Differences in the prospective association between individual plasma phospholipid saturated fatty acids and incident type 2 diabetes: the EPIC-InterAct case-cohort study.

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Summary

Background Conflicting evidence exists regarding the association between saturated fatty acids (SFAs) and type 2 diabetes. In this longitudinal case-cohort study, we aimed to investigate the prospective associations between objectively measured individual plasma phospholipid SFAs and incident type 2 diabetes in EPIC-InterAct participants.

Methods The EPIC-InterAct case-cohort study includes 12,403 people with incident type 2 diabetes and a representative subcohort of 16,154 individuals who were selected from a cohort of 340,234 European participants with 3.99 million person-years of follow-up (the EPIC study). Incident type 2 diabetes was ascertained until Dec 31, 2007, by a review of several sources of evidence. Gas chromatography was used to measure the distribution of fatty acids in plasma phospholipids (mol%); samples from people with type 2 diabetes and subcohort participants were processed in a random order by centre, and laboratory staff were masked to participant characteristics. We estimated country-specific hazard ratios (HRs) for associations per SD of each SFA with incident type 2 diabetes using Prentice-weighted Cox regression, which is weighted for case-cohort sampling, and pooled our findings using random-effects meta-analysis.

Findings SFAs accounted for 46% of total plasma phospholipid fatty acids. In adjusted analyses, different individual SFAs were associated with incident type 2 diabetes in opposing directions. Even-chain SFAs that were measured (14:0 [myristic acid], 16:0 [palmitic acid], and 18:0 [stearic acid]) were positively associated with incident type 2 diabetes (HR [95% CI] per SD difference: myristic acid 1.15 [95% CI 1.09–1.22], palmitic acid 1.26 [1.15–1.37], and stearic acid 1.06 [1.00–1.13]). By contrast, measured odd-chain SFAs (15:0 [pentadecanoic acid] and 17:0 [heptadecanoic acid]) were inversely associated with incident type 2 diabetes (HR [95% CI] per 1 SD difference: 0.79 [0.73–0.85] for pentadecanoic acid and 0.67 [0.63–0.71] for heptadecanoic acid), as were measured longer-chain SFAs (20:0 [arachidic acid], 22:0 [behenic acid], 23:0 [tricosanoic acid], and 24:0 [lignoceric acid]), with HRs ranging from 0.72 to 0.81 (95% CIs ranging between 0.61 and 0.92). Our findings were robust to a range of sensitivity analyses.

Interpretation Different individual plasma phospholipid SFAs were associated with incident type 2 diabetes in opposite directions, which suggests that SFAs are not homogeneous in their effects. Our findings emphasise the importance of the recognition of subtypes of these fatty acids. An improved understanding of differences in sources of individual SFAs from dietary intake versus endogenous metabolism is needed.

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Previous studies of dietary SFA intake have had inconclusive results, limited by measurement error of dietary assessment, and have focused on total SFA intake without analysis of SFA intake varying by carbon chain lengths. The objective measurement of SFAs with different carbon chain lengths in blood fractions enables assessment of individual SFAs. SFAs in blood can be directly interpreted as dietary SFAs for fatty acids that are good biomarkers of intake, such as 15:0 (pentadecanoic acid) and 17:0 (heptadecanoic acid), which are exogenously derived from dietary dairy fats. However, interpretation is more complex for SFAs like palmitic acid (16:0) and stearic acid (18:0), which are synthesised endogenously through de-novo lipogenesis stimulated by increased intake of carbohydrates and alcohol, and which might only partly represent dietary intake. The extent to which different dietary components can induce de-novo lipogenesis varies. Additionally, uncertainties remain about the extent to which dietary SFAs are incorporated into blood SFAs, and the relative contribution of de-novo lipogenesis versus habitual diets to the amounts of SFAs circulating in the blood. However, the varying effects of different blood SFAs on the risk of type 2 diabetes are of scientific and public health interest. Only a few small studies have assessed a range of circulating SFAs, and evidence supporting associations of different blood SFAs with the incidence of type 2 diabetes is scarce.

In this large longitudinal study of the European Prospective Investigation into Cancer and Nutrition Study (EPIC)-InterAct study, we aimed to investigate the prospective associations between objectively measured individual SFAs in the plasma phospholipid fraction and incident type 2 diabetes. We also investigated associations of food consumption with circulating SFAs, and studied SFA metabolism indirectly by analysing ratios of relevant fatty acids.

Methods

Study design and population

The methods of the InterAct project have previously been described in detail. To summarise, we did a case-cohort study that combines the temporal sequence and the measurement efficiency of a case-control study. From the EPIC cohort of 27779 participants, we excluded 483 for whom no fatty acid data were available, leaving 27296 adults, among whom there were 12132 cases of type 2 diabetes and 15919 subcohort participants (including 755 incident cases of type 2 diabetes within the subcohort; figure 1). All participants provided written informed consent and the study was approved by all local ethics committees.

Procedures

Incident type 2 diabetes was ascertained up until December 31, 2007, through a review of several sources of evidence, reported previously, self-report, linkage to primary care registers, secondary care registers, medication use (drug registers), hospital admissions, and mortality data. No diabetes cases were ascertained solely by self-report and we sought further evidence for all cases with information about incident type 2 diabetes from fewer than two independent sources at a minimum, including a review of individual medical records in some centres. Cases in Denmark and Sweden were identified from local and national diabetes and pharmaceutical registers and were judged to be verified.

Figure 1: Case-cohort design of the InterAct study and the number of participants included in this analysis

* A random sample of 2053 type 2 diabetes cases from Denmark was included after the exclusion of 2577 cases.
Fatty acids were profiled at the Medical Research Council Human Nutrition Research (Cambridge, UK); profiling involved analysis of plasma samples stored at baseline at −196°C (or −150°C in Denmark)—a temperature at which fatty acids remain stable. The assay methods have previously been described and included hydrolysis and methylation to convert phospholipid fatty acids into more volatile fatty acid methyl esters and separation of the different fatty acids by gas chromatography ([J&W HP-88, 30 m length, 0.25 mm internal diameter [Agilent Technologies, CA, USA]) equipped with flame ionisation detection (7890N GC [Agilent Technologies]). Samples from people with type 2 diabetes and subcohort participants were processed in random order by centre, and laboratory staff were masked to all participant characteristics by the use of anonymised aliquots.

We identified 37 different fatty acids by their retention times compared with those of commercial standards and expressed each level as percentage of total phospholipid fatty acids (mol%). These fatty acids included nine SFAs with relative concentrations higher than 0.05%: myristic acid (14:0), coefficient of variation 9.4%; pentadecanoic acid (15:0), 11.9%; palmitic acid (16:0), 1.6%; heptadecenoic acid (17:0), 4.2%; stearic acid (18:0), 2.0%; arachidic acid (20:0), 15.3%; behenic acid (22:0), 10.3%; tricosanoic acid (23:0), 18.9%; and lignoceric acid (24:0), 14.7%. We used human and equine plasma (Sera Laboratories International, West Lockhart, CA, USA) to calculate BMI (in kg/m²). Waist circumference was

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<th>Age (years)</th>
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<th>Distribution of fatty acids (mol%) by type 2 diabetes status</th>
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Data are mean (SD). The total subcohort of 15 919 people includes 755 people with incident type 2 diabetes as per the design of a case-cohort study. The 15 164 non-cases represent the subcohort minus the 755 incident cases of type 2 diabetes in this subcohort; this approach enables a comparison of non-cases with cases of type 2 diabetes. *110 individuals in the subcohort had missing BMI values, so BMI data are available for only 15 809 people. 15FA group 1=from 14 or 16, 0, and 18.0. 15FA group 2=from 15.0 and 17.0. 15FA group 3=from 20.0, 22.0, 23.0, and 24.0. 15FA=saturated fatty acid.

Table 1: The distribution of plasma phospholipid saturated fatty acids by categories of age, sex, and BMI in the subcohort of the EPIC-InterAct study, and by type 2 diabetes status
measured by staff, with the exception of French participants and a subset of participants from Oxford, UK, who self-reported their measurements, and in Umeå, Sweden, where this parameter was not recorded.

We used lifestyle questionnaires to assess demographics, smoking status, medical history, and education level. Physical activity was assessed by a validated questionnaire from which a four-point ordinal category of activity was derived. We assessed habitual diet during the past 12 months at baseline using country-specific validated food frequency questionnaires or diet histories. Total energy and nutrient intakes were based on the standardised EPIC Nutrient Database.24 HbA1c was measured in the erythrocyte fraction from samples stored at –196°C using the Tosoh-G8 HPLC analyser (Tosoh Bioscience, Japan) at Stichting Huisartsen Laboratorium (Etten-Leur, Netherlands).

Statistical analysis
We analysed the distribution of individual plasma phospholipid saturated fatty acids and expressed them as mol%.

We estimated country-specific hazard ratios (HRs) and 95% CIs for associations per one standard deviation (SD, as the underlying timescale), study centre, sex, physical activity index, smoking status, and education level. Model 2 included these parameters plus total energy intake, alcohol intake, and BMI. After recording patterns of association for the nine individual SFAs, we made a post-hoc decision to create three additional exposures based on groupings of SFAs that fit with potential biological action: group 1 (sum of the even-chain SFAs 14:0, 16:0, and 18:0); group 2 (sum of the odd-chain SFAs 14:0, 17:0, and 19:0); and group 3. Since stearoyl-CoA desaturase-1 catalyses the desaturation of 16:0 to 16:1(n-7) and of 18:0 to 18:1(n-9) through the de-novo lipogenesis pathway, we also estimated stearoyl-CoA desaturase-1 activity using product-to-precursor ratios (ratio of 16:1[n-7] to 16:0 and of 18:1[n-9] to 18:0)25,26 and assessed each ratio for its association with incident type 2 diabetes.

In a sensitivity analysis based on model 2, we analysed the effects of adjustment for dietary variables (intakes of meat, fruit and vegetables, soft drinks, total dairy products, and carbohydrates [g/day]). We also did an analysis that further accounted for baseline HbA1c value, as a covariate.

To minimise the possibility of reverse causality, we also excluded 2348 people with HbA1c of 6.5% or higher at baseline or those confirmed as cases of type 2 diabetes and dietary intake;9,11,14 group 2 (sum of the odd-chain SFAs) and dietary intake;9,11,14 group 3 (sum of the even-chain SFAs). Since stearoyl-CoA desaturase-1 catalyses the desaturation of 16:0 to 16:1(n-7) and of 18:0 to 18:1(n-9) through the de-novo lipogenesis pathway, we also estimated stearoyl-CoA desaturase-1 activity using product-to-precursor ratios (ratio of 16:1[n-7] to 16:0 and of 18:1[n-9] to 18:0)25,26 and assessed each ratio for its association with incident type 2 diabetes.

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We postulated that circulating SFAs would be derived from diet and through de-novo lipogenesis, and associated with carbohydrate and alcohol consumption.\(^9,11,12\) Within the subcohort, we studied associations between each circulating SFA and food intakes, using Pearson correlation coefficients and 95% CI adjusted for age, sex, BMI, and energy intake. We used Stata, version 13.1 for all analyses.

### Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Results

In the subcohort participants (mean age 52.3 years [SD 9.2]), SFAs comprised 46% (SD 1.2%) of the total phospholipid fatty acids, with the greatest contributors being the even-chain fatty acids 16:0 (mean 30.1% [SD 1.7%]) and 18:0 (14.3% [1.3%]), whereas 15:0, 17:0, and 20:0–24:0 were present in low relative concentrations (all <0.5%) (table 1). Older adults, those with higher BMI, and men had higher relative concentrations of even-chain SFAs (14:0, 16:0, and 18:0), whereas we noted the opposite for longer-chain SFAs (20:0, 22:0, 23:0, and 24:0) (table 1). Relative concentrations of odd-chain SFAs (15:0 and 17:0) were higher in people with a lower BMI and in women, but were similar across different age groups (table 1). Appendix p 2 shows SFA distribution by country.

Model 2 in table 2 shows that even-chain SFAs were each positively associated with type 2 diabetes risk individually and when combined (SFA group 1 HR 1.43 [95% CI 1.29–1.58]). By contrast, odd-chain SFAs were each inversely associated with type 2 diabetes individually and when combined (SFA group 2 HR 0.69 [95% CI 0.60–0.80]). Longer-chain SFAs were each inversely associated with type 2 diabetes individually and when combined (SFA group-3 HR 0.70 [95% CI 0.66–0.74]). The results were similar when 23:0 was included in SFA group 2 and removed from group 3 (data not shown). Sensitivity analyses showed the same results (table 2 and appendix p 3). Analyses with quintiles of the fatty acid distributions led to similar conclusions in terms of direction and significance of associations (appendix p 5).

Among estimated markers of stearoyl-CoA desaturase-1 activity, the ratio of 16:1(n-7) to 16:0 was significantly positively associated with type 2 diabetes, but the ratio of 18:1(n-9) to 18:0 was not (table 2; appendix pp 3–5).
All three groups of SFAs were modestly or weakly correlated with foods, but with overall distinct patterns across fatty acid groups (figure 3). Even-chain SFAs were positively associated with alcohol, soft drinks, margarine, and potatoes, and negatively associated with fruit and vegetables, and both olive oil and vegetable oil (figure 3A). By contrast, odd-chain SFAs generally showed positive associations with dairy products, cakes and cookies, nuts and seeds, and fruit and vegetables, but negative associations with red and processed meat, soft drinks, alcohol, and margarine (figure 3B). The overall correlations for longer-chain SFAs were similar to, but weaker than, those for odd-chain SFAs (figure 3C).

**Discussion**

In this large prospective case-cohort study, we investigated the association of nine individual SFAs with the risk of type 2 diabetes, and recorded distinct patterns of association. Even-chain SFAs (14:0, 16:0, and 18:0) were positively associated with the incidence of type 2 diabetes, whereas odd-chain SFAs (15:0 and 17:0) and longer-chain SFAs (20:0, 22:0, 23:0, and 24:0) were inversely associated with type 2 diabetes. Our findings of the differential associations between nine individual SFAs and type 2 diabetes therefore provide the strongest evidence so far for differential associations between nine individual SFAs and type 2 diabetes (panel).

The existing evidence around this topic is inconclusive and is based on only a few studies with small sample size, in which the number of participants with type 2 diabetes ranged from 34 in a Finnish study to 673 in a German study. Null associations have been recorded for the even-chain SFAs 14:0, 16:0, and 18:0, although positive associations have also been reported for 16:0 and 18:0. Some studies reported an inverse association between type 2 diabetes risk and odd-chain SFAs 15:0 and 17:0, whereas one showed an inverse association only for the sum of 15:0 and 17:0, and one a non-significant association with 15:0. Only one study reported a significant association with longer-chain SFAs, but showed a positive association for 24:0 (unlike the inverse association recorded in our study). Our findings with 12,132 cases of type 2 diabetes therefore provide the strongest evidence so far for differential associations between nine individual SFAs and type 2 diabetes (panel).
labeled dietary fatty acids show that some incorporation of these SFAs into phospholipids occurs,\(^{11}\) and evidence also indicates that de-novo lipogenesis is reduced when dietary starch is substituted for sugar, which suggests the importance of carbohydrate type.\(^{11}\) The extent to which de-novo lipogenesis might also contribute to the circulating levels of these SFAs could be quite low under conditions of relatively high-fat habitual diets in free-living Western populations.\(^{11}\) Because existing knowledge is insufficient, future research is needed to quantify the effects of habitual diets and metabolic state on blood concentrations of SFAs. Importantly, measurements of even-chain SFAs in the blood should not be used to directly interpret dietary SFAs.

Given that de-novo lipogenesis is the underlying process for increased levels of even-chain SFAs, this pathway might increase the risk of type 2 diabetes through hepatic steatosis and related mechanisms.\(^{11}\)

Direct effects of even-chain SFAs could also promote the development of type 2 diabetes: biochemical studies indicate toxic effects specifically of 16:0, including activation of inflammatory cytokines and lipotoxicity to pancreatic β cells.\(^{11}\) Epidemiological research cannot address biological mechanisms and should therefore be complemented by experimental studies.

Our finding of positive correlations of dairy products with odd-chain SFAs (15:0 and 17:0) is consistent with previous evidence that these SFAs are markers of exogenous origin dairy fat intake.\(^{6,14}\) Although residual confounding by other dairy components such as vitamin D or calcium cannot be ruled out, and nor can the possible effects of processes related to fermentation of dairy products,\(^{6,10}\) our strong findings for odd-chain SFAs in the blood lend support to accumulating evidence of an inverse association between dairy products and type 2 diabetes risk.\(^{4,14}\)

Our report of an inverse association between the very-long-chain SFAs and type 2 diabetes provides the largest appraisal so far of an as-yet under-researched group of SFAs that might undergo distinct fatty acid metabolism through peroxisomal fatty acid oxidation.\(^{21}\) To our knowledge, the sources and metabolic effects of very-long-chain SFAs are largely unknown, and our new findings should provide the impetus for further research into this group of fatty acids.

The main limitation of our study was that SFAs were measured at one timepoint only, and intra-individual variation over time is likely. However, we would not expect any errors to be differential with respect to case status. We could only assess relative—not absolute—concentrations of fatty acids (in mol%), but this valid approach is often used in epidemiological research and tends to provide a better interpretation of metabolic inter-relationships than do absolute measurements.\(^{6}\) Fatty acids were measured in long-term stored samples, but stability is likely given that samples were stored at −196°C.\(^{6}\) Plasma phospholipid SFAs might be indicative of both dietary and metabolic influences, affected by interplay of complex exogenous and genetic factors, which we were unable to tease out. This raises caution against inferring dietary consumption on the basis of measurements of SFAs that are not exclusively exogenously derived. Our indirect estimation of fatty acid desaturation ratios has limited use as a biomarker of stearoyl-CoA desaturase-1 activity,\(^{6}\) but direct measures involving liver biopsies or tracer techniques are not feasible in epidemiological studies. Our outcome ascertainment was complete for the entire cohort and therefore does not have the problem of ascertainment bias consequent on a requirement for attendance at a follow-up visit, but was limited by a reliance on clinically incident diabetes. However, we minimised false positives by applying rigorous verification criteria to ensure that no case was included unless verified by at least two independent sources. Although false negatives were also possible because of undiagnosed incident diabetes, such
misclassification can be assumed to be non-differential with regard to the exposure and therefore any potential bias would be unlikely to change our conclusions based on a relative scale (HR) in our analysis. We also minimised the potential for reverse causation bias from undiagnosed prevalent diabetes by applying three separate sensitivity analyses to test the robustness of our results: adjustment for baseline HbA1c, exclusion of those with raised HbA1c at baseline, and exclusion of those with diabetes diagnosed within 2 years of baseline.

The strengths of our study include the large sample size, prospective study design and hence the ability to study the temporality of association with type 2 diabetes, long follow-up, the inclusion of populations from eight European countries with diverse dietary intakes, comprehensive type 2 diabetes case ascertainment and verification, adjustment for many potential confounders, and a series of sensitivity analyses. We were able to analyse the associations of type 2 diabetes with a large number of objectively measured SFAs.

In conclusion, our findings indicate that different individual plasma phospholipid SFAs are differentially associated with risk of type 2 diabetes, which supports the importance of recognising that individual blood SFAs exert heterogeneous effects. Further research into an increased understanding of dietary versus endogenous metabolic sources of circulating individual SFAs will help to inform updated public health messages about the dietary intake of saturated fats and their sources.

Contributors
SJS and NGF had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. AK was responsible for the measurement of fatty acids, LW and K5 did the fatty acid measurements, SJS did the statistical analyses, and NGF drafted the report. Working group members helped to improve the report (AK, SJS, FT, JK, MBS, FC, JMH, MG, JWB, GJvW, CL, and NJW). NJW is the co-ordinator of the InterAct project. All authors contributed to interpretation of data, critically revised the article for important intellectual content, and approved the final version.

Declaration of interests
JMH has received grants from the Health Research Fund (Spanish Ministry of Health) and from the Murcia Regional Government (grant no. 6236). The other authors declare no competing interests.

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