

## PAPER

# Dysregulations in circulating sphingolipids associate with disease activity indices in female patients with systemic lupus erythematosus: a cross-sectional study

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**Objective:** The objective of this study was to investigate the association of clinical and renal disease activity with circulating sphingolipids in patients with systemic lupus erythematosus. **Methods:** We used liquid chromatography tandem mass spectrometry to measure the levels of 27 sphingolipids in plasma from 107 female systemic lupus erythematosus patients and 23 controls selected using a design of experiment approach. We investigated the associations between sphingolipids and two disease activity indices, the Systemic Lupus Activity Measurement and the Systemic Lupus Erythematosus Disease Activity Index. Damage was scored according to the Systemic Lupus International Collaborating Clinics damage index. Renal activity was evaluated with the British Island Lupus Activity Group index. The effects of immunosuppressive treatment on sphingolipid levels were evaluated before and after treatment in 22 female systemic lupus erythematosus patients with active disease. **Results:** Circulating sphingolipids from the ceramide and hexosylceramide families were increased, and sphingoid bases were decreased, in systemic lupus erythematosus patients compared to controls. The ratio of C<sub>16:0</sub>-ceramide to sphingosine-1-phosphate was the best discriminator between patients and controls, with an area under the receiver-operating curve of 0.77. The C<sub>16:0</sub>-ceramide to sphingosine-1-phosphate ratio was associated with ongoing disease activity according to the Systemic Lupus Activity Measurement and the Systemic Lupus Erythematosus Disease Activity Index, but not with accumulated damage according to the Systemic Lupus International Collaborating Clinics Damage Index. C<sub>16:0</sub>- and C<sub>24:1</sub>-hexosylceramides were able to discriminate patients with current versus inactive/no renal involvement. All dysregulated sphingolipids were normalized after treatment. **Conclusion:** We provide evidence that sphingolipids are dysregulated in systemic lupus erythematosus and associated with disease activity. This study demonstrates the utility of simultaneously targeting multiple components of a pathway to establish disease associations. *Lupus* (2016) 0, 1–11.

**Key words:** Systemic lupus erythematosus; sphingolipids; disease activity

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with heterogeneous clinical manifestations. Despite major efforts to phenotype the different sub-types of SLE, there is still a need to identify biomarkers that can serve to track disease progression as well as treatment efficacy.

Sphingolipids are a family of bioactive signalling compounds that are involved in several cellular processes including apoptosis, cell proliferation,

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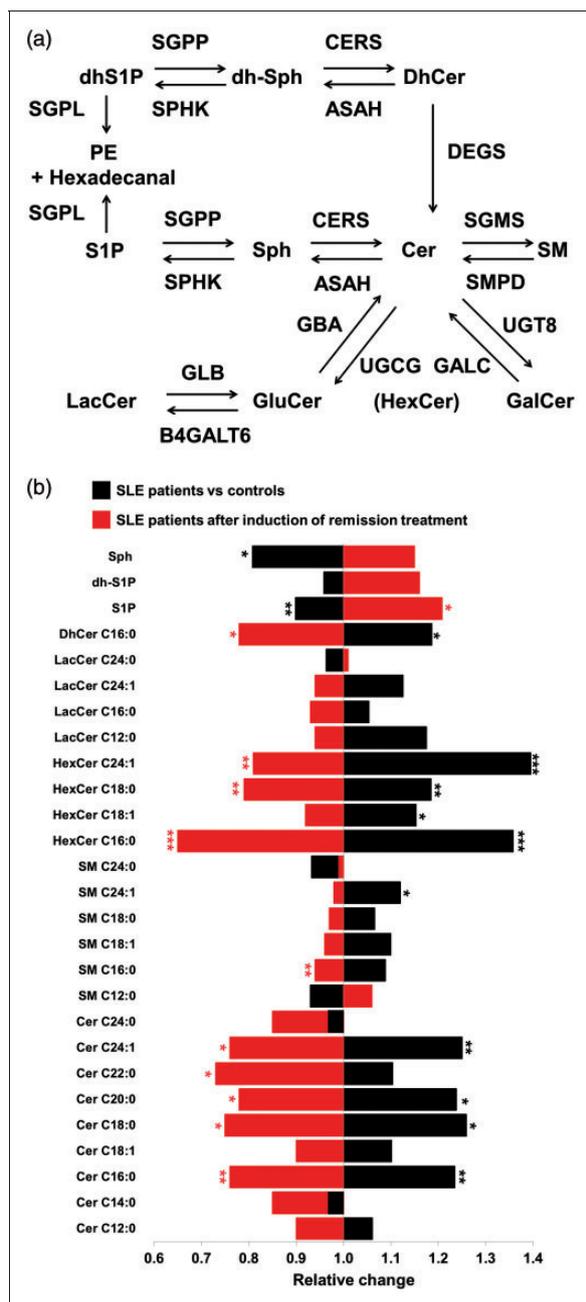
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migration and differentiation.<sup>1</sup> Ceramides constitute the central core of the sphingolipid pathway as they can be synthesized from any other sphingolipid<sup>2</sup> (Figure 1(a)). Dysregulation of sphingolipids has been described in several conditions,<sup>3</sup> especially

in inflammatory and immune mediated diseases,<sup>4</sup> including SLE.<sup>5</sup> Specifically, alterations in compounds of the sphingolipid pathway have been described in SLE and some of its related comorbidities in both humans and murine models.<sup>5-7</sup> This includes alterations in sphingomyelins, ceramides, glycosphingolipids and sphingosine-1-phosphate (S1P). Therefore, the sphingolipid pathway has arisen as an interesting target in the search for biomarkers of SLE. Circulating sphingolipids have been suggested both as diagnostic markers and compounds of interest to monitor the activity of the disease.<sup>5</sup> Because all compounds in the pathway are highly correlated, the simultaneous measurement of several sphingolipids in a pathway-based analysis is expected to provide more information than the determination of individual compounds.<sup>8</sup> Herein, levels of representative components of the sphingolipid pathway were determined in patients with SLE and compared to matched population controls. The ability of dysregulated compounds to track disease progression, both globally and more specifically in patients with renal involvement, was evaluated. Additionally, the same compounds were screened in 22 patients with SLE before and after initiation of immunosuppressive treatment.



**Figure 1** Sphingolipid profiles in systemic lupus erythematosus (SLE) patients relative to controls and after immunosuppressive treatment. (a) Compounds included in the study and related enzymes. (b) Levels of sphingolipids of patients with SLE (Cohort 1,  $n = 107$ ) relative to controls (Cohort 1,  $n = 23$ ) were normalized after induction of remission treatment

Continued

## Materials and methods

### Study design and clinical cohort

A cross-sectional study was performed at the Karolinska University Hospital, Stockholm, Sweden. All patients fulfilled at least four of the classification criteria of SLE.<sup>9</sup> The Karolinska

### Figure 1 Continued

(Cohort 2,  $n2 = 3$ ). Black bars = (compound average in the SLE group/compound average in the control group). Red bars = (compound average in SLE patients before treatment/compound average in SLE patients after treatment). For Cohort 1, Student's t-test or Mann-Whitney U-test were performed for normally and non-normally distributed data (Shapiro-Wilk test), respectively. For Cohort 2, pairwise comparisons before and after treatment were performed using the Wilcoxon signed-rank test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Details on the levels and 95% confidence intervals of each sphingolipid for controls and SLE patient groups are provided in Supplementary Table 1.

Cer = ceramide; DhCer = dihydroceramide; dh-S1P = dihydrosphingosine-1-phosphate; HexCer = hexosylceramide; GalCer = galactosylceramide; GluCer = glucosylceramide; LacCer = lactosylceramide; PE = phosphoethanolamine; SM = sphingomyelin; Sph = sphingosine; S1P = sphingosine-1-phosphate. See Supplementary Table 2 for enzyme abbreviation. **[AQ6]**

SLE cohort comprises 320 SLE patients and 320 population based age and gender matched controls enrolled between September 2004 and March 2010. Only females were included in this study. A multivariate design of experiments (DoE) approach<sup>10</sup> was applied to select samples representative for the entire cohort using clinical data as the basis for selection. This method ensures that selected samples are representative of the multidimensional space determined by the full SLE cohort, while simultaneously reducing the number of samples to be analyzed. First, principal component analysis (PCA) was used to summarize all clinical, personal and biochemical data for the full cohort (205 variables). The scores plot for the resulting PCA model is shown in Supplementary Figure 1. To ensure that all proposed SLE sub-phenotypes (ANA neg, SM/RNP, APS-like, SS-like and other SLE)<sup>11,12</sup> were evenly represented, a two factorial experimental design was applied to the scores plot displayed in Supplementary Figure 1. Five samples were selected from each of the four corners of the design as well as

three samples around the centre point for each sub-phenotype (Supplementary e-method 1). A schematic representation of this analysis strategy is shown in Supplementary Figure 2. This method gave 23 selected samples for each sub-phenotype to give a total of 115 selected samples from the full SLE cohort, as well as 23 controls. Of the 115 samples, seven were no longer available from the cohort bio-bank. The distribution of these seven individuals across the selected samples was visually examined in the PCA scores plot and determined to not bias the DoE model (Supplementary Figure 3). Of the remaining 108 SLE patients, one individual was later excluded post-analysis due to being diagnosed with vasculitis. Accordingly the final selected SLE sub-group analysed herein consisted of the 107 individuals described in Table 1. A more detailed description of the sample selection procedure is provided in Supplementary e-method 1. The clinical characteristics, disease activity and renal function of the 107 selected SLE patients as well as controls are presented in Table 1.

**Table 1** Demographics and levels of controls and patients with SLE included in the cross-sectional study. Only female individuals were included in the study

	SLE (n = 107)	Controls (n = 23)	FC <sup>a</sup>	p-value <sup>b</sup>
Age (years)	48.2 [45.1–51.1]	48.4 [40.6–56.2]	0.99	0.946
BMI	25.1 [24.1–26.0]	25.4 [23.3–27.5]	0.99	0.756
SLEDAI total	4.6 [3.7–5.5]	—	—	—
SLEDAI ≥ 6	40	—	—	—
SLAM total	7.8 [6.9–8.7]	—	—	—
SLAM ≥ 7	60	—	—	—
SLICC total	1.7 [1.3–2.1]	—	—	—
SLICC ≥ 2	45	—	—	—
Prednisolone dose (mg/day)	5.9 [4.5–7.2]	—	—	—
Number on immunosuppressants	48	N.A.	—	—
	MMF (n = 7)			
	CYC (n = 11)			
	AZA (n = 25)			
	MTX (n = 5)			
Number on antimalarials	HCQ (n = 32)	N.A.	—	—
Number on biologics	RTX (n = 5)	N.A.	—	—
Serum creatinine (μM) <sup>c</sup>	74.7 [69.5–79.9]	71.0 [67.4–74.6]	1.05	0.238
Albumin (g/L) <sup>d</sup>	38.6 [37.7–39.5]	42.1 [40.3–43.9]	0.92	0.002
Cholesterol (mM) <sup>c</sup>	5.2 [4.9–5.4]	5.3 [4.7–5.8]	0.98	0.704
LDL (mM) <sup>c</sup>	3.1 [2.9–3.3]	3.3 [2.8–3.9]	0.94	0.304
HDL (mM) <sup>c</sup>	1.4 [1.3–1.5]	1.4 [1.2–1.6]	1.00	0.538
TG (mM) <sup>c</sup>	1.2 [1.1–1.4]	1.3 [0.9–1.7]	0.92	0.895

SLE: systemic lupus erythematosus; BMI: body mass index; SLEDAI: systemic lupus erythematosus disease activity index; SLAM: systemic lupus activity measurement; SLICC: Systemic Lupus International Collaborating Clinics; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglycerides; MMF: mycophenolate mofetil; CYC: cyclophosphamide; AZA: azathioprine; MTX: methotrexate; HCQ: hydroxychloroquine; RTX: rituximab; N.A.: not applicable.

Average [95% confidence interval] values are presented.

<sup>a</sup>FC: fold change (SLE/controls).

<sup>b</sup>Student's t-test.

<sup>c</sup>SLE (n = 106); control (n = 21).

<sup>d</sup>SLE (n = 105); control (n = 21).

**Table 2** Clinical characteristics in patients with active SLE before and after immunosuppressive treatment

	Before (n = 22)	After (n = 22)	p-value <sup>a</sup>
Age (years)	36.8 [31.5–41.0]	37.3 [32.0–42.6]	1.000
Serum creatinine (μM)	75.1 [60.2–89.9]	74.5 [61.5–87.4]	0.754
SLEDAI	13.5 [10.2–16.7]	7.8 [4.0–7.6]	<0.0001
Major organ manifestations requiring increased therapy	Nephritis (n = 20)	N.A.	—
	Arthritis (n = 1)		
	CNS/lung (n = 1)		
Prednisolone (mg/day)	15.5 [11.8–19.2]	9.4 [8.3–10.5]	0.005
Ongoing immunosuppressants before increased therapy	11	N.A.	—
	MMF (n = 7)		
	CYC (n = 1)		
	AZA (n = 2)		
	MTX (n = 1)		
Number on antimalarials	HCQ (n = 7)	N.A.	—
Treatment given <b>AQ9</b>	RTX (=14)	N.A.	—
	MMF (n = 7)		
	CYC (n = 1)		

Average [95% confidence interval] values are presented.

SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus disease activity index; CNS: central nervous system; MMF: mycophenolate mofetil; CYC: cyclophosphamide; AZA: azathioprine; MTX: methotrexate; HCQ: hydroxychloroquine; RTX: rituximab; N.A.: not applicable.

<sup>a</sup>Wilcoxon signed-rank test.

In order to investigate the effects of immunosuppressive treatment on sphingolipids, additional plasma samples were taken between from 22 female patients with active SLE before and after treatment. **AQ1** Samples were collected between December 2004 and September 2013. For information on these patients and their treatment, see Table 2.

For blood collection, 10 mL of whole blood was collected in EDTA tubes after overnight fasting. Samples were left standing for 1 h before centrifugation at room temperature for 20 min at 1450 g. After centrifugation samples were aliquoted and immediately stored at  $-80^{\circ}\text{C}$  until use.

At the inclusion in the Karolinska SLE cohort, disease activity was assessed by the SLE Disease Activity Index (SLEDAI)<sup>13</sup> and the Systemic Lupus Activity Measurement (SLAM).<sup>14</sup> Accumulated organ damage was evaluated by the Systemic Lupus International Collaborating Clinics (SLICC)<sup>15</sup> damage index. Being a cross-sectional cohort, patients had varying disease activity at the inclusion time-point. For evaluation of the renal activity at sampling, the British Island Lupus Activity Group (BILAG) was used for subdividing the current renal activity into different grades (A–E).<sup>16</sup> Patients with some extent of renal activity at sampling were here grouped as grade A + B + C, depending on the grade of proteinuria and renal

function. Grade D + E represented patients with renal disease in full remission (D) or no previous renal involvement (E).

The study was approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki's principles. Signed consent forms were collected from all sample donors.

#### *Liquid chromatography tandem mass spectrometry analysis of sphingolipids in plasma*

Sphingolipids were determined in plasma using a previously described method with some modifications.<sup>17</sup> An aliquot of 25 μL was used for the determination of sphingomyelins (SM), ceramides (Cer), hexosylceramides (HexCer), lactosylceramides (LacCer) and dihydroceramides (DhCer). Sphingoid bases were determined using an independent aliquot of 50 μL. Details of the extraction protocol are provided as Supplementary e-method 2. Chromatographic and mass spectrometry details have been published elsewhere.<sup>17</sup> The reproducibility of calculated concentrations within the analysis was evaluated by the simultaneous analysis of a laboratory plasma reference material (n = 6) randomized both during the extraction and across the sequence. Coefficients of variation obtained for each compound are reported in Supplementary Table 1.

#### *Affinity proteomics*

Antibodies from the Human Protein Atlas project (www.proteinatlas.org) were utilized in order to screen sphingolipid-related proteins using antibody suspension bead arrays. The suspension bead array, consisting of covalently bonded antibodies to carboxylated beads (MagPlex Microspheres, Luminex), was created according to the manufacturer's protocol with minor modifications. The coupling of beads, labelling of samples and assay were performed as previously described.<sup>18</sup> A brief description is provided as Supplementary e-method 3.

#### *Statistical analysis*

Student's t-test or non-parametric Mann–Whitney U-test were performed for normally and non-normally distributed data, respectively. Paired samples were analysed using the Wilcoxon signed-rank test. Correlations were assessed by Pearson product–moment or Spearman's correlation for normally and non-normally distributed data, respectively. Differences were considered significant at  $p < 0.05$ . For Cohort 1, the area under the

receiver operating characteristic (ROC) curve (AUC) was used to assess the discrimination between SLE patients and controls. Statistical analyses were performed with SSPS V.22.0.

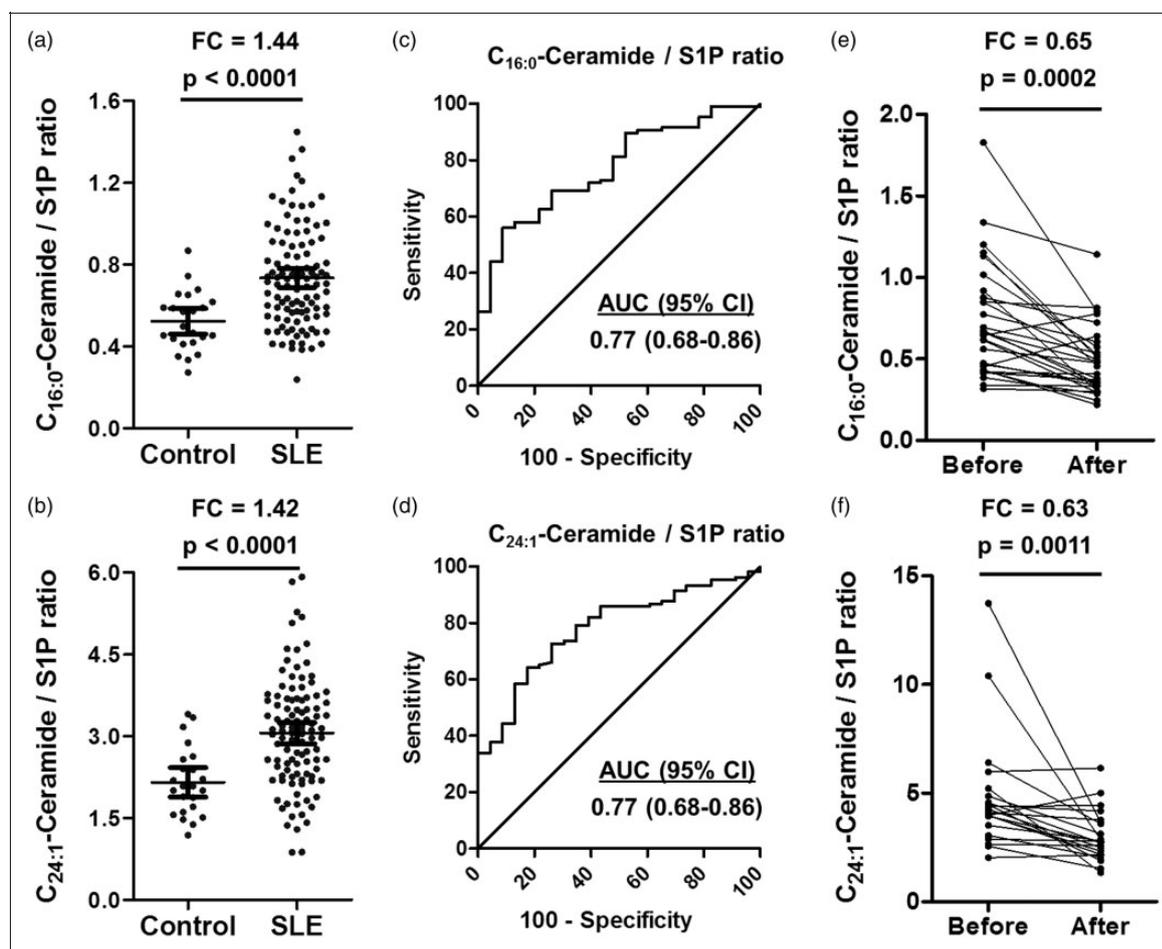
## Results

### *SLE patients exhibit an altered circulating sphingolipid profile*

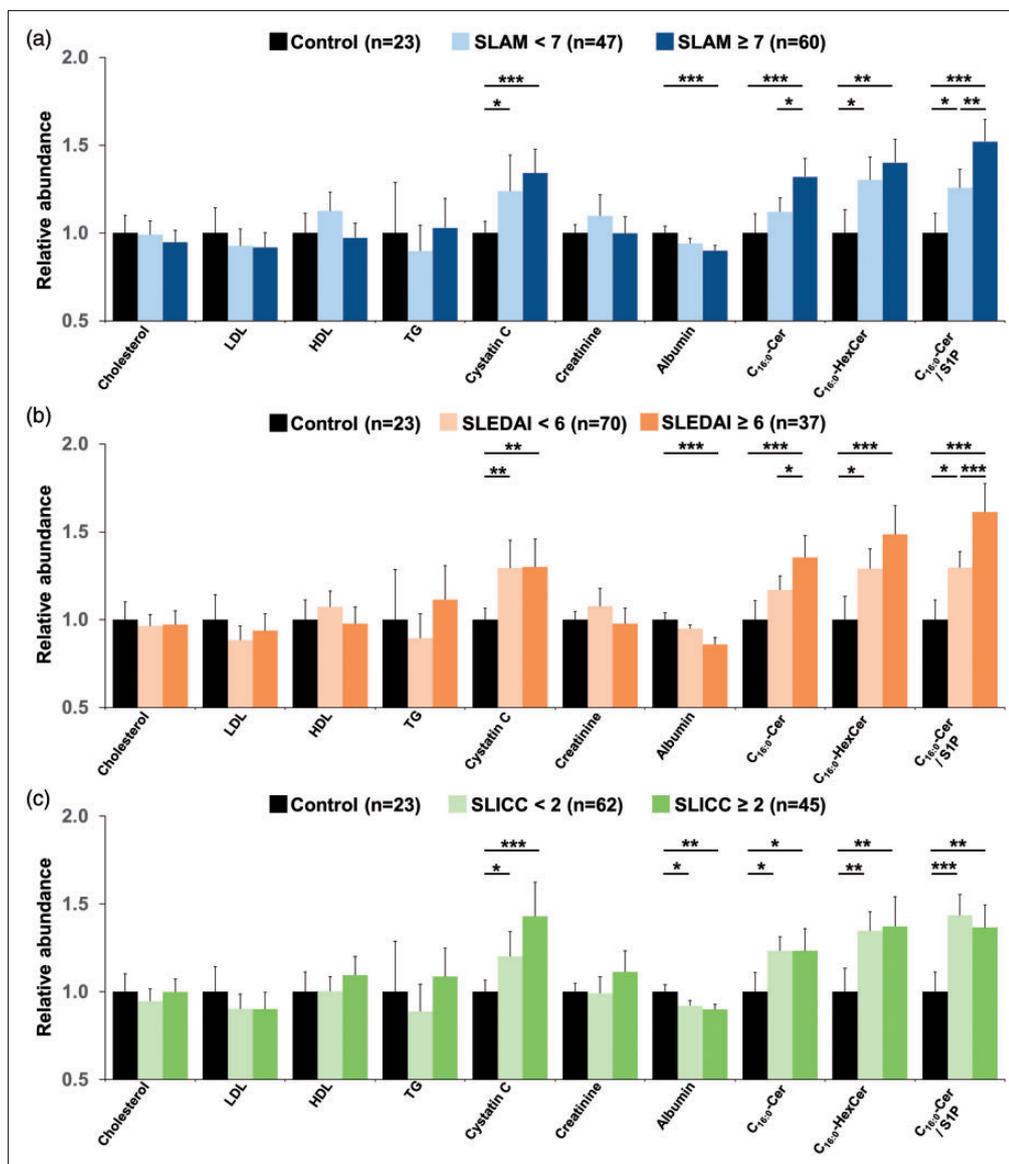
Sphingolipids are discussed in terms of the lipid class (e.g. ceramides) and the associated fatty acid chain (e.g. palmitic acid). The fatty acid nomenclature depends upon the length of the alkyl chain and degree of unsaturation. For example, palmitic acid contains a 16 carbon saturated alkyl chain (C16:0) and nervonic acid possesses a 24 carbon alkyl chain with a single double bond (C24:1) to give the

species C<sub>16:0</sub>-ceramide and C<sub>24:1</sub>-ceramide, respectively. In this cross-sectional study, circulating levels of sphingolipids were altered in female patients with SLE ( $n=107$ ) relative to controls ( $n=23$ ) with significant increases in ceramides (C<sub>16:0</sub>, C<sub>18:0</sub>, C<sub>20:0</sub> and C<sub>24:1</sub>), hexosylceramides (C<sub>16:0</sub>, C<sub>18:0</sub>, C<sub>18:1</sub> and C<sub>24:1</sub>), C<sub>24:1</sub>-sphingomyelin and C<sub>16:0</sub>-dihydroceramide and decreased levels of sphingosine and S1P (Figure 1(b), Supplementary Table 1). The ratio between altered ceramide species and their enzymatically-produced derivative, S1P, increased in patients with SLE (Figure 2(a–b)). In addition, the ratio evidenced increased ability to differentiate between SLE controls and patients (Figure 2(c–d)) relative to the sphingolipid species individually (Supplementary Table 1).

A total of four out of eight sphingolipid-related enzymes screened using an affinity-based proteomics platform were altered in SLE patients



**Figure 2** Ceramide to S1P ratios are increased in systemic lupus erythematosus (SLE) patients. ((a)–(b)) Ratios of C<sub>16:0</sub>- and C<sub>24:1</sub>-ceramide to S1P in controls ( $n=23$ ) and patients with SLE ( $n=107$ ) from Cohort 1. ((c)–(d)) receiver operating characteristic curves obtained for C<sub>16:0</sub>- and C<sub>24:1</sub>-ceramide to S1P ratios in cohort 1. (C–D) Decreases of C<sub>16:0</sub>- and C<sub>24:1</sub>-ceramide to S1P ratios after induction of remission treatment in Cohort 2. [AQ7] FC = Fold change (SLE vs controls). For Cohort 1, Student's t-test was performed. For Cohort 2, pairwise comparisons before and after treatment were performed using the Wilcoxon signed-rank test.



**Figure 3** Association between levels of dysregulated sphingolipids and other biochemical markers with clinical indices accounting for ongoing disease activity [Systemic Lupus Activity Measurement (SLAM) (a) and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (b)] and accounting for the accumulated damage [Systemic Lupus International Collaborating Clinics (SLICC)(c)]. Vertical bars represent the average of the compound within its subgroup normalized to the average of the control group (Control group value = 1) and whiskers represent 95% confidence intervals. Statistical significance for multiple comparisons was determined using one-way ANOVA with Bonferroni’s post-hoc correction or Kruskal–Wallis with Dunn’s post-hoc correction for normally and non-normally distributed data, respectively. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . The number of samples per group indicates the number of individuals included in the sphingolipid measurements. The number of individuals included in the other clinical measurements is shown in Table 1.

relative to controls. Ceramide synthase 5 (CERS5) and sphingosine-1-phosphate lyase (SGPL1) were upregulated in SLE patients relative to controls (Supplementary Table 3). In contrast, glucosylceramidase (GBA) and UDP-galactose-ceramide galactosyltransferase (UGCG) were downregulated in SLE patients relative to controls (Supplementary Table 3).

#### *Dysregulated sphingolipids association with disease activity indices*

The association between levels of dysregulated sphingolipids with clinical indices accounting for ongoing disease activity (SLAM and SLEDAI) and for the accumulated damage (SLICC) (Figure 3, Supplementary Figure 4) was examined.

Patients were stratified according to the current disease activity, for which a cut-off point of SLAM  $\geq 7$  and SLEDAI  $\geq 6$  to define active disease was used. For damage, a SLICC score  $\geq 2$  was used to define high organ damage. Other lipid and renal markers of SLE (cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TG), cystatin C, creatinine and albumin) were also included in the comparison (Figure 3).  $C_{24:1}$ -ceramide, hexosylceramides ( $C_{16:0}$  and  $C_{24:1}$ ) and ( $C_{16:0}$ - and  $C_{24:1}$ -) ceramide/S1P ratios as well as cystatin C were increased in the SLAM, SLEDAI and SLICC groups relative to controls. Both  $C_{16:0}$ -ceramide and the  $C_{16:0}$ -ceramide to S1P ratio were able to discriminate patient groups with higher disease activity, while damage was not associated with ceramide levels (Figure 3). [AQ2]

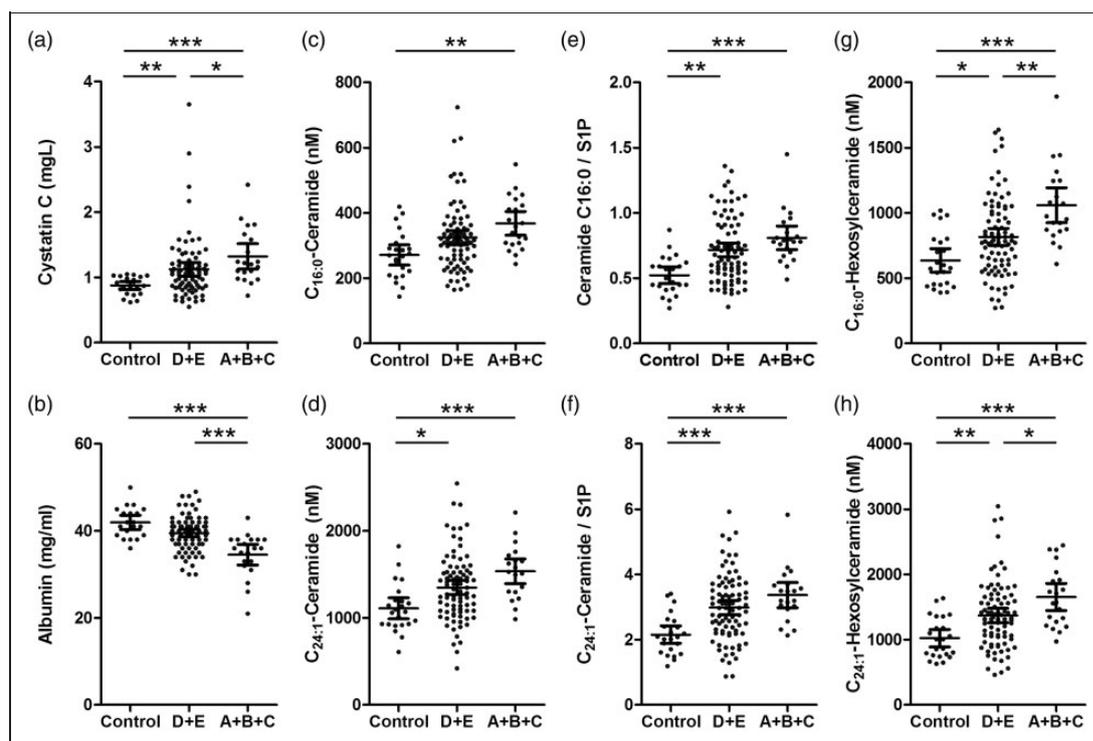
*Sphingolipid levels before and after immunosuppressive treatment in patients with active SLE*

In the longitudinal patient cohort ( $n=22$ ), initiation of immunosuppressive treatment in active

SLE patients resulted in normalization of the circulating sphingolipid profile at follow-up after  $9.5 \pm 3.3$  months, with significant decreases in ceramides ( $C_{16:0}$ ,  $C_{18:0}$ ,  $C_{20:0}$ ,  $C_{22:0}$  and  $C_{24:1}$ ), hexosylceramides ( $C_{16:0}$ -,  $C_{18:0}$ - and  $C_{24:1}$ -),  $C_{16:0}$ -sphingomyelin and  $C_{16:0}$ -dihydroceramide (Figure 1(b)). Sphingoid bases were elevated after treatment with significant increases for S1P (Figure 1(b)). The ratios of  $C_{16:0}$ -ceramide and  $C_{24:1}$ -ceramide with S1P decreased ( $p < 0.01$ ) after treatment (Figure 2(e-f)), with 22 and 18 patients exhibiting lower levels, respectively.

*Association of hexosylceramides with renal BILAG*

$C_{16:0}$  and  $C_{24:1}$ -hexosylceramides were increased in SLE patients with current renal involvement according to renal BILAG (A + B + C,  $n=21$ ) relative to patients with inactive or no previous renal involvement (BILAG D + E,  $n=86$ ) or controls ( $n=23$ ) (Figure 4(g-h)). These hexosylceramides presented the same trend as cystatin C (Figure 4(a)). The other dysregulated sphingolipids, including  $C_{16:0}$ - and  $C_{24:1}$ -ceramides and their ratio with



**Figure 4** Circulating markers of renal function and sphingolipids in relation to renal British Island Lupus Activity Group (BILAG). ((a)–(h)) Comparisons between circulating levels of renal function markers cystatin C, albumin and  $C_{16:0}$ - and  $C_{24:1}$ -sphingolipids. Systemic lupus erythematosus (SLE) patients were subphenotyped as presenting no current renal activity (Renal BILAG E + D) and SLE patients with current renal activity (Renal BILAG C + B + A). Each dot represents one individual. Average with 95% confidence intervals are presented for each subgroup. Statistical significance for multiple comparisons was determined using one-way ANOVA with Bonferroni’s post-hoc correction or Kruskal–Wallis with Dunn’s post-hoc correction for normally and non-normally distributed data, respectively. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  [AQ8].

S1P, did not differ between renal and non-renal activity (Figure 4(c-f)).

## Discussion

We report dysregulations in circulating bioactive sphingolipids in patients with SLE. We also demonstrate positive associations between a subset of sphingolipids in general as well as renal SLE activity. Furthermore, we observed that levels of dysregulated sphingolipids were normalized after immunosuppressive treatment.

Abnormal sphingolipid metabolism is linked to the development and progression of several diseases. Specifically the modulation of the sphingolipid pathway is used as a therapeutic intervention in immune-mediated diseases such as psoriasis<sup>19</sup> and multiple sclerosis<sup>20</sup> and has also been suggested in SLE.<sup>5</sup> SLE related research has shown that alterations in the sphingolipid pathway are not restricted to a single enzyme or compound, but instead evidences effects across the pathway.<sup>5,7</sup> Additionally, the involvement of different sphingolipids in SLE-related comorbidities, such as renal<sup>7,21,22</sup> or cardiovascular<sup>23,24</sup> complications, has been described. It is therefore challenging to select a single sphingolipid species as a biomarker of disease. The current work demonstrates instead the utility of performing a pathway-based<sup>8</sup> approach that targets the different families of molecules in a biochemical pathway. In this exploratory cohort, control and patient groups were balanced in terms of potential confounders that may affect sphingolipid levels, especially cholesterol, TG, LDL and HDL<sup>3,25</sup> as well as renal function.

Patients with SLE presented increased levels of ceramides with differing fatty acid chain lengths, especially C<sub>16:0</sub>- and C<sub>24:1</sub>-ceramides. This shift was accompanied by a concomitant decrease in the levels of sphingoid bases. The observed higher levels of the ceramide to S1P ratios in SLE patients (Figure 2) may thus be due to either increased biosynthesis of ceramides or enhanced degradation of S1P. Both SGPL1, the enzyme that degrades S1P, and CerS5, one of the enzymes responsible for increased biosynthesis of C<sub>16:0</sub>-ceramide, were increased in the SLE group.

The correlations of dysregulated sphingolipids with SLE disease activity measurements suggest that their alterations may be useful as markers of disease activity. It is complex to monitor the disease activity in multifactorial diseases such as SLE. There are, accordingly, several different SLE

disease activity indices. There is presently no general agreement on which one is best, but each index has its own strengths and caveats.<sup>16</sup> This situation has led to a constant search for more robust objective biomarkers. In this study, C<sub>16:0</sub>-ceramide and its ratio with S1P were associated with both SLAM and SLEDAI. It is also of note that these elevated lipid levels were independent of the amount of irreversible accumulated damage, as evaluated by SLICC scoring. Taken together, our data suggest that the circulating level of C<sub>16:0</sub>-ceramide is a possible biomarker for disease activity in SLE.

Increased levels of ceramides have also been reported in children<sup>26</sup> and adults<sup>25</sup> with chronic kidney disease. Additionally, ceramides have been shown to be responsible for hyperhomocysteinemia-induced glomerular injury when produced by de novo formation in rats<sup>27</sup> or via acid sphingomyelinase in mice.<sup>28</sup> In our cohort, SLE patients with ongoing renal involvement presented slightly higher C<sub>16:0</sub>-ceramide levels relative to patients with non-renal involvement, though these increases were not statistically significant. Larger patient cohorts are thus required to confirm the findings.

Glycosphingolipids (GSL) represent another part of the sphingolipid pathway that has been linked to SLE.<sup>6</sup> The basic structure of these compounds consists of a ceramide linked to a sugar moiety of varying complexities. The basic GSL, lactosylceramides and hexosylceramides, were measured in the present study. In the current study, hexosylceramides comprise the families of glucosyl- and galactosyl-ceramides, because these two compounds share the same parent mass and could not be chromatographically resolved under the experimental conditions. Patients with SLE presented higher circulating levels of hexosylceramides relative to controls. The cause of these elevated levels may be related to either one of the species (glucosyl- or galactosyl-) or both. Interestingly, the two sphingolipid-related enzymes found downregulated by affinity proteomics are related to GSL. Glucosylceramidase (GBA) is responsible for the degradation of glucosylceramides into ceramides and 2-hydroxyacylsphingosine 1-beta-galactosyltransferase (UGT8) is involved in the transfer of a galactose moiety to the ceramide backbone (Figure 1). The abundance of galactosyl species in plasma is low, suggesting that the observed changes are due to increases in the glucosyl species regulated by GBA. Additionally, increased GSL biosynthesis has been described in CD4<sup>+</sup>T cells from patients with SLE, leading to their accumulation both in the plasma membrane and intracellular compartments.<sup>6</sup> Dysregulations of GSL have also been

linked to kidney diseases<sup>29</sup> and in particular to lupus nephritis.<sup>7</sup> The lack of change in circulating levels of lactosylceramides described by Nowling *et al.*<sup>7</sup> was reproduced in our larger cohort. However, in our cohort, even though C<sub>16:0</sub>- and C<sub>24:1</sub>-hexosylceramides were not associated with any of the clinical indices, they were the only sphingolipids that differed between patients with current renal and non-renal disease activity (Figure 4). The behaviour of these hexosylceramides paralleled circulating increases of cystatin C and decreases of albumin, two other markers used to assess renal dysfunction. The higher relative change between renal subgroups for C<sub>16:0</sub>-hexosylceramide relative to cystatin C or creatinine suggests in patients that this hexosylceramide may be an earlier prognostic marker of lupus nephritis.<sup>7</sup>

Using a longitudinal cohort, we observed that initiation of immunosuppressive treatment resulted in a normalization of dysregulated sphingolipids. Thus, the global sphingolipid profile returned to levels observed in the healthy controls following treatment and accompanied by a decrease in SLEDAI. However, the limited number of patients did not allow comparisons between the different treatment regimens used or the evaluation of response data.

Despite the fact that the number of controls was low, our cohort was matched in terms of TG, LDL and HDL between patients and controls. This matching is of importance as the SLE population is characterized by dyslipoproteinemia, including higher TG and lower HDL levels,<sup>30,31</sup> which may have an impact on sphingolipid levels. Thus the actual differences relative to the healthy population are expected to be even higher, as ceramides and hexosylceramides are associated with TG<sup>32,33</sup> while S1P is transported by the ApoM fraction of HDL.<sup>3</sup> The experimental design ensured that there were no significant differences between levels of cholesterol and lipoproteins between SLE patients and controls, which enabled us to account for possible increases in TGs and decreases in HDL that are associated with disease activity and to some extent prednisolone usage.<sup>31</sup>

One caveat of the study is the source of S1P dysregulations. Following blood collection, samples were left in the EDTA tubes for one hour before centrifugation. It is possible that S1P could leak from erythrocytes over time, which could lead to artefactual increases in the levels of S1P and dh-S1P. However, this would result in a global increase in S1P levels, because all samples were collected using the same protocol (see Materials and Methods section). Accordingly, there could be a bias in the absolute reported concentrations of

S1P, but the relative differences between groups should still be accurate. The findings should also be tempered within the context of the S1P carrier proteins. A recent study suggested that other sources, such as the plasma protein fraction, could be the source of plasma S1P dysregulation in disease processes.<sup>34</sup> S1P is bound to either HDL-associated apolipoprotein M or albumin in plasma,<sup>35</sup> with the different carriers exerting different biological effects.<sup>3</sup> In our study, both S1P (Supplemental Figure 4) and albumin (Figure 3) were decreased between controls and SLE patients (Figure 1, Table 1) as well as in patients with ongoing disease activity (Supplemental Figure 4, Figure 3). However, no correlation between plasma S1P and albumin levels was found (Supplemental Figure 5), indicating that in our data S1P dysregulations are independent of albumin levels. This may have implications for SLE given that Hammad *et al.* reported that different plasma pools of S1P may have different contributions to S1P signalling. It would be of significant interest to further investigate the source of S1P dysregulations in order to clarify the present findings. **[AQ3]**

Taken together, our findings demonstrate that a pathway-based approach unravels strong dysregulations in circulating sphingolipids among patients with SLE. Our results suggest that the sphingolipid pathway plays an important role and should be further explored in the search for biomarkers of disease activity in SLE. It is, in particular, of interest to further examine if increased circulating C<sub>16:0</sub>-hexosylceramide can be used as a marker of active renal disease in SLE patients and to evaluate potential associations between sphingolipid levels and response to treatment.

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## Supplemental Material

Supplementary material for this paper can be found at <http://journals.sagepub.com/doi/suppl/10.1177/0961203316686707>.

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