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Decreased plasma levels of select very long chain ceramide species Are associated with the development of nephropathy in type 1 diabetes

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Abstract

Objective—Sphingolipid metabolism is altered in diabetes and we analyzed the plasma concentrations of sphingolipid species to investigate their association with the development of albuminuria in type 1 patients with diabetes.

Materials and Methods—Samples were collected from 497 type 1 diabetic patients during their enrollment into the Diabetes Control and Complications Trial (DCCT). We determined plasma concentrations of multiple ceramide species and individual sphingoid bases and their phosphates using high performance liquid chromatography-tandem mass spectrometry and

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Author contributions

RLK and SMH directed sample analyses, analyzed the data, and wrote the manuscript. KJH and NLB conducted the statistical analyses of the data and wrote the relevant sections. MMAIG effected sample selection, processing, and contributed to data analysis. PAC provided liaison between MUSC and the DCCT/EDIC Research Group. GV and MFL-V guided patient selection, contributed to the discussion, and reviewed/edited the manuscript. MLV directed the project.

Conflict of interest

The authors have no conflicting interests to disclose.

investigated their association with the development of albuminuria during 14–20 years of follow-up.

Results—Patients exhibited normal albumin excretion rates (AER <40 mg/24 h) at the time of plasma sampling. Although the majority of patients (N = 291; 59%) exhibited normal levels of albuminuria throughout follow-up, 141 patients (28%) progressed to microalbuminuria (40 mg/24 h ≤ AER < 300 mg/24 h), while 65 (13%) progressed to macroalbuminuria (AER ≥ 300 mg/24 h). To test the association of log transformed plasma sphingolipid level with the development of albuminuria, generalized logistic regression models were used where normal, micro- and macroalbuminuria were the outcomes of interest. Models were adjusted for DCCT treatment group, baseline retinopathy, gender, baseline HbA1c %, age, AER, lipid levels, diabetes duration, and the use of ACE/ARB drugs. Increased plasma levels of very long, but not long chain ceramide species measured at DCCT baseline were associated with decreased odds to develop macroalbuminuria during the subsequent nineteen years (DCCT Baseline to EDIC year 8).

Conclusion—These studies demonstrate, prospectively, that decreased plasma levels of select ceramide species are associated with the development of macroalbuminuria in type 1 diabetes.

Keywords

Sphingolipids; Sphingosine; Albuminuria; Microalbuminuria; Macroalbuminuria

1. Introduction

Diabetic nephropathy is the major cause of end-stage renal failure and one of the major causes of morbidity and mortality in diabetes [1]. Overt nephropathy is usually preceded by increased albuminuria [2] which is not only associated with the risk of developing renal insufficiency [3,4] but also with the development and progression of cardiovascular disease [5] in patients with diabetes. Thus, there is considerable interest in determining the mechanisms responsible for albuminuria and in identifying early markers that may be associated with this complication of diabetes.

The pathological mechanism(s) that are related to the development of increased urine albumin are not well defined. It has been suggested that dyslipidemia is a contributing factor to complications of diabetes and that modification in the metabolism of lipids may ultimately contribute to renal and other complications in diabetic subjects [6]. Circulating lipoproteins (HDL, LDL, and VLDL) and albumin are the carriers of sphingolipids in plasma. Sphingolipids are important constituents of cell membranes and in the last decade their role in cell signaling and activation has been extensively studied. Due to the complexity of the analyses and because of the expense of the technique and instrumentation necessary for quantitation of the different species of sphingolipids, efforts to measure blood sphingolipids and to use these measures as diagnostic and prognostic tools have been very limited. We [7–9] and others [10–12] have investigated human sphingolipidomics in plasma and in lipoproteins isolated from non-diabetic subjects using advanced mass spectroscopy techniques.

Although sphingolipids comprise only a small fraction of plasma lipids and hence, lipoprotein lipids, there is mounting evidence that sphingolipid metabolism is altered in diabetes and specific sphingolipid classes may contribute to diabetic complications [6]. In a cross sectional study of 326 type 1 diabetes patients enrolled in the Finnish Diabetic nephropathy Study (FinnDiane), plasma sphingomyelin levels emerged as a biochemical covariate of urinary albumin excretion rate [13]. Unfortunately, plasma ceramide levels were not investigated in that study. Most of the evidence demonstrating the role of sphingolipids in diabetes has arisen from studies using animal models of diabetes [14] with the focus on ceramide metabolism as it relates to insulin sensitivity [15–20]. The concentrations of plasma sphingosine also were reportedly elevated in a group of type 2 diabetic patients compared with levels in healthy control subjects suggesting that the rate of ceramide metabolism in the cells of diabetic patients was elevated [21]. Plasma ceramide levels are elevated in type 2 diabetic patients compared to levels determined in control subjects, with diabetic patients exhibiting elevated levels of the C18:0, C20:0, C24:1 ceramide species [20].

We investigated the potential association of the plasma concentrations of select ceramide species and of individual sphingoid bases and their phosphates with the future development of nephropathy in a subgroup of a well characterized group of type 1 diabetes patients, the DCCT/EDIC cohort.

2. Methods

2.1. Study subjects

The DCCT was a randomized, clinical trial of 1,441 patients who were 13–39 years of age and had type 1 diabetes for 1–15 years at study entry [22]. In this manuscript we studied a subgroup of patient from the DCCT who had diabetes for 1–15 years and normal albumin excretion rates (<40 mg/24 h) at entry into the study. Some of the patients were free from clinically evident retinopathy (Early Treatment Diabetic Retinopathy Study score, ETDRS = 1), some had mild to moderate non-proliferative diabetic retinopathy (ETDRS 2–9). The participants were randomized into groups that received either intensive or conventional insulin therapy and were followed for an average of 6.5 years. At the baseline DCCT examination, each participant received a complete physical examination which included a medical history, an electrocardiogram, and routine laboratory analyses to determine serum creatinine, lipid profile, and HbA_{1c} levels [22]. Four-hour urine collections for measurement of albumin excretion rate (AER) and creatinine clearance were also obtained during EDIC on alternate years [23]. The study was terminated early, in 1993, because of the observed major beneficial effect of intensive therapy on retinal, renal, and neurologic complications. In 1994, approximately 95% of the DCCT participants were enrolled into an observational study, the Epidemiology of Diabetes Interventions and Complications (EDIC) study. The goal of the EDIC study was to assess the long-term effects of prior separation of glycemic levels on micro- and macrovascular outcomes in type 1 diabetes [23]. During EDIC, all patients were under the care of their personal physicians and encouraged to practice intensive insulin therapy.

The present study was performed on plasma samples collected from type 1 diabetes patients at the time of their entry into the DCCT (Baseline) before they were randomized into one of the two study treatment arms. Our main aim was to determine whether plasma levels of ceramide species and of sphingoid bases and their phosphates measured at DCCT Baseline would be associated with the development of abnormal albuminuria after 14–20 years of disease progression (Year 8 of the EDIC study). Participants included in the study had normal (<40 mg/24 h) AER at DCCT Baseline and a sufficient volume of plasma in stored specimens to perform the measurements. Some participants exhibited background or mild retinopathy (ETDRS score of 2–9). AER levels were measured annually during the DCCT study and every other year during the first 8 years of the EDIC follow up study. Study endpoints were persistent normal AER throughout DCCT and up to EDIC year 8 (all AER <40 mg/24 h); incident microalbuminuria (40 mg/24 h ≤ AER < 300 mg/24 h); and macroalbuminuria (AER ≥ 300 mg/24 h).

2.2. Blood sample collection for plasma sphingolipid analysis

Fasting plasma samples collected in EDTA and obtained at DCCT Baseline were sent at the time of collection to the DCCT/EDIC central laboratory for standard lipid analysis. Aliquots of these samples were archived for future research purposes. In 1999–2000, as part of a NIH/JDF Program Project Grant awarded to the Medical University of South Carolina, plasma samples collected during DCCT were provided by the DCCT/EDIC Coordinating Center and NIDDK to complement studies conducted during the Program Project. These plasma samples were stored at –70 °C and refreezing effects were minimized by preparing aliquots of the plasma when thawed for the first time and by using a new, frozen aliquot for the assay of plasma sphingolipids. The IRB at Medical University of South Carolina and all participating DCCT/EDIC centers approved the sample collection procedures. Written informed consent was obtained from all participants.

2.3. Sphingolipid extraction and analysis

Analyses of plasma levels of sphingoid bases, sphingoid base 1-phosphates, and ceramide (Cer) species were conducted in the Lipidomics Core Facility at the Medical University of South Carolina as previously described [7–9]. Briefly, 100 µl of plasma from each patient was spiked with internal standards and the sphingolipid complement in each sample was quantitatively extracted. The sphingolipids in plasma extracts were separated and their masses quantitated using high performance liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) as described previously [7–9]. Lipids eluted during chromatography were detected and quantitated using a Thermo Scientific Quantum Access triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI) operating in multiple reaction monitoring (MRM) positive ion mode. Chromatographic separations were obtained under a gradient elution of a Peeke Scientific (Redwood City, CA), Spectra C8SR 150×3.0 mm; 3-µm particle size column. Quantitative analyses were based on calibration curves generated by injecting known amounts of the target analytes and an equal amount of the internal standards. A listing of the internal standards used and of the sphingolipids with available calibration standards was previously published by our group [7]. The calibration standards were obtained predominately from the MUSC Lipidomics Share Resource facility, and from commercially available sources, Avanti Polar Lipids Inc.

and Matreya LLC. The following sphingolipids had available standards for plotting calibration curves: sphingosine, dihydro-sphingosine, sphingosine 1-phosphate, dihydro-sphingosine 1-phosphate, and C14, C16, dihydro-C16, C18, C18:1, C20, C22, C24, and C24:1 ceramide species. The C20:1, C22:1, C26, and C26:1 molecular species of ceramide were quantified using the calibration curve of the closest eluting counterpart. The final concentrations of analytes in samples were determined using the appropriate corrections for sample loss based on internal standard recovery calculations. The resulting data were then normalized to the volume of sample analyzed. Final results are reported as the nanomolar concentration in plasma.

2.4. Statistical analysis

The concentrations of sphingoid bases and their phosphates and of multiple ceramide species were measured in plasma collected at DCCT baseline and used to determine if plasma sphingolipid levels could predict elevated risk to develop abnormal albuminuria. All marker levels were assessed for model residual normality and homoscedasticity and transformed when necessary. Following data normalization, all biomarkers were standardized and the analysis results represent the association between a one standard deviation change in each biomarker and the odds to develop micro- and macroalbuminuria.

Baseline covariates for the current analyses were obtained from DCCT baseline history, physical examination and laboratory data (fasting lipids and renal function). Model endpoints were persistent normal AER (all AER <40 mg/24 h); incident microalbuminuria (40 mg/24 h AER < 300 mg/24 h); and macroalbuminuria (AER ≥ 300 mg/24 h). Standard descriptive statistics were used to summarize the general demographic and clinical data. The Kruskal-Wallis test was used to evaluate continuous baseline demographic and clinical measures across albuminuria outcomes; the Pearson chi-square test was used to assess the association for categorical variables. Similarly, a Kruskal-Wallis test was used to test unadjusted group differences between baseline biomarker levels and the albuminuria severity classification. Parallel, pair-wise comparisons of biomarker levels were also assessed between the normoalbuminuria, microalbuminuria, and macroalbuminuria groups.

Generalized logistic regression models were used to quantify the association of increased baseline marker levels on the subsequent development of micro- and macroalbuminuria. The primary parameter of interest in the logistic regression models was the change in the log-odds (with 95% Wald CI) for the progression to micro- or macroalbuminuria as compared to those that remained normoalbuminuric throughout follow up; initial design models were adjusted for DCCT randomized treatment, baseline levels of AER, and the use of any ACE/ARB drugs during the study. Covariate adjusted models additionally contain baseline retinopathy status (none vs. mild/moderate), gender, and baseline measures of age, HbA1c %, BMI, triglycerides, AER and diabetes duration. Modifying effects of DCCT treatment group, baseline retinopathy status, and ACE/ARB drug use on the effects of the association between ceramide levels and albuminuria were examined in all models. Additionally, linear regression models (Table 4) were developed to assess the relationship between baseline plasma ceramide levels and AER levels. During the study, 46% of participants measured for this analysis had taken ACE/ARB drugs for the treatment of either hypertension or

nephropathy. This treatment may cause changes in measured AER following treatment, thus peak study AER was chosen as the outcome of interest. Due to non-normality in the model residuals, both plasma ceramides and AER levels were transformed using the natural logarithm prior to analysis.

All statistical analyses were performed using the SAS System version 9.3. Significance for all planned comparisons was set at a 2-sided p-value of 0.05 and no correction for multiple testing has been applied to reported *p* values.

3. Results

The concentrations of multiple sphingolipid species were measured in plasma samples collected from 497 participants who had normal AER values at DCCT baseline (<40 mg/24 h; 78.5% of 633 participants). Demographic and clinical differences at DCCT Baseline between study subjects who exhibited persistent normal albuminuria and those that developed micro- or macroalbuminuria are summarized in Table 1. At DCCT Baseline, the mean age of subjects in the study cohort was 26.8 ± 7.4 years with an average duration of diabetes of 5.8 ± 4.3 years; 250 (50.3%) of the 497 subjects were male and 246 (49.5%) were assigned to the DCCT intensive treatment group. Although both genders were equally represented, there were more male subjects in the group who ultimately developed macroalbuminuria during the follow-up period. Also, the subjects included in our protocol were similarly distributed into both treatment arms (intensive and conventional) of the DCCT. Regardless of similar representation in both groups, a significantly lower percentage of the group who ultimately developed macroalbuminuria were part of the intensive therapy arm of the DCCT.

There were statistically significant trends across disease progression groups in several clinical measures – participants who progressed to more severe levels of albuminuria during the study had higher BMI, and exhibited higher HbA1c levels at DCCT baseline and throughout the follow-up period. Plasma and lipoprotein cholesterol levels, at DCCT baseline, did not differ among subjects in each of the three albuminuria groups, but total triglyceride levels were significantly higher in the group who ultimately developed macroalbuminuria. The use of ACE/ARB medications was significantly greater in the group of subjects which ultimately developed macroalbuminuria, but statin therapy did not differ among the groups. Although all subjects included in this study had normal AER at DCCT Baseline, the level of AER at baseline was significantly different among the three albuminuria outcome groups.

Prior to final analysis, model residuals were assessed for normality through the use of histograms and quantilequantile (Q-Q) plots. Results show that all the measured sphingolipids including ceramides were positively skewed; therefore natural logarithm transformations were applied and resulted in normally distributed biomarkers. The unadjusted concentrations of sphingolipids measured in the plasma samples obtained from 497 type 1 diabetic patients at the time of their enrollment into the DCCT are summarized in Table 2. Regardless of the level of albuminuria, there were no significant differences in the plasma concentrations of the measured sphingolipids other than ceramides. Similarly, there

were no significant differences in plasma concentrations of the long chain ceramide species between the three albuminuria groups. In contrast, there was a significant decrease in the plasma concentrations of the very long chain (C20, C20:1, C22:1, C24:1, C26, and C26:1) ceramide species in patients who progressed to macroalbuminuria compared to plasma levels in diabetic patients who remained normoalbuminuric during the follow-up period.

The design and covariate adjusted logistic regression results are shown in Table 3. In the initial design adjusted models, a one standard unit increase in levels of both C16 and C18 were moderately associated with a nearly 30% decrease in odds to develop macroalbuminuria during the study period. Additionally, one standard unit increases in multiple very long chain ceramides (C20, C20:1, C22:1, C24, C24:1, C26, and C26:1) were associated with significantly decreased odds to develop macroalbuminuria. Following adjustments for additional covariates of interest (i.e., gender and DCCT baseline measures of age, HbA1c%, BMI, and triglyceride levels), the associations of the long chain ceramide species with macroalbuminuria were no longer statistically significant. However, many of the very long chain ceramide species (C20, C22:1, C24, C26, and C26:1) maintained a significant association with decreased odds (31–38%) to progress to macroalbuminuria. In the study population, there were no significant modifying effects of DCCT treatment group assignment, baseline retinopathy status, or the use of ACE/ARB drugs on the relationships between sphingolipids or ceramides with albuminuria outcomes. In addition to logistic regression models, time to event analysis were investigated to assess the impact of changes in concentrations of sphingoid bases and their phosphates and of multiple ceramide species on the time taken to develop macroalbuminuria. Results from the analysis were similar to results from the logistic regression analysis (data not shown).

The linear regression model results are shown in Table 4. In the design adjusted models, a majority of the long and very long chain ceramide species were significantly and negatively associated with increases in peak study AER levels (C16, C18, C18:1, C20, C20:1, C22:1, C24, C24:1, C26, and C26:1). Following adjustments for additional covariates, C16 as well as several of the very long chain ceramide species remained significantly associated with peak study AER levels (C20, C20:1, C22:1, C24, C26, C26:1). Similar to the results of the analysis of the association between plasma sphingolipid level and disease progression, none of the sphingoid bases, sphingoid base 1-phosphates were significantly associated with increased study AER.

4. Discussion

We investigated the plasma concentrations of select sphingolipid classes including multiple ceramide species and the individual sphingoid bases and their phosphates. We determined that high plasma levels of the very long chain ceramide species (C20, C22:1, C24, C26, and C26:1) were significantly associated with lower frequency to progress to macroalbuminuria in a subgroup of patients of the DCCT/EDIC cohort. The lower frequency to progress to macroalbuminuria was observed with high levels of certain very long ceramides even in patients enrolled in the intensive glucose control arm of the study although there was a relative lower percentage of patients developing macroalbuminuria in this particular subgroup. This was expected since it is well known that optimized glucose control will

protect against the development of nephropathy. Also interesting is the fact that progression to macroalbuminuria is more frequent in patients placed on ACE inhibitors/ARBs thus indicating that the patients who had low levels of very long chain ceramides are more likely to develop hypertension and require drug treatment. We believe that this novel and potentially clinically important, negative association between high plasma levels of select very long chain ceramide species and the development of macroalbuminuria may reflect a regulatory role of ceramides in pathways which may ultimately lead to a loss of renal function.

Ceramides are an integral part of cell membrane structure where they participate in membrane lipid raft formation. Lipid rafts are sphingolipid and cholesterol-rich domains of the plasma membrane, which contain a variety of signaling and transport proteins [24]. Sphingomyelin in membranes readily associates with cholesterol and together they form liquid-ordered domains (i.e., lipid rafts) with other sphingolipids and with some raft-associated proteins also located within these liquid-ordered domains. Ceramide within rafts alters the structure of these domains and the lateral partitioning of membrane components [25]. The distinct biophysical properties of ceramides also may play a major role in the formation of these rafts and as a result, in the regulation of ceramide-dependent signaling pathways [26]. Ceramide molecules also can associate with other ceramide molecules in biological membranes to form small ceramide-enriched, gel-phase domains which tend to fuse into larger ceramide-enriched membrane platforms which, in turn, may be important for the lateral clustering of select proteins [27]. The formation of ceramide-enriched domains and platforms is largely dependent on the ceramide structure. Ceramides are formed from the covalent association between a sphingosine molecule and a fatty acid. Recently, properties of ceramide with different fatty acyl chains including long chain (C16 and C18), very long chain (C24) and unsaturated (C18:1 and C24:1) ceramides were examined in phosphatidylcholine model membranes [28]. It was determined that saturated ceramides increase the order of fluid membranes and promotes its gel/fluid phase separation whereas unsaturated ceramides have a lower (C24:1) or no (C18:1) ability to form gel domains at 37 °C. Additional evidence gathered using animal models [29] or cultured cells *in vitro* [30] clearly demonstrate that very long chain ceramides influence membrane biophysical properties which may compromise cellular processes that critically depend on membrane structure such as trafficking, sorting, and cell proliferation. Thus, the presence of different ceramide species in cell membranes has a distinct biophysical impact with acyl chain saturation dictating membrane lateral organization and influencing cell signaling and functionality [28–32].

In addition to their structural role in cell membranes, ceramides also act as signaling molecules and have been shown to activate extracellular signal-related kinase 2, p38, JNK, and I κ B kinases (I κ Ks) in various cell types [33,16,17]. Most recently, ceramides have been associated with altered metabolism and have been strongly implicated in the pathogenesis of insulin resistance [15–21]. Increased concentrations of total ceramides and select ceramide species have been reported in plasma from type 2 diabetes patients and correlate with the severity of insulin resistance [20]. Ceramide also has been postulated as a primary lipid mediator of insulin resistance in skeletal muscle [19,34–36]. The mechanism whereby ceramides may influence insulin sensitivity are unclear but changes in membrane levels of

very long chain ceramide species in mice have been associated with insulin resistance through their role in modifying insulin receptor translocation into liver cell membrane microdomains [32]. The association between ceramides and insulin resistance potentially is clinically important as the presence of insulin resistance and the metabolic syndrome are accepted risk markers for the development of diabetes complications and for macrovascular disease in patients with and without type 2 diabetes. To this end, insulin resistance, calculated using an estimated glucose disposal rate (eGDR), strongly predicted the development of nephropathy in the DCCT cohort [37]. However, the correlation of plasma concentrations of ceramide species with insulin resistance in the DCCT/EDIC cohort remains to be determined.

The mechanism(s) whereby the ceramide composition of kidney cell membranes may become altered in diabetic patients with albuminuria remains to be determined. The ceramide composition of the membranes of kidney cells presumably may become altered by metabolizing or directly interacting with plasma lipoproteins, which themselves contain an altered ceramide composition. These abnormal lipoproteins could then deliver their altered ceramide complement to the cell. The tissue origin of the ceramide species in these altered lipoproteins remains to be determined, but presumably would originate primarily from the liver or intestine. Ceramide generation and/or assembly may also occur in the circulation by enzyme-mediated exchanges among the lipoproteins. Alternatively, the modifications of lipoproteins frequently associated with diabetes (e.g. glycation, oxidation, changes in apolipoprotein composition, association with antibodies forming immune complexes) may alter the interaction of the lipoprotein with cell lipoprotein receptors. These changes in cell-lipoprotein interactions may activate cell signaling pathways which then may alter intracellular sphingolipid metabolism. The mechanisms whereby lipoproteins in diabetic patients may alter kidney cell sphingolipid metabolism, and, additionally, the effects of altered cell sphingolipid composition on kidney cell physiology, remain to be determined but clearly, additional studies are warranted.

We previously analyzed plasma levels of sphingolipids in a limited (n = 10) group of well characterized, healthy, non-diabetic subjects [7–9]. However, data of the distribution of sphingolipids in plasma from diabetic patients is limited and this study represents the first comprehensive examination to be conducted in type 1 diabetic patients. While the limited number of healthy subjects which were investigated prohibits formal statistical analyses of the sphingolipid composition of these diabetic patients compared to the healthy subjects, it must be noted that there was a striking average 2.5-fold increase in plasma sphingosine 1-phosphate (S1P) concentration in the cohort of type 1 diabetic patients (752 ± 369 nM) compared to S1P levels previously observed in plasma from healthy, non-diabetic subjects (305 ± 54 nM) [7]. Again, additional studies are warranted to investigate the potential for this additional, biologically important sphingolipid class to alter cell metabolism in type 1 diabetes patients.

Little is known concerning the possible role of ceramides in the development of diabetic complications. To our knowledge, this study is the first to examine the association of plasma sphingolipid levels with diabetes complications and to demonstrate, prospectively, that plasma levels of select ceramide species are associated with the development of

macroalbuminuria in type 1 diabetes. However, determination of the precise functions which are affected at the glomerular level by changes in the distribution of ceramide species is a critical question that remains to be investigated. It should be also noted that lipoproteins transport numerous additional sphingolipid types in addition to those analyzed in this study [7]. The association of the plasma levels of these additional sphingolipid classes with the development of macroalbuminuria in type 1 diabetes, and with other complications associated with diabetes, is unknown and remains to be determined. Clearly, additional studies are warranted.

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Abbreviations

DCCT	Diabetes Control and Complications Trial
EDIC	Epidemiology of Diabetes Interventions and Complications
AER	Albumin excretion rate
ETDRS	Early treatment diabetic retinopathy study score
Cer	Ceramide
LC-ESI-MS/MS	High performance liquid chromatography-tandem mass spectrometry with an electrospray ion source
MRM	Multiple reaction monitoring
BMI	Body mass index
eGDR	Estimated glucose disposal rate
S1P	Sphingosine 1-phosphate
ACE/ARB	Angiotensin-converting enzyme inhibitors/Angiotensin II Receptor Blockers
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases

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Table 1

Baseline demographics and categorization by albuminuria status during the follow-up period.

Demographic	Overall n = 497	Albuminuria status			P-value
		Normal n = 291	Micro n = 141	Macro n = 65	
Age (y)	26.8 ± 7.4	27.6 ± 7.0	25.7 ± 7.8	25.5 ± 7.7	0.028
Male % (n)	50.3 (250)	48.8 (142)	45.4 (64)	67.7 (44)	0.049
Intensive treatment group % (n)	49.5 (246)	57.0 (166)	48.9 (69)	16.9 (11)	<0.001
Absent retinopathy % (n)	49.9 (248)	54.3 (158)	46.8 (66)	36.9 (24)	0.008
Duration of T1DM (months)	69.1 ± 51.0	65.6 ± 50.6	72.0 ± 53.0	78.2 ± 47.4	0.101
Body Mass Index (kg/m ²)	23.3 ± 2.8	23.3 ± 2.8	22.9 ± 2.9	24.2 ± 3.0	0.009
Weight (kg)	68 ± 12	69 ± 12	66 ± 12	71 ± 13	0.007
Diastolic Blood Pressure (mm Hg)	71 ± 9	71 ± 9	71 ± 9	73 ± 8	0.378
Systolic blood Pressure (mm Hg)	113 ± 12	112 ± 12	113 ± 11	114 ± 11	0.354
Total cholesterol (mg/dl)	179 ± 34	181 ± 34	178 ± 35	177 ± 32	0.513
HDL cholesterol (mg/dl)	51 ± 12	51 ± 12	52 ± 12	48 ± 12	0.076
LDL cholesterol (mg/dl)	112 ± 29	113 ± 29	110 ± 29	110 ± 28	0.349
Triglycerides (mg/dl)	82 ± 47	81 ± 50	81 ± 40	93 ± 44	0.035
HbA1c (%) (baseline)	9.0 ± 1.6	8.6 ± 1.4	9.2 ± 1.6	10.2 ± 1.7	<0.001
HbA1c (%) (study mean)	8.3 ± 1.2	7.9 ± 1.0	8.5 ± 1.2	9.5 ± 1.0	<0.001
Use of ACE/ARB during study [% (n)]	45.9 (228)	33.0 (96)	53.2 (75)	87.7 (57)	<0.001
Use of statins during study [% (n)]	34.2 (170)	33.3 (97)	30.5 (43)	46.2 (30)	0.173
Serum creatinine (mg/dl)	0.80 ± 0.15	0.81 ± 0.15	0.77 ± 0.15	0.81 ± 0.15	0.033
Albumin excretion rate (Baseline)	12.7 ± 8.5	10.9 ± 7.2	16.0 ± 9.9	13.7 ± 8.3	<0.001
Albumin excretion rate (study mean)	66.3 ± 210.1	10.2 ± 3.7	27.4 ± 16.2	401.6 ± 458.0	<0.001

Italicized bold numbers indicate $p < 0.05$.

Data are expressed as mean ± SD except where indicated.

Table 2

Plasma ceramide level at DCCT baseline and by progression to micro- or macroalbuminuria during follow-up period.

Sphingolipid (nM)	Overall n = 497	Albuminuria		P-value ^a
		Normal n = 291	Macro n = 141	
Sphingosine	15.8 ± 17.7	16.6 ± 20.9	15.2 ± 13.0	0.892
dh-Sphingosine	7.3 ± 6.1	7.7 ± 6.7	6.6 ± 5.2	0.201
Sphingosine 1-Phosphate	751.6 ± 368.8	742.9 ± 342.8	768.9 ± 420.3	0.981
dh-Sphingosine 1-Phosphate	114.3 ± 68.6	112.7 ± 63.4	117.8 ± 78.7	0.973
dh-C16 Ceramide	30.2 ± 16.4	30.9 ± 17.6	29.6 ± 13.9	0.304
Long chain ceramide species				
C14	26.2 ± 12.0	26.6 ± 11.5	25.4 ± 12.3	0.336
C16	374.3 ± 169.0	386.7 ± 181.9	367.1 ± 152.0	0.141
C18	105.5 ± 63.0	106.6 ± 60.0	110.1 ± 72.3	0.068
C18:1	26.7 ± 16.5	27.0 ± 15.3	27.9 ± 19.7	0.059
Very long chain ceramide species				
C20	171.8 ± 100.3	178.6 ± 107.1	171.6 ± 93.9	0.029
C20:1	13.9 ± 7.3	14.6 ± 7.8	13.6 ± 6.6	0.005
C22	227.8 ± 60.0	230.5 ± 61.7	225.8 ± 61.3	0.692
C22:1	57.5 ± 15.7	59.0 ± 15.9	56.4 ± 15.4	0.027
C24	2963 ± 993	3028 ± 1029	2950 ± 999	0.089
C24:1	945.9 ± 281.1	969.1 ± 289.8	929.6 ± 274.0	0.037
C26	122.8 ± 72.7	126.3 ± 73.7	125.3 ± 70.4	0.009
C26:1	42.8 ± 24.3	44.3 ± 24.6	43.0 ± 22.7	0.007

Italicized bold numbers indicate $p < 0.05$.

Data are expressed as mean ± SD.

Abbreviations: dh-dihydro; C-carbon length of ceramide chain.

^aUnivariate analysis conducted using Kruskal-Wallis Test statistic for overall group differences.

^b $p < 0.05$ vs. microalbuminuria.

normal vs. $d_c > 0.50$

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Table 3

Odds of abnormal albuminuria for patients with increased plasma levels of sphingolipid species.

Sphingolipid species	Design adjusted ^a		Covariate adjusted ^b	
	Microalbuminuria Odds ratio (95 % CI)	Macroalbuminuria Odds ratio (95 % CI)	Microalbuminuria Odds ratio (95 % CI)	Macroalbuminuria Odds ratio (95 % CI)
Sphingosine	0.94 (0.76–1.16)	0.97 (0.71–1.32)	0.96 (0.78–1.19)	1.06 (0.75–1.50)
dh-Sphingosine	0.85 (0.69–1.05)	0.98 (0.71–1.35)	0.88 (0.71–1.08)	1.07 (0.73–1.58)
Sphingosine 1-Phosphate	0.99 (0.80–1.22)	0.96 (0.71–1.30)	1.00 (0.80–1.24)	0.97 (0.69–1.36)
dh-Sphingosine 1-Phosphate	1.02 (0.83–1.27)	0.96 (0.71–1.30)	1.05 (0.84–1.30)	1.00 (0.71–1.40)
dh-C16 Ceramide	0.91 (0.73–1.12)	0.77 (0.67–1.06)	0.90 (0.72–1.13)	0.74 (0.52–1.05)
Long chain ceramide species				
C14	0.85 (0.68–1.05)	0.90 (0.66–1.23)	0.87 (0.70–1.09)	0.85 (0.60–1.19)
C16	0.87 (0.70–1.07)	0.71 (0.52–0.98)	0.87 (0.69–1.08)	0.76 (0.54–1.07)
C18	0.99 (0.80–1.22)	0.72 (0.53–0.98)	1.00 (0.80–1.26)	0.81 (0.58–1.14)
C18:1	0.95 (0.77–1.18)	0.74 (0.53–1.01)	0.97 (0.77–1.21)	0.87 (0.61–1.24)
Very long chain ceramide species				
C20	0.92 (0.74–1.13)	0.63 (0.46–0.86)	0.93 (0.74–1.16)	0.63 (0.44–0.89)
C20:1	0.95 (0.77–1.17)	0.67 (0.49–0.90)	0.99 (0.79–1.24)	0.73 (0.52–1.03)
C22	0.92 (0.74–1.14)	0.89 (0.65–1.23)	0.92 (0.74–1.16)	0.75 (0.52–1.09)
C22:1	0.81 (0.66–1.01)	0.64 (0.47–0.88)	0.84 (0.67–1.06)	0.64 (0.45–0.92)
C24	0.91 (0.74–1.13)	0.70 (0.50–0.98)	0.90 (0.72–1.13)	0.62 (0.43–0.91)
C24:1	0.86 (0.69–1.06)	0.72 (0.53–0.99)	0.89 (0.72–1.12)	0.77 (0.54–1.10)
C26	0.98 (0.79–1.22)	0.70 (0.52–0.95)	0.97 (0.78–1.22)	0.62 (0.44–0.87)
C26:1	0.93 (0.74–1.15)	0.69 (0.51–0.93)	0.95 (0.75–1.19)	0.69 (0.49–0.96)

Italicized bold numbers indicate $p < 0.05$.

Abbreviations used are same as Table 2.

^aDesign adjusted models contain standardized Marker level, DCCT treatment group assignment, baseline AER measure and treatment with ACE/ARB drugs during study period.^bCovariate adjusted models contain standardized biomarker level, DCCT Treatment Group, baseline retinopathy status, use of ACE/ARB drugs during study period, gender, and baseline measures of duration of T1DM, age, HbA1c %, BMI, triglyceride levels, and AER.

Table 4

Linear regression of baseline plasma ceramides on peak study AER values.

Sphingolipid species	Design adjusted ^a		Covariate adjusted ^b	
	Beta (SE)	P value	Beta (SE)	P value
Sphingosine	−0.027 (0.066)	0.687	−0.007 (0.061)	0.909
dh-Sphingosine	−0.034 (0.066)	0.606	−0.018 (0.062)	0.774
Sphingosine 1-Phosphate	0.021 (0.066)	0.749	0.008 (0.062)	0.892
dh-Sphingosine 1-Phosphate	0.014 (0.066)	0.835	0.019 (0.062)	0.754
dh-C16 Ceramide	−0.103 (0.067)	0.121	−0.108 (0.064)	0.094
Long chain ceramide species				
C14	−0.056 (0.066)	0.393	−0.047 (0.062)	0.454
C16	−0.177 (0.066)	0.008	−0.143 (0.062)	0.021
C18	−0.162 (0.066)	0.014	−0.114 (0.064)	0.073
C18:1	−0.177 (0.066)	0.007	−0.010 (0.063)	0.115
Very long chain ceramide species				
C20	−0.226 (0.066)	<0.001	−0.206 (0.063)	0.001
C20:1	−0.237 (0.065)	<0.001	−0.163 (0.064)	0.011
C22	−0.070 (0.066)	0.288	−0.100 (0.063)	0.115
C22:1	−0.201 (0.066)	0.002	−0.163 (0.063)	0.010
C24	−0.146 (0.066)	0.027	−0.163 (0.062)	0.009
C24:1	−0.169 (0.066)	0.011	−0.113 (0.063)	0.074
C26	−0.192 (0.066)	0.004	−0.185 (0.061)	0.003
C26:1	−0.233 (0.065)	<0.001	−0.182 (0.062)	0.003

Peak study AER is transformed using the natural logarithm, individual ceramides were natural logarithm transformed prior to standardization.

Italicized bold numbers indicate $p < 0.05$.

Abbreviations used are same as Table 2.

^aDesign adjusted models contain standardized Marker level, DCCT treatment group assignment, baseline AER measure and treatment with ACE/ARB drugs during study period.

^bCovariate adjusted models contain standardized biomarker level, DCCT Treatment Group, baseline retinopathy status, use of ACE/ARB drugs during study period, gender, and baseline measures of duration of T1DM, age, HbA1c %, BMI, triglyceride levels, and AER.