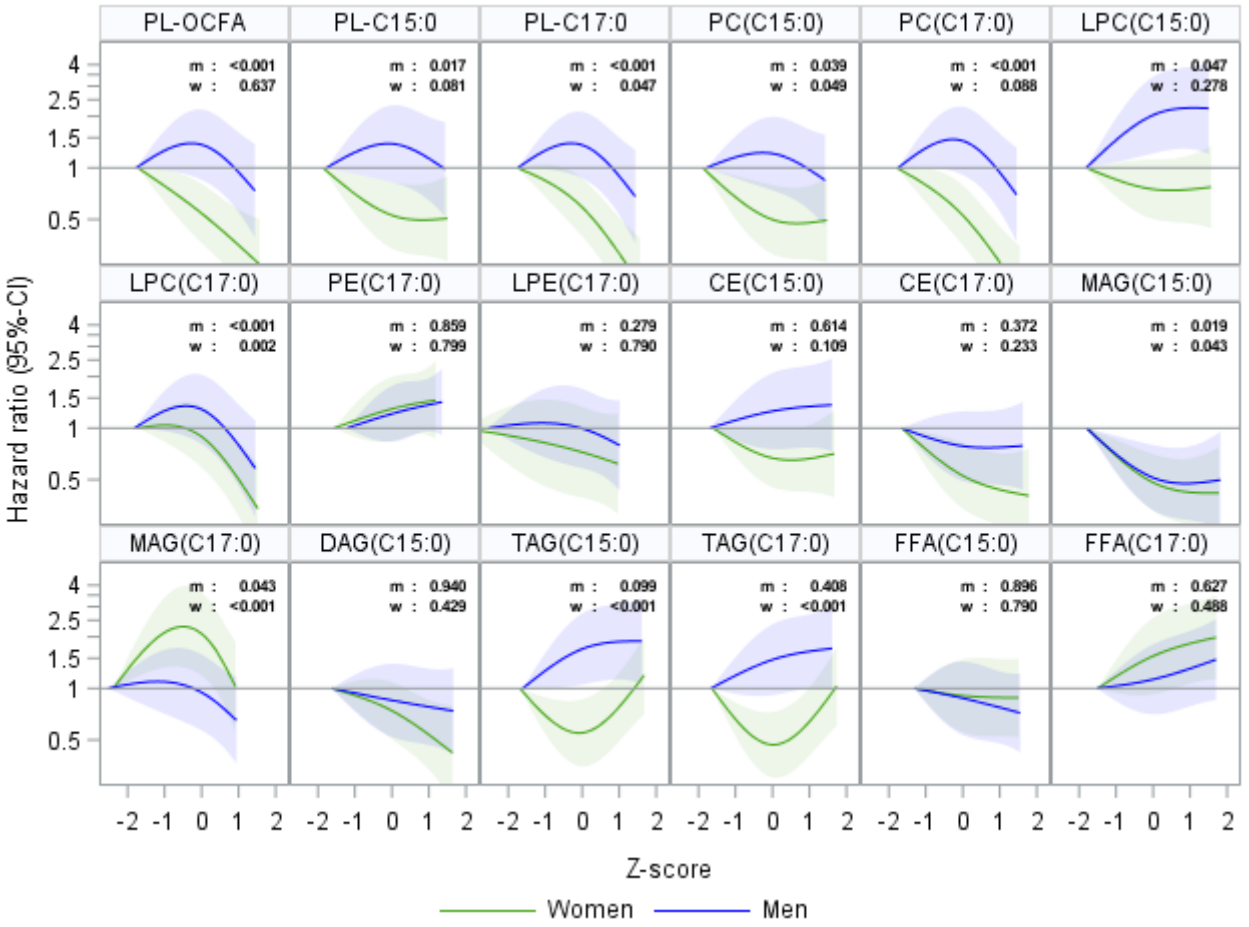
OTHER CLASSES				
CE				
Continuous analysis	0.90 (0.79-1.01)	0.68 (0.57-0.80)	0.83 (0.73-0.95)	0.59 (0.50-0.69)
Q2 vs Q1	1.31 (0.86-1.98)	0.81 (0.53-1.24)	1.14 (0.76-1.71)	0.74 (0.48-1.15)
Q3 vs Q1	1.11 (0.72-1.70)	0.92 (0.60-1.40)	0.59 (0.38-0.92)	0.52 (0.34-0.81)
Q4 vs Q1	0.82 (0.52-1.27)	0.48 (0.31-0.75)	0.85 (0.56-1.29)	0.32 (0.20-0.52)
Q5 vs Q1	0.81 (0.53-1.26)	0.40 (0.25-0.63)	0.50 (0.32-0.79)	0.28 (0.18-0.45)
MAG				
Continuous analysis	0.77 (0.67-0.89)	0.76 (0.65-0.89)	0.93 (0.82-1.07)	0.95 (0.84-1.07)
Q2 vs Q1	0.86 (0.57-1.28)	0.58 (0.38-0.87)	1.11 (0.75-1.66)	1.23 (0.80-1.88)
Q3 vs Q1	0.49 (0.32-0.74)	0.37 (0.24-0.57)	0.88 (0.57-1.35)	1.06 (0.69-1.61)
Q4 vs Q1	0.51 (0.33-0.77)	0.31 (0.20-0.48)	0.79 (0.51-1.21)	0.86 (0.56-1.32)
Q5 vs Q1	0.48 (0.31-0.74)	0.56 (0.37-0.86)	0.72 (0.47-1.10)	0.52 (0.32-0.84)
DAG				
Continuous analysis	0.75 (0.65-0.86)	0.58 (0.50-0.69)	-	-
Q2 vs Q1	0.85 (0.56-1.28)	0.92 (0.64-1.32)		
Q3 vs Q1	0.76 (0.51-1.13)	0.41 (0.27-0.63)		
Q4 vs Q1	0.42 (0.27-0.67)	0.36 (0.22-0.56)		
Q5 vs Q1	0.58 (0.38-0.89)	0.26 (0.16-0.45)		
TAG				
Continuous analysis	1.07 (0.95-1.21)	0.87 (0.74-1.02)	1.10 (0.97-1.24)	0.90 (0.76-1.05)
Q2 vs Q1	1.53 (0.97-2.40)	1.20 (0.79-1.84)	1.38 (0.88-2.15)	0.81 (0.51-1.27)

Q3 vs Q1	1.37 (0.89-2.10)	0.57 (0.36-0.91)	1.61 (1.03-2.52)	0.78 (0.51-1.21)
Q4 vs Q1	1.39 (0.89-2.17)	0.87 (0.57-1.34)	1.37 (0.87-2.15)	0.57 (0.37-0.90)
Q5 vs Q1	1.35 (0.87-2.10)	0.72 (0.46-1.13)	1.41 (0.91-2.18)	0.80 (0.51-1.24)
FFA				
Continuous analysis	0.90 (0.78-1.03)	0.90 (0.78-1.04)	1.03 (0.90-1.18)	0.98 (0.85-1.14)
Q2 vs Q1	0.75 (0.49-1.17)	1.03 (0.66-1.62)	0.88 (0.56-1.37)	0.97 (0.60-1.57)
Q3 vs Q1	1.06 (0.70-1.60)	0.87 (0.55-1.37)	0.81 (0.52-1.26)	1.28 (0.80-2.03)
Q4 vs Q1	0.80 (0.52-1.22)	0.91 (0.59-1.43)	0.99 (0.65-1.50)	0.99 (0.63-1.57)
Q5 vs Q1	0.61 (0.39-0.95)	0.76 (0.48-1.20)	0.97 (0.63-1.49)	0.98 (0.61-1.56)

[1] Lipids derived from lipidomics mass spectrometry analysis
[2] Values are Hazard Ratios (HR) and 95% confidence intervals derived from a multivariable Cox regression model adjusted for age (strata)

C15:0, pentadecanoic acid; **C17:0**, heptadecanoic acid; **CE**, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **LPC, l**ysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG,** monoacylglycerols; **OCFA**, odd-chain saturated fatty acids; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **PL,** phospholipids; **PL-OCFA**, phospholipid species containing odd-chain fatty acids; **Q**, quintile; **TAG**, triacylglycerols.

LPC, lysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines



Supplemental Figure 2. Associations between lipids and incidence of T2D modelled using a restricted cubic spline function with 3 knots placed at the 5th, 50th and 95th percentiles. Shaded areas are 95% confidence intervals. Numbers in the top right of each panel are p-values for men (m) and women (w). Multivariable model adjusted for: age, sex, waist circumference, BMI, cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), education (in or no training, vocational training, technical school, or technical college or university degree), smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), occupational activity (light, moderate, or heavy), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or

.40.0 g/d), coffee intake (energy-adjusted), fiber intake (energy-adjusted), red meat intake (energy-adjusted), total energy intake (kJ/day), fasting status and prevalent hypertension.

CE, cholesteryl esters; **DAG**, diacylglycerols; **FFA**, free fatty acids; **LPC, l**ysophosphatidylcholines; **LPE**, lysophosphatidylethanolamines; **MAG,** monoacylglycerols; **OCFA**, odd-chain saturated fatty acids; **PC**, phosphatidylcholines; **PE**, phosphatidylethanolamines; **PL,** phospholipids; **PL-C15:0**, phospholipid species containing C15:0; **PL-C17:0**, phospholipid species containing C17:0; **PL-OCFA**, phospholipid species containing odd-chain fatty acids; **TAG**, triacylglycerols.

Supplementary Table 7. Associations between free fatty acids (FFA) and incident diabetes among men and women in the EPIC-Potsdam case-cohort study [1,2]

	N	HR (95% CI) [1]	
		C15:0	C17:0
Reference (All participants)	1886	0.91 (0.79-1.04)	1.20 (1.06-1.37)
Fasting	274	1.80 (1.28-2.53)	1.71 (1.08-2.70)
Non-fasting	1333	0.83 (0.70-0.97)	1.19 (1.03-1.38)

[1] Models adjusted for age, sex, waist circumference, BMI, cycling (0, 0.1–2.4, 2.5–4.9, or >=5 h/week), sports activity (0, 0.1–4.0, or >4.0 h/week), education (in or no training, vocational training, technical school, or technical college or university degree), smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), occupational activity (light, moderate, or heavy), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or .40.0 g/d), coffee intake (energy-adjusted), fiber intake (energy-adjusted), red meat intake (energy-adjusted), total energy intake, and prevalent hypertension, total FFA

[2] Men (n = 132) and women (n = 147) who only drank (did not eat) were not included

Supplemental Table 8. Partial correlations coefficients of phospholipid species containing OCFA (PL-OCFA) with fiber-rich foods, adjusted for dairy food variables (total dairy fat, butter, full-fat dairy) in the subcohort population (n=1137)

	Whole grain bread, grain flakes, grains, muesli			**Fruits and vegetables**		
	PL-OCFA	PL-C15:0[1]	PL-C17:0[1]	PL-OCFA	PL-C15:0[1]	PL-C17:0[1]
Reference[2]	0.17	0.15	0.16	0.07	0.04	0.08
+ total dairy fat	0.15	0.13	0.14	0.08	0.05	0.08
+ butter	0.23	0.20	0.21	0.11	0.07	0.12
+ full-fat dairy	0.17	0.15	0.16	0.08	0.05	0.09

[1] PL-C15:0 is the sum of phospholipids PC(C15:0) and LPC(C15:0), and PL-C17:0 is the sum of phospholipids LPE(C17:0), PE(C17:0), PC(C17:0), LPC(C17:0)

[2] Adjusted for age, sex, total energy intake

Supplemental Table 9. Dairy foods, fat from dairy and fiber intake according to quintiles of phospholipid odd chain fatty acids in the subcohort population (n=1137). Values are median (Interquartile range)

		Quintiles of phospholipid species containing OCFA					Total subcohort
		1	2	3	4	5	
Butter (g/1000kcal)	Men	0.65 (0.03-2.92)	0.86 (0.08-4.32)	1.06 (0.07-5.97)	3.58 (0.90-7.18)	7.97 (2.77-13.85)	1.32 (0.15-5.74)
	Women	0.51 (0.06-2.54)	0.93 (0.28-2.73)	0.94 (0.17-6.02)	1.73 (0.36-6.37)	5.18 (1.79-10.88)	1.59 (0.34-6.37)
Low-fat dairy products (g/1000kcal)	Men	5.71 (0-33.69)	17.41 (0-58.75)	7.8 (0-31.88)	3.65 (0-35.84)	1.16 (0-34.19)	5.99 (0-40.76)
	Women	13.4 (1.73-54.34)	19.12 (0.86-84.35)	24.46 (0.74-74.56)	29.37 (1.00-110.4)	14.12 (0-95.89)	21.59 (1-83.92)
Full-fat dairy products (g/1000kcal)	Men	24.15 (14.02-45.25)	29.39 (10.62-52.52)	32.74 (16.23-60.45)	40.40 (20.89-78.18)	35.94 (20.43-79.12)	31.18 (16.04-59.39)
	Women	38.52 (20.52-92.23)	40.45 (17.86-73.12)	54.94 (24.56-91.75)	40.66 (21.38-87.24)	57.26 (25.06-107.00)	48.42 (21.38-92.34)
Low-fat cheese (g/1000kcal)	Men	0 (0-0.32)	0 (0-3.44)	0 (0-0.92)	0 (0-0)	0 (0-0.92)	0 (0-0.84)
	Women	0 (0-2.27)	0 (0-4.55)	0 (0-4.1)	0 (0-2.21)	0 (0-4.54)	0 (0-3.48)
Full-fat cheese (g/1000kcal)	Men	12.03 (4.14-18.35)	12.9 (3.95-21.24)	11.52 (4.88-18.04)	11.62 (5.37-17.45)	14.53 (5.56-18.51)	12.4 (4.57-19.19)
	Women	12.18 (5.25-0)	12.91 (5.02-0)	12.19 (6.1-0)	14.13 (7.62-0)	13.22 (7.01-0)	13 (6.26-0)
Total yogurt (g/1000kcal)	Men	7.21 (0-20.77)	10.95 (1.45-26.67)	8.19 (0.99-30.27)	14.19 (4.02-27.37)	6.27 (0.97-24.34)	8.86 (1.01-24.53)
	Women	25.08 (5.84-43.31)	24.04 (7.25-50.48)	28.93 (8.28-57.03)	28.4 (11.64-57.83)	21.68 (7.4-55.98)	25.13 (8.29-54.35)
Low-fat yogurt (g/1000kcal)	Men	0 (0-0.74)	0 (0-6.98)	0 (0-0)	0 (0-2.31)	0 (0-0)	0 (0-0.6)
	Women	0 (0-5.07)	0 (0-21.86)	0 (0-13.91)	0 (0-28.5)	0 (0-5.94)	0 (0-13.3)
Full-fat yogurt (g/1000kcal)	Men	0 (0-0)	0 (0-4.99)	0 (0-7.24)	0 (0-18.84)	0 (0-8.95)	0 (0-7.47)
	Women	0 (0-15.92)	0 (0-10.3)	0 (0-32.07)	0 (0-16.95)	0 (0-22.35)	0 (0-21.44)

Milk <= 1.5% (g/1000kcal)	Men	0 (0-6.03)	0 (0-32.66)	0 (0-6.58)	0 (0-5.28)	0 (0-0)	0 (0-7.09)
	Women	0 (0-12.47)	0 (0-20.97)	0 (0-18.28)	0 (0-18.28)	0 (0-15.44)	0 (0-18.12)
Milk 3.5% (g/1000kcal)	Men	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-10.86)	0 (0-4.54)	0 (0-0)
	Women	0 (0-1.56)	0 (0-0)	0 (0-1.26)	0 (0-0)	0 (0-4.58)	0 (0-0.92)
Fat from dairy products (g/1000kcal)	Men	3.90 (2.66-5.33)	4.72 (3.2-6.68)	4.32 (2.4-6.28)	5.06 (3.6-6.66)	5.12 (3.93-7.10)	4.50 (2.98-6.31)
	Women	6.31 (3.95-7.72)	5.87 (3.91-7.82)	5.64 (4.2-7.86)	6.40 (4.5-8.36)	6.46 (4.47-8.82)	6.06 (4.28-8.13)
Fiber intake (g/1000kcal)	Men	9.41 (8.04-11.31)	9.83 (8.32-11.3)	9.61 (8.42-11.11)	9.96 (8.34-11.54)	9.4 (8.34-11.26)	9.65 (8.26-11.31)
	Women	10.88 (9.23-12.40)	10.97 (9.52-12.94)	11.07 (9.66-13.06)	11.29 (9.77-13.23)	11.04 (9.59-12.99)	11.09 (9.61-12.90)

Manuscript 3: Plasma lipidomic n-6 Polyunsaturated Fatty Acids and Type 2 Diabetes Risk in the EPIC-Potsdam Prospective Cohort Study

Plasma lipidomic n-6 Polyunsaturated Fatty Acids and Type 2 Diabetes Risk in the EPIC Potsdam Prospective Cohort Study

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ABSTRACT

Aims/hypothesis: The evidence on plasma n-6 polyunsaturated fatty acids (PUFAs) and type 2 diabetes (T2D) risk is inconsistent. We examined the associations of lipid class-specific PUFA concentrations with T2D risk.

Methods: In a case-cohort study nested within the prospective European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort (subcohort=1,137; T2D cases=775; follow-up median 6.5 years), we measured plasma 18:2, 20:3, and 20:4 concentration in 12 lipid classes, arguably reflecting the plasma concentrations of linoleic acid (18:2n-6), dihomo-gamma-linolenic acid (20:3n-6), and arachidonic acid (20:4n-6). Delta-5 desaturase (D5D) activity was estimated as 20:4/20:3-ratio. Associations with T2D were estimated with Cox proportional-hazards models per SD, adjusted for potential risk factors and respective class sum to separate the association from the total of the class.

Results: Higher concentrations of 18:2 were inversely associated with T2D risk, particularly in diacylglycerols (HR 0.68; 95% CI 0.50-0.93), triacylglycerols (HR 0.52; 95% CI 0.36-0.74), and lysophosphatidylcholines (HR 0.69; 95% CI 0.54-0.90), however, monoacylglycerol(18:2) was positively associated (HR 1.40; 95% CI 1.16-1.67). Higher concentrations of 20:3 in phospholipids (HRs 1.14–1.63), free fatty acids (HR 1.28; 95% CI 1.05-1.55), cholesteryl esters (HR 1.45; 95% CI 1.13-1.86), and monoacylglycerols (HR 1.22; 95% CI 1.06-1.41) were linked to higher T2D incidence. Higher 20:4 concentrations were positively associated in free fatty acids (HR 1.30; 95% CI 1.05-1.55) and phosphatidylethanolamines (HR 1.36; 95% CI 1.22-2.17) but not associated in other lipid classes. The estimated D5D activity in phospholipids and cholesteryl esters was associated with lower T2D risk (total phospholipids HR 0.84 95% CI 0.73-0.96 and CE HR 0.78; 95% CI 0.68-0.89). SNPs in the D5D-encoding FADS genes explained relatively high proportions of variation of estimated D5D activity in those lipid classes.

Conclusion: n-6 PUFAs concentrations in plasma were associated differently with T2D incidence depending on FA and the lipid class.

Keywords:

desaturases, diabetes, fatty acids, linoleic acid, lipidomics, lipid metabolism, n-6, omega-6

RESEARCH IN CONTEXT

What is already known about this subject?

- In total plasma phospholipids, higher concentrations of linoleic acid (18:2n-6) are linked to lower type 2 diabetes risk, whereas dihomogamma-linolenic acid (DGLA, 20:3n-6) is associated with higher diabetes risk, and arachidonic acid (AA, 20:4n-6) has not been associated with diabetes risk.
- Estimated delta-5 desaturase activity (AA/DGLA ratio) in total phospholipids is associated with lower type risk.

What is the key question?

- Are the associations of n-6 polyunsaturated fatty acids (PUFAs) concentrations and estimated desaturase activities with T2D risk consistent across sub-classes of phospholipids and other lipid classes determined by plasma lipidomics?

What are the new findings?

- N-6 PUFAs were associated differently with T2D incidence depending on the specific FA and the lipid class.
- Higher estimated D5D activity was associated with reduced T2D risk when determined in phospholipid classes and cholesteryl esters, but not in other lipid classes.

How might this impact clinical practice in the foreseeable future?

- The identified class-specific associations of PUFAs with T2D contribute to the understanding of the role of PUFA intake and lipid metabolism in the aetiology of type 2 diabetes. They provide evidence for the preventive role of plant-based PUFA and potentially provide novel targets for diabetes prevention.

INTRODUCTION

Current dietary recommendations to reduce coronary heart disease risk suggest consuming polyunsaturated fatty acids (PUFAs) in exchange for saturated fatty acids (FA) (1). However, recommendations for type 2 diabetes (T2D) prevention do not clearly emphasise PUFAs as a component of diet quality so far (2). In recent years, prospective studies have evaluated the association of the most abundant dietary and circulating PUFA, linoleic acid (LA, 18:2n-6), with T2D incidence. According to a recent meta-analysis of prospective cohorts, higher 18:2n-6 intake and higher concentrations in adipose tissue and different blood compartments were associated with a decreased T2D risk (3).

Besides the dietary intake of 18:2n-6, its downstream metabolism to arachidonic acid (AA, 20:4n-6) may be relevant for diabetes risk (4). Phospholipid 20:4n-6 was not related to T2D risk in the two largest cohort investigations, European Prospective Investigation into Cancer (EPIC)-InterAct and the FORCE cohort consortium (5, 6). However, associations vary substantially across individual cohorts and may also differ for other blood fractions(6). In contrast, dihomo-gamma-linolenic acid (DGLA, 20:3n-6) has been consistently related to an increased risk of T2D in prospective cohort studies (5). Critical enzymes of PUFA conversion are the delta-5 (D5D) and delta-6 desaturases (D6D) (4). Product-to-precursor FA ratios in phospholipids to estimate the activity of these desaturases were associated with T2D risk in prospective studies, but in opposite directions: estimated D5D activity was associated with lower and estimated D6D activity with higher risk (5, 7). Mendelian randomisation analysis using variants in *FADS1* (encoding D5D) and *FADS2* (encoding D6D) supports a causal role of desaturase activity in T2D development (8).

We have previously observed that associations of FA with T2D risk vary across plasma lipid classes (9, 10). Very few studies have examined T2D risk associations of n-6 PUFAs in different lipid compartments simultaneously. Plasma 20:3n-6 levels were positively associated with T2D risk in phospholipids and cholesteryl esters (CE) but not in triglycerides (TG) in a study of Finnish men (11), whereas another study observed 20:3n-6 positively associated in CE but not in phospholipids (12). Apart from phospholipids, CE, and TG, other n-6 PUFA-containing lipid classes in plasma include, for example, free fatty acids (FFA), other glycerolipids (monoacylglycerols (MG) and diacylglycerols (DG)), and several sphingolipids (ceramides (Cer), dihydroceramides (dhCer), lactosylceramides (LacCer), hexosylceramides (HexCer), sphingomyelins (SM)). In addition, the phospholipid types include phosphatidylcholines (PC), phosphatidylethanolamines (PE), lysophosphatidylethanolamines

(LPE), lysophosphatidylcholines (LPC), and phosphatidylinositol (PI). The T2D risk associations of n-6 PUFAs across these lipid (sub)classes have not been systematically examined. Furthermore, whether the estimated desaturase activity is similar in all lipid classes is still largely unexplored, and, to our knowledge, lipid class-specific associations of estimated desaturase activities with T2D risk have not been comprehensively reported. Moreover, while FADS variants have been associated with desaturase activities in phospholipids (8, 13) and CE (14), other plasma lipid classes have been little explored.

We aimed to evaluate associations of n-6 PUFAs concentrations and estimated desaturase activities with T2D risk across twelve lipid classes from plasma lipidomics profiles in a prospective cohort study. We additionally aimed to evaluate the influence of *FADS* genomic variation on estimated D5D activity across the lipid classes.

MATERIALS AND METHODS

Design and population

The EPIC-Potsdam cohort recruited 27,548 participants (16,644 women aged mainly 35-65 years and 10,904 men aged 40-65 years mostly) from the general population in Potsdam, Germany, between 1994-1998 (15). Consent was obtained from all participants, and approval was given by the Ethical Committee of the State Brandenburg, Germany.

The baseline examination included blood sampling, anthropometry, blood pressure measurements, and the collecting of information on prevalent diseases and sociodemographic and lifestyle characteristics. Blood plasma was stored in liquid nitrogen tanks at -196°C or deep freezers at -80°C until analysis. Follow-up questionnaires were administered every two-three years to identify incident T2D cases. Response rates for follow-up rounds 1, 2, 3 and 4 were 96%, 95%, 91% and 90%, respectively. We considered the questionnaires returned by August 31, 2005.

A case-cohort study nested within the prospective EPIC-Potsdam study was designed for efficient molecular phenotyping (10, 16). From all participants who provided blood samples (n=26,437), we randomly selected the subsample (subcohort, n=1,248) and considered all cases with incident T2D identified during follow-up period (median 6.5 years, interquartile range 6.0-8.7 years) (n=801) (**Supplementary Figure S1**). After excluding participants with missing follow-up information, prevalent diabetes at recruitment, and insufficient blood, the

analytical sample involved 1886 participants, including 775 participants with incident T2D from whom 26 were part of the random subcohort.

Type 2 diabetes ascertainment

Participants with incident T2D were detected by self-report of the diagnosis, antidiabetic medication use, or diabetes dietary treatment, but also based on information from death certificates or sources such as the tumor centers, physicians, and clinics. All potential cases were verified by questionnaires sent to the treating physicianOnly cases diagnosed after the baseline examination date and verified by a physician as T2D (International Classification of Diseases, 10th revision code: E11). were included as incident cases.

Fatty acid and lipidomic profiling

To compare with the concentrations of PUFAs derived from the lipidomics analyses by mass spectrometry (MS), we measured PUFAs in total plasma phospholipids by gas chromatography (GC) with a flame ionisation detector as previously described (9). The proportions of the FA were expressed as a percentage of the total FA present in the chromatogram (13 FAs, including 18:2n-6, 20:3n-6, and 20:4n-6).

The lipidomics analysis was performed by Metabolon using the Metabolon Complex Lipid Panel, as previously described (10, 16). Briefly, lipids were extracted from plasma samples in the presence of deuterated internal standards using an automated BUME extraction. Extracts were infused, and MS analysed with a Shimadzu LC with nano PEEK tubing and a Sciex SelexION−5500 QTRAP mass spectrometer. Upon ionisation, the lipids passed through a SelexIon differential mobility spectrometry (DMS), which separates lipids by class. After the DMS filtering, lipids enter the multiple reaction monitoring, in which both the mass of lipid and the mass of its characteristic fragments are measured. Individual lipid species were quantified by taking the ratio of the signal intensity of each target compound to that of its assigned internal standard, then multiplying by the concentration of internal standard added to the sample.

This platform quantified a total of 940 distinct molecular species concentrations and FA composition across 15 lipid classes (FFA, CE, MG, Cer, dhCer, LacCer, HexCer, SM, LPE, LPC, PI, DG, TG, PC, PE). Two subclasses of PE were also available: phosphatidylethanolamine ether (PEO) and phosphatidylethanolamine plasmalogen (PEP), presented separately from other PE. Measured concentrations of lipid species were used to calculate within-class FA sums (summing concentrations of all lipid species having a specific

FA within each lipid class). We screened 111 within-class FA sums, of which we identified the ones including PUFAs (18:2, 18:3, 20:2, 20:3, 20:4, 22:2, 22:4, 22:5). When summing all the lipids containing each PUFA, total 18:2 was the most abundant (46.8 μM), the second was total 20:4 (13.5 μM), followed by total 20:3 (3.3 μM) (**Supplementary Table S1**). We compared n-6 PUFAs concentration assessed in total plasma phospholipids by the traditional GC with the PUFA relative concentration in all the phospholipid classes measured by MS. The high correlations (18:2n-6(GC)-18:2(MS) r=0.96, 20:3n-6(GC)-20:3(MS) r=0.93 and 20:4n-6(GC-20:4(MS) r=0.91) indicate n-6 conformation. Besides the sum in all phospholipid classes, the class-specific correlations are presented in **Supplementary Figure S2**. Of note, lipids containing 18:3 were also relatively abundant (2.7 μM), however, we did not consider them for our analysis because it remained unclear in our data if they reflect gamma-linolenic acid (18:3n-6 or alpha-linolenic acid (18:3n-3).

Within-class FA sums are synonymous with molecular species level in lipid classes containing only one FA per molecule (FFA, CE, MG, Cer, dhCer, LacCer, HexCer, SM, LPE, LPC). For lipid classes with two or three FA (PC, PE, DAG, TAG), the sum within a class was used for calculations, e.g., all lipids in the class that carry 18:2 were summed. This resulted in 36 lipid variables from 12 lipid classes. For each FA, we also calculated total phospholipid concentration by summing all the phospholipid species with that FA and total plasma by summing all lipids with that FA.

D5D activity was estimated as product-to-precursor ratio (20:4/20:3), calculated for each lipid class.

Genotyping

We genotyped rs174546, a single nucleotide polymorphism (SNP) strongly associated with estimated D5D activity (8) and in strong linkage disequilibrium with other SNPs in that region. We, therefore, used rs174546 to reflect genomic influence on estimated lipid class-specific D5D activity. Detailed information on array types, quality control, and software packages were reported elsewhere (8).

Statistical analyses

We imputed missing information on covariates by sex-specific median imputation (height: n=1) and model-based single imputation (BMI: n=4, blood pressure: n=116). There were no missing values of GC plasma phospholipids FA concentrations GC. We assumed that missing values in lipidomics-based lipid species concentrations were below the limit of quantification.

Lipids with more than 70% values missing were excluded. The remaining were imputed using the "Quantile Regression Imputation of Left-Censored data" approach from the R package imputeLCMD (17). We log-transformed to stabilise skewed distributions and z-scaled (mean=0, SD=1) the lipid species concentrations and ratios.

All descriptive analyses were based on the random subcohort. We evaluated and visualised intercorrelations among the lipidomics-measured lipids using gaussian graphical models (18). In this network model, the edges represent covariance between two lipids that could not be explained by adjustment for any subset of other lipids. The reported correlation coefficients are adjusted for all other lipids.

The longitudinal associations between lipids and diabetes risk were evaluated with Cox proportional hazards models stratified by age in the case-cohort study population, accounting for the oversampling of cases by Prentice weighting (19). We estimated multivariable-adjusted hazard ratios (HR) and 95% confidence intervals (CI), considering lipid concentrations as continuous variables standardised to 1-SD increments in the log-scale. Cox models were adjusted for age, sex, waist circumference, height, leisure-time physical activity, highest achieved education level, smoking status, alcohol intake, fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), standard clinical blood lipid markers: total cholesterol, high-density cholesterol, and TG (except in models using TG class as exposure), medication (antihypertensive, lipid-lowering, acetylsalicylic acid), and respective class sum (to separate the association from the total of the class). In a sensitivity analysis, models were further adjusted for other FA associated with T2D: the odd chain FAs 15:0 and 17:0 (9), and FAs in the de novo lipogenesis pathway (16:0, 18:0, 16:1n-7c, 18:1n-7c) (20). Additionally, we performed multiplicative interaction analyses for sex by including the cross-product to the fully-adjusted Cox proportional hazards models.

Association of rs174546 with estimated D5D activity were assessed assuming an additive genetic model, adjusted for age at recruitment and sex.

All analyses were performed with SAS Enterprise Guide 7.1 and R version 4.1.0.

RESULTS

Baseline characteristics of subcohort participants of the EPIC-Potsdam cohort are presented in **Table 1**. Subcohort participants were 61% women and had a median age of 49 years and a

BMI of 25. Almost two-thirds had secondary education or higher, most were never smokers, and half reported a diagnosis of hypertension.

Table 1. Baseline characteristics of subcohort (n=1137*)

Variable	Median (IQR) or percentage
Women	60.6%
Age (years)	49.4(42.1-57.6)
Body Mass Index (kg/m²)	25.4(23.0-28.0)
Waist circumference (cm)	85.0(75.0-93.5)
Leisure time activity: sport, biking, gardening (hours/week)	5 (2-8)
Education	
Primary school	38.6%
Sec./high school	23.8%
College/higher	37.6%
Smoking	
Never	48.5%
Former	31.5%
Current smoker (<20 U/day)	14.8%
Current smoker (≥20 U/day)	5.2%
Antihypertensive medication	20.0%
Lipid-lowering medication	5.1%
Acetylsalicylic acid medication	10.0%
Alcohol intake (per day)	
0	2.8%
1-6 g	39.3%
6.1-12 g	19.4%
12.1-24 g	19.4%
24.1-60 g	16.8%
60.1-96 g	2.0 %
96 g or more	0.2%
Total energy intake (kJ/day)	8446 (6791-10300)
Systolic blood pressure (mmHg)	127.5 (116.5-140.0)
Diastolic blood pressure (mmHg)	83.0 (76.0-90.5)
Cholesterol (mg/dl)	204.6 (177.9-231.3)
Triglyceride (mg/dl)	106.8 (75.13-162.8)
HDL cholesterol (mg/dl)	55.0 (46.3-64.8)
Prevalent hypertension (self-report)	49.5%

*No participants had missing data

PUFAs concentrations by lipid classes

The distribution of PUFAs (18:2, 20:3, and 20:4) among the lipid classes was heterogeneous, and some of the lipid classes contained none of them (Cer, dhCer, LacCer, HexCer, SM) (**Figure 1**). In the phospholipid classes, 18:2 accounted for 10% (PEO) to 45% (PC) of the total FA concentration in each class (sum of all compounds containing 18:2 within the lipid

class). TG and CE had a median proportion of 46% and 39% of 18:2, respectively, while other classes had considerably lower proportions. In all classes, 20:3 had less than 8% abundance. 20:4 was particularly abundant in PI (48%) and PE subclasses (40% of PEP, 32% of PEO, and 26% of other PE), whereas lower concentrations were found in CE (8%) and FFA (0.4%).

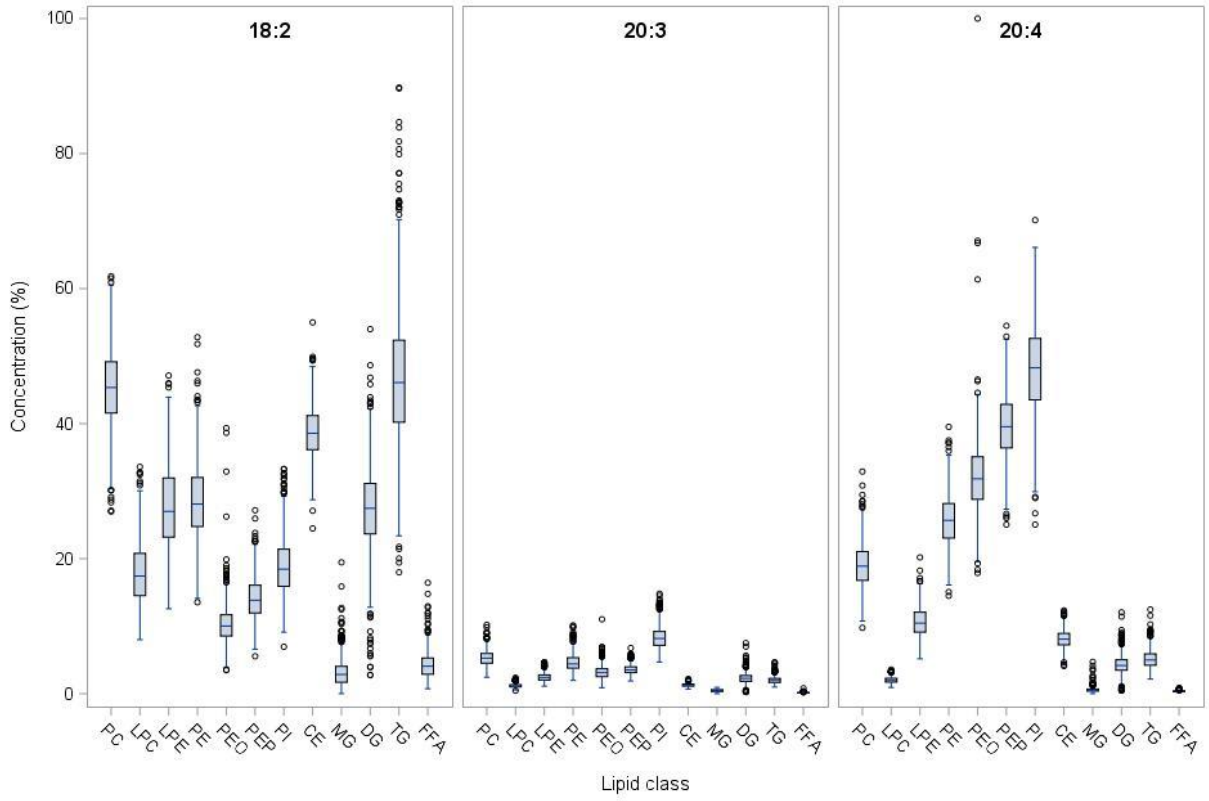

Figure 1. Fatty acid abundance (%) in each lipid class (subcohort n=1137)

Correlation of PUFAs concentrations across lipid classes

Correlations between PUFAs within a class appeared class-dependent (**Supplementary Figure S3A**). For example, 20:3 and 20:4 (biochemically connected through a desaturation step) were most strongly positively correlated in CE (r=0.83) and PI (r=0.79), but less so in PE, MG and PEO (**Supplementary Figure S3B**). Correlations of individual PUFAs across lipid classes were strongest between DG and TG. For example, DG(18:2) was positively correlated with TG(18:2) (r=0.88), as well as DG(20:4) with TG(20:4) (r=0.84). However, these lipids were not significantly correlated with the same FA in the phospholipid classes.

Longitudinal associations with T2D risk

In Cox regression models adjusted for potential confounders and corresponding lipid class sum (thus, reflecting FA abundance within lipid classes), the associations of PUFAs with incident T2D differed according to the lipid class (**Figure 2, Supplementary Table S2**). Higher 18:2 abundance was inversely associated with T2D risk, particularly in LPC(18:2), DG(18:2), and TG(18:2), however, MG(18:2) was positively associated with T2D risk. Total plasma 18:2, total phospholipids 18:2, and some phospholipids classes, traditionally measured to evaluate 18:2 concentrations, were weakly inversely associated with T2D. Higher 20:3 abundance was positively associated with T2D risk in most classes (PC(20:3), PE(20:3), PEP(20:3), PI(20:3), FFA(20:3), CE(20:3), and MG(20:3)), except for DG(20:3) and TG(20:3). The concentration of 20:4 in total plasma, total phospholipids, and most lipid classes appeared not associated with T2D risk, but PEO(20:4) and FFA(20:4) were positively associated with T2D risk. These associations were not influenced by sex (all p-interaction >0.1) or further adjustments for odd chain FA or FA in the de novo lipogenesis pathway (**Supplementary Figure S4**).

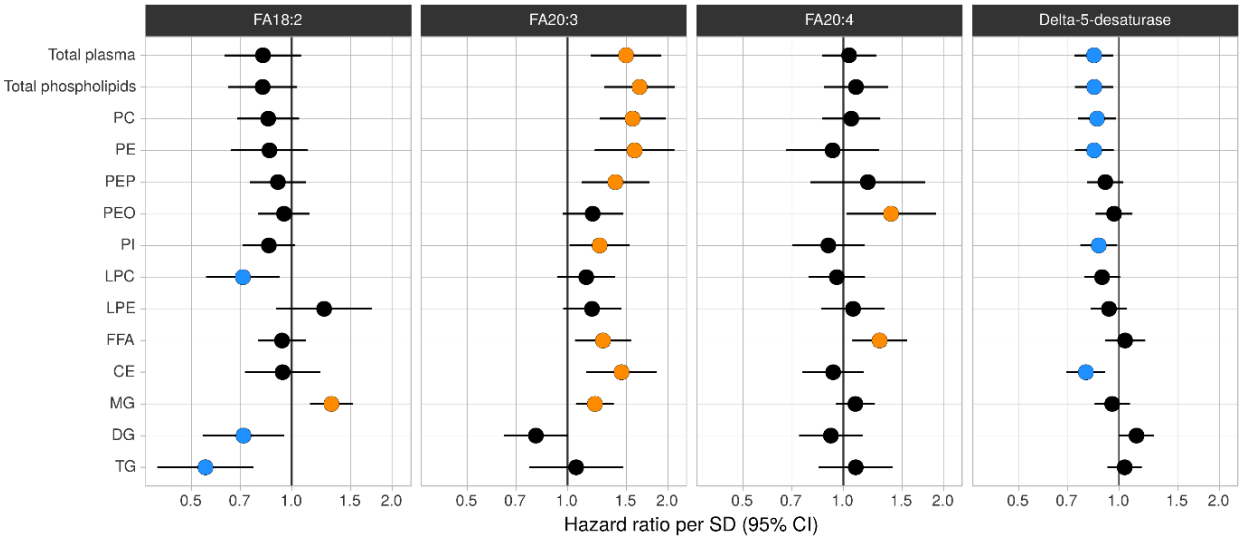

Figure 2. Associations of PUFAs and delta-5 desaturase activities with type 2 diabetes.

Hazard ratios per 1 standard deviation higher plasma concentration, derived from models adjusted for age (as underlying time variable), sex, waist circumference, height, leisure-time physical activity (hours/week), highest achieved education level (in or no training, skilled worker, technical school, or university degree), smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG -except in the TG class), antihypertensive medication, lipid-lowering medication, acetylsalicylic acid medication, and respective lipid class sum (subcohort n=1137, cases=775)

Estimated desaturases activity, *FADS* genotypes, and T2D risk

We found the highest estimated D5D activity (20:4/20:3 ratio) in PE and the subclasses PEP, PEO, followed by CE and other phospholipids classes (PI, LPE, PC), whereas it was lowest in MG (**Supplementary Figure S5**). When correlating estimated D5D activity between different classes, highest correlations were among PC, LPC, and CE (r=0.81-0.87), while the correlations of estimated D5D activity in MG with those in all the other classes were low (r≤0.10) (**Supplementary Figure S6**). Higher estimated D5D activity was significantly associated with reduced T2D risk when determined in phospholipid classes (PC, PE, PEP, PI) and CE, but not in the other lipid classes (**Figure 2**). The inverse association appeared strongest in CE (HR per SD: 0.78, 95% CI: 0.68-0.89) (**Supplementary Table S2**).

Genetic analysis revealed that SNPs within the *FADS* gene region explain variance in the estimated D5D activity, depending on lipid class: 10-18% in phospholipid classes (PC, PI, LPC, PEP, and PE), 15% in CE, 9% in FFA, 5-7% in LPE and PEO, and ≤3% in glycerolipids (**Supplementary Table S3**).

DISCUSSION

In this cohort study, we evaluated associations of n-6 PUFA in the lipidome, including 15 lipid classes and two subclasses, with T2D risk. 18:2 was highly abundant in phospholipid classes (particularly PC), CE, and TG; 20:3 represented a small fraction of FA in most lipid classes, whereas 20:4 accounted for a large proportion of circulating PI and PE. DG(18:2) and TG(18:2) were highly intercorrelated and both inversely associated with T2D risk. While LPC(18:2) was also significantly inversely associated with T2D risk, potentially inverse associations of 18:2 in other phospholipids classes did not reach statistical significance. In contrast, a positive association was observed for MG(18:2). Higher concentrations of 20:3 were associated with higher diabetes risk across most lipid classes, except for DG(20:3) and TG(20:3). 20:4 was unrelated to risk in most lipid classes, although positive associations were observed for PEO(20:4) and FFA(20:4). Explained variance of estimated D5D activity by genomic variation in the *FADS* locus was the highest in PC, PI, LPC, PEP, PE and CE, in which the estimated D5D activity was inversely associated with T2D.

The heterogeneous integration of n-6 PUFAs into lipids across different lipid classes is influenced by diet, endogenous synthesis, preferential oxidation of certain FAs, and the

adipose tissue's metabolic regulation of uptake and release. In the case of phospholipids, the different FA compositions and, therefore, varying membrane properties are regulated by the specificity/selectivity of the enzymes in the phospholipid biosynthesis or remodelling pathways (21). Our results align with previous evidence showing that PI had the highest enrichment of 20:4n-6 of all phospholipids (22), attributed to the acyl chain remodelling by acyltransferases (acyl-CoA:l-acyl-GPC acyltransferase) that selectively incorporate 20:4n-6 into PI (23). In contrast, 20:4n-6 is a poor substrate for TG synthesis (22), as reflected in the low concentrations of TAG(20:4) in our data. Another example of acyltransferase is the lecithin-cholesterol acyltransferase (LCAT), catalyser of the synthesis of CE by transferring one FA from PC to CE, with specificity for 18:2n-6 (24), which explains our observation of a high concentration of 18:2 in CE (39%) and the positive correlation between CE(18:2) and PC(18:2). The LCAT specificity is lower for 20:4n-6, explaining the lower proportion of 20:4 in CE (8%). The diverse distribution of PUFAs across the lipid classes supports the notion of a differential role and metabolic control of PUFAs in the different lipid compartments.

Associations of n-6 PUFAs with T2D risk varied in direction, strength, and precision across different lipid classes. The 18:2n-6 levels in total phospholipids and total serum have been previously related to reduced risk of T2D in prospective cohort studies (5, 6). Our findings indicate that the inverse association of phospholipid 18:2 and T2D is mainly observable in LPC. Other prospective cohort studies have linked higher concentrations of LPC(18:2) with a lower T2D risk (25-28). Noteworthy, LPC(18:2) has also been associated with lower coronary heart disease risk (29). Although the mechanisms underlying the protective effects of LPC(18:2) are not well understood, LPC(18:2) was inversely associated with C-reactive protein and plasminogen activator inhibitor 1 in humans (29). Other LPC metabolites have been reported to stimulate glucose uptake in adipocytes improving glycemia in mice (30).

Our observation of an inverse association of TG(18:2) with T2D risk is in line with a previous prospective analysis in Finnish men (HR= 0.87), although this association was not statistically significant (11). Stable isotope studies indicate that FA in TG reflect dietary FA intake (31), and we have recently shown that higher intake (10% vs 4% energy) of (mainly) n-6 PUFAs increased the concentrations of TG(18:2) in a randomised trial (10). In another intervention study, a diet rich in n-6 PUFA influenced plasma TG(18:2), along with total phospholipids(18:2), CE(18:2), and FFA(18:2) (32). Even though DG(18:2) was not reported in those studies, DG(18:2) was strongly positively correlated with TG(18:2) in our study. Consequently, the inverse associations observed for different 18:2 containing lipids likely reflect the beneficial effects of dietary intake of 18:2n-6 (2, 3).

Noteworthy, MG(18:2) was positively associated with T2D incidence, consistent with previous prospective cohort studies (33). MGs, in general, have been reported to have adverse effects even when containing FA that are protective in other classes (10). MGs have been linked to the control of insulin secretion (34), but mechanistic studies are warranted to explain the opposite T2D risk association of MG(18:2) compared to other 18:2-containing lipid metabolites. Noteworthy, MG(18:2) was linked to a higher coronary heart disease risk, supported by Mendelian randomisation analysis (29).

In our study, higher concentrations of 20:3 were related to an increased T2D risk in most lipid classes. These findings are consistent with prospective studies using total plasma phospholipids (5, 11), total serum (35), and CE (11, 12). We did not find significant associations of 20:3 in TG, similar to one previous study (11). In contrast to the widely available 18:2n-6, dietary intake of 20:3n-6 is negligible. The fact that plasma 20:3n-6 was not increased after diets supplemented with 18:2n-6 (36, 37) suggests that endogenous regulation has more impact on plasma 20:3n-6 concentrations than the dietary intake of the substrate for its endogenous production. In previous analyses, we detected minor influence of dietary PUFA content on 20:3-containing plasma lipids (10). Lower concentrations of 20:3n-6 may result from a reduced enzymatic conversion of its precursors (18:2n-6 and 18:3n-6) or increased desaturation of 20:3n-6 to form 20:4n-6 (4).

Our finding that 20:4 enrichment in most phospholipids classes and CE was not associated with T2D risk is consistent with other prospective cohort studies that measured 20:4n-6 in total phospholipids and CE (5, 6). Nonetheless, in our research, PEO(20:4) was associated with higher T2D risk. The exact mechanism of this association is not evident. We also observed a positive association of plasma FFA(20:4) with T2D risk. In a cross-sectional study, individuals with T2D had higher concentrations of FFA(20:4) compared to controls (38), and FFA(20:4) was higher in subjects with impaired glucose tolerance (39). Still, large prospective cohort studies to compare our results are lacking. Higher secretion of 20:4n-6 from adipose tissue in fasting state and reduced 20:4n-6 uptake and turnover are possible explanations for higher plasma FFA(20:4) concentration (22). FFA(20:4n-6) concentration may be more regulated than other predominant FFA, as suggested by greater stability in its levels (40).

Apart from its role in eicosanoid signalling, 20:4n-6 is involved in gene expression and, because it is part of phospholipids in cell membranes, it influences the cell properties. Endogenous production of 20:4n-6 (and therefore the decrease in the concentration of 20:3n-

6) is regulated by the enzyme D5D, whose activity is commonly estimated with product-precursor fatty acid ratios in different lipid pools in epidemiological studies. The high intercorrelations between the estimated D5D activities in CE and phospholipids classes in the present study confirm results from a previous study (41). In another study, the correlation between D5D in serum total phospholipids and serum FFA was moderate (r=0.31) (42), similar to our observed correlations between plasma phospholipids classes and plasma FFA (r=0.25-0.48). The estimated D5D activities in CE and in adipose tissue were reported to be only moderately correlated (r=0.36) (14). Assuming FFA in plasma are a marker of adipose tissue FA composition, our results are comparable (correlation CE and FFA: r=0.42).

Previous prospective cohort studies observed inverse associations of the estimated D5D activity with T2D risk for total phospholipids (5, 11). Our study confirms and extends these findings by detailing PC, PE, and PI as the phospholipid classes with stronger inverse associations. Estimated D5D activity in CE was also inversely associated in our study as in earlier research (11). We also found that the estimated D5D activities in phospholipids classes and CE were more strongly influenced by genomic variance within the *FADS* locus than the activity estimated in other lipid classes. *FADS1* variants have previously been associated with the estimated D5D activities in plasma phospholipids (8), individual phospholipids species (PC, PE, PI) (13) and CE (14). The consistent associations of estimated D5D activities in plasma phospholipids and CE with T2D risk and with *FADS* variants support that FA composition in these lipid classes reflects the pathogenic involvement of PUFA metabolism in T2D aetiology. In our study, *FADS* variants were not related to D5D activity in glycerolipids (MG, DG, and TG). Thus, FA in glycerolipids may not reflect desaturase activity.

A major strength of the present study is the comprehensive lipidomics data, with three PUFA concentrations that likely reflect 18:2n-6, 20:3n-6, and 20:4n-6 determined across 13 lipid classes in the context of a large prospective cohort study on incident diabetes. However, our study has some limitations. As we focused on relative concentrations of specific PUFA-containing lipids, higher concentrations of one particular lipid may actually be reflecting lower concentrations of other lipids within the class. Still, in our sensitivity analysis, we adjusted for FA commonly associated with T2D (odd-chain SFA, FA from de novo lipogenesis), and the associations remained largely unchanged. Our lipidomics data did not provide information about the conformation (n-3 versus n-6) of the lipids. However, we examined the correlations with several n-6 PUFA measured in total phospholipids by GC in the same study population and found high correlations, supporting that our exposures reflect n-6 PUFA. For lipids with more than one fatty acid (PC, PE, DG, TG), we only considered

one FA, thus, our modelling approach is not sensitive to potential interaction between the FA bound in the same lipid metabolite. Lastly, the population was predominantly white from one region in Germany, limiting the generalisability of the data.

In conclusion, our results indicate a complex variability in the n-6 PUFAs incorporation into different plasma lipid classes. We provide evidence that n-6 PUFAs are associated differently with T2D incidence depending on the specific FA and the lipid class. 18:2 was mostly related to lower T2D risk, most strongly in LPCs, DGs, and TGs. In contrast, MG(18:2) was related to higher T2D risk. While 20:3 was linked to higher risk in most lipid classes, 20:4 was rather neutral – with the exception of PEO(20:4) and FFA(20:4) being associated with higher T2D risk. . Evaluation of estimated D5D activities and *FADS* gene variants further support that the plasma lipidome is an important reflection of T2D-related PUFA metabolism.

SUPPLEMENTARY MATERIALS

Table S1: Concentrations of the sum of each polyunsaturated fatty acid (PUFA) across all the different lipid classes measured in total plasma from the subcohort participants (n = 1137)

Table S2: Prospective associations of PUFAs and desaturases activities with type 2 diabetes (subcohort n = 1137, cases= 775)

Table S3: Associations between rs174546* (*FADS1*) and estimated delta-5 desaturase activity in different classes in subcohort participants (n = 1137)

Figure S1: Participant flow chart

Figure S2: Correlations between n-6 polyunsaturated fatty acids concentrations measured in plasma phospholipids by gas chromatography with the concentrations of lipids containing the same fatty acid in each lipid class (measured by mass spectrometry, Metabolon Complex Lipid Panel), based on the subcohort population (n=1137)

Figure S3: (A) Network showing intercorrelations between lipids containing polyunsaturated fatty acids and (B) Intraclass correlations (lipids from the same class with different FA) and interclass correlations (lipids from different classes containing the same FA), based on the subcohort population (n=1137)

Figure S4: Sensitivity analysis of n-6 PUFAs in different classes and their associations with type 2 diabetes

Figure S5. Estimated desaturases activity (ratio of 20:4/20:3) in each lipid class

Figure S6. Intercorrelations between D5D estimated activity (ratio of 20:4/20:3) in different lipid classes

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Conceptualisation, M.P., F.E. and M.B.S.; Methodology, M.P., F.E., C.W, and O.K.; Software, M.P., F.E., C.W. and O.K.; Formal Analysis, M.P. and F.E.; Investigation, M.P. and F.E.; Resources, M.B.S.; Data Curation, M.P., F.E., C.W. and O.K.; Writing – Original Draft Preparation, M.P.; Writing – Review & Editing, M.B.S, F.E., C.W and O.K.; Visualization, F.E.; Supervision, M.B.S.; Project Administration, M.B.S.; Funding Acquisition, M.B.S.

All authors critically revised the manuscript and approved the final version to be published. MP takes responsibility for the integrity of the data and the accuracy of the analyses.

DATA AVAILABILITY STATEMENT

The datasets analysed during the current study are not publicly available due to data protection regulations. In accordance with German Federal and State data protection regulations, epidemiological data analyses of EPIC-Potsdam may be initiated upon an informal inquiry addressed to the secretariat of the Human Study Center (office.hsz@dife.de). Each request will then have to pass a formal process of application and review by the respective principal investigator and a scientific board.

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SUPPLEMENTARY MATERIAL

Plasma lipidomic n-6 Polyunsaturated Fatty Acids and Type 2 Diabetes Risk in the EPIC Potsdam Prospective Cohort Study

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Table S1. Concentrations of the sum of each polyunsaturated fatty acid (PUFA) across all the different lipid classes measured in total plasma from the subcohort participants (n = 1137)

Variable	Median (µM)	25th Percentile	75th Percentile
Total 18:2	46.82	41.01	55.05
Total 18:3	2.69	2.08	3.58
Total 20:2	0.50	0.39	0.62
Total 20:3	3.27	2.67	3.99
Total 20:4	13.49	11.31	15.95
Total 22:2	0.02	0.02	0.03
Total 22:4	0.34	0.28	0.42
Total 22:5	1.27	1.04	1.51

Table S2. Prospective associations of PUFAs and desaturases activities with type 2 diabetes (subcohort n = 1137, cases= 775)

Parameter		Hazard Ratios (95% Confidence Intervals) *			
		18:2	20:3	20:4	D5D
Total plasma	Model 1	1.53 (1.37-1.71)	2.05 (1.81-2.33)	1.47 (1.32-1.65)	0.60 (0.54-0.67)
	Model 2	0.81 (0.62-1.06)	1.48 (1.16-1.89)	1.03 (0.85-1.24)	0.84 (0.73-0.96)
Total phospholipids	Model 1	0.95 (0.84-1.08)	1.90 (1.66-2.16)	1.30 (1.16-1.47)	0.62 (0.56-0.70)
	Model 2	0.81 (0.64-1.03)	1.65 (1.29-2.10)	1.08 (0.87-1.35)	0.84 (0.73-0.96)
PC	Model 1	0.96 (0.85-1.09)	1.86 (1.63-2.11)	1.27 (1.14-1.43)	0.62 (0.56-0.69)
	Model 2	0.83 (0.66-1.05)	1.61 (1.27-2.05)	1.05 (0.85-1.30)	0.84 (0.73-0.96)
PE	Model 1	1.37 (1.21-1.54)	1.84 (1.63-2.07)	1.49 (1.32-1.67)	0.69 (0.62-0.76)
	Model 2	0.84 (0.64-1.12)	1.63 (1.22-2.17)	0.92 (0.65-1.29)	0.82 (0.72-0.94)
PEP	Model 1	0.89 (0.81-0.99)	1.27 (1.15-1.41)	1.14 (1.03-1.26)	0.81 (0.73-0.91)
	Model 2	0.91 (0.74-1.11)	1.42 (1.13-1.79)	1.19 (0.80-1.76)	0.86 (0.76-0.98)
PEO	Model 1	0.90 (0.82-1.00)	1.21 (1.09-1.34)	1.07 (0.97-1.18)	0.81 (0.73-0.90)
	Model 2	0.95 (0.79-1.13)	1.19 (0.96-1.46)	1.36 (1.00-1.84)	0.95 (0.83-1.09)
PI	Model 1	1.08 (0.97-1.21)	1.56 (1.39-1.76)	1.37 (1.21-1.54)	0.78 (0.70-0.86)
	Model 2	0.83 (0.69-1.01)	1.28 (1.02-1.59)	0.91 (0.70-1.19)	0.86 (0.76-0.98)
LPC	Model 1	0.62 (0.56-0.69)	1.06 (0.95-1.18)	0.83 (0.75-0.93)	0.71 (0.63-0.79)
	Model 2	0.69 (0.54-0.90)	1.14 (0.94-1.40)	0.95 (0.77-1.16)	0.88 (0.78-1.00)
LPE	Model 1	0.84 (0.75-0.93)	1.07 (0.96-1.18)	0.94 (0.84-1.04)	0.83 (0.74-0.93)
	Model 2	1.22 (0.88-1.71)	1.21 (0.98-1.48)	1.06 (0.85-1.34)	0.93 (0.82-1.05)

FFA	Model 1	1.14 (1.04-1.26)	1.38 (1.25-1.53)	1.36 (1.22-1.51)	0.89 (0.81-0.98)
	Model 2	0.93 (0.80-1.09)	1.28 (1.05-1.55)	1.30 (1.06-1.59)	1.01 (0.88-1.16)
CE	Model 1	1.24 (1.12-1.37)	1.61 (1.43-1.80)	1.21 (1.08-1.35)	0.60 (0.53-0.67)
	Model 2	0.95 (0.72-1.24)	1.45 (1.13-1.86)	0.92 (0.74-1.15)	0.78 (0.68-0.89)
MG	Model 1	1.87 (1.64-2.13)	1.51 (1.36-1.68)	1.51 (1.36-1.69)	1.15 (1.05-1.26)
	Model 2	1.40 (1.16-1.67)	1.22 (1.06-1.41)	1.11 (0.95-1.29)	0.95 (0.84-1.07)
DG	Model 1	1.95 (1.74-2.19)	1.76 (1.58-1.96)	1.92 (1.72-2.14)	1.35 (1.21-1.50)
	Model 2	0.68 (0.50-0.93)	0.77 (0.61-0.98)	0.89 (0.70-1.12)	1.09 (0.96-1.23)
TG	Model 1	1.86 (1.67-2.09)	2.04 (1.82-2.29)	2.14 (1.91-2.40)	1.07 (0.97-1.19)
	Model 2	0.52 (0.36-0.74)	1.04 (0.73-1.49)	1.06 (0.80-1.40)	1.01 (0.90-1.14)

*Hazard ratio (HR) and 95% confidence interval (CI) per 1 standard deviation higher plasma concentration of each parameter, derived from covariable-adjusted models:
Model 1: for age (as underlying time variable) and sex
Model 2: for age (as underlying time variable), sex, waist circumference, height, leisure-time physical activity (hours/week), highest achieved education level (in or no training, skilled worker, technical school, or university degree), smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG -except in the TG class), antihypertensive medication, lipid-lowering medication, acetylsalicylic acid medication, and respective lipid class sum

Table S3. Associations between rs174546* (*FADS1*) and estimated delta-5 desaturase activity in different classes in subcohort participants (n = 1137)

Lipid Class	Beta estimate	p-value	R squared (Explained variance)
PC	-0.53	7.54E-51	0.18
PI	-0.75	1.3E-47	0.16
LPC	-0.24	4.26E-45	0.16
CE	-0.57	8.11E-44	0.15
PEP	-1.30	2.85E-42	0.15
PE	-0.95	2.45E-28	0.10
FFA	-0.14	2.04E-25	0.09
LPE	-0.44	2.43E-20	0.07
PEO	-1.34	6.48E-16	0.05
TG	-0.11	7.62E-09	0.03
DG	0.00	0.90544	0.0003
MG	0.00	0.963385	0.0002

***** rs174546 was a lead SNP selected as a proxy of *FADS1* SNPs

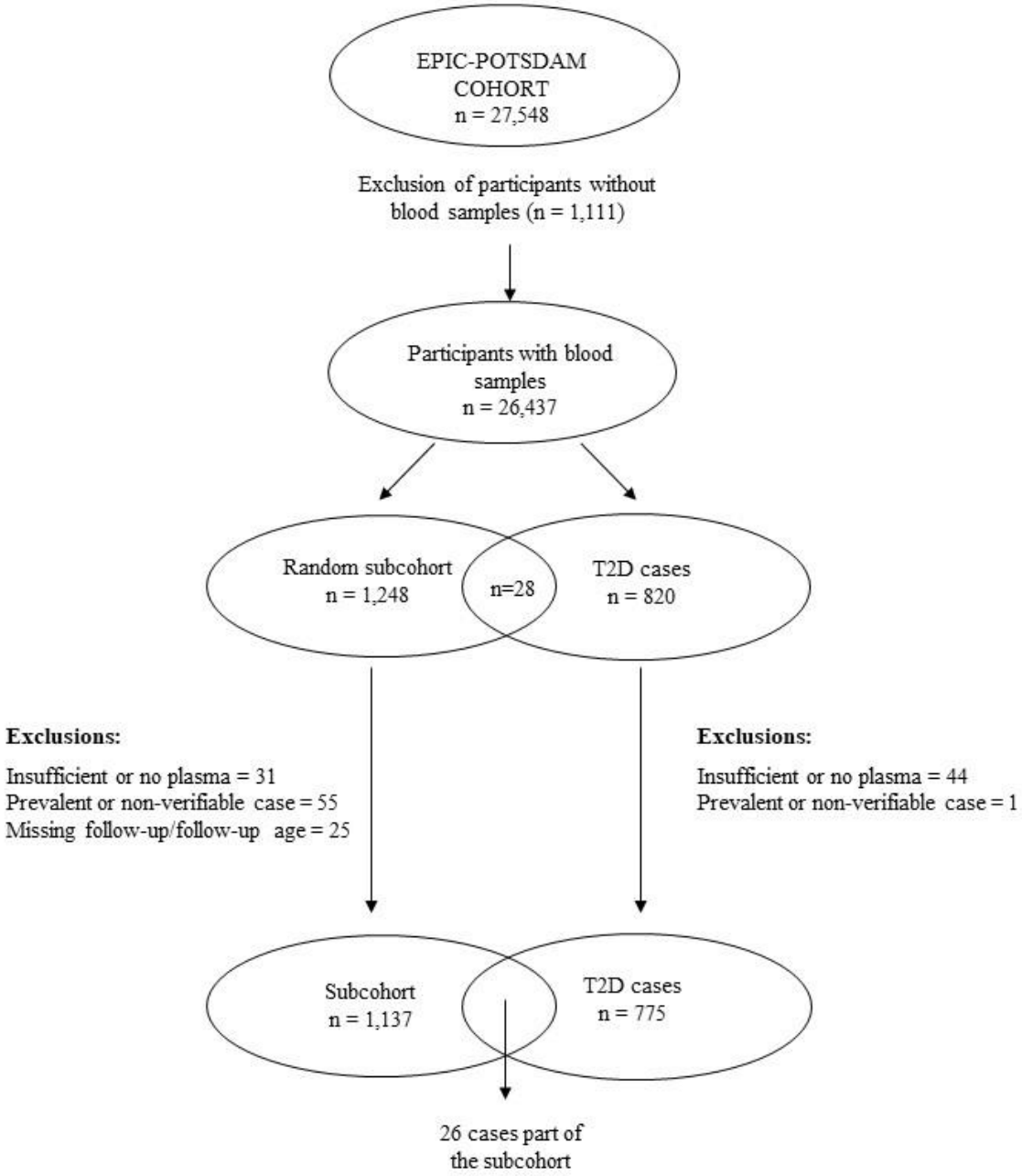

Figure S1. Participant flow chart
T2D, Type 2 diabetes

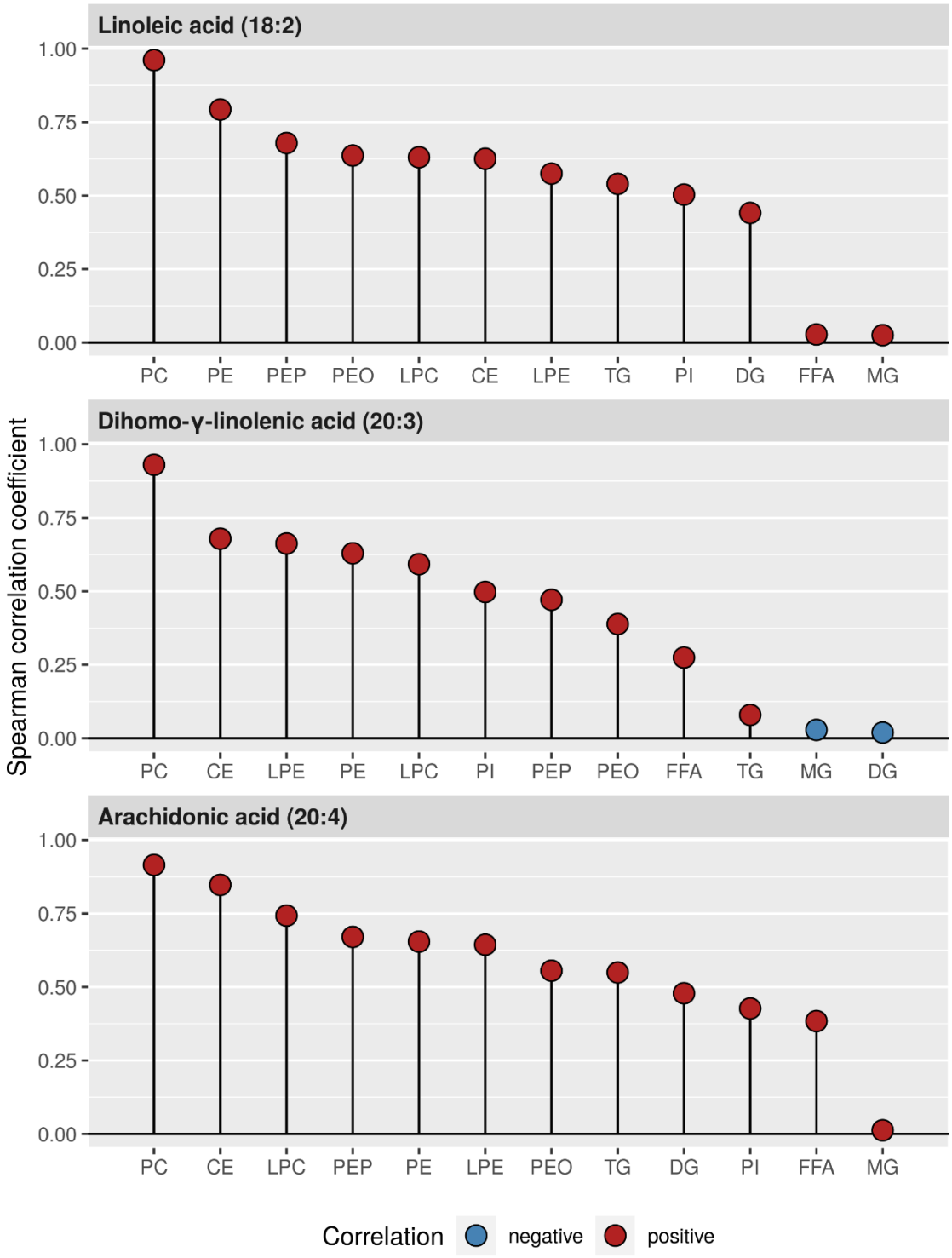

Figure S2. Correlations between n-6 polyunsaturated fatty acids concentrations measured in plasma phospholipids by gas chromatography [1] with the concentrations of lipids containing the same fatty acid in each lipid class (measured by mass spectrometry, Metabolon Complex Lipid Panel) [2], based on the subcohort population (n=1137)

[1] Relative proportion of total plasma phospholipids

[2] Relative proportion of total fatty acids within each class (%)

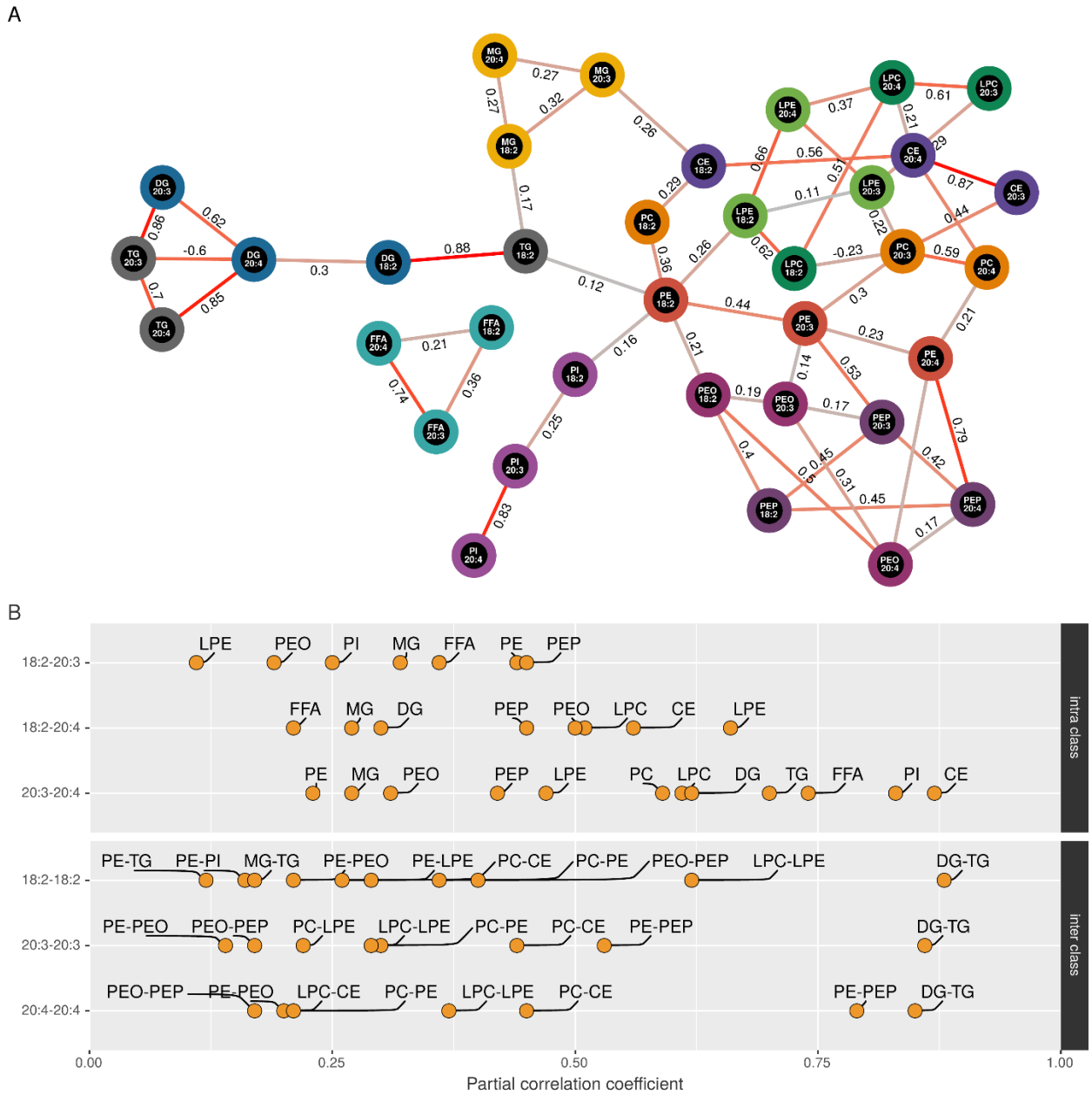

Figure S3. (A) Network showing intercorrelations between lipids containing polyunsaturated fatty acids and **(B)** Intraclass correlations (lipids from the same class with different FA) and interclass correlations (lipids from different classes containing the same FA), based on the subcohort population (n=1137)

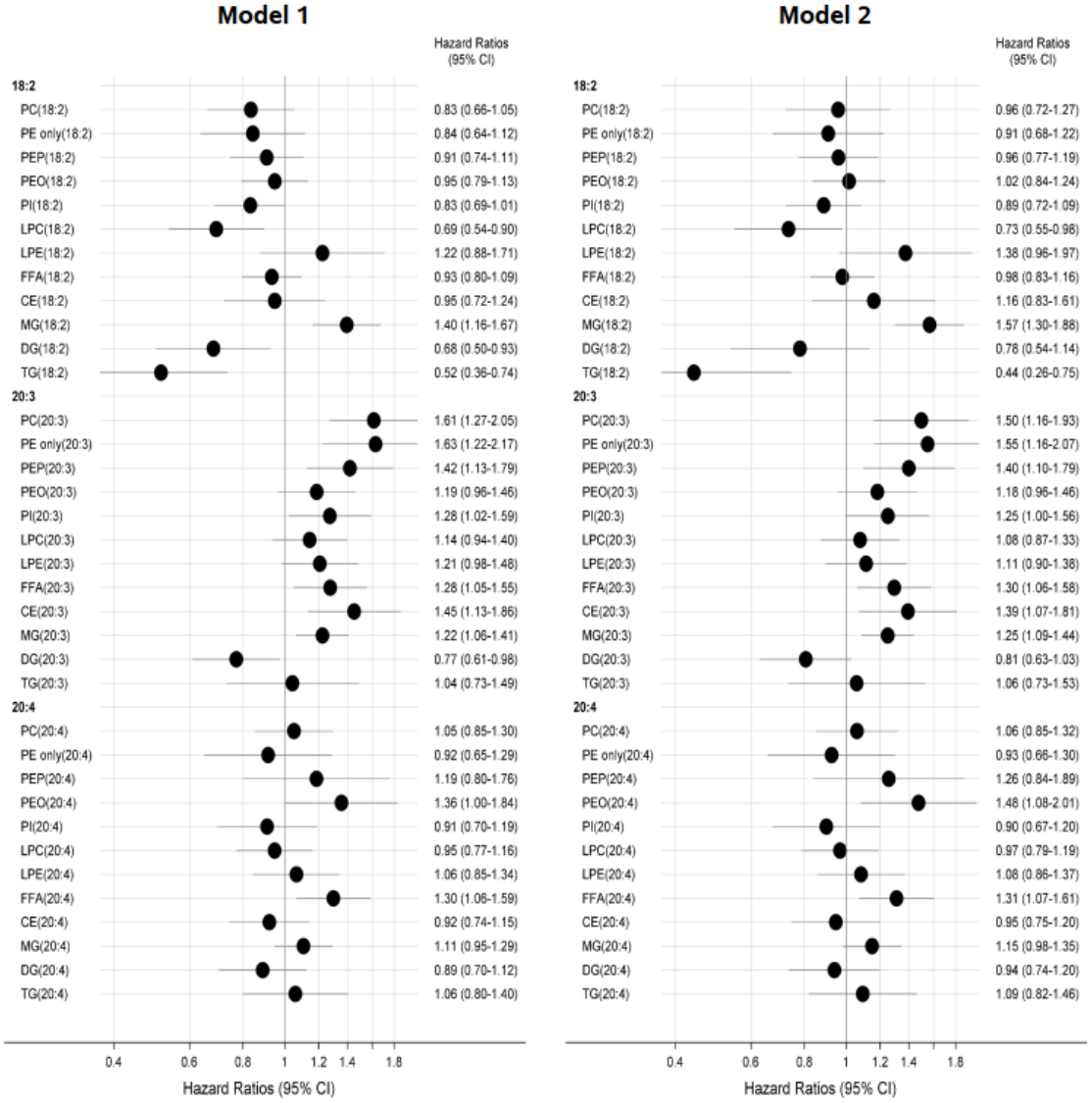

Figure S4. Sensitivity analysis of n-6 PUFAs in different classes and their associations with type 2 diabetes

Model 1: Fully adjusted model age (as underlying time variable), sex, waist circumference, height, leisure-time physical activity (hours/week), highest achieved education level (in or no training, skilled worker, technical school, or university degree), smoking status (never, past, current, <20 cigarettes/day, or current >20 cigarettes/day), alcohol intake (0, 0.1–5.0, 5.1–10.0, 10.1–20.0, 20.1–40.0, or >40.0 g/d), fasting status at blood draw, total energy intake, blood pressure (systolic and diastolic), blood lipids (TC, HDL-C and standard clinical TG -except in the TG class), antihypertensive medication, lipid-lowering medication, acetylsalicylic acid medication, and respective lipid class sum

Model 2: Model 1 with further adjustment for FAs: 15:0, 17:0, 16:0, 18:0, 16:1n-7c, 18:1n-7c

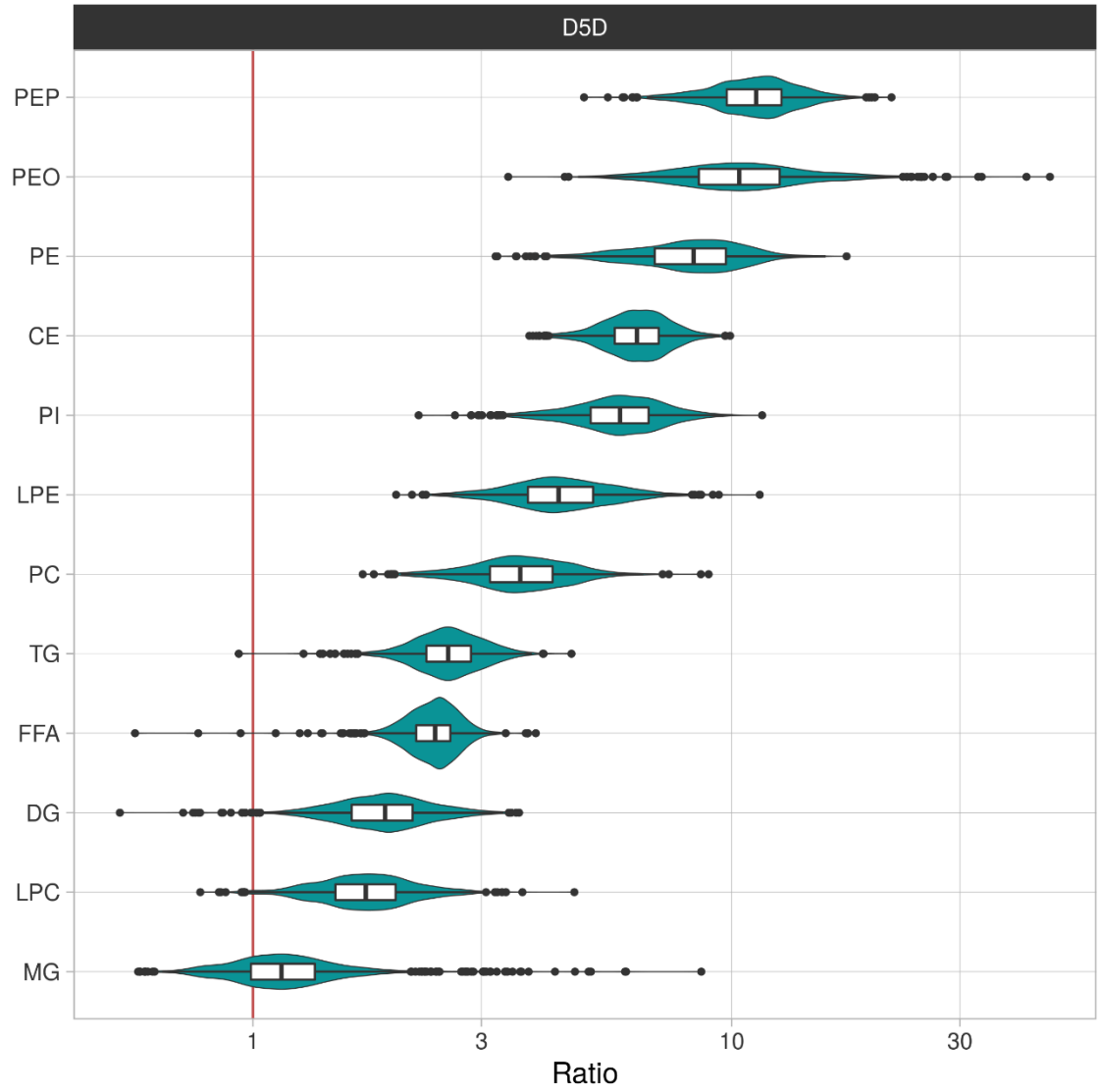

Figure S5. Estimated desaturases activity (ratio of 20:4/20:3) in each lipid class

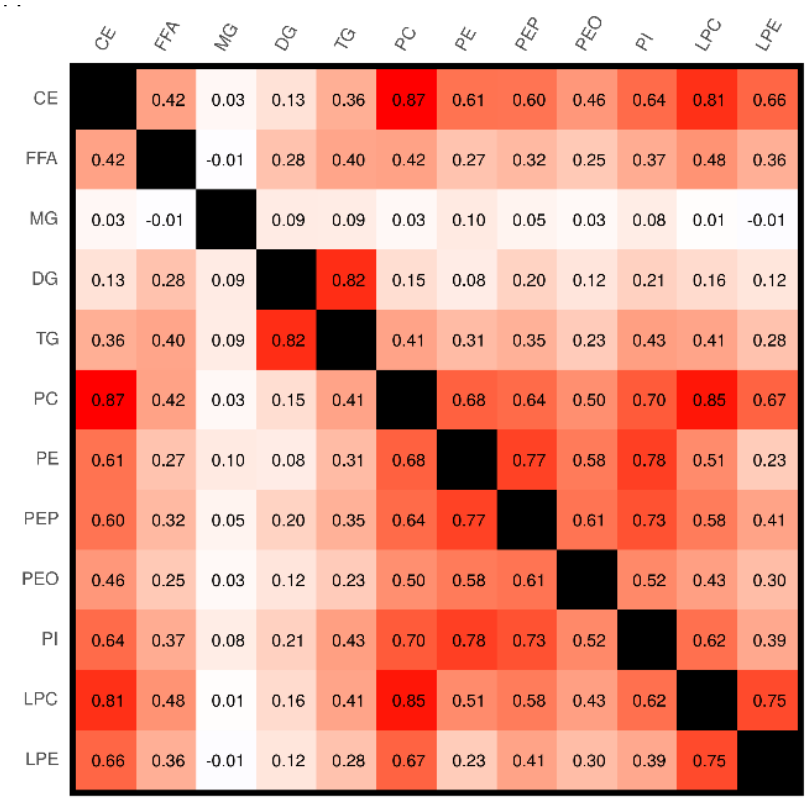

Figure S6. Intercorrelations between D5D estimated activity (ratio of 20:4/20:3) in different lipid classes

5. Discussion

Plasma FAs have been associated with T2D risk, but associations remain poorly understood. Whether industrial TFAs and ruminant TFAs exert the same influence on T2D risk has been unclear. Higher plasma OCFA concentrations were inversely associated with T2D incidence when analysed in phospholipids, however, the OCFA distribution across a range of lipid classes was unknown, and evidence of the associations of the abundance of OCFAs in lipid classes with incident T2D was lacking. Similarly, several circulating n-6 PUFAs were linked to higher or lower T2D risk in prospective studies, depending on their conformation. An open question was whether the associations with T2D depend on the plasma fraction where n-6 PUFAs are measured.

To further understand these associations, this thesis includes the systematical analysis of the association of FAs (TFAs, OCFAs, and n-6 PUFAs) with self-reported dietary intake and prospective T2D risk. The novelty of my thesis relied on the depth of molecular profiling data. It included seven types of TFAs in plasma phospholipids and deep lipidomics profiling data from fifteen lipid classes in prospective nested case-cohort studies within the EPIC Potsdam Study. In each manuscript, the distribution and intercorrelation of the FAs and the prospective association with T2D risk in unprecedented detail and depth were examined. Moreover, the cross-sectional association of TFAs and OCFAs in plasma phospholipids with self-reported dietary intake was analysed. My results extend the evidence on the relationship between diet, lipid metabolism, and subsequent T2D risk. In addition, my work generated several potential new biomarkers of dietary intake and prospective T2D risk and provided insight into early diabetogenic pathways. The three manuscripts contributed to current research by (1) identifying contrasting robust associations of ruminant TFAs subtypes with T2D risk, (2) describing the abundance of OCFAs and PUFAs in diverse lipid classes, (3) discovering class-specific associations of OCFAs and PUFAs with T2D incidence, (4) analysing ruminant TFAs and OCFAs as potential biomarkers of dairy intake.

5.1 Trans Fatty Acids (Manuscript 1)

TFA subtypes

In plasma phospholipids, we identified seven TFAs subtypes. Although most TFAs are found in ruminant and industrial fats, the proportions are different (22). Therefore, we classified

16:1n-7*t*, 18:1n-7*t*, *c*9*t*11-CLA, *t*10*c*12-CLA as ruminant TFA and 18:1n-6*t*, 18:1n-9*t*, 18:2n-6,9*t* as industrial TFA. From the ruminant TFAs, the most abundant was *c*9*t*11-CLA, followed by 18:1n-7*t*, and only a small portion was 16:1n-7*t*. The most abundant of the industrial TFAs was 18:1n-9*t*. The TFAs subtypes were intercorrelated with the other TFAs, other FAs, and different food sources. 16:1n-7t, 18:1n-7t, and c9t11-CLA were positively correlated with the intake of fat-rich dairy foods. Industrial TFA 18:1 isomers were positively correlated with margarine.

Not all ruminant TFAs are the same

In previous meta-analyses of prospective studies, total TFA intake was not associated with T2D risk (26, 61). Total TFA intake includes TFAs industrially and ruminant-derived subtypes. This thesis revealed that the association of TFA subtypes with T2D risk is heterogeneous. Numerous reports indicate that industrial TFA intake is linked with a higher risk of coronary heart disease and all-cause mortality (26), but prospective cohort studies of industrial TFAs and T2D incidence were lacking. When analysing circulating TFA subtypes in total plasma phospholipids, we found no indication of a harmful effect of the three industrial TFA subtypes on T2D risk. Still, it remains justified to recommend reducing industrial TFA intake and from the food supply as part of public health initiatives (62) because of their link with other outcomes (26).

The results in this thesis exposed diverse significant associations of ruminant TFAs with T2D risk. Ruminant TFA intake has been inversely associated with T2D risk in a meta-analysis of prospective cohort studies (26); however, the five analysed studies only examined 16:1n-7*t* (63-67). In our study, 16:1n-7*t* was not associated with T2D risk when accounting for other TFAs and confounders in the regression models. Hence, the previously reported inverse associations of 16:1n-7t with T2D risk may have been confounded by unmeasured TFAs.

Moreover, novel and contrasting associations of the other three ruminant TFAs with T2D risk were identified. After adjustment for confounders and other TFAs, we found inverse associations of 18:1n-7*t* and *t*10*c*12-CLA with T2D incidence, whereas *c*9*t*11-CLA was associated positively. The directions of the associations remained unchanged when models were further adjusted for other FA biomarkers of dairy fat (15:0, 17:0), FAs in the de novo lipogenesis pathway, n-6 PUFAs, and major food correlates (butter, total dairy, margarine). These results provide novel evidence of the association of two CLA isomers in plasma with T2D incidence, as it has not been investigated in other large prospective cohort studies. Furthermore, the inverse associations between 18:1n-7*t* and T2D risk after accounting for

other TFAs are also novel. 18:1n-7*t* was not associated with T2D risk in the Cardiovascular Health Study (67), but their models did not account for other TFAs. Previous evidence on specific ruminant TFA subtypes, their effect on the risk of incident T2D, and the underlying mechanisms for these associations is sparse. The findings in this thesis indicate that further research should consider the TFA conformation carefully.

TFA as potential biomarkers of dairy intake

We found that *c*9*t*11-CLA was correlated with the same dairy fat sources (full-fat dairy, full-fat cheese, butter, desserts) as the other ruminant TFAs. 80% of CLA in dairy products is *c*9*t*11-CLA (68). In intervention studies, *c*9*t*11-CLA plasma concentration increased after consumption of cheddar cheese (69) and dairy products naturally enriched in CLA (by altering cattle feedings) (70-73). Besides dairy intake, the circulating *c*9*t*11-CLA concentration may be influenced by other factors, like endogenous synthesis or oxidation rate. *c*9*t*11-CLA (= *c*9,*t*11-18:2) is likely endogenously synthesised from 18:1n-7*t* (= *t*11-18:1) (74, 75). The strong association with increased T2D risk in our analysis provides a strong rationale to study determinants of plasma *c*9*t*11-CLA availability and its metabolic effects in depth.

Full-fat dairy intake was correlated with plasma phospholipid concentrations of 18:1n-7*t* and *t*10*c*12-CLA, which were associated with a lower risk of T2D. Adjustment for total dairy intake had only a minor impact on the TFA-T2D risk associations. Hence, these FAs may reflect T2D risk factors independent of total dairy intake. However, an alternative explanation for the robust T2D risk association is that FA biomarkers provide a more precise estimate of dairy intake than self-reported habitual consumption in FFQs, and thus, accounting for estimated dairy intake had little impact on the associations. In addition, these FAs may be mediators of potential protective effects of dairy intake (35) or indirect markers of other dairy components (e.g., calcium, vitamin D, proteins, fermentation products) (76). Controlled feeding trials assessing the effects of different dairy types on circulating TFA subtypes may provide conclusive evidence of their applicability as dairy intake biomarkers.

The fact that some ruminant TFA subtypes were associated with T2D in opposite directions could be attributable to being derived from different types of dairy products o dairy products with different TFA profiles. It has been reported that the type of dairy food consumed has different effects on T2D risk (35). For example, significant inverse associations were reported for cheese and yoghurt, while milk consumption showed no association with diabetes risk (35). In addition, the FA profile of the same type of dairy products can be modified through altered cattle feeding regimens. For example, the milk from cows that eat fresh forages, whole

oilseeds, or oil supplements have higher concentrations of *c*9*t*11-CLA (77), and cows eating fish and sunflower oil have higher 18:1n-7*t* (78). Other aspects influencing FA profile are cow genetics, season, farming conditions, and food processing (79). To validate these TFA as biomarkers of intake, it remains crucial to distinguish what exactly they are reflecting (total dairy, dairy fat, or specific dairy subgroups) and what determines their content in food sources and plasma.

5.2 Odd Chain Fatty Acids (Manuscript 2)

Plasma OCFAs distribution across the different lipid classes

The comprehensive lipidomics approach in this study detected OCFAs in the lipid classes of TG, FFA, CE, PC, PE, LPC, LPE, MG, and DG, but not in PI, Cer, dhCER, LacCer, HexCer, and SM. The integration of 15:0 and 17:0 in these lipid classes was heterogeneous. For example, although MGs represent merely 0.1% concentration of total plasma lipids, the relative OCFA abundance in MGs was more than ten times higher than other lipid classes and had a particular enrichment of 15:0. In phospholipid classes (LPC, PC, LPE, PE), 17:0 was more abundant than 15:0. The heterogeneous distribution of OCFAs across the examined lipid classes indicates complex metabolic regulation. Possible mechanisms include differences in the FA-selectivity of the synthesis and degradation enzymes across lipid classes.

Class-specific associations of OCFAs with T2D risk

Pooled analyses of prospective studies have reported inverse associations of 15:0 and 17:0 in total plasma phospholipids with T2D risk (27). Our results extend these findings through detailed analyses of the OCFA-T2D risk associations in specific phospholipid classes. We observed pronounced inverse associations of PC(15:0), PC(17:0), and LPC(17:0) with T2D risk, suggesting enrichment in these specific lipid classes as potential drivers of the inverse association of total plasma phospholipid OCFAs with T2D risk in previous reports. Noteworthy, PC was the most abundant phospholipid and LPC, derived from PC (80), was the third most abundant.

Phospholipids, the main components of cell membranes and part of the surface layer of lipoproteins, play an essential role in cellular signalling (19). The FA composition of PC (and hence a large part of cell membranes) may modulate insulin action (81). LPC are predominantly part of high-density lipoproteins (HDL). The lipid profile of the HDL surface

may influence their polarity, which could contribute to HDL dysfunctionality (82), an aspect that has been implicated with T2D (83). In a cross-sectional analysis, LPC(17:0) was significantly lower in individuals with T2D than in individuals without T2D (84), and in a prospective cohort study, LPC(17:0) was inversely associated with T2D risk (85). Moreover, LPC(17:0) was also inversely associated with incident myocardial infarction (86). Future research on LPC(17:0) as a potential cardiometabolic risk biomarker is warranted.

Moreover, plasma concentrations of the glycerolipids MG(15:0) and DG(15:0) were associated with lower T2D risk. It is unknown if MG(15:0) and DG(15:0) correlate with 15:0 in plasma phospholipids and reflect the same protective effects for T2D incidence (27, 31). Surprisingly, it was not the case for 15:0 as part of TG. In fact, TG(15:0) was associated with a higher risk in men. TG(15:0) was inversely correlated to 15:0 in plasma phospholipids in a cross-sectional study (87). These contrasting associations of 15:0 in plasma glycerolipids is a novel finding that merits further research.

Besides the heterogeneity across lipid classes, the T2D risk associations of OCFAs in this study differed by sex. Higher abundance of 15:0 in PC and DG, and 17:0 in PC, LPC, and CE were inversely associated with T2D risk in women. Higher 15:0 in MG was inversely associated with T2D in men and women. However, higher 15:0 abundance in LPC and TG was associated with higher T2D risk in men, while women with a higher 17:0 FFA concentration were at higher T2D risk. Stronger inverse associations of circulating metabolites involving 15:0 and 17:0 with T2D risk in women were previously reported in a meta-analysis of sixteen prospective cohorts (27). In addition to the potential sex differences in OCFA metabolism, another possible explanation for the sex differences is that OCFA biomarkers reflect different dietary exposures in men and women.

Most prospective studies on T2D risk only measured OCFAs in total phospholipids or CE (16), limiting the comparisons that may be made between multiple lipid classes. The results of this thesis expand the previous evidence by providing class-specific and sex-specific associations that differ in direction and strength of association.

15:0 and 17:0 as potential biomarkers of dairy intake

Plasma phospholipid OCFAs have been proposed as biomarkers of total dairy and dairy fat intakes (33). In our analyses of phospholipid classes, full-fat dairy intake was correlated with the abundance of 15:0 and 17:0. Thus, considering the inverse associations with T2D risk, OCFAs could also be mediators of the previously reported protective effects of dairy intake

(35) or markers of some other dairy components (76). We found that participants with higher yoghurt intake had stronger inverse associations between OCFAs and T2D. The associations of the OCFA biomarkers with T2D may reflect the contributions of specific sources of dairy fat, such as yoghurt. Future studies should aim to investigate associations of OCFA in plasma lipid classes with a diversity of dairy food types.

Their value as dairy biomarkers has been questioned because small concentrations are found in other foods like meats, for example, (38) and because of the possible endogenous synthesis of 17:0 from fibre-rich foods (36). In our study, both 15:0 and 17:0 in different phospholipid classes correlated negatively with red meat intake but were positively correlated with dairy fat intake and fibre-rich foods (whole grains). It should also be noted that, compared to 17:0, 15:0 has been more strongly correlated with dairy fat (33) and, in some studies, 17:0 was more strongly correlated with fibre intake than with dairy intake (88). However, in the phospholipid classes analysed in this thesis, differences between 15:0 and 17:0 in their association with dairy or fibre-rich foods intake were not apparent.

We, however, found stronger inverse associations of concentrations of 17:0 than 15:0 in lipid classes, which was the case in earlier studies with phospholipids and CE (27, 31). We also found stronger inverse associations of OCFAs with T2D in people with higher fibre intakes, and fibre is inversely related to T2D risk (89). The extent to which the inverse associations of OCFAs reflect dairy or fibre intake should be further explored. It thus seems sensible to encourage future research of 15:0 and 17:0 independently, considering the different strengths in associations with T2D and potential differences in food sources.

5.3 N-6 Polyunsaturated Fatty Acids (Manuscript 3)

Class distribution of PUFAS

In this thesis, n-6 PUFAs were quantified as part of TG, DG, MG, FFA, CE, PC, PE, PI, LPC, and LPE. The percentage of 18:2 was high in phospholipid classes, particularly PC. Similar concentrations were found in TG and CE. CE had higher concentrations of 18:2 than of 20:4, also found in a previous study (90). The enzyme lecithin-cholesterol acyltransferase (LCAT) catalyses the synthesis of CE by transferring one FA from PC to CE and has a greater preference for 18:2 compared to 20:4 (91). PI had the highest proportions of 20:4 among all phospholipids, as previous evidence has pointed (92), by the selective incorporation of 20:4 into PI (93). In contrast, TG contained low concentrations of 20:4 because 20:4 is a poor

substrate for TG synthesis (92). 20:3 represented only a small fraction of the FAs in most lipid classes. The heterogeneous distribution of PUFAs across the lipid classes highlights a differential role and metabolic control of PUFAs in the different lipid classes.

Class-specific associations of PUFAs with T2D risk

Pooled analyses of prospective cohort studies have reported inverse associations of 18:2n-6 in total plasma phospholipids (49). Our results extend these findings by identifying LPC as the phospholipid group with the stronger inverse associations for 18:2. Previous prospective cohort studies have also linked higher LPC(18:2) with decreased T2D risk (85, 94, 95). LPC(18:2) was also inversely associated with the risk of developing impaired glucose tolerance (94). Our observations of LPC(18:2) associated with a lower risk of T2D corroborated previous findings. It is noteworthy that LPC(18:2) was also inversely associated with incident myocardial infarction (86) and with incident coronary heart disease (96). The accumulating evidence on the protective role of LPC(18:2) calls for research into biological mechanisms.

We also found higher relative concentrations of 18:2 in DG, and TG associated with lower T2D incidence. TG has been proved to reflect dietary FA intake (97, 98), and specifically, TG(18:2) increased after higher n-6 PUFA intake in randomised trials (17, 99). It is unknown if DG(18:2) also reflects LA intake, however, it was strongly correlated with TG(18:2). Thus, the observations of TG(18:2) and DG(18:2) may reflect the inverse associations of LA intake with T2D that were reported in a large prospective study (100). Nonetheless, the associations between 18:2n-6 intake and T2D are inconsistent when considering other studies (101), whereas the associations of 18:2n-6 biomarkers were more robust (40). Previous observational studies on circulating 18:2n-6 have primarily investigated total plasma/serum, erythrocyte phospholipids, plasma phospholipids, and cholesteryl esters (40). This thesis extends the evidence by showing that DG and TG were also inversely associated in our cohort. It should be noted that higher concentrations of the same FA, 18:2, in MG were associated with higher T2D risk. The reason why MG(18:2) is associated with T2D is currently unknown but stands in line with previous prospective cohort studies that revealed that MG(18:2) was positively associated with T2D incidence (102) and even coronary heart disease (96). These results highlight that the abundance of 18:2n-6 is associated with T2D risk in different directions depending on the lipid class.

Besides the higher concentrations of 18:2 in MG, higher concentrations of 20:3 in MG were also linked to higher T2D incidence, as was 20:3 abundance in other lipids: the phospholipids

(PC, PE, PEP, PI), FFA, and CE. These findings are consistent with different prospective studies using plasma concentrations in total phospholipids (41-44, 47), and CE (30, 42, 103). Our findings provide additional evidence of positive associations of 20:3 in specific phospholipid classes and FFA with higher T2D incidence. The exact biological mechanisms behind these associations are unclear, but CE(20:3) has been associated with insulin resistance (104, 105) and, in total serum, 20:3 was associated with lower insulin sensitivity (106).

The concentrations 20:3 and 20:4 are influenced by the activity of D5D, enzyme that catalyses the insertion of a double bond into 20:3. Unlike the observations for 20:3, we noted that higher concentrations of 20:4 in most PL classes and CE were not associated with T2D risk, as it was also observed in a pooled analysis of prospective cohort studies (49). However, the estimated D5D activities (represented by the ratio 20:4/20:3) in CE and PL classes, which were highly intercorrelated in our study and a previous one (107), were inversely associated with T2D risk. These results align with prospective cohort studies that observed inverse associations of higher estimated D5D activity with T2D risk in total PL (42, 52) and CE (42). Our results confirm and extend these previous findings by highlighting that, among the phospholipid classes, the estimated D5D activity in the PC, PE, and PI had the strongest inverse associations.

Some factors have been reported to be involved in regulating D5D activity, for example, glucagon, adrenaline, glucocorticoids, and thyroxin may depress D5D activity (108). Moreover, accumulating evidence has shown that the estimated D5D activity and circulating n-6 PUFAs concentration are affected by genetic variation (109). The estimated D5D activity in PC, PI, LPC, PEP, PE, and CE were stronger associated with genetic variants within *FADS* genes (desaturase encoding genes), more strongly than in other classes. In agreement with our results, *FADS1* variants were strongly associated with the estimated D5D activities in individual phospholipids species (PC, PE, PI) (56). Similarly, findings from Swedish cohorts also revealed that the lead SNP was significantly inversely associated with estimated D5D activity in serum CE (60). The estimated D5D activity in phospholipid classes and CE was inversely associated with T2D risk, suggesting that the plasma lipidome may reflect T2D-related endogenous PUFA metabolism. In contrast, in glycerolipids (MG, DG, and TG), which were not associated with T2D incidence, the explained variance of estimated D5D activity by genomic variation in the *FADS* locus was not notable, therefore, they may not be good proxies of D5D enzyme activity.

Overall, our results point in the same direction as those who noted an increased risk of T2D associated with a low estimated D5D activity. Furthermore, our results support the notion that the associations of PUFA intake with T2D are likely to be attributable not only to dietary intake but also to genetic variation. The metabolic pathways explaining the differences observed for different lipid classes on their association with T2D risk must be confirmed.

5.4 Strengths and Limitations

The analyses forming the basis of this thesis offered several strengths. **(1)** We had a large sample size within a prospective cohort study. The prospective design decreased the risk of reverse causation because the FA biomarker analyses were conducted before T2D was diagnosed, which allows for the definition of temporal sequence. The longitudinal nature also has the advantage that the outcome is unknown at baseline, preventing selection bias. Because of the detailed baseline assessment of the participants (demographics, metabolic markers, clinical factors, lifestyle), we could account for a wide range of potential confounders. By adjusting the models for confounding factors, the effects of the confounders that could distort the observed associations between the FA biomarkers and T2D risk are reduced. **(2)** The availability of a comprehensive plasma TFA profile with seven different subtypes allowed the identification of TFA biomarkers, an objective exposure measurement (both diet and biological processes). Other studies on TFAs that used self-reported questionnaires (64, 110-115) have limitations in measuring these TFAs intake accurately at very low levels. Furthermore, many studies have only measured one or two TFAs or have collapsed them (total trans 18:1(64)). With the seven TFAs subtypes, we were able to compare the strength of their association with T2D independently of each other, as the concentrations of the other TFAs could be accounted for in the fully adjusted models. **(3)** The comprehensive metabolite profiling approach allowed us to investigate the abundance of OCFAs and PUFAs across lipid classes and their association with T2D. These analyses refined previously reported associations of OCFAs and PUFAs generated commonly from the total phospholipids or total plasma. **(4)** Our study profited from information on dietary intake by combining classical methods of dietary assessments by self-report and the objective biomarkers of intake.

Several limitations must also be acknowledged. **(1)** The observational design cannot provide causal evidence of the effect of any biomarker on the development of T2D. Although the models accounted for extensive relevant risk factors as confounders, the potential for residual confounding remains a possibility that limits causal interpretation. **(2)** The interpretation of the results as biomarkers of intake is limited in terms of how well the FA reflects intake and

to what extent other factors might influence the circulating metabolites. Examples of other factors affecting the concentrations are genetic factors, endogenous synthesis, different rates of digestion and metabolism, and FA storage sites. **(3)** For analysis of OCFAs and PUFAs in different lipid classes, we only considered the PUFA or OCFA concentration in each lipid class for our exposure classification. However, some lipid molecules have more than one FA (PC, PE, DG, and TG). Thus, the diversity of other FAs present in these lipids was not considered. **(4)** We quantified the FAs concentration as percentages instead of absolute amounts. TFAs were expressed as a percentage of total FA concentration in plasma phospholipids. The OCFAs and PUFAs were evaluated as a percentage of the total FA abundance in each class. Such an approach overlooks decreases in other FAs as a consequence of higher percentages of the FA of interest —a higher concentration of a FA is linked to a FA that is reduced, which can be heterogeneous **(4)** The lack of specification of the n-structure of molecular species involving PUFAs (i.e., the position and configuration cis/trans of double bonds) does not allow to be sure of the exact characterisation (n-6 vs. n-3). Still, we evaluated the correlations of PUFAs abundance in lipid classes with several n-6 PUFAs measured previously in total phospholipids, and they were high, supporting that the exposures reflect n-6 PUFAs. **(5)** It should also be acknowledged that not all participants provided fasting blood samples. However, fasting time or most recent food intake before sampling could bias T2D risk associations if linked to the disease status. However, because of the prospective study design, it is unlikely that the future disease status influenced the fasting state. Still, random variation in food eaten before sampling could have contributed to random variation in measured lipids and, therefore, a higher chance of false-negative results. **(6)** The dietary measurements were self-reported with an FFQ, which is limited by recall bias. Still, FFQ is a reliable and valid method (116) and is suitable for ranking participants within a study population according to intake, as was the case in this research. Even so, assessing total dairy intake is complicated by the hidden dairy sources in sauces, dressings, desserts, mixed preparations, and cooking fat. Also, we collapsed subtypes of products into a category. For example, types of cheeses are all included in the cheese category. This could obscure the identification of associations of heterogeneous food groups with the FAs. **(7)** As our study participants were middle-aged people from one region in Germany, the generalisability of these results to other groups is limited.

6. Conclusions and Future Perspectives

Overall, this thesis presented novel evidence about novel lipid biomarkers of T2D risk. The quantification of seven TFAs subtypes in plasma phospholipids and OCFAs and n-6 PUFAs across plasma 15 lipid classes allowed the identification of their altered plasma concentrations preceding the onset of T2D by a median of 6.5 years. Higher plasma concentrations of 18:1n-7*t* and *t*10*c*12-CLA in total phospholipids, MG(15:0), LPC(18:2), DG(18:2), TG(18:2) and, in women, PC(15:0), DG(15:0), PC(17:0), LPC(17:0), CE(17:0) were associated with a lower T2D risk. Higher plasma concentrations of phospholipid *c*9*t*11-CLA, MG(18:2), PC(20:3), PE(20:3), PEP(20:3), PI(20:3), FFA(20:3), CE(20:3), MG(20:3), PEO(20:4), FFA(20:4), and FFA(17:0) in women, and LPC(15:0) and TG(15:0) in men, were associated with a higher T2D risk. The findings confirmed that the conventional classification of FAs into TFAs, n-6 PUFAs, or SFAs is not determining the direction or strength of the associations with T2D risk and disregards biological differences between FAs with different structures. Moreover, the direction and strength of associations depended on the FA and lipid class evaluated.

The results indicated that plasma phospholipid TFAs' relationship with T2D risk depends on their specific conformation. No harmful associations between industrial TFAs and incident T2D were detected, which provides new prospective evidence for the relationship between industrial TFAs and T2D development. Previous studies have already highlighted that a distinction should be made between ruminant and industrial sources. The presented results have further expanded the evidence by showing that some ruminant TFAs have opposing relations to diabetes risk, thus, additional distinctions should be made among ruminant TFAs and even among CLAs. The mild difference in structure (*t*10*c*12-CLA vs. *c*9*t*11-CLA) resulted in significant differences in their impact on T2D incidence. The role of CLA isomers and their determinants could represent a new direction for TFA research.

TFAs and OCFAs in plasma phospholipids associated with reduced T2D risk were also correlated with total dairy intake. A question to be further investigated is whether the analysed TFAs and OCFAs metabolites have causal benefits or if they are markers for other beneficial substances in dairy, specific types of dairy, or other foods associated with dairy intake. Further research should investigate the associations of TFA subtypes and OCFAs in different lipid classes with dairy subgroups (types of cheese, yoghurt) in controlled feeding studies. Furthermore, understanding their determinants and involvement in T2D aetiology may be relevant for food production because of the possible FA profile modification by the cattle feed and other factors related to production.

The findings indicated a heterogeneous distribution of OCFAs and n-6 PUFAs across lipid classes. These FAs were differently linked to T2D risk depending on lipid class and, in the case of OCFAs, sex, which indicates that these variables are essential to consider in epidemiological studies investigating these FA biomarkers. The identified prospective associations of the abundance of OCFAs and n-6 PUFAs across plasma lipidomics profiles with T2D diabetes risk highlight the potential of a detailed approach that characterises diverse lipid metabolites in further prospective studies.

We observed that ratios of 20:4/20:3 (as proxies for D5D activity) across PC, PE, PI, and CE were strongly associated with T2D incidence and were more strongly influenced by genomic variance than the activity estimated in glycerolipids. These findings suggest that the PUFA composition in phospholipid classes and CE may reflect T2D-related PUFA metabolism.

The identified FA abundances associated positively or negatively with the risk of T2D are candidate biomarkers of T2D risk to be confirmed in future prospective observational and interventional research. If their pathogenic importance is confirmed, a better characterisation of their determinants and metabolic pathways involved with T2D is necessary. With the corroboration of the present findings in future research, monitoring these biomarkers will allow identifying individuals at risk, providing evidence-based recommendations and, thus, preventing or delaying the development of diabetes.

In conclusion, my results have contributed to the open question of how TFA subtypes in plasma phospholipids from different sources are associated with T2D risk. The industrial TFAs were not associated with T2D, and the direction of association of ruminant TFAs with T2D depended on the exact conformation. The OCFAs and PUFAs were heterogeneously distributed in plasma lipid classes, and the direction and strength of the longitudinal associations with T2D risk depended on the lipid class. The findings from this thesis expand the growing literature showing that complex FA metabolism is associated with T2D risk and that deeper characterisation through a detailed lipidomics profile is desirable to unravel the associations. The findings of this thesis are a new foundation on which future research can build to explain the mechanisms and causality of the associations between diet, plasma biomarkers, and diabetes.

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This page contains personal data. Hence, it is not part of this online publication.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst und ausschließlich die angegebenen Quellen genutzt habe. Weiterhin versichere ich, die Arbeit bei keiner anderen Hochschule eingereicht zu haben.

Potsdam, den 15.06.2022 Marcela Prada Acuña