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Circulating metabolites and lipids are associated with glycaemic measures in South Asians

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Abstract

Background: South Asians are at higher risk for diabetes (DM) than many other racial/ethnic groups. Circulating metabolites are measurable products of metabolic processes that may explain the etiology of elevated risk. We characterized metabolites associated with prevalent DM and glycaemic measures in South Asians.

Methods: We included 717 participants from the Mediators of Atherosclerosis in South Asians Living in America (MASALA) study, aged 40–84 years. We used baseline fasting serum for metabolomics and demographic, behavioral, glycaemic data from baseline and at 5 years. We performed LC-MS untargeted metabolomic and lipidomic analysis with targeted integration of known signals. Individual linear and ordinal logistic regression models were adjusted for age, sex, BMI, diet, exercise, alcohol, smoking and family history of DM followed by elastic net regression to identify metabolites most associated with the outcome.

Results: There were 258 metabolites with detectable signal in >98% of samples. Thirty-four metabolites were associated with prevalent DM in an elastic net model. Predominant metabolites associated with DM were sphingomyelins, proline (OR 15.86; 95% CI 4.72, 53.31) and betaine (OR 0.03; 0.004, 0.14). Baseline tri and di-acylglycerols [DG (18:0/16:0) (18.36; 11.79, 24.92)]

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CONTRIBUTION STATEMENT

The roles for each author are as follows. MDG conceived of the analytic design and performed the analysis; CS, ML, DH contributed to the analytic plan, CS, ML, AMK, NRK and DH contributed to the interpretation of the results and reviewed and edited the manuscript; MDG wrote the manuscript; and AMK conceived of the project idea. MDG had primary responsibility for final content. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors do not have financial conflicts of interest to disclose.

were positively associated with fasting glucose and long-chain acylcarnitines [CAR 26:1 (−0.40; −0.54,−0.27)] were inversely associated with prevalent DM and HbA1c at follow-up.

Discussion: A metabolomic signature in South Asians may help determine the unique etiology of diabetes in this high-risk ethnic group. Future work will externally validate our findings and determine the effects of modifiable risk factors for DM.

Keywords

diabetes; metabolomics; South Asian

INTRODUCTION

South Asians are at higher risk for diabetes (DM) than many other racial/ethnic groups despite a lower average body mass index.[1] The prevalence and incidence of type 2 diabetes (T2D) in South Asians is increasing, both in their native countries and in the diaspora.[2] While typical risk factors such as obesity, added dietary sugar, and red or processed meat intake are not common in this population, other factors such as high sedentary time, significant family history of DM and insulin resistance do not fully explain the increased prevalence.[3] Understanding biological mechanisms underlying this higher prevalence may shed light on the reasons for these significant and worsening health disparities.

Metabolites are small molecules that are measurable products of metabolic processes found in blood, urine and tissue.[4] They reflect endogenous, lifestyle and environmental exposures and represent the metabolic changes occurring in response to these exposures. Profiling metabolites can help to clarify areas of dysregulated metabolism in diabetes, which may, in turn, help unravel the etiology behind the elevated prevalence of DM in South Asians.

Metabolites have previously been associated with DM incidence and prevalence in other populations. Increased concentrations of branched-chain amino acids and their breakdown products, C3 and C5 acylcarnitines, have been associated with diabetes.[5, 6] Lysophosphatidylcholine [7, 8] and glycine[6, 8] have been inversely associated with incident T2D in meta-analyses. Serum betaine was inversely associated with incident T2D in the Diabetes Prevention Program, and its plasma levels increased with the intensive lifestyle intervention.[9]

We aimed to characterize metabolites associated with prevalent DM and fasting glucose and hemoglobin A1c after 5-years of follow-up in U.S. South Asians in the Mediators of Atherosclerosis in South Asians Living in America (MASALA) cohort study.

METHODS

Participants

We used data from South Asians who participated in the MASALA community-based cohort study and had complete diet and metabolomic data. The detailed methods have been described elsewhere.[10] Briefly, MASALA is a prospective cohort study in which we

enrolled community-dwelling individuals living in the San Francisco Bay Area and the greater Chicago areas from 2010–2013. Participants self-identified as having South Asian ancestry were aged 40–84 years and had no known cardiovascular disease. Those on nitroglycerin, with active cancer, with impaired cognitive ability, a life expectancy less than five years, who lived in a nursing home, or who had plans to relocate were excluded. The University of California, San Francisco and Northwestern University Institutional Review Board approved the study protocol and all study participants provided written informed consent.

After approximately 4.8 years of follow-up, 749 (83%) of the cohort returned to complete Exam 2.

Demographic and diet data

Each participant underwent in-person interviews to determine age, sex, medical history, physical activity and smoking status and alcohol intake. Family history of diabetes for parents and siblings was determined by self-report. Food group intake was collected with the Study of Health Assessment and Risk in Ethnic (SHARE) groups South Asian Food Frequency Questionnaire, which was developed and validated in South Asians in Canada.[11] The Alternative Healthy Eating Index (AHEI) score was calculated as a sum of food components; each food component is scored from 0 (worst) to 10 (best), for a maximum score of 110.

Metabolic Profiling by UPLC-MS

A total of 754 serum samples obtained at the baseline exam (2010–2013) were analyzed by ultra-performance liquid chromatography mass spectrometry (UPLC-MS) using previously described analytical and quality control procedures.[12, 13] Sample analysis was performed in an order designed to be orthogonal to clinical and demographic data (metadata) of potential significance with respect to the study design and outcome (participant acrostic, age, gender, BMI, diabetes medication use). For quality control assessment and data pre-processing, a study reference (SR) sample was prepared by pooling equal parts of each study sample.

Serum samples were prepared and analyzed using UPLC-MS as previously published.[12, 13] In brief, 50 μL aliquots were taken from each sample, diluted 1:1 with ultrapure water for lipid profiling and 1:1.4 for small molecule profiling. Protein was removed by addition of organic solvent to the diluted sample (four volumes isopropanol per volume of diluted sample for lipidomic profiling and three volumes of acetonitrile per volume of diluted sample for small molecule profiling) followed by mixing and centrifugation to yield a homogenous supernatant. Aliquot sets of prepared samples were subjected to chromatographic separation using an ACQUITY UPLC (Waters Corp., Milford, MA, USA) system. Lipidomic profiling was performed using reversed-phase chromatography (RPC) with a 2.1 × 100 mm Acquity BEH C8 column maintained at 55°C. Polar metabolite profiling was performed using hydrophilic interaction liquid chromatography (HILIC) with a 2.1 × 150 mm Acquity BEH HILIC column maintained at 40°C. Both separation types were coupled to high resolution mass spectrometry (Xevo G2-S TOF mass spectrometers, Waters

Corp., Manchester, UK) via a Z-spray electrospray ionisation source. The lipidomic profiling assay was conducted in both positive and negative ion modes (generating lipid RPC + and RPC- datasets respectively), while the HILIC assay was performed in the positive ion mode only (generating the HILIC+ dataset). A SR sample was acquired every 10 study samples throughout the analysis. In addition, a dilution series was created from the SR and analyzed immediately prior to and after the study sample analysis for use in signal filtering as described previously [13].

Raw data was converted to the mzML open source format and signals below an absolute intensity threshold of 100 counts were removed using the MSConvert tool in ProteoWizard. [14] Metabolite signal extraction was performed using an open-source package to detect, integrate and report pre-defined and annotated lipids and metabolites from an in-house database (https://doi.org/10.5281/zenodo.3523406). An open-source package was applied for elimination of potential run-order effects and filtering of the extracted metabolites.[15] Only those measured with high accuracy (relative coefficient of variance in SR samples less than 20%) and high precision (correlation to dilution in SR dilution series greater than 0.8) were retained and put forward for biological analysis. Of the 754 total study samples, 32 were not included in our analysis due to insufficient sample volume and five were excluded due to missed injection in the HILIC+ assay.

Metabolic measures at baseline:

Weight was determined using a digital scale, height with a stadiometer, and waist circumference using a measuring tape halfway between the lower ribs and the anterior superior iliac spine, at the site of greatest circumference. Blood samples were obtained after a requested 12-hour fast. Fasting plasma glucose was measured using the hexokinase method (Quest diagnostics, San Jose, CA). An oral glucose tolerance test was performed, in which participants consumed a 75g oral glucose solution, and blood samples for plasma glucose and insulin were taken after 120 minutes. Type 2 diabetes was defined as a fasting glucose ≥126 mg/dl or use of a glucose-lowering medication.

Metabolic measures at 5-year follow-up:

We assessed fasting plasma glucose and hemoglobin A1c at 5-year follow-up using the methods described above.

Statistical methods

Before modeling, relative abundance of metabolites were log-transformed to reduce the potential for outliers to influence the model. Baseline covariates of age, sex, body mass index (BMI), diet score, exercise, alcohol consumption (yes/no), smoking and family history of diabetes were selected *a priori* based on current understanding of confounders in the literature. We additionally adjusted for baseline fasting glucose in the analysis of hemoglobin A1c at 5-year follow-up.

Multiple logistic regression analyses were used to determine associations of relative abundance of each independent metabolite with prevalent diabetes at enrollment, adjusted for age, sex, caloric intake and BMI. We used multiple linear regression analyses to

determine associations of each metabolite with continuous fasting glucose or HbA1c at 5-year follow-up, after excluding those with prevalent diabetes at baseline. We applied the Benjamini-Hochberg method to adjust for multiple comparisons, with an FDR<0.05 deemed significant. To adjust for unreliable parameter estimates that may occur when using multiple regression models in the setting of multicollinearity, we performed an elastic net regularized regression model to evaluate metabolites that were significant in independent analyses. The elastic-net model allowed for a penalized logistic regression on all biomarkers simultaneously to identify the most predictive metabolites for prevalent diabetes.

The analysis was completed using STATA (version 11.2, 2009, College Station, TX, USA).

RESULTS

We included 717 MASALA cohort participants with full metabolomics data at baseline in this analysis. This sample of participants had a mean age of 56 years of age and were 44% were female. Average BMI was 26 kg/m^2 and they were predominantly nonsmokers. Only one-third of participants reported consuming any alcohol. At enrollment, 16% had diabetes defined by the use of glucose-lowering medications or on the basis of fasting glucose ≥126 mg/dl at baseline. Sixteen percent of participants used glucose-lowering medications, 26% of participants used statin medications and 5% used fibrate medications at baseline. (Table 1)

There were 258 metabolites with detectable signal in >98% of samples. Using logistic regression analyses adjusted for age, sex and BMI, we found 61 metabolites associated with prevalent DM [FDR < 0.05]. An elastic net regression model identified a subset of 34 metabolites that most contributed to the DM outcome, taking into consideration the likely collinearity of metabolites along the same pathway. Using this subset of metabolites, we additionally adjusted for diet score, physical activity, smoking, alcohol use and family history of diabetes, and 21 metabolites remained significantly associated with odds of prevalent diabetes. (Table 2). Adjustment for cholesterol-lowering medications (statins) did not appreciably change the results. We performed sensitivity analyses stratifying by sex (Supplemental Table) and found that certain sphingolipids, triglycerides and were common, but that proline and betaine associations in the full cohort were driven by men, who comprised 70% of the diabetes cases.

In further analyses, we excluded participants with diagnosed prevalent diabetes for assessment of linear association of baseline metabolites with continuous glycaemic measures after 5-year follow-up (N=692). Eight metabolites were positively and significantly associated with fasting glucose after 5-year follow-up including triacylglycerols, diacylglycerols and a ceramide (FDR<0.05) (Table 3). In analysis with 5-year HbA1c as the dependent outcome, we identified seven metabolites, predominantly long-chain acylcarnitines that were inversely associated with HbA1c. (Table 4)

DISCUSSION

This analysis is a first step to define a serum metabolite signature for prevalent diabetes and glycaemic measures in South Asians. We found strong, positive, odds of association between

triacylglycerol, two phospholipids, and proline with prevalent diabetes. Notably, there were inverse associations between the presence of long-chain acylcarnitines and both odds of prevalent diabetes and HbA1c at 5-year follow-up, which contrasts with some prior findings [16, 17] but has previously been shown in South Asians and the PREDIMED cohort.[18, 19]

A pattern of serum metabolites can help define the metabolic processes disrupted in diabetes and help to explain the etiology of diabetes in different individuals and populations.[4] South Asians are at higher risk for diabetes than many other racial or ethnic groups [1] at a leaner body mass index, however the causes of this disparity are still unclear and are not completely explained by traditional risk factors such as obesity, smoking, physical inactivity and family history.[1] Early decline in beta cell function, causing a decrease in insulin section, compounded by higher rates of insulin resistance by low skeletal muscle mass and in addition to traditional lifestyle risk factors is a strong hypothesis to the higher rates of diabetes in South Asians.[20] Categorization of the metabolic changes reflected in metabolite profiling in South Asians may help inform prevention and treatment targets.

In this analysis, we found an inverse relationship between betaine and odds of prevalent diabetes. Betaine can be acquired through diet intake, from foods sources including wheat bran and germ, beets and spinach. It can also be created through the oxidation of choline, which is found in red meat, poultry, eggs. Betaine is a major determinant of plasma homocysteine concentration.[21] Lower levels of betaine have previously been cross-sectionally linked to diabetes and obesity[21] in observational studies in a variety of populations, most recently shown in a systematic review.[8] Direct supplementation with betaine in humans with prediabetes has not been shown to significantly decrease glucose or insulin levels[22], although it has been shown to decrease fasting glucose and liver fat in mice.[23] In the Diabetes Prevention Program, Walford et al. demonstrated that glycine betaine measured at baseline was inversely associated with incident diabetes.[9] Importantly, the level of betaine increased with the intensive lifestyle intervention in the Diabetes Prevention Program, showing that it may be an indicator for the success of modifiable risk factors. The findings presented in our Supplemental Table of a significant relationship between betaine and prevalent DM in men but not in women are likely due to low power and deserve further assessment in a larger sample size.

Altered protein metabolism and elevated circulating levels of branched-chain amino acids are present in states of insulin resistance, diabetes and obesity.[5] We did not find higher levels of branched-chain amino acids associated with diabetes or glycaemic measures in our cohort, nor did we find elevations of short and medium-chain C3 and C5 metabolites, which are metabolic degradation products of branched-chain amino acids. Given the preponderance of prior data linking higher levels of branched-chain amino acids with diabetes and insulin resistance, it is possible that the untargeted nature of our metabolomics analysis may not have captured these metabolites in sufficient relative abundance. Additionally, a prior analysis found lower levels of amino acids in South Asian compared to European populations in the UK.[24] We did find that proline was positively associated with odds of prevalent diabetes. Previously, increased levels of proline has been associated with insulin resistance in South Asian and Chinese men of low body mass, suggesting that this

metabolite may reflect metabolic differences underlying diabetes independent of those caused by obesity.[25]

Medium and long-chain acylcarnitines are generally elevated in the fasting state in obesity, insulin resistance and diabetes, in animal and human studies.[16, 26, 27] Mechanistically, obesity and extra intake of unhealthy fats may increases beta oxidation, overwhelming the TCA cycle and leading to incomplete fatty acid oxidation and intramitochondrial metabolite accumulation in skeletal muscle.[28] Release of long-chain acylcarnitines into skeletal muscle and subsequently plasma may be an attempt to relieve this intra-mitochondrial stress.[27] Glucose-lowering therapies, specifically metformin, have been shown to decrease levels of circulating long-chain acylcarnitines, which may be a key to the mechanism.[29, 30] We found an inverse odds of diabetes with medium- and long-chain acylcarnitine concentration, which may perhaps be reflective of the use of glucose-lowering therapies in those diagnosed with diabetes since all participants diagnosed with DM at baseline were using one or more of these medications.

The association of acylcarnitines prospectively with diabetes is less clear. In the MASALA cohort, we observed an inverse association between long-chain acylcarnitines (C16, C18 and C26) and both prevalent diabetes and HbA1c at follow-up. In an investigation comparing Dutch and South Asian Surinamese participants without diabetes living in Amsterdam, South Asian men progressed to T2D from insulin resistance at a higher rate than their Dutch counterparts.[31] In this cohort, long-chain acylcarnitines had a small but significant inverse association with T2D and HbA1c,[19] mirroring our findings. Similarly, an analysis of the PREDIMED cohort found an inverse association of long-chain acylcarnitines with incident diabetes[18]. Conversely, Sun et al. found an positive association between long-chain acylcarnitines and incident type 2 diabetes in a Chinese cohort.[16]

Serum ceramides have previously been associated with diabetes and insulin resistance,[32] and tissue-specific elevation in lactosylceramides are linked to mitochondrial dysregulation in the hearts of diabetic mice.[33] In humans, lactosylceramides have been associated with non-alcoholic steatohepatitis in the context of insulin resistance.[34] In the current analysis, increases in LacCer(d18:1/16:0) and HexCer(d18:1/22:0) were inversely associated with odds of diabetes, while Cer(d16:1/20:0) was positively correlated with fasting glucose at 5-year follow-up. It is possible that use of glucose-lowering therapies that improve circulating hyperglycemia may also affect the abundance of ceramides in a cross-sectional analysis, while the association with future fasting glucose may be evidence of early glucose dysregulation.

LPC (19:0/0:0) and LPC (20:0/0:0) were inversely associated with lower HbA1c after 5-years of follow-up, which is a novel finding. This is a molecule in which the acyl group has a fully saturated C19 or C20 chain and is attached to a glycerol moiety. Plasma lysophosphatidylcholines (LPC) are implicated in changes in lipid homeostasis. In mice models, LPCs are inversely associated with obesity and diabetes and altered rapidly after initiation of a high-fat diet.[35] Lysophosphatidylcholines, specifically 18:2, have been inversely associated with diabetes,[8] which is in line with our findings associating metabolites with prevalent DM.

Triglyceride elevation in the context of prevalent diabetes is well-known. We found a positive association between abundance of one triglyceride (54:6_2) and prevalent DM, which was unaffected by adjustment for statin use. Triglycerides have also been associated with diabetes; a lipid cluster enriched with triglycerides aided prediction of progression to diabetes in Finnish men who were normoglycaemic or dysglycaemic at baseline.[36] In this study, a lipid signature containing triglycerides and diacylglycerophosphides helped to differentiate those who went on to develop diabetes from those who did not. In our analysis, a group of lipids containing triglycerides, diacylglycerol and one ceramide were positively associated with fasting glucose at 5-year follow-up.

The strengths of this study are the unique data from a longitudinal, U.S-based South Asian cohort with robust measures of diabetes, demographic measures and metabolomics data. Limitations include the cross-sectional design of the prevalent diabetes analysis which cannot clarify cause and effect of metabolites associated with diabetes, and the potential influence of diabetes medication use at baseline on metabolite profiles. We were unable to assess metabolites associated with incident diabetes due to a relatively small sample size. However, this analysis did include glycaemic measures to assess prospective associations of metabolites and markers of diabetes in a large population without diabetes at baseline.

Sphingomyelins, lysophosphatidylcholines and proline are associated with prevalent diabetes while long-chain acylcarnitines and betaine were inversely associated with prevalent diabetes. Long-chain acylcarnitines and a lysophosphatidylcholine was inversely associated with prevalent diabetes and HbA1c. Our future directions include assessment of metabolites associated with incident diabetes and validation of these metabolites in an independent South Asian cohort.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NOVELTY STATEMENT

- South Asians are at higher risk of diabetes than many other racial/ethnic minorities, however the etiology is not clear
- Circulating metabolites are small molecules reflecting metabolic processes
- This paper explores the metabolites associated with prevalent diabetes and follow-up glycaemia for the first time in a South Asian population, and found that sphingomyelins, proline, betaine, di-and tri-acylglycerols and long-chain acylcarnitines were associated with these metabolic changes
- The conflicting associations of long-chain acylcarnitines with diabetes in different populations may reflect intra-mitochondrial stress as part of a potential mechanism for glycaemic change in South Asians

Table 1:

Baseline Characteristics for MASALA Study Sample (n=717) †

Baseline characteristics for masala study sample (n=717) †	
Age (years)	56 (9)
Women (%)	44
BMI (kg/m^2)	26 (4)
Reported calories per day (kcal/day)	1675 (515)
AHEI score	70 (7)
Reported exercise (MET-minutes/week) *	983 (1612)
Smoking (%)	
Never	83
Current	3
Former	14
Reported alcohol use (any) (%)	34
GFR (mL/min)	60 (2)
CKD-EPI (mL/min/1.73 m^2)	92 (14)
Diabetes at enrollment exam (%)	16
Fasting glucose (mg/dl)	133(30)
Fasting insulin (mg/dl	108(193)
HOMA-IR	6.3(13.2)
Normoglycaemic or prediabetic	
Fasting glucose (mg/dl)	98 (18)
Fasting insulin (mg/dl	69 (43)
HOMA-IR	2.9 (2.7)
Diabetes medication use (%)	16
Statin use (%)	26
Fibrate use (%)	5

† Mean (SD) unless otherwise specified

* Median(IQR)

Table 2:

Metabolites associated with prevalent diabetes in an elastic-net regularized logistic regression model[†‡ *]

Metabolite	Unadjusted OR	Adjusted OR	95% CI (upper)	95% CI (lower)
Sphingomyelins				
SM(d18:2/14:0)	0.10	0.09	0.04	0.2
SM(d18:1/16:0)	0.01	0.01	0.001	0.04
SM(d18:2/24:1)	0.04	0.03	0.01	0.09
SM(d18:2/23:0)	0.09	0.09	0.04	0.22
SM(d18:2/22:0)	0.06	0.07	0.03	0.20
Cholesterol and triglycerides				
TG(54:6_2)	3.75	4.41	2.56	7.60
Cholesterol	0.001	0.001	0.0002	0.009
Phospholipids				
PC(O-16:0/18:2)	0.08	0.09	0.04	0.19
PC(18:0/18:0)	0.07	0.08	0.04	0.18
PC(18:2/18:2)	0.12	0.12	0.06	0.24
LPC(19:0/0:0)	0.18	0.16	0.08	0.30
LPC(22:0/0:0)	0.32	0.24	0.13	0.44
LPE(20:4/0:0)	6.15	7.11	3.24	15.64
LPE(22:4/0:0)	1.76	2.24	1.59	3.18
Ceramides				
LacCer(d18:1/16:0)	0.06	0.06	0.03	0.14
HexCer(d18:1/22:0)	0.08	0.08	0.04	0.16
Carnitines				
Carnitine	0.12	0.08	0.02	0.28
CAR(26:0)	0.18	0.12	0.06	0.24
CAR(26:1)	0.19	0.14	0.08	0.27
Amino acids				
Proline	19.66	15.86	4.72	53.31
Betaine	0.17	0.03	0.004	0.14

[†]Adjusted for age, sex, BMI, calories, diet quality, exercise, smoking, alcohol use (yes/no), family history of diabetes

[‡]LPC: lysophosphocholine; PC: phosphocholine; LPE: lysophosphatidylethanolamine

[*]P<0.0002 for all associations

Table 3:

Baseline metabolites associated with fasting glucose at 5-year follow-up among those without known diabetes at baseline [†‡]

Metabolite	Unadjusted ß	Adjusted ß	95% CI (upper)	95% CI (lower)
TG(50:1)	5.00	3.25	1.64	4.86
TG(54:1)	5.54	4.66	2.51	6.80
TG(54:2)	8.23	6.04	2.82	9.25
TG(52:1)	5.70	4.94	2.70	7.18
DG(16:0/16:0)	7.54	6.47	3.81	9.12
DG(18:0/18:1)	6.92	5.87	3.42	8.32
DG(18:0/16:0)	19.77	18.36	11.79	24.92
DG(16:0/18:1)	6.47	5.19	2.46	7.92
Cer(d16:1/20:0)	11.34	9.94	5.90	13.97

[†]Adjusted for age, sex, BMI, calories, diet quality, exercise, smoking, alcohol use (yes/no), family history of diabetes

[‡]P<0.0002 for all associations

Table 4:

Baseline metabolites associated with HbA1c at 5-year follow-up among those without known diabetes[†‡]

Metabolite	Unadjusted ß	Adjusted ß	95% CI (upper)	95% CI (lower)
CAR(18:0)	−0.23	−0.30	−0.42	−0.17
CAR(18:3)	−0.11	−0.17	−0.25	−0.09
CAR(20:1)	−0.12	−0.19	−0.29	−0.10
CAR(26:0)	−0.15	−0.23	−0.34	−0.12
CAR(26:1)	−0.19	−0.24	−0.34	−0.14
LPC(19:0/0:0)	−0.77	−0.24	−0.35	−0.14
LPC(20:0/0:0)	−0.21	−0.20	−0.31	−0.10

[†]Adjusted for age, sex, BMI, calories, diet quality, exercise, smoking, alcohol use (yes/no), family history of diabetes, baseline fasting glucose (mg/dl)

[‡]P<0.0002 for all associations