Omega-6 Fatty Acid Biomarkers and Incident Type 2 Diabetes: A Pooled Analysis of 20 Cohort Studies

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Abstract

Background
Metabolic effects of omega-6 polyunsaturated fatty acids (n-6 PUFA) remain contentious, and evidence is limited regarding their potential role in primary prevention of type 2 diabetes (T2D).

Methods
Global consortium of 20 prospective cohort studies with new, harmonized, individual-level analyses. Each study measured biomarker linoleic acid (LA, the major dietary PUFA) and arachidonic acid (AA, a precursor of metabolic- and inflammation-mediating metabolites) at baseline among adults without prevalent T2D. Associations of n-6 PUFA biomarkers with T2D risk were assessed prospectively using a pre-specified analytic plan for exposures, covariates, and effect modifiers. Findings were pooled using inverse-variance weighted meta-analysis.

Findings
This study comprised 39,740 adults from 10 countries, with age (range of cohort means) 49-76yrs and BMI, 23.3-28.4kg/m², including 4,347 incident T2D cases during 366,073 person-years of follow-up. In multivariable-adjusted pooled analyses, higher LA biomarker associated with 35% lower risk of T2D (RR per interquintile range: 0.65, 95%CI: 0.60-0.72, P<0.001, I²=54%). Findings for LA biomarker were generally similar in different lipid compartments including phospholipids, plasma, cholesterol esters, and adipose tissue. Levels of AA biomarker were not significantly associated with T2D risk overall (RR per interquintile range: 0.96, 95%CI: 0.88-1.05, P=0.38, I²=63%). Relations of LA and AA biomarker with T2D were not significantly modified by age, BMI, sex, race, aspirin use, n-3 PUFA levels, or FADS genetic variants (all P-interaction≥0.13).

Interpretation
Findings suggest long-term benefit of LA, and no harms of AA, for the prevention of T2D.

Funding
Funding is outlined in Appendix Table 1.
RESEARCH IN CONTEXT

Evidence before this study
We conducted electronic searches via Pubmed, manual searches of reference lists of previous original publications and systematic reviews, and expert contacts to identify prospective observational studies that had assessed the relation between the major dietary omega-6 polyunsaturated fat (n-6 PUFA), linoleic acid (LA), and its downstream metabolite arachidonic acid (AA) and their relation with risk of incident type 2 diabetes. We identified few prior studies that have investigated LA and AA in relation to T2D; and most relied on estimated consumption levels from self-reported questionnaires, for which evidence has been considered weak. While biomarkers of LA and AA offer objective assessment of exposure free of recall bias, only a handful of prospective studies have evaluated associations of LA or AA biomarkers and T2D, with potential limitations of publication bias, and inadequate power to evaluate interactions by population characteristics.

Added value of this study
We formed a consortium including 20 prospective cohorts across 10 countries and assessed, using new harmonized individual-level analyses within each cohort, the relation between biomarker levels of LA and AA and risk of incident T2D. During 366,703 person-years of follow-up, 4,347 new cases of T2D were identified. Meta-analysis of findings across cohorts found that biomarker levels of LA were inversely associated with T2D in a linear fashion, with comparable findings across different lipid compartments. Conversely, overall biomarker levels of AA were not significantly associated with T2D. The magnitude of association for LA was meaningful – 35% lower risk per interquintile range. To our knowledge, this is the largest and most detailed assessment of objective n-6 PUFA biomarkers and incidence of T2D. The breadth and scope of the cohorts further allowed assessment of heterogeneity. Despite the diversity of the 20 contributing cohorts, there was little evidence that the associations differed by age, BMI, sex, race, omega-3 PUFA levels, aspirin use, or variation in the FADS gene.
Implications of all the available evidence

With T2D reaching alarming levels around the world, the identification of dietary and other modifiable risk factors for the prevention of T2D is of clinical, scientific, and public health importance. Several dietary guidelines recommend increased LA consumption to improve blood cholesterol levels and reduce cardiovascular risk. Our investigation provides novel findings that, when combined with in vitro experimental and shorter-term interventions on metabolic risk factors, strongly support an additional role of LA for prevention of T2D in generally healthy populations. In addition, our findings do not support hypothesized concerns about potential harmful effects of AA. Consistent with this, a recent systematic review found that biomarker levels of AA were associated with lower incidence of coronary heart disease.
INTRODUCTION

The influence on health of n-6 polyunsaturated fatty acids (PUFA), in particular linoleic acid (LA), the predominant n-6 PUFA, remains disputed.\textsuperscript{1, 2} Most major guidelines, such as those from the American Heart Association and Dietary Guidelines for Americans, recommend 5%-10% of energy come from LA, primarily derived from vegetable oils.\textsuperscript{3, 4} On the other hand, some have hypothesized that LA may be harmful due to competition with n-3 PUFA or due to harmful effects of its metabolite, arachidonic acid (AA).\textsuperscript{5, 6} For instance, due to such concerns, French national guidelines recommend limiting LA consumption to no more than 4% of energy.\textsuperscript{7}

While many studies have investigated cardiovascular effects of n-6 PUFA,\textsuperscript{4, 8} less is known regarding their influence on other major outcomes, in particular type 2 diabetes (T2D). A recent meta-analysis of randomized controlled feeding studies indicated that total PUFA consumption (predominantly LA) improves both glycaemia and insulin resistance.\textsuperscript{9} Yet, whether such short-term benefits translate to primary prevention of T2D remains unclear. Most prior longitudinal studies of LA and incident T2D relied on self-reported dietary estimates of intake which may be limited by errors or bias in recall.\textsuperscript{10} In contrast, because LA cannot be synthesized by humans, biomarker measurements of LA can provide objective assessments free of memory errors, recall bias, or inaccuracies of food databases.\textsuperscript{11} Biomarker measurements are also crucial for studying effects of AA, for which levels are tightly regulated and less correlated with dietary intake.\textsuperscript{12} Yet, only a handful of prospective studies have evaluated associations between LA or AA biomarkers and T2D, creating potential limitations of publication bias and inadequate power to evaluate interactions by demographic, medical or genetic characteristics.\textsuperscript{10} Thus, the potential effects of n-6 PUFA, including LA and its metabolite AA, on T2D remain unresolved and are of considerable clinical, scientific, and public health importance. To address these questions, we performed a consortium analysis of new, harmonized, individual-level investigations within the Fatty Acids and Outcomes Research Consortium (FORCE).\textsuperscript{13} Our primary aim was to assess the association of LA and AA biomarkers with incident T2D, with additional aims to assess factors that might modify these associations. We hypothesized that LA, but not AA biomarkers, would be inversely associated with T2D risk.
METHODS

Identification of cohorts

Prospective studies were identified via expert contacts and online searches which had assessed circulating or tissue biomarkers of LA and AA, and incidence of T2D. Twenty of 26 identified studies (77%) agreed to participate by February 2016.

Uniform analysis protocol

A standardized analysis protocol was developed and provided to each participating cohort. To reduce heterogeneity, the analysis plan included harmonized specifications for population inclusion, exposures, covariates, effect modifiers, outcomes, and analysis; and for methods for pooling results. Individual scientists from each cohort performed analysis using individual-level data, with results provided in pre-specified standardized electronic forms to one investigator (J.Wu) for pooling.

Population. Each cohort evaluated all adults (≥18yrs) with LA and AA biomarker measurements and free of prevalent T2D at baseline.

Fatty acid measurements. Fatty acid (FA) levels were assessed in each study in various lipid compartments and expressed as % of total FAs. (Appendix and eTable1).

Ascertainment of incident diabetes. Incident T2D was defined by fasting glucose ≥126 mg/dL (7.0mmol/L), 2-hour post oral glucose tolerance test glucose ≥200 mg/dL (11.1mmol/L), new use of insulin or oral hypoglycemic medication, fasting or non-fasting HbA1C concentration≥6.5% and/or in some cohorts by self-reported physician diagnosis; whichever occurred first (eTable2).

Covariates. Based on biological interest and well-established relations with type 2 diabetes risk, prespecified covariates included age, sex, race, field/clinical center if applicable, body mass index (BMI), education, smoking, physical activity, alcohol intake, prevalent coronary heart disease, treated hypertension, treated hypercholesterolemia, and biomarker n-3 PUFA concentrations (eTable3 and 4). Participants with missing continuous covariates were excluded (maximum exclusion=3.3%); those with missing categorical covariates were included via missing indicator categories.
Effect modification. To minimize concerns over multiple-testing, we pre-specified all potential sources of heterogeneity based on demographic, anthropometric, or biologic significance. Cohort-specific stratified analyses were performed by age, sex, race, BMI, long-chain n-3 PUFA biomarker concentrations, aspirin use (which may promote formation of AA-derived resolvers of inflammation), and common genetic variation in the FA desaturase genes (FADS, OMIM 606149; single nucleotide polymorphism (SNP) rs174547), which most strongly associates with n-6 PUFA levels (eTable5).14

Cohort analyses. For prospective cohorts with time-to-event data, Cox proportional hazards were used to obtain the hazard ratio (HR) and its standard error (SE). For studies with a case-cohort design, weighted Cox models were used.15 Participants were followed from time of FA measurement to time of T2D diagnosis, death, or censoring at end of follow-up, whichever occurred first. For a prospective case-cohort16 and prospective case-control study17 without time-to-event data, logistic regression (weighted for case-cohort studies) was used to obtain the odds ratio (OR) and its SE for incident T2D. All analyses utilized robust standard errors.

Sensitivity analyses. To reduce likelihood of reverse causation due to prevalent subclinical disease, sensitivity analyses were conducted in each cohort excluding cases in the first 2 years of follow-up. To minimize exposure misclassification due to changes in FA levels over time, each cohort also performed a sensitivity analysis censoring participants after the initial 6 years of follow-up.

Pooling and meta-analysis

HRs and ORs were considered to approximate relative risks (RRs) and pooled to generate summary results using inverse-variance weighted meta-analysis. Random effects models were also performed in sensitivity analyses.18 Because FAs were measured in different lipid compartments (phospholipids, plasma, cholesterol esters, adipose) using differing methods, LA and AA were evaluated continuously per study-specific interquintile range (the distance between the midpoint of the first and fifth quintiles) to facilitate pooling. Results were pooled separately for each lipid compartment and across all studies. If a study had multiple measures, the overall pooled analysis prioritized adipose tissue>erythrocyte phospholipids>plasma phospholipids>total plasma/serum>cholesterol esters, based on considerations of better reflecting long-term intakes.19
Potential non-linear relationships were assessed by pooling the HR/OR for each study-specific quintile, evaluated as an indicator variable against the lowest quintile as the reference; and also in each compartment by multivariate inverse-variance weighted meta-regression, modelling the FA quintile results using restricted cubic splines. Because findings across compartments could not be pooled using restricted cubic splines, these latter analyses were considered exploratory. Heterogeneity was assessed using the I² statistic. Statistical significance of differences between prespecified subgroups was assessed using inverse-variance weighted meta-regression. All meta-analyses were performed using STATA13, two-sided alpha=0.05.

**Role of the funding sources**

Cohort specific funding is outlined in Appendix Table 1. Unilever also provided Tufts University with a restricted grant (‘epidemiological research on circulating polyunsaturated fatty acids in relation to cardiometabolic health within the CHARGE-consortium’) to partly support this analysis. The funders had no role in study design, study conduct, data analysis, manuscript preparation, or decision to submit.
Results

Study and participant characteristics

Twenty cohorts included 39,740 participants in 10 countries, including the United States, several European countries, Australia, and Taiwan (Table 1). Most studies (n=17) were prospective cohorts, with three prospective case-cohorts (n=2) or nested case-control (n=1) studies. The ranges of mean cohort ages (49 to 76 years) and BMI (23 to 28 kg/m²) were wide; within cohorts, much broader ranges of ages and BMI were represented (eTable 3). Most participants were of European descent (eTable 4); although several cohorts had sizeable numbers of participants of African (CHS, IRAS, MESA: 11·1%-24·5%), Asian (CCCC, MESA: 25·6%-100%), or Hispanic (IRAS, MESA, 22·2%-33·2%) descent.

Biomarker FAs were measured in phospholipids (n=14 cohorts), total plasma/serum (n=6), cholesterol esters (n=4), and adipose tissue (n=1), with 6 cohorts having measurements in 2+ lipid compartments. Except for ULSAM-50 (1970-1973), baseline blood sampling was performed between 1987-1989 and 2002-2006. All studies used gas chromatography, with inter-assay CVs ≤15% (Appendix). Median LA levels varied from ~10% in erythrocyte phospholipids to ~50% in plasma cholesterol esters (eFigure 1A); and AA, from <1% in adipose tissue to ~13% in erythrocyte phospholipids (eFigure 1B). Spearman correlations across lipid compartments within studies having more than one measure ranged from 0·59-0·84 for LA and 0·53-0·91 for AA (eTable 1).

n-6 PUFA and incident T2D

During 366,073 person-years of follow-up, 4,347 new cases of T2D occurred (eTable 2). In pooled analyses, LA levels were inversely associated with incidence of T2D, with 35% lower risk in continuous analyses per interquintile range (RR: 0·65, 95% CI=0·60-0·72, P<0·001) and 43% lower risk comparing the top to the bottom quintile in categorical analysis (RR=0·57, 95% CI=0·51-0·64, P<0·001) (Table 2). Findings were similar across lipid compartments (Figure 1), although not statistically significant in adipose tissue (only 1 study with 99 incident T2D cases). Heterogeneity in the overall pooled analysis was moderate (I²=53·9% for continuous analyses, 46·3% for quintile analyses).
AA biomarker was not associated with incidence of T2D overall (RR per interquintile range=0·96, 95% CI=0·88-1·05, P=0·38, Table 2 and Figure 2). AA biomarker was also not associated with T2D in separate lipid compartments except total plasma where an inverse association was seen (per interquintile range, RR=0·73, 95% CI=0·62-0·86, P<0·001, I²=63·8%).

Evaluated categorically across quintiles, the dose-response for LA biomarker and T2D appeared monotonic (Figure 3, eFigure 2A and 2B). Compared to the lowest quintile, participants in each of the higher quintiles of LA biomarker had significantly lower risk.

Restricted cubic spline analysis
In exploratory analyses within each lipid compartment, there was little evidence for nonlinearity in the relationship between LA biomarker and incident T2D in cholesterol esters or total plasma (eFigure 3, P-nonlinearity≥0·4 each; P-linearity<0·001 each). A potentially nonlinear association was identified in erythrocyte phospholipids (P-nonlinearity=0·005) and plasma phospholipids (P-nonlinearity=0·03) (eFigure 3), each with initially steeply declining risk that plateaued (but did not significantly increase) at very high levels. For AA, total plasma biomarker levels were associated with lower risk (P-linearity≤0·001), with little evidence for non-linear associations within any of the compartments (P-nonlinearity≥0·47) (eFigure 4). While overall phospholipids AA levels were not associated with T2D (Table 2, Figure 2), exploratory restricted cubic splines separately evaluating erythrocyte phospholipids and plasma phospholipids suggested divergent linear associations with T2D.

Effect modification
The associations of LA and AA biomarkers with incident T2D did not significantly vary according to any pre-specified potential sources of heterogeneity (P-heterogeneity≥0·13 each; eTable 6). In 12 cohorts with available genetic data, no significant interaction was identified by FADS desaturase gene variants for either LA or AA biomarker and incident T2D (P-interaction≥0·47; eTable 7).

Sensitivity analyses
Compared with main analyses, similar results were observed for LA and AA biomarkers after exclusion of T2D cases identified in the first 2 years of follow up, and censoring follow-up at 6 years after baseline (eTable 8).
Discussion

In this consortium including 20 prospective studies across 10 countries, biomarker levels of LA were inversely associated with T2D, while AA levels were not associated with T2D. The magnitude of association for LA biomarker was substantial: nearly 45% lower risk across quintiles. To our knowledge, this is the largest and most detailed biomarker assessment of n-6 PUFA and T2D, including across multiple lipid compartments. Despite the breadth and scope of the cohorts, there was little evidence that associations differed by age, BMI, sex, race, n-3 PUFA levels, aspirin use, or FADS gene variation.

With T2D reaching alarming levels around the world, the identification of dietary and other modifiable risk factors for the prevention of T2D is of great clinical, scientific, and public health importance. Several dietary guidelines recommend increased LA consumption to improve blood cholesterol and reduce cardiovascular risk.\(^4\) Incorporation of LA into phospholipids alters membrane fluidity and may modulate insulin receptor activity.\(^22\) In a meta-analysis of 102 randomized controlled feeding trials, dietary PUFA (predominantly LA) improved glycaemia, insulin resistance, and insulin secretion capacity, whether compared to carbohydrate, saturated fat, or (for some endpoints) even monounsaturated fat.\(^9\) In other randomized controlled trials, LA-rich vegetable oil reduced markers of inflammation, visceral fat deposition, and hepatic steatosis.\(^23\) Because dietary LA intake correlates with circulating and tissue LA,\(^12\) our biomarker-based findings extend and expand these prior results by providing evidence suggesting long-term benefits of LA for preventing onset of T2D, supporting clinical recommendation to increase dietary intake of LA-rich vegetable oils. Our novel findings also support the need for future studies, including (a) establishing the potential influence and clinical effects of other (e.g. pharmacologic) influences on these FA biomarkers, (b) identifying the downstream biologic mediating pathways of effects of these FA biomarkers on risk of T2D, and (c) investigating potential novel (e.g. pharmacologic and lifestyle) influences on these downstream biologic mediating pathways. Mendelian randomization studies\(^24\) should also assess how common genetic variants that influence levels of these and other FA associate with T2D.
Even with established benefits for blood cholesterol levels and glucose-insulin homeostasis, some scientists continue to describe n-6 PUFA as being harmful for health. A main theorized harm relates to conversion of LA to AA, which has been considered as pro-inflammatory and potentially harmful for glucose metabolism, weight regulation, and eating behaviour. Yet, multiple studies demonstrate that variations in both dietary LA and AA have little effect on circulating AA levels, indicating close endogenous regulation of the latter. In addition, AA is gives rise to important metabolites that actively resolve inflammation; while systematic reviews of trials have not identified pro-inflammatory effects of LA consumption. Indeed, a recent systematic review found that biomarker AA levels were associated with lower incidence of coronary heart disease. We also did not find evidence for harms of AA biomarker for T2D. Together with the experimental and interventional studies on metabolic risk factors described above, our findings do not support evidence for harms of high dietary n-6 PUFA. In addition, while n-3 and n-6 PUFA has been hypothesized to compete, we did not identify any evidence for physiologic relevant interaction in this large, well-powered consortium analyses.

A recent nested case-cohort analysis from the European EPIC cohort, published during the preparation of our manuscript, found an inverse association between plasma phospholipid LA and T2D (per SD, HR:0·80; 95% CI=0·77-0·83), and no significant association for AA (HR:1·02; 95% CI=0·98-1·06). Our findings are consistent with this recent report but include a worldwide perspective, using data from multiple lipid compartments, and detailed assessment of potential effect modification including by variation in the FADS gene. Our findings also appreciably reduce the possibility of chance findings or publication bias, compared to any individual cohort report, given our inclusion from the outset of all available cohorts with measured FA biomarkers and assessment of incident T2D. Addition of EPIC-InterAct is unlikely to materially alter the major conclusions of the current study, given the similarities in findings.

Surprisingly, relatively little is known about the health relevance of FA function between different lipid compartments. The current analyses provided novel assessment of associations and dose-response between n-6 PUFA and T2D in different compartments. For LA biomarker, all
compartments (except adipose tissue, n=1 study) demonstrated significant linear inverse associations with T2D, suggesting a class effect of LA rather than primacy of any single compartment. In exploratory analyses, LA’s protective association appeared linear in cholesterol esters and total plasma, but nonlinear in phospholipids, with a plateauing of benefit at very high levels. The biologic and clinical relevance of this discrepancy deserves further investigation. Studies are also needed to define the precise dose-response relationship between a broad range of LA intake and biomarker levels in different lipid compartments. For AA biomarker, there was little evidence for non-linearity for any of the lipid compartments. The opposing associations of erythrocyte phospholipids vs. plasma phospholipids AA in semi-parametric analyses deserves further research; this could be due to chance since AA levels in these two compartments are highly correlated and known to readily interexchange.30 Consistent with this, plasma phospholipid AA in EPIC was not associated with T2D.29 Our new findings of a protective association between AA in total plasma and incident T2D, based on findings in 6 cohorts, should be explored further.

Our investigation has important strengths. We included prospective cohorts, which minimized the likelihood of selection bias. Use of biomarkers avoided recall bias associated with self-reported intake and allowed objective assessment of LA and AA. Collaboration between 20 cohorts enabled simultaneous investigation of multiple lipid compartments, which could be cost-prohibitive for any single study. Harmonized, pre-defined analysis protocols standardized exposures, outcomes, covariates, and statistical modelling, reducing posthoc-driven reporting and heterogeneity across studies. The prespecified analytic plan and inclusion of most global cohorts greatly reduced publication bias. The large numbers of participants and events increased statistical power to explore effect modification. Results were consistent in sensitivity analyses, increasing confidence in the robustness of findings and underlying model assumptions. Inclusion of multiple cohorts and nations having diverse demographic, lifestyle, and dietary characteristics enhanced generalizability.

Potential limitations should be considered. Few data were available on adipose tissue, reducing power and precision to assess its relevance for T2D. While multiple races/ethnicities were included, most participants were of European origin and statistical power was limited to confirm differences in other
groups, although central risk estimates for LA biomarkers were protective in each. FA biomarker levels were assessed at baseline, and changes over time would attenuate findings toward the null, causing underestimation of magnitudes of true associations. LA biomarkers reflect dietary intake and other factors such as metabolism, therefore differences in T2D risk should not be interpreted as entirely attributable to dietary LA. We did not assess other fatty acid biomarkers which should be the subject of future investigations, in particular saturated fatty acids such as palmitic acid which have pro-diabetogenic effects in experimental studies. Reliability of T2D ascertainment likely differed across the cohorts, which may have caused some outcome misclassification and underestimation of true associations. Our findings are relevant to incidence of T2D, not T1D. Residual confounding by unmeasured or imprecisely measured covariates cannot be fully excluded, including by other fatty acid biomarkers. Yet, the magnitude of the observed relationship between LA biomarker and T2D, consistency across biomarker compartments, inclusion of varied populations with diverse underlying characteristics, and supportive biologic plausibility from interventional trials of risk factors argue against statistical chance and uncontrolled confounding as the sole explanations for the results.

In summary, in this international collaboration of 20 prospective cohorts, biomarker levels of LA, the major dietary n-6 PUFA, were inversely related to the risk of incident T2D, while levels of AA were not significantly associated with risk.
References


Figure legends

**Figure 1.** Pooled relative risks (RR) of type 2 diabetes mellitus (T2D) per interquintile range of linoleic acid (18:2n6), which is the distance between the mid-points of the first and fifth quintiles. Association was assessed in multivariable models for each cohort, and results pooled using inverse-variance weighted meta-analysis. If multiple biomarkers were available within a study, one was chosen for the overall analysis with order of preference based on the biomarker that may best reflect long-term dietary intake, i.e. adipose tissue > phospholipids > total plasma > cholesterol esters. Similarly, erythrocyte phospholipids was preferred over plasma phospholipids if both were available from a cohort.

**Figure 2.** Pooled relative risks (RR) of type 2 diabetes mellitus per interquintile range of arachidonic acid (20:4n6), which is the distance between the mid-points of the first and fifth quintiles. Association was assessed in multivariable models for each cohort, and results pooled using inverse-variance weighted fixed effects meta-analysis. If multiple biomarkers were available within a study, one was chosen for the overall analysis with order of preference based on the biomarker that may best reflect long-term dietary intake, i.e. adipose tissue > phospholipids > total plasma > cholesterol esters. Similarly, erythrocyte phospholipids was preferred over plasma phospholipids if both were available from a cohort.

**Figure 3.** Pooled relative risks (RR) of type 2 diabetes mellitus by quintiles of linoleic acid (18:2n6) and arachidonic acid (20:4n-6). Association of linoleic acid (treating the lowest quintile as the reference group) with T2D was assessed in multivariable models for each cohort, results were subsequently pooled using inverse-variance weighted meta-analysis. If multiple biomarkers were available within a study, the order of preference were based on which biomarker may best reflect long-term dietary intake, i.e. adipose tissue > phospholipids > total plasma > cholesterol esters. Similarly, erythrocyte phospholipids was preferred over plasma phospholipids if both were available from a cohort. AGES-Reykjavik was excluded from these analyses due to the limited number of incident T2D cases, so the effect estimates were pooled from the other 19 cohorts.