Identification of Niemann-Pick C1 (NPC1) disease biomarkers through sphingolipid profiling

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Abbreviations used: CER, ceramide; CSF, cerebrospinal fluid; CNS, central nervous system; DC, dihydroceramide; ESI, electrospray ionization; FA, formic acid; GM(1/2/3), gangliosides; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; HPLC, high performance liquid chromatography; LC, lactosylceramide; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MC, monohexosylceramide; NIH, National Institutes of Health; NPC1, Niemann-Pick type C1; QC, quality control; SPH, sphingosine; S'PH, sphinganine; S1P, sphingosine-1-phosphate; S'1P, sphinganine-1-phosphate; SM, sphingomyelin; ST, sulfatide
ABSTRACT
Niemann-Pick C1 (NPC1) is rare neurodegenerative disease for which treatment options are limited. A major barrier to development of effective treatments has been the lack of validated biomarkers to monitor disease progression or serve as outcome measures in clinical trials. Using targeted metabolomics to exploit the complex lipid storage phenotype that is the hallmark of NPC1 disease, we broadly surveyed Npc1−/− mouse tissues and identified elevated species across multiple sphingolipid classes that increased with disease progression. There was striking accumulation of sphingoid bases, monohexosylceramides and GM2 gangliosides in liver, and sphingoid bases and GM2 and GM3 gangliosides in brain. These lipids were modestly decreased following miglustat treatment, but markedly decreased in response to treatment with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), two drugs that have shown efficacy in NPC1 animal models. Extending these studies to human subjects led to identification of sphingolipid classes that were significantly altered in the plasma of NPC1 patients. Plasma monohexosylceramides and ceramides were elevated, whereas sphingoid bases were reduced in NPC1 subjects. Intervention with miglustat in NPC1 patients was accompanied by striking alterations in plasma (reductions in GM1 and GM3 gangliosides) and CSF (increased monohexosylceramides) sphingolipids. Similar alterations were observed in the CSF from the NPC1 feline model following HP-β-CD treatment. Our findings suggest that these lipid biomarkers may prove useful as outcome measures for monitoring efficacy of therapy in clinical trials.

Key words: Biomarkers, Metabolomics, Neurodegeneration, Niemann-Pick C, Sphingolipids
Niemann-Pick type C (NPC) disease is a rare, progressive neurodegenerative disorder with an estimated incidence in Western Europeans of 1 in 100,000 (1). This number is likely an underestimate given the difficulty in establishing the diagnosis and analysis of NPC1 carrier frequency from large-scale exome sequencing that would predict an incidence of 1 in 20,000 (H. Runz, personal communication). Approximately 95% of NPC cases are caused by mutations of the NPC1 gene located on chromosome 18q11 (2), the remaining 5% are caused by mutations in the NPC2 gene mapped to chromosome 14q24.3 (3, 4). Affected individuals typically present in early childhood with ataxia and progressive impairment of motor and intellectual function, and usually die in adolescence, though increasingly NPC disease is being recognized among the adult population with cognitive defects.

At the cellular level, the underlying mutations in NPC1 and NPC2 genes profoundly affect the intracellular trafficking of cholesterol and, as a consequence, lead to lysosomal accumulation of multiple lipid species (5). The lipid storage phenotype is observed in almost all tissues examined and is characterized by marked elevations of unesterified cholesterol and complex sphingolipids (e.g., mono- and dihexosylceramides and gangliosides) (6, 7). The lysosomal lipid accumulation in NPC1 animal models is accompanied by cellular oxidative stress, as evidenced by increased levels of reactive oxygen species (ROS), marked elevation of cholesterol oxidation products, and hallmarks of chronic oxidative damage (8-10). These complex lipid storage events in NPC1 disease, like other sphingolipidoses, are also associated with alterations in neuronal morphology, including formation of meganeurites, ectopic dendrites, and axonal spheroids (11). NPC disease is also characterized by neurodegeneration, including the presence of intracellular protein aggregates in the form of neurofibrillary tangles in cerebral cortex, neuroinflammation, and a well-defined patterned loss of cerebellar Purkinje cells (11, 12).

Many of the prominent neuropathological features of human NPC disease (e.g., neuronal lipid storage and progressive loss of Purkinje cells) are recapitulated in the BALB/c Npc<sup>ahl</sup> (Npc1<sup>−/−</sup>) mouse, a spontaneously occurring inbred model that harbors a retroposon insertion in the Npc1 gene (13-15). In the Npc1<sup>−/−</sup> mice, accumulation of unesterified cholesterol and gangliosides occurs in morphologically normal neurons at birth and thus precedes neuronal injury and cell loss (11, 16). Concomitant with the lipid accumulation, brains of Npc1<sup>−/−</sup> mice exhibit microglial activation and infiltration and expression of pro-inflammatory mediators (16-18). The clinical, neuropathological and biochemical abnormalities present in juvenile-onset patients are similarly faithfully modeled in a naturally-occurring feline model of NPC disease, which has a missense
mutation in the \( Npc1 \) gene (19, 20). Intervention with miglustat, an inhibitor of glycosphingolipid biosynthesis, or 2-hydroxypropyl-\( \beta \)-cyclodextrin (HP-\( \beta \)-CD), a cholesterol-binding agent, alleviates neuronal ganglioside (miglustat and HP-\( \beta \)-CD) and cholesterol (HP-\( \beta \)-CD) storage in the NPC1 mouse model, and markedly prolongs survival in NPC1 mouse and cat models (21-26). These promising pre-clinical studies have led to administration of HP-\( \beta \)-CD in four NPC1 patients through investigator-sponsored single-patient Investigational New Drugs (INDs) in the U.S. and two NPC1 patients in Japan (27), and laid the groundwork for a Phase 1 trial for HP-\( \beta \)-CD that was initiated in January 2013 at the NIH.

A major challenge for developing treatments for rare diseases is extending promising results from early phase clinical results to Phase 2/3 efficacy studies due to the limited number of study subjects and, in the case of NPC1 disease, the reliance on clinical outcome measures that require long-term observation and are difficult to quantify (28). The availability of rigorous, quantifiable, non-invasive, biochemical disease markers would provide outcome measures that potentially could facilitate clinical study design by increasing power and accelerating evaluation of therapeutic efficacy in clinical trials. In the present study, we hypothesized that the complex sphingolipid storage phenotype in NPC1 disease would provide opportunity for discovery of disease biomarkers for guiding evaluation of therapeutics for this disorder. In the present study, we performed mass spectrometry-based targeted metabolomic profiling for sphingolipid species in blood and tissue samples from \( Npc1^- \) mice and cerebrospinal fluid (CSF) samples from the feline NPC1 model, and extended these discovery efforts to analysis of plasma and CSF samples obtained from human NPC1 subjects enrolled in an NIH-sponsored natural history study. We found several sphingolipid species were significantly altered in the plasma of NPC1 subjects as compared to age-matched controls, and that these complex lipids responded to therapeutic intervention both in the murine and feline models and in the plasma and CSF of human subjects. We propose that these sphingolipid biomarkers may prove useful as outcome measures for monitoring efficacy of therapy and accelerating approval of new therapies.
MATERIALS AND METHODS

Animals
BALB/c Npc<sup>nth</sup> (<i>NpcI<sup>−/−</sup></i>) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and have been maintained in a breeding colony at Washington University. NPC1 cats were raised in the animal colony of the School of Veterinary Medicine at the University of Pennsylvania under NIH and USDA guidelines for the care and use of animals in research (25). Experimental procedures were approved by the Washington University and University of Pennsylvania Animal Studies Committees and were conducted in accordance with the USDA Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

Human subjects
The human studies adhere to the principles of the Declaration of Helsinki, as well as to Title 45, US Code of Federal Regulations, Part 46, Protection of Human subjects. Fasting NPC1 plasma samples were obtained from individuals enrolled in NIH protocol 06-CH-0186 (Evaluation of Biochemical Markers and Clinical Investigation of Niemann-Pick Disease, type C; PI: F.D. Porter). This clinical protocol was approved by the NICHD Institutional Review Board and the analysis of coded human samples was approved by the Human Studies Committee at Washington University. Informed written consent and assent, when possible, were obtained and documented in the medical record.

Reagents
Internal sphingolipid standards were purchased from commercial sources if available (Matreya Inc, Pleasant Gap, PA, USA; AVANTI Polar Lipids Inc, Alabaster, AL, USA). Two deuterated ceramides (d18:1 C22:0 d<sub>4</sub>, d18:1 C24:0 d<sub>4</sub>) were prepared in house.

Administration of HP-β-CD and miglustat to animals
<i>NpcI<sup>−/−</sup></i> and <i>NpcI<sup>+/+</sup></i> mice were treated with HP-β-CD, miglustat, or a combination of the two drugs. Starting at postnatal day 7 (P7), some mice were given a subcutaneous injection of HP-β-CD at a dose of 4000 mg/kg. The volume administered was based on body weight (20 µL/ 1 gm mouse), such that a 5 gram mouse would receive 0.1 cc HP-β-CD solution. Injections were given using a 0.5 cc or 1 cc insulin syringe. Mice receiving miglustat were administered an intraperitoneal injection of 300 mg/kg miglustat starting at P10 and continuing daily until...
weaning at approximately P23. Again, the volume of administration was based on body weight (10 µL/1 gm mouse) such that a 5 gram mouse would receive 0.05 cc miglustat solution. Injections were given using a 0.5 cc insulin syringe. At weaning, mice were switched to an oral dose of 1200 mg/kg, which was given daily in their powdered chow (29). Oral administration continued until mice were sacrificed. Finally, some mice received a combination of the two treatments as listed above. All mice in this study were housed in individual cages and fed powdered chow daily starting at weaning. Treatment of NPC1 cats with HP-β-CD was performed as described previously (25). The experimental protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Sample preparation

Mouse tissue samples (approximately 50mg) were homogenized in 1 ml of PBS. To 100 µL of homogenate sphingolipid internal standards were added, followed by protein precipitation with methanol, and collection of the supernatant. Extraction efficiency and recovery was tested with different organic solvents. Recoveries were assessed using internal standards for each lipid class due to unavailability of many authenticated standards for sphingolipids. For the protein precipitation method, methanol provided the optimal extraction efficiency for most lipid classes: 90.8% for CER 22:0 d4, 92.1% for CER 24:0 d4, 93.4% for LC 16:0 d35, 77.5% for SPH C17, 81.2% for S1P C17, 101.2% for SM C17 and 105.5% for MC 18:0 d3. The supernatant was adjusted to 1 mL of 1:1 (v/v) methanol/water solution for HPLC/MS/MS. Human plasma samples (50 µL each) were prepared by spiking in sphingolipid internal standards followed by addition of methanol for plasma protein precipitation. After removing the proteins, the supernatant was also adjusted to 1 mL of 1:1 (v/v) methanol/water. Human and cat CSF samples (100 µL each) were prepared by spiking in sphingolipid internal standards followed by addition of methanol and water to adjust the volume to 500 µL of 1:1 (v/v) methanol/water. A pooled lipid extract from samples was used as quality control (QC) sample, and injected between every 8 samples to verify the instrumental consistency.

Instrumentation

An online column trapping HPLC/MS/MS system was established using an API-4000 mass spectrometer (Applied Biosystems, Foster City, CA, USA), connected with a Valco electro-actuated valve (Valco Instruments Co, Houston, TX, USA), two LC systems (Shimadzu 20AR: Shimadzu Scientific Instruments, Columbia, MD, USA; Agilent 1100: Agilent Technologies, Santa Clara, CA, USA), and a Leap HTC PAL autosampler (Leap Inc, Carrboro, NC, USA). Thermo Betasil C18 trapping columns (2.1 x 10 mm and 4.6 x 10
mm, 5 µm parcel size (Thermo Scientific Co., Waltham, MA, USA)) and four analytical HPLC columns (Thermo Betasil C18, 2 x 100 mm, 5 µm; Agilent Eclipse XDR-C18, 3 x 100 mm, 3.5 µm; Agilent Zorbax Eclipse C18, 3 x 50 mm, 3.5 µm; Varian Metasil C18, 2 x 100 mm, 3 µm (Varian Co, Palo Alto, CA, USA)) were used for sphingolipid analysis. Mobile phases of 1.5% formic acid (FA) in water and acetonitrile were mainly used for trapping. Mobile phases for analytical HPLC are described in the quantification of sphingolipids sections below. Multiple reaction monitoring (MRM) with Q₁ and Q₃ ions using positive (or negative) ion electrospray (ESI) mode was applied, and Analyst 1.51v software (Applied Biosystems) was used to quantify each sphingolipid.

**Quantification of sphingosine (SPH), sphinganine (S'PH), sphingosine-1-phosphate (S1P), sphinganine-1-phosphate (S'1P)**

Samples containing spiked C17-SPH and C17-S1P as internal standards were injected onto the online trapping HPLC/MS/MS system for SPH, S'PH, S1P and S'1P. Calibration samples of analytes, containing the same amount of the internal standards, were also analyzed by the system. An Agilent Eclipse XRD-C18 column was used for the analysis with a solvent gradient of 70% B (2% FA in 1:1 methanol/acetonitrile) and 30% A (2% FA in water) to 100% B in 5 min. Positive Q₁ and Q₃ ions for all four analytes including the internal standard were determined by analyte infusion.

**Quantification of monohexosylceramides (MC) and lactosylceramides (LC)**

Samples spiked with deuterated monohexosylceramide (d18:1 C18:0 d₁₅) and deuterated lactosylceramide (d18:1 C16:0 d₁) as internal standards were analysed for glycosylceramides using the online trapping HPLC/MS/MS system. Calibration samples of the analytes, containing the same amount of internal standards, were also analyzed by the system. A Varian Metasil C18 column was used for analysis with a solvent gradient of 85% B (10 mM ammonium acetate (NH₄OAc) and 1% FA) in 1:1 methanol/isopropanol and 15% A (10 mM NH₄OAc and 1% FA in 3:7 acetonitrile/water) to 100% B in 5 min. Published positive Q₁ and Q₃ ions of these analytes were used for these analyses (30).

**Quantification of gangliosides and sulfatides (ST)**

Samples spiked with deuterated GM1 (d18:1 C18:0 d₁₅) and deuterated ST (d18:1 C18: 0 d₁) as internal standards were used for ganglioside and ST analyses on the online trapping HPLC/MS/MS system. An Agilent Zorbax Eclipse C18 column was used for analysis with a solvent gradient of 85% B (10 mM ammonium acetate (NH₄OAc) and 1% FA) in 1:1 methanol/isopropanol and 15% A (10 mM NH₄OAc and 1% FA in 3:7 acetonitrile/water) to 100% B in 5 min. Published positive Q₁ and Q₃ ions of these analytes were used for these analyses (30).
fluoride (NH$_4$F) in 1:1 methanol/isopropanol) and 15% A (10 mM NH$_4$F in water) to 100% B in 5 min. MRM with negative ion ESI was applied. No calibration samples were used for quantification due to a lack of available standards. Thus, peak area comparison of each analyte with the internal standard was applied for ganglioside and ST quantification. Published negative Q$_1$ and Q$_3$ ions of these analytes were used for these analyses (31).

**Quantification of ceramides (CER) and sphingomyelins (SM)**

Samples spiked with each of three CER (d18:1 C17:0, d18:1 C22:0 d$_4$, and d18:1 C24:0 d$_4$) and SM (d18:1 C17:0) as internal standards were directly injected into a Thermo Electron Betasil C18 analytical column, bypassing the trapping column. The solvent gradient was 90% B (10 mM NH$_4$OAc and 1% FA in 1:1 methanol/isopropanol) and 15% A (10 mM NH$_4$OAc and 1% FA in 3:7 acetonitrile/water) to 100% B in 5 min. Calibration samples of the CER, containing the same amount of the internal standards, were also prepared for CER quantification. Calibration standards for SM were not used so that peak area comparison of each analyte with the internal standard was applied for SM quantification. Published positive Q$_1$ and Q$_3$ ions of these analytes were used for these analyses (30).

**Analysis of lipid mass spectrometry data**

For preparation of heat maps, Z-scores [(observed value – analyte mean)/ analyte standard deviation] were calculated across experimental conditions. For the Z-score transformations, each analyte over the entire mouse population has a median value of 0 with a standard deviation of 1. Color-coding of heat maps was performed on Microsoft Excel. Sphingolipids were grouped according to the following lipid classes: sphingoid bases, ceramides (CER), dihydroceramides (DC), lactosylceramides (LC), monohexosylceramides (MC), sphingomyelin (SM), gangliosides (GM1, GM2, and GM3), and sulfatides (ST). For presentation of plasma and CSF data, analyte concentrations are presented either as absolute quantification (ng/ml) or as peak area ratios (analyte/ internal standard normalized to QC).

**Statistical analyses**

Results are expressed as mean ± SEM. For group comparisons, the statistical significance of differences in mean values was determined by a two-tailed single-factor ANOVA or Student’s t test using Graphpad Prism 5 for Mac OS X (version 5.0d). A p value of 0.05 or less was considered significant. A Bonferroni posttest correction was used to adjust for multiple comparisons.
RESULTS

Sphingolipid profiling in the BALB/c Npc<sup>.nih</sup> mouse model

Previous studies have shown accumulation of sphingolipid species in the tissues of BALB/c Npc<sup>nih</sup> (Npc<sup>1<sup>−/−</sup></sup>) mice, including increased total sphingomyelin in liver and total GM2 and GM3 gangliosides in both liver and brain tissues (15, 32, 33). To explore whether this complex lipid phenotype might provide distinct molecular species that could serve as biomarkers for NPC1 disease, we harvested tissues from pre-symptomatic (4 weeks), symptomatic (7 weeks) and pre-terminal (9 weeks) Npc<sup>1<sup>+/+</sup></sup>, Npc<sup>1<sup>+</sup>−</sup> and Npc<sup>1<sup>−/−</sup></sup> mice. We used LC-MS/MS-based metabolomics to perform a broad survey of ~100 metabolites in the sphingolipid de novo and recycling pathways in liver, spleen and brain tissues from all three genotypes of mice at each of the time points in the disease progression. Lipid classes profiled included sphingoid bases (SPH, S'PH, S1P and S'1P), ceramides (CER), sphingomyelins (SM), dihydroceramides (DC), glucosyl- and galactosylceramides (MC), lactosylceramides (LC), gangliosides (GM1, GM2 and GM3) and sulfatides (ST) (Fig. 1). We identified 36 lipid species that were present in each of the tissue samples, were sufficiently abundant to be accurately quantified using available deuterated or odd-chain internal standards, and were altered by the presence of disease and during the course of disease progression. Since several of the lipids had a range of values of over two orders of magnitude and exhibited vastly different dynamic ranges, data for liver, spleen and brain tissues are represented as Z-score transformed values in heat map format (Fig. 2A-C). When the Bonferroni posttest correction for multiple comparisons was applied, no significant differences were observed between Npc<sup>1<sup>+/+</sup></sup> and Npc<sup>1<sup>+</sup>−</sup> in any of the tissues at any of the time points examined. By contrast, multiple sphingolipid species were markedly elevated in the Npc<sup>1<sup>−/−</sup></sup> mice in all tissues (liver > spleen >> brain), even in pre-symptomatic postnatal day 28 (P28) mice. In general, tissue sphingolipid concentrations increased with disease progression, with the most striking accumulation in liver tissue in P49 and P63 mice among LC (177-fold increase for 16:0, p<0.05), sphingoid bases (22.3-fold increase for SPH, p<0.001; 126-fold increase for S'PH, p<0.01), MC (20.1-fold increase for 16:0, p<0.005; 16.0-fold increase for 24:1, p<0.001), GM2 (87.8-fold increase for 16:0, p<0.001; 16.0-fold increase for 22:0, p<0.001), GM3 (9.6-fold increase for 16:0, p<0.001; 7.1-fold increase for 18:0, p<0.001; 14.2-fold increase for 18:0, p<0.001; 16.3-fold increase for 22:0, p<0.001), and ST (41.4-fold increase for 16:0, p<0.001; 21.4-fold increase for 18:0, p<0.001; 14.2-fold increase for 20:0, p<0.001). In brain tissue of P63 mice, there was marked storage of sphingoid bases (2.7-fold increase for SPH, p<0.01; 3.7-fold increase for
SPH, p<0.01) and, GM2 (20.5-fold increase for 18:0, p<0.001; 25.4-fold increase for 20:0, p<0.001; 40.1-fold increase for 22:0, p<0.01), and GM3 gangliosides (14.3-fold increase for 18:0, p<0.001). These are consistent with previously reported lipid class measurements in the Npc1 mouse model and provides new data for specific molecular species that may track with disease severity (15, 21, 32-34). Intriguingly, significant tissue-specific reductions in select species, not reported previously, were observed in brain tissue (98.5% reduction in CER 18:1, p<0.05; and 54% reduction in MC 24:1, p<0.001).

Effect of disease-modifying treatments in the BALB/c Npc<sup>nth</sup> mouse models

Both miglustat, an inhibitor of glycosphingolipid biosynthesis, and HP-β-CD have been shown in previous studies to reduce neuronal ganglioside storage in NPC1 animal models (21-23, 26, 29). To explore whether these interventions act more broadly to correct the widespread alterations in the sphingolipid profiles of Npc<sup>1−/−</sup> mice, we measured sphingolipid levels in liver, spleen and brain tissues in P66-67 Npc1<sup>+/−</sup> and Npc1<sup>−/−</sup> mice, untreated and treated with miglustat, HP-β-CD, or the drugs in combination. Sphingolipid content of liver tissue is presented in Fig. 3, and percent change in lipid content in response to drug therapy (monotherapy and combined) in the Npc1<sup>−/−</sup> mice for all three tissues is shown in Table 1. The effect of miglustat on sphingolipid metabolism in vivo was surprisingly modest. The only significant changes were 52% and 37% reductions in S1P and LC 20:0 (p<0.05), respectively, in the liver, and 1.4- and 1.3-fold elevations in brain MC 18:0 and 20:0 (p<0.001), respectively.

By contrast, peripheral administration of HP-β-CD in the NPC1 mouse model resulted in broad and highly significant reductions across multiple sphingolipid classes (liver > spleen >> brain). In liver, HP-β-CD was effective in reducing storage of sphingoid bases (88% reduction in SPH, p<0.01; 95% reduction in S'PH, p<0.05; 80% reduction in S1P, p<0.001; 85% reduction in S'1P, p<0.001), LC (82-91% reduction for all species, all p<0.001), MC (82-97% reduction for all species, all p<0.001), DC (67% reduction in 24:1, p<0.001), SM (60-70% reduction in 16:0, 18:0 and 18:1, all p<0.001), gangliosides (96% reduction for GM1 16:0, p<0.005; 88% reduction for GM1 18:0, p<0.01; 89% reduction for GM3 16:0, p<0.05; 76% reduction in GM3 18:0, p<0.01; 87% reduction for GM3 22:0, p<0.05), and sulfatides (92% reduction in 16:0, p<0.05; 77% reduction in 18:0, p<0.001; 81% reduction in 20:0, p<0.001; 62% reduction in 22:0, p<0.05). Combined treatment with miglustat and HP-β-CD did not appear to affect sphingolipid profiles beyond HP-β-CD monotherapy in any of the tissues examined.
Plasma sphingolipid profiles in human NPC1 subjects

Alterations in the tissue sphingolipids, similar to that observed in the Npc1−/− mouse model, have been reported in post-mortem human NPC1 tissues, but – with the exception of a single case report – circulating sphingolipids in NPC1 subjects have not previously been characterized (6, 7). Since sphingolipids are well represented in the plasma, principally incorporated into circulating lipoproteins (35), we hypothesized that the marked sphingolipid alterations in the tissues might be reflected in the plasma of human NPC1 subjects and potentially identify novel NPC1 disease biomarkers. To test this hypothesis, we obtained fasting plasma samples from human NPC1 subjects enrolled in an observational study at the U.S. National Institutes of Health. We measured plasma sphingolipid levels in a cohort of 56 fasting NPC1 subjects and 56 age-matched control subjects (subjects were not matched for gender since no differences were observed between male and female subjects).

We found significant elevations in multiple sphingolipid species in the circulation of the NPC1 subjects (Fig. 4). Most striking were the MC species, with 2.1-2.5-fold elevations in all species examined, as well as a 1.7-fold elevation in CER 16:0 (p<0.005). Unexpectedly, we observed significant reductions in all sphingoid bases: SPH (48%, p<0.01), S1P (34%, p<0.001), S’PH (53%, p<0.01), S’1P (31%, p<0.005). Interestingly, no differences between the groups were observed for plasma LC concentrations, species that accumulate to high levels in multiple tissues in murine and human NPC1 disease (6).

Response of plasma and CSF sphingolipids to miglustat therapy in human NPC1 subjects

While there are currently no FDA approved therapies for NPC1 disease, miglustat, which is approved in the U.S. for treatment of Gaucher disease, is used off-label to treat NPC1 patients. Approximately half of the NPC1 subjects (52%) enrolled in the NIH observational study are receiving treatment with miglustat, thus providing an opportunity for us to examine the plasma sphingolipid markers cross-sectionally in treated and untreated subjects. Total CER (22%, p<0.05), GM1 (32%, p<0.01) and GM3 (26%, p<0.05), as well specific sphingolipid species (CER 22:0, 27% reduction, p<0.05; LC 16:0, 35% reduction, p<0.005; and GM1 16:0, 41% reduction, p<0.005) were significantly lower among treated compared to untreated subjects (Fig. 5). No significant differences were found among the sphingoid bases or MC species, even though these lipids differed between the NPC1 subjects and control cohorts (not shown).

Within the subpopulation of patients receiving miglustat in the longitudinal NIH study, 31% of the patients (n=9) were initiated on therapy subsequent to their initial visit. This allowed us to examine longitudinally the response to miglustat therapy in these subjects. Comparison of the post-treatment to the pre-treatment plasma
concentrations revealed uniform reductions in all GM ganglioside species and in some CER (20:0, p<0.05; 22:0, p<0.05; 24:0, p<0.05) and LC (16:0, p<0.05; 22:0, p<0.05) species (Fig. 6A-D). For all three lipid classes, maximal reductions were generally observed among the shorter chain 16:0 species. When analyzed by individual response to miglustat therapy, all subjects showed significant reductions in plasma GM1 16:0 (mean 49% reduction, range 11-75%, p<0.001) and GM3 20:0 (mean 28%, range 7-59%, p<0.005) (Fig. 6E-H). Miglustat therapy also was accompanied by alterations in the CSF sphingolipid profile. Following initiation of treatment, mean MC concentrations were significantly increased for 18:0 (182%, p<0.01), 20:0 (127%, p<0.01), and 24:0 (59%, p<0.01) (Fig. 7A), and the intra-subject comparison of post-treatment to pre-treatment CSF concentrations similarly revealed significant increases in MC 18:0 (p<0.01), 20:0 (p<0.01), 24:0 (p<0.01), 24:1 (p<0.01) (Fig. 7B-F). The elevation of the MC species in human CSF closely mirrors the accumulation of these MC species in brain tissue of miglustat-treated mice (Table 1). The lack of response to therapy in two subjects, NPC 40 and NPC 54, was likely due to dose-limiting toxicity (i.e. diarrhea) in NPC 40 and under-dosing based on body mass in NPC 54, resulting in suboptimal miglustat treatment.

Response of CSF sphingolipids to HP-β-CD therapy
Based on the striking alterations in the sphingolipid profile in the brain tissue of the HP-β-CD-treated Npc1^{-/-} mice, we reasoned that metabolomics profiling of the CSF might identify lipid biomarkers that are readily accessible and could serve as biomarkers for HP-β-CD therapy. Obtaining CSF from the treated animals, however, is challenging due to difficulty in accessing CSF in mice and the limited quantities of CSF available from an individual mouse. Therefore, we employed the NPC1 feline model for these studies. Three-week-old NPC1 cats were administered 120 mg HP-β-CD intrathecally and CSF samples were obtained immediately prior to drug delivery and three days post-treatment. Sphingolipid profiling demonstrated uniform increases in MC (all species, p<0.05) and LC (20:0, p<0.05; 24:0, p<0.01) classes (Fig. 8), as well as select ganglioside species (GQ1 20:0, p<0.05).
DISCUSSION

At present there are no FDA approved therapies for NPC1 disease. Based on a recent controlled trial for miglustat, the glycosphingolipid synthesis inhibitor has gained approval for treatment of NPC1 disease in over 40 countries. Nonetheless, FDA approval has proven elusive, stemming primarily from the limited number of available study subjects and the reliance on clinical outcome measures that require long-term observation and are difficult to quantify (28). There is a pressing need to identify reliable, rigorously quantifiable, disease specific biomarkers to facilitate clinical evaluation of promising drugs that are emerging from the pre-clinical pipeline. In the present study, we used mass spectrometry-based metabolomic profiling to identify a panel of sphingolipids that are elevated in the plasma and CSF of human NPC1 subjects, and that respond to treatment both in animal models and in NPC1 patients. We propose these lipids can serve as disease biomarkers to provide important new tools for monitoring efficacy of therapy in clinical trials.

The abnormalities in sphingolipid metabolism that accompany NPC1 disease are well established. Our findings of marked elevation of sphingoid bases, MC, LC, GM gangliosides and ST in the Npc1+/− mouse tissues are consistent with previous characterization of the murine model by Pentchev and colleagues (15, 32, 33). While our goal extended earlier analyses – in particular by identifying specific molecular species within sphingolipid classes, the primary objective of our study was to exploit the altered sphingolipid profile to identify relatively abundant and quantifiable species that have the potential to serve as biomarkers for clinical monitoring of disease progression or response to drug intervention. The sphingolipidosis occurring in NPC1 disease is generally believed to be a consequence of the trapping of unesterified cholesterol in lysosomes, which prevents normal recycling of sphingolipids that complex with cholesterol in membranes. Conversely, genetically restricting synthesis of higher order gangliosides in Npc1−/− mice results in less cholesterol accumulation in neurons, suggesting a more complex sequestration process (36). Molecular profiling carried out in the present study also revealed profound, yet previously unrecognized, reductions in select sphingolipid species in brain tissue (e.g., CER 18:1 and MC 24:1). While the significance of the reduction in CER 18:1 is uncertain, the reduction in the MC 24:1 likely reflects demyelination that accompanies NPC1 disease (37).

Sphingolipid storage has similarly been described in viscera and brain of human NPC1 patients (6, 7). In the present study, we extend these earlier observations by providing the first systematic examination of circulating molecular sphingolipid species. In agreement with an earlier case report (38), plasma MC were significantly elevated in the NPC1 subjects, although the reported increase in LC was not observed in our cohort (38).
the two major MC species – glucosylceramide and galactosylceramide – are isobaric, we were not able to differentiate between the lipids using LC-MS/MS. However, the previously described elevation of glucosylceramide in human tissues suggests that this sphingolipid likely accounts for the bulk of the circulating MC. An unexpected finding was the significant decrease in plasma sphingoid bases in the plasma of the human NPC1 cohort, since these species are elevated in the Npc1−/− mouse tissues (33). The lower concentrations of the circulating sphingoid bases, which are a minor constituent (~0.08%) of the total circulating sphingolipid pool (35) appear to reflect altered sphingolipid synthesis – S’PH is an intermediate in the de novo synthetic pathway – and sphingolipid remodeling – SPH is exclusively derived from SM and CER turnover by sequential actions of sphingomyelinases and ceramidases. The latter finding is supported by the defective sphingomyelinase activity that is well documented in human NPC1 disease, a consequence of inactivation of the enzyme caused by cellular cholesterol accumulation (33, 39, 40).

Sphingolipids are major constituents of the plasma lipidome and are predominantly associated with circulating lipoproteins. During lipoprotein synthesis, sphingolipids, such as free sphingoid bases, CER, SM and MC, are incorporated into nascent VLDL particles and are secreted into the plasma, equilibrating across all major lipoprotein classes (e.g., VLDL, IDL, LDL and HDL) (41, 42). The most significant elevations in plasma sphingolipids in the human NPC1 subjects were for MC and CER species, the latter known to accumulate up to 40-fold in human liver tissue (6). Given that circulating sphingolipids are predominantly of hepatic origin (41, 42), the increased representation of these species in the plasma of NPC1 patients reflects the storage of these two abundant lipid classes. Exocytosis of membrane vesicles (“exosomes”) provides another route for secretion of sphingolipids into the circulation. Exosomes, which are formed during biogenesis of multivesicular bodies, are enriched in lipid raft domains that contain cholesterol and glycolipid components, such as GM1 (43). In lipid overload states, both lipoprotein secretion and exocytosis provide potential mechanisms for shedding excess lipid to maintain cellular lipid homeostasis. In NPC1 cells, which exhibit marked endolysosomal lipid storage, enhanced exocytosis results in increased exosomal cholesterol release (44). Whether exocytosis similarly provides a route for export of excess sphingolipids, which could contribute to the increased plasma glycolipids in NPC1 subjects, remains to be determined.

Intervention with therapeutic agents led to profound alterations in the sphingolipid profiles in animal models and in humans. In human NPC1 subjects, miglustat treatment resulted in significant reductions in multiple circulating sphingolipid species, including plasma LC and GM1 and GM3 ganglioside species, as would be
expected in the setting of inhibition of the hepatic glucosylceramide synthase (Figure 1). This effect of miglustat on complex sphingolipid synthesis in the brain is similarly reflected by the reduced CSF concentrations of CER, LC, and gangliosides. On the other hand, MC species, which accumulated in the brain tissue of miglustat-treated Npc1–/– mice, were elevated in the plasma and more profoundly in the CSF following initiation of miglustat. While elevation of plasma and CSF MC species at first seems counterintuitive in light of miglustat’s proposed mechanism of action through inhibition of glucosylceramide synthase, the enzyme responsible for conversion of ceramide to glucosylceramide (29), a number of reports have shown an increase of glucosylceramide in brain tissue in response to treatment with iminosugar-based inhibitors (e.g., miglustat). (45-47) There is mounting evidence that the increased glucosylceramide is due to inhibition of GBA2, a glucosylceramidase that is abundant particularly in the brain and is responsible for maintenance of glycosphingolipid homeostasis.

In comparison to miglustat, HP-β-CD was considerably more effective in ameliorating sphingolipid storage in the Npc1–/– mouse model. This is consistent with the relative effects of these drugs on preserving neurological function and prolonging survival (21, 29). The HP-β-CD-induced changes in the mouse tissues and the uniform increase in MC and LC concentration in the CSF following intrathecal HP-β-CD administration in the NPC feline model are consistent with exosomal secretion of lysosomal contents, possibly through HP-β-CD-triggered exocytosis (48). These findings raise the possibility that sphingolipids in the MC and LC classes may provide sensitive biochemical markers for monitoring the acute CNS response to HP-β-CD therapy in human patients. HP-β-CD has already been administered both peripherally and intrathecally to human NPC1 subjects in the U.S. under a single patient IND, and intracerebroventricular administration is currently being studied in Phase 1 trial initiated in January 2013 at the NIH Clinical Center. Moreover, it is anticipated that approximately half of the NPC1 subjects who eventually will be enrolled in the Phase 1 trial will already be receiving miglustat (through off-label treatment), thus providing an opportunity to investigate the potential synergistic effects of combined HP-β-CD/miglustat administration in humans, as well as the utility of these biomarkers as outcome measures for planned Phase 2/3 clinical trials.

A highly desirable feature of effective biomarkers is the ability to sample non-invasively from body fluids. The primary goal of this study was to identify candidate sphingolipid species that could serve as NPC1 disease biomarkers for monitoring response to therapy. We found that treatment with HP-β-CD alone or in combination
with miglustat corrected to a significant extent the tissue lipid abnormalities in the Npc1−/− mouse model. HP-β-CD was significantly more effective than miglustat at reducing lipid storage, which is consistent with the greater efficacy of HP-β-CD in preserving neurological function and prolonging survival in the NPC1 animal models (21, 22, 29). Although multiple sphingolipid species were altered in response to miglustat – suggesting broad trends – only a few of these changes reached significance after correcting for multiple comparison error, largely due to the limited number of animals per group (n=4-5) and the large intra-group variation, particularly among less abundant species. It is possible that the clinical benefit afforded by miglustat therapy results from regional changes in sphingolipid metabolism (e.g., in neuronal or select neuronal populations) that are not detectable by bulk tissue analysis. Alternatively, miglustat may exert its effects through non-lipid storage or downstream pathways, or by enhancing neuronal survival through other mechanisms. Indeed, a recent study examining the efficacy of miglustat in the feline NPC1 model revealed dramatic rescue of Purkinje neurons despite only modest effects on sphingolipid storage (26). Nonetheless, treatment of NPC1 patients with the sphingolipid synthesis inhibitor was associated with significant reductions (LC and GM1 and GM3 gangliosides) and elevations (MC) in multiple circulating sphingolipid species. Lipids in the latter sphingolipid class – which accumulated in the brain tissue of miglustat-treated Npc1−/− mice – were similarly elevated in the CSF following initiation on miglustat therapy, indicating a direct effect of miglustat on CNS sphingolipid metabolism and suggesting that CSF sampling and quantification of MC species may provide a non-invasive outcome measures for monitoring drug intervention.

The NIH Observational NPC Study has proven to be a rich repository of specimens for biomarker discovery. Previous metabolomic profiling efforts in our laboratory using these specimens led to discovery of cholesterol oxidation products (i.e., oxysterols) as NPC1 disease-specific markers (10). These oxysterols, which reflect the unique intersection of oxidative stress and excess intracellular free cholesterol in this disorder, were elevated in all human NPC1 subjects studied, and led to development of a sensitive and specific liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for quantifying the oxysterols in human plasma (49). The oxysterol assay offers considerable advantages over the existing diagnostic tools (e.g., filipin staining for cholesterol) and is being implemented as the diagnostic standard in major reference labs worldwide that diagnose NPC1 disease. More recently, gene expression profiling using samples from the Observational Study demonstrated increased levels of galectin-3, a pro-inflammatory molecule, and cathepsin D (CTSD), a lysosomal aspartic protease, in the serum of NPC1 patients (50). These proteins were reduced in the tissues of
Npc1−/− mice administered HP-β-CD, suggesting that they might provide useful outcome measures, particularly in the setting of HP-β-CD treatment.

NPC1 disease pathogenesis is complex, involving proteostatic, lipid trafficking, inflammatory, oxidative stress, autophagic and pro-apoptotic pathways. It is, therefore, unlikely that any one biomarker, or even class of biomarkers, will provide a quantifiable, biochemical measure to diagnose disease, to follow disease progression, and to assess the response to intervention. For example, plasma oxysterols, which are exceptional diagnostic biomarkers, appear to have limited ability to monitor interventions, such as miglustat treatment, that neither reduce oxidizable cholesterol stores nor ameliorate oxidative stress (Ory and Porter, unpublished). Rather, it is far more likely that a panel of biomarkers that correspond to different aspects of the pathogenic cascade – or relates to the biological pathway targeted by a therapeutic – will be most effective in following the course of disease and response to therapy. The sphingolipid species identified by our study are readily detectable in the circulation and the CSF, easily quantifiable using tandem mass spectrometry and provide a non-invasive window into the tissue lipid storage that is central to the disease process. Further studies are needed to determine whether the altered plasma sphingolipid profile is specific for NPC1 disease or is shared with other lysosomal storage diseases. In light of the challenges presented by the rare disease status of NPC1 and the need for surrogate outcome measures to obtain meaningful and timely results in a clinical trial setting, we propose that these biomarkers will be an important addition to the toolbox being developed to evaluate new therapeutics for this disease.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Sphingolipid biosynthetic pathway. Precursors, intermediates and glycosylated sphingolipids are shown in black. Enzymes involved and synthesis and degradation are labeled in red and blue, respectively. Abbreviations: DC, dihydroceramide; GalCer, galactosylceramide; GluCer, glucosylceramide; LC, lactosylceramide; SM, sphingomyelin, and SPH, sphingosine.

Fig. 2. Heat map of sphingolipid content in Npc1−/− mouse tissues. Heat maps depict sphingolipid species in (A) liver, (B) spleen and (C) brain tissues from Npc1+/+, Npc1+/− and Npc1−/− mice harvested at 28, 49 and 63 days. Lipid content is quantified using Z-score and displayed under conditional formatting. Rows represent different sphingolipids and column groups represent genotype and age of tissue harvest. Row Z-score ranges from -3 to +3, which is shown by a gradient of green through black to red. Lipid classes profiled included sphingoid bases (SPH, S’PH, and S1P), ceramides (CER), sphingomyelins (SM), dihydroceramides (DC), lactosylceramides (LC), monohexosylceramides (MC), gangliosides (GM1, GM2, and GM3), and sulfatides (ST).

Fig. 3. Effect of drug therapy on sphingolipid accumulation in Npc1+/+ and Npc1−/− mouse liver tissue. Plots show sphingolipid content of P66-67 Npc1+/+, untreated Npc1−/−, and Npc1−/− treated with miglustat (Mig), HP-β-CD (CD), or both (n=4-5/group). Lipid content is presented for (A) sphingoid bases (SPH, S’PH, S1P, S’1P), (B) lactosylceramides (LC), (C) monohexosylceramides (MC), (D) dihydroceramides (DC), (E) sphingomyelins (SM), (F) GM series gangliosides (GM), (G) sulfatides (ST), and (H) ceramides (CER). Data is presented as pmoles/mg protein. Percent change in treated Npc1−/− mice and p values are presented in Table 1.

Fig. 4. Plasma sphingolipid profiles in NPC1 subjects. Plasma concentrations (peak area ratios) are shown for (A) monohexosylceramides, (B) ceramides, (C) sphingoid bases, (D) lactosylceramides, and (E) gangliosides for age-matched control and NPC1 subjects (n=56/group). Data is shown as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

Fig. 5. Comparison of plasma sphingolipid concentrations in untreated and miglustat-treated NPC1 subjects. Mean group plasma concentrations (peak area ratios) for untreated (n=27) and miglustat-treated (n=29) NPC1 subjects are shown for (A) total ceramide, GM1 and GM3, (B) ceramides (CER 20:0, 27% reduction, p<0.05), (C) lactosylceramides (LC 16:0, 35% reduction, p<0.005), and (D) gangliosides (GM1 16:0, 41% reduction,
p<0.005). Data is shown as mean ± SEM; *p<0.05, **p<0.01.

**Fig. 6.** Effect of miglustat treatment on plasma sphingolipid concentrations in NPC1 subjects. Mean group plasma concentrations (peak area ratios) for NPC1 subjects (n=8) pre- and post-initiation of miglustat treatment are shown for (A) ceramides (20:0, p<0.05; 22:0, p<0.05; 24:0, p<0.05), (B) monohexosylceramides (p=NS), (C) lactosylceramides (16:0, p<0.05; 22:0, p<0.05), and (D) gangliosides (p<0.05 for all species). Plasma concentrations (peak area ratios) for individual NPC1 subjects (n=8) pre- and post-initiation of miglustat treatment are shown for (E) GM1 16:0 (F) GM3 20:0, (G) ceramide (CER) 20:0, and (H) lactosylceramide (LC) 16:0. For GM1 16:0 and GM3 20:0, p<0.001 for pre vs. post concentrations; for CER 20:0 and LC 16:0, p<0.05 for pre vs. post concentrations.

**Fig. 7.** Effect of miglustat treatment on CSF sphingolipids in individual NPC1 subjects. (A) Mean group CSF concentrations (peak area ratios) for NPC1 subjects (n=8) pre- and post-initiation of miglustat treatment are shown for MC species. CSF concentrations (peak area ratios) for individual NPC1 subjects (n=9) pre- and post-initiation of miglustat treatment are shown for (B) MC 18:0, (C) MC 20:0, (D) MC 22:0, (E) MC 24:0 and (F) MC 24:1. For MC 18:0, MC 20:0, MC 24:0 and MC 24:1, p<0.01 for pre vs. post concentrations.

**Fig. 8.** Effect of HP-β-CD administration on CSF sphingolipids concentrations in the NPC1 cat model. 3-week-old NPC1 cats were treated intrathecally with 120 mg HP-β-CD, and CSF samples were obtained immediately pre- and 3 days post-treatment. Mean group CSF concentrations (nM) are shown for (A) MC and (B) LC species. For all MC species, p<0.05; LC 20:0, p<0.05; LC 24:0, p<0.01 for pre vs. post concentrations.
Table 1. Percent change in tissue sphingolipid content in Npc1<sup>−/−</sup> mice following HP-β-CD (CD) and miglustat (Mig) monotherapy and combined treatment

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Figure 1

Serine + Palmitoyl CoA
\[\text{Serine Palmitoyltransferase}\]

3-Keto Sphinganine
\[\text{3-Keto S'PH Reductase}\]

Sphinganine
\[\text{DC Synthase}\]

Dihydroceramide
\[\text{DC Desaturase}\]

Galactosylceramide \rightarrow \text{Gal Cer Synthase} \rightarrow \text{Cer Synthase} \rightarrow \text{Sulfotransferase}

Cerebroside

Sphingosine
\[\text{S1Pase} \rightarrow \text{SPH Kinase}\]

Galatosylceramide \rightarrow \text{Glu Cer Synthase} \rightarrow \text{Glucocerebrosidase (GBA1/GBA2)}

Ceramide

Ceramide \rightarrow \text{SM Synthase} \rightarrow \text{SMase}

Sphingosine-1-Phosphate

Lactosylceramide \rightarrow \text{LC Synthase}

Ganglio-Series

Sphingomyelin
### Figure 2A

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Figure 2C
Figure 3

A

B

C

D

Figure 3
Figure 4

A

B

C

D

E

Peak Area Ratio

Peak Area Ratio

Peak Area Ratio

Peak Area Ratio

MC 16:0  MC 18:0  MC 20:0  MC 22:0  MC 24:0

Cer 16:0  Cer 20:0  Cer 22:0  Cer 24:0

S1  S1P  SPH  S1PH

LC 16:0  LC 18:0  LC 20:0  LC 22:0  LC 24:0

GM1 18:0  GM1 16:0  GM3 18:0  GM3 16:0  GM3 16:1  GM3 20:0

CTRL

NPC1

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**

**
Figure 6

A

B

C

D

Peak Area Ratio

Peak Area Ratio

Peak Area Ratio

Peak Area Ratio

Pre

Post

Pre

Post

Pre

Post

Pre

Post
Figure 7

A

MC 18:0  MC 24:0
MC 20:0  MC 24:1
MC 22:0

Peak Area Ratio

Pre  Post

B

Peak Area Ratio MC 18:0

Pre  Post

C

Peak Area Ratio MC 20:0

Pre  Post

NPC 5  NPC 7  NPC 19  NPC 20  NPC 21  NPC 23  NPC 40  NPC 49  NPC 54

D

Peak Area Ratio MC 22:0

Pre  Post

NPC 5  NPC 7  NPC 19  NPC 20  NPC 21  NPC 23  NPC 40  NPC 49  NPC 54

E

Peak Area Ratio MC 24:0

Pre  Post

NPC 5  NPC 7  NPC 19  NPC 20  NPC 21  NPC 23  NPC 40  NPC 49  NPC 54

F

Peak Area Ratio MC 24:1

Pre  Post

NPC 5  NPC 7  NPC 19  NPC 20  NPC 21  NPC 23  NPC 40  NPC 49  NPC 54
Figure 8

A

B

MC 16:0
MC 18:0
MC 20:0
MC 22:0
MC 24:0
MC 24:1

LC 16:0
LC 18:0
LC 20:0
LC 22:0
LC 24:0
LC 24:1

Quantity (nM)

Pre (0 Hour)  Post (72 Hour)

Pre (0 Hour)  Post (72 Hour)