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Targeted lipidomics reveal derangement of ceramides in major depression and bipolar disorder

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ABSTRACT

Changes of sphingolipid metabolism were suggested to contribute to the patho-etiology of major depression (MD) and bipolar disorder (BD). In a pilot study we assessed if lipid allostasis manifested in pathological plasma concentrations of bioactive lipids i.e. endocannabinoids, sphingolipids, ceramides, and lysophosphatidic acids.
Methods: Targeted and untargeted lipidomic analyses were performed according to GLP guidelines in 67 patients with unipolar or bipolar disorders (20–67 years, 36 male, 31 female) and 405 healthy controls (18–79 years, 142 m, 263 f), who were matched according to gender, age and body mass index. Multivariate analyses were used to identify major components, which accounted for the variance between groups and were able to predict group membership.
Results: Differences between MD and BP patients versus controls mainly originated from ceramides and their hexosyl-metabolites (C16Cer, C18Cer, C20Cer, C22Cer, C24Cer and C24:1Cer; C24:1GluCer, C24LacCer), which were strongly increased, particularly in male patients. Ceramide levels were neither associated with the current episode, nor with the therapeutic improvement of the Montgomery Åsberg Depression Rating Scale (MARDS). However, long-chain ceramides were linearly associated with age, stronger in patients than controls, and with high plasma levels of diacyl- and triacylglycerols. Patients receiving antidepressants had higher ceramide levels than patients not taking these drugs. There was no such association with lithium or antipsychotics except for olanzapine.
Conclusion: Our data suggest that high plasma ceramides in patients with major depression and bipolar disorder are indicative of a high metabolic burden, likely aggravated by certain medications.

1. Introduction

Major depression (MD) and bipolar disorder (BD) are the second leading cause of disability worldwide leading to high costs for healthcare systems [1], and up to 50% of all patients with major depression do not sufficiently respond to multiple antidepressant treatment [2]. Major depression is unipolar whereas a diagnosis of bipolar disorder is given if a patient has experienced at least one episode of mania with or without a prior depressive episode, or has had at least one depressive episode and a period of hypomania. MD and BD are often co-morbid with obesity and metabolic syndrome, i.e. a clustering of dyslipidemia, abdominal obesity, hypertension and diabetes [3], and it has been suggested that a "metabolic" MD may represent a depression subtype with unique pathophysiologic mechanisms, which negatively impact on the outcome of medical treatment and cognitive functions in these patients [4]. Above being sources of energy, distinct lipids regulate receptor-mediated signaling processes and cellular and subcellular membrane compositions that are crucial for the maintenance of transmembrane- and vesicle transport, compartmentalization and organelle functions. These signaling lipids include sphingolipids, ceramides, eicosanoids, endocannabinoids and lysophosphatidic acids.

In particular, convincing evidence suggests that sphingomyelin and ceramide metabolism are deregulated in MD and BD to an extent

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that such changes manifest in alterations of plasma concentrations of specific subtypes [5]. For example, long-chain (C16-C22Cer) and very long-chain ceramides (≥C24Cer) were elevated in patients with a recent episode of major depression in comparison to healthy controls [5]. In addition, peripheral mononuclear cells of patients with major depression had high activity of acidic sphingomyelinase (ASM), an enzyme which generates ceramides from sphingomyelin. Upon treatment of the cells with antidepressants, which act as functional ASM inhibitors [6], ASM activity dropped to normal, suggesting that sphingomyelin and ceramide metabolism may be relevant for mood stabilization. In support, similar ASM deregulations were found in the rodent brain in models of depression [7–10], and the authors suggested that depression-like behavior was induced by ceramide accumulation in the hippocampus resulting in a suppression of neurogenesis and slowing of neuroprogenitor proliferation [10,11]. It was further hypothesized that increased oxidative and inflammatory stresses in depressive patients lead to an overactivation of ASM and deregulation of the hypothalamic-pituitary axis of corticoid homeostasis [7]. In turn, elevated ceramides or defective ER to Golgi transfer of ceramides may impose oxidative stress owing to a depolarization and permeabilization of mitochondria, generation of reactive oxygen species, cytochrome *c* release and disruption of iron homeostasis [12,13].

Ceramides are formed as intermediates in the biosynthesis of complex sphingolipids such as sphingomyelins, cerebrosides and gangliosides [14,15]. They are generated through three major pathways (i) de novo synthesis by serine palmitoyl transferase and ceramide synthases [16], (ii) the salvage pathway through reacylation of sphingosine [17] and (iii) by hydrolysis of sphingomyelins via neutral, alkaline and acidic sphingomyelinases (SMases) [15], which localize to cell membranes (neutral and alkaline SM) or lysosomal membranes (acidic, ASM). The generation of ceramides is stimulated under conditions of cellular stresses or in response to inflammatory or pro-death stimuli [18].

It is of note that several antidepressants and antipsychotic agents are amphiphilic lysosomotropic drugs, which act as functional ASM inhibitors [19,20]. These compounds get trapped in the lysosome owing to their lipophilic and weakly basic properties and impair binding of ASM to the lysosomal membrane, leading to its detachment and subsequent inactivation [19,21]. Mild ASM inhibition with functional inhibitors has been shown to stimulate the autophagic flux, hence promoting waste removal [22], which is particularly important for neuronal longevity. However, stronger or persistent ASM inhibition result in accumulation of sphingomyelin accompanied by lysosomal damage and late stage block of autophagy [23], suggesting a bell-shaped response and necessity for tight control of ceramide turnover.

It is therefore not surprising that alterations of ceramides were found in the context of a variety of diseases including insulin resistance [24], obesity [25], cardiovascular disease [26], Alzheimer's disease [12] and breast cancer [27] all pointing to a common metabolic deregulation. Considering ceramides' multiple molecular functions including regulation of cell growth, viability, redox homeostasis [13,28], cell barrier integrity, differentiation, and senescence [29] and putative specificity of short versus long-chain ceramides [30] it is conceivable that ceramides point to disease-specific pathologies and disease specific patterns, and that they are mechanistically relevant for the course of the disease and response to medication.

To address this hypothesis we performed an observational pilot lipidomic study encompassing lipids of four classes (endocannabinoids, sphingolipids, ceramides and lysophosphatidic acids). Multivariate analyses revealed that ceramides accounted for most of the variance, so that we focused on this class to analyze the associations of ceramide patterns with disease-specific aspects, medication, metabolic status and demographic data.

Table 1A
Demographic data of patients with major depression or bipolar disorder and healthy control subjects.

Age & BMI		Controls		Patients (samples)	
		Male	Female	Male	Female
Age (class)	<30	45	134	3 (6)	9 (18)
	31–40	29	45	9 (12)	5 (9)
	41–50	22	30	12 (25)	8 (15)
	51–60	26	33	10 (19)	7 (15)
	61–70	11	18	2 (5)	2 (4)
	>70	9	3	0	0
	Total	142	263	36	31
BMI (class)	≤20	8	50	0	4 (8)
	20.1–25	59	134	18 (31)	12 (24)
	25.1–30	59	44	13 (26)	9 (18)
	>30	16	31	5 (10)	6 (11)
	Missing data		4		
	Total	142	263	36	31

2. Methods

2.1. Patients and healthy controls

Patients with major depression or bipolar disorder ($n = 67$, aged 20–67 years, 36 men, 31 women) and healthy controls ($n = 405$, aged 18–79 years, 142 men, 263 women) were consecutively recruited from inpatients of the Department of Psychiatry, Psychotherapy and Psychosomatic Medicine (patients) and from students and staff members of the University Hospital Frankfurt (controls), who routinely

Table 1B
Patient subgroups and medication.

		Patients	
		Male	Female
Patient subgroups			
Type	Bipolar	16	18
	Unipolar	20	15
Episode	Depressive	30	22
	Mixed	4	5
	Manic	13	2
Smoker	Unknown	2	0
	Yes	14	19
	No	17	11
Comorbidities & medication			
Hypertension	No	25	24
	Yes	7	7
Diabetes	No	28	30
	Yes	4	1
Thyroid dysfunct.	No	27	23
	Yes	5	8
High cholesterol	No	32	30
	Yes	0	1
Antidepressant	No	7	10
	Yes	25	21
Antipsychotic	No	16	13
	Yes	16	18
Antiepileptic	No	25	25
	Yes	7	6
Lithium	No	24	21
	Yes	8	10
Melatonin	No	31	29
	Yes	1	2
Esketamine	No	31	31
	Yes	1	0
Electroconvusive therapy	No	28	28
	Yes	4	3
Number of different drugs	0	1	0
	1	14	12
	2	9	13
	3	6	5
	4	2	1

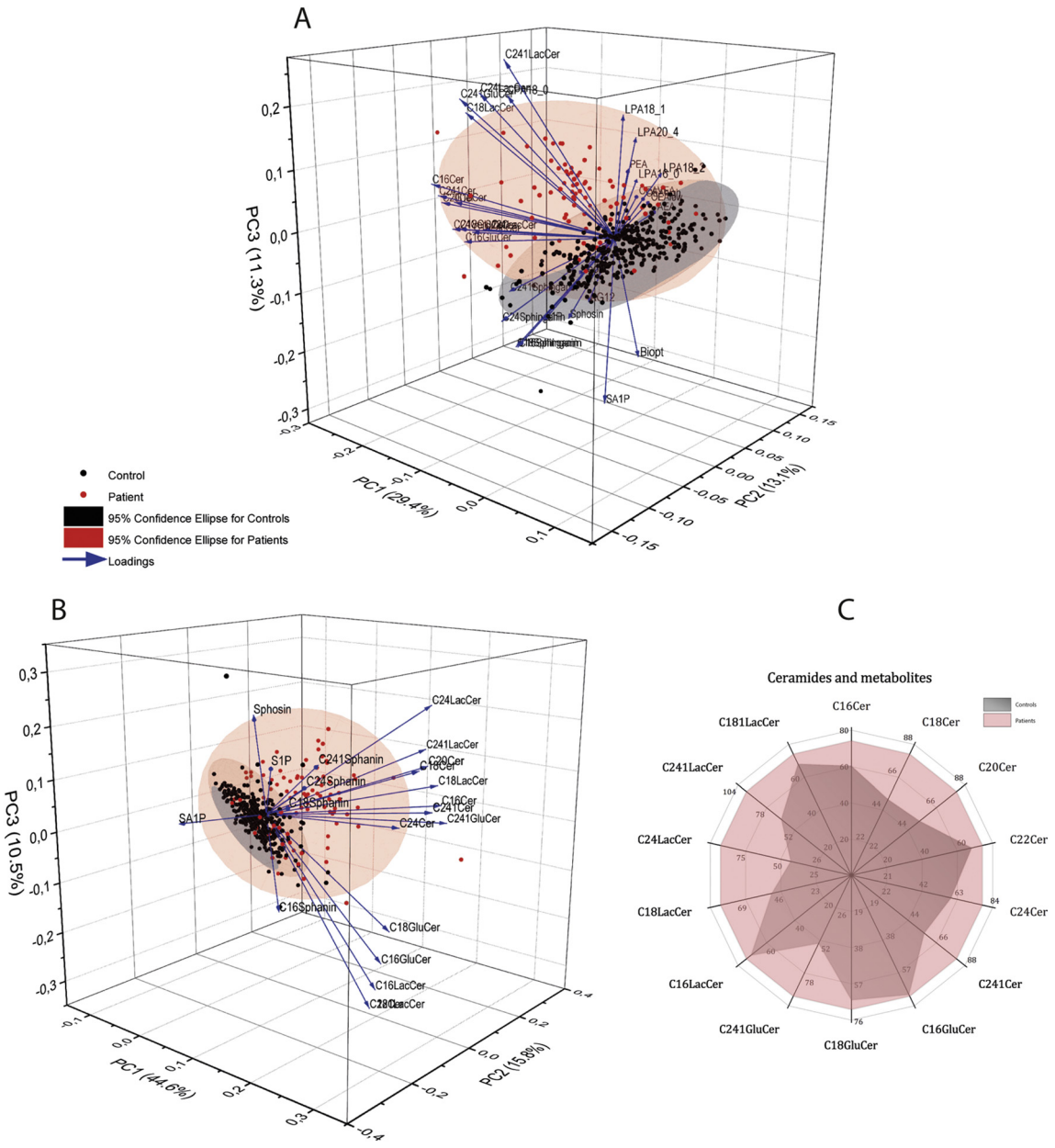


Fig. 1. Principal component analysis of lipids in patients with unipolar or bipolar disorders and controls. A: PCA score biplots of the first three PCA components extracted from 2 pterins and 32 lipid species including sphingolipids, ceramides, lysophosphatidic acids (LPA) and endocannabinoids. Normalized lipid concentrations i.e. percentages of the 90% quantile were used as PCA input. Eigenvalues, loadings and scores are presented in Suppl. Table 1A. Dots show the individual samples, the shaded area shows the 95% confidence intervals (CI), and the blue arrows show the loading. B: PCA biplots of the first three PCA factors extracted from ceramides, glucosylceramides, lactosylceramides and sphingolipids including sphingosine, sphinganins, sphingosine-1-phosphate (S1P) and sphinganin-1-phosphate (SA1P). Dots show the individual samples, the shaded area shows the 95% confidence intervals (CI), and the blue arrows show the loading. Eigenvalues, loadings and scores are presented in Suppl. Table 1B. C: Polar plots showing the means of normalized ceramides, glucosylceramides and lactosylceramides (percentages of the 90% quantile) in patients and controls.

reported at the institutional occupational health service. Samples from aged controls were available from a concurrent study addressing lipid homeostasis during aging. The estimate of the number of patients and controls to be included was based on a previous study, in which we have analyzed serum lipids in patients with multiple sclerosis [31]. Informed written consent was obtained from all subjects. It is a pilot study following an observational parallel group design. Inclusion criteria for patients were age ≥ 18 years and a clinically verified diagnosis of major depression or bipolar disorder based on ICD10 criteria and validated by specialists independently (SKS and AR). Most patients ($n = 57$) provided two consecutive samples, one at onset of inpatient care and one before discharge. Patients were treated after guidelines for their respective disorder and episode with antidepressants, antimanic and/or mood stabilizers as well as electroconvulsive therapy (ECT) in a small subgroup ($n = 7$). For controls, inclusion criteria were age > 18 years, no current medical condition queried by medical interview, and no drug intake for at least one week except contraceptives, vitamins and L-Thyroxin. Acetylsalicylic acid 100 mg and antihypertensive drugs were no exclusion criteria in controls >50 years. Each control subject provided one sample. Human blood samples were collected in K^+-EDTA tubes (Microvette Sarstedt), kept on ice, and centrifuged in a tabletop centrifuge (Eppendorf) at 3000 rpm and 4 °C for 10 min. Plasma aliquots were then stored immediately at −80 °C until analysis. Data and blood collection adhered to the Declaration of

Helsinki and were approved by the Ethics Committee of the Medical Faculty of the Goethe University. Demographic data are summarized in Tables 1A and 1B.

2.2. Clinical assessment

Severity of depression was assessed with the MADRS (Montgomery Åsberg Depression Scale) at onset of inpatient care and shortly before discharge. MADRS consists of 10 items describing the main symptoms of depression within the last 7 days. Patients with bipolar disorder were additionally queried with the Young Mania Rating Scale (YMRS) to assess the severity of manic symptoms. The test encompasses 11 major clinical symptoms characterizing the last 48 h [32].

The Pittsburgh sleep quality index (PSCI) was used to assess sleep quality and quantity. It is a self-assessment, 19-item questionnaire [33], which was filled in by the patients at the onset of inpatient care and before discharge. A score >5 indicates poor sleep. The chronotype was measured with help of the Morningness Eveningness Questionnaire. The MEQ is a self-assessment questionnaire, which consist of 19 items [34].

2.3. Analysis of lipid signaling molecules

2.3.1. Targeted analysis of signaling molecules

Bioactive lipids including sphingolipids and ceramides, lysophosphatidic acids, endocannabinoids and pterins were analyzed in plasma by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as described in detail in the supplementary material. All analytical methods were further developed based on methods reported in our previous studies [31,35–38].

In brief, the analytes were extracted using liquid-liquid-extraction except for pterins, which were extracted using solid phase extraction. Plasma sample volumes were 10 μl for sphingolipids, 50 μl each for LPA and pterins and 100 μl for endocannabinoids. The quantification of all analytes was performed using a hybrid triple quadrupole-ion trap mass spectrometer QTRAP 5500 or 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in positive ESI mode for sphingolipids and endocannabinoids and in negative ESI mode for LPA and pterins.

Sphingolipids were separated using an Agilent 1200 HPLC system equipped with a Zorbax C18 Eclipse Plus UHPLC column (50 × 2.1 mm, 1.8 μm, Agilent technologies, Waldbronn, Germany) and the analysis of LPA was done on the same HPLC system using a Luna C18 column (50 × 2 mm, 5 μm, Phenomenex, Aschaffenburg, Germany). For the chromatographic separation of pterins, a Synergi Hydro RP column (250 × 2 mm, 4 μm, Phenomenex, Aschaffenburg, Germany) coupled to an Agilent 1200 HPLC system was used while analysis of the endocannabinoids was done using an Agilent 1290 Infinity I UHPLC system equipped with an Acquity UPLC BEH C18 UPLC column (100 × 2.1 mm, 1.7 μm, Waters, Eschborn, Germany).

Quality control samples of three different concentration levels (low, middle, high) were run as initial and final samples of each run. For all analytes, the concentrations of the calibration standards, quality controls and samples were evaluated by Analyst software 1.6 and MultiQuant Software 3.0 (Sciex) using the internal standard method (isotope-dilution mass spectrometry). Calibration curves were calculated by linear or quadratic regression with 1/x weighting or 1/x2 weighting. Variations in accuracy of the calibration standards were <15% over the range of calibration, except for the lower limit of quantification (LLOQ), where a variation in accuracy of 20% was accepted. For the acceptance of the analytical run, the accuracy of the QC samples had to be between 85% and 115% of the nominal concentration for at least 67% of all QC samples. The lower and upper limits of quantification for each analyte are given in the Suppl. Methods.

2.3.2. Untargeted lipidomics

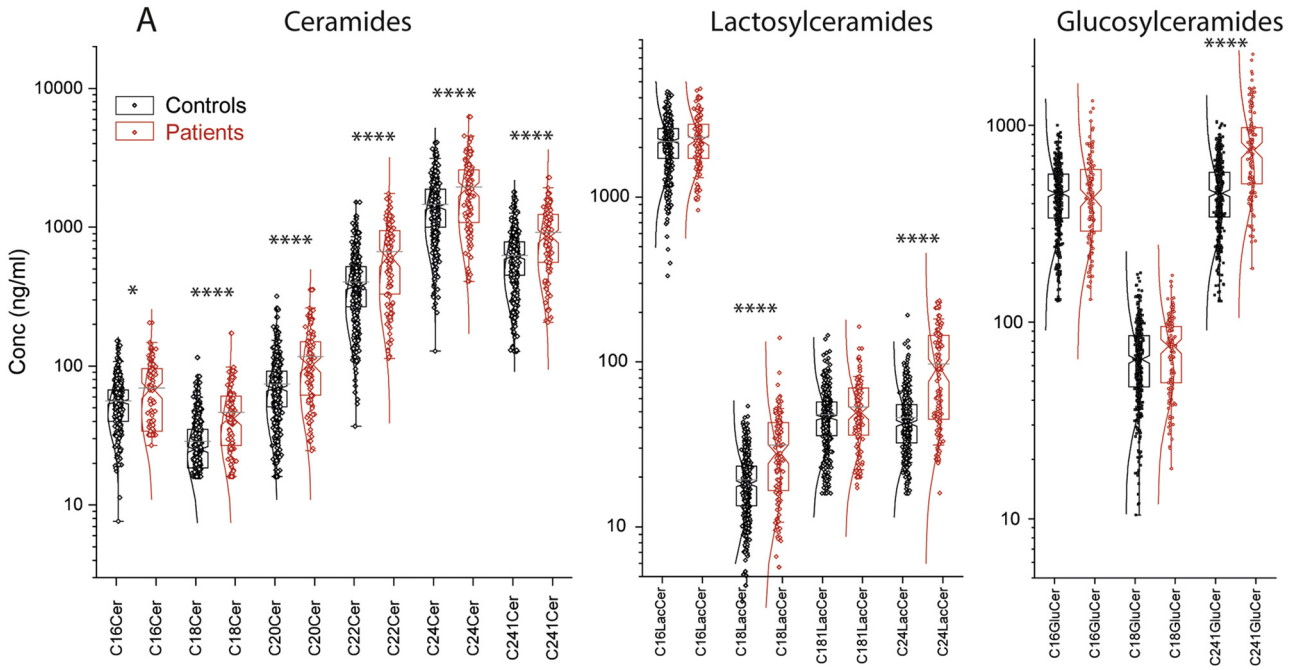
Plasma samples (20 μl) were extracted using methyl-tert-butyl-ether [39]. The organic phase was split into two aliquots, one for analysis in negative ion mode and the other in positive ion mode. After drying under a nitrogen stream at 45 °C, the aliquots were reconstituted in 120 μl methanol or stored at −40 °C until analysis. LC-MS analysis was performed on a Nexera X2 system (Shimadzu Corporation, Kyoto, Japan) coupled to a TripleTOF 6600 (Sciex, Darmstadt, Germany). The chromatographic separation was done on a Zorbax RRHD Eclipse Plus C8 1.8 μm 50 × 2.1 mm ID column (Agilent, Waldbronn, Germany) with a SecurityGuard Ultra C8 pre-column (Phenomenex, Aschaffenburg, Germany), using a binary gradient with 40 °C column temperature and a flow rate of 0.3 ml/min. For the positive mode, the mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in water and mobile phase B of 0.1% formic acid in acetonitrile:isopropanol 2:3 (v/v). For measurement in negative mode 1 mM ammonium formate and 0.1% formic acid in water was used as mobile phase A. The MS analysis encompasses a TOF MS Scan from 100 to 1000 m/z with six data dependent acquisitions per cycle and a mass range of 50–1000 m/z. The identification of the lipid species was based on the exact mass (±5 ppm), the isotope ratio and the comparison of the MS/MS spectra with the reference spectra according to LIPID MAPS (http://www.lipidmaps.org), METLIN (http://metlin.scripps.edu) or the Human Metabolome Database (HMDB, version 4.0).

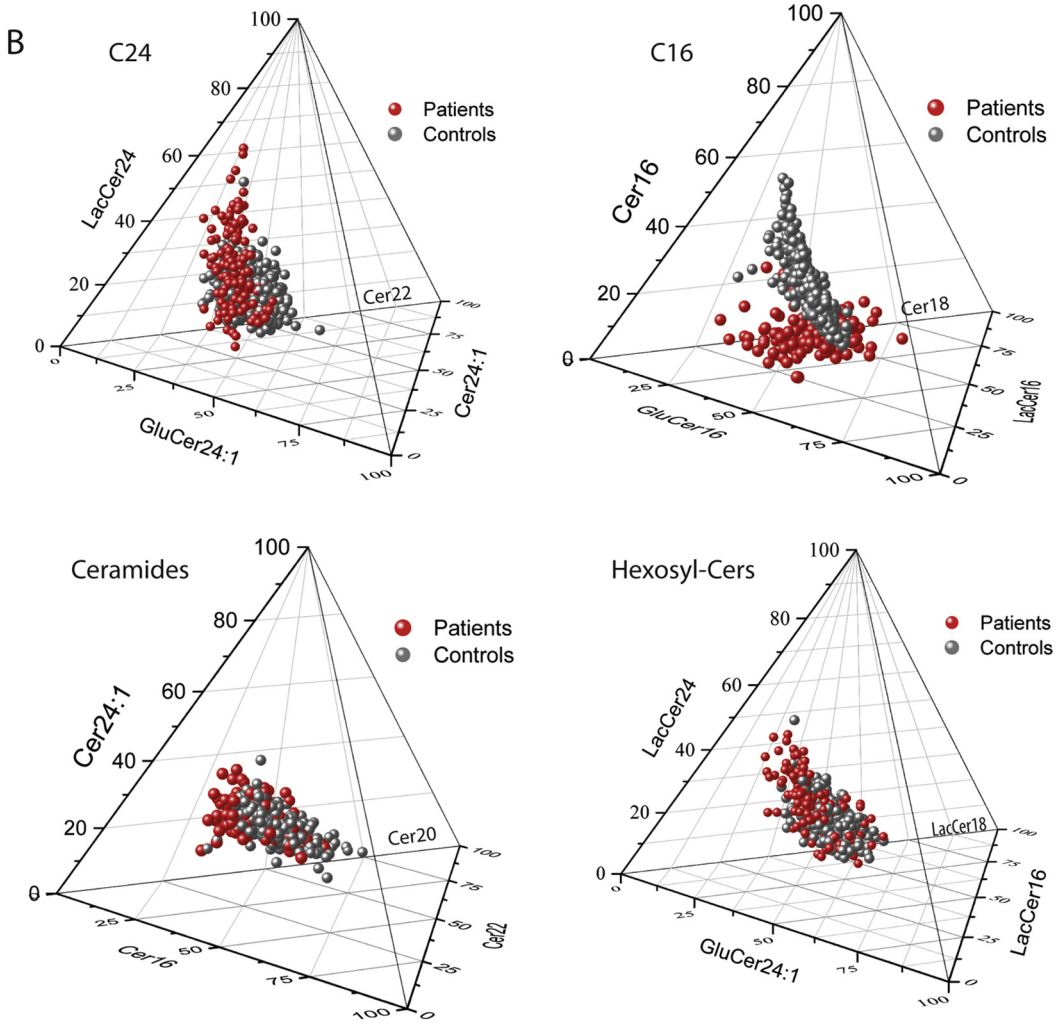
To reduce the impact of small variations in instrument sensitivity during the measurements all samples were randomized prior to analysis. Quality control samples were injected at the start and at the end of a run and after every 10th sample to verify system stability.

2.4. Statistics

Lipid concentrations are presented as scatter plots with mean ± standard deviation (SD) or box-scatter plots, where the box is the interquartile range and the whiskers show minimum to maximum, or the 95% confidence interval (CI), specified in the figure legend. Data were analyzed with SPSS 25, Origin Pro 2019 and GraphPad Prism 7.0. The frequency distributions of ceramides or glucosylceramides were fitted according to Lorentizian (C24:1Cer, GluCer24:1) or the sum of two Lorentizian curves (C24LacCer), based on the best fit values. Principal component analysis (PCA) and partial least square analysis (PLS) were used for targeted lipid and untargeted lipidomic data, respectively to reduce dimensionality and identify the factors, which contributed most to the variance, and discriminated best between groups. Group (i.e. patient versus control), gender, age and BMI were considered as independent factors. In addition, linear canonical discriminant analysis (DA) was used to assess the predictability of group membership based on DA scores. DA was performed without and with bootstrapping, the latter

Fig. 2. Plasma concentrations of ceramides, glucosylceramides and lactosylceramides. A: Box/scatter plots of ceramides in plasma, analyzed with targeted LC-MS/MS. The notched box shows the interquartile range, the line is the median, the whisker show the 95% CI and the dots are individual results of samples from unipolar or bipolar patients (red) and controls (black). The line shows the log-distribution curve. Concentrations were submitted to 2-way ANOVA for "lipid" × "group" after normalization to percentages. Groups were subsequently compared per *t*-tests for each lipid individually using an adjustment of alpha according to Šidák. Asterisks indicate significant differences between groups. $^{*}P < 0.05$, $^{****}P < 0.0001$. Scatter/boxplots of normalized concentrations are shown in Suppl. Fig. 4A. B: Tetraeders showing scatter clouds of normalized long-chain C24 ceramides, short chain C16 ceramides, ceramides of different chain length (C16, C20, C22, C24:1), or glucosyl or lactosyl ceramides (LacCer16, LacCer18, LacCer24, GluCer24:1). The most strongly regulated ceramides were selected for tetraeder analysis. The dots show individual samples of patients (red) and controls (grey).


A
Ceramides
Lactosylceramides
Glucosylceramides
Controls
Patients
Conc (ng/ml)
C16Cer
C18Cer
C20Cer
C22Cer
C24Cer
C241Cer
C16LacCer
C18LacCer
C181LacCer
C24LacCer
C16GluCer
C18GluCer
C241GluCer


B
C24
C16
Ceramides
Hexosyl-Cers
Patients
Controls
LacCer24
Cer22
GluCer24:1
Cer24:1
Cer16
Cer18
GluCer16
LacCer16
Cer20
LacCer18

using a stratified random sampling approach considering gender and age, and 100 iterations.

Plasma concentrations were compared between groups or subsamples of groups using analyses of variance (ANOVA), or *t*-tests according to the data subgroup structure and distribution. Age, gender and body mass index (BMI) were introduced as covariates, and if significant, further analyses of group differences were performed after matching controls to patients based on age and gender. In particular, a random subset of females <30 years was drawn to adjust the numbers in this control subgroup. For cluster analyses and principal component analysis, lipid concentrations were log2 transformed or normalized to the 90% quantile of the respective lipid to allow for a combined analysis.

Patients who provided two consecutive samples are represented twice in some scatter plots, which is specified in the figure legend. Statistical analyses were done for samples and repeated for "one sample per subject" i.e. taking either only the first or the second sample of a patient or the mean of consecutive samples, and DA analyses were performed using initial samples as training data and final samples as tests data, or vice versa, each with 50% of the controls. Further analyses consisted in χ^2 statistics and linear regression analyses. Time courses including samples at onset of inpatient care and before discharge were submitted to paired *t*-tests or multivariate analysis of variance (2-way ANOVA) using the subject factors 'time' and 'lipid'. Further 2-way ANOVAs compared 'group' × 'gender'. In case of significant results of ANOVAs, groups were mutually compared using *t*-tests, and *P*-values were adjusted according to the procedures of Dunnett (versus one control group) or Šidák. The alpha level was set at 0.05 for all comparisons and asterisks in the figures refer to adjusted *P*-values.

3. Results

3.1. Principal component analysis reveals deregulations of ceramides

To assess lipid patterns and reduce dimensionality we performed standard PCA analyses with input of 32 lipids plus biopterin and neopterin, the latter two included as oxidative parameters. Because of the broad concentration range of different lipids, normalized data were used as PCA input, i.e. percentages of the respective 90% quantile. Detailed PCA results are included as Suppl. Table 1A. The first three PCA components accounted for 53.8% cumulative variance (Fig. 1A), and major group differences were caused by ceramides of different chain lengths and their hexosyl metabolites, particularly long-chain C24 lactosylceramide and C24:1 glucosylceramide. The PCA was repeated with input of sphingolipids and ceramides only (Fig. 1B, Suppl. Table 1B) to better identify the regulated candidates. The strongest group differences were caused by C16Cer, C18Cer, C20Cer, C22Cer, C24Cer, C24:1Cer, C24:1GluCer and C24LacCer. Hence, further analyses focused on these ceramides. The group means are depicted in the polar plot (Fig. 1C), showing that most of these ceramide species were increased in patients, except C16GluCer, C16LacCer and C18GluCer. Descriptive statistics of plasma concentrations are shown in Suppl. Table 2A for sphingolipids including ceramides, and in Suppl. Table 2B for endocannabinoids, lysophosphatidic acids, biopterin and neopterin. According to the canonical discriminant analysis using ceramides, glucosylceramides and lactosylceramides as independent factors, 82.8% of the original grouped cases were correctly classified.

3.2. Associations of long and short-chain ceramides

Analyses of variance confirmed significant differences of all ceramides and of C18LacCer, C24LacCer and C24:1GluCer between controls and patients (Fig. 2A), and analyses of covariates pointed to an influence of gender and age for some of the candidates. Statistical analyses were therefore repeated using subsets of controls, where two controls were matched with one patient sample. Despite some loss of power, the results were not affected (not shown), so that further analyses were done with all controls. The tetraeder analysis (Fig. 2B) of each 4 selected candidate lipids reveals the shift of the patient scatter cloud relative to the controls, which is also evident in the multi regression matrix (Suppl. Fig. 1), which shows that most ceramides have as linear relationship with each other and are normally or log-normally distributed.

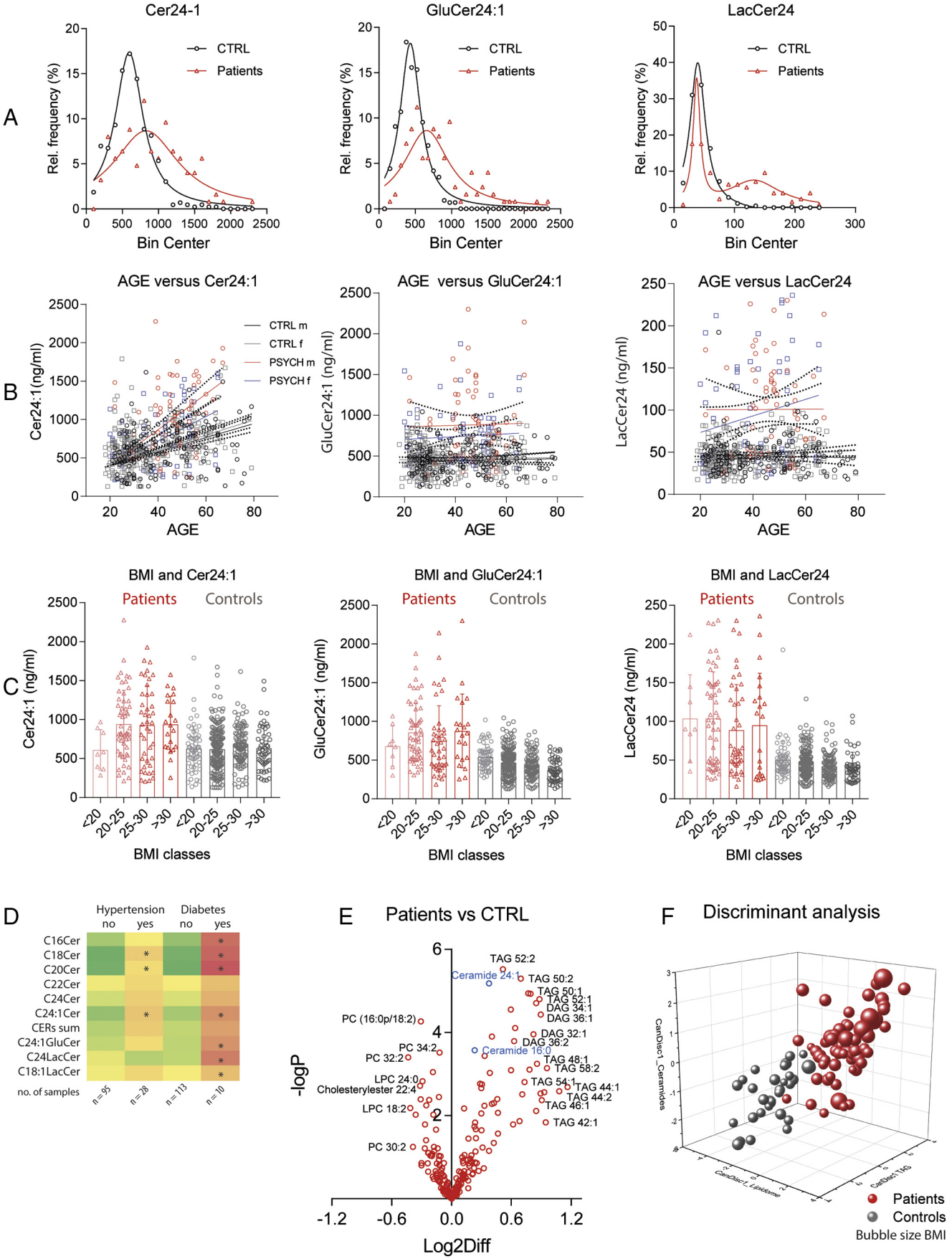
3.3. Influence of age, gender and body mass index

Because ANOVAs pointed to influences of age and gender, we assessed the subgroups and associations in more detail, focusing on the long-chain ceramides, which were most strongly regulated. The frequency distributions show the flattening and right shift of the Gauss-curve in the patients relative to controls (Fig. 3A), and a summed Gauss-curve for C24LacCer for patients indicating two subpopulations, either with normal or strongly elevated levels. Linear regression analyses of age versus ceramides shows a significant association of C24:1Cer with age, with a steeper regression line in patients than controls (Fig. 3B). There was no association of age with C24:1GluCer or C24LacCer. A detailed analysis of associations of all ceramides and hexosyl-ceramides with age (Suppl. Fig. 2A) revealed an increase of all ceramides with age in both controls and in patients, but the regression line was significantly steeper in patients than controls for Cer18, Cer20, Cer22, Cer24:1 and GluCer16.

The association of ceramides with the body mass index (BMI) was less obvious (Fig. 3C; Suppl. Fig. 2B), but the levels of C18Cer, C20Cer and C24:1Cer were positively associated with the BMI, either in both patients and controls (C18) or in patients only (C20, C24:1) (Suppl. Fig. 2B). In controls, hexosyl-ceramides tended to decrease with increasing BMI, which did not occur in patients (Suppl. Fig. 2B).

Further analysis of patient subgroups according to comorbidities revealed higher ceramide levels in patients with a diagnosis of arterial hypertension (14 patients with 28 samples) and/or with diabetes mellitus (5 patients with 10 samples), pointing to a metabolic syndrome as relevant confounding factor (Fig. 3D heat map). Therefore, we studied the nutrient/metabolic lipidome by untargeted lipodomics and observed a strong increase of multiple diacylglycerols (DAG) and

Fig. 3. Frequency distributions of C24 ceramides in patients and controls and associations with age and BMI. A: Frequency distribution of C24:1Cer, C24:1 GluCer and C24LacCer in bipolar and unipolar patients and controls. Each bin of the frequency distribution contains the number of values that lie within the range of values that define the bin. The dots show the frequency versus the bin centers, the line shows the distribution curve, which was fitted according to Lorentizian (C24:1Cer, GluCer24:1) or the sum of two Lorentizian curves (C24LacCer). The best-fit values for amplitude, center and width differed significantly between groups ($P < 0.0001$). B: XY scattergrams and linear regression analyses of age versus C24:1Cer, C24:1GluCer and C24LacCer in male and females patients and respective controls. The line is the regression line, the dotted lines show the 95% CIs. There was a significant association of age with C24:1Cer in all groups ($P < 0.0001$). The slope was significantly steeper in patients versus controls. The CIs of the slopes were for male controls 7.386 to 10.84, female controls 8.418 to 11.4, male patients 19.44 to 27.14 and female patients 12.01 to 19.89. There was no association of age with GluCer24:1 or LacCer24. C: Bar/scatter plots of BMI classes versus C24:1Cer, C24:1GluCer and C24LacCer. The bar shows the mean, the whisker SD and the dots are individual samples. GluCer24:1 decreases with increasing BMI in controls but not patients. There was no positive association of all tested lipids with the BMI. D: Heat map of the mean lipid concentrations in patients without and with hypertension or diabetes. The colour scale ranges from low (green) to red (high) levels for each lipid individually. Significant differences between the subgroups are marked with asterisks within the respective colour field. E: Volcano plot showing the log2-fold difference of lipid concentrations (x-axis) versus the negative logarithm of the *P*-value (y-axis) comparing patients (combined unipolar and bipolar) versus controls. Lipids reduced in patients occur on the left side of the y-axis, increased lipids on the right side. Untargeted lipidomic analyses were performed in a random subset of 30 controls, 30 bipolar and 30 unipolar patients by HPLC/TOF mass spectrometry. Each scatter is one lipid. (−logP >1.5 plus 1.5fold change was considered as significant). F: The 3D bubble plot shows the individual scores for the respective first CanDisc factor for lipidome (220 species, X-axis) triglycerides (TAG, Y-axis), and ceramides (*Z*-axis). The size of the bubble is the body mass index.


A
Cer24-1
GluCer24:1
LacCer24
CTRL
Patients
Rel. frequency (%)
Bin Center
B
AGE versus Cer24:1
AGE versus GluCer24:1
AGE versus LacCer24
CTRL m
CTRL f
PSYCH m
PSYCH f
Cer24:1(ng/ml)
GluCer24:1 (ng/ml)
LacCer24 (ng/ml)
AGE
C
BMI and Cer24:1
BMI and GluCer24:1
BMI and LacCer24
Patients
Controls
BMI classes
D
Hypertension
Diabetes
no
yes
C16Cer
C18Cer
C20Cer
C22Cer
C24Cer
C24:1Cer
CERs sum
C24:1GluCer
C24LacCer
C18:1LacCer
no. of samples
n=95
n=28
n=113
n=10
E
Patients vs CTRL
-logP
Log2Diff
TAG 52:2
Ceramide 24:1
TAG 50:2
TAG 50:1
TAG 52:1
DAG 34:1
DAG 36:1
DAG 32:1
DAG 36:2
Ceramide 16:0
TAG 48:1
TAG 58:2
TAG 54:1
TAG 44:1
TAG 44:2
TAG 46:1
TAG 42:1
PC (16:0p/18:2)
PC 34:2
PC 32:2
LPC 24:0
Cholesterylester 22:4
LPC 18:2
PC 30:2
F
Discriminant analysis
CanDisc1_Ceramides
Patients
Controls
Bubble size BMI

triacyglycerols (TAG) of various chain length along with increases of C24Ceramide and C16Ceramide in patients versus controls (Fig. 3E). Canonical discriminant analysis using 220 different lipid species (TAG, DAG, cholesterylesters, LPC, PI, PC, PE, fatty acids of different chain lengths and saturation) and ceramides as independent factors, was able to classify 95.5% of the original grouped cases to the correct control or patient group. The 3D scatter plot using the respective first CanDisc factor scores for triacylglycerols (TAG), ceramides (CERs) and untargeted lipidome clearly separates control and patient scatter clouds (Fig. 3F).

3.4. Influence of major depression versus bipolar disorder and current episode

We further analyzed the influence of disease subtype by gender, i.e. unipolar versus bipolar in males and females (Fig. 4), and the impact of the current episode, i.e. euthymic, depressive or mixed/manic (Suppl. Fig. 3), the latter based on clinical assessment and MADRS and YMRS rating scales. There was no significant effect of gender for any of the ceramides in control subjects but in patients, males had higher levels of ceramides than females, which was true for unipolar (major depression) and bipolar patients. It is noteworthy that male patients had higher BMI levels than females (CTRL m 25.5 ± 3.7; CRTL f 23.7 ± 4.6; Patients m 27.0 ± 3.1, Patients f 25.2 ± 5.6). There was no obvious difference between patients with major depression (unipolar) and bipolar patients (Fig. 4). There was also no difference between patients with a current euthymic, depressive or manic episode (Suppl. Fig. 3). Some ceramides tended to be lower in mixed/manic patients, which was caused however by the lower number of patients in this subgroup, and it was not statistically significant.

3.5. Association of ceramides with depression score and therapeutic outcome

As shown in Fig. 5A, almost all depressed patients experienced a substantial reduction of the MADRS upon inpatient therapy. However, levels of ceramides were not associated with the MADRS (Fig. 5B) and there was no unidirectional change of any of the ceramides between the first sample and the final sample before discharge (Fig. 5C). A number of putative determinants were considered to differentiate patients with dropping ceramides versus patients with raising levels including gender, loss/gain of body weight, number of previous episodes, years since first diagnosis, improvement of sleep, switch of medication, or electroconvulsive therapy (ECT), but none of these factors was positively or negatively associated with the individuals' ceramide-course. ECT patients ($n = 7$) had high levels of C16Cer and C24Cer but the low numbers of these patients only allows for an exploratory analysis (Suppl. Table 3). The effects of medication were further assessed by comparing no/yes subgroups for medication classes i.e. antidepressants, antipsychotics and lithium (Fig. 5D for ceramides, Suppl. Fig. 4 for hexosyl-ceramides), and in case of significant effects further analyses were done for individual compounds (Suppl. Table 3). The analysis revealed that patients receiving antidepressants had higher levels of C18Cer, C22Cer and C24Cer than patients not receiving drugs of this group. Venlafaxine partly recapitulated this effect, whereas antipsychotics and lithium had no effect, except olanzapine, which was associated with high levels (Suppl. Table 3, Suppl. Fig. 4C). The analysis did not consider that several patients were receiving more than one medication, but there was no association of the number of different drugs with any of the ceramides (Suppl. Table 3), suggesting that indeed specific compounds tended to contribute to the raise of plasma ceramides.

4. Discussion

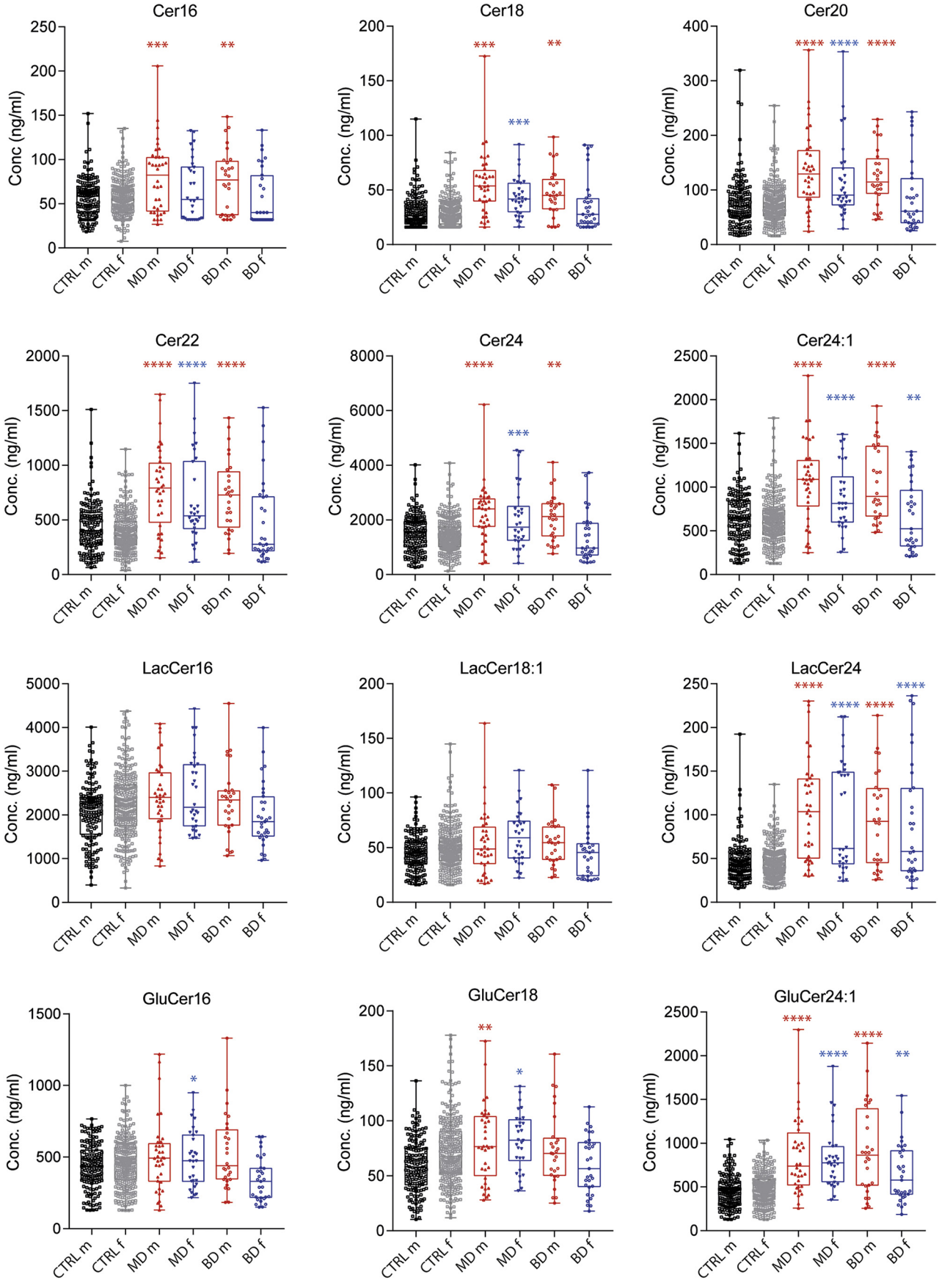
We show in the present pilot study that plasma ceramides are substantially increased in patients with major depression and bipolar disorder irrespective of the severity of symptoms in the current episode. The high ceramide levels clearly indicate, and likely contribute to the high metabolic burden and increased cardiovascular risk in these patients [40,41]. High plasma or serum ceramide levels have also been associated with other diseases including diabetes mellitus [42], Alzheimer's disease [43], breast cancer [27,44] and colon cancer [45,46], all contributed by metabolic deregulations. Obesity is a risk factor for all of them [47], and interestingly, C20Cer and C24:1Cer were positively associated with the BMI in patients but not in the controls. Further, ceramides showed a steeper raise with increasing age in patients than in controls, all suggesting that the MD and BD patients are at a higher risk of accumulating unfavorable metabolic age- and diet-associated pro-aging effects. It is of note that MD and BD patients also had higher levels of a number of monounsaturated triglycerides suggesting a misbalancing of mono and polyunsaturated fatty acids. Particularly the latter are supposed to be of protective value in the context of aging and age-associated disease [48,49], and omega-3 lipids were indeed reduced in the postmortem brain of MD patients [50,51].

Because of the multiple disease associations, ceramides cannot classify as biomarkers for major depression or bipolar disorder because they lack specificity, but the ceramide pattern considering the individual's fingerprint of ceramide subtypes, metabolites and precursors may still reveal a complex disease-specific lipid phenotype [35,52], and metalipidomic analyses may address this question in the future.

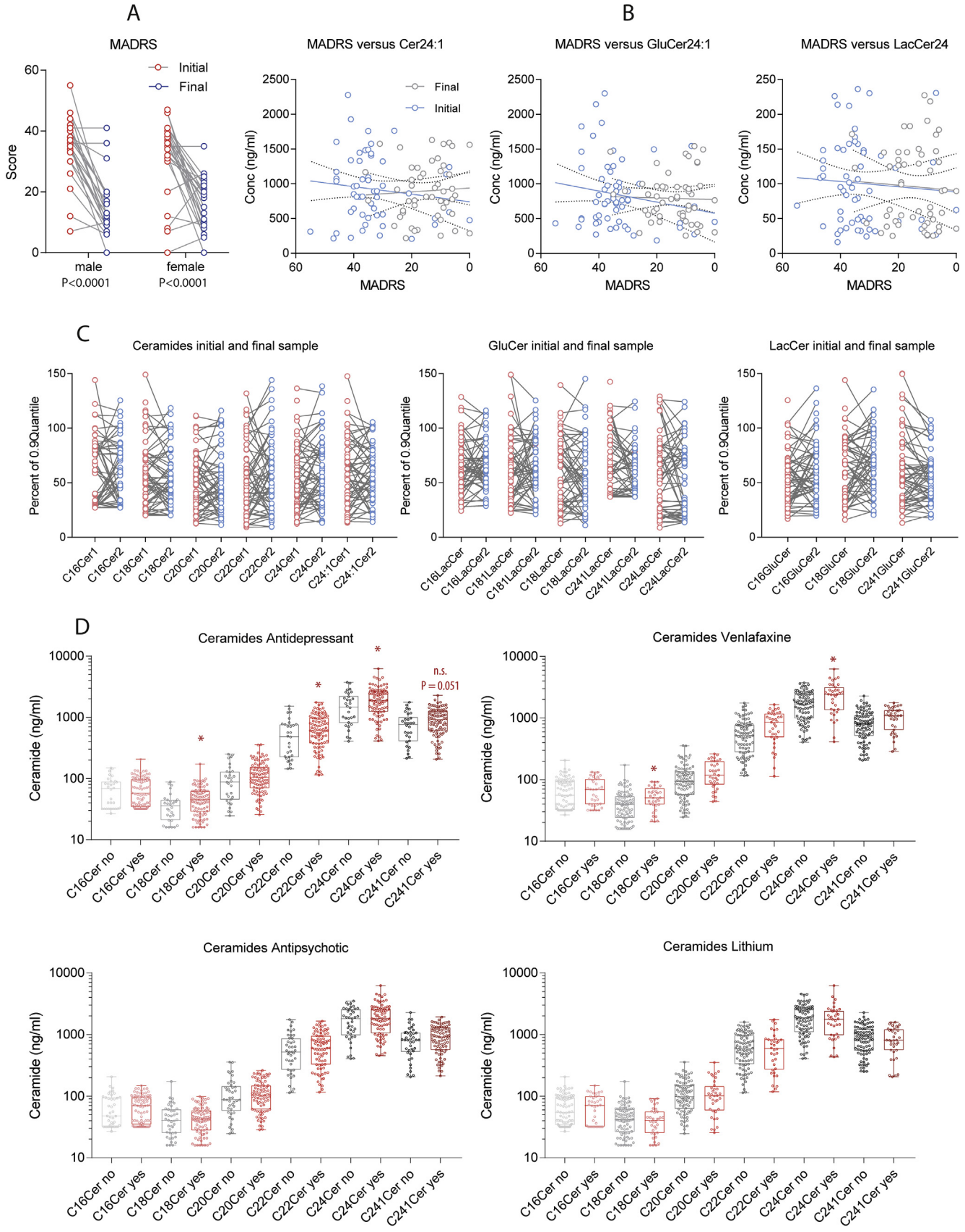
The study has some limitations owing to the diversity of the patients and medications, and owing to the confounding factors age, metabolic status and gender. To address and balance these factors we had to include a large cohort of control subjects to match patients with controls according to age, gender and BMI. Therefore, the number of controls is about 3-fold higher than the number of patients. The data were robust in that statistically significance was maintained with randomly drawn subsets of controls and canonical discriminant analysis based on ceramides and long-chain glucosyl and lactosyl ceramides allowed for predictability of group membership of 82%, and in combination with triacylglycerides of 88%. The strength of our study relies in the multivariate assessment of multiple lipid species without a priori selection of candidates.

High brain ceramides have been observed previously in rodent models of depression-like behavior [8,10] and were associated mechanistically with suppression of adult neurogenesis [8,11], autophagy [22] and deregulation of corticosteroid homeostasis [7]. All of these deregulations were restored upon treatment of the animals with amitriptyline or similar antidepressants, in parallel with an attenuation of depression-like behavior. Hence, therapeutic efficacy of these drugs was ascribed to lowering of elevated ceramide levels [53], which was confirmed by analysis of ceramides in brain tissue, and was further substantiated by human genetic studies. The latter revealed that the abundance of SMPD1 (gene encoding acidic sphingomyelinase, ASM) splice variants, which encode for enzymatically low-active "protective" ASM variants were rarely found in MD patients [54]. In line with this conclusion, a number of antidepressants and antipsychotic agents are functional inhibitors of ASM, called FIASM [53,55], which converts sphingomyelin to ceramides at the inner lysosomal membrane and plasma membrane, hence contributing to sphingomyelin degradation

Fig. 4. Gender and subgroup specific plasma concentrations of ceramides, glucosylceramides and lactosylceramides. The box/scatter plots show plasma concentrations of individual ceramides, which were most strongly regulated. The box shows the interquartile range, the line is the median, the whiskers show the 95% CI, and each scatter represents an individual sample. Patients who provided two consecutive samples are represented twice. Groups were compared per 2-way ANOVA for "group" × "gender", and subsequent *t*-tests using an adjustment of alpha according to Šidák. Statistical analyses were repeated for "one sample per subject" i.e. taking either only the first or the second sample of a patient or the mean of consecutive samples. Despite the loss of power, the results did not change. Asterisks indicate significant differences between groups as indicated, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.


Cer16
Cer18
Cer20
Cer22
Cer24
Cer24:1
LacCer16
LacCer18:1
LacCer24
GluCer16
GluCer18
GluCer24:1
Conc. (ng/ml)
CTRL m
CTRL f
MD m
MD f
BD m
BD f

and ceramide production in the lysosome [15,56]. Although loss of function mutations of ASM in Niemann-Pick disease are obviously detrimental and lead to a lysosomal storage disease [57], it has been proposed that moderate or transient inhibition of ASM is of therapeutic value for a number of diseases including major depression [53] and cancer [58,59].



Considering these studies, we expected to find lower ceramides in patients receiving drugs with FIASM properties, but opposite to expectations, ceramides were higher in patients taking antidepressants or the antipsychotic olanzapine than in patients not receiving these drugs. The plasma is not representative for what happens at crucial sites in the brain and our observation therefore does not contradict the putative molecular mechanism of FIASMs but points to an add-on metabolic risk associated with these drugs, which more or less all lead to a gain of body weight [60,61].

Considering the postulated effect of antidepressants on ceramide metabolism and earlier findings of altered plasma levels in patients with MD or BD, we further surmised that ceramide level would decrease significantly during recovery. However, again this expectation was not met. Instead, ceramides changed in some patients from the initial to the recovery sample, the latter taken before discharge, but not in a unidirectional manner, and a number of putative explanations such as change of medication, body weight gain or loss, number of previous episodes or disease years were all not associated with either a reduction or increase. In addition, the ceramides were not associated with MADRS scores, which in most depressed patients dropped in parallel with the clinical recovery. Hence, the data suggest that the plasma ceramides are indicative of the complex metabolic disease, which is aggravated by some medications, but they are apparently no predictors of the disease course or response to medication.

In summary, analysis of plasma ceramides may be useful as a diagnostic tool to identify those patients with a particularly high risk of cardiovascular disease or metabolic syndrome who require close monitoring of glucose, blood pressure and body weight to prevent such sequelae of the psychiatric disorder.

Conflict of interest

The authors declare that there are no conflicts of interest. The funding organizations had no role in data acquisition, analysis or decision to publish the results.

Author contributions

NBK, KKS, KSJH collected samples from patients or controls, performed experiments and analyzed data. ST, DT and LH performed mass spectrometry lipid analyses, and DT and GG managed the Lipidome-Lab. SW recruited healthy controls and organized sample collection. SKS organized and supervised the phenotypic databank and collecting of blood samples. SKS and AR diagnosed the patients and contributed clinical insight and discussed the data. IT and NBK initiated the study, organized sample collection and analysis, and analyzed data. NBK recruited patients and recorded detailed demographic data. IT made the figures and wrote the manuscript. All authors contributed to writing or revising of the manuscript and agreed to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.04.002.

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Fig. 5. Association of ceramide plasma levels with effects of treatments. A, B: Montgomery–Åsberg Depression Rating Scale (MADRS) at the onset of inpatient care and before discharge in male and female patients (left) and association of the MARDS with long-chain ceramides and their hexosyl-metabolites (right panels). The analysis was done with all patients who provided two consecutive samples, and corresponding scores at onset of inpatient care and before discharge. The lines are the linear regression lines for the initial (blue) and final (grey) samples and the dotted lines show the 95 CI bands. Despite the MARDS reduction in most patients upon treatment (shown in A), ceramide levels did not change accordingly. The association of MADRS with other clinical scores is presented in Suppl. Fig. 4D. C: Before/after analysis of all ceramides and their hexosyl-metabolites including patients who provided two consecutive samples. Each symbol and line is one patient. The plasma concentrations were normalized to the respective 90% quantile and are presented as percentages to show all ceramides in one figure. There was no unidirectional change of the ceramides from sample-1 to sample-2 (2-way ANOVA of "time" × "ceramide" was n.s. for time). D: Effect of medication on plasma concentrations of ceramides. Ceramides tended to be higher in patients taking antidepressants (upper left). A single antidepressant associated with high levels was venlafaxine (upper right). There was no significant association with lithium or antipsychotics except for olanzapine (Suppl. Fig. 4C). The corresponding effects of medication on lactosyl- and glucosylceramides are shown in Suppl. Fig. 4B and a detailed analysis of medication effects and comorbidities is presented in Suppl. Table 3.

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