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Plasma 1-deoxysphingolipids are predictive biomarkers for type 2 diabetes mellitus

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ABSTRACT

Objective: Serine palmitoyltransferase (SPT) catalyzes the condensation of serine and palmitoyl coenzyme A, the first step in the de novo sphingolipid synthesis. Apart from these canonical substrates, SPT can also metabolize alanine and other acyl coenzyme As. This forms a spectrum of atypical sphingoid bases which are altered in the context of the metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM). We investigated whether atypical sphingolipids can be used as prospective markers to predict the incidence of T2DM.

Research design and methods: Using liquid chromatography/mass spectrometry, we analyzed the sphingoid base profile in a prospective cohort with 339 individuals. All individuals were followed up for a period of 8 years.

Results: Confirming earlier results, we found 1-deoxysphingolipids (1-deoxySLs) to be significantly elevated in patients with MetS, impaired fasting glucose, and T2DM. Patients who developed T2DM during the follow-up period (n=32) showed significantly higher 1-deoxySL levels at baseline compared with those who did not develop T2DM until the end of the study (n=70). 1-Deoxysphingosine levels were independent predictors for T2DM even after adjusting for glycated hemoglobin (standardized adjusted OR=2.1, CI 95% (1.19 to 3.71); p=0.010), MetS (standardized adjusted OR=1.97, CI 95% (1.13 to 3.43); p=0.017), and other risk factors such as age, sex, BMI, and lipid-lowering drugs. Similar results were observed for the 1-deoxysphinganine levels.

Conclusions: Our results support a novel role for 1-deoxySL as predictive biomarkers for the development of T2DM in risk patients and warrants further larger prospective trials in lower risk cohorts.

Key messages

- 1-Deoxysphingolipids (1-deoxySLs) are significantly elevated in patients with impaired fasting glucose, metabolic syndrome (MetS), and T2DM.
- 1-DeoxySLs are independent predictors for the risk to develop T2DM even after adjusting for glycated hemoglobin and the presence of MetS.
- Although plasma triglycerides and 1-deoxySLs showed a high collinearity, stepwise logistic regression analysis revealed 1-deoxySLs as independent predictors for T2DM.

INTRODUCTION

Diabetes mellitus type 2 (T2DM) remains a major cause of mortality and morbidity worldwide, despite the advances in risk prediction, diagnosis, and management. Traditional risk factors such as impaired fasting glucose (IFG) and impaired glucose tolerance fail to predict almost 50% of the patients who will develop T2DM.[1 2] This fact has fueled an expanding research pursuit to identify risk factors and novel biomarkers to improve risk prediction.[3 4] With the progress in mass spectrometry and nuclear magnetic resonance spectroscopy, many blind spots in the human plasma metabolome and lipidome are becoming visible and quantifiable. In fact, several metabolites such as acylcarnitines, amino acids, phospholipids, and also sphingolipids have become candidates of biomarkers for the prediction of future T2DM.[5–7]

Sphingolipids comprise a heterogeneous class of lipids including free sphingoid bases, ceramides, sphingomyelins, and glycosphingolipids. Sphingoid bases are the shared structural element in all sphingolipids and typically formed by the condensation of L-serine and palmitoyl coenzyme A. This reaction is catalyzed by the enzyme serine palmitoyltransferase (SPT)[8–10] forming C18-sphinganine (C_{18}SA), which is then subsequently *N*-acylated to (dihydro)-ceramides. Ceramides are bioactive lipids which serve as building blocks for the synthesis of complex sphingolipids like sphingomyelins or gylcosphingolipids.

There is increasing evidence that sphingolipids are actively involved in the development of T2DM.[11] Ceramides were shown to

counteract the insulin action on glucose uptake and glycogen synthesis by inhibiting protein kinase B/Akt through different mechanisms,[12] and it was demonstrated recently that many of the beneficial effects of adiponectin on β-cell survival in T2DM are linked to its lowering effect on intracellular ceramide levels.[13] Moreover, plasma levels of ceramides were found to be elevated in patients with T2DM[14] and to correlate with the degree of insulin resistance in these patients. In contrast, sphingomyelin and gylcosphingolipids were found to be decreased in plasma of diabetic monkeys.[15] Myriocin, a potent SPT inhibitor, was shown to improve insulin resistance and preclude the development of T2DM in animal models.[16] Apart from the canonical substrates, SPT is also able to metabolize other acyl coenzyme As in the range of C_{12} to C_{18}, which results in a spectrum of sphingoid bases with variable carbon chain length. Under certain conditions, SPT can also use other amino acids such as L-alanine and partly also glycine as alternative substrates.[17] This generates an atypical class of neurotoxic 1-deoxysphingolipids (1-deoxySL) which lack the C_1 hydroxyl group of canonical sphingolipids. SPT is positioned at a metabolic cross-point which metabolically interconnects fatty acid, amino acid (serine, alanine, and glycine), and thereby indirectly also the carbohydrate metabolism.

We showed previously that 1-deoxyspingolipids (1-deoxySLs) are significantly elevated in the plasma of individuals with metabolic syndrome (MetS)[18] and T2DM.[19] In this follow-up study, we aimed to investigate the ability of 1-deoxySLs to predict incident diabetes by analyzing the sphingoid base profile in a prospective cohort with 339 individuals.

RESEARCH DESIGN AND METHODS

Patients and study design

For this study, a group of 339 patients were selected from a prospective cohort study of patients who underwent coronary angiography for the evaluation of established or suspected stable coronary artery disease (CAD).[20] The group was selected based on the availability of baseline and follow-up data for T2DM. The study participants were enrolled into the study between September 1999 and October 2000. Anthropometric data were collected, clinical chemistry laboratory parameters were measured, and coronary angiography (Judkin's technique) was performed on all study participants at baseline. Coronary artery stenoses with lumen narrowing of 50% or more were considered significant, and coronary arteries were defined as normal in the absence of any visible lumen narrowing at angiography.

All participants gave written informed consent.

Diagnosis

Diabetes at baseline was diagnosed according to the WHO criteria, that is, fasting glucose levels ≥7 mmol/L (126 mg/dL), plasma glucose levels ≥11.1 mmol/L (200 mg/dL) 2 h after an oral glucose challenge (75 g), or a previous physician diagnosis of T2DM.

Metabolic syndrome at baseline was defined according to the criteria of the National Cholesterol Education Program-Adult Treatment Panel III criteria, if three or more of the following criteria were met: waist circumference >102 cm in men or >88 cm in women; triglycerides (TGs) ≥1.7 mmol/L (150 mg/dL); high-density lipoprotein (HDL) cholesterol <1.0 mmol/L (40 mg/dL) in men or <1.3 mmol/L (50 mg/dL) in women; blood pressure ≥130/≥85 mm Hg; and fasting glucose ≥6.1 mmol/L (110 mg/dL). Patients with fasting glucose ≥7 mmol/L (126 mg/dL) at baseline were diagnosed with T2DM, patients with fasting glucose between 5.6 and 6.9 mmol/L (100–125 mg/dL) were diagnosed as IFG while those with fasting glucose <5.6 mmol/L were diagnosed as normal fasting glucose (NFG).

Prospective study

For the diagnosis of incident T2DM, blood samples were collected 2, 4, 6 and 8 years after enrollment. Individuals were considered to have incident diabetes if one of the three criteria was met at any of the follow-up examinations: fasting glucose levels ≥7 mmol/L (126 mg/dL), plasma glucose levels ≥11.1 mmol/L (200 mg/dL) 2 h after an oral glucose challenge (75 g), or the clinical diagnosis of T2DM by a physician during the follow-up period. Individuals were classified to be free from incident T2DM if *both* conditions were met; fasting glucose levels <7 mmol/L (126 mg/dL) *and* plasma glucose levels <11.1 mmol/L (200 mg/dL) 2 h after an oral challenge with 75 g glucose at *all* follow-up visits. Individuals with missing values for fasting plasma glucose or 2 h post-glucose challenge were excluded. From the total cohort, 105 participants fulfilled either the criteria of incident T2DM (n=32) or the absence of incident T2DM (n=70) and were included in the prospective analysis.

Clinical chemistry

Venous blood samples were collected after overnight fasting and at least 12 h before angiography. Laboratory analysis was performed on fresh serum or blood samples. Glycated hemoglobin (HbA1c) was analyzed by high-performance liquid chromatography on a Menarini-Arkray KDK HA 8140 (Arkray KDK, Kyoto, Japan). Triacylglycerols, total cholesterol, HDL, and other clinical chemistry variables were measured on a Hitachi 717 or 911 system (Roche).

Sphingolipid analysis

The sphingoid base profile was analyzed as described before[18] with some modifications. Briefly, 0.5 mL methanol including 200 pmol of the internal standards d7-sphingosine and d7-sphinganine (d7SA, d7SO; Avanti Polar Lipids, Alabaster, Alabama, USA) was added to 100 µL of plasma and extracted for 1 h under agitation on a thermomixer at 37°C. Precipitated proteins were

pelleted by centrifugation and the supernatant transferred to a new tube. For lipid hydrolysis, 75 µL of methanolic HCl (1 N HCl and 10 M H_2O in methanol) was added to the supernatant and incubated for 16 h at 65°C. This was followed by the addition of 100 µL of 10 M KOH to neutralize the HCl and hydrolyze the phospholipids. To this mix, 625 µL chloroform was added. Then, 100 µL 2N ammonium hydroxide and 0.5 mL alkaline water were added to complete the phase separation. The mix was then vortexed and centrifuged at 16 000*g* for 5 min. After centrifugation, the upper phase was discarded and the lower organic phase was washed 2–3 times with alkaline water (pH 10.3). Finally, the organic phase was dried under N_2 and kept at −20° C until analysis.

The sphingoid bases were separated on a C_{18} column (Uptispere 120 Å, 5 µm, 125×2 mm, Interchim, Montluçon, France) and analyzed on a TSQ Quantum Ultra mass spectrometer (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton. Intra assay and inter assay coefficient of variation (%) of the method was between 5% and 20%.

Table 1 Baseline characteristics of patients with MetS and individuals free of MetS

	No MetS at baseline (n=192)	MetS at baseline (n=147)	p Value
Age (years)	64.2±10.1	61.2±9.7	0.006
Sex (female)	65 (33.85%)	43 (29.25%)	0.367
Hypertension (WHO)	**78 (40.63%)**	**98 (66.67%)**	**1.98E-06**
T2DM (baseline)	**31 (16.15%)**	**77 (52.38%)**	**1.28E-12**
History of smoking	106 (55.21%)	97 (65.99%)	0.045
Coronary artery stenosis >50%	112 (58.33%)	98 (66.67%)	0.117
BMI	**26±4.1**	**28.9±4.2**	**4.59E-11**
Waist circumference (cm)	**91.6±10.7**	**101.6±11.4**	**3.92E-14**
Waist-to-hip ratio	**0.9±0.1**	**1.0±0.1**	**2.91E-08**
Cholesterol (mmol/L)	5.6±1	5.6±1.2	0.478
LDL (mmol/L)	3.4±0.8	3.3±0.9	0.068
HDL (mmol/L)	**1.4±0.4**	**1±0.3**	**4.49E-19**
Triglycerides (mmol/L)	**1.4±0.5**	**2.5±1.6**	**3.18E-22**
Glucose (mmol/L)	**6±1.8**	**7.7±2.5**	**9.56E-20**
HbA1c (%)	**6±0.8**	**6.7±1.4**	**2.06E-07**
Insulin (µU/mL)	**9.1±7.6**	**16.7±15.5**	**2.98E-13**
HOMA-IR	**2.4±2.2**	**5.5±4.5**	**2.0E-18**
Systolic BP	**130.8±21.1**	**145±21**	**4.82E-10**
Diastolic BP	**76.3±12.3**	**82.2±12.2**	**8.29E-06**
CRP	**0.7±1**	**1.1±1.7**	**2.65E-05**
Serum potassium	4.4±0.4	4.4±0.5	0.656
Creatinine (mg/dL)	1.1±0.5	1.2±0.7	0.053
GFR (Mayo)	86.6±18.6	85±21.4	0.973
T2DM treatment	**31 (16.15%)**	**77 (52.38%)**	**1.28E-12**
Diuretics	58 (30.21%)	60 (40.82%)	0.042
Antihypertensive drugs	149 (77.6%)	127 (86.39%)	0.039
Lipid-lowering drugs	90 (46.88%)	79 (53.74%)	0.210
Plasma sphingolipids			
C16SO (µmol/L)	15.32±5.38	14.19±5.4	0.022
C16SA (µmol/L)	0.45±0.19	0.5±0.29	0.220
C17SO (µmol/L)	8.13±2.73	7.22±2.45	0.002
C18PhytoSO (µmol/L)	0.12±0.04	0.11±0.05	0.066
C18SAdiene (µmol/L)	28.22±7.71	26.22±7.88	0.014
C18SO (µmol/L)	94.77±18.48	89.79±22.77	0.019
C18SA (µmol/L)	3.21±1.04	3.66±1.84	0.065
C19SO (µmol/L)	2.88±1.2	2.65±1.19	0.043
C20SO (µmol/L)	0.17±0.05	0.18±0.06	0.039
C20SA (µmol/L)	**0.02±0.01**	**0.03±0.01**	**2.81E-04**
1-deoxySO (µmol/L)	**0.15±0.09**	**0.21±0.14**	**5.65E-06**
1-deoxySA (µmol/L)	**0.07±0.03**	**0.09±0.06**	**3.26E-05**

Values are shown as mean±SD for the continuous variables and numbers and per cent of total for the categorical variables. p Values were calculated using the unpaired two-sided t test on the log-transformed continuous variables. For the categorical variables, the p value was calculated using the χ^2 test. Variables in bold font have significant differences after the Bonferroni correction (p<0.001).
BMI, body mass index; CRP, C reaction protein; GFR (Mayo), Glomerular filtration rate (Mayo equation); Hb, hemoglobin; HbA1c, glycated Hb; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, low-density lipoprotein; MetS, metabolic syndrome; SA, sphinganine; SO, sphingosine; T2DM, type 2 diabetes mellitus.

Sphingoid bases in plasma are typically *N*-acylated and conjugated to different headgroups.[21] It is important to note that 1-deoxySLs lack the C_1 hydroxyl group of typical SLs and therefore cannot form complex sphingolipids such as sphingomyelin or glycosphingolipids. Plasma 1-deoxySLs can be present as 1-deoxysphinganine (1-deoxySA) or 1-deoxysphingosine (1-deoxySO)) and in the *N*-acylated forms (1-deoxy-dihydro-ceramides and 1-deoxyceramides, respectively).[22] We performed an acid/base hydrolysis prior to analysis and quantified the total amount of all long-chain bases (which reflects the SPT activity). At that stage, we cannot distinguish between the free base and *N*-acyls anymore. Therefore, the reported sphingoid base concentrations do not reflect the original levels of free

Table 2 Baseline characteristics of patients with NFG, IFG, and T2DM

	NFG at baseline (n=124)	IFG at baseline (n=107)	T2DM at baseline (n=108)	p Value
Age (years)	62.5±10.3	63.6±10.1	62.7±9.7	0.708
Sex (female)	48 (38.71%)	27 (25.23%)	33 (30.56%)	0.085
Hypertension (WHO)	56 (45.16%)	64 (59.81%)	56 (51.85%)	0.085
History of smoking	70 (56.45%)	61 (57.01%)	72 (66.67%)	0.218
Coronary artery stenosis >50%	71 (57.26%)	65 (60.75%)	74 (68.52%)	0.202
MetS ATP III Definition	**26(20.97%)**	**44 (41.12%)***	**77 (71.3%)*§**	**1.01E-13**
BMI	26.4±4.6	27.3±4	28.2±4.3	0.006
Waist circumference (cm)	**91.9±12**	**97±10.7**	**99.4±12.2***	**9.54E-06**
Waist-to-hip ratio	**0.9±0.1**	**1±0.1**	**1±0.1***	**1.60E-04**
Cholesterol (mmol/L)	5.6±1.1	5.7±1	5.4±1.2	0.133
LDL (mmol/L)	3.4±0.8	3.5±0.8	3.1±0.9	0.001
HDL (mmol/L)	1.3±0.4	1.3±0.4	1.1±0.4	0.001
Triglycerides (mmol/L)	**1.7±1.1**	**1.7±0.8**	**2.3±1.7***	**3.39E-05**
Glucose (mmol/L)	**5.3±0.9**	**6.2±0.5***	**8.9±2.8*§**	**7.81E-55**
HbA1c (%)	**5.8±0.5**	**5.9±0.5**	**7.5±1.4***	**4.44E-44**
Insulin (µU/mL)	**9.4±13.5**	**12.9±10.5***	**15.4±11.8*§**	**2.39E-08**
HOMA-IR	**1.9±1.2**	**3.6±3.1***	**5.9±4.9*§**	**3.64E-23**
Systolic BP	133.3±22.7	138.7±21.3	139.6±22	0.040
Diastolic BP	77.9±12.6	79.7±11.2	79.2±13.9	0.457
CRP	0.7±1.1	0.9±1.7	1±1.2	0.058
Serum potassium	4.4±0.4	4.3±0.4	4.5±0.5	0.070
Creatinine (mg/dL)	1.2±0.9	1.1±0.2	1.2±0.4	0.640
GFR (Mayo)	86.2±19.7	86.8±17.3	84.7±22.4	0.496
Diuretics	33 (26.61%)	35 (32.71%)	50 (46.3%)	0.006
Antihypertensive drugs	93 (75%)	87 (81.31%)	96 (88.89%)	0.025
Lipid-lowering drugs	58 (46.77%)	49 (45.79%)	62 (57.41%)	0.162
Plasma sphingolipids				
C16SO (µmol/L)	15.26±6.46	14.67±4.38	14.5±5.03	0.695
C16SA (µmol/L)	0.44±0.26	0.48±0.21	0.49±0.23	0.096
C17SO (µmol/L)	8.11±3	7.74±2.43	7.31±2.38	0.165
C18PhytoSO (µmol/L)	0.12±0.05	0.11±0.04	0.11±0.04	0.792
C18SAdiene (µmol/L)	28.64±8.57	27.21±6.86	26.01±7.68	0.033
C18SO (µmol/L)	94.56±22.14	92.67±19.29	90.3±19.85	0.250
C18SA (µmol/L)	3.16±1.44	3.38±1.15	3.7±1.69	0.027
C19SO (µmol/L)	2.88±1.33	2.82±1.15	2.62±1.08	0.262
C20SO (µmol/L)	0.17±0.06	0.18±0.05	0.18±0.05	0.028
C20SA (µmol/L)	**0.02±0.01**	**0.03±0.01**	**0.03±0.02***	**1.33E-05**
1-deoxySO (µmol/L)	**0.14±0.1**	**0.17±0.09**	**0.22±0.14***	**2.22E-06**
1-deoxySA (µmol/L)	0.07±0.05	0.08±0.03	0.09±0.05	0.001

Values are shown as mean ±SD for the continuous variables and numbers and percentage of total for the categorical variables. p Values were calculated using the ANOVA followed by the Bonferroni correction on the log transformed continuous variables. For the categorical variables, the p value was calculated using the χ^2 test. Variables in bold font have significant differences after the Bonferroni correction ($p=4.1\times10^{-4}$). The three groups were compared with each other.
*Represent a significant difference from NFG.
§Represents a significant difference from IFG.
ANOVA, analysis of variance; ATP, Adult Treatment Panel; BMI, body mass index; CRP, C reaction protein; GFR (Mayo) Glomerular filtration rate (Mayo equation); Hb, hemoglobin; HbA1c, glycated Hb; HDL, high-density lipoprotein; HOMA-IR, Homeostatic model assessment-insulin resistance; IFG, impaired fasting glucose; LDL, low-density lipoprotein; MetS, metabolic syndrome; NFG, normal fasting glucose; SA, sphinganine; SO, sphingosine; T2DM, type 2 diabetes mellitus.

sphingoid bases in plasma but refer to the sphingoid base composition of all plasma sphingolipids (independent whether free or *N*-acylated).

Statistical analysis

Continuous variables were log-transformed and the means were compared using the two-sided t test or analysis of variance followed by the Bonferroni correction. Categorical variables were compared using the χ^2 test. Prospective analysis for the development of T2DM was performed using binary logistic regression. Mean comparisons were done between those individuals who developed T2DM and those who did not. A two-sided t test was performed on the log-transformed variables. Univariate binary logistic regression models were calculated to evaluate the predictive role of each of the measured variables. Multivariate logistic regression models were then performed to adjust for the traditional predictors. Since the number of cases in the incident T2DM group is 32, we limited the number of independent variables to two to avoid overfitting. Log-transformed and normalized variables in SD units were used for the logistic regression models. Statistical analyses were performed in SPSS V.16.0 (IBM, Zurich, Switzerland).

RESULTS

In a cross-sectional design, we first compared the sphingoid base plasma profiles from patients with and without MetS (table 1) as well as from participants with NFG, IFG, and T2DM (table 2).

At baseline, plasma concentrations of 1-deoxySLs and C_{20}SA-based sphingolipids were significantly higher in patients with MetS (table 1; $p=5.56\times10^{-6}$ for 1-deoxySO, $p=3.26\times10^{-5}$ for 1-deoxySA, and $p=2.81\times10^{-4}$ for C_{20}SA). Other sphingoid bases such as C_{16}SO, C_{17}SO, C_{18}SAdiene, C_{18}SO, and C19SO were lower but did not reach statistical significance after correction for multiple comparisons.

Both 1-deoxySO and 1-deoxySA were elevated in patients with T2DM (table 2) and in patients with IFG but only 1-deoxySO remained significantly elevated after the Bonferroni correction for multiple comparisons ($p<4.1\times10^{-4}$). It should be noted that Bonferroni correction is a very rather conservative correction method that increases the likelihood of false negatives. Therefore, the lack of significance for 1-deoxySA in patients with T2DM after correction cannot be necessarily interpreted as no difference in 1-deoxySA levels. It is rather likely that this is a false-negative finding as two independent cohorts showed a significant increase in 1-deoxySA and 1-deoxySO in patients with T2DM.[18 23] Interestingly, C20SA was also found to be significantly elevated in patient with T2DM. Other sphingoid bases were not significantly different.

Correlation analysis (table 3) revealed a strong positive correlation between 1-deoxySLs, TGs, glucose, HbA1c, insulin, and homeostatic model assessment-insulin

Table 3 Spearman correlation coefficients for the analyzed sphingoid bases, clinical chemistry, and anthropometric variables ($p<0.05$ in bold)

	C_{16}SO	C_{16}SA	C_{17}SO	C_{18}PhytoSO	C_{18}SAdiene	C_{18}SO	C_{18}SA	C_{19}SO	C_{20}SO	C_{20}SA	1-DeoxySO	1-DeoxySA
Age	**0.14**	−0.02	**0.25**	**0.11**	**0.11**	**0.11**	−0.05	**0.27**	−0.03	−0.04	**−0.13**	**−0.17**
BMI	0.06	**0.16**	−0.07	−0.03	0.03	−0.03	**0.19**	−0.08	0.09	**0.18**	**0.29**	**0.26**
Waist circumference	−0.10	0.04	**−0.24**	**−0.15**	−0.11	**−0.13**	0.10	**−0.20**	0.04	**0.11**	**0.33**	**0.26**
WHR	**−0.24**	−0.08	**−0.38**	**−0.19**	**−0.26**	**−0.19**	−0.02	**−0.27**	0.01	0.02	**0.27**	**0.16**
Cholesterol	**0.43**	**0.39**	**0.40**	**0.43**	**0.53**	**0.57**	**0.40**	**0.19**	**0.22**	**0.20**	**0.19**	**0.26**
LDL-C	**0.34**	**0.34**	**0.36**	**0.37**	**0.43**	**0.50**	**0.31**	**0.21**	**0.17**	**0.14**	0.05	**0.13**
HDL-C	**0.41**	0.07	**0.41**	**0.32**	**0.45**	**0.34**	0.08	**0.19**	−0.09	−0.07	−0.07	−0.01
TG	−0.09	**0.13**	**−0.17**	−0.02	−0.07	−0.09	**0.13**	**−0.14**	**0.14**	**0.20**	**0.42**	**0.39**
Glucose	−0.05	0.07	**−0.16**	−0.06	**−0.19**	**−0.12**	**0.14**	−0.10	**0.14**	**0.24**	**0.32**	**0.24**
HbA1c	0.01	−0.01	−0.01	0.09	−0.04	0.02	0.10	−0.04	0.04	**0.13**	**0.20**	**0.22**
Insulin	0.03	**0.18**	−0.07	−0.04	−0.07	−0.06	**0.17**	0.00	**0.21**	**0.19**	**0.23**	**0.19**
HOMA	0.02	**0.19**	−0.10	−0.01	−0.11	−0.07	**0.18**	−0.03	**0.21**	**0.24**	**0.29**	**0.25**
Systolic BP	0.06	0.09	0.05	0.08	0.05	0.01	0.09	0.02	0.07	**0.15**	0.04	0.09
Diastolic BP	0.03	0.10	−0.03	0.02	−0.01	−0.04	0.07	−0.01	0.05	0.09	0.11	**0.14**
CRP	**−0.13**	−0.03	−0.07	−0.02	−0.02	**0.13**	**0.15**	0.01	**0.14**	0.03	0.05	0.10
Potassium	−0.06	−0.06	−0.10	−0.01	0.01	0.02	0.01	−0.09	−0.01	−0.04	**0.16**	**0.15**
Serum creatinine	**−0.11**	**−0.17**	−0.11	−0.08	**−0.16**	−0.10	**−0.20**	−0.02	−0.04	**−0.13**	0.08	0.05
GFR (Mayo)	**−0.12**	0.08	**−0.23**	−0.07	**−0.11**	−0.07	**0.11**	**−0.22**	0.06	**0.11**	0.06	0.06

BMI, body mass index; CRP, C reaction protein; GFR (Mayo) Glomerular filtration rate (Mayo equation); Hb, hemoglobin; HbA1c, glycated Hb; HDL-C, high-density lipoprotein cholesterol; HOMA, Homeostatic model assessment; LDL-C, low-density lipoprotein cholesterol; SA, sphinganine; SO, sphingosine; TG, triglycerides; WHR, waiste-to-hip ratio.

resistance while the serine-derived sphingolipids correlated with low-density lipoprotein cholesterol and total cholesterol.

The potential of plasma sphingoid bases as biomarkers to predict the risk to develop T2DM was assessed by comparing baseline levels between individuals who developed T2DM during the follow-up period of the study (incident T2DM, n=32) and those who did not (no incident T2DM, n=70). Baseline 1-deoxySLs were significantly higher in the incident T2DM group (table 4) as were HbA1c and TGs, (p=0.004 for 1-deoxySO, p=0.011 for 1-deoxySA, p=0.006 for HbA1c, and p=0.024 for TGs).

Univariate binary logistic regression (table 5) revealed that 1-deoxySLs, along with typical risk factors such as HbA1c, TGs, hypertension, and the presence of an MetS, were significant predictors for the development of T2DM. Surprisingly also, serum potassium was a predictor for incident T2DM in the univariate analysis , which is in accordance with a recent report from the Atherosclerosis Risk in Communities (ARIC) study.[24]

The predictive potential of the 1-deoxySLs was further evaluated in a bivariate binary logistic regression model by using each of the significant variables from the univariate models as covariates (table 5) as well as other

Table 4 Baseline values of clinical variables and sphingolipid levels in the incident T2DM group and the group which did not develop T2DM until the end of the study period (8 years)

	No iT2DM (n=70)	iT2DM (n=32)	p Value
Age (years)	61.3±9.1	64.8±10.7	0.130
Sex (female)	26 (37.14%)	11 (34.38%)	0.787
Hypertension (WHO)	28 (40%)	19 (59.38%)	0.069
History of smoking	37 (52.86%)	14 (43.75%)	0.393
Coronary artery stenosis >50%	38 (54.29%)	23 (71.88%)	0.093
MetS ATP III Definition	**15 (21.43%)**	**14 (43.75%)**	**0.020**
BMI	26.4±3.3	27±5.3	0.722
Waist circumference (cm)	92.5±10.5	96.6±13.8	0.162
Waist-to-hip ratio	0.9±0.1	1±0.1	0.150
Cholesterol (mmol/L)	5.6±1	5.8±1.3	0.670
LDL (mmol/L)	3.5±0.8	3.4±0.9	0.659
HDL (mmol/L)	1.3±0.4	1.3±0.4	0.900
Triglycerides (mmol/L)	**1.6±0.9**	**2.2±1.6**	**0.024**
Glucose (mmol/L)	5.8±1.1	5.8±0.9	0.793
HbA1c (%)	**5.7±0.4**	**6±0.5**	**0.006**
Insulin (µU/mL)	9±4.6	16±26.6	0.249
HOMA-IR	2.3±1.2	2.9±2.2	0.576
Systolic BP	**131.6±19.1**	**140.5±21.1**	**0.041**
Diastolic BP	76.5±11	79.8±14.1	0.295
CRP	0.7±1	0.8±1.5	0.773
Serum potassium	**4.3±0.4**	**4.5±0.3**	**0.027**
Creatinine (mg/dL)	1.1±0.1	1.2±1	0.194
GFR (Mayo)	89.5±15.3	85±23	0.111
Diuretics	20 (28.57%)	12 (37.5%)	0.367
Antihypertensive drugs	**57 (81.43%)**	**31 (96.88%)**	**0.035**
Lipid-lowering drugs	37 (52.86%)	16 (50%)	0.789
C16SO (µmol/L)	15.27±5.34	16.29±8.99	0.937
C16SA (µmol/L)	0.46±0.19	0.52±0.42	0.518
C17SO (µmol/L)	7.96±2.78	8.66±3.88	0.874
C18PhytoSO (µmol/L)	0.11±0.04	0.13±0.09	0.284
C18SAdiene (µmol/L)	28.37±7.55	30.76±12.14	0.566
C18SO (µmol/L)	93.08±19.41	101.83±32.38	0.188
C18SA (µmol/L)	3.28±1.12	3.66±2.17	0.518
C19SO (µmol/L)	2.8±1.14	3.07±1.48	0.470
C20SO (µmol/L)	0.17±0.05	0.19±0.08	0.187
C20SA (µmol/L)	0.02±0.01	0.03±0.02	0.613
1-deoxySO (µmol/L)	**0.14±0.06**	**0.2±0.17**	**0.004**
1-deoxySA (µmol/L)	**0.07±0.03**	**0.09±0.08**	**0.011**

p Values are calculated using the t test on the log transformed variables (variables in bold font are p<0.05).
ATP, Adult Treatment Panel; BMI, body mass index; CRP, C reaction protein; GFR (Mayo) Glomerular filtration rate (Mayo equation); Hb, hemoglobin; HbA1c, glycated Hb; HDL, high-density lipoprotein; HOMA-IR, Homeostatic model assessment-insulin resistance; iT2DM, incident type 2 diabetes mellitus; LDL, low-density lipoprotein; MetS, metabolic syndrome; SA, sphinganine; SO, sphingosine.

Table 5 Binary logistic regression models results for incident T2DM showing the ORs for the univariate and multivariate models

		OR (95% CI)	p Value
Univariate binary logistic regression models			
	1-deoxySO	**2.09 (1.22–3.58)**	**0.007**
	1-deoxySA	**1.83 (1.12–2.99)**	**0.016**
	TG	**1.69 (1.05–2.7)**	**0.030**
	HbA1c	**4.11 (1.42–11.85)**	**0.009**
	Systolic BP	**1.64 (1.01–2.65)**	**0.044**
	Potassium	**1.78 (1.05–3.03)**	**0.03**
	MetS	**2.85 (1.16–7.03)**	**0.023**
Multivariate binary logistic regression models			
Model 1	**1-deoxySO**	**2.32 (1.31–4.12)**	**0.004**
	Age	1.63 (0.99–2.69)	0.054
Model 2	**1-deoxySO**	**2.13 (1.23–3.7)**	**0.007**
	Sex	1.17 (0.46–2.96)	0.746
Model 3	**1-deoxySO**	**2.05 (1.2–3.5)**	**0.008**
	BMI	1.09 (0.68–1.75)	0.731
Model 4	**1-deoxySO**	**1.97 (1.13–3.43)**	**0.017**
	MetS	2.37 (0.93–6.06)	0.071
Model 5	**1-deoxySO**	**2.1 (1.22–3.59)**	**0.007**
	Lipid-lowering drugs	0.85 (0.36–2.04)	0.720
Model 6	1-deoxySO	1.76 (0.94–3.27)	0.076
	TG	1.31 (0.75–2.27)	0.344
Model 7 (stepwise logistic regression)	**1-deoxySO**	**2.03 (1.19–3.49)**	**0.010**
	Zlog_TG		0.342
Model 8	**1-deoxySO**	**2.1 (1.19–3.71)**	**0.010**
	HbA1c	**4.11 (1.37–12.34)**	**0.012**
Model 9	**1-deoxySO**	**2.13 (1.22–3.72)**	**0.008**
	Systolic BP	**1.73 (1.04–2.86)**	**0.033**
Model 10	**1-deoxySO**	**1.88 (1.03–3.42)**	**0.039**
	Potassium	1.62 (0.94–2.78)	0.083
Model 1	**1-deoxySA**	**2.0 (1.19–3.36)**	**0.009**
	Age	1.6 (0.99–2.61)	0.057
Model 2	**1-deoxySA**	**1.83 (1.12–3)**	**0.017**
	Sex	0.93 (0.37–2.29)	0.869
Model 3	1-deoxySA	1.82 (1.12–2.97)	0.016
	BMI	1.13 (0.71–1.8)	0.607
Model 4	**1-deoxySA**	**1.68 (1.01–2.79)**	**0.048**
	MetS	2.26 (0.88–5.79)	0.088
Model 5	1-deoxySA	1.83 (1.12–3)	0.016
	Lipid-lowering drugs	0.88(0.37–2.09)	0.770
Model 6	1-deoxySA	1.56 (0.9–2.71)	0.112
	TG	1.38 (0.81–2.36)	0.234
Model 7 (stepwise logistic regression)	**1-deoxySA**	**1.81 (1.1–2.96)**	**0.019**
	TG	–	0.230
Model 8	**1-deoxySA**	**1.76 (1.06–2.92)**	**0.030**
	HbA1c	**3.78 (1.29–11.13)**	**0.016**
Model 9	**1-deoxySA**	**1.76 (1.07–2.89)**	**0.025**
	Systolic BP	1.57 (0.96–2.56)	0.071
Model 10	1-deoxySA	1.62 (0.94–2.77)	0.082
	Potassium	1.69 (0.99–2.9)	0.055

Variables are log transformed and standardized in SD units.
ORs are reported per increase of 1 SD unit. Variables in bold font are significant (p<0.05).
BMI, body mass index; BP, blood pressure; HbA1c, glycated Hb; MetS, metabolic syndrome; SA, sphinganine; SO, sphingosine; T2DM, type 2 diabetes mellitus; TG, triglycerides.

risk factors (age, sex, BMI) and the use of lipid-lowering drugs. Baseline 1-deoxySO remained significant predictors for the development of T2DM even after adjustment for age, sex, BMI, HbA1c, MetS, systolic blood pressure, serum potassium, or the use of lipid-lowering drugs (table 5). Interestingly, adjusting for TGs made both TGs and 1-deoxySO non-significant predictors while the regression model was still significant, possibly due to the

high colinearity between TGs and 1-deoxySO. However, using stepwise logistic regression, plasma 1-deoxySO remained significantly included in the regression model while TGs became non-significant (table 5). 1-DeoxySA showed a similar independent predictive role with significant OR after adjusting for the same risk factors, apart from serum potassium which again had a high colinearity with 1-deoxySA.

CONCLUSIONS

In the current study, we showed that plasma 1-deoxySLs are elevated in T2DM and are novel and independent predictors of T2DM. In two previous cross-sectional studies, we found that 1-deoxySLs are elevated in the plasma of patients with MetS[18] and T2DM.[19] These previous findings were confirmed in this study. In extension of our previous findings, we also showed that 1-deoxySLs are elevated in patients with IFG, suggesting that these lipids are already elevated in an early phase of T2DM. We also found that elevated 1-deoxySLs plasma levels are significantly and independently associated with an increased risk of developing T2DM after adjusting for several traditional risk factors and lipid-lowering drugs.

Pathologically elevated plasma 1-deoxySLs were originally found in the context of a rare inherited sensory neuropathy (Hereditary sensory and autonomic neuropathy type I, HSAN1) which is associated with several missense mutations in SPT.[25–27] These mutations induce a shift in the substrate specificity of the enzyme that results in an increased 1-deoxySL formation.[27] Further studies showed that 1-deoxySLs are neurotoxic and accumulate in the peripheral nerves but not in the brain or other organs of transgenic HSAN1 animals.[27 28] However, it is not fully understood why 1-deoxySLs are also elevated in conditions of metabolic disorders such as MetS or T2DM. Cell culture studies showed that the increased availability of alanine alone is not sufficient to induce a significant 1-deoxySL formation. In contrast, cells which were either cultured at high density or which were treated with the CerS inhibitor fumonisin B1 (FB1) generate significant amounts of 1-deoxySLs.[17] This suggests that a yet unknown regulatory mechanism is underlying the formation of these lipids in metabolic diseases. Elevated 1-deoxySL levels were also found in plasma of high fat and high fructose fed prediabetic and diabetic monkeys.[29] This corroborates our observation that 1-deoxySLs are already elevated early in the course of T2DM development. Since 1-deoxySLs are cytotoxic, it is conceivable that they could play a role in the pathogenesis of T2DM such as β-cell failure and other T2DM-related sequelae, like retinopathy or diabetic neuropathy. Indeed, the addition of 1-deoxySA is toxic to β cells in culture and results in cytoskeletal aberrations and cell death.[30] These effects were at least partly mediated though JNK and p38 MAPK pathways. In the same work, we showed that 1-deoxySA compromises glucose-stimulated insulin secretion from primary β cells in vitro. These findings further support the notion that 1-deoxySLs are not only early biomarkers in the course of T2DM development but might also be involved in the pathogenesis of T2DM. Given their reported neurotoxic activity in HSAN1, it is also feasible that 1-deoxySLs play a role in the pathogenesis of the diabetic neuropathy. Recently, we showed that lowering plasma 1-deoxySL with L-serine was associated with a significant improvement in the neuropathy in a diabetic rat model.[31] This indicates that 1-deoxySLs act as both predictive biomarkers and potential therapeutic targets in T2DM.

We also found that plasma C_{20}SA levels are increased in patients with IFG and T2DM. The origin and mechanisms by which plasma C_{20}SA is formed are not fully clear yet. It has been shown that stearoyl coenzyme A is used by SPT as a substrate to form C_{20} sphingoid bases.[32] The mechanism that regulates such a substrate shift is not clearly elucidated but several factors could play a role. The small SPT subunits (ssSPTa/b)[8] which interact with SPT can modulate the affinity of SPT for various acyl coenzyme A substrates. Moreover, we have shown that the SPTLC3 subunit influences the affinity of SPT for various acyl coenzyme As.[10 33] Whether these proteins play a role in the pathogenesis of T2DM or whether plasma C_{20}SA levels are associated with other pathological processes related to T2DM is currently not clear.

The availability of 8-year follow-up data including biannual oral glucose tolerance tests is a substantial strength of this study. However, the total number of patients who developed incident T2DM during the follow-up period was limited and did not allow us to adjust for all possible confounders without the risk of over-fitting. At that point, we cannot fully exclude a possible influence of other confounders than HbA1c and MetS which were adjusted for. Furthermore, the investigated individuals were hospitalized with chest pain and underwent coronary angiography for the evaluation of established or suspected CAD. Our study cohort may therefore be at a higher risk to develop T2DM than the general population. Despite these limitations, the study showed that 1-deoxySO and 1-deoxySA levels are significant, independent, and predictive risk markers and comparable to other established risk factors like HbA1c and MetS.

However, larger and optimally interventional studies are needed to further confirm the clinical relevance of 1-deoxySLs as prognostic biomarkers and to explore their potential in the risk management of patients with MetS and T2DM.

Contributors AO performed the lipid extraction, mass spectrometric analysis and statistical analysis, and wrote the manuscript. AM and AV were involved in study design, sample collection, and patient characterization. CHS and HD were involved in study design, sample collection and patient characterization, and critically revised the manuscript. AvE and TH contributed to study design, data interpretation, and critically revised the manuscript. TH is the guarantor.

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