Interictal, circulating sphingolipids in women with episodic migraine

A case-control study

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ABSTRACT

Objective: To evaluate interictal, circulating sphingolipids in women migraineurs.

Methods: In the fasting state, serum samples were obtained pain-free from 88 women with episodic migraine (EM; $n = 52$) and from controls ($n = 36$). Sphingolipids were detected and quantified by high-performance liquid chromatography coupled with tandem mass spectrometry using multiple reaction monitoring. Multivariate logistic regression was used to examine the association between serum sphingolipids and EM odds. A recursive partitioning decision tree based on the serum concentrations of 10 sphingolipids was used to determine the presence or absence of EM in a subset of participants.

Results: Total ceramide (EM 6,502.9 ng/mL vs controls 10,518.5 ng/mL; $p < 0.0001$) and dihydroceramide (EM 39.3 ng/mL vs controls 63.1 ng/mL; $p < 0.0001$) levels were decreased in those with EM as compared with controls. Using multivariate logistic regression, each SD increase in total ceramide (odds ratio [OR] 0.07; 95% confidence interval [CI]: 0.02, 0.22; $p < 0.001$) and total dihydroceramide (OR 0.05; 95% CI: 0.01, 0.21; $p < 0.001$) levels was associated with more than 92% reduced odds of migraine. Although crude sphingomyelin levels were not different in EM compared with controls, after adjustments, every SD increase in the sphingomyelin species C18:0 (OR 4.28; 95% CI: 1.87, 9.81; $p = 0.001$) and C18:1 (OR 2.93; 95% CI: 1.55, 5.54; $p = 0.001$) was associated with an increased odds of migraine. Recursive partitioning models correctly classified 14 of 14 randomly selected participants as EM or control.

Conclusion: These results suggest that sphingolipid metabolism is altered in women with EM and that serum sphingolipid panels may have potential to differentiate EM presence or absence.

Classification of evidence: This study provides Class III evidence that serum sphingolipid panels accurately distinguish women with migraine from women without migraine. *Neurology*® 2015;85:1214-1223

GLOSSARY

BMI = body mass index; **C1P** = ceramide-1-phosphate; **CI** = confidence interval; **DHC** = dihydroceramide; **DHSM** = dihydro-sphingomyelin; **DSM-IV** = *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition); **EM** = episodic migraine; **HIT-6** = Headache Impact Test-6; **LacCer** = lactosylceramide; **MHC** = monohexosylceramide; **OPLS-DA** = orthogonal partial least squares discriminant analysis; **OR** = odds ratio; **PHQ-9** = Patient Health Questionnaire-9; **S1P** = sphingosine-1-phosphate; **SM** = sphingomyelin.

Although the full pathophysiology of migraine is not known, current theories suggest that migraine is largely an inherited brain disorder associated with a sterile, neurogenic inflammation and alterations in neuronal excitability and the cerebrovasculature.^{1,2} Several lines of evidence also indicate that migraineurs have a greater risk of stroke and disorders related to lipid metabolism including hypercholesterolemia, impaired insulin sensitivity, and obesity.³⁻⁶

Sphingolipids (e.g., sphingomyelins, ceramides) are a group of bioactive lipids that are critical components of membrane microdomains.^{7,8} In addition to important structural roles, these

Supplemental data
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Table 1 Demographic, headache, and laboratory characteristics

	Controls (n = 36)	EM (n = 52)	p Value
Age, y	30.9 (8.5)	33.4 (8.0)	0.156
Race, n (%)			
White	31 (86.1)	41 (78.9)	0.663
Asian	3 (8.3)	6 (11.5)	
Black	2 (5.6)	5 (9.6)	
Marital status, n (%)			
Single	21 (58.3)	31 (59.6)	0.048
Married	15 (4.7)	14 (26.9)	
Divorced/separated/widowed	0	7 (13.5)	
Education, n (%)			
HS	14 (38.9)	17 (32.7)	0.737
College	13 (36.1)	23 (44.2)	
Postgrad	9 (25.0)	12 (23.1)	
Income, n (%)			
<\$20,000	11 (31.4)	7 (13.5)	0.131
\$20,000–\$49,000	10 (28.6)	17 (32.7)	
\$50,000–\$100,000	6 (17.1)	17 (32.7)	
>\$100,000	8 (22.9)	11 (21.1)	
m-BMI, kg/m ²	27.7 (8.1)	26.0 (6.2)	0.249
PHQ-9 ≥15, n (%)	1 (2.8)	3 (5.9)	0.644
Smoking history, n (%)			
None	32 (88.9)	46 (88.5)	1.000
<1/2 PPD	3 (8.3)	4 (7.7)	
≥1/2 PPD	1 (2.8)	2 (3.9)	
HA characteristics			
28-d HA frequency	0 (0, 0)	5.6 (3.1)	<0.001
HA disability (HIT-6)	36.6 (1.8)	62.9 (7.8)	<0.001
Aura, n (%)	0	25 (48.1)	<0.001
Standard laboratories			
Total chol, mg/dL	177.4 (36.0)	177.4 (39.8)	0.768
HDL, mg/dL	57.9 (15.5)	54.5 (12.9)	0.200
LDL, mg/dL	102.5 (31.2)	105.0 (28.8)	0.452
TG, mg/dL	88.2 (46.1)	94.0 (49.2)	0.508
Glucose, mg/dL	85.7 (9.0)	85.8 (8.1)	0.811
Insulin, μU/mL	13.3 (6.0)	12.7 (8.2)	0.898

Abbreviations: chol = cholesterol; EM = episodic migraine; HA = headache; HDL = high-density lipoprotein; HIT-6 = Headache Impact Test-6; HS = high school; LDL = low-density lipoprotein; m-BMI = measured body mass index; PHQ-9 = Patient Health Questionnaire-9; Postgrad = postgraduate; PPD = packs per day; TG = triglycerides. Data are presented as mean (SD) except where indicated as n (%).

lipids function as second messengers in a multitude of cellular processes regulating energy homeostasis, apoptosis, and inflammation.^{7–9}

Neurologic disorders that are the result of severe deficiencies in enzymes that regulate sphingolipid metabolism have long been

described (e.g., Gaucher disease).¹⁰ Recent human research suggests that even subtle changes of sphingolipid balance may be involved in neurologic disorders (e.g., dementia, multiple sclerosis) and obesity.^{11–14} Furthermore, emerging basic science evidence suggests that sphingolipids may participate in neuronal functions and signaling pathways associated with pain.^{9,15,16}

Recently, we provided evidence that bioactive products of adipose tissue (e.g., adiponectin) are also associated with migraine, suggesting that alterations in lipid metabolism may be linked to a more generalized inflammatory response.^{6,17} Based on the data that the metabolism of the bioactive sphingolipid, ceramide, is regulated by adipokines,^{18,19} inflammation,^{20,21} obesity,^{22,23} and insulin resistance,^{24,25} we hypothesized that circulating ceramide levels would be decreased and downstream sphingolipid levels (e.g., sphingosine-1-phosphate [S1P]) increased in women with episodic migraine (EM).

METHODS **Participants.** A total of 88 women were recruited between December 2009 and March 2014 from Drexel University College of Medicine, the University of Toledo, and The Johns Hopkins University School of Medicine. Women were eligible for the study if they were aged 18 years or older, had a diagnosis fulfilling the *International Classification of Headache Disorders*, 2nd edition, for migraine as determined by a headache specialist or were nonpain, nonheadache controls.²⁶ In addition, migraine subgroups were characterized based on aura status. Participants with migraine with aura alone or in conjunction with migraine without aura were classified as having aura. Participants with diabetes, chronic immune or inflammatory disorders, thyroid, renal, or cerebrovascular disease, and chronic pain other than migraine were excluded.

All participants underwent vital sign evaluations including body mass index (BMI) and a neurologic examination and completed a standardized questionnaire to ascertain demographics (e.g., age, sex), medical and headache characteristics including disability evaluated using the Headache Impact Test-6 (HIT-6), and comorbidities such as depression evaluated using the Patient Health Questionnaire-9 (PHQ-9) as described below. Fasting serum blood samples were collected in all participants. Participants with EM were required to have no changes in daily medications within 4 weeks and to be pain-free for 24 hours at the time of the blood draw.

Standard protocol approvals, registrations, and patient consents. The study protocol was approved by institutional review boards at each institution. Written informed consent was obtained from all participants.

HIT-6. The HIT-6 is a validated questionnaire that consists of 6 items covering various areas that reflect quality of life.²⁷ Higher scores (range of 36–78) indicate an increasing impact of headaches on daily functioning.

Table 2 Crude, circulating sphingolipid levels (ng/mL) in women with EM and nonpain controls

	Controls (n = 36)	EM (n = 52)	p Value ^a
Ceramide			
C16:0	63.1 (21.6)	52.8 (17.7)	0.017
C18:0	44.7 (19.7)	46.2 (20.1)	0.743
C20:0	113.5 (65.6)	117.1 (38.6)	0.747
C22:0	1,510.0 (768.3)	1,301.6 (488.4)	0.124
C24:0	8,354.4 (2,257.2)	4,090.1 (2,471.9)	<0.0001 ^a
C26:0	128.5 (57.3)	109.8 (38.2)	0.070
C22:1	21.5 (20.0)	17.3 (9.9)	0.201
C24:1	282.9 (127.0)	317.9 (126.1)	0.204
Total	10,518.5 (2,932.5)	6,052.9 (2,633.4)	<0.0001 ^a
Dihydroceramide			
C22:0	20.5 (10.8)	18.5 (6.5)	0.283
C24:0	42.6 (14.5)	20.8 (10.5)	<0.0001 ^a
Total	63.1 (22.5)	39.3 (13.2)	<0.0001 ^a
Monohexosylceramide			
C16:0	729.2 (202.5)	749.9 (211.3)	0.648
C20:0	31.2 (12.0)	34.1 (8.9)	0.192
C22:0	689.0 (419.8)	768.8 (276.2)	0.285
C24:0	1,448.1 (599.6)	1,272.0 (430.1)	0.112
C26:0	17.0 (4.9)	9.6 (5.1)	<0.0001 ^a
C16:1	77.9 (21.1)	76.9 (24.5)	0.832
C18:1	5.8 (2.1)	6.0 (1.6)	0.656
C22:1	42.5 (27.1)	40.5 (17.2)	0.670
C24:1	77.0 (55.2)	58.8 (28.9)	0.047
Total	3,011.0 (818.5)	3,242.4 (548.5)	0.189
Lactosylceramide			
C16:0	1,928.2 (415.4)	1,795.2 (428.3)	0.151
C22:0	96.1 (51.8)	90.7 (33.2)	0.557
C24:0	69.3 (37.1)	61.7 (22.8)	0.241
C22:1	8.6 (3.4)	8.7 (3.5)	0.827
C24:1	218.7 (107.7)	245.1 (92.0)	0.220
Total	2,320.7 (460.1)	2,201.4 (501.4)	0.260
Sphingomyelin			
C16:0	379,312.8 (64,402.5)	384,908.3 (66,282.7)	0.695
C18:0	109,560.0 (33,236.7)	128,732.0 (34,197.5)	0.011
C20:0	105,385.9 (27,866.4)	94,571.9 (28,820.5)	0.083
C22:0	112,659.1 (25,740.7)	109,593.2 (34,521.7)	0.652
C24:0	51,989.1 (12,475.8)	50,816.2 (15,952.9)	0.713
C16:1	57,870.7 (12,535.3)	56,216.9 (10,543.9)	0.505
C18:1	126,841.2 (39,244.2)	148,866.1 (40,926.1)	0.013
C20:1	24,178.0 (6,713.5)	28,161.5 (8,306.7)	0.019
C22:1	59,428.4 (12,784.2)	60,877.3 (13,804.0)	0.619
C24:1	193,725.3 (42,188.1)	184,158.1 (42,035.8)	0.298
Total	1,220,951.0 (233,670.3)	1,246,901.0 (225,239.9)	0.731

Continued

PHQ-9. The PHQ-9 is a diagnostic measure for current depression.²⁷ Previous research has shown that a score of ≥ 15 on the PHQ-9 is associated with a 68% sensitivity and 95% specificity for diagnosing “major depressive disorder” using the *DSM-IV* criteria; thus, depression was classified as a PHQ-9 score ≥ 15 .²⁸

Anthropometrics. Height was measured to the nearest 0.5 in with a stadiometer. Weight was measured with a standard scale to the nearest 0.5 lb. BMI was then calculated.

Laboratory methods. Blood was collected in BD serum tubes with no additives (Becton Dickinson, Franklin Lakes, NJ), chilled on ice, centrifuged to remove cells, aliquoted, and stored at -80°C until assayed.

Routine labs. Glucose, insulin, total cholesterol, high-density lipoprotein, calculated low-density lipoprotein, and triglycerides were evaluated using standard techniques in hospital core labs at each study site.

Sample preparation and sphingolipidomic analysis. A modified Bligh and Dyer liquid-liquid extraction method was used for the extraction of total lipids from the serum as previously described.^{12,29} Sphingolipid analysis was conducted using high-pressure liquid chromatography coupled with electrospray ionization tandem mass spectrometry (API3000s; AB SCIEX Inc., Toronto, Canada) using methods similar to those previously described.^{12,29} Individual species of dihydroceramide (DHC), ceramide, monohexosylceramide (MHC), dihexosylceramide, and sphingomyelin (SM) (C16:0–C26:1) were separated by gradient elution using a C18 reverse-phase column (Phenomenex, Torrance, CA). The eluted sample was injected into the ion source, and the detection and quantitation of each sphingolipid species was performed by electrospray ionization tandem mass spectrometry by multiple reaction monitoring. Instrument control was performed using Analyst 1.4.2 (AB SCIEX Inc.). Concentrations were calculated by fitting sample data to 8-point calibration standard curves as previously described.^{12,29}

Outcomes. Primary outcomes included (1) the odds of migraine per SD increase in each sphingolipid serum concentration and (2) the potential of sphingolipid concentrations to classify those with migraine vs controls.

Statistical analyses. Continuous variables were summarized as mean (SD) and categorical variables as count (percent). Differences between migraineurs and controls were evaluated using *t* tests and χ^2 tests. The relationships between the plasma sphingolipids and EM were examined using multiple methods, including logistic regression, orthogonal partial least square discriminant analysis, and a nonparametric machine learning approach of random forest to ascertain consistency of results in the first analysis of this relationship. We first utilized logistic regression to examine the relationship between the sphingolipids, per SD increase, and odds of having EM. We adjusted for potential confounders of migraine and ceramide metabolism including age, BMI, and total cholesterol. In addition, we adjusted for marital status given the significant difference across migraineurs and controls. As we examined 40 sphingolipid species, we used Bonferroni correction and only considered the group differences with $p \leq 0.001$ as being significant. These analyses were conducted using STATA version 12.1 (StataCorp, College Station, TX).

Next, differential expression of lipid species was analyzed by hierarchical clustering of the $\log_2$ values, and displayed in a heatmap. Clustering was performed using the “pheatmap” package in

Table 2 Continued

	Controls (n = 36)	EM (n = 52)	p Value ^a
Dihydrosphingomyelin			
C16:0	12,695.4 (2,778.9)	12,212.4 (4,036.3)	0.572
C18:0	452,663.2 (121,214.3)	428,354.4 (122,983.7)	0.362
C20:0	860,112.3 (305,068.9)	843,874.4 (298,346.5)	0.804
C22:0	54,785.1 (14,220.4)	47,339.4 (17,493.5)	0.037
C24:0	5,835.1 (1,820.5)	5,657.5 (1,822.1)	0.657
Total	1,386,091 (428,233.7)	1,354,180 (419,690)	0.682
S1P	400.0 (119.8)	404.1 (117.7)	0.834

Abbreviations: EM = episodic migraine; S1P = sphingosine-1-phosphate. Data are presented as mean (SD).

^aOnly p values ≤ 0.001 are significant after Bonferroni corrections.

R software (pheatmap version 0.7.7, R version 3.1.1). An orthogonal partial least squares discriminant analysis (OPLS-DA) was generated to cluster patients in a disease-dependent manner (SIMCA-P+ 13.0; Umetrics, Umeå, Sweden). This multivariate statistical technique uses a classed-based principal component analysis-like algorithm. This allows the model to generate separation based on disease state as opposed to other factors such as differences in age, sex, or medication.³⁰ A model is considered significant if the q^2 is greater than 0.4.

Lastly, we used the nonparametric machine learning approach of random forest to classify EM by defining serum concentrations of particular sphingolipid species that best described separation between the control and EM groups. Ten thousand individual decision trees were generated and then combined into one overall model using the TreeBagger function (MATLAB 2014b; MathWorks, Natick, MA). The overall model was validated by fitting a random selection of 6 control and 8 EM samples that were not used in the initial model building, back into the model.

RESULTS Participants. A total of 88 women (52 with EM, 36 controls) were included in the analyses. The mean age of those with migraine was 33.4 (8.0) years with a range of 20 to 50 years and controls had a mean age of 30.9 (8.5) years with a range of 18 to 46 years ($p = 0.156$). Women with migraine were more likely to be divorced, separated, or widowed as compared with controls (table 1). However, there were no significant group differences in other demographic factors or health characteristics, including BMI, serum glucose, insulin, total cholesterol, triglycerides, high-density lipoprotein, and calculated low-density lipoprotein. Of the 52 participants with migraine, the mean (SD) monthly headache frequency was 5.6 (3.1) headache days per month, with a mean (SD) disability (HIT-6) score of 62.9 (7.8). Aura was reported by 25 (48%) of the participants with migraine (table 1).

Reduced very long chain ceramides and increased long chain sphingomyelins are associated with migraine. We analyzed 40 individual sphingolipid species related to the bidirectional metabolism of SM to ceramide,

and S1P to ceramide (figure e-1 on the *Neurology*[®] Web site at Neurology.org). Table 2 displays these sphingolipids separated by class into ceramides, DHCs, MHCs, lactosylceramides (LacCers), SMs, dihydrosphingomyelins (DHSMs), and S1P.

EM was associated with lower serum levels of total ceramide and total DHC with specific reductions in very long chain ceramides C24:0, DHC C24:0, and MHC C26:0 (table 2). Using multivariable logistic regression, every 1-SD increase in total ceramide (odds ratio [OR] 0.07; 95% confidence interval [CI]: 0.02, 0.22; $p < 0.001$) and total DHC (OR 0.05; 95% CI: 0.01, 0.21; $p < 0.001$) was associated with more than 92% decreased odds of EM (table 3). These associations were largely driven by reductions in the very long chain ceramides C24:0 (OR 0.05; 95% CI: 0.01, 0.21; $p < 0.001$), DHC C24:0 (OR 0.03; 95% CI: 0.005, 0.14; $p < 0.001$), and MHC C26:0 (OR 0.11; 95% CI: 0.04, 0.32; $p < 0.001$). There was no association between any of the LacCer species with migraine (tables 2 and 3).

Although crude total SM was not associated with migraine, mean levels of the long chain SM species C18:0 and C18:1 were higher in those with migraine as compared with controls (table 2). Using multivariate logistic regression, SM C18:0 (OR 4.28; 95% CI: 1.87, 9.81; $p = 0.001$) and C18:1 (OR 2.93; 95% CI: 1.55, 5.54; $p = 0.001$) were associated with more than 2.5-fold increased odds of migraine for each 1-SD increase in each SM species. DHSM and S1P levels were not associated with EM (tables 2 and 3). None of the sphingolipid concentrations differed with headache frequency or across migraine subgroups of those with vs without aura.

Identification of the sphingolipid cluster that best separates EM and controls. Following use of the logistic regression models to determine which individual sphingolipids were most associated with EM, we next evaluated which cluster of sphingolipids best separated those individuals with EM from controls using an OPLS-DA. OPLS-DA modeling showed a clear separation ($q^2 = 0.59$) between patients with EM and controls (figure 1A). We then used a hierarchical clustering analysis to identify which group of sphingolipids drove these group separations. Results of these analyses were consistent with primary findings from the logistic regression analyses. Specifically, EM was associated with decreased concentrations in 13 sphingolipids that included 3 very long chain MHCs (C24:0, C24:1, and C26:0), 3 very long chain ceramides (C22:1, C24:0, C26:0), 3 long chain ceramides (C16:0, C:20:0, C20:1), 2 DHCs (C22:0, 24:0), 1 DHSM (C22:0), and 1 SM (C20:0). These were accompanied by increases in 7 sphingolipids that

Table 3 Univariate and multivariate logistic regression analyses evaluating the odds ratio of migraine compared with controls with each SD increase in total and individual species of sphingolipid

Sphingolipid	Model 1	p Value ^a	Model 2	p Value ^a	Model 3	p Value ^a	Model 4	p Value ^a
Ceramide								
C16:0	0.58 (0.37, 0.92)	0.022	0.59 (0.37, 0.94)	0.025	0.62 (0.38, 1.02)	0.060	0.63 (0.38, 1.03)	0.065
C18:0	1.08 (0.70, 1.66)	0.740	1.07 (0.69, 1.65)	0.770	1.53 (0.85, 2.73)	0.154	1.52 (0.85, 2.73)	0.157
C20:0	1.07 (0.70, 1.66)	0.744	1.04 (0.67, 1.61)	0.876	1.22 (0.75, 1.97)	0.429	1.28 (0.76, 2.13)	0.351
C22:0	0.71 (0.46, 1.10)	0.128	0.70 (0.45, 1.10)	0.126	0.77 (0.47, 1.26)	0.294	0.75 (0.44, 1.28)	0.297
C24:0	0.09 (0.03, 0.25)	<0.001 ^a	0.09 (0.03, 0.25)	<0.001 ^a	0.09 (0.03, 0.26)	<0.001 ^a	0.05 (0.01, 0.21)	<0.001 ^a
C26:0	0.67 (0.43, 1.04)	0.076	0.62 (0.39, 0.99)	0.045	0.66 (0.40, 1.09)	0.106	0.61 (0.34, 1.09)	0.095
C22:1	0.75 (0.48, 1.18)	0.213	0.68 (0.43, 1.10)	0.113	0.75 (0.45, 1.24)	0.259	0.76 (0.45, 1.28)	0.302
C24:1	1.34 (0.85, 2.12)	0.205	1.38 (0.87, 2.19)	0.176	1.35 (0.85, 2.14)	0.203	1.45 (0.89, 2.37)	0.134
Total	0.11 (0.04, 0.28)	<0.001 ^a	0.11 (0.04, 0.28)	<0.001 ^a	0.10 (0.04, 0.28)	<0.001 ^a	0.07 (0.02, 0.22)	<0.001 ^a
Dihydroceramide								
C22:0	0.79 (0.51, 1.22)	0.283	0.77 (0.50, 1.20)	0.251	0.86 (0.53, 1.40)	0.535	0.86 (0.51, 1.45)	0.579
C24:0	0.07 (0.03, 0.22)	<0.001 ^a	0.07 (0.02, 0.22)	<0.001 ^a	0.07 (0.02, 0.22)	<0.001 ^a	0.03 (0.005, 0.14)	<0.001 ^a
Total	0.12 (0.05, 0.31)	<0.001 ^a	0.11 (0.04, 0.30)	<0.001 ^a	0.10 (0.03, 0.29)	<0.001 ^a	0.05 (0.01, 0.21)	<0.001 ^a
Monohexosylceramide								
C16:0	1.11 (0.72, 1.70)	0.644	1.10 (0.71, 1.70)	0.660	1.11 (0.72, 1.73)	0.634	1.09 (0.70, 1.71)	0.691
C20:0	1.35 (0.86, 2.10)	0.192	1.29 (0.82, 2.04)	0.276	1.30 (0.81, 2.07)	0.271	1.29 (0.80, 2.07)	0.289
C22:0	1.27 (0.82, 1.97)	0.282	1.24 (0.79, 1.94)	0.344	1.23 (0.79, 1.93)	0.360	1.25 (0.79, 1.99)	0.340
C24:0	0.70 (0.44, 1.09)	0.117	0.69 (0.44, 1.10)	0.119	0.71 (0.45, 1.13)	0.151	0.69 (0.42, 1.13)	0.142
C26:0	0.18 (0.08, 0.39)	<0.001 ^a	0.18 (0.08, 0.39)	<0.001 ^a	0.18 (0.08, 0.39)	<0.001 ^a	0.11 (0.04, 0.32)	<0.001 ^a
C16:1	0.95 (0.62, 1.46)	0.829	0.89 (0.57, 1.39)	0.601	0.88 (0.56, 1.38)	0.576	0.88 (0.55, 1.42)	0.596
C18:1	1.12 (0.68, 1.86)	0.651	1.11 (0.66, 1.87)	0.691	1.10 (0.65, 1.86)	0.729	1.08 (0.60, 1.93)	0.792
C22:1	0.91 (0.59, 1.39)	0.666	0.90 (0.58, 1.39)	0.629	0.94 (0.60, 1.45)	0.770	0.94 (0.59, 1.48)	0.777
C24:1	0.62 (0.38, 1.02)	0.061	0.60 (0.37, 0.99)	0.045	0.64 (0.39, 1.08)	0.094	0.62 (0.36, 1.09)	0.097
Total	1.45 (0.83, 2.51)	0.190	1.52 (0.86, 2.66)	0.146	1.55 (0.88, 2.73)	0.126	1.51 (0.85, 2.69)	0.165
Lactosylceramide								
C16:0	0.73 (0.47, 1.12)	0.152	0.71 (0.45, 1.10)	0.126	0.70 (0.45, 1.10)	0.122	0.68 (0.43, 1.08)	0.105
C22:0	0.88 (0.57, 1.35)	0.553	0.81 (0.52, 1.26)	0.354	0.82 (0.52, 1.29)	0.390	0.79 (0.49, 1.27)	0.328
C24:0	0.77 (0.50, 1.19)	0.241	0.75 (0.48, 1.17)	0.201	0.78 (0.50, 1.22)	0.273	0.77 (0.49, 1.22)	0.266
C22:1	1.05 (0.68, 1.61)	0.824	1.03 (0.66, 1.60)	0.900	1.00 (0.64, 1.57)	0.996	0.99 (0.62, 1.60)	0.977
C24:1	1.32 (0.85, 2.08)	0.220	1.30 (0.82, 2.05)	0.259	1.26 (0.79, 2.00)	0.326	1.26 (0.79, 2.02)	0.331
Total	0.78 (0.51, 1.20)	0.258	0.75 (0.48, 1.17)	0.201	0.74 (0.47, 1.16)	0.191	0.72 (0.45, 1.14)	0.161
Sphingomyelin								
C16:0	1.09 (0.71, 1.68)	0.691	1.08 (0.69, 1.70)	0.738	1.08 (0.69, 1.71)	0.728	1.17 (0.65, 2.11)	0.590
C18:0	1.89 (1.13, 3.15)	0.015	1.84 (1.09, 3.11)	0.022	2.40 (1.32, 4.35)	0.004	4.28 (1.87, 9.81)	0.001 ^a
C20:0	0.68 (0.44, 1.06)	0.087	0.66 (0.42, 1.04)	0.075	0.69 (0.44, 1.09)	0.112	0.47 (0.24, 0.92)	0.027
C22:0	0.91 (0.59, 1.39)	0.648	0.87 (0.56, 1.35)	0.540	0.89 (0.57, 1.39)	0.610	0.80 (0.45, 1.42)	0.445
C24:0	0.92 (0.60, 1.41)	0.709	0.88 (0.57, 1.37)	0.587	0.87 (0.56, 1.35)	0.533	0.77 (0.44, 1.35)	0.357
C16:1	0.86 (0.56, 1.32)	0.501	0.80 (0.51, 1.27)	0.348	0.89 (0.54, 1.46)	0.648	0.85 (0.47, 1.55)	0.601
C18:1	1.82 (1.11, 2.98)	0.018	1.81 (1.09, 3.02)	0.022	2.69 (1.45, 4.97)	0.002	2.93 (1.55, 5.54)	0.001 ^a
C20:1	1.79 (1.08, 2.97)	0.025	1.78 (1.06, 3.00)	0.030	2.09 (1.20, 3.64)	0.009	2.83 (1.45, 5.54)	0.002
C22:1	1.12 (0.73, 1.72)	0.615	1.04 (0.65, 1.65)	0.875	1.11 (0.69, 1.79)	0.666	1.17 (0.63, 2.17)	0.623
C24:1	0.79 (0.51, 1.23)	0.297	0.76 (0.48, 1.21)	0.241	0.73 (0.46, 1.16)	0.183	0.65 (0.37, 1.14)	0.131
Total	1.12 (0.73, 1.73)	0.598	1.09 (0.69, 1.71)	0.722	1.16 (0.73, 1.86)	0.527	1.39 (0.70, 2.74)	0.348

Continued

Table 3 Continued

Sphingolipid	Model 1	p Value ^a	Model 2	p Value ^a	Model 3	p Value ^a	Model 4	p Value ^a
Dihydrosphingomyelin								
C16:0	0.88 (0.58, 1.35)	0.569	0.84 (0.54, 1.32)	0.452	0.80 (0.51, 1.26)	0.337	0.72 (0.41, 1.26)	0.253
C18:0	0.82 (0.53, 1.26)	0.359	0.78 (0.50, 1.22)	0.283	0.74 (0.47, 1.17)	0.200	0.65 (0.36, 1.16)	0.144
C20:0	0.95 (0.62, 1.45)	0.801	0.93 (0.60, 1.44)	0.749	0.88 (0.56, 1.38)	0.580	0.85 (0.48, 1.54)	0.599
C22:0	0.62 (0.39, 0.99)	0.046	0.59 (0.36, 0.97)	0.038	0.57 (0.35, 0.95)	0.029	0.45 (0.24, 0.84)	0.012
C24:0	0.91 (0.59, 1.39)	0.652	0.84 (0.54, 1.32)	0.453	0.84 (0.53, 1.33)	0.454	0.76 (0.44, 1.32)	0.331
Total	0.93 (0.60, 1.42)	0.727	0.90 (0.58, 1.40)	0.639	0.85 (0.54, 1.34)	0.484	0.76 (0.41, 1.41)	0.388
Sphingosine-1-phosphate	1.04 (0.65, 1.65)	0.881	1.03 (0.65, 1.64)	0.893	1.04 (0.65, 1.68)	0.860	1.16 (0.69, 1.92)	0.579

Data are presented as odds ratio (95% confidence interval). Model 1 = univariate analyses; model 2 = multivariate analyses adjusting for age and marital status; model 3 = multivariate analyses adjusting for model 2 variables + body mass index; model 4 = multivariate analyses adjusting for model 3 variables + total cholesterol.

^a Only p values ≤ 0.001 are significant after Bonferroni corrections.

included 3 SMs (C18:0, C18:1, and C20:1), 2 MHCs (C20:0 and C22:0), 1 very long chain LacCer (C24:1), and 1 very long chain ceramide (C24:1) (figure 1B).

A combination of particular blood sphingolipids identifies EM. Using the recursive partitioning approach of random forest, we defined a series of 10 sphingolipid species that classified the EM group. This analysis created a series of decision rules that identified an interdependent group of risk factors for EM. These included Cer C18:0, Cer C18:1, MHC C18:1, MHC C26:0, LacCer C18:1, LacCer C24:1, DHSM C18:0, DHSM C20:0, and SM C18:0, SM C18:1, SM C20:1, and SM C22:0 (figure 2). Validity of the final model was confirmed by secondary analysis by randomly selecting 6 control participants and 8 participants with EM who were not included in the group used to train the model. The model correctly identified 100% of the selected participants as control or EM.

DISCUSSION We conducted an interictal, lipidomic analysis of circulating sphingolipids and sphingolipid metabolites in a cohort of women with EM as compared with healthy, nonpain controls. Only one previous study has evaluated any circulating sphingolipids in patients with headache disorders.³¹ In this prior study, and in agreement with our present findings, no difference in interictal total sphingomyelin levels was found in 7 patients who had migraine without aura compared with 10 controls. However, in contrast to the current study, none of the individual sphingomyelin species or other sphingolipids included in our current study were evaluated.³¹ In addition, although data from experimental systems support a role for ceramides in nociceptive processing,^{9,15,16,32} no previous study has evaluated the relationship between circulating total or individual

species of ceramides and its metabolites (DHC, MHC, DHMHC, LacCer, DHDHC, and S1P) in human participants with any headache disorder, including migraine.

Our cross-sectional study has 3 primary findings. First, we found that serum levels of total ceramide and its precursor, DHC, were *decreased* in women episodic migraineurs as compared to controls. These changes were largely driven by reductions in the very long chain ceramides (ceramide C24:0, DHC C24:0, and MHC C26:0) and remained significant even after controlling for covariates (including BMI and cholesterol) and multiple comparisons. Second, we found *increased* levels of very long chain sphingomyelin species C18:0 and C18:1 in those with migraine. Notably, the primary changes in sphingolipids were consistent with both logistic regression and hierarchical clustering analyses. These findings suggest that migraineurs have decreased de novo synthesis of ceramides as well as an independent downstream increase in the conversion of ceramide metabolic products (e.g., sphingomyelin).

Finally, we demonstrated that a forest model decision tree, based on the concentrations of a panel of sphingolipids, was able to correctly differentiate those women with migraine from controls in a small subset of participants (n = 14). While this finding should be cautiously interpreted given the small sample size, it suggests that sphingolipid panels may have the potential to be utilized as a migraine diagnostic tool. However, additional research is needed to both validate and build on these initial findings before we will be able to determine this.

Ceramides are important lipid mediators that serve as signaling molecules capable of regulating multiple cellular functions including apoptosis, atherosclerosis, insulin resistance, and inflammation.^{33,34} Ceramides are generated either de novo (starting with the

Figure 1 Multivariate analyses and hierarchical clustering of sphingolipids separate women with EM from healthy controls

(A) Three-dimensional OPLS-DA plots demonstrating significant ($q^2 = 0.59$) separation between those with EM (red spheres) and controls (yellow spheres). (B) Heat map (represented by range of color intensity) and hierarchical clustering (depicted by dendrogram) showing fold changes in the indicated sphingolipid species in those with EM and controls. Cer = ceramide; Con = controls; DHCer = dihydroceramide; DHSM = dihydrosphingomyelin; EM = episodic migraine; LacCer = lactosylceramide; MonohexCer = monohexosylceramide; OPLS-DA = orthogonal partial least squares discriminant analysis; SM = sphingomyelin.

condensation of palmitoyl-CoA with serine) or through hydrolysis (breakdown) of sphingomyelin and other sphingolipids (e.g., monohexylceramides such as glucosylceramide and galactosylceramide)²⁰ (figure e-1).

While the current study was not able to determine mechanisms for the association of low total ceramides and DHC with migraine, there are several possibilities. First, it is possible that an upstream mediator stimulates ceramide catabolism to a greater extent in

those with migraine than in controls. Animal data in mice have demonstrated that adiponectin potently stimulates ceramidase activity, resulting in decreased ceramide levels.¹⁹ In addition, the administration of recombinant adiponectin to mice has been shown to universally decrease all hepatic ceramide and DHC species.¹⁹ Given previous human research that supports that adiponectin is increased in those with migraine,^{6,35,36} it is possible that adiponectin could be one such upstream mediator stimulating ceramide

Figure 2 Random forest model decision tree showing EM and control classifications for participants based on a panel of sphingolipids

Boxes in blue show the sphingolipid variable; the adjacent values are the serum cutoff concentrations used to define the groups. Boxes in gray show classification decisions. The inset shows the results of the model validation in which a randomly selected subset of participants (6 controls, 8 EM) were correctly classified by the model. Cer = ceramide; DHSM = dihydrosphingomyelin; EM = episodic migraine; LacCer = lactosylceramide; MonoHexCer = monohexosylceramide; SM = sphingomyelin.

catabolism in migraineurs. Future studies are warranted to test this hypothesis.

Second, it is possible that alterations in enzymatic activity of sphingolipid hydrolysis or phosphorylation contributed to our findings of low total ceramide and DHC in migraineurs. One such example would be if there was an increase in enzymatic activity of sphingomyelin synthase, it would be possible that sphingomyelin levels would be increased and ceramide levels decreased.²⁰ Consistent with this hypothesis, in the current study, along with the decreased total ceramide, at least 2 individual sphingomyelin species (C18:0, C18:1) were increased.

Another possibility that could be consistent with our finding of low total ceramide and DHC would be an increase in ceramide kinase activity in those

with migraine. In such a case, it could be hypothesized that increases in ceramide kinase activity would be associated with an increase in the proinflammatory ceramide metabolite ceramide-1-phosphate (C1P) (figure e-1).^{37–39} This hypothesis is particularly attractive given that C1P activation is associated with activation of several processes implicated in migraine pathophysiology, including arachidonic acid release, increases in prostaglandin E₂, and macrophage chemotaxis.²⁰ Although we were not able to evaluate C1P levels in the current study, the lack of differences in total DHSM, sphingomyelin, LacCer, or S1P (ceramide metabolites) in migraineurs as compared with controls in the current study would be compatible with such a hypothesis. Finally, it is possible that the alterations in sphingolipids in our current study may reflect chronic activation of a compensatory system in the face of repetitive and chronically increased neurogenic inflammation.

There are several limitations of our study that warrant consideration. First, although our EM and control groups were carefully characterized and described, our sample size was relatively small. Larger confirmatory studies to validate these initial findings are needed. In addition, because we did not include those with chronic migraine in this study, our findings are limited to individuals with EM. This study was also not designed to capture differences across the menstrual cycle; thus, we are unable to determine whether differences in sphingolipids in women migraineurs are present in the luteal vs the follicular phase. Finally, although we were able to evaluate several targeted sphingolipids and sphingolipid metabolites, we did not attempt to evaluate all ceramide precursors and metabolites or to evaluate any of the enzymes that participate in ceramide anabolism and catabolism. Future studies evaluating potential upstream sphingolipid mediators (e.g., adiponectin), sphingolipid enzymes, as well as targeted downstream metabolites (e.g., C1P), and their association with migraine are warranted.

Taken together, our findings suggest it is possible that migraine is a neurologic disorder of “minor” sphingolipid dysmetabolism. Further research, validating the ceramide and sphingomyelin associations with migraine, as well as research examining mechanisms for these associations, may advance our understanding of migraine pathophysiology and open possibilities of the identification of novel migraine biomarkers and targeted drug therapies directed against sphingolipid pathways.

AUTHOR CONTRIBUTIONS

B. Lee Peterlin: study conception and design, data interpretation, primary authorship of manuscript, approval of final manuscript. Michelle M.

Mielke: study design, data analysis, data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Alex M. Dickens: data analysis, data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Subroto Chatterjee: data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Paul Dash: data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Guillermo Alexander: data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Rebeca V.A. Vieira: acquisition of data, data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Veera Venkata Ratnam Bandaru: acquisition of data, data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Joelle M. Dorskind: acquisition of data, data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Gretchen E. Tietjen: data interpretation, revision of manuscript for intellectual content, approval of final manuscript. Norman H. Haughey: study conception and design, data analysis and interpretation, revision of manuscript for intellectual content, final manuscript approval.

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Comment: Altered sphingolipid metabolism— A marker for episodic migraine?

A biological marker for migraine would be of great value in clinical practice. This collaborative study evaluated interictal, circulating sphingolipids in 52 female patients with episodic migraine compared with 36 women who did not have headaches. Total ceramide and its precursor, dihydroceramide, were decreased in the migraineurs as compared with controls, and, furthermore, some very long sphingomyelin species were increased in those with migraine. The authors suggest that serum sphingolipid panels have the potential to differentiate patients with episodic migraine from controls.

The study¹ was properly performed, based on compelling and clear thoughts on the pathophysiologic basis of the disease. There are some limitations. Only female patients with migraine were included. Chronic migraine was not studied. Also, there was an unusually high frequency of patients who reported having symptoms of aura. Medical therapy is only briefly mentioned. The use of contraceptive pills in this female study population and the eventual confounding role of any antimigraine prophylactic medication might have been of some interest when discussing the import of the results.

It should be stressed that a diagnosis of migraine is still a clinical one, based on a careful history of the patient and utilizing internationally recognized clinical criteria. In many persons, a clinical diagnosis may be difficult. Specific markers of the disease are therefore of particular interest. However, before claiming that an altered sphingolipid metabolism might provide a diagnostic tool for migraine, a comparison should be made with other types of headache, e.g., cluster headache.

To summarize, this study is an important contribution to our understanding of the pathophysiology of migraine and may have vast practical clinical and therapeutic implications if it is supported by further studies.

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DISCLOSURE

B. Peterlin received current investigator-initiated grant support from Luitpold Pharmaceuticals and a past investigator-initiated grant from GSK within the past 5 years for studies unrelated to the current manuscript. Dr. Peterlin serves on the editorial boards for the journals *BMC Neurology*, *Headache*, and *Neurology*[®]. M. Mielke has received funding through NIH for studies unrelated to the current manuscript; serves as associate editor for *Alzheimer's and Dementia: Journal of the Alzheimer's Association* and *Journal of Alzheimer's Disease*. A. Dickens reports no disclosures relevant to the manuscript. S. Chatterjee has received NIH funding unrelated to the current manuscript. Dr. Chatterjee serves as an editor of the *World Cardiology Journal*. P. Dash, G. Alexander, R. Vieira, V. Bandaru, and J. Dorskind report no disclosures relevant to the manuscript. G. Tietjen received past investigator-initiated grants from GSK within the past 5 years for studies unrelated to the current manuscript. Dr. Tietjen serves as an associate editor for the journal *Headache*. N. Haughey reports no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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