



Urinary sphingolipids in adolescents and young adults with youth-onset diabetes

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Abstract

Background This study evaluated urinary sphingolipids as a marker of diabetic kidney disease (DKD) in adolescents and young adults with youth-onset type 1 and type 2 diabetes.

Methods A comprehensive panel of urinary sphingolipids, including sphingomyelin (SM), glucosylceramide (GC), ceramide (Cer), and lactosylceramide (LC) species, was performed in patients with youth-onset diabetes from the SEARCH for Diabetes in Youth cohort. Sphingolipid levels, normalized to urine creatinine, were compared in 57 adolescents and young adults with type 1 diabetes, 59 with type 2 diabetes, and 44 healthy controls. The association of sphingolipids with albumin-to-creatinine (ACR) ratio and estimated glomerular filtration rate (eGFR) was evaluated.

Results The median age (interquartile range [IQR]) of participants was 23.1 years (20.9, 24.9) and the median duration of diabetes was 9.3 (8.5, 10.2) years. Urinary sphingolipid concentrations in patients with and without DKD (ACR ≥ 30 mg/g) were significantly elevated compared to healthy controls. There were no significant differences in sphingolipid levels between participants with type 1 and type 2 diabetes. In multivariable analysis, many sphingolipid species were positively correlated with ACR. Most significant associations were evident for the following species: C18 SM, C24:1 SM, C24:1 GC, and C24:1 Cer (all $p < 0.001$). Sphingolipid levels were not associated with eGFR. However, several interaction terms (diabetes type*sphingolipid) were significant, indicating diabetes type may modify the association of sphingolipids with eGFR.

Conclusion Urinary sphingolipids are elevated in adolescents and young adults with youth-onset diabetes and correlate with ACR. Urinary sphingolipids may therefore represent an early biomarker of DKD.

Keywords Pediatrics · Youth-onset diabetes · Sphingolipids · Diabetic kidney disease

Introduction

Diabetes is a leading cause of kidney disease, accounting for approximately 45% of cases of kidney failure in the United States [1]. The management of diabetic kidney disease (DKD) remains challenging, as sensitive and specific markers of disease progression are lacking. While albuminuria has traditionally been clinically used to identify patients at risk for disease progression, it has significant limitations. Up to 50% of patients with diabetes and albuminuria can regress to normoalbuminuria [2], and 29–41% of patients with diabetes may experience a decline of kidney function despite having normal urinary albumin excretion [3–5]. Therefore, a pressing need for the development of more sensitive and specific biomarkers of disease progression in DKD has been widely recognized [6].

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Sphingolipids, a class of lipids involved in intracellular signaling and cellular metabolism, have been identified as a potential mediator of DKD in both type 1 and type 2 diabetes mellitus. Urinary sphingolipids may therefore represent a novel early biomarker of kidney injury and disease progression in DKD. Dysregulation of sphingolipid metabolism occurs in diabetes, resulting in increased de novo synthesis of sphingolipids in kidney tissue [7, 8]. Subsequent accumulation of sphingolipid species has been shown to mediate early changes of DKD including glomerular hypertrophy and extracellular matrix productions [9, 10]. Sphingolipids have also been identified as mediators of particular comorbidities exclusively associated with type 2 diabetes including insulin resistance, inflammation, and hepatic steatosis [11–13]. Furthermore, obesity-related fatty acid dysregulation and inflammation are potential causes of increased sphingolipid production [14]. Sphingolipids may therefore play a more important role in the pathophysiology of DKD in type 2 diabetes.

In the current study, we investigated urinary sphingolipid profiles in a sub-cohort of participants with youth-onset diabetes in the SEARCH for Diabetes in Youth (SEARCH) study. We aimed to 1) compare urinary sphingolipid profiles in youth-onset type 1 and type 2 diabetes, and 2) investigate the association of urinary sphingolipids with traditional markers of DKD. We hypothesized that urinary sphingolipids would be more elevated and associated with albuminuria in adolescents and young adults with youth-onset type 2 diabetes mellitus.

Methods

Study cohort and design

This is a cross-sectional study that compares urinary sphingolipids in a sub-cohort of adolescents and young adults with youth-onset type 1 and type 2 diabetes in the SEARCH study. SEARCH is a multicenter observational cohort study of youth with diabetes mellitus, and the methods of the SEARCH study have been previously reported [15, 16]. Briefly, youths less than 20 years of age who received a new diagnosis of non-gestational diabetes were identified from six recruitment centers from 2000–2010. A subset of participants at least 10 years of age and with 4 years diabetes duration were recruited for an outcome visit between 2011 and 2015. From this cohort, urine samples from 57 participants with type 1 diabetes and 59 with type 2 diabetes were randomly selected with serum creatinine and cystatin C values available for estimation of glomerular filtration rate (eGFR). Clinical characteristics of study participants and laboratory data were provided

by the SEARCH data coordinating center, including age, race, diabetes type and duration, medications, body mass index (BMI), hemoglobin A1C levels, lipid profiles, serum creatinine and cystatin C, and urinary albumin and creatinine. As trends in diabetes incidence vary by race, race was determined via self-report by the parent or participant, depending on age. eGFR was estimated using the average of the recently derived cystatin C and creatinine U25 equation, as this provides a more accurate and precise measurement of GFR compared to single-marker estimates [17]. DKD was defined as a urinary albumin-to-creatinine ratio (ACR) ≥ 30 mg/g. To provide a comparison group of healthy youth without diabetes, 43 healthy control samples were selected from a local biorepository at Cincinnati Children's Hospital Medical Center (CCHMC). This cohort was frequency matched according to age and sex to the study cohort. The study was approved by the local Institutional Review Board.

Sphingolipid analysis

A comprehensive panel of urinary sphingolipids was performed that included the following sphingolipid species: ceramide (Cer) species (C16, C18, C20, C24, C24:1); glucosylceramide (GS) species (C16, C18, C20, C22, C24, C24:1, C26); lactosylceramide (LC) species (C16, C18, C20, C24, C24:1, C26); and sphingomyelin (SM) species (C16, C18, C20, C22, C24:1, C24). Individual species of the sphingolipid panel were selected based on previous studies demonstrating these species were elevated in obesity and diabetes [10, 18]. Testing was performed at the CCHMC Clinical Mass Spectroscopy Laboratory (Director, Kenneth Setchell, PhD), as previously reported [18]. Individual sphingolipid species were detected and quantified in urine specimens by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) using a Waters Xevo TQ-S triple-quadrupole mass spectrometer interfaced with Acquity UPLC system (Waters, Milford, MA). Urine was extracted twice with the Folch procedure. Optimized parameters were determined with individual standard compounds. Sphingolipids were separated by an Acquity UPLC CSH C18 column and quantified under positive ion mode. Reference standards of all the sphingolipid species quantified were obtained from Avanti Lipids (Alabaster, Alabama) and used to construct calibration curves. For quantification, the precursor and product ion pairs for multiple reaction monitoring were selected by MS/MS spectra of standards or calculated theoretically if the standard for a given sphingolipid was not available. Quantification of sphingolipid species with various fatty acid chain lengths was performed using the curve of each sphingolipid species with the closest number of chain length.

Statistical analysis

Descriptive statistics were summarized and comparisons between the groups made using chi-squared testing for categorical variables and Kruskal–Wallis testing for continuous variables. Comparisons were made within each individual sphingolipid species, as well as for aggregate levels for each category. Linear regression modeling was used to investigate the association of urinary sphingolipids levels with ACR and eGFR. Multivariable analysis was then performed to assess the independent associations while controlling for clinically relevant covariates, including age, BMI, diabetes duration, serum triglyceride level, hypertension, HbA1c, sex, and diabetes type. Interactions between diabetes type and sphingolipid levels were investigated to determine if the relationship between urinary sphingolipids and outcomes was modified by diabetes type. Log transformation was used as appropriate to fulfill the assumptions of linear regression modeling. All analyses were performed with SAS statistical software, version 9.4.

Results

Demographic and clinical characteristics

The study cohort included 116 adolescents and young adults with youth-onset diabetes: 57 with type 1 diabetes and 59 with type 2 diabetes. The median age (IQR) of participants was 23.1 years (20.9, 24.9) and the median duration of diabetes

was 9.3 (8.5, 10.2) years. Overall, 53% were females, 38.8% were Black, 37% were white, 21.6% were Hispanic, and 2.6% were Asian American/Pacific Islander. Demographic characteristics of the control subjects were similar to study subjects: mean age 23 years (21, 25), and 49% female.

Comparison of clinical and demographic characteristics between participants with type 1 and type 2 diabetes are presented in Table 1. There were no differences in sex or race between participants with type 1 and type 2 diabetes. There were no differences in indices of glycemic control or diabetes duration between those with type 1 and type 2 diabetes, though participants with type 2 diabetes were slightly older. Those with type 2 diabetes also had an increased prevalence of cardiovascular risk factors, including higher BMI, triglyceride levels, blood pressure, and lower HDL cholesterol levels. eGFR and ACR were similar in participants with type 1 and type 2 diabetes.

Table 2 Sphingolipid levels according to patient sex

Sphingolipid class	Study subjects (diabetes)	
	Males (n=55)	Females (n=61)
Sphingomyelin	108.9 (78.1, 147.8)	*167.4 (138.8, 217.8)
Ceramide	4.9 (3.3, 10.1)	*21.5 (11.6, 39.5)
Glucosylceramide	12.3 (9.5, 17.9)	*28.9 (17.6, 40.6)
Lactosylceramide	6.4 (4.6, 15.2)	*26.7 (11.4, 70.2)

Levels expressed as nmol/gm creatinine and as median (interquartile range)*

all p values were <0.0001 compared to males

Table 1 Clinical characteristics

Variable	Type 1 diabetes (n=57)	Type 2 diabetes (n=59)	P-value
Age	22.2 (19.9, 23.6)	24.2 (21.4, 25.8)	0.0007
Gender	52.6%	52.5%	0.99
Diabetes duration	9.5 (8.6, 10.2)	9.2 (8.4, 10.2)	0.34
BMI, kg/m ²	25.8 (22.2, 31.8)	35.6 (29.0, 40.1)	<0.0001
Hypertension (%)	35.1%	61.0%	0.005
SBP	112 (103, 120)	118 (110, 126)	0.012
DBP	72 (67, 78)	78 (72, 83)	0.001
HbA1c (%)	9.5 (8.3, 11.3)	9.0(6.2, 11.7)	0.16
Glucose (mg/dL)	200 (130, 283)	190 (111, 293)	0.56
LDL (mg/dL)	89 (73, 115)	93 (76, 123)	0.48
HDL (mg/dL)	55 (43, 66)	39 (33, 44)	<0.0001
TG (mg/dL)	84 (56, 125)	118 (79, 223)	0.0006
Prescribed insulin	94.7%	50.9%	<0.0001
ACR	7.2 (4.3, 40.3)	10.1 (4.3, 309.2)	0.24
Serum creatinine (mg/dL)	0.66 (0.57, 0.89)	0.64 (0.51, 0.77)	0.13
Serum cystatin C (mg/L)	0.70 (0.58, 0.87)	0.73 (0.62, 0.83)	0.71
eGFR (ml/min/1.73 m ²)	107.7 (95.7, 117.6)	112.6 (98.0, 134.3)	0.21

Data expressed as median (interquartile range)

BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; LDL low density lipoprotein; HDL high density lipoprotein; TG triglyceride; ACR albumin: creatinine ratio; eGFR estimated glomerular filtration rate

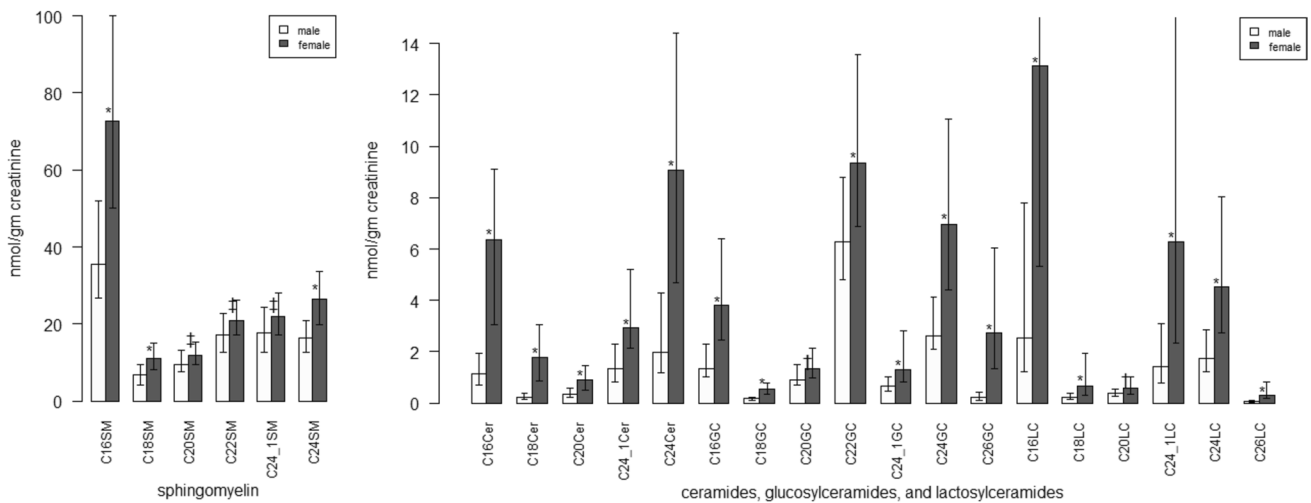


Fig. 1 Urinary sphingolipid levels in participants with diabetes by sex, * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$

Total (Table 2) and individual levels (Fig. 1) of different subspecies of SM, Cer, GC, and LC were markedly higher in females compared to males among children with diabetes. There was no significant difference among sphingolipid classes according to race/ethnicity. BMI was not associated with total levels of any sphingolipid class. Increase in age was associated with a weak but statistically significant decrease in total LC ($r = -0.21$, $p = 0.027$) and total SM levels ($r = -0.29$, $p = 0.002$). There was a significant correlation of HbA1c with total Cer ($r = 0.27$), GC ($r = 0.24$), and LC ($r = 0.27$) levels (all $p < 0.01$). Sphingolipid levels did not correlate with LDL and HDL cholesterol, or serum triglycerides.

Among control subjects, total levels of LC were higher in females (4.9 vs. 3.2 nmol/gm, $p = 0.002$), but other sphingolipid subspecies did not reach statistical significance.

Urinary sphingolipid profiles

All sphingolipid species were significantly higher in adolescents and young adults with type 1 and type 2 diabetes compared to healthy controls (Fig. 2). The most significant differences were seen for all C16 species (C16SM, C16Cer, C16GC, and C16LC), C24:1Cer and C24:1LC, and C24Cer, C24GC, and C24LC. Results remained similar when stratified by sex, though some species did not reach statistical significance (Supplemental Fig. 1). There were no differences in any sphingolipid species between participants with type 1 and type 2 diabetes. Therefore, subsequent analyses were performed using a single dataset combining type 1 and type 2 diabetes.

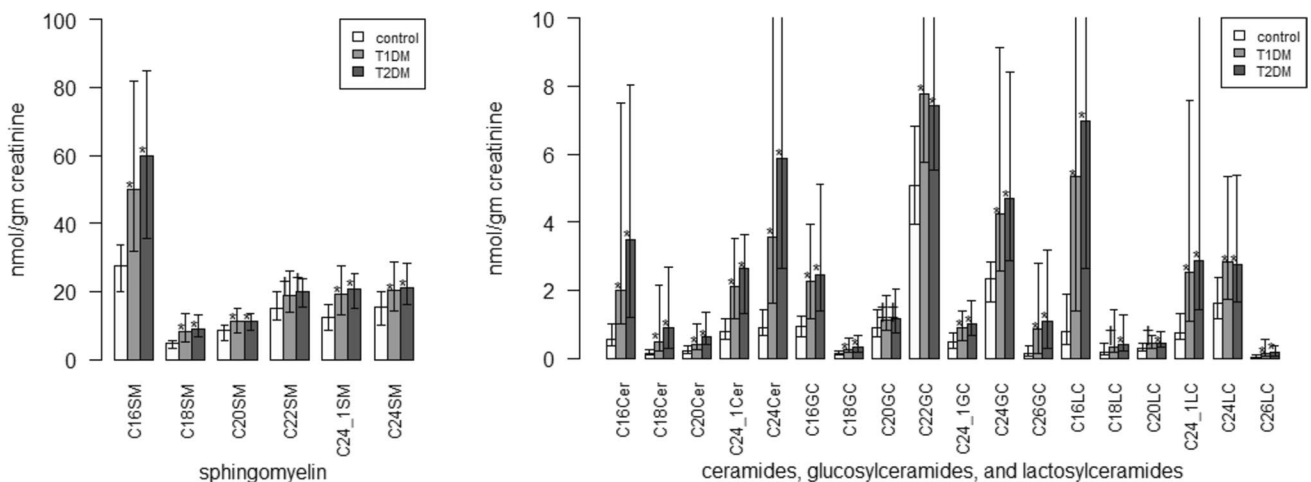


Fig. 2 Urinary sphingolipid levels in participants with type 1 and type 2 diabetes, * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ (all p-values are compared to control groups)

Comparison of sphingolipid levels in adolescent and young adults with DKD (ACR ≥ 30), those without DKD, and healthy controls is shown in Fig. 3. In general, sphingolipids were higher in those with DKD compared to those without, and those without DKD had elevated levels compared to healthy controls. This pattern was most prominent and significant for C16SM, C24:1SM, C24Cer, C24:1Cer, C22 GC, C24 GC, and C24:1 GC.

Association of urinary sphingolipid species with ACR and eGFR

In adjusted analyses, all SM species as well as many Cer and GC species were significantly associated with ACR (Fig. 4). Lactosylceramide species were not associated with ACR. The most significant associations were seen for C18SM, C24:1 SM, C24:1Cer, and C24:1 GC species (all $p < 0.001$), where there was more than a twofold increase in ACR per IQR increase in sphingolipid levels. To investigate if the association of sphingolipids with ACR varied according to diabetes type, interaction terms were tested for each species (diabetes type \times sphingolipid species). All interaction terms were not significant.

There was no association of any sphingolipid species with eGFR in adjusted analyses (Supplemental Fig. 2). However, several interaction terms were significant, indicating the association of urinary sphingolipids with eGFR varied according to diabetic type (Supplemental Fig. 3). Interaction terms were most significant for C24:1 Cer, C24:1 GC, C16 LC, C18 LC, C20 LC, and C24:1 LC. For these sphingolipid species, there was a significant positive association of species C20 LC and C24 LC with eGFR in type 1 diabetes. However, in type 2 diabetes, negative associations were observed between eGFR and the following sphingolipid species: C24:1 Cer, C24:1 GC, C18 LC, and C24:1 LC.

Discussion

We demonstrated that urinary sphingolipids are significantly elevated in adolescents and young adults with youth-onset diabetes compared to healthy controls. Many individual urinary sphingolipid species were associated with increased ACR, indicating these species may represent early biomarkers of kidney injury. Sphingolipid profiles were similar in both type 1 and type 2 diabetes, indicating that diabetes per se, rather than obesity-related metabolic abnormalities such as insulin resistance, inflammation, and fatty acid dysregulation, is primarily responsible for increased urinary sphingolipid levels.

Sphingolipid dysregulation has been implicated in the pathogenesis of a variety of kidney diseases, including lupus nephritis, Fabry's disease, and diabetes [19]. Though the exact mechanism of sphingolipid-mediated kidney injury has yet to be elucidated, it may occur via the disruptive effects of sphingolipid accumulation on podocyte integrity or mitochondrial function [20, 21]. As dysregulated lipid metabolism has been implicated in the pathogenesis of diabetes, particular attention has been given to the role of sphingolipids in DKD [22]. Previous studies have demonstrated that increased plasma sphingolipid species correlate with albuminuria in type 1 and type 2 diabetes [23–25]. Urinary levels of ceramide were investigated by Morita et al. in adults with type 2 diabetes mellitus, and they found elevated levels in those with more advanced DKD [26]. Our study similarly showed that sphingolipid species were elevated in adolescents and young adults with DKD, and many species demonstrated strong correlation with albuminuria. Together, these findings indicate sphingolipids may represent a useful biomarker of DKD.

Despite increasing evidence linking altered sphingolipid metabolism to DKD, the clinical application of these

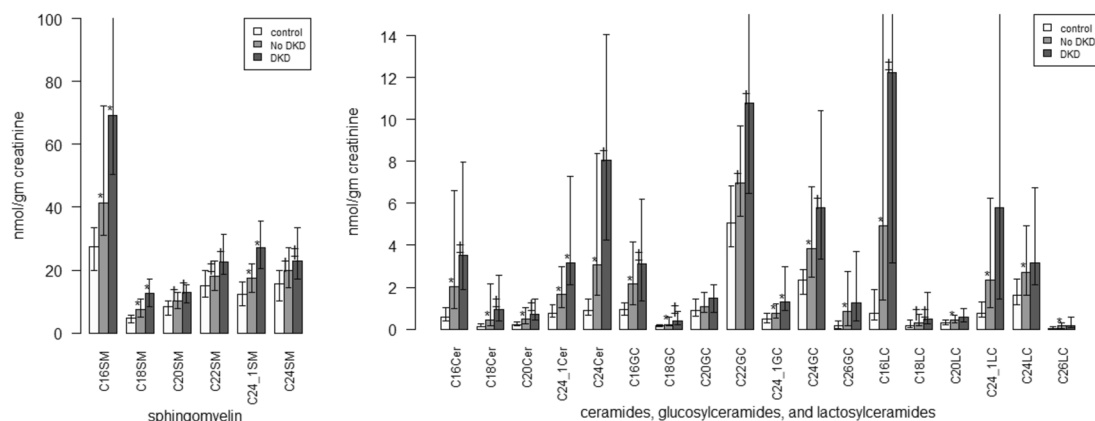


Fig. 3 Urinary sphingolipid levels in participants with and without DKD, * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$ (p -values compare No DKD to controls, and DKD to No DKD)

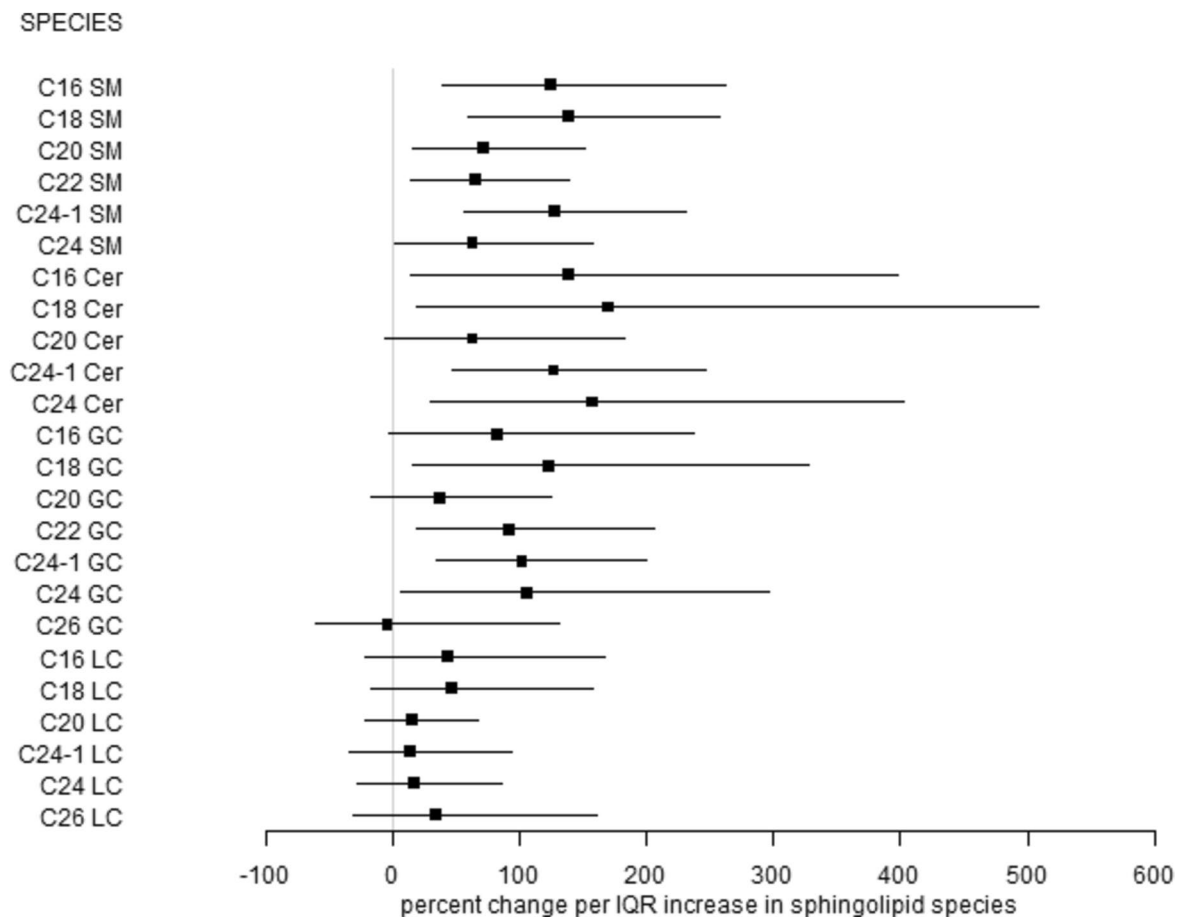


Fig. 4 Association of urinary sphingolipid with ACR

biomarkers for disease progression has not been established. In our cohort, urinary sphingolipids were elevated even among subjects without albuminuria, suggesting these may represent a more sensitive marker of early kidney injury. Increased production of sphingolipids in kidney tissue may cause elevation in urinary levels in the absence of more global injury to the glomerular basement membrane and subsequent albuminuria. Therefore, urinary sphingolipids may identify kidney injury in those patients with DKD but with normal albumin excretion. However, we were unable to assess the long-term risk of kidney disease progression among those adolescents with elevated urinary sphingolipid levels, and longitudinal studies are therefore needed to evaluate the prognostic value of urinary sphingolipids in DKD. It is possible that increased urinary sphingolipids may be a result of early kidney injury but not causally related. Intracellular levels of all sphingolipid species may increase in proportion to diabetic severity, with subsequent release into the urine in proportion to the extent of diabetic injury from other processes. Future studies are needed to assess whether urinary sphingolipids are more predictive than urinary albumin in assessing the risk of progression of kidney disease.

Considering the well-established associations between obesity, inflammation, and sphingolipid synthesis [14, 27, 28], the finding of similar urinary sphingolipid profiles in type 1 and type 2 diabetes was not in agreement with our hypothesis and was somewhat surprising. Hyperglycemia as the main cause of increased urinary sphingolipids, rather than metabolic dysfunction, was further supported by the following findings: 1) significant correlations existed between HbA1c and urinary Cer, GC, and LC species, and 2) no correlation was seen between sphingolipid levels and BMI or serum triglycerides. A possible explanation is that hyperglycemia induces metabolic pathways that increase production of intermediates needed for sphingolipid production. For example, reduced nicotinamide adenine dinucleotide phosphate (NADPH) can be generated from glucose shunting to the pentose phosphate pathway, and this is a required cofactor for both fatty acid and dihydrosphingosine synthesis [9, 29]. Aerobic glycolysis also can generate acetyl CoA and serine, both of which are substrates for de novo sphingolipid synthesis [30]. Thus, increased sphingolipid production in response to hyperglycemia may represent a common pathophysiologic pathway of DKD in both type 1 and type 2 diabetes.

An interesting finding of this study was the interaction between diabetes type and eGFR for several sphingolipid species, most notably C24:1 Cer, C24:1 GC, and several LC species. Specifically, these urinary sphingolipids tended to be associated with an increase in eGFR in type 1 diabetes, while they were associated with a decrease in eGFR among those with type 2 diabetes. This might indicate that urinary sphingolipids are associated with hyperfiltration in type 1 diabetes, which is presumed to be the earliest manifestation of kidney injury [31]. Conversely, urinary sphingolipids may associate with more advanced kidney injury (i.e., a decline in eGFR) in type 2 diabetes. It may also be that obesity-associated hyperfiltration, which occurs primarily in type 2 diabetes, is a confounding factor that modifies the relationship between urinary sphingolipids and eGFR between those with type 1 and type 2 diabetes.

To our knowledge, this is the first study to report sex-related differences in urinary sphingolipids, with females consistently demonstrating higher levels across all individual species. Other studies have shown elevations of serum sphingolipids levels in females, and similarly hypothesized this may be attributed to sex-related differences in metabolism-related genes [32, 33]. Considering sex-related differences in control subjects were much less apparent (only LC species were significantly higher), this may indicate that sphingolipid production in response to hyperglycemia is more pronounced in females. Evidence suggests women with diabetes may be at higher risk for progression of kidney disease [34, 35], and females with youth-onset diabetes have increased prevalence of hyperfiltration and proteinuria [36–38]. Therefore, increased urinary sphingolipid production in females with diabetes may explain these sex-related differences in DKD. Future studies with long-term follow-up are needed to further investigate the interplay between sex, urinary sphingolipid levels, and risk for progression of DKD.

This study has many strengths. Participants were selected from a large, multicenter, representative population of adolescents and young adults with diabetes [16]. Our cohort included only patients with both a serum creatinine and cystatin C value, thereby enabling us to estimate GFR using the U25 estimating equations, which have been validated in both children and young adults [17]. However, several limitations deserve mention. First, the observational nature of the study prevents any inference regarding the causative effect of urinary sphingolipids in the pathogenesis of DKD, though a growing body of evidence suggests the pathogenic role of glomerular sphingolipid accumulation in kidney disease [30]. Second, ACRs were obtained using a single spot urine, rather than repeated measurements that are recommended to diagnose persistent albuminuria due to the day-to-day variation that can occur in urine albumin excretion [39].

Finally, although we investigated a comprehensive panel of urinary sphingolipids, it is possible a downstream product of sphingolipids may be responsible for mediating DKD. For example, GM3 ganglioside, a higher order glycosphingolipid, antagonizes insulin resistance and is increased in the kidney tissue of rats with type 1 diabetes [40, 41].

In conclusion, we demonstrated that urinary sphingolipids are similarly elevated in both type 1 and type 2 diabetes. This indicates that hyperglycemia, rather than obesity associated-metabolic dysfunction, is responsible for increased urinary sphingolipid levels. Several urinary sphingolipids were independently associated with albuminuria and were increased even in those with normal urinary albumin excretion. Urinary sphingolipids may therefore represent an early biomarker of DKD.

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SEARCH 3/4

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention and the National Institute of Diabetes and Digestive and Kidney Diseases.

Author contributions EN wrote the manuscript and performed the analysis. NS, SM, and MM contributed to the discussion and reviewed/edited the manuscript. KDRS and WZ developed and validated the urinary sphingolipid analyses and performed laboratory analyses and data interpretation. EN is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior presentation This data was presented in abstract form at the American Society of Nephrology meeting, 2020 (virtual only).

Data availability Data from the SEARCH for Diabetes in Youth that were used in this study are available for request at the NIDDK Central Repository (NIDDK-CR) website, Resources for Research (R4R), <https://repository.niddk.nih.gov/>.

Declarations

Conflict of interest There are no conflicts of interest to disclose.

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