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Cerebrospinal Fluid Calbindin D Concentration as a Biomarker of Cerebellar Disease Progression in Niemann-Pick Type C1 Disease

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ABSTRACT

Niemann-Pick type C (NPC) 1 disease is a rare, inherited, neurodegenerative disease. Clear evidence of the therapeutic efficacy of 2-hydroxypropyl-β-cyclodextrin (HPβCD) in animal models resulted in the initiation of a phase I/IIa clinical trial in 2013 and a phase IIb/III trial in 2015. With clinical trials ongoing, validation of a biomarker to track disease progression and serve as a supporting outcome measure of therapeutic efficacy has become compulsory. In this study, we evaluated calcium-binding protein calbindin D-28K (calbindin) concentrations in the cerebrospinal fluid (CSF) as a biomarker of NPC1 disease. In the naturally occurring feline model, CSF calbindin was significantly elevated at 3 weeks of age, prior to the onset of cerebellar dysfunction, and steadily increased to >10-fold over normal at end-stage disease. Biweekly intrathecal administration of HPβCD initiated prior to the onset of neurologic dysfunction completely normalized CSF calbindin in NPC1 cats at all time points analyzed when followed up to 78 weeks of age. Initiation of HPβCD after the onset of clinical signs (16 weeks of age) resulted in a delayed reduction of calbindin levels in the CSF. Evaluation of CSF from patients with NPC1 revealed that calbindin concentrations were significantly elevated compared with CSF samples collected from unaffected patients. Off-label treatment of patients with NPC1 with miglustat, an inhibitor of glycosphingolipid biosynthesis, significantly decreased CSF calbindin compared with pretreatment concentrations. These data suggest that the CSF calbindin concentration is a sensitive biomarker of NPC1 disease that could be instrumental as an outcome measure of therapeutic efficacy in ongoing clinical trials.

Introduction

Niemann-Pick type C (NPC) disease is an inherited lysosomal storage disorder that results in the accumulation of cholesterol and multiple sphingolipids, leading to progressive neurologic and visceral dysfunction (Pentchev et al., 1985; Patterson et al., 2001; Vanier, 2010). NPC disease is inherited in an autosomal recessive manner and is caused by a mutation in either the *NPC1* or *NPC2* gene. The majority of mutations (approximately 95%) occur in the *NPC1* gene (Vanier, 2010), which encodes a large, 13-transmembrane domain protein that is responsible for binding and transporting cholesterol to the late endosomal/lysosomal compartment (Higgins et al., 1999; Neufeld et al., 1999). The *NPC2* gene encodes a small soluble lysosomal protein; approximately 5% of patients have mutations in this gene. The age of onset of neurologic disease is highly variable and correlates with disease progression and lifespan, with the juvenile, or classic, form being the most commonly diagnosed (Vanier, 2010). Neurologic symptoms include cerebellar ataxia, dysarthria, dysphagia, progressive dementia, vertical supranuclear gaze palsy, cataplexy, seizures, and dystonia. Death commonly occurs by 20 years of age (Vanier, 2010).

In addition to the intracellular accumulation of cholesterol and sphingolipids, a hallmark of neuropathology is the death of Purkinje cells (Walkley and Suzuki, 2004). The therapeutic efficacy of 2-hydroxypropyl-β-cyclodextrin (HPβCD) to ameliorate Purkinje cell death and cerebellar dysfunction and to increase lifespan was recently described in animal models of NPC1 disease (Camargo et al., 2001; Liu et al., 2008, 2009; Davidson et al., 2009; Taylor et al., 2012; Pontikis et al., 2013;

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ABBREVIATIONS: CNS, central nervous system; CSF, cerebrospinal fluid; HC, hydroxycholesterol; HPβCD, 2-hydroxypropyl-β-cyclodextrin; IL, interleukin; NIH, National Institutes of Health; NPC, Niemann-Pick type C.

Lopez et al., 2014). Clear evidence of the therapeutic potential of HPβCD in animal models of NPC1 disease led to a phase I/IIa National Institutes of Health (NIH) human clinical trial in 2013 (ClinicalTrials.gov identifier NCT01747135) and a phase IIb/III trial (ClinicalTrials.gov identifier NCT02534844) that began in 2015. A major obstacle in clinical trials is the paucity of validated surrogate markers of brain disease that can be monitored as secondary clinical endpoints (Platt and Lachmann, 2009). It has previously been shown that 24(*S*)-hydroxycholesterol (HC), a sterol that is synthesized exclusively in neurons in the central nervous system (CNS) and exported to the plasma, is decreased in the plasma of patients with NPC1 (Porter et al., 2010). It was therefore hypothesized that treatment with HPβCD would reduce cholesterol sequestered in the lysosome and increase cerebrospinal fluid (CSF) and plasma levels of 24(*S*)-HC. Evaluation of one patient showed that after the first five administrations of HPβCD, plasma levels of 24(*S*)-HC significantly increased, indicating that the therapy is appropriately redistributing cholesterol (Maarup et al., 2015). Studies conducted in the feline model of NPC1 disease demonstrated an increase in 24(*S*)-HC in the CSF and plasma after the initial administration of HPβCD; however, attenuation of the response in treated cats was seen with subsequent doses, suggesting that 24(*S*)-HC may not serve as an effective marker to monitor therapy long term (Tortelli et al., 2014).

Calcium-binding protein calbindin D-28K (calbindin) is present at high levels in the cerebellum, where it is predominantly localized in dendrites, soma, and axons of Purkinje cells (Baimbridge et al., 1982; Kurobe et al., 1992). Immunostaining of Purkinje cells with antibodies to calbindin aides in quantifying Purkinje cell loss in various disease processes (de Barry and Gombos, 1989; Iacopino et al., 1990; Kurobe et al., 1992; Ferrer et al., 1993; Ishikawa et al., 1995; Maguire-Zeiss et al., 1995; Nakagawa et al., 1996; Nag and Wadhwa, 1999; Barski et al., 2003; Haworth et al., 2006; Laure-Kamionowska and Maślińska, 2009; Lee et al., 2010; Verdes et al., 2010; Wierzba-Bobrowicz et al., 2011). Indeed, decreases in calbindin immunoreactivity may be seen in disease prior to the loss of Purkinje cells and the onset of ataxia (Vig et al., 1998; Barski et al., 2003). In both the murine and feline models of NPC1 disease, decreased calbindin immunoreactivity in Purkinje cells and Purkinje cell loss have been described (Davidson et al., 2009; Vite et al., 2015). Furthermore, elevations in CSF calbindin concentrations have been found in numerous neurologic diseases, specifically those with prevalent cerebellar involvement (Kiyosawa et al., 1993; Craig-Schapiro et al., 2011).

In this study, we evaluated calbindin concentrations in the CSF as a biomarker of NPC1 disease progression. First, we measured calbindin concentrations in cats with naturally occurring NPC1 disease, which closely resemble the biochemistry, pathology, and clinical presentation of juvenile NPC1 disease (Somers et al., 2003; Vite et al., 2008). Next, calbindin concentrations were determined in NPC1 cats that received intrathecal HPβCD into the cerebellomedullary cistern. We previously reported that presymptomatic intrathecal HPβCD administration to cats with NPC1 disease prevented the onset of cerebellar tremor and resulted in Purkinje cell survival; postsymptomatic therapy similarly slowed the progression of disease (Ward et al., 2010; Vite et al., 2015). Finally, we determined CSF calbindin concentrations from subjects with NPC1 who were untreated or received off-label substrate reduction therapy with miglustat, an inhibitor of glycosphingolipid biosynthesis (Platt et al., 1994).

Materials and Methods

Animals. NPC1 cats were produced by breeding male and female cats heterozygous for the *NPC1* missense mutation. All cats were raised in the National Referral Center for Animal Models of Human Genetic Disease of the University of Pennsylvania School of Veterinary Medicine (NIH OD P40-10939; University of Pennsylvania, Philadelphia, PA) under National Institutes of Health and U.S. Department of Agriculture guidelines for the care and use of animals in research. The experimental protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. There was an equal male/female distribution. Whole blood from cats was tested for the *NPC1* missense mutation using the TaqMan (Life Technologies, Grand Island, NY) real-time polymerase chain reaction–based DNA test to identify affected, normal, and heterozygote cats. The custom TaqMan single-nucleotide polymorphism genotyping assay included forward primer CTGGATCGACGATTACTTTGATTGG and reverse primer CGATCGGTGCTGTTGTAGACT, with VIC/MGB-NFQ and FAM/MGB-NFQ as the reporter/quencher for alleles 1 and 2, respectively. Using a TaqMan Genotyping Master Mix (4371353; Life Technologies), the assay was run on an Applied Biosystems 7500 platform (Applied Biosystems, Foster City, CA). Untreated and treated NPC1 cats were euthanized at a humane endpoint, when they were no longer able to remain in sternal recumbency without support, or at a predetermined endpoint. Euthanasia was performed using an overdose of intravenous barbiturate. Immediately prior to euthanasia, cats were given an intravenous dose of 200 U heparin to prevent blood clotting during the tissue harvest. After euthanasia, animals were perfused through the left ventricle with 750 ml 0.9% cold saline and tissues were collected.

HPβCD. HPβCD-C0926 (Sigma Aldrich, St. Louis, MO) or Kleptose HPB (Janssen Research & Development, Beerse, Belgium) was used for all intracisternal administrations. HPβCD was administered in a 20% (w/v) solution dissolved in 0.9% saline (Hospira Inc., Lake Forest, IL). The presymptomatic treatment group received 120 mg HPβCD (4000 mg/kg brain weight) intrathecally at the cerebellomedullary cistern every 14 days, beginning at 3 weeks of age. This dose was found to be most effective at reducing Purkinje cell death in NPC1 cats in our previous study (Vite et al., 2015) and is similar to the 4000-mg/kg body weight dose used subcutaneously in NPC1 mice (Davidson et al., 2009). The postsymptomatic treatment group received 120 mg HPβCD intrathecally at the cerebellomedullary cistern every 14 days, beginning at 16 weeks of age. All intracisternal dosing and CSF collection was performed in cats anesthetized with propofol (up to 6 mg/kg intravenously; Abbott Laboratories, Chicago, IL).

CSF Collection in Cats. Approximately 1 ml CSF was collected from the cerebellomedullary cistern at the time of each dosing in HPβCD-treated cats and every 2 weeks from untreated NPC1 and normal cats and was frozen at −80°C until analyzed. CSF collection time points and animal numbers per cohort were as follows: normal cohort: 3 weeks (n = 5), 6–10 weeks (n = 5), 12–16 weeks (n = 5), and 20–24 weeks (n = 5); NPC1 untreated cohort: 3 weeks (n = 5), 6–10 weeks (n = 5), 12–16 weeks (n = 6), and 20–24 weeks (n = 5); NPC1 HPβCD presymptomatic cohort: 6–10 weeks (n = 6), 12–16 weeks (n = 5), 20–24 weeks (n = 5), 54–58 weeks (n = 6), and 74–78 weeks (n = 5); and NPC1 HPβCD postsymptomatic cohort: 20–26 weeks (n = 6) and endpoint at 40–51 weeks (n = 4).

CSF Collection in Patients with NPC1. Patient CSF was collected by lumbar puncture between August 2006 and January 2011 as part of an ongoing longitudinal natural history study conducted at the NIH Clinical Center (06-CH-0186; ClinicalTrials.gov NCT00344331). Twenty patients with NPC1 were untreated, whereas 16 patients with NPC1 received off-label substrate reduction therapy with miglustat. Miglustat was dosed in 100-mg capsules at a

dose of 200 mg three times a day adjusted for body surface area in children [(body surface area/1.8) × 600]. This study was approved by the NIH *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Institutional Review Board. CSF was collected in polystyrene tubes, aliquoted, frozen on dry ice, and stored at −80°C. Control CSF was obtained from 31 pediatric patients who were undergoing a lumbar puncture for another clinical indication.

Calbindin Assay. Each well of a 96-well high-bind plate (L15XB-3; Mesoscale, Rockville, MD) was coated with 5 μl monoclonal anti-human calbindin D antibody (MAB3320; R&D Systems, Minneapolis, MN) at 20 μg/ml and incubated overnight uncovered at room temperature to dry spots. The plate was then blocked with 150 μl 3% Blocker A (R93AA-2; Mesoscale) per well and incubated for 1 hour at room temperature on a plate shaker. The plate was then washed five times with phosphate-buffered saline containing 0.05% Tween-20. A standard curve was created using a recombinant human calbindin D (custom order, stock 2.34 mg/ml; R&D Systems) diluted in 1% Blocker A with the highest standard at 234 ng/ml followed by 4-fold serial dilutions. Then, 25 μl of standards and samples (neat) was added to the plate in duplicate and incubated for 1 hour at room temperature on a plate shaker. After five washes, sTAG donkey anti-goat (R32AG-1; Mesoscale) premixed 1:1 with goat polyclonal anti-human calbindin D antibody (AF3320; R&D Systems) combined to 1 μg/ml in 1% Blocker A was added to the plate and incubated for 1 hour at room temperature on a plate shaker. The plate was then washed five times followed by the addition of 150 μl of 1× Read Buffer (R92TC-3; Mesoscale) and read on a Mesoscale plate reader (Sector Imager 2400; Mesoscale).

Statistical Analysis. Statistical analyses were conducted with two-tailed *t* tests. One-way analysis of variance was used to demonstrate significance of the NPC1 patient CSF calbindin concentration after initiation of miglustat therapy. P values < 0.05 were considered statistically significant.

Results

CSF Calbindin Concentrations over Time in NPC1 Cats. To determine whether the release of calbindin from degenerating Purkinje cells could be used as a reliable biomarker of cerebellar disease progression, we measured calbindin concentrations in the CSF of untreated NPC1 cats, produced by breeding male and female cats heterozygous for the *NPC1* missense mutation, and we compared these data with those from normal control cats of the same age (Table 1) using a highly sensitive electrochemiluminescence-based assay. Clinical signs of untreated NPC1 cats include cerebellar ataxia and intention tremors that appear at approximately 6 weeks of age and progress to the inability to ambulate and finally the incapacity to remain in sternal recumbency without support, which warrants euthanasia at a mean age of 20.7 ± 5 weeks ($n = 39$; range, 9–29 weeks) (Vite et al., 2015). At all time points analyzed, untreated NPC1 cats had significantly higher levels of calbindin in the CSF (Fig. 1). Specifically, at 3 weeks of age, calbindin was 19.6 ± 3.7 ng/ml in untreated NPC1 cats ($n = 5$) compared with 11.2 ± 2.2 ng/ml in normal cats at the same age ($n = 5$) ($P = 0.0046$). At 6–10 weeks of age, calbindin in untreated NPC1 cats ($n = 5$) increased to 51.0 ± 15.4 ng/ml compared with 11.3 ± 1.7 ng/ml in normal age-matched cats ($n = 5$) ($P = 0.0009$). In untreated NPC1 cats aged 12–16 weeks ($n = 6$), calbindin further increased to 86.0 ± 26.4 ng/ml and was significantly higher than normal control cats at the same age ($n = 5$) at 9.5 ± 3.3 ng/ml ($P = 0.0003$). Finally, at the age of the humane endpoint for untreated NPC1 cats (20–24 weeks of age; $n = 5$), calbindin in the CSF was elevated to 95.8 ± 29.7 ng/ml, whereas normal age-matched cats ($n = 5$) remained at 9.0 ± 2.0 ng/ml ($P = 0.0004$).

Within the untreated NPC1 cohort, calbindin levels significantly increased between 3 and 6–10 weeks ($P = 0.013$) and between 6–10 and 12–16 weeks ($P = 0.037$). No difference was determined between 12–16 and 20–24 weeks ($P = 0.613$) (Fig. 1), suggesting a plateau in the levels with time. In contrast, no significant differences were found in CSF calbindin concentrations in normal control cats among the ages evaluated.

We hypothesized that presymptomatic administration of HPβCD at 3 weeks of age would decrease CSF calbindin concentrations in NPC1 cats. At 6–10 weeks of age, HPβCD-treated NPC1 cats ($n = 6$) had calbindin concentrations of 8.3 ± 3.8 ng/ml, which were significantly lower than age-matched untreated NPC1 cats ($P = 0.0002$) and similar to those found in normal control cats ($P = 0.201$). At 12–16 weeks ($n = 5$) and 20–24 weeks ($n = 5$) of age, calbindin remained indistinguishable from normal at 7.9 ± 0.8 ng/ml ($P = 0.393$) and 8.5 ± 2.0 ng/ml ($P = 0.728$), respectively, and significantly decreased compared with untreated NPC1 cats ($P = 0.0002$ and $P = 0.0004$, respectively). HPβCD-treated cats evaluated at 54–58 weeks of age ($n = 6$) continued to show low calbindin levels of 5.7 ± 2.2 ng/ml; at the predetermined endpoint of the

TABLE 1
Treatment groups in feline NPC

Genotype	Treatment Group	Dosing	CSF Calbindin Analyzed	Subjects
			wk	*n*
Normal	Untreated		3	5
			6–10	5
			12–16	6
			20–24	5
NPC	Untreated		3	5
			6–10	5
			12–16	5
			20–24 (endpoint)	5
	Presymptomatic HPβCD 120 mg IC	Initiated at 3 wk, every 14 d	6–10	6
			12–16	5
			20–24	5
			54–58	6
			74–78	5
	Postsymptomatic HPβCD 120 mg IC	Initiated at 16 wk, every 14 d	20–26	6
			40–51 (endpoint)	4

IC, intrathecal administration at the cerebellomedullary cistern.

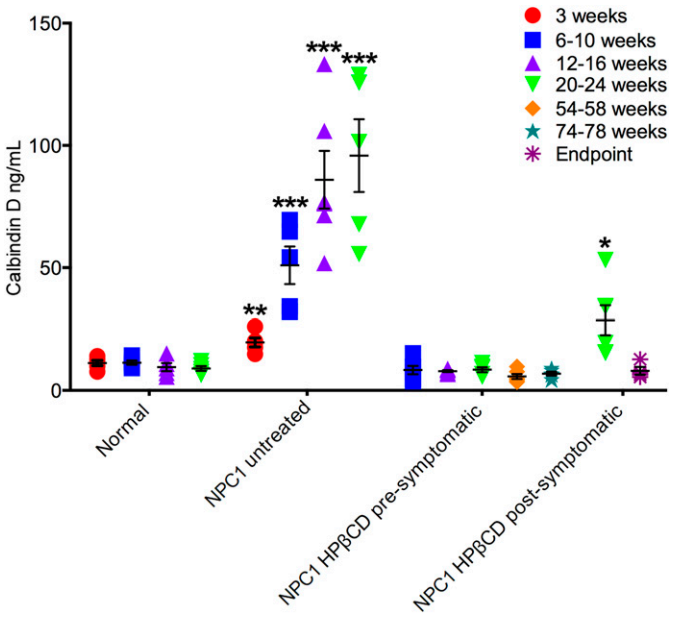

Fig. 1. CSF calbindin levels in NPC1 cats. CSF calbindin levels were significantly increased in NPC1 cats relative to normal age-matched control samples. Initiation of HPβCD prior to symptom onset (3 weeks of age) resulted in normalization of CSF calbindin at all time points analyzed. Cats treated with HPβCD after the onset of symptoms (16 weeks of age) had significantly elevated CSF calbindin levels at the first time point analyzed (20–24 weeks of age) but levels were normalized by the endpoint. Time points were as follows: normal cohort: 3 weeks (n = 5), 6–10 weeks (n = 5), 12–16 weeks (n = 5), and 20–24 weeks (n = 5); NPC1 untreated cohort: 3 weeks (n = 5), 6–10 weeks (n = 5), 12–16 weeks (n = 6), and 20–24 weeks (n = 5); NPC1 HPβCD presymptomatic cohort: 6–10 weeks (n = 6), 12–16 weeks (n = 5), 20–24 weeks (n = 5), 54–58 weeks (n = 6), and 74–78 weeks (n = 5); and NPC1 HPβCD postsymptomatic cohort: 20–26 weeks (n = 6) and endpoint at 40–51 weeks (n = 4). Data are presented as means and S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the normal cohort.

study at 74–78 weeks of age (n = 5), calbindin levels remained below normal levels at 6.8 ± 1.6 ng/ml.

In contrast with NPC1 cats that first received HPβCD prior to the onset of clinical signs, cats that first received HPβCD postsymptomatically at 16 weeks of age had calbindin levels of 28.6 ± 13.5 ng/ml at 20–26 weeks (n = 6), which was significantly higher than normal ($P = 0.022$) but significantly lower than untreated cats ($P = 0.004$). At 20–26 weeks, cats in the postsymptomatic treatment cohort also had significantly higher CSF calbindin levels than cats in the presymptomatic treatment cohort ($P = 0.015$). Four animals in this treatment cohort were allowed to reach the humane endpoint, which occurred between 40 and 51 weeks of age. At the endpoint, calbindin levels were normalized in the CSF of cats treated postsymptomatically at 8.0 ± 2.8 ng/ml, which was not significantly different from cats treated presymptomatically at the 54- to 58-week time point.

CSF Calbindin Concentrations Compared with Clinical Signs and Histopathology. The cats evaluated in this calbindin study were the same cats evaluated in a previously published article in which both clinical signs and histopathology were evaluated (Vite et al., 2015). As previously described, untreated NPC1 cats demonstrated progressive cerebellar ataxia, which was apparent in the first 10 weeks of life and reached maximum severity (a score of 4 on an ataxia scale) by 24 weeks of age. Onset and progression of head tremors were similar, with a maximum score of 3 on a tremor scale by the age of 24 weeks. Untreated cats were euthanized when they were nonambulatory and no longer able to remain in sternal recumbency without support, which occurred at a mean age of 20.7 ± 5 weeks (range, 9–29 weeks) (Vite et al., 2015). Histopathological data from NPC1 cats at the endpoint showed significantly fewer calbindin-positive Purkinje cells of the cerebellum compared with age-matched normal control cats (Vite et al., 2015). In this study, CSF calbindin concentrations were significantly elevated by 3 weeks of age, prior to the onset of cerebellar ataxia and intention tremor, with levels dramatically increasing by 10 weeks of age and rising with disease progression.

In contrast with untreated NPC1 cats, it has been previously detailed that NPC1 cats that received intrathecal administration of HPβCD that began presymptomatically (Table 1) had ataxia and head tremor severity scores that remained normal (score of 0) at 24 weeks of age. Furthermore, NPC1 cats in the presymptomatic HPβCD treatment group showed significantly greater Purkinje cell numbers compared with age-matched untreated NPC1 cats ($P < 0.05$) and were not significantly different from normal cats at 24 weeks of age (Vite et al., 2015). In corroboration with these clinical and histopathological findings, herein the CSF calbindin concentration of NPC1 cats that first began HPβCD prior to the onset of clinical signs remained in the normal range at all time points analyzed.

Vite et al. (2015) also showed that NPC1 cats that received intrathecal administration of HPβCD starting at 16 weeks of age (Table 1) had intermediate ataxia and tremor scores of approximately 2 at 24 weeks of age. Similarly, cats in the postsymptomatic treatment group had significantly fewer Purkinje cells at 24 weeks compared with normal control cats (Vite et al., 2015). Likewise, CSF calbindin concentrations at this time point were intermediate between untreated and normal control cats.

CSF Calbindin Concentrations in Subjects with NPC1. Calbindin CSF concentrations were measured in a cohort of 36 subjects with NPC1 being followed in a natural history trial. Longitudinal values were available for 15 subjects. NPC1 subjects ranged in age from 1.8 to 51.3 years (mean 11.4 ± 10.4 years; median 7.5 years), sex distribution was 16 males and 20 females, and NIH Neurologic Severity Scores (Yanjanin et al., 2010) ranged from 3 to 43 with mean and median values of 17.8 ± 11.6 and 18.0, respectively. At the initial evaluation, 16 subjects (44%) received off-label miglustat therapy, and CSF calbindin levels before and after initiation of miglustat therapy were available for 5 subjects. Figure 2A shows the significant ($P < 0.0001$) elevation of CSF calbindin concentration in subjects with NPC1 (4.78 ± 4.25 ng/ml) compared with control values (0.76 ± 0.34 ng/ml). Calbindin levels poorly correlated but decreased with age ($r^2 = 0.6$, $P = 0.4$; Fig. 2B). Calbindin levels did not correlate with either neurologic disease severity ($r^2 = 0.03$, $P = 0.16$; Fig. 2C) or age-adjusted neurologic severity ($P = 0.87$). No significant difference ($P = 0.28$) was observed when comparing untreated versus miglustat-treated subjects with NPC1 (Fig. 2D). However, we did observe a decrease in CSF calbindin levels when evaluating the subset of subjects with NPC1 for which we had paired CSF samples before and after miglustat therapy. On a percentage basis, CSF calbindin decreased on average to 66.6% ± 6.6% of pretreatment levels 6–15 months after initiation of off-label

miglustat therapy. This marked decrease in CSF calbindin was only observed when evaluating serial pre- and postmiglustat samples. Figure 2F shows a comparison between serial paired samples for subjects who were untreated at both time points ($n = 16$), subjects who were treated at both time points ($n = 15$), and the five pre- and postmiglustat sample pairs. One-way analysis of variance was used to demonstrate significance ($P = 0.011$) of the CSF calbindin concentration after initiation of miglustat therapy.

Discussion

Clear evidence of the therapeutic efficacy of HPβCD in murine and feline models of NPC1 disease led to the initiation of a phase I NIH human clinical trial in 2013 (ClinicalTrials.gov identifier NCT01747135). As safety studies have concluded and larger-scale efficacy studies have begun (ClinicalTrials.gov identifier NCT02534844), the need for a reliable, repeatable, and sensitive biomarker has become increasingly important. An ideal biomarker is one that continues to increase or decrease with disease progression and then ceases to change or is improved with effective therapeutic intervention. Because of the neurodegenerative nature of NPC1 disease, a valuable biomarker must also be capable of detecting changes in the CNS.

Numerous biomarker studies have been conducted in both animal models and human patients with NPC1 disease. One study demonstrated an increase in CSF levels of total tau resulting from axonal degeneration in patients with NPC1. Treatment with miglustat, a glycosphingolipid synthesis inhibitor, was capable of decreasing CSF levels of total tau in subjects with NPC1 (Mattsson et al., 2011). In addition, altered expression of glutathione *S*-transferase, superoxide dismutase, and fatty acid binding protein 3 have also been found in the CSF of subjects with NPC1, and treatment with miglustat appeared to partially correct fatty acid binding protein 3 levels in a subset of subjects with NPC1 (Cologna et al., 2012). Various inflammatory markers have also been determined to have altered levels in the CSF of patients with NPC1, including increased levels of interleukin (IL)-3, chemokine (C-X-C motif) ligand 5, IL-16, and chemokine ligand 3 and decreased levels of IL-4, IL-10, IL-13, and IL-12p40 in the CSF. Treatment with miglustat had only a modest effect on CSF levels of IL-3, IL-10, and IL-13 (Cologna et al., 2014). Increased serum levels of galectin-3, a proinflammatory molecule, and cathepsin D, a lysosomal aspartic protease, have also been established in patients with NPC1. Treatment

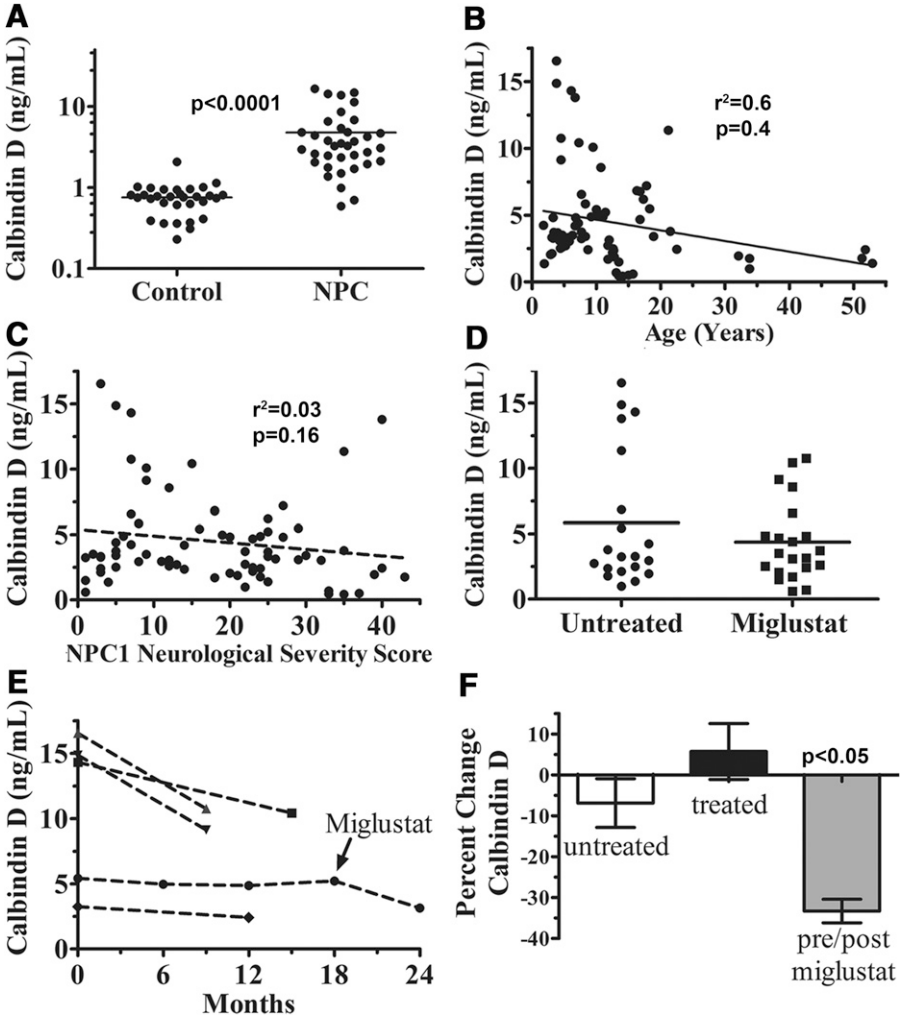

Fig. 2. CSF calbindin levels in patients with NPC1. (A) CSF calbindin levels were significantly ($P < 0.0001$) increased in subjects with NPC1 relative to pediatric control values. (B) Lack of a significant correlation between CSF calbindin levels and age in subjects with NPC1 ($r^2 = 0.6$, $P = 0.4$). (C) CSF calbindin levels also did not significantly correlate ($r^2 = 0.03$, $P = 0.16$) with NPC1 neurologic severity scores. (D) No significant difference noted between subjects treated with miglustat (mean untreated 5.82 ± 5.20 ng/ml, $n = 20$; mean miglustat-treated 4.36 ± 3.05 ng/ml, $n = 16$). (E and F) However, for five subjects for whom pre- and postmiglustat therapy samples were available, CSF calbindin levels decreased ($P < 0.05$) after initiation of miglustat therapy. In (E), miglustat therapy was initiated after the time 0 sample, except for the one subject for which four pretreatment samples were available and miglustat therapy was initiated at 18 months (arrow).

of NPC1 knockout mice with HPβCD, but not miglustat, normalized liver expression of galectin-3 and cathepsin D (Cluzeau et al., 2012). Using high-performance liquid chromatography and tandem mass spectrometry, various lipids including monohexosylceramides, ceramides, sphingoid bases, GM1 and GM3 gangliosides (Fan et al., 2013), and lysosphingomyelin-509 (Giese et al., 2015) have been evaluated and suggested as potential biomarkers for NPC1. Despite the abundance of studies, none of the above biomarkers were considered reliable, sensitive, and specific enough to validate the effectiveness of HPβCD in the established clinical trial.

Currently, 24(*S*)-HC, a sterol that is synthesized exclusively in neurons in the CNS and exported to the plasma, is decreased in the plasma of patients with NPC1 (Porter et al., 2010) and has demonstrated the most potential as a pharmacodynamic biomarker of target engagement in NPC1 disease (Maarup et al., 2015). Extensive studies conducted in the feline model of NPC1 demonstrated an increase in 24(*S*)-HC in the CSF and plasma after the initial administration of HPβCD; however, attenuation of the response was seen and 24(*S*)-HC was not elevated in response to any subsequent doses (Tortelli et al., 2014). As a result of the mitigation after initial dosing in cats, 24(*S*)-HC may not be effective as a marker to monitor therapy long term.

Calcium homeostasis is crucial for neuronal function and the calcium-binding protein calbindin has demonstrated differential expression, including the cerebellum, hippocampus, cingulate cortex, and parietal cortex, and contributed to selective neuronal vulnerability (Mattson et al., 1991; Mithbaokar et al., 2016) in adverse conditions such as ischemia (Goodman et al., 1993) and Alzheimer disease (Greene et al., 2001; Iritani et al., 2001). Isolated areas of the aging rat and human brain including the cerebellum and corpus callosum, as well as discrete areas of human brain tissue afflicted with Parkinson, Huntington, and Alzheimer disease, have shown significant reductions in calbindin mRNA and protein levels (Iacopino and Christakos, 1990). CSF calbindin has been shown to be elevated in Alzheimer disease (Craig-Schapiro et al., 2011) and in patients with cerebellar lesions including multiple system atrophy, subacute cerebellar degeneration with lung cancer, and Wernicke-Korsakoff syndrome (Kiyosawa et al., 1992, 1993). Together, these studies suggest that degenerating neurons, predominately Purkinje cells in the cerebellum, leak calbindin into the CSF, which can then be reliably measured by an immunoassay and serve as a potential marker of neurodegenerative cerebellar disease.

Progressive Purkinje cell death is the hallmark CNS pathology of NPC disease. It has previously been shown that NPC1 cats revealed a significant loss of calbindin-positive Purkinje cells at end-stage disease (approximately 24 weeks of age). Presymptomatic HPβCD treatment attenuated the loss of Purkinje cells, and all cats in this study cohort lived beyond the 76-week study period or were euthanized due to nondisease-related causes. However, postsymptomatic HPβCD treatment was less effective at offsetting cell death, and significantly fewer Purkinje cells remained compared with normal age-matched cats. Survival corroborated these findings because no single cat in the postsymptomatic cohort lived to 1 year of age (43.5 $\pm$ 5.8 weeks of age) (Vite et al., 2015).

In this study, we evaluated calbindin levels in the CSF as a biomarker of disease progression in untreated cats affected with NPC1 disease and as a measure of therapeutic efficacy in NPC1 cats treated with HPβCD both before and after the onset of symptoms. We found CSF calbindin to be a convincing marker of disease progression, increasing steadily with age, and it was significantly higher than normal age-matched controls at all time points analyzed. CSF calbindin was drastically elevated (>10-fold normal) at end-stage disease. This corresponds with the severe loss of calbindin-positive Purkinje cells, as previously seen by histopathology (Vite et al., 2015). Importantly, CSF calbindin was significantly elevated at the earliest time point analyzed (3 weeks of age), which was prior to the onset of cerebellar dysfunction, signifying calbindin as a sensitive marker providing early detection of subtle CNS changes. CSF calbindin did not significantly differ between age groups in normal cats, confirming that alterations are not a result of development or aging. Presymptomatic HPβCD treatment completely normalized CSF calbindin at all ages analyzed, which ranged from 6 to 78 weeks of age. These results are consistent with the preservation of calbindin-positive Purkinje cells, previously determined by immunohistochemistry (Vite et al., 2015). Finally, 4–8 weeks after postsymptomatic HPβCD treatment, CSF calbindin levels were significantly higher than normal. Again, this correlated with a significant loss of Purkinje cells compared with normal control cats (Vite et al., 2015). However, when animals reached the humane endpoint due to neurologic NPC1 disease, CSF calbindin levels had been normalized.

Our data support that disease-related death of Purkinje cells results in a release of calbindin into the CSF, which can then be readily quantified by an immunoassay (Kiyosawa et al., 1992, 1993; Craig-Schapiro et al., 2011). Although the exact mechanism of HPβCD in unknown, it is clear that presymptomatic treatment leads to clearance of cholesterol and sphingolipid storage and preservation of calbindin-positive Purkinje cells in the cerebellum (Vite et al., 2015). The postsymptomatic treatment group may provide more clarity of the shortcomings of HPβCD and also the role of calbindin. Four to 8 weeks after postsymptomatic treatment, calbindin levels remained elevated in the CSF, possibly suggesting that Purkinje death was ongoing or that the accumulation of calbindin was long-lasting in CSF. Interestingly, all cats in the postsymptomatic treatment group had normalized levels of CSF calbindin at the humane endpoint. Postsymptomatic treatment was capable of thwarting further Purkinje cell death and slowing disease progression; however, late intervention was less profound at improving neurologic function and increasing survival. These data confirm that presymptomatic therapy is most effective and that a threshold of Purkinje cell death had been reached by 16 weeks of age that had irreversible effects on neurologic function.

Finally, we evaluated CSF calbindin in patients with NPC1. As seen in NPC1 cats, calbindin concentrations were significantly elevated in the CSF of untreated patients with NPC1. In addition, 16 patients with NPC1 were undergoing off-label treatment with miglustat, an aminosugar that inhibits glucosylceramide synthase (Platt et al., 1994). Miglustat has been shown to stabilize, but not reverse, neurologic disease progression in children, juveniles, and adults (Patterson et al., 2007, 2010; Pineda et al., 2009). In this study, we demonstrated that after the initiation of substrate reduction therapy with miglustat, CSF calbindin levels were significantly decreased compared with pretreatment concentrations. These

data suggest that CSF calbindin concentrations could serve as an outcome measure for ongoing clinical trials for NPC1, including the phase I/II study of vorinostat therapy (ClinicalTrials.gov identifier NCT02124083) and the phase IIb/III study of HPβCD (ClinicalTrials.gov identifier NCT02534844).

In summary, this study identified CSF calbindin as a novel and sensitive biomarker of NPC1 disease progression and of therapeutic efficacy of HPβCD in the feline model of NPC1. Most importantly, calbindin was detectable early in the disease stage prior to onset of clinical signs, steadily increased with disease progression, and was normalized after effective therapeutic intervention. CSF calbindin was also elevated in subjects with NPC1 and was decreased after substrate reduction therapy with miglustat. These data suggest implications for CSF calbindin concentrations as a measure of disease progression and therapeutic efficacy in ongoing human clinical trials.

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Authorship Contributions

Participated in research design: Bradbury, Bagel, Sampson, Remaley, Porter, Vite.

Conducted experiments: Bradbury, Bagel, Sampson, Farhat, Ding, Swain, Prociuk, O'Donnell, Porter, Vite.

Contributed new reagents or analytic tools: Sampson, Wassif, Remaley.

Performed data analysis: Bradbury, Farhat, Drobatz, Gurda, Porter.

Wrote or contributed to the writing of the manuscript: Bradbury, Sampson, Farhat, Wassif, Remaley, Porter, Vite.

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