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Associations of NAFLD with circulating ceramides and impaired glycemia

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ABSTRACT

Aim: Determine the association of circulating ceramides with NAFLD and glycemic impairment.
Methods: Sample: 669 participants in the Mediators of Atherosclerosis in South Asians Living in America (MASALA) cohort aged 40–84 years without cardiovascular disease, cirrhosis, or significant alcohol intake.
Clinical measures: Computed tomography scans at baseline for hepatic attenuation. Fasting serum specimens at baseline and after 5 years. Lipidomics: LC-MS-based analysis of 19 known ceramide signals.
Statistical analysis: Linear and logistic regression models of log-transformed ceramides, hepatic attenuation and glucose adjusted for age, sex, calories, study site, BMI, exercise, diet quality, alcohol, saturated fat, lipid-lowering medications and fasting glucose.
Results: Average age was 55 years, 44% were women, mean BMI was 25.9 kg/m2, and 8% had NAFLD. In adjusted models, Cer(d16:1/20:0) and Cer(d18:1/18:0) were associated with lower mean hepatic attenuation (increased liver fat) (β −4.29; 95% CI [-5.98, −2.59]) and (β −3.40; 95% CI [-5.11, −1.70]), and LacCer(d18:1/16:0) with higher attenuation (β 4.44; 95% CI [2.15, 6.73]). All three ceramides partially mediated the relationship between hepatic attenuation and fasting glucose by 16%, 11% and 5%, respectively, after 5-years.
Conclusions: Three circulating ceramides were strongly associated with NAFLD and fasting glucose after 5 years, and partially mediated this association.

1. Introduction

The burden of type 2 diabetes in South Asians is exceedingly high, estimated at over 20% for those living in the United States [1], causes major morbidity and mortality annually and is rising in incidence each year [2–4]. This elevated risk cannot be fully explained by traditional factors such as obesity, tobacco use, diet and physical activity [1]. Investigations into the etiology of this increased risk are ongoing and likely due to both modifiable and non-modifiable factors, however early clinical markers of concern are not well-established.

Ectopic fat deposition, particularly fatty liver, is an emerging risk factor for cardiometabolic disease [5]. Non-Alcoholic Fatty Liver Disease (NAFLD) is present in approximately 25% of the worldwide population and prevalence of NAFLD has been shown to be higher in South Asians as compared to people of many other races and ethnicities [6]. NAFLD includes the spectrum of disease from fatty liver (NAFL) to liver inflammation with or without fibrosis (NASH). Given the higher risk of type 2 diabetes in South Asian individuals, determination of biomarkers and intermediate metabolic changes associated with nontraditional risk factors such as NAFLD may help to clarify mechanisms of risk and identify areas for intervention.

NAFLD and type 2 diabetes have both been associated with circulating metabolites called ceramides, which are a type of bioactive lipid present in plasma and tissue [7]. Ceramides have been implicated in the pathogenesis of diabetes in animal models and human studies [8–10], and have a strong association with impaired glucose homeostasis. Prior work within the Mediators of Atherosclerosis in South Asians Living in America (MASALA) study has shown an association between specific ceramides (16, 18 and lactosyl ceramides) and prevalent type 2 diabetes [11]. Separately, the presence of ceramides has been associated with

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NAFLD [8]. It is hypothesized that when nutrient intake exceeds the body's storage capacity, increases in circulating free fatty acids lead to the production of ceramides and promote triglyceride deposition in the liver [12]. This, in turn, leads to elevated liver fat and hepatic insulin resistance [12]. Ceramides may be both a cause and consequence of impaired glucose homeostasis, but it is unclear whether they have a mediating role in the association between NAFLD and diabetes.

Clarifying the relationship between circulating ceramide metabolism, NAFLD and glucose dysregulation in South Asian may illuminate new pathways and mechanisms of risk to inform treatment and prevention. The objective of this study was to determine the association of individual circulating ceramide species abundance with presence and severity of hepatic steatosis and fasting glucose at 5-year follow-up.

2. Methods

2.1. 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 [13]. Briefly, MASALA is a prospective cohort study which enrolled community-dwelling individuals living in the San Francisco Bay Area and the greater Chicago areas from 2010 to 2013. Participants self-identified as having South Asian ancestry and were aged 40–84 years and without 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. Participants with self-reported cirrhosis (n = 1) and alcohol consumption of greater than 7 drinks/week (n = 41) were excluded to restrict the analysis sample to those with likely non-alcoholic fatty liver disease. There are no participants taking HIV anti-retroviral therapy, valproic acid or amiodarone, and we excluded participants with routine use of the following steatosis-inducing medications at the baseline exam: tamoxifen (n = 1), methotrexate (n = 2) or glucocorticoids (n = 4). Participants with prevalent diabetes at the baseline exam were excluded for a prespecified subgroup analysis (n = 178). After approximately 4.8 years of follow-up, 749 (83%) participants from the entire cohort returned to complete Exam 2.

The University of California, San Francisco and Northwestern University Institutional Review Board approved the study protocol and all study participants provided written informed consent.

2.2. Demographic data

Each participant underwent in-person interviews to determine age, sex, medical history, medication use, physical activity, smoking status, and alcohol intake. 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 [14].

2.3. 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 [15,16]. Sample analysis was performed in an order designed to be orthogonal to clinical and demographic data metadata. For quality control assessment and data preprocessing, 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 [15,16]. In brief, 50 μL aliquots were taken from each sample, diluted 1:1 with ultrapure water, and protein was removed by addition of organic solvent to the diluted sample (four volumes isopropanol per volume of diluted sample) 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. The chromatographic separation was performed using a binary mobile phase system consisting of (A) a 50:25:25 mixture of water:acetonitrile:isopropanol with 5 mm ammonium acetate, 0.05% acetic acid, and 20 μM phosphoric acid and (B) 50:50 acetonitrile:isopropanol with 5 mm ammonium acetate, 0.05% acetic acid. This was coupled to high resolution mass spectrometry (Xevo G2-S TOF mass spectrometers, Waters Corp., Manchester, UK) via a Z-spray electrospray ionisation source conducted in both positive and negative ion modes (generating Lipid RPC + and Lipid RPC- datasets). A SR sample was analyzed every 10 study samples throughout the analysis. In addition, a dilution series was created from the neat (non-diluted) SR and analyzed immediately prior to and after the study sample analysis for use in signal filtering as described previously [15].

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 [17]. Metabolite signal extraction was performed using PeakPantheR, an open-source package to detect, integrate and report pre-defined and annotated lipids and metabolites from an in-house database [18]. Elimination of potential run-order effects and filtering of the extracted metabolites was performed using the nPYc-Toolbox, an open-source package for data pre-processing [19]. 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.

2.4. Cardiometabolic factors

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. Hip circumference was measured at the maximum girth of the buttocks. 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 75 g oral glucose solution, and blood samples for plasma glucose and insulin were taken after 120 min. Type 2 diabetes was defined as a fasting glucose $\geq$ 126 mg/dl, 2-hour post-challenge glucose $\geq$ 200 mg/dl or use of a glucose-lowering medication.

2.4.1. Metabolic measures at 5-year follow-up

We assessed incident diabetes and fasting plasma glucose at 5-year follow-up study visit using an oral glucose tolerance test with the methods described above. The change in glucose was calculated as the difference between fasting glucose measurement (mg/dL) at Exam 2 and at Exam 1.

Assessment of hepatic attenuation and ectopic fat was done with non-contrast computed tomography (CT) images obtained at baseline exam with electron-beam or multidetector CT scanners as previously described [6]. Non-contrast cardiac CT images were obtained to quantify hepatic attenuation and pericardial fat volume. The CT scan range encompassed the entire heart and provided information on 45 mm of adipose tissue around the proximal coronary arteries. There were nine regions of interest read within homogenous portions of the liver at two levels. Lower values of hepatic attenuation measured in Hounsfield Units (HU) correspond to greater quantity of liver fat. Fatty liver was defined as a dichotomous variable with hepatic fat attenuation < 40 HU.

A trained CT technician obtained a lateral scout image of the

abdomen to establish position between the L4 and L5 vertebrae. Medical Image Processing, Analysis, and Visualization (MIPAV) software (Center for Information Technology and National Institutes of Health 1999) was used to interrogate CT images at vertebral levels L4-L5. For the abdominal, visceral fat, intermuscular fat and subcutaneous fat measurements. The subcutaneous tissue compartment included tissue outside the visceral cavity but within the body contour, and visceral fat was defined as fat with the appropriate HU within the visceral cavity. Measurements from four abdominal and back muscle groups (psoas, paraspinous, oblique and rectus muscles) were summed to obtain total intermuscular fat area.

2.5. Statistical methods

Relative abundance of metabolites were log-transformed to diminish the impact of extreme outliers and insure that the distributional features of the data were well suited to modeling using conventional parametric methods. Multivariable linear regression analyses were used to determine associations of relative abundance of each independent ceramide and mean hepatic attenuation and ectopic fat depots. Multivariable logistic regression analysis examined the association of ceramide abundance with the presence or absence of fatty liver and prevalent diabetes. The analyses were adjusted for age (years), sex and study site, body mass index (BMI), diet score (AHEI-2010), energy intake, exercise (MET-minutes/week), alcohol intake (none vs. 1–7 drinks/week) (Model 1) and further adjusted for statin use (yes/no), saturated fat intake (g/day) and baseline fasting glucose (mg/dL) (Model 2). Change in BMI and waist circumference from Exam 1 to Exam 2 was found not to be significant in the model and was not included. An *a priori* determined subgroup analysis excluding those with prevalent diabetes was completed, and Model 2 did not include baseline fasting glucose.

The conservative Bonferroni method was applied to adjust for multiple comparisons for 19 ceramide outcomes, with an alpha < 0.003 deemed significant. Where hepatic attenuation was associated with fasting glucose at follow-up exam, formal mediation analyses were conducted using percentile bootstrap to estimate the standard error of the mediation effect for ceramides identified in the previous step. Causal mediation analysis creating a simulation of predicted values of the mediator or outcome variable, followed by calculation of the average causal mediation, direct effects, and total effects was performed to assess whether the inclusion of metabolites partially mediates the association of hepatic attenuation on subsequent fasting glucose in those participants without baseline diabetes [20]. Interaction by sex was tested and none found.

The analysis was completed using STATA (version 16.1, 2021, College Station, TX, USA).

3. Results

3.1. Demographic characteristics

This investigation included 669 MASALA study participants with metabolite and clinical data. The average age was 55 years, 44% were women, mean BMI was 25.9 kg/m2, and 20% of participants had diabetes at the baseline exam. Approximately 8% of participants had fatty liver, defined as hepatic attenuation < 40 HU (Table 1).

3.2. Ceramides and associations with hepatic attenuation

Cer(d16:1/20:0) and Cer(d18:1/18:0) were strongly and consistently associated with lower hepatic attenuation (β −4.29; 95% CI [-5.98, −2.59]) and (β −3.40; 95% CI [−5.11, −1.70]) in all regression models, with lower hepatic attenuation denoting higher quantity of liver fat (Table 2). Cer(d16:1/20:0) and Cer(d18:1/18:0) were also associated with presence of fatty liver (OR 4.25; 95% CI [1.93, 9.32]), (OR 3.69; 95% CI [1.69, 8.06]) in models adjusting for age, sex, exercise, diet quality, BMI, alcohol use (Model 1) but not those further adjusting for fasting glucose, saturated fat intake and lipid-lowering medication use (Model 2) (Table 2). LacCer(d18:1/16:0) was associated with higher hepatic attenuation and lower liver fat (4.44; 95% CI [2.15, 6.73]) in a fully adjusted model, and lower odds of fatty liver (OR 0.20; 95 %CI [0.08, 0.53]) in Model 1.

Table 1
Baseline characteristics of MASALA cohort, 2010–2013 (N = 669); Mean (SD) unless otherwise specified.

Characteristic	Mean (SD)
Women	308 (46%)
Age [mean (SD)]	55 (9)
Body mass index (BMI, kg/m^2)	25.9 (4.0)
Waist circumference (cm)	
Women	89 (10)
Men	96 (9)
Hip circumference (cm)	
Women	103 (10)
Men	102 (8)
Exercise (met-minutes/ week) [median, IQR]	952 (323–1890)
Calories [mean (SD)]	1662 (500)
Cholesterol medication use	283 (42%)
Statin medication use	188 (26%
Fibrates, Niacin, ezetimibe	35 (5%)
Smoking (current)	18 (3%)
Alcohol use (current)	214 (31%)
Diabetes	140 (20%)
Hypertension	284 (40%)
eGFR	60 (2)
ASCVD-10 year (pooled cohort)	7.8% (0.9%)
Total cholesterol (mg/dL)	188 (37)
LDL-c (mg/dL)	111(32)
HDL-c (mg/dL)	50(13)
AST	21 (7)
ALT	21 (14)
Visceral fat cm^2	135 (55)
Pericardial fat cm3	59 (30)
Subcutaneous fat cm^2	238 (95)
Intermuscular fat cm^2	21 (9)
Fatty liver prevalence	57 (8%)
Fatty liver prevalence by diabetes status	
Normoglycemic	23 (6%)
Impaired glycemia	12 (8%)
Diabetes	22 (17 %)

In an *a priori* subgroup excluding those participants with prevalent diabetes (Supplemental Table 1), Cer(d16:1/20:0) and Cer(d18:1/18:0) were associated with mean hepatic attenuation (-4.79; 95% CI [-6.68, −2.90]), (-3.97; 95% CI (-5.85, −2.10) (Model 2)] and presence of fatty liver (OR 4.98; 95% CI [1.75, 14.15]) (Model 1), (OR 5.63; 95% CI [2.03, 15.65]) (Model 2)], respectively. LacCer(d18:1/16:0) was associated with higher mean hepatic attenuation (4.61; 95% CI [1.98, 7.24]), but not associated with presence of fatty liver.

3.3. Ceramides and associations with other ectopic fat

Cer(d16:1/20:0) was associated with greater visceral fat area (cm^2) (13.99; 95% CI [6.32, 21.66]) in a model adjusted for age, sex, study site, BMI, alcohol use, exercise, diet quality, caloric intake, lipid-lowering medication use, saturated fat intake, fasting glucose. There were no associations of ceramide abundance with other ectopic fat depots, including pericardial volume, intermuscular, subcutaneous fat areas or hip circumference (representing gluteofemoral fat). (Supplemental Table 2) Pearson correlation coefficients between liver fat and each type of ectopic fat ranged from −0.22 for hip circumference to −0.50 for visceral fat (Supplemental Table 3). Adjustment for other ectopic fat depots modestly attenuated the association between liver fat area and Cer(d16:1/20:0), Cer(d18:1/18:0) and LacCer(d18:1/16:0) however they remained significant (Supplemental Table 4).

Table 2
Associations of Ceramide Abundance with Mean Hepatic Attenuation (Hounsfield Units) and Fatty Liver (Hepatic attenuation < 40 HU), MASALA study (N = 669).

	Hepatic Attenuation (HU)				Fatty Liver (<40 HU)			
	Model 1[a] [β (95% CI)]	*P* value	Model 2[b] [β (95% CI)]	*P* value	Model 1[a][OR (95% CI)]	*P* value	Model 2[b][OR (95% CI)]	*P* value
Cer(d16:1/20:0)	**−5.01 (-6.68, −3.33)**	**6.93 e-09**	**−4.29 (-5.98, −2.59)**	**8.63 e-07**	**4.25 (1.93, 9.32)**	**0.0003**	**2.86 (1.27, 6.43)**	**0.01**
Cer(d16:1/22:0)	−2.52 (-4.31, −0.72)	0.006	−1.92 (-3.70, −0.15)	0.03	1.74 (0.82, 3.71)	0.1487	1.33 (0.61, 2.90)	0.48
Cer(d16:1/24:0)	−1.22 (-3.07, 0.64)	0.20	−0.92 (-2.75, 0.91)	0.32	1.12 (0.52, 2.43)	0.78	0.93 (0.42, 2.09)	0.87
Cer(d16:1/24:1)	−0.43 (−2.20, 1.34)	0.63	−0.51 (-2.24, 1.22)	0.56	1.71 (0.82, 3.57)	0.15	1.59 (0.76, 3.32)	0.22
Cer(d18:1/18:0)	**−4.11 (-5.83, −2.39)**	**3.37 e-06**	**−3.40 (−5.11, −1.70)**	**9.73 e-05**	**3.69 (1.69, 8.06)**	**0.0010**	2.97 (1.31, 6.74)	0.01
Cer(d18:1/20:0)	−3.10 (−5.35, −0.84)	0.0071	−2.81 (-5.01, −0.61)	0.01	3.25 (1.22, 8.64)	0.018	2.86 (1.03, 7.91)	0.04
Cer(d18:1/22:0)	−3.00 (−5.56, −0.43)	0.02	−2.56 (-5.11, −0.01)	0.05	2.18 (0.74, 6.44)	0.16	1.95 (0.63, 6.05)	0.25
Cer(d18:1/24:0)	−1.45 (-4.40, 1.47)	0.33	−1.36 (-4.26, 1.55)	0.36	1.28 (0.36, 4.51)	0.70	1.32 (0.35, 5.00)	0.69
Cer(d18:1/24:1)	0.80 (-1.60, 3.19)	0.51	0.46 (-1.88, 2.80)	0.70	1.54 (0.57, 4.19)	0.40	1.67 (0.59, 4.69)	0.33
Cer(d18:1/25:0)	1.93 (-0.08, 3.96)	0.06	1.48 (-0.54, 3.49)	0.14	0.79 (0.33, 1.87)	0.59	0.88 (0.35, 2.20)	0.79
Cer(d18:1/26:1)	−0.14 (-1.86, 1.57)	0.874	0.03 (−1.66, 1.71)	0.97	1.67 (0.81, 3.46)	0.17	1.46 (0.69, 3.09)	0.24
Cer(d18:2/22:0)	0.79 (-1.30, 2.85)	0.46	0.80 (-1.25, 2.84)	0.45	0.83 (0.35, 2.00)	0.68	0.82 (0.33, 2.05)	0.67
Cer(d18:2/24:0)	2.56 (0.24, 4.87)	0.03	2.28 (-0.01, 4.56)	0.05	0.48 (0.18, 1.24)	0.13	0.51 (0.18, 1.41)	0.19
Cer(d 19:1/24:0)	1.17 (-0.42, 2.75)	0.15	1.00 (-0.59, 2.60)	0.22	0.78 (0.40, 1.53)	0.48	0.76 (0.38, 1.52)	0.44
HexCer(d18:1/20:0)	2.83 (0.97, 4.79)	0.003	1.68 (-0.25, 3.62)	0.09	0.53 (0.24, 1.16)	0.11	0.79 (0.34, 1.84)	0.59
HexCer(d18:1/22:0)	2.98 (0.89, 5.07)	0.005	1.67 (-0.53, 3.86)	0.12	0.37 (0.16, 0.88)	0.03	0.56 (0.22, 1.41)	0.22
HexCer(d18:1/24:0)	2.52 (0.30, 4.75)	0.03	1.02 (-1.29, 3.32)	0.39	0.40 (0.16, 1.01)	0.05	0.64 0.24, 1.72)	0.37
Lac Cer(d18:1/16:0)	**5.97 (3.84, 8.09)**	**7.41 e-08**	**4.44 (2.15, 6.73)**	**0.0001**	**0.20 (0.08, 0.53)**	**0.001**	0.41 (0.14,1.15)	0.09

Bolded = p < 0.003.
[a] Adjusted for age, sex, study site, body mass index, diet score (AHEI-2010), energy intake, exercise (MET-minutes/week), alcohol intake (none vs. 1–7 drinks/week).
[b] Additionally adjusted for cholesterol lowering medication use, saturated fat intake and fasting glucose.

3.4. Associations between hepatic attenuation, ceramide abundance and diabetes

Lower quantity of hepatic fat was associated with lower odds of prevalent diabetes (OR 0.94; 95% CI [0.92, 0.96]) and incident diabetes (0.96; 95% CI [0.93, 0.99]) and associated with lower fasting glucose at 5-year follow-up (-0.23; 95% CI [-0.34, −0.12]) as well as change in glucose from baseline to 5-year follow-up exam (-0.16; 95% CI [-0.28, −0.05]). (Table 3) Cer(d16:1/20:0) and Cer(d18:1/18:0) were positively associated with prevalent DM, while LacCer(d18:1/16:0) was inversely associated with prevalent DM, after adjusting for age, sex, study site, diet, exercise, alcohol intake, saturated fat intake and BMI (p < 0.003). Ceramides were not associated with incident diabetes or change in fasting glucose between baseline and Year 5 (Table 3). In the *a priori* analysis excluding participants with prevalent diabetes at baseline, Cer(d16:1/20:0) and Cer(d18:1/18:0) were positively associated and LacCer(d18:1/16:0) was inversely associated with fasting glucose at 5-year follow-up (4.91; 95% CI [2.43, 7.39]), (4.56; 95% CI [2.08, 7.04]) and (-5.01; 95% CI [-8.32, −1.71]), respectively (Table 3).

3.5. Mediation analysis

In a subgroup analysis excluding those with prevalent diabetes at baseline, Cer(d16:1/20:0), Cer(d18:1/18:0) and LacCer(d18:1/16:0) were partial mediators of the association of NAFLD with year 5 glucose levels, with a mediation effect of −0.04 (-0.08, −0.02), −0.03 (-0.06, −0.01) and −0.01 (-0.03, 0.00), respectively, corresponding to 16%, 11% and 5% of the total effect mediated through each ceramide (Table 4).

3.6. Discussion

In a population of South Asians in the United States, Cer(d16:1/20:0) and Cer(d18:1/18:0) abundance was directly associated with liver fat, while LacCer(d18:1/16:0) was inversely associated with liver fat. These ceramides, which have a well-established association with impaired glucose tolerance and diabetes in both animal and human models, were also associated with prevalent diabetes and future fasting glucose [21]. All three ceramides partially mediated the relationship between hepatic attenuation and fasting glucose after 5-years of follow-up in participants without baseline diabetes.

The presence of NAFLD in South Asians has been estimated at

Table 3
Association of Hepatic Attenuation and Ceramide Abundance with Prevalent DM and Fasting Glucose at 5-year Follow-up[a].

	Prevalent Diabetes (OR; 95% CI)	*P* value	Incident Diabetes (OR; 95% CI)	*P* value	Fasting glucose at Exam 2[b] (Mean; 95% CI)	*P* value	Change in Glucose[b] (Mean; 95% CI)	*P* value
Fatty liver, HU < 40	**2.97 (1.57, 5.61)**	**0.0008**	**2.04 (0.68, 6.09)**	**0.20**	**7.60 (3.43, 11.77)**	**0.0004**	**5.11 (0.79, 9.44)**	**0.02**
Mean hepatic attenuation, HU	**0.94 (0.92, 0.96)**	**0.0000**	**0.96 (0.93, 0.99)**	**0.01**	**−0.23 (-0.34, −0.12**	**0.0001**	**−0.16 (-0.28, −0.05)**	**0.01**
Cer(d16:1/20:0)	**2.44 (1.44, 4.14)**	**0.0009**	1.73 (0.75, 4.02)	0.20	**4.91 (2.43, 7.39)**	**0.0001**	3.25 (0.67, 5.84)	0.01
Cer(d18:1/18:0)	**2.53 (1.49, 4.29)**	**0.0006**	1.84 (0.82, 4.13)	0.37	**4.56 (2.08, 7.04)**	**0.0003**	3.03 (0.45, 5.62)	0.02
Lac Cer(d18:1/16:0)	**0.09 (0.05, 0.19)**	**0.0000**	0.51 (0.17, 1.51)	0.23	**−5.01 (-8.32, −1.71)**	**0.003**	−0.11 (-3.55, 3.33)	0.94

Bolded = p < 0.003.
[a] Adjusted for age, sex, study site, BMI (WHO Asian categories), diet score (AHEI-2010), energy intake, exercise (MET-minutes/week), alcohol intake (none vs. 1–7 drinks/week), saturated fat intake.
[b] Restricted to participants without prevalent DM at baseline.

Table 4
Mediation Analysis Investigating Ceramides as Mediators between Hepatic Attenuation and Fasting Glucose at Exam 2.

	Total Effects	Direct effects	ACME	Percent of Total Effect Mediated
Cer(d16:1/20:0)	−0.26 (-0.37, −0.15)	−0.22 (-0.34, −0.10)	−0.04 (-0.08, −0.02)	16
Cer(d18:1/18:0)	−0.26 (-0.38, −0.15)	−0.24 (-0.35, −0.12)	−0.03 (-0.06, −0.01)	11
Lac Cer (d18:1/16:0)	−0.26 (-0.38, −0.14)	−0.25 (-0.37, −0.13)	−0.01 (-0.03, 0.00)	5

The association of mean liver fat and fasting glucose at 5-year follow-up directly AND through each ceramide is represented by "Total Effects." The direct association between liver fat and fasting glucose removing the mediator is represented by "Direct Effects." The "ACME" represents the portion of the association of liver fat and fasting glucose that is mediated through each ceramide.

18–30% in surveys of various rural and urban populations in countries in South Asia [22]. In a cohort of South Asian people with diabetes, the prevalence of NAFLD or NASH is 50–80% [22]. Despite the high burden of NAFLD, it is often identified only during an evaluation for abnormal blood liver function tests, or incidentally on imaging studies of the abdomen for other reasons; there is currently no comprehensive strategy for clinical screening. The high prevalence of NAFLD, especially in Asian and South Asian populations with low or normal BMI, and its association with diabetes in a population at great risk for this condition [13] makes it even more important to investigate the mechanisms of and potential biomarkers for this condition.

NAFLD affects and is affected by hepatic and peripheral insulin resistance, and impairment of this finely tuned system promotes higher risk for diabetes. Even in populations without prevalent diabetes, NAFLD has been associated with higher fasting insulin levels [21]. In a Korean population with existing prediabetes, 45% had NAFLD which was associated with a relative risk of 1.8 for the development of diabetes during 5-year follow-up [23]. The presence of NAFLD promotes hepatic insulin resistance, affects hepatic glucose and insulin regulation, and promotes increased lipogenesis which may further exacerbate NAFLD severity [24]. In this analysis of the MASALA study, NAFLD was associated with prevalent diabetes, incident diabetes and with higher fasting glucose 5 years after enrollment, and has previously been independently associated with glycemic progression [25]. The prevalence of NAFLD in our cohort was lower than in other studies of South Asians, and may reflect the specificity of CT-derived measures. The prevalence of CT-defined fatty liver was higher in South Asians in the MASALA cohort than in 4 other racial and ethnic groups in the Multi-Ethnic Study of Atherosclerosis, using the same CT methods [6].

Ceramides are a type of biologically active lipid with strong associations with diabetes risk [26]. In animal models, an upregulation of adipocyte lipolysis, caused by increased inflammation, saturated fat intake, or insulin resistance, leads to an influx of free fatty acids into the circulation and liver [27]. As the storage capacity for fatty acids is exceeded, circulating free fatty acids pair with a sphingoid backbone to form ceramides, which then increase intrahepatic triglyceride deposition [12]. Inhibition of ceramide synthetase, and decrease in ceramide 16:0 decreased hepatic steatosis and insulin resistance in mice [8]. Ceramide concentrations are higher in those with NASH than NAFLD, and may serve as a marker of progression by correlating with hepatic inflammation and with whole-body insulin resistance [9]. In this investigation, ceramides were not associated with incident diabetes, which may be due to a small number of incident diabetes cases (n = 50), but were significantly associated with fasting glucose at 5-year follow-up. Long-chain ceramides partially mediated the relationship between fatty liver and future rise in fasting glucose, suggesting that while ceramides likely have a role in multiple stages of metabolic dysfunction, accumulation of circulating ceramides may indicate significantly worsening glycemic impairment.

Ectopic fat depots apart from fatty liver, in particular visceral fat, have also been linked with cardiometabolic disease. A prior investigation in MASALA found that both visceral adiposity and liver fat were associated with glycemic progression [25]. In our analysis, visceral fat and liver fat were independently associated with Cer(d16:1/20:0). Inclusion of liver fat and other ectopic fat depots as covariates rendered the association between Cer(d16:1/20:0) and visceral fat area insignificant, while only modestly attenuating the association between Cer (d16:1/20:0), Cer(d18:1/18:0) and LacCer(d18:1/16:0) and liver fat. This finding suggests that there is a unique relationship between liver fat and ceramides that is unexplained by the presence of other ectopic fat depots.

Ceramides with long-chain fatty acids, C16 and C18, have well-established associations with insulin resistance and diabetes [10]. In a Hispanic population at high risk for T2D in the SOL study, a ceramide score including C18 ceramides was significantly associated with risk for diabetes [28]. In a population-based cohort study from China, 13 sphingolipids, including several C18 ceramides, were associated with higher risk of future diabetes. This association was thought to be mediated through beta-cell dysfunction [29]. In a population with Polycystic Ovary Syndrome (PCOS), C16 and C18 ceramides were increased in PCOS cases, while LacCer was only increased in non-obese, non-insulin-resistant cases of PCOS. In prior work in the MASALA study, concentrations of Cer(d16:1/20:0) at baseline exam was associated with higher fasting glucose at 5-year follow-up [11]. Taken together, the abundance of long-chain ceramides in the circulation may indicate that metabolic abnormalities are taking place that are worsening hepatic fat deposition and leading to diabetes. The presence of these markers may warrant special intervention for the prevention of diabetes [12].

Saturated fatty acids, specifically palmitate, are a precursor for *de novo* synthesis of C16:0 and C18:0 sphingolipids, which are associated with insulin resistance and hepatic steatosis [27]. In a trial comparing overfeeding with saturated fat, unsaturated fat or carbohydrates, saturated fat intake increased intrahepatic triglycerides, total plasma ceramides and HOMA-IR [30]. In the same trial, saturated fat intake increased adipose tissue lipolysis, a possible mechanism for the increase in intrahepatic triglycerides. This suggests that intake of saturated fat may have a role in increasing production of ceramides and may be a target for dietary intervention to reduce diabetes risk.

The strengths of this study include the robust baseline phenotyping of the only longitudinal cohort population of South Asians in the United States, a group at extremely high risk for diabetes and cardiovascular disease. Limitations include the absence of repeat measures of metabolomics and liver fat assessment such that longitudinal associations of liver fat and ceramides cannot be firmly established. Due to lack of a measurement of liver histology, this study is also unable to differentiate between NAFLD and NASH. In this study, the prevalence of fatty liver was lower than estimated in other South Asian populations. While the CT-based methods used in this study are accurate, it is possible that the prevalence of fatty liver was underestimated and MRI-based methods would have shown stronger relationships with circulating ceramides [31]. Despite these limitations, this is the first longitudinal characterization of ceramide abundance, liver fat and diabetes in South Asians.

Fatty liver is associated with prevalent and incident diabetes and future fasting glucose in South Asians and may be an underexplored factor to identify high-risk South Asian individuals with a lower BMI. Circulating ceramides likely play a role in the relationship between NAFLD and glycemic impairment, and in the onset and progression of clinically significant cardiometabolic disease. In further investigations, measurement of changes in ceramide abundance and liver fat can help to determine if ceramide abundance may be a biomarker of clinical utility in South Asians for whom traditional risk factors are inadequate for risk stratification for cardiometabolic disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

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

Data sharing

The data that support the findings of this study are available from MASALA study but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the MASALA Steering Committee.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2022.109829.

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