Plasma neurofilament light, glial fibrillary acidic protein and lysosphingolipid biomarkers for pharmacodynamics and disease monitoring of GM2 and GM1 gangliosidoses patients

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ABSTRACT

GM2 and GM1 gangliosidoses are genetic, neurodegenerative lysosomal sphingolipid storage disorders. The earlier the age of onset, the more severe the clinical presentation and progression, with infantile, juvenile and late-onset presentations broadly delineated into separate phenotypic subtypes. Gene and substrate reduction therapies, both of which act directly on sphingolipidosis are entering clinical trials for treatment of these disorders. Simple to use biomarkers for disease monitoring are urgently required to support and expedite these clinical trials. Here, lysosphingolipid and protein biomarkers of sphingolipidosis and neuropathology respectively, were assessed in plasma samples from 33 GM2 gangliosidosis patients, 13 GM1 gangliosidosis patients, and compared to 66 controls. LysoGM2 and lysoGM1 were detectable in 31/33 GM2 gangliosidosis and 12/13 GM1 gangliosidosis patient samples respectively, but not in any controls. Levels of the axonal damage marker Neurofilament light (NF-L) were highly elevated in both GM2 and GM1 gangliosidosis patient plasma samples, with no overlap with controls. Levels of the astrocytosis biomarker Glial fibrillary acidic protein (GFAP) were also elevated in samples from both patient populations, albeit with some overlap with controls. In GM2 gangliosidosis patient plasma NF-L, Tau, GFAP and lysoGM2 were all most highly elevated in infantile onset patients, indicating a relationship to severity and phenotype. Plasma NF-L and liver lysoGM2 were also elevated in a GM2 gangliosidosis mouse model, and were lowered by treatment with a drug that slowed disease progression. These results indicate that lysosphingolipids and NF-L/GFAP have potential to monitor pharmacodynamics and pathogenic processes respectively in GM2 and GM1 gangliosidoses patients.

1. Introduction

The number of active drug development programs and approvals for rare diseases has increased during the last decade [1]. A trend that is in part due to many rare disorders being monogenic, with the resultant known disease causes, tractable mechanisms for therapies and relatively clear-cut diagnostic methodologies, all contributing to an increased chance of success. However, several intrinsic challenges must be overcome, including reducing diagnostic delay to enable early enrollment and the design of the clinical trials themselves. Biomarkers have long played an important role in disease diagnosis and are now increasingly utilized as drug development tools to support mechanism and efficacy

claims in clinical trials. For biomarkers to deliver this value, it is imperative to gain knowledge of the biomarkers' performance and their relation to clinical parameters prior to initiating interventional clinical trials. Plasma Neurofilament light chain (NF-L) is a biomarker which is gaining traction as a monitoring and prognostic biomarker in several prevalent central nervous system disorders [2,3]. Here we aimed to investigate plasma NF-L and other biomarkers in GM2 and GM1 gangliosidoses, two severe, pan-ethnic, genetic, neurodegenerative, closely related lysosomal storage disorders (LSD) [4,5]. Absent or diminished activity of the lysosomal hydrolases β-hexosaminidase A (EC 3.2.1.52) or β-galactosidase (EC 3.2.1.23), respectively, underlie the two aforementioned disorders.

GM2 gangliosidosis is caused by autosomal recessive deleterious mutations in one of the three genes (*HEXA*, *HEXB*, *GM2A*) that comprise the lysosomal β-hexosaminidase system [4,6]. Patients harboring deleterious mutations in both alleles of the *HEXA*, *HEXB* or *GM2A* genes are also referred to as having Tay-Sachs disease (OMIM #272800), Sandhoff disease (OMIM #268800) and GM2 gangliosidosis AB-variant (OMIM #272750) respectively. Common to all 3 subtypes is the absence of a fully functioning lysosomal β-hexosaminidase A, which comprises of one α (encoded by *HEXA*) and one β (encoded by *HEXB*) subunit, with the GM2 activator protein (encoded by *GM2A*) acting to present the GM2 ganglioside (GM2) substrate. The absence of hexosaminidase activity leads to substrate accumulation, most notably of the eponymous glycosphingolipid GM2 [4,6]. The most severely affected patients present as infants and suffer from a developmental arrest and unfortunately the majority die before 3.5 years of age [7]. Juvenile onset patients have a slower rate of decline, however many still have both motor and cognitive impairments, and few reach their third decade [8,9]. The more attenuated adult onset form is variable in presentation, with ataxia being a common symptom [4]. Although patients are often grouped in 3 phenotypic classes by age at onset (infantile, juvenile and late onset), in reality the presentations can be considered as a continuous spectrum.

Similarly, GM1 gangliosidosis (OMIM# 230500) is caused by mutations in the lysosomal β-galactosidase gene (*GLB1*). Loss of lysosomal β-galactosidase activity results in accumulation of GM1 ganglioside and related glycoconjugates in the CNS and visceral organs [10]. The early infantile onset form is particularly severe, with patients afflicted by severe psychomotor retardation, hepatomegaly, splenomegaly and skeletal abnormalities [5,11], while the juvenile and later onset forms progress more slowly [12].

Currently, there are no approved treatments for either GM1 or GM2 gangliosidoses. Therapeutic approaches explored preclinically include enzyme replacement therapy, gene therapy, pharmacological chaperones and substrate reduction therapy with small molecule enzyme inhibitors [4,6,13,14]. Several prospective therapeutics are now transitioning into early clinical testing. However, similar to many rare disorders, there are no commonly recognised clinical efficacy endpoints and physicians are faced with extreme clinical heterogeneity. Gaps which heighten the need for biomarkers, for reliable monitoring of disease and measurement of treatment effects. A pharmacodynamic biomarker is one which indicates that a biological response has occurred in an individual exposed to a medicinal product and can be used to support dosing [15]. While, a monitoring biomarker can be assessed serially to assess disease status including an effect of a medicinal product.

In LSDs in which the activity of sphingolipid hydrolases are deficient, secondary to accumulation of the more abundant N-acylated sphingolipid, there is a large fold increase of the equivalent N-deacylated lysosphingolipid [16]. Lysosphingolipid formation is thought to occur via direct action of acid ceramidase, which in a normal cell presumably barely hydrolyzes glycosphingolipids due a lower local concentration. Plasma levels of the lysosphingolipids glucosylsphingosine (GlcSph) and globotriaosylsphingosine (lysoGb3) have been studied in-depth as both diagnostic and pharmacodynamic markers for therapies acting directly on the lysosomal sphingolipid accumulation in Gaucher Disease and

Fabry Disease, respectively [17–19]. Beyond their use as biomarkers, lysosphingolipids have also been implicated as having a major role in the pathogenic process [16,20], with the most clear-cut evidence in galactosylceramidase deficiency (Krabbe disease) [21]. Although the presence of lysoGM2 in the CNS of GM2 gangliosidosis patients was first investigated more than 30 years ago [22,23], study of plasma lysoGM2 and lysoGM1 in their respective gangliosidosis patient populations has been limited, and little is known about their relationship to disease severity [24–27].

Neurofilament light chain (NF-L) is a neuronal cytoskeletal protein which is highly expressed in large calibre myelinated axons, where it is part of the neuroaxonal scaffold [3]. NF-L expression is largely restricted to the CNS, and NF-L in plasma strongly correlates with its levels in cerebrospinal fluid (CSF) in pathological states [28–30]. NF-L levels increase in CSF and blood proportionally to the degree of axonal damage. Consequently, spurred by the recent availability of sensitive assay technology, plasma NF-L is increasingly finding utility as both a monitoring and prognostic biomarker of neuronal damage and degeneration in a variety of disorders [2,3]. In multiple sclerosis (MS), a disorder in which the immune system attacks the myelin sheath, plasma NF-L is elevated, correlates with MRI measured lesions, and is reduced by approved treatments [29,31,32]. Recently, it was demonstrated that plasma NF-L is also elevated in pediatric patients with two neurodegenerative LSDs: neuronal ceroid lipofuscinosis type 2 (CLN2) [33] and mucopolysaccharidosis type II [34]. Notably, in CLN2 patients receiving intracerebroventricular infusions of the enzyme replacement therapy cerliponase alfa, which attenuated the rate of motor decline, plasma NF-L was reduced by 50%/year over a 3-year treatment period [35].

Beyond NF-L, measurement of other brain derived proteins in blood to gain in-sights into other pathological aspects would be of utility. Similar to NF-L, increases of the neuronal cytoskeletal protein Tau in biofluids are a consequence of neuronal degeneration and axonal damage [36]. Glial fibrillary acidic protein (GFAP) is an astrocyte intermediate cytoskeletal filament, the expression of which is increased in reactive astrocytosis [37]. Increased levels of full length and proteolytic fragments of GFAP in CSF and blood are biomarkers of reactive astrocytes. Patient blood and/or CSF levels of GFAP are elevated in amongst others MS, traumatic brain injury and various cerebrovascular pathologies [38]. Immunostaining of GFAP has been typically used as a marker of astrocytosis in GM2 and GM1 gangliosidosis patients and animal models [4,14,39].

As GM1 [40,41] and GM2 gangliosidoses [39,42], are associated with neurodegeneration, dysmyelination and neuroinflammation, we hypothesized that plasma NF-L, Tau and GFAP could be biomarkers for monitoring disease and treatment effects in these disorders. Here we aimed to investigate plasma levels of NF-L, GFAP and Tau and compare them to sphingolipidosis biomarkers which arise proximal to the enzymatic defect in GM2 and GM1 gangliosidoses. Additionally, we used a GM2 gangliosidosis mouse model to examine whether plasma NF-L and lysoGM2 would be amenable to change upon treatment with an exploratory drug with a mechanism known to alter disease progression in the model.

2. Methods

2.1. Plasma samples

Control pediatric plasma samples came from BioIVT (West Sussex, UK) (*n* = 20) and LeeBiosolution Inc. (Missouri, USA) (*n* = 35). Subjects with neurodegenerative disease and traumatic brain injury were excluded. An additional 11 adult control samples came from BIOBANK der Blutspender (Blood donation service, red cross Munich) or Idorsia Blood Donation (ethical permission EC Ref.Nr 249.002). In both cases written informed consent was obtained before sample collection. Plasma from patients with lysosomal storage disorders was collected at SphinCS (Hochheim, Germany) and LSD Brazil Network (Porto Alegre, Brazil).

Both collections were approved by the local Ethical committees (Ethik-Kommission bei der Landesärztekammer Hessen, Comitê de Ética em Pesquisa do Grupo de Pesquisa e Pós-Graduação do Hospital de Clínicas de Porto Alegre). Written informed consent was obtained from all patients studied. For the children who participated in this study, written informed consent was obtained from their legal guardians.

2.2. Measurement of plasma lysosphingolipids

Lysosphingolipids lysoGM2 and lysoGM1 were quantified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on an ABSCIEX QTRAP6500 with a Dionex Ultimate 3000 HPLC system. LysoGM2 and lysoGM1 were undetectable in control plasma, so all calibrants and quality control (QC) were made in control plasma. Calibrants (12 points, in the range from 0.5 nM to 1 μM) were prepared in pooled human plasma (BioIVT). Three sets of quality control samples were made using different matrices, two sets using EDTA-plasma and one set in heparin plasma spiking lysoGM2 and lysoGM1 at 0, 5, 10, 33 and 100 nM. Each calibrant and QC was run in duplicate and triplicate respectively. All calibrant and QC samples were processed as study samples and the LC-MS/MS injection order of all samples was randomized.

Each plasma sample (10 μL), calibrant and QC sample, was thawed at room temperature. Proteins in the sample were precipitated with methanol (40 μL). After vortexing for 10 s, the samples were centrifuged at 4000g for 10 min and the supernatant (30 μL) was transferred to 96-well polypropylene plates, from which 5 μL of sample was injected on the LC-MS/MS system.

LysoGM2 and lysoGM1 were separated on an ACQUITY UPLC BEH column (C18, 2.1 × 50 mm, 1.7 μm) with buffer A (water with 0.1% formic acid) and buffer B (acetonitrile:acetone (50:50) with 0.1% formic acid) running at 1 mL/min with a column temperature of 50 °C. A multi-step gradient was run; from 10% to 75% of buffer B over the first 2.5 min; from 75% to 100% of buffer B over 2.7 min; staying at 100% buffer B over 3.00 min and then re-equilibration. The following source parameters were used: curtain gas (20); collision gas (medium) Q1 and Q3 resolution (unit); ion spray voltage (−4500 V); temperature (500 °C); gas1 (50) and gas2 (25). Other parameters (EP, DP, CE, and CXP) were optimized per transition using standard procedures. The multiple reaction monitoring transitions (Q1 in Da/Q3 in Da) were: lysoGM1 (1278.4/290.1); lysoGM2 (116.4/290.1). LC-MS/MS data was processed using Multiquant3.0.

The LLOQ was the lowest accurately calibrant with an acceptable signal to noise. The QC samples had a mean precision for triplicates of 4.8% for lysoGM1 and 4.3% for lysoGM2 respectively. The carryover for both lysoGM2 and lysoGM1 was below 0.5%. For 28 GM2 gangliosidosis patient samples measured in 2 independent batches 170 days apart variability was calculated (variability = 100*(repeat-original)/mean)). The median variability (%) and (% samples variability with-in ±30%) was 6% (94%) for lysoGM2.

2.3. Measurement of neurology protein biomarker in human plasma

Plasma concentrations of NF-L, GFAP, Tau and UCH-L1 were measured in human samples using the Neuro 4-Plex A Kit (Cat#102153), the microplate washer, the thermo-regulated plate shaker and the SR-X platform, all from Quanterix. The assay was performed following the manufacturer's instruction. Stability of plasma protein biomarkers to different plasma storage conditions was assessed. The mean CV of duplicate intra-batch assessment of samples from 12 subjects were: NF-L, 5.2%; Tau, 5.2%; GFAP, 12.2%. For 30 samples measured in 2 independent batches 160 days apart variability was calculated (variability = 100*(repeat-original)/mean)). The median variability (%) and (% samples variability with-in ±30%) was: NF-L, −0.7% (83%); Tau, 17.3 (77%); GFAP, −9.9% (93%). The UCH-L1 data was not further analyzed because it failed repeatability criteria

(to pass >66% of samples with a variability with-in ±30%). During the study 3 control samples and 1 GM2-Tay-Sachs patient sample could not be measured for protein markers, additionally GFAP was missing for 1 GM2-Sandhoff (Supplemental table 1). The missing values were caused by the SR-X not being able to collect a valid image (too many debris, not enough valid beads or optical signal interference). EDTA- and heparin-plasma from two donors and 5 different conditions before storing the samples at −20 °C were monitored: plasma stored 4 h at 4 °C, plasma stored 24 h at 4 °C, plasma stored 4 h at room temperature, plasma stored 24 h at room temperature and plasma directly at −20 °C. NF-L, GFAP and Tau were stable in the tested conditions, the inter-condition CV were below 15%. UCH-L1 showed high variation over the storage conditions: 24% for EDTA-plasma and 35% heparin plasma.

2.4. Hexb$^{-/-}$ mice

Mixed sex $Hexb^{-/-}$ mice (hexβ^{tm1R1p}) and corresponding WT littermates were born as a result of heterozygote:heterozygote matings at Albert Einstein College of Medicine, NY, USA. $Hexb^{-/-}$ mice received chronic treatment with sinbaglustat ((($2S,3R,4R,5S$)-2-(hydroxymethyl)-1-pentylpiperidine-3,4,5-triol)) (Idorsia Pharmaceuticals Ltd., Allschwil, Switzerland) at the nominal doses of 30 and 300 mg/kg/d provided as part of pelleted mice feed (Provimi Kliba, Kaiseraugst, Basel, Switzerland) starting at 4 weeks of age. WT controls received the same feed formulation without sinbaglustat. Mice were kept group-housed at the Kennedy Center animal facility of Albert Einstein College of Medicine until they were euthanized at 105 ± 1 days (i.e. 15 weeks) of age by ketamine/xylazine overdose. Retro-orbital blood was sampled in EDTA containing tubes. Blood was centrifuged for 10 min at 2500g, plasma was removed and stored at −80 °C and then shipped to Idorsia for analysis. There were 11 males and 8 females in the untreated WT group, 4 males and 6 females in the untreated $Hexb^{-/-}$ group, 6 males and 8 females in the $Hexb^{-/-}$ mice group treated with 30 mg/kg/d of sinbaglustat, and 4 males and 8 females in the $Hexb^{-/-}$ mice group treated with 300 mg/kg/d. All animal handling procedures were approved by the Institutional Animal Care and Use Committee of Alert Einstein College of Medicine. Full details of the mouse study will be published elsewhere. Concentrations of NF-L were measured in mouse plasma samples using a customized Meso Scale Discovery assay as previously described [43]. The left lateral liver lobe (~110 mg each) was homogenized in 1 mL methanol using a Tissue-Lyzer II (300 Hz, 3 min) and centrifuged. The supernatant was further 2-fold diluted and lysoGM2 analyzed as described in section 2.2.

2.5. Analysis

Values in patient samples below the lowest accurate calibrant (the LLOQ) were replaced with the lowest calibrant concentration. Patient and biomarker data were analyzed using R version 4.0.3. The GM2 gangliosidosis patients were also grouped by their age at onset, a proxy for severity (vide supra). Patients who were diagnosed before 2 years of age were placed in the age of onset 0–2 years, even when the precise age at onset was not available. Statistical tests utilized are indicated in the text and figure legends. The R-package modules ggplot2, pheatmap and corrplot were used to generate figures. For heatmaps the data was first z-normalized.

3. Results

3.1. Cohort

The cohort was assembled retrospectively from samples taken for research purposes, with an initial aim to assess the protein biomarkers. The cohort consisted of 33 GM2 gangliosidosis and 13 GM1 gangliosidosis patients coming from two independent centers. The majority of the patients were pediatric (median age < 18 years) and they had been

diagnosed either by standard enzymatic testing, genetics or both (Table 1). For some patients age at diagnosis and are at onset were available. The median time in years (range) between diagnosis and the first plasma sampling was: GM2-Tay-Sachs, 3 years (0–13); GM2-Sandhoff, 7 years (1.2–19); GM1 gangliosidosis, 2.1 years (0.8–15). The median time in years (range) between onset and the first plasma sampling was: GM2-Tay-Sachs ($N = 14$), 5 years (1.2–33); GM2-Sandhoff ($N = 4$), 13 years (1.7–33); GM1 gangliosidosis ($N = 3$), 10 years (7.6–15). In GM2 gangliosidosis patients age at sampling correlated with age at diagnosis (R 0.9) and age at neurological onset (R 0.76) (Supplemental fig. 1). In the GM1 gangliosidosis patients, age at diagnosis and age at sampling were correlated (R 0.76), not enough data was available to perform a meaningful analysis for age at onset. The GM2 gangliosidosis patients were also grouped by their age at onset, a proxy for severity classes (vide supra). The grouping by age at onset was not performed for GM1 gangliosidosis as only 1 patient had an age of onset not in the lowest 0–2 year range. Additionally, a small number of available patients with the related LSDs Gaucher disease type 3 (GD3) or Niemann Pick disease type C (NPC), were included to aid with initial specificity assessment of the protein biomarkers. A control group was assembled using plasma from two different commercial suppliers ($N = 20$ and 35). Additional samples in the control group ($N = 11$) came from a blood donation. All plasma samples analyzed came from different subjects, except for six GM2 gangliosidosis patients (3 Tay-Sachs and 3 Sandhoff), each of which contributed 2 plasma samples taken between 6 and 12 months apart.

3.2. Potential diagnostic and pharmacodynamic biomarkers plasma lysoGM2 and lysoGM1 are elevated in patients with the respective gangliosidosis

The plasma levels of lysoGM2 and lysoGM1 were measured in the patient samples using an LC-MS/MS assay (Fig. 1). LysoGM2 was only detected in the plasma of the GM2 gangliosidosis patients (31/33 patients), in all other plasma samples there was no detectable chromatographic peak (Supplemental fig. 2). The range of observed plasma lysoGM2 concentrations was similar in Tay-Sachs and Sandhoff disease sub-groups (Fig. 1A). Plasma lysoGM2 in the patients was strongly negatively correlated with age at sampling (Fig. 1B). With the non-detectable values in adult patients. Using the lower limit of quantification (LLOQ) as a cut-off, the sensitivity and specificity of lysoGM2 for detection of GM2-gangliosidosis in this cohort was 94% and 100% respectively. The median elevation of lysoGM2 above the LLOQ was 40.5-, 2.1- and 2.3-fold for GM2 gangliosidosis patients with an age of onset in the ranges 0–2, 3–4 and 5–10 years respectively, representing a lower estimate for the actual change. There was a significant difference in lysoGM2 based on the age at onset groupings (Fig. 1C). LysoGM1 was detected in the plasma of 12/13 GM1 gangliosidosis patients, as well as in 5 of the GM2 gangliosidoses patients, which had the most elevated lysoGM2 (Fig. 1D). Using the LLOQ as a cut-off, the sensitivity and specificity of lysoGM1 for detection of GM1 gangliosidosis in this cohort was 92% and 95% respectively. The level of lysoGM1 in some GM2

gangliosidosis patients was higher than that in the older GM1 gangliosidosis patients. Plasma lysoGM1 significantly correlated with age at sampling in the GM1 gangliosidosis patients (Spearman R 0.58, p 0.038), the comparatively weak correlation may be due to the narrow age range (10/13 patients <5 years) of this group (Fig. 1F). The median elevation of lysoGM1 above the LLOQ in GM1 gangliosidosis patients with an age of onset 0–2 years was 104-fold. The patient with a non-quantifiable value was the oldest in the cohort.

3.3. Potential disease-monitoring biomarkers NF-L, tau and GFAP are elevated in plasma from GM2 and GM1 gangliosidoses patients

Plasma levels of the protein biomarkers NF-L, Tau and GFAP were measured using the SR-X platform (Fig. 2 and Fig. 3). All GM2 and GM1 gangliosidoses patients had elevated plasma NF-L with no overlap with the control population (Fig. 2A). Plasma NF-L was not correlated with age at sampling in the controls, while it was correlated in both GM2 and GM1 gangliosidoses patients (Fig. 2B & C). Median plasma NF-L was 42.5-, 13.0- and 4.3-fold elevated above the median of all controls in GM2 gangliosidosis patients with an age at onset 0–2, 3–4 and 5–10 years respectively (Fig. 2D). In GM1 gangliosidosis patients with an age of onset of 0–2 years, the median elevation of plasma NF-L was 92.3-fold. Plasma levels of Tau were moderately elevated in GM2 and GM1 gangliosidoses patients, particularly in younger patients with an early onset (Fig. 2E-H). There was a significant overlap in the range of values between the gangliosidoses and control subjects.

GFAP was also elevated in plasma from GM2 and GM1 gangliosidoses patients (Fig. 3A), albeit to a lesser extent than NF-L. There was a strong negative correlation of plasma GFAP with age at sampling in the GM2 gangliosidosis patients and in subjects in the control population who were less than 10 years old at sampling (Fig. 3B). GM2 gangliosidosis patients with an age of onset 0–2 years had the highest plasma GFAP (Fig. 3D).

The 2 NPC patients had elevated plasma NF-L, while plasma GFAP and Tau were in the normal range. None of the 3 protein markers were elevated in the plasma from the two GD3 patients.

3.4. A limited number of pairs of plasma biomarkers correlate to one another

To investigate similarity and differences of the biomarkers, inter-biomarker correlations were assessed. As all biomarkers correlated with age at sampling, correlations between biomarkers were assessed in each disorder in patients with-in a limited age range (Fig. 4). In the heatmap of GM2 gangliosidosis patients younger than 7 years old at sampling, biomarkers clustered in two distinct groups, one group with NF-L and Tau, and a second with the lysosphingolipids and GFAP (Fig. 4A). The biomarkers with-in these subgroups significantly correlated with one another (Fig. 4B). In plasma from GM1 gangliosidosis patients younger than 7 years old at sampling, the biomarkers did not cluster together and there was no significant correlation between any pair of biomarkers (Fig. 4B & D).

Table 1

Cohort characteristics. For patients with 2 samples, the age at the first sampling was used. n.a. = not applicable. Age at sampling was available for all patients. *For GM2 gangliosidosis, GM2 gangliosidosis and GD biochemical test was enzymatic, while for NPC, one of the current blood biomarkers was used [44].

Disease	N	N (%) female	Mean ± SD age at sampling (years)	Median age (years)	N (%) diagnosis, biochemical*	N (%) diagnosis, biochemical* and genetics	N (%) age at diagnosis available	N (%) age at onset available
Control	66	39 (59.1)	11.1 ± 8.0	10	n.a.	n.a.	n.a.	n.a.
GM2-Tay-Sachs	27	11 (40.7)	10.3 ± 10.4	7	14 (51.9)	13 (48.1)	27 (100.0)	14 (51.9)
GM2-Sandhoff	6	2 (33.3)	13.2 ± 8.6	13	4 (66.7)	2 (33.3)	5 (83.3)	4 (66.7)
GM1 gangliosidosis	13	7 (53.8)	5.2 ± 4.6	3	11 (84.6)	2 (15.4)	13 (100.0)	3 (23.1)
GD3	2	1 (50.0)	11.0 ± 0.0	11	0 (0.0)	2 (100.0)	2 (100.0)	2 (100.0)
NPC	2	2 (100.0)	17.0 ± 8.5	17	0 (0.0)	2 (100.0)	2 (100.0)	2 (100.0)
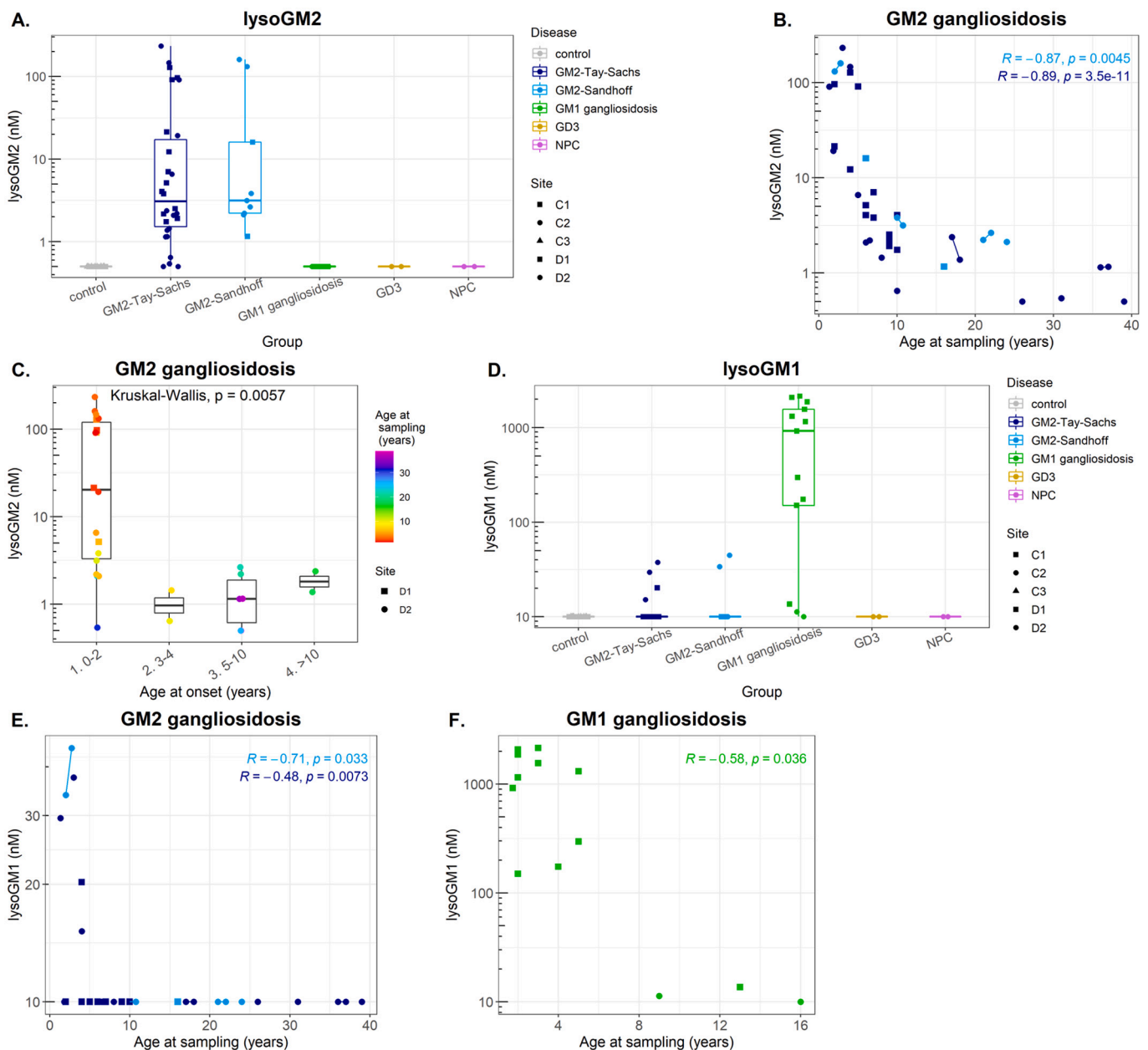
Fig. 1. Plasma levels of lysosphingolipid biomarkers, lysoGM2 and lysoGM1. (A) and (D): Boxplot showing respective biomarker levels per disease. LysoGM2 and lysoGM1 values which were below the respective LLOQ, were replaced with the LLOQ (10 and 0.5 nM respectively). (B), (E) and (F): scatter plots for biomarker levels vs age at sampling for GM2 and GM1 gangliosidoses. The Spearman correlations are given in the scatter plots. When longitudinal values were measured in samples from a patient, the points for that patient are connected by a line in the scatter plot. (C): Biomarker levels by age at onset, with points colored by age at sampling, only the first sample from each patient is included. The same color scheme for disease is used in A,B,D,E and F. Points are shaped by the different sites the samples came from (3 shapes for control, and 2 for the patient samples). Fig. 1B is redrawn with coloring by age at onset in Supplemental fig. 3. Summary statistics of the biomarker per group and per age of onset grouping are provided in the supplement (Supplemental table 1 and Supplemental table 2).

3.5. Analysis of biomarkers in longitudinal plasma samples from GM2 gangliosidosis patients

For 6 GM2 gangliosidosis patients, serial samples taken 6–12 months apart were analyzed (Supplemental fig. 4). All biomarkers appeared to be relatively similar in the 6 pairs of serial samples, with no statistically significant differences observed between the two time points (paired *t*-test).

3.6. Plasma NF-L is reduced by treatment in Hexb$^{-/-}$ mice

To assess whether plasma NF-L could be used as a biomarker of

treatment effects, *Hexb*$^{-/-}$ mice, a commonly used mouse model of GM2 gangliosidosis, was utilized [45]. *Hexb*$^{-/-}$ mice have been described to have neurological and pathological findings consistent with Sandhoff disease [46]. Dual inhibitors of the sphingolipid metabolizing enzymes non-lysosomal glucocerebrosidase (GBA2) and glucosylceramide synthase (GCS) have been shown to result in therapeutic benefit in this model [47] and when used off-label in patients [48]. To investigate the response of plasma NF-L to treatment, *Hexb*$^{-/-}$ mice were treated with 30 or 300 mg/kg/d of sinbaglustat (ACT-519276), a dual inhibitor of GBA2 and GCS which has completed phase I clinical studies [49], as food-admix or were left untreated from 30 days of age until euthanasia at 105 days. The *Hexb*$^{-/-}$ mice had significantly elevated plasma NF-L at
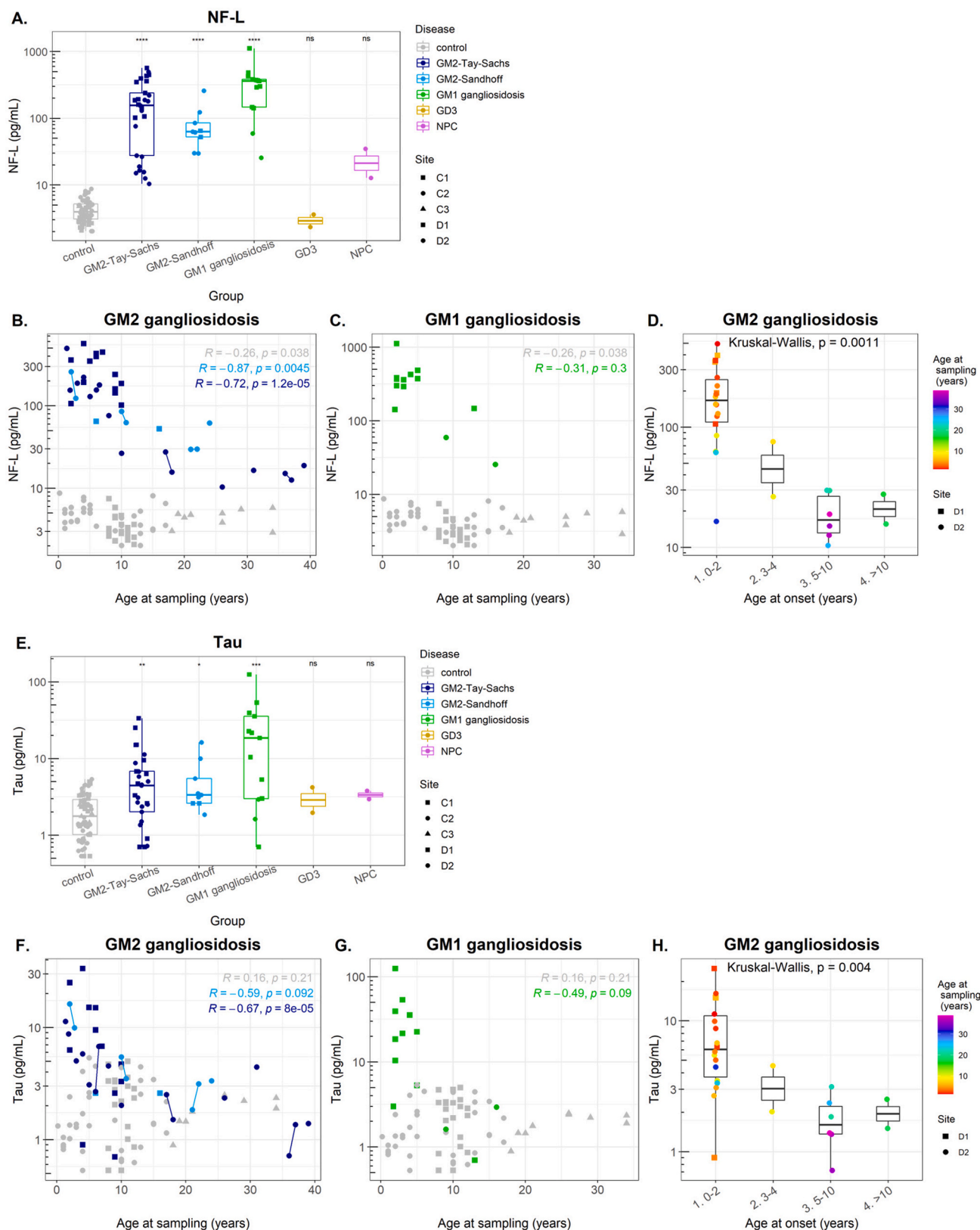
Fig. 2. Plasma levels of the neuronal damage markers NF-L and Tau. (A) and (E) boxplots of each biomarker per disease group. Each group was compared to the control group using a non-parametric Wilcoxon test and *p* values corrected using the method of Bonferroni. *P* < 0.0001, ****; *p* < 0.001, ***; *p* < 0.01, **; *p* < 0.05, *; ns, not significant. (B,C,F,G) Scatter plots for biomarker levels vs age at sampling for GM2 and GM1 gangliosidoses, with the control group included in both cases. The Spearman correlations are given in the scatter plots. When longitudinal values were measured in samples from a patient, the points for that patient are connected by a line in the scatter plot. (D) and (H) Biomarker levels by age at onset, with points colored by age at sampling, only the first sample from each patient is included. The same color scheme for disease is used in A-F. Points are shaped by the different sites the samples came from (3 shapes for control, and 2 for the patient samples). Figs. B and F are redrawn with coloring by age at onset in Supplemental fig. 3.
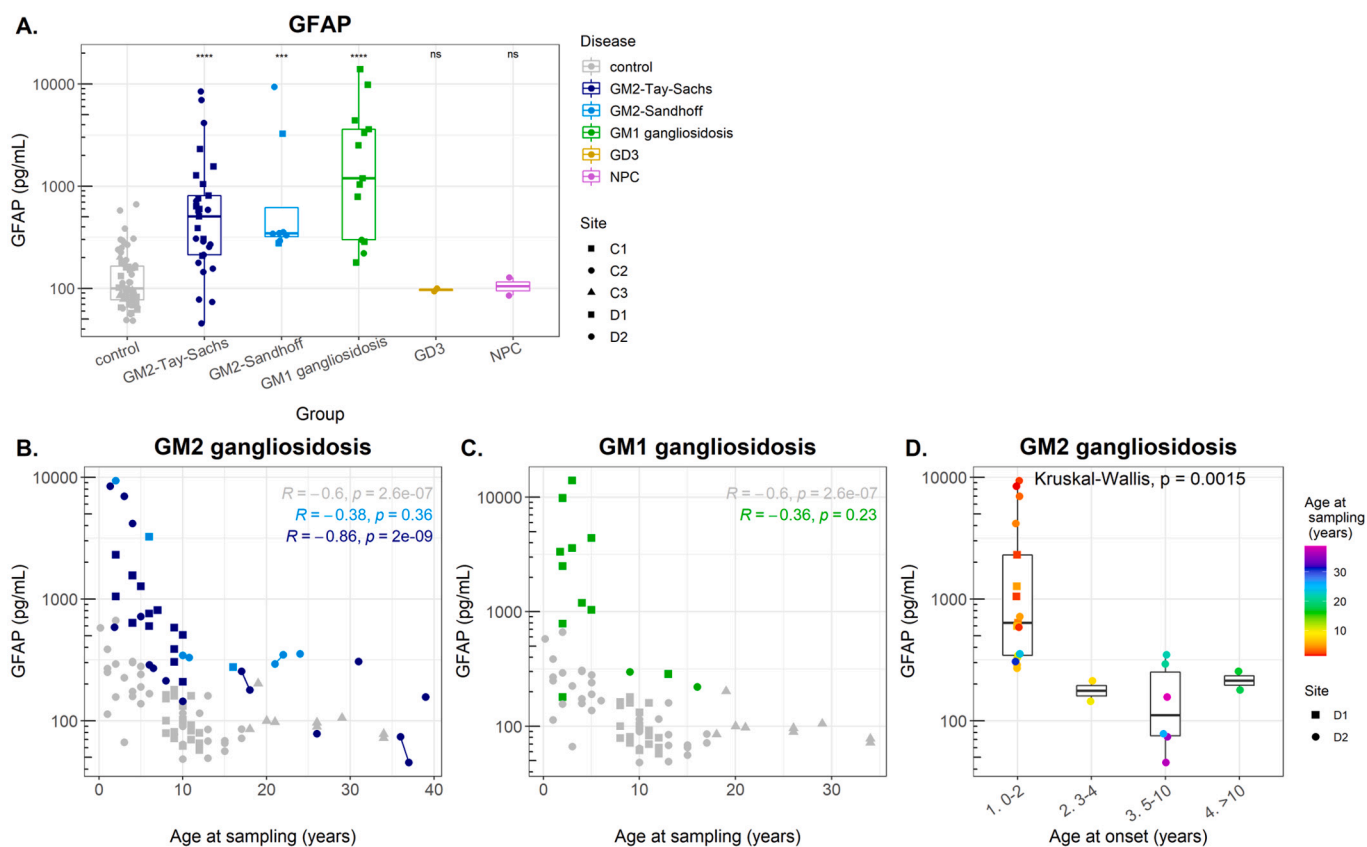
Fig. 3. Plasma levels of the astrocytosis marker GFAP. (A) boxplot of GFAP levels per disease group. Each group was compared to the control group using a non-parametric Wilcoxon test and *p* values corrected using the method of Bonferonni. $P < 0.0001$, ****; $p < 0.001$,***; $p < 0.01$, **; $p < 0.05$, *; ns, not significant. (B) and (C): scatter plots for biomarker levels vs age at sampling for GM2 and GM1 gangliosidoses, with the control group included in both cases. The Spearman correlations are given in the scatter plots. When longitudinal values were measured in samples from a patient, the values for that patient are connected by a line in the scatter plot. (D): Biomarker levels by age at onset, with points colored by age at sampling only the first sample from each patient is included. The same color scheme for disease is used in A,B and C. Points are shaped by the different sites the samples came from (3 shapes for control, and 2 for the patient samples). Fig. 3B is redrawn with coloring by age at onset in Supplemental fig. 3.

105 days an age where the first behavioural deficits were already present, which was reduced dose dependently by treatment with sinbaglustat (Fig. 5). Similarly, lysoGM2 was elevated in the liver of the untreated *Hexb*$^{-/-}$ mice and reduced by treatment with sinbaglustat.

4. Discussion

In rare diseases like GM2 and GM1 gangliosidoses, biomarkers reflecting different aspects of pathology are needed to help expedite clinical development of therapeutics and enable patient monitoring. The lysosphingolipids studied could be used to measure pharmacodynamic effects of therapies acting directly on the lysosomal sphingolipid accumulation, while the presented protein neuropathology biomarkers may report on CNS related disease severity and/or treatment effects. Importantly, all biomarkers were measurable in an accessible sample type: plasma, utilizing comparatively simple methodology which can be centralized for multicenter clinical trials. In the case of NF-L, solid preanalytical performance [2], and the possibility of a regulatory approved test for more prevalent disorders will likely also pave the road for implementation in rare disorders. The 5 biomarkers assessed were significantly increased in patients and only in a few cases correlate to one another in defined patient subsets, suggesting that in-line with their biology they may provide complementary information on different aspects of pathogenesis.

Plasma lysoGM2 and lysoGM1 were elevated in almost all patients with the respective gangliosidosis, with the dependency on age, and age at onset better delineated than in earlier studies [24,25,27].

Consequently, these markers could be used to as early pharmacodynamic biomarkers of glycosphingolipid lowering therapies and/or complement existing diagnostics, at least in the infantile and juvenile populations where the level is always clearly elevated. The diagnostic specificity of lysoGM2 and lysoGM1 is supported by other studies covering a wider range of LSDs where no healthy or diseased controls had measurable values [25,26]. Neither here, nor in the published studies was lysoGM2 detectable in NP-C patients, a disorder with secondary GM2 accumulation. Noting that in diagnosis it is essential to simultaneously measure lysoGM1 and lysoGM2, as here some GM2 gangliosidosis patients had elevations in both. The utility of lysoGM2 and lysoGM1 as pharmacodynamic markers is supported by our and published mouse model studies [13], as well as analogous usage of the equivalent biomarkers in both Gaucher disease (GlcSph) and Fabry disease (lysoGb3) [18,19]. The negative correlation of lysoGM2 and lysoGM1 with age at sampling and onset mirrors that of the hexosaminidase and galactosidase enzyme activity tests currently used as diagnostic tests for these disorders [5,6]. Results that fit with the model where-by less enzyme activity generally results in more substrate accumulation, and consequently greater production of the lysoGM2 and lysoGM1 secondary lipid products. Notably, as lysoGM2 and lysoGM1 are produced in vivo, they are not susceptible to the in vitro artifacts of enzyme activity testing (artificial substrate, acidification of non-lysosomal protein). LysoGM2 was similarly elevated in both the Tay-Sachs and Sandhoff forms of GM2 gangliosidosis, consistent with deficiency of Hexosaminidase A (αβ subunit conformation) activity, and not Hexosaminidase B (ββ) or S (αα), being the primary cause of lysoGM2
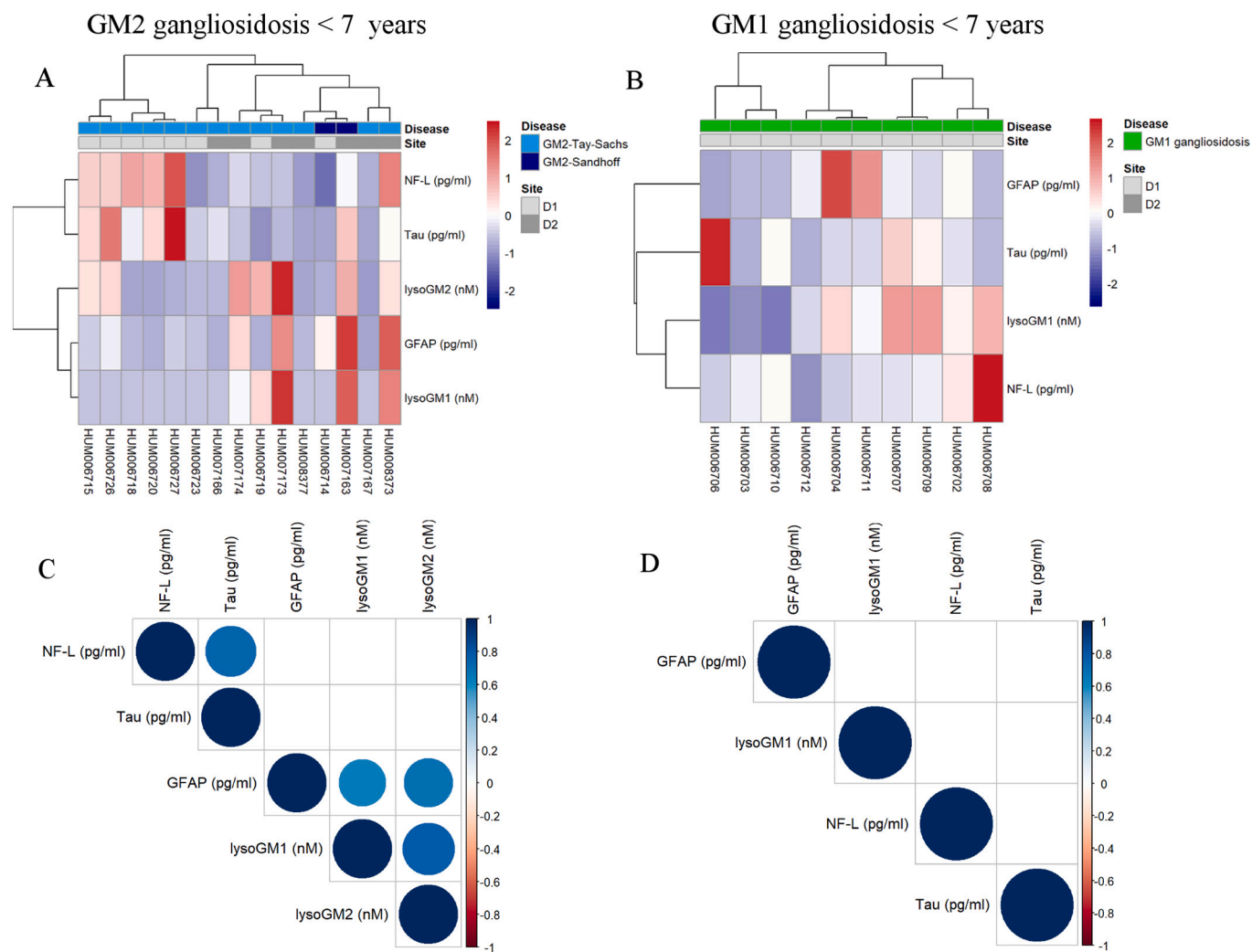
Fig. 4. Limited correlation of plasma biomarkers to one another in GM2 and GM1 gangliosidoses patients. (A) and (B) heatmaps with hierarchical clustering and (C) and (D) correlation matrices were generated using GM2 and GM1 gangliosidoses patient samples when age at sampling was <7 years, with only the first sample from each patient included. For the heatmaps clustering is based on Pearson correlation, with patients on the x-axis and biomarkers on the y-axis. For correlation matrices correlations are Spearman, and r (filled circle) is displayed only if $p < 0.05$.
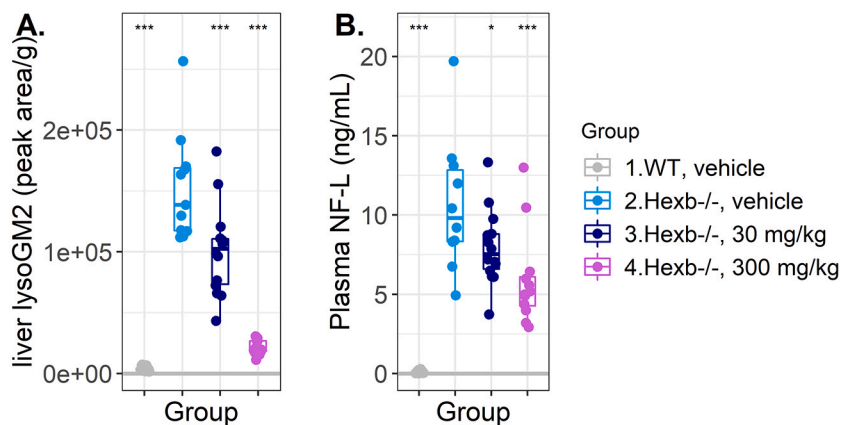
Fig. 5. Treatment effect of sinbaglustat on plasma NF-L and liver lysoGM2 in $Hexb^{-/-}$ mice at 105 days of age. Data was analyzed using Dunnett's test to compare each group to $Hexb^{-/-}$, vehicle. $P < 0.001$,***; $p < 0.01$ **; $p < 0.05$, *; ns, not significant. As no lysoGM2 standard was available at time of study the units for lysoGM2 are peak area/ unit wet weight. LysoGM2 was measured in liver rather than plasma, due to the limited quantity of available plasma being used for other measurements.

elevation. In plasma from GM2 gangliosidosis patients with very high lysoGM2, lysoGM1 could also be detected, probably as a result of secondary lysosomal accumulation of GM1. Secondary ganglioside accumulation is observed in some LSDs, including NPC and mucopolysaccharidoses [50,51]. Although direct conversion of lysoGM2 to lysoGM1 would be an alternate plausible mechanism, to our knowledge there is currently no supportive evidence.

Plasma NF-L was highly elevated in both GM2 and GM1 gangliosidosis patients, with levels >100 pg/mL in many patients, a level generally only observed in patients with severe neurodegenerative diseases such as CLN2 and amyotrophic lateral sclerosis [2,30,33]. Importantly, in *Hexb*$^{-/-}$ mice, plasma NF-L was lowered by a potential future treatment modality for GM2 gangliosidosis, dual pharmacological GBA2/GCS inhibition, highlighting the potential to utilize plasma NF-L to longitudinally monitor neuropathology in GM2 and GM1 gangliosidoses patients.

NF-L was previously observed to be moderately elevated in cerebrospinal fluid of 3 adult NPC patients [52]. The elevation of plasma NF-L in two pediatric NPC patients presented here corroborates this finding and highlights and is further supported by a very recent, extensive study of plasma NF-L in NPC patients [53]. Although plasma NF-L was normal in the two GD3 patients in this study, elevated plasma NF-L has been observed in the 4L/PS-NA model of neuronopathic Gaucher disease, suggesting further study is still warranted, perhaps in patients with a more severe presentation [54].

Literature on Tau in the gangliosidoses is limited, but tauopathy has been observed in the Sandhoff mouse model [55]. As Tau, like NF-L, is a cytoskeletal protein, the elevation in the infantile patients may be a result of general neuronal damage rather than tauopathy. The elevation of plasma Tau was more modest than that for NF-L. Given the scarce data on Tau in gangliosidoses and the correlation observed between Tau and NF-L in GM2 gangliosidoses, further studies would be necessary to elucidate if it really could be of additional utility.

Previous work on protein biomarkers in gangliosidosis identified a number of inflammatory markers in the CSF, but only a limited number of patients had elevated plasma levels [56]. Plasma GFAP was elevated above the level in controls in GM2 and GM1 gangliosidoses patients. Substrate reduction and gene therapies reduce astrocytosis in animal models of GM2 and GM1 gangliosidoses [13,47,57], suggesting that the plasma measurement employed here in patients could be of use to measure treatment effects in clinical trials.

Plasma lysoGM2, NF-L, Tau and GFAP negatively correlated with age at sampling in GM2 gangliosidosis with a similar trend observed in GM1 gangliosidosis. The higher plasma biomarker levels in younger patients is most likely a result of the well documented more severe disease in earlier onset patients. Given the rapid degeneration in the infantile populations, it is unlikely that intra-patient values would decrease with age. A conclusion supported by the relatively stable with-in patient elevated values observed for lysoGM2, NF-L and GFAP in the GM2 gangliosidosis patients, implying that these biomarkers report on chronic ongoing pathological processes. LysoGM2, NF-L, Tau and GFAP also significantly differed by the age of neurological onset in GM2 gangliosidosis patients, which is a predictor (although imperfect) for disease phenotype(/severity). Plasma NF-L appeared to offer the best separation of groups by age of neurological onset. MRI studies have indicated that in both GM2 and GM1 gangliosidoses, CNS atrophy and its progression are strongly linked to the age at onset [9,12]. Plasma NF-L may offer a simple, relatively non-invasive method to monitor this degeneration. Interestingly the two GM2 gangliosidosis patients with an infantile onset who have lived into their twenties had comparatively low levels of all 4 biomarkers. The general lack of correlation between lysosphingolipid biomarkers and the protein neurology markers in defined patient cohorts, indicates that the different pathophysiological processes they represent (sphingolipidosis, neurodegeneration and astrocytosis) may not develop in tandem, with neuronal degeneration and inflammation presumably being downstream of sphingolipidosis.

The lack of correlation could also be related to a greater fraction of plasma lysosphingolipids being periphery derived, as suggested by the elevation in *Hexb*$^{-/-}$ mouse liver observed here and in literature [13]. This further underscores the challenge of phenotypic variability, and the benefit of utilizing multiple biomarkers for patient monitoring.

NF-L and GFAP are not specific, and consequently cannot be used to diagnose a specific disease. For lysosphingolipid biomarkers the levels should also be assessed in other disorders with secondary ganglioside accumulation such as the mucopolysaccharidoses [51] to further assess diagnostic specificity, with the sensitivity being excellent only for infantile and juvenile presentations. The presented study was retrospective, using samples from different sites, without predefined harmonization of sample collection. Most of the discussed links of the biomarkers to distinct pathological processes discussed are based on analogy to other disorders. To confirm the potential of the biomarkers for disease monitoring and pharmacodynamics further work should focus on prospective longitudinal studies that link biomarker levels to more detailed clinical and pathological information on each patient, including in the context of therapeutic interventions. Detailed work in observational studies to assess the prognostic value of plasma NF-L, as has been performed in other diseases [2], would also be warranted. A comparison of plasma biomarkers to levels in cerebrospinal fluid would offer additional insights, although the burden of sampling may preclude such a study.

In conclusion, the different biomarkers presented could be utilized in clinical trials in both GM2 and GM1 gangliosidoses, to monitor disease and treatment effects.

Contributions

Designed research RW, MS, DS, CS, PG, RG, EM. Set-up assays and run experiments, HF, MG. Analyzed data HF, MG, RW. Led collection of human samples for research RG, YA, EM. Led animal work KD. Wrote paper, RW. Reviewed paper contributing to lay-out and interpretation MS, KD, DS, PG, YA, EM, RG.

Declaration of Competing Interest

RW, HF, MS, MG, DS, CS and PG are current or past employees of Idorsia pharmaceuticals which has an interest in developing pharmaceuticals for the treatment of lysosomal storage disorders.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2022.100843.

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