Diastereomer-specific quantification of bioactive hexosylceramides from bacteria and mammals

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Abstract  Mammals synthesize, cell-type specifically, the diastereomeric hexosylceramides, β-galactosylceramide (GalCer) and β-glucosylceramide (GlcCer), which are involved in several diseases, such as sphingolipidosis, diabetes, chronic kidney diseases, or cancer. In contrast, Bacteroides fragilis, a member of the human gut microbiome, and the marine sponge, Agelas mauritianus, produce α-GalCer, one of the most potent stimulators for invariant natural killer T cells. To dissect the contribution of these individual stereoisomers to pathologies, we established a novel hydrophilic interaction chromatography-based LC-MS² method and separated (R > 1.5) corresponding diastereomers from each other, independent of their lipid anchors. Testing various bacterial and mammalian samples, we could separate, identify (including the lipid anchor composition), and quantify endogenous β-GlcCer, β-GalCer, and α-GalCer isomers without additional derivatization steps. Thereby, we show a selective decrease of β-GlcCers versus β-GalCers in cell-specific models of GlcCer synthesize-deficiency and an increase of specific β-GlcCers due to loss of β-glucoceramidase 2 activity. Vice versa, β-GalCer increased specifically when cerebroside sulfotransferase (Gal3st1) was deleted. We further confirm β-GalCer as substrate of globotriaosylceramide synthase for galabiaosylceramide synthesis and identify additional members of the human gut microbiome to contain immunogenic α-GalCers. Finally, this method is shown to separate corresponding hexosylphosphoinositol standards, promoting its applicability in further investigations.—von Gerichten, J., K. Schlosser, D. Lamprecht, I. Morace, M. Eckhardt, D. Wachten, R. Jennemann, H-J. Gröne, M. Mack, and R. Sandhoff. Diastereomer-specific quantification of bioactive hexosylceramides from bacteria and mammals. J. Lipid Res. 2017. 58: 1247–1258.

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Abbreviations: AP, Nα-hydroxy acyl phytosphingosine; AS, Nα-hydroxy acyl sphingosine; BS, Nβ-hydroxy acyl sphingosine; Cer, ceramide; CST, cerebroside sulfotransferase (Gal3st1); Fa2h, fatty acid 2-hydroxylase; GALC, galactosylceramidase; GalCer, galactosylceramide; GalSph, galactosylphosphoinositol; GBA, glucosylceramidase; Gb2, β-glucosylceramide 2; Gb3β, globotriaosylceramide synthase; GlcCer, glucoceramide; GlcSph, glucosphingosine; GSL, glucosylphospholipid; HexCer, hexosylceramide; HexSph, hexosylphosphoinositol; HPTLC, high-performance TLC; iNKT, invariant natural killer T; NP, N-nonhydroxy acyl phytosphingosine; NS, N-nonhydroxy acyl sphingosine; UGCC, glucosylceramide synthase; Ugt8α, β-galactosylceramide synthase; CST/ Pax8Cre, deletion of cerebroside sulfotransferase by Cre (cycization recombinase) under control of the paired box 8 (Pax8)-promotor in renal tubular epithelial cells; (Hex)Cer (d18:1/24:1), (hexosyl)ceramide with a C18-sphingosine (d18:1) and nervonic acid (24:1) (i.e., a N-nonhydroxy acyl sphingosine-type hexosylceramide); Ugc8/AbCer or Ugc8/Pax8Cre, deletion of Ugc8 (UDP-glucose ceramide glucosyltransferase) by Cre (cycization recombinase) under control of the albumin (Ab)-promotor in hepatocytes or the paired box 8 (Pax8)-promotor in renal tubular epithelial cells.

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Mammals produce a large variety of glycosphingolipid (GSL) structures, based on the initial synthesis of either glucocerebrosides [the term is used here only for β-glucosylceramides (GlcCers)] or cerebrosides [the term is used here only for β-galactosylceramides (GalCers)] by β-GlcCer synthase (UGCG) or β-GalCer synthase (UGT8A), respectively (1–3). Due to the Golgi and ER location of their synthesis and the topology of the corresponding enzymes, β-GlcCer and β-GalCer are in contrast to more complex GSLs also located on the cytosolic side of cell membranes (4–6), by that widening their terrain of biological action to cytosolic binding partners. Whereas glucocerebrosides appear to be omnipresent in almost all cell types of the mammalian body, cerebrosides have mainly been associated to brain, especially myelin structures, and to kidneys (7). Likewise, systemic deficiency of β-GlcCer-based GSLs due to mutation of Uggl is embryonically lethal at E6.5 (8) and organ-specific functions of β-GlcCer, including its derivatives, have been addressed with cell-specific deletion of the Uggl-gene function (9–19). In contrast, mice with a systemic deletion of Ugt8a pass embryogenesis. However, Ugt8a-deficiency results in severe demyelination and motor dysfunction before most of the mice die by the age of 5 weeks (20, 21).

The catabolism of GSLs includes the lysosomal degradation of β-GlcCer and β-GalCer by β-glucosylceramidase (GBA) and galactosylceramidase (GALC) with the help of activator proteins (22, 23). Mutations of GBA and of GALC resulting in substantial loss of corresponding enzyme activities cause Gaucher disease and Krabbe disease, respectively (24–28). Symptoms of defective cerebroside catabolism caused by GALC mutations are motor deterioration, cognitive decline, and the presence of numerous, often multinucleated, glooid cells. The lysosomal storage of glucocerebroside especially affects phagocytic cells (“Gaucher cells”) and goes along with hepatosplenomegaly, which is currently treated with enzyme replacement and/or substrate reduction (29–32). Some patients on enzyme replacement therapy have been reported to develop type 2 diabetes or metabolic syndrome, malignancies, and central nervous system disorders (33), which underlines the biological activity of glucocerebroside and its metabolites. Likewise, increased levels of glucocerebroside correlate multidrug resistance in several tumor cell lines and tumor specimens (34, 35). In general, sphingolipid metabolism defects further promote complications like cardiovascular events via oxidant stress in chronic kidney diseases (36) and downregulation of GSL synthesis appears to help reverse symptoms/phenotypes (37), including polycystic kidney disease (38).

Enzymes catalyzing the production of corresponding α-anomers in mammals have not been described so far, although recently it has been proposed that, at very low levels, α-GalCer is endogenously present and necessary for invarient natural killer T (iNKT) cell homeostasis (39). Furthermore, α-GalCer, which was described initially in the marine sponge, Agelas mauritianus (40), is a highly potent activator of iNKT cells, when presented via CD1d (structurally related to the major histocompatibility complex proteins) by antigen-presenting cells (41). Currently, the potential of α-GalCer-stimulated iNKT cells in tumor treatment is under investigation (42–45). The natural stimulation of iNKT cells via CD1-presented α-GalCer may derive from members of the gut microbiome, such as Bacteroides fragilis, which was shown to produce α-GalCer and thereby probably influences host immune homeostasis (46). Although this is, so far, the only strain investigated to produce α-GalCer, the majority of bacteroides strains were described to produce precursor Cers and Cer phospholipids (47).

Differentiation of β-GlcCer and β-GalCer from complex biological samples has, so far, been achieved by normal phase TLC in the presence of borate (48), by normal phase HPLC with (49) or without derivatization (50, 51), and quite recently by isocratic or zwitterionic hydrophilic interaction chromatography (HILIC)-coupled MS (52, 53). On the contrary, discrimination of β-GalCer from α-GalCer with high sensitivity from complex samples, so far, is based on TLC-immuno-overlays with antibodies specific for the α-glycosidic linkage (39).

To set up a method capable of quantitatively and sensitively distinguishing the four diastereomeric hexosylceramides (HexCers), β-GlcCer, β-GalCer, α-GlcCer, and α-GalCer (Fig. 1A), we used a synthetic mixture of α- and β-GlcCer and a synthetic mixture of α- and β-GalCer, both with an α/β-ratio of about 15/85 and with an identical Cer backbone containing nervonic acid amid-bound C18-sphingosine, i.e., HexCer(d18:1/24:1). Here we show separation of these α/β-HexCer mixtures with HILIC using propionitrile as a major component of solvent A. Further investigations demonstrate that β-HexCers with α-hydroxy fatty acid moieties, which are either of 2R- or 2S-configuration, separated from each other. We transferred the method to biological samples. Addressing murine organs, we identified and quantified complex mixtures of β-GlcCers and β-GalCers and successfully showed specific changes of either β-GlcCers or β-GalCers in mice lacking either β-glucosylceramidase 2 (Gba2, systemically), Uggg (cell specifically), globotriaosylceramide synthase (Gb3S; A4galt, systemically), or cerebroside sulfotransferase (CST; Gal3st1, cell specifically). Whereas these murine samples did not contain detectable amounts of α-GalCer, we confirmed the presence of α-GalCers in B. fragilis and identified low levels of identical α-GalCers in at least two more bacterial strains of the human gut microbiome.

**MATERIALS AND METHODS**

**HexCer standards**

β-GlcCer(d18:1/16:0), β-GlcCer[d18:1/24:1(15Z)], α-GalCer (d18:1/16:0), α-GalCer[d18:1/24:1(15Z)], β-GalCer(d18:1/16:0), β-GalCer[d18:1/24:1(15Z)], β-GalCer[d18:1/α(R)h18:0], β-GalCer[d18:1/α(S)h18:0], and synthetic mixtures of α/ β-GlcCer[d18:1/24:1(15Z)]and/or β-GalCer[d18:1/24:1(15Z)] with an α/β-ratio of about 15/85, and α-galactosylsphingosine (GalSph) (d18:1) were purchased from Avanti Polar Lipids. β-GalSph(d18:1) was obtained from Abcam. β-glucosylsphingosine (GlSph)(d18:1), keratin [Nα-hydroxy acyl sphingosine (NS)-type β-GalCers from bovine brain], and phrenosin [Nα-hydroxy
(AS)-type β-GalCers from bovine brain] were purchased from Matreya. KRN7000/α-GalCer(18:0/26:0) was obtained from AdipoGen Life Sciences. A previously published mix of β-GalCer(d18:1/14:0, 19:0, 25:0) and β-GalCer(d18:1/31:0) was used as internal standard (10).

**Solvents and additives**

Chloroform, methanol, isopropanol, water, all LC-MS Chromasolv®-grade, and 2-butanol (≥99.5%) for GC were from Honeywell Speciality Chemicals Selcze GmbH; propionitrile for synthesis was from Merck Darmstadt; formic acid (99–100%) was from VWR International; and ammonium formate (≥99%) for MS-analytics was from Sigma-Aldrich.

**Mice**

Tissue was isolated from euthanized mice of recently published mouse strains: Gbn+/− (54), Gbs+/− (55), Uggf/AlbCre (11), Uggf/Pax8Cre, CST−/−Pax8Cre and (Uggf/−+CSTf/−)Pax8Cre (16), and fatty acid 2-hydroxylase (Fa2h)−/− (56). All animal procedures were approved and performed in accordance with federal laws.

**Bacteria**

*B. fragilis* (DSM-2151), *Bacteroides vulgaris* (DSM-1447), *Bacteroides ovatus* (DSM-1896), *Bacteroides thetaotaomicron* (DSM-2079), *Bacteroides intestinalis* (DSM-17393), *Bacteroides caccae* (DSM-19024), *Bacteroides uniformis* (DSM-6597), *Prevotella copri* (DSM-18205), *Lactobacillus reuteri* (DSM-20016), and *Bifidobacterium longum subsp. infantis* (DSM-20088) were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

**Bacteroides culture**

According to a previous publication (57), *Bacteroides* were grown anaerobically on Columbia agar plates with 5% sheep blood and *Bifidobacterium* on MRS medium plates containing L-cysteine-HCl (0.5 g/l) for 48 h. *Lactobacillus* was grown on MRS medium plates in an aerobic overnight culture. One culture plate was harvested and transferred in a tube containing 200 µl methanol.

**Lipid extraction from mouse tissue**

Mouse tissue was homogenized in water on ice using an Ultra Turrax T25 and homogenates were lyophilized. GSLs were isolated as previously described (58). Briefly, dried tissue samples were extracted three times with mixtures of chloroform/methanol/water. Supernatants were pooled, dried, and subjected to methanolic mild alkaline hydrolysis (0.1 M potassium hydroxide) for 2 h at 37°C to eliminate glycerophospholipids and subsequently were neutralized with acetic acid. Saponified extracts were then desalted using reverse phase C-18 columns.

**Lipid extraction from bacteria**

Dried bacteria samples were homogenized in methanol using the Quiagen TissueLyser II. One stainless steel bead (5 mm) per tube was agitated for 2 min at 25 Hz. Lysates were extracted in 1 ml chloroform/methanol (2/1) at 37°C for 15 min with occasional sonication. Lipid-containing supernatants were collected following centrifugation at 2,000 g for 10 min and pellets were then re-extracted once with chloroform/methanol/water (10/10/1) and once more with chloroform/methanol/water (30/60/8). Supernatants were pooled and dried.

**HILIC- and reversed phase LC-MS² analysis of HexCers**

Aliquots corresponding to 2 mg mammalian tissue wet weight or, rather, 2 mg dried bacteria were mixed with internal lipid standards for analysis by LC-MS² using an Aquty I-class ultra-performance
LC and a Xevo TQ-S “triple-quadrupole” instrument, both from Waters. Using a CSH C18 column (2.1 × 100 mm, 1.7 μm; Waters), lipids were measured in reversed phase LC mode with a gradient between 57% solvent A (50% methanol) and 99% solvent B (1% methanol, 99% isopropanol), both containing 0.1% formic acid and 10 mM ammonium formate as additives (supplemental Table S1). In HILIC mode, lipids were separated on a CORTECS HILIC column (2.1 × 150 mm, 1.6 μm; Waters) using a gradient between 100% solvent A (97% propionitrile, 2% 2-butanol, and 1% water) and 100% solvent B (97% methanol, 2% 2-butanol, 1% water, and 10 mM ammonium formate), both containing 0.1% formic acid as additive (supplemental Table S2). The lipids analyzed are listed in supplemental Table S3 and were detected by multireaction monitoring (supplemental Table S4). To increase the sensitivity of the mass spectrometric detection, a solution of 120 mM ammonium formate in methanol was co-injected with the eluent of the column for ESI at a flow rate of 10 μl/min. GlcCer(d18:1;14:0), GlcCer(d18:1;19:0), GlcCer(d18:1;25:0), and GalCer(d18:1;31:0) were used as internal standards to quantify α- and β-anomers of GlcCer and GalCer with NS-, AS-, N-nonhydroxy acyl sphingosine (NP)-, and N-hydroxy acyl sphingosine (AP)-type Cer anchors. For quantification of NP- and AP-sphingolipids with the NS-type standard, the previously determined empirical factor of 12.2 was taken into account (12). Student’s t-tests were conducted with GraphPad Prism (GraphPad Software, San Diego, CA) and P ≤ 0.05 was considered to be statistically significant. Data are presented as the mean ± standard deviation.

RESULTS

Separation of β-GlcCer, β-GalCer, and α-GalCer by HILIC

The diastereomeric GSLs, β-GlcCer, β-GalCer, α-GlcCer (not shown), and α-GalCer are not distinguishable by their fragmentation patterns in triple quadrupole-based MS² (Fig. 1A, B) (59). Furthermore, they are not separated by C18-reversed phase mode with LC (Fig. 1C). The latter is predictable, as the structural differences of these glycolipids are within the hydrophilic moiety and not within the hydrophobic part, which interacts with the reversed phase. With normal-phase high-performance TLC (HPTLC), all four diastereomeric HexCer standards can be separated, if the HPTLC plate is impregnated prior to separation with borate (39, 48) and if they contain identical Cer anchors (supplemental Fig. S1). To quantitatively differentiate all four isomers in biological samples, chromatography, therefore, has to be combined with information about the Cer anchor. Therefore, we set up a method for HILIC coupled MS². Initially, we used solvent gradient systems starting with 95–97% acetonitrile, but did not observe separation of our diastereomeric HexCer mixtures on HILIC columns used in our laboratory (data not shown). Consequently, we increased the hydrophobicity of the starting solvent (solvent A): acetonitrile was exchanged by propionitrile and 2% of water by 2% of 2-butanol (because methanol in solvent A widened the peaks). In a gradient of increasing polarity, solvent A was replaced successively by increasing proportions of solvent B (97% methanol, 2% 2-butanol, 1% water) in a nonlinear fashion with increasing steepness. As a result, β-GlcCer eluted first followed by β-GalCer, α-GlcCer, and finally α-GalCer, all of them containing the identical Cer anchor, Cer(d18:1/24:1). Importantly, β-GlcCer and β-GalCer (R = 1.7), as well as β-GalCer and α-GlcCer (R = 2.3), separated by baseline (R ≥ 1.5, Fig. 2A). Keeping β-GalCer constant at a concentration of 0.1 μg/ml, a dilution series of β-GlcCer followed a linear decreasing peak area and β-GlcCer was detected with a LOD of 2 ng/ml (25 fmol injected), corresponding to 1% of the abundant β-GalCer (Fig. 2B). Vice versa, keeping β-GlcCer constant (0.1 μg/ml), a dilution series of β-GalCer was detected down to a LOD of 7 ng/ml (86 fmol injected), corresponding to 5% of the abundant β-GlcCer (Fig. 2C). Furthermore, α-GalCer (75 ng/ml) could be quantified in the presence of a 110-fold concentration of the β-anomer (8 μg/ml) (Fig. 2D).

Identifying HexCers of different Cer compositions

Next, we compared retention times of endogenous HexCers from mouse organs with our initial and some further HexCer-standards: β-GlcCer [d18:1/(14 or 16 or 19 or 25:0)], keratin [a mixture of NS-type β-GalCers with non-hydroxy fatty acids (N) and sphingosine (S)], and phrenosine [a mixture of AS-type β-GalCers with α(R)-hydroxy fatty acids (A) and sphingosine (S)]. Liver contains mainly NS-type β-GlcCers (60); stomach contains, in addition, substantial amounts of AS- and NP-type β-GlcCers (61); and intestine contains predominantly the AP-type besides AS- and NP-type β-GlcCers (12, 62, 63). Kidney is also enriched in β-GalCers (49, 64) of the NS- and AS-type (60, 65).

Combining data of retention times and associated MS² information (revealing the type of Cer anchor) for standards and biological samples, we observed decreasing retention times with longer acyl chain length (roughly −0.016 ± 0.005 min/CH₂-unit). As compared with NS-β-HexCer, corresponding AS-β-HexCers were delayed by +0.242 ± 0.009 min, NP-HexCer by +0.084 ± 0.004 min, and AP-HexCer by +0.282 ± 0.008 min (Fig. 2E).

Comparison of the fatty acid chain length distribution in β-GlcCer with that in β-GalCers of various mouse tissues

Applying the HILIC-MS²-based separation of β-GlcCer and β-GalCer, we determined the distribution of both compounds in various mouse tissues and, by that, confirming enrichment of β-GalCer mainly in brain (97%) and kidney (42%), and additionally revealing its abundance in lymph nodes (85%) and auricles (59%) (Fig. 3). Furthermore, results confirmed enrichment of nervonic acid within brain cerebrosides, whereas corresponding glucocerebrosides were enriched in stearic acid. The latter is the main acyl chain of neuronal gangliosides. Although not always as clear cut, the acyl chain length distribution of cerebrosides and glucocerebrosides was not identical in any tissue investigated.

The method confirms pathological changes of β-GlcCer and β-GalCer in mouse organs

Gba2 catalyzes the hydrolysis of β-GlcCer to glucose and Cer at the cytosolic side of intracellular membranes (66). Mice lacking Gba2 activity were reported with 2- to 3-fold increased levels of β-GlcCer in liver (67, 68). Analysis of
HexCers from liver, kidney, and intestine of WT and Gba2-deficient mice revealed an increase of β-GlcCers, specifically, in Gba2-deficient mice, but not corresponding β-GalCers (Fig. 4A–C). Interestingly, the increase of intestinal β-GlcCers was restricted to the rather minor NS-type, whereas the AS-type, NP-type, and dominant AP-type of β-GlcCers were not affected. Furthermore, HILIC-MS² signals corresponding to β-GlcCers, but not to β-GalCers, were significantly reduced in liver and kidney samples of mice with a hepatocyte-specific and renal proximal tubulus-specific deficiency of the UGCG (Ugcg<sup>−/−</sup>/AlbCre and Ugcg<sup>−/−</sup>/Pax8Cre), respectively (Fig. 4A, D). However, mainly NS-type β-GlcCers were decreased and AS-type β-GlcCers even increased a little bit in hepatocyte-Ugcg-deficient liver samples. Vice versa, β-GalCer accumulated more than 10-fold in kidneys of CST-deficient mice due to a block in further conversion to sulfatides (Fig. 4D). Here, β-GlcCer concentrations did not change. Likewise, analysis of male kidney cortex samples from Gb3S-deficient mice revealed a more than 2-fold increase of β-GalCers, which was almost exclusively due to an increase of NS-type cerebrosides (Fig. 4E). Levels of β-GlcCers were also not affected by this model. We expected increased levels of cerebrosides (β-GalCer) in kidneys of this model, as murine Gb3S also catalyzes the conversion of β-GalCer into galabiaosylceramide (Gal<sub>d</sub>Cer/Galα4Galβ1Cer/GalOse<sub>3</sub>Cer) (55), a gal-series GSL enriched in male kidneys (69); altogether, these data supported the correct association of chromatographic peaks to cerebrosides (β-GalCers) and glucocerebrosides (β-GlcCers), respectively.

**AS-type cerebrosides with 2R-hydroxyacyl chains are separated from those with 2S-hydroxyacyl chains**

Cerebrosides from brain are enriched in cerebrosides of the AS-type (70), which contain 2R-hydroxy fatty acids (71) and depend on FA2H (56, 72), which specifically incorporates acyl chains with an α-hydroxy group in R-configuration (73). Here, we compared the retention...
times of AS-type cerebrosides with corresponding synthetic standards containing a 2-hydroxyacyl chain with either R- or S-stereochemistry. The β-GalCer standard with a 2R-hydroxystearoyl chain separated from the corresponding compound with 2S-hydroxy fatty acids eluted significantly earlier (−0.27 ± 0.02 min) than AS-type glucocerebrosides (β-GlcCer) with 2R-configuration at the α-hydroxy group and, thus, could be distinguished by this HILIC method. In contrast to brain, stomach contains AS-type β-GlcCer. Normal levels depend on synthesis by FA2H as corresponding enzyme deficiency (Fig. 5H) reduces the signal to about one tenth. Due to the specificity of the enzyme (73), this dependence implies the α-hydroxy acyl group of AS-β-GlcCers from stomach to be of 2R-configuration.

The α-GalCer of B. fragilis is identified by the HILIC-MS² method

B. fragilis has been reported to produce Cers and α-GalCers containing an iso- or anteiso-branched C17-, C18-, and C19-sphinganin and a β(R)-hydroxylated and iso-branched C17-fatty acid (46, 74). Here, we observed α-GalCers from B. fragilis to elute 0.03 min before our calculated retention times (tR = 4.00–4.03 min) for corresponding AS-type α-GalCers, but definitely beyond AS-type β-GalCers (tR = 3.92–3.95 min, Δ = 0.05–0.06 min). This slight forward shift may be due to the hydroxy group of the acyl chain, which is, for B. fragilis, in the 3(β)-position, but not in the 2(α)-position, of the fatty acid. This shift was consistent for all three compounds reported by B. fragilis (Fig. 6A, B).

Corresponding α-GalCers are common to further bacteria of the human gut microbiome

In addition to B. fragilis, we analyzed lipid extracts of nine further bacteria of the human gut microbiome. In P. copri and B. vulgatus, we detected the identical HexCer peaks as for the N-β-hydroxy acyl sphingosine (BS)-type α-GalCers of B. fragilis (Fig. 6C). However, overall levels of these compounds were at least 100-fold lower than in B. fragilis, respectively. The other seven species investigated did not contain quantifiable amounts of α-GalCer.
Nevertheless, *B. ovatus, B. thetaiotaomicron,* and *B. caccae* contained signals for \( \alpha \)-GalCers, which were above the LOD in both the HILIC and the reversed phase LC method (supplemental Table S5).

**Diastereomeric hexosylsphingosines elute differently from the HILIC column**

Finally, we addressed the elution behavior of hexosylsphingosines (HexSphs) with standards for glucosylsphingosine (\( \beta \)-GlcSph), psychosine (\( \beta \)-GalSph), and \( \alpha \)-GalSph, all with a C18-sphingosine as long chain base. Although not baseline separated, the \( \beta \)-GlcSph eluted before \( \beta \)-GalSph \((R = 1.0)\) and \( \alpha \)-GalSph came off the column last \((R = 0.7)\) in the same order as the corresponding HexCers (Fig. 7). Hence, this method may also be useful to identify the type of HexSph present.

**DISCUSSION**

Lipidomics is a fast growing field of interest impacting more and more medical research. Often all-in-one analysis is demanded and high throughput methods like direct infusion into high resolution mass spectrometers with MS\(^2\)-scans and subsequent data processing software try to fulfill these requests (75). These MS-only techniques, however do not distinguish stereoisomeric compounds with qualitatively identical fragmentation behaviors. Separation and quantification of such stereoisomers therefore requires combination of MS with orthogonal methods, such as chromatography or ion mobility. LC, especially, is a widespread technique, often coupled to MS to increase specificity and sensitivity of detection.

Here, we present a HILIC-based method, which enables the separation of \( \alpha \) - and \( \beta \) -anomers of GlcCer and GalCer comprising identical Cer anchors, which was not possible with reversed phase LC. HexCers are amphipathic molecules with their structural differences buried in the polar head group, but not the hydrophobic anchor, the latter interacting with the stationary phase in reversed phase LC and the former in HILIC. Coupling to MS\(^2\) allows identification of differences in Cer anchor composition to unambiguously ascribe NS-, AS-, NP-, and AP-type HexCer-derived HILIC-peaks of biological samples to either GlcCer or GalCer with either \( \alpha \) - or \( \beta \) -glycosidic linkage. The method can be used to analyze complex biological lipid extracts, as demonstrated by the application to various mouse organs and bacteria.

**Fig. 4.** HILIC-MS\(^2\)-based quantification of \( \beta \)-GlcCers and \( \beta \)-GalCers in liver of WT, *Gba2\(^{−/−}\)*, and *Uggg\(^{−/−}\)/AlbCre* mice (A); kidney of WT and *Gba2\(^{−/−}\)* mice (B); small intestine of WT, and *Gba2\(^{−/−}\)* mice (C); kidney of WT, *Uggg\(^{−/−}\)/Pax8Cre*, *CST\(^{−/−}\)/Pax8Cre*, (Uggg and CST\(^{−/−}\)/Pax8Cre* mice (D); and in kidney cortex of WT and *Gb3s\(^{−/−}\)* mice. Note the specific increase of NS-\( \beta \)-GlcCer in liver, kidney and small intestine of *Gba2\(^{−/−}\)* mice, the selective decrease of NS-GlcCer in liver and of NS- and AS-GlcCer in kidney of *Uggg\(^{−/−}\)/AlbCre* and *Uggg\(^{−/−}\)/Pax8Cre* and (Uggg and CST\(^{−/−}\)/Pax8Cre* mice, respectively (E). Vice versa, NS- and AS-GalCer accumulates in kidneys with CST-deficiency [CST\(^{−/−}\)/Pax8Cre* and (Uggg and CST\(^{−/−}\)/Pax8Cre* as well as NS-\( \beta \)-GalCer in cortex of *Gb3s\(^{−/−}\)* mice. n \( ≥ \) 3, except for (D) WT = 1.
The correct assignment of peaks was supported by measuring a selective increase or decrease of β-GlcCer over β-GalCer in various tissues with GBA2 or UGCG deficiencies, respectively. Loss of GBA2 activity leads to the accumulation of the substrate β-GlcCer. In contrast, the lack of UGCG activity either in hepatocytes or in renal tubular cells omitted corresponding production of β-GlcCer, thereby reducing the overall β-GlcCer concentration of liver or kidney, as expected (11, 16, 67, 76). Further confirmation was obtained by detecting a selective increase of β-GalCer over β-GlcCer in organs lacking activity of either CST or Gb3S, for both of which β-GalCer is the substrate to produce either sulfatide SM4s or galabiosylyseramide, respectively (16, 55, 77).

Interestingly, however, the levels of not all β-GlcCer species went up with Gbn2 deficiency and the levels of AS-type β-GlcCers remained unchanged. There are two possible explanations, which are: i) GBA2 may not be expressed in cells that express the AS-type; or ii) the AS-type β-GlcCer resides in lipid layers with no access to the cytosolic activity of GBA2. Whereas loss of UGCG activity in renal tubular epithelial cells caused a decrease of NS- and AS-type β-GlcCers in kidney, the hepatocyte-specific loss of UGCG activity, causing NS-types to go down, even led to an increase of minor AS-type β-GlcCers. Because tissues are multi cell-type structures, the loss of enzyme activity in one specific cell type will always lead only to a decrease of a product and not to complete disappearance, if this product is synthesized by several cell types of the respective organ. An increase of a subspecies may imply either an increased relative abundance of the corresponding synthesizing cell type in mutant tissue or a reactive increased production or decreased catabolism of these compounds within the corresponding cells.

The method on top separates AS-type cerebrosides containing the α-hydroxy group of the acyl chain either in 2R- or 2S-configuration. Both configurations may appear in biological samples and AS-type cerebrosides of the brain were reported to contain basically the 2R-configuration (71), which is confirmed with this HILIC-MS² method.

Screening different mouse tissues with this method, we further confirmed high abundance of cerebrosides in brain and kidney, whereas most other tissues, including liver and spleen, are dominated by the presence of glucocerebrosides.
Two exceptions were identified: auricles and, especially, lymph nodes are relatively abundant in cerebrosides, as compared with glucocerebrosides, and further investigations need to address from which cell types they originate.

As this method delivers data for individual species, it allows detection of differences in Cer anchor compositions between cerebrosides and glucocerebrosides or following of changes in these compositions upon aging, activation, or disease progression. Here, we plotted the relative acyl chain length distributions of cerebrosides against those of glucocerebrosides found in WT mouse tissues. As expected, brain cerebrosides are rich in nervonic acid, a major component of myelin sheaths (60, 78, 79). In contrast, the small amount of brain glucocerebrosides is synthesized mainly with stearic acid. This points to neuronal cell origin, as these cells convert this compound to complex gangliosides, such as GM1a, GD1a, GD1b, or GT1b, all of which are known to basically contain stearic acid in their lipid anchor (80, 81). Likewise, nervonic acid was the major fatty acid incorporated into cerebrosides of colon, small intestine, thymus, and lymph nodes, whereas glucocerebrosides of these organs were enriched with the shorter palmitic acid. In summary, this points to different cell types or differentiation stages of cells forming either cerebrosides or glucocerebrosides and expressing different pattern of Cer synthases, which remains to be investigated.

It may be noted that this investigation did not include HexCers with other sphingoid bases than C18-sphingosine and C18-phytosphingosine or acyl chains shorter than C16 or longer than C26, excluding, thereby, compounds such as those with C20-sphingosine that are described for aging brain (82) or renal papillae (65) or the well-known sphingolipids with ultra-long acyl chains (>C26) specific for epidermis or male germ cells (83).

In contrast to the afore-discussed cerebrosides and glucocerebrosides, iNKT cells are activated when recognizing GalCer with an α-glycosidic linkage presented on the cell surface receptor, CD1d, of antigen-presenting cells (41, 84). One natural source of this compound in contact with the human body is B. fragilis, a bacterial member of the human gut microbiome (46). Here, we show detection of α-GalCers from B. fragilis by HILIC-MS2 with unique retention times, different from the β-glycosidic mammalian counterparts. How many other bacteria of the human gut microbiome may also express these immune stimulatory compounds has to be elucidated, as well as their potential role in colitis, when the intestinal barrier gets leaky for bacteria or in sepsis. To elucidate the use of HILIC-MS2 for screening of bacteria that express α-GalCer, we analyzed an initial set of 10 bacteria of the human gut microbiome. At least two other bacteria were detected with quantifiable amounts of these compounds and another three strains delivered signals corresponding to α-GalCer, which were above the lower detection limit. Although concentration of α-GalCer in all five of these strains was at least 100-fold lower than in B. fragilis, the results show that there might be a remarkably high number of bacteria present in our gut, which is capable of synthesizing the immunogenic α-anomeric variant of GalCer. These results underline the power of the method to screen for such compounds and further support the idea that α-GalCer is a natural CD1d-ligand for iNKT cell activation.

Very low amounts of endogenously produced α-GalCer (and eventually α-GlcCer) have also been discussed to contribute to successive iNKT cell maturation (39). In this context, endolysosomal catabolism of α-GalCer has been discussed to regulate levels of α-GalCer on antigen-presenting cells. Cerebrosidase and GBA are specific for β-glycosylceramides and seem not to turn over α-GalCer. Catabolism rather appears to be initiated by acidic ceramidase releasing the lyso-compound, α-GalSph, which, in contrast to α-GalCer, is a substrate for α-galactosidase. Either pharmacological inhibition of these enzymes or
defective activity in Fabry disease thereby induces a pro-inflammatory phenotype of iNKT cells (39, 85–87). Under pathological conditions of Krabbe disease, metachromatic leukodystrophy, or Gaucher disease, when either cerubrósidae or GBA is dysfunctional, significant amounts of β-GalSph or β-GlcSph accumulate due to turnover by acidic ceramidade, and these compounds contribute to the pathology of the disease (25, 26, 88, 89). Here, significantly different retention times were achieved for the three types of HexSphs, making the HILIC-MS² method a tool to identify the type of HexSph appearing and to study concentration changes of these cell-toxic or immune-stimulatory compounds.

In summary, the HILIC method presented here in combination with detection by MS² is useful to selectively identify and quantify small amounts of the diastereomers, β-GlcCer, β-GalCer, α-GalCer, and corresponding lyso-compounds from complex biological lipid extracts, without any prior derivatization step.

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REFERENCES

1. Schulte, S., and W. Stoffel. 1993. Ceramide UDPgalactosyltransferase from myelinating rat brain: purification, cloning, and expression. Proc. Natl. Acad. Sci. USA. 90: 10260–10269.
2. Ichikawa, S., N. Nakajo, H. Sakiyama, and Y. Hirabayashi. 1994. A mouse B16 melanoma mutant deficient in glycolipids. Proc. Natl. Acad. Sci. USA. 91: 2703–2707.
3. Kolter, T., and K. Sandhoff. 1999. Sphingolipids—their metabolic pathways and the pathobiology of neurodegenerative diseases. Angew. Chem. Int. Ed. 38: 1532–1568.
4. Jackel, D., A. Karrenbauer, K. N. Burger, G. van Meer, and F. Wieland. 1992. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. J. Cell Biol. 117: 259–267.
5. Futerman, A. H., and R. E. Pagano. 1991. Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. Biochem. J. 280: 295–302.
6. Burger, K. N., P. van der Bijl, and G. van Meer. 1996. Topology of sphingolipid galactosyltransferases in ER and Golgi: transbilayer movement of monohexosyl sphingolipids is required for higher glycosphingolipid biosynthesis. J. Cell Biol. 133: 15–28.
7. Makita, A., and N. Taniguchi. 1985. Glycosphingolipids. In Glycolipids. H. Wiegandt, editor. Elsevier, Amsterdam, 1–99.
8. Yamashita, T., R. Wada, T. Sasaki, C. Deng, U. Bierfreund, K. Sandhoff, and R. L. Proia. 1999. A vital role for glycosphingolipid synthesis during development and differentiation. Proc. Natl. Acad. Sci. USA. 96: 9142–9147.
9. Jennemann, R., R. Sandhoff, S. Wang, E. Kiss, N. Greitz, C. Zuliani, A. Martin-Villalba, R. Jager, H. Schorle, M. Kenzelmann, et al. 2005. Cell-specific deletion of glucosylceramide synthase in brain leads to severe neural defects after birth. Proc. Natl. Acad. Sci. USA. 102: 12459–12464.
10. Jennemann, R., R. Sandhoff, L. Langbein, S. Kaden, U. Rothermel, H. Gallala, K. Sandhoff, H. Wiegandt, and H. J. Grone. 2007. Integrity and barrier function of the epidermis critically depend on glucosylceramide synthesis. J. Biol. Chem. 282: 3083–3094.
11. Jennemann, R., U. Rothermel, S. Wang, R. Sandhoff, S. Kaden, R. Out, T. J. van Berkel, J. M. Aerts, K. Ghaouharali, C. Sticht, et al. 2010. Hepatic glycosphingolipid deficiency and liver function in mice. Hepatology. 51: 1799–1809.
12. Jennemann, R., S. Kaden, R. Sandhoff, V. Nordström, S. Wang, M. Volz, S. Robine, N. Amen, U. Rothermel, H. Wiegandt, et al. 2012. Glycosphingolipids are essential for intestinal endocytic function. J. Biol. Chem. 287: 32909–32916.
13. Amen, N., D. Mathow, M. Rabionet, R. Sandhoff, L. Langbein, N. Greitz, C. Jackel, H. J. Grone, and R. Jennemann. 2013. Differentiation of epidermal keratinocytes is dependent on glucosylceramide: ceramide processing. Hum. Mol. Genet. 22: 4164–4179.
14. Nordström, V., M. Wilkershauser, S. Herzet, J. Rozman, O. von Bohlen Und Halbach, S. Meldner, U. Rothermel, S. Kaden, F. C. Roth, C. Waldeck, et al. 2013. Neuronal expression of glucosylceramide synthase in central nervous system regulates body weight and energy homeostasis. PLoS Biol. 11: e1001506.
15. Rabionet, M., A. Bayerle, R. Jennemann, H. Heid, J. Fuchs, C. Marsching, S. Porubsky, C. Bolenz, F. Guillou, H. J. Grone, et al. 2015. Male meiotic cytokinesis requires ceramide synthase 3-dependent sphingolipids with unique membrane anchors. Hum. Mol. Genet. 24: 4792–4808.
16. Stettner, P., S. Bourgeois, C. Marsching, M. Traykova-Brauch, S. Porubsky, V. Nordström, C. Hopf, R. Koesters, R. Sandhoff, H. Wiegandt, et al. 2013. Sulfatides are required for renal adaptation to chronic metabolic acidosis. Proc. Natl. Acad. Sci. USA. 110: 9998–10003. [Erratum. Proc. Natl. Acad. Sci. USA. 110: 14813.]
17. Yamashita, T., M. L. Allende, D. N. Kalkofen, N. Werth, K. Sandhoff, and R. L. Proia. 2005. Conditional LoxP-flanked glucosylceramide synthase allele controlling glycosphingolipid synthesis. Genesis. 43: 175–180.
18. Watanabe, S., S. Endo, E. Oshima, T. Hoshi, H. Higashi, K. Yamada, K. Tohyama, T. Yamashita, and Y. Hirabayashi. 2010. Glycosphingolipid synthesis in cerebellar Purkinje neurons: roles in myelin formation and axonal homeostasis. Glia. 58: 1197–1207.
19. Seito, N., T. Yamashita, Y. Tsukuda, Y. Matsu, A. Urita, T. Onodera, T. Mizutani, H. Haga, N. Fujitani, Y. Shinohara, et al. 2012. Interruption of glycosphingolipid synthesis enhances osteoarthritis development in mice. Arthritis Rheum. 64: 2579–2588.
20. Rosio, A., E. Binczek, and W. Stoffel. 1996. Functional breakdown of the lipid bilayer of the myelin membrane in central and peripheral nervous system by disrupted galactocerebrosides synthesis. *Proc. Natl. Acad. Sci. USA.* 93: 13280–13285.

21. Coetzee, T., N. Fujita, J. Dupree, R. Shi, A. Blight, K. Suzuki, and B. Popko. 1995. Myelination in the absence of galactocerebrosides and sulfatide: normal structure with abnormal function and regional instability. *Cell.* 86: 209–219.

22. Sandhoff, K. 2013. Metabolic and cellular bases of sphingolipidoses. *Biochem. Soc. Trans.* 41: 1562–1568.

23. Sandhoff, K. 2016. Neuronal sphingolipidoses: membrane lipids and sphingolipid activator proteins regulate lysosomal sphingolipid catabolism. *Biochim. Biophys. Acta.* 1858: 146–151.

24. Beutler, E., and G. A. Grabowski. 2001. Gaucher disease. In *The Metabolic and Molecular Bases of Inherited Disease.* C. R. Scriver, A. L. Beaudet, W. S. Sly, et al., editors. McGraw-Hill, New York. 3653–3668.

25. Grabowski, G. A., G. A. Petsko, and E. H. Kolodny. 2017. Gaucher disease. In *The Online Metabolic and Molecular Bases of Inherited Disease.* D. Valle, A. L. Beaudet, B. Vogelstein, et al., editors. McGraw-Hill Education, New York.

26. Wenger, D. A., M. Escolar, P. Luzi, and M. A. Rafi. 2017. Krabbe disease (globoid cell leukodystrophy). In *The Online Metabolic and Molecular Bases of Inherited Disease.* D. Valle, A. L. Beaudet, B. Vogelstein, et al., editors. McGraw-Hill Education, New York.

27. Wenger, D. A., K. Suzuki, Y. Suzuki, and K. Suzuki. 2001. Galactosylceramide lipidosis. Globoid cell leukodystrophy (Krabbe disease). In *The Metabolic and Molecular Bases of Inherited Disease.* C. R. Scriver, A. L. Beaudet, W. S. Sly, et al., editors. McGraw-Hill, New York. 3669–3694.

28. Sidransky, E. 2004. Gaucher disease: complexity in a “simple” disorder. *Nat. Genet.* 36: 8–13.

29. Thomas, A. S., A. Mehta, and D. A. Hughes. 2014. Gaucher disease: haematological presentations and complications. *Br. J. Haematol.* 165: 427–440.

30. Smid, B. E., M. J. Ferraz, M. Verhoek, M. Mirzaian, P. Wisse, H. S. Overkleeft, C. E. Hollak, and J. M. Aerts. 2016. Biochemical response to substrate reduction therapy versus enzyme replacement therapy in Gaucher disease type I patients. *Orphanet J. Rare Dis.* 11: 28.

31. Deegan, P., D. Fernandez-Sasso, P. Giraldo, H. Lau, Z. Panahlool, and A. Zimran. Treatment patterns from 647 patients with Gaucher disease: an analysis from the Gaucher Outcome Survey. *Blood Cells Mol. Dis.* Epub ahead of print. October 20, 2016; doi:10.1016/j.bcmd.2016.10.014.

32. Futerman, A. H., and F. M. Platt. 2017. The metabolism of glucocerebrosides - from 1965 to the present. *Mol. Genet. Metab.* 120: 22–26.

33. Ilan, Y., D. Elstein, and A. Zimran. 2009. Glucocerebrosidase: an evolutionary advantage for patients with Gaucher disease and a new immunomodulatory agent. *Immunol. Cell Biol.* 87: 514–524.

34. G. Aerts, J. C. Allegood, S. Kelly, and E. Webb. 2004. Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods.* 36: 207–224.

35. Shaver, R. L., J. C. Allegood, H. Park, E. Wang, S. Kelly, C. A. Haynes, M. C. Sullivan, and A. H. Merrill. Jr. 2009. Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole time-of-flight mass spectrometers. *J. Lipid Res.* 50: 1592–1707.

36. Boutin, M., Y. Sun, J. J. Shacka, and C. Auvray-Blais. 2016. Tandem mass spectrometry multiplex analysis of glucosylceramide and galactosylceramide isoforms in brain tissues at different stages of Parkinson disease. *Anal. Chem.* 88: 1856–1863.

37. Nakajima, K., H. Akiyama, K. Tanaka, A. Kohyama-Kogane, P. Greimel, and Y. Hirabayashi. 2016. Separation and analysis of mono-glucosylated lipids in brain and skin by hydrophilic interaction chromatography based on carbohydrate and lipid moiety. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1031: 146–155.

38. Yildiz, Y., H. Matern, B. Thompson, J. C. Allegood, R. L. Warren, D. M. Ramirez, R. E. Hammer, F. K. Hamra, S. Matern, and D. W. Russell. 2006. Mutation of betaglucocerebrosidase 2 causes glycogenesis, lipodystrophy, and impaired male fertility. *Clin. Invest.* 116: 2983–2994.

39. Porubsky, S., A. O. Speak, M. Salio, R. Jennemann, M. Bonrouhi, R. Zafarulla, Y. Singh, J. Dyson, B. Luckow, A. Lehen, et al. 2012. Globosides but not isoglobosides can impact the development of invariant NKT cells and their interaction with dendritic cells. *J. Immunol.* 189: 5007–5017.

40. Zoller, I., M. Meixner, D. Hartmann, H. Bussow, R. Meyer, V. Gieselman, and M. Eckhardt. 2008. Absence of 2-hydroxylated sphingolipids is compatible with normal neural development but causes late-onset axon and myelin sheath degeneration. *J. Neurosci.* 28: 9741–9754.

41. Fley, A., D. Greenwood, and F. O’Grady. 1985. Comparative growth of Bacteroides species in various anaerobic culture media. *J. Med. Microbiol.* 19: 195–201.

42. Kabioni, M., A. C. van der Spoel, C. C. Chuang, B. von Tunpling-Radosta, M. Lijtens, D. Bouwmeester, C. C. Hellbusch, C. Korner, H. Wiegandt, K. Gorgas, et al. 2008. Male germ cells require polysaccharide sphingolipids with complex glycosylation for completion of meiosis: a link to ceramide synthesis-2. *J. Biol. Chem.* 283: 13557–13560.

43. Hsu, F. F., and J. Turk. 2001. Structural determination of glycosphingolipids as lihiated adducts by electrospray ionization mass spectrometry using low-energy collisional-activated dissociation on a triple stage quadrupole instrument. *J. Am. Soc. Mass Spectrom.* 12: 619–79.

44. Imgrund, S., D. Hartmann, H. Farwana, M. Eckhardt, R. Sandhoff, J. Degen, V. Gieselman, K. Sandhoff, and K. Willecke. 2009. Increased sphingolipid turnover in the spleen of MRL/lpr mice. *Lab. Anim.* 43: 135–140.
Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. 

Bouhours, D., and J. F. Bouhours. 1985. Developmental changes of the lipidic part of the neutral glycosphingolipids of the rat stomach. J. Biol. Chem. 260:2172–2177.

Umesaki, Y., A. Suzuki, T. Kasama, K. Tolyama, M. Mutai, and T. Yamakawa. 1981. Presence of asialo GM1 and glucosylceramide in the intestinal mucosa of mice and induction of fucosyl asialo GM1 by conventionalization of germ-free mice. J. Biochem. 90:1731–1738.

Okabe, K., R. W. Keenan, and G. Schmidt. 1968. Phosphoglycosine groups as quantitatively significant components of the sphingolipids of the muscosa of the small intestines of some mammalian species. Biochem. Biophys. Res. Commun. 31:137–143.

Adams, E. P., and G. M. Gray. 1968. The carbohydrate structures of the neutral ceramide glycolipids in kidneys of different mouse strains with special reference to the ceramide dehydroxosides. Chem. Phys. Lipids. 2:147–155.

Marching, C., M. Rabionet, D. Mathow, R. Jennemann, C. Kremser, S. Porubsky, C. Bolenz, K. Willecke, H. J. Grone, C. Hopf, et al. 2014. Renal sulfatides: sphingoid base-dependent localization and region-specific compensation of CerS2 dysfunction. J. Lipid Res. 55:2354–2369.

Körschen, H. G., Y. Yildiz, D. N. Raju, S. Schonauer, W. Bonigk, V. Jansen, E. Kremmer, U. B. Kaupp, and D. Wachtel. 2013. The non-lysosomal beta-glucosidase GBA2 is a non-integral membrane-associated protein at the endoplasmic reticulum (ER) and Golgi. J. Biol. Chem. 288:3381–3393.

Yildiz, Y., P. Hoffmann, S. Vom Dahl, B. Breiden, R. Sandhoff, C. Niederau, M. Horwitz, S. Karlsson, M. Filacamo, D. Elstein, et al. 2013. Functional and genetic characterization of the non-lysosomal glucosylceramidase 2 as a modifier for Gaucher disease. Orphanet J. Rare Dis. 8:151.

Gonzalez-Carmona, M. A., R. Sandhoff, F. Tacke, A. Vogt, S. Weber, A. E. Canbay, G. Rogler, T. Sauerbruch, F. Lammert, and Y. Yildiz. 2012. Beta-glucosidase 2 knockout mice with increased glucosylceramide show impaired liver regeneration. Liver Int. 32:1534–1536.

Coles, L., J. B. Hay, and G. M. Gray. 1970. Factors affecting the glycosphingolipid composition of murine tissues. J. Lipid Res. 11:158–165.

Kishimoto, Y., and N. S. Radin. 1963. Occurrence of 2-hydroxy fatty acids in animal tissues. J. Lipid Res. 4:139–143.

Hammarström, S. 1969. Configuration of 2-hydroxy acids from brain cerebrosides determined by gas chromatography. FEBS Lett. 5:192–195.

Eckhardt, M., A. Yaghootfam, S. N. Fewou, I. Zoller, and V. Gieselman. 2005. A mammalian fatty acid hydroxylase responsible for the formation of alpha-hydroxylated galactosylceramide in myelin. Biochem. J. 388:249–254.

Guo, L., X. Zhang, D. Zhou, A. L. Okunade, and X. Su. 2012. Stereospecificity of fatty acid 2-hydroxylase and differential functions of 2-hydroxy fatty acid enantiomers. J. Lipid Res. 53:1327–1335.

Miyagawa, E., R. Azuma, T. Suto, and I. Yano. 1979. Occurrence of free ceramides in Bacteroides fragilis NCTC 9343. J. Biochem. 86:311–320.

Simons, B., D. Kauhanen, T. Sylvanne, K. Tarasov, E. Duchoslav, and K. Ekroos. 2012. Shotgun lipidomics by sequential precursor ion fragmentation on a hybrid quadrupole time-of-flight mass spectrometer. Metabolites. 2:195–213.

Walden, C. M., R. Sandhoff, C. C. Chuang, Y. Yildiz, T. D. Butters, R. A. Dwek, F. M. Platt, and A. C. van der Spoel. 2007. Accumulation of glucosylceramide in murine testis, caused by inhibition of beta-glucosidase 2: implications for spermatogenesis. J. Biol. Chem. 282:32655–32664.

Ogawa, D., K. Shikata, K. Honke, S. Sato, M. Matsuda, R. Nagase, A. Tone, S. Okada, H. Ushii, J. Wada, et al. 2004. Cerebroside sulfotransferase deficiency ameliorates L-selectin-dependent monocyte infiltration in the kidney after ureteral obstruction. J. Biol. Chem. 279:2085–2090.

Kishimoto, Y., and N. S. Radin. 1959. Composition of cerebroside acids as a function of age. J. Lipid Res. 1:79–82.

Radin, N. S., and Y. Akahori. 1961. Fatty acids of human brain cerebrosides. J. Lipid Res. 2:335–341.

Ginkel, C., D. Hartmann, K. Vom Dorp, A. Zlomuzica, H. Farwanah, M. Eckhardt, R. Sandhoff, J. Degen, M. Rabionet, E. Dere, et al. 2012. Ablation of neuronal ceramide synthase 1 in mice decreases ganglioside levels and expression of myelin associated glycoprotein in oligodendrocytes. J. Biol. Chem. 287:41888–41902.

Kishimoto, Y., and N. S. Radin. 1966. Determination of brain gangliosides by determination of ganglioside stearic acid. J. Lipid Res. 7:141–145.

Rosenberg, A., and N. Stern. 1966. Changes in sphingosine and fatty acid components of the gangliosides in developing rat and human brain. J. Lipid Res. 7:122–131.

Sandhoff, R. 2010. Very long chain sphingolipids: tissue expression, function and synthesis. FEBS Lett. 584:1907–1913.

Brossay, L., M. Chiòfa, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J. Exp. Med. 188:1521–1528.

Pereira, C. S., O. Azevedo, M. L. Maia, A. F. Dias, C. So-Miranda, and M. F. Macedo. 2013. Invariant natural killer T cells are phenotypically and functionally altered in Fabry disease. Mol. Genet. Metab. 108:241–248.

Darmoise, A., S. Teneberg, L. Bouzonville, R. O. Brady, M. Beck, S. H. Kaufmann, and F. Winau. 2010. Lysosomal alpha-galactosidase controls the generation of self lipid antigens for natural killer T cells. Immunity. 33:216–228.

Kim, J., J. H. Kim, and F. Winau. 2014. Thinking inside the box: endogenous alpha-anomeric lipid antigens. Immunity. 41:505–506.

Gieselman, V., and I. Krägeloh-Mann. 2017. Metachromatic leukodystrophy. In The Online Metabolic and Molecular Bases of Inherited Disease. D. Valle, A. L. Beaudet, B. Vogelstein, et al., editors. McGraw-Hill Education, New York.

Toda, K., T. Kobayashi, I. Goto, K. Ohno, Y. Eto, K. Inui, and S. Okada. 1990. Lysosulfatide (sulfogalactosylsphingosine) accumulation in tissues from patients with metachromatic leukodystrophy. J. Neurochem. 55:1585–1591.