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Application of N-Palmitoyl-O-Phosphocholineserine for Diagnosis and Assessment of Response to Treatment in Niemann-Pick Type C disease

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Abstract

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Niemann-Pick type C (NPC) disease is a rare lysosomal storage disorder caused by mutations in either the *NPC1* or the *NPC2* gene. A new class of lipids, N-acyl-O-phosphocholineserines were recently identified as NPC biomarkers. The most abundant species in this class of lipid, N-palmitoyl-O-phosphocholineserine (PPCS), was evaluated for diagnosis of NPC disease and treatment efficacy assessment with 2-hydroxypropyl-β-cyclodextrin (HPβCD) in NPC. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were developed and validated to measure PPCS in human plasma and cerebrospinal fluid (CSF). A cutoff of 248 ng/mL in plasma provided a sensitivity of 100.0% and specificity of 96.6% in identifying NPC1 patients from control and NPC1 carrier subjects. PPCS was significantly elevated in CSF from NPC1 patients, and CSF PPCS levels were significantly correlated with NPC neurological disease severity scores. Plasma and CSF PPCS did not change significantly in response to intrathetical (IT) HPβCD treatment. In an intravenous (IV) HPβCD trial, plasma PPCS in all patients was significantly reduced. These results demonstrate that plasma PPCS was able to diagnose NPC1 patients with high sensitivity and specificity, and to evaluate the peripheral treatment efficacy of IV HPβCD treatment.

Keywords

LysoSM-509; N-palmitoyl-O-phosphocholineserine; Niemann-Pick disease type C; diagnosis; 2-hydroxypropyl-β-cyclodextrin; treatment assessment

INTRODUCTION

Niemann-Pick type C (NPC) disease is an autosomal recessive neurovisceral lysosomal storage disorder with an estimated minimal incidence of 1/120,000 live births (1). Approximately 95% of the NPC cases result from loss-of-function of NPC1 protein caused by genetic mutations (2), with the remainder involving deficiency of NPC2 protein (3). Impairment of either protein leads to the accumulation of unesterified cholesterol and glycosphingolipids in lysosome and late endosomes (1).

The heterogeneous NPC phenotype includes hepatosplenomegaly, cholestatic jaundice, developmental delay, gait ataxia, clumsiness, cataplexy, epilepsy, dystonia, supranuclear gaze palsy, dysarthria, dysphagia, cerebellar ataxia, psychiatric illnesses, or cognitive decline, with differing ages of onset and rates of disease progression (4). As a result, the diagnosis of NPC is challenging (5). Biomarker profiling and genetic tests are currently used as first-line diagnostic tests for NPC; the time-consuming filipin staining test is used to facilitate diagnosis in uncertain cases (6). Biomarkers currently used for NPC diagnosis include cholestane-3β,5α,6β-triol (7–20), 7-ketocholesterol (7, 8, 17), N-palmitoyl-O-phosphocholineserine (PPCS, referred to as lysoSM-509 prior to structural identification) (21–26), lysosphingomyelin (27), and 3β,5α,6β-trihydroxycholanoyl-glycine (28, 29). Miglustat (N-butyl-deoxynojirimycin), which inhibits the synthesis of glucosylceramide, the building block of more complex glycosphingolipids, is approved outside the US for treatment of NPC but shows only limited efficacy in slowing the disease progression (30). Treatment with 2-hydroxypropyl-β-cyclodextrin (HPβCD) significantly slowed progression of neurological manifestations, and increased lifespan in NPC1 mouse and cat models (31–

34). Intrathecal (IT) HPβCD treatment slowed neurodegenerative disease progression in NPC1 patients in Phase 1/2a trial, and is currently being studied in Phase 2b/3 trial and extension study (NCT# 02534844) (35–37). Clinical trials of intravenous (IV) delivery of HPβCD for treatment of visceral manifestations in NPC disease (NCT#s 03471143, 03887533) are also in progress.

PPCS was originally misassigned as an isoform of lysosphingomyelin (21). We recently identified it as the most abundant species in a new class of lipid, N-acyl-O-phosphocholineserines (38). After synthesizing a standard compound and a deuterated internal standard, we developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for accurate and reliable quantification of PPCS. Here we applied PPCS to NPC diagnosis and assessment of treatment efficacy with HPβCD.

MATERIALS AND METHODS

Clinical studies

Human studies adhered 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. Informed consents were obtained from the participants and their guardians. The clinical protocols were approved by the Institutional Review Boards of NICHD/NIH, Rush University Medical Center, Universitätsklinikum Münster, Hospital de Clínicas de Porto Alegre, Centro Universitario Estácio de Ribeirão Preto, St. Louis Children's Hospital, Boston Children's Hospital, Asante Pediatric Hematology and Oncology – Medford, and Children's Hospital of Orange County. All the clinical samples were de-identified, and the analysis of de-identified human samples was approved by the Human Studies Committee at Washington University.

Collection of plasma samples for diagnostic assay development

All plasma samples were collected in ethylenediamine tetraacetic acid dipotassium salt containing tubes. The NPC1 and NPC1 carrier plasma samples were collected from affacted and obligate heterozygote study participants, respectively, at NICHD/NIH, Rush University Medical Center, Universitätsklinikum Münster, and Centro Universitario Estácio de Ribeirão Preto. The longitudinal natural history study of NPC disease was conducted at the NIH. NIH also provided plasma samples from study participants with familial hypercholesterolemia (FH) and Batten resulting from mutations in CLN3. Hospital de Clínicas de Porto Alegre collected plasma samples from patients affected with mucopolysaccharidosis (MPS) type I, II, IIIA, IIIB, IIIC, IVA, VI, VII, GM1 gangliosidosis (GM1), GM2 gangliosidosis (GM2) including Tay-Sachs and Sandhoff, Batten resulting from mutations in CLN1 and CLN2 genes, mucolipidosis (ML) type II/III, Fabry, Krabbe, and Gaucher diseases. Centro Universitario Estácio de Ribeirão Preto also provided plasma samples from individuals affected with acid-sphingomyelinase deficiency (ASMD), Wolman, Tay-Sachs, cerebrotendinous xanthomathosis (CTX), Fabry, and spastic paraplegia type 5 (SPG5). Universitätsklinikum Münster collected plasma samples from individuals affected with ASMD and cholesteryl ester storage disease (CESD), and ASMD carriers. Normal plasma samples were obtained from anonymized residual samples at St. Louis Children's Hospital.

Collection of plasma and CSF samples from monthly IT HPβCD treatment of NPC disease

The Phase 1/2a open-label, dose-escalation study of monthly intrathecal doses of 50–1200 mg of HPβCD was performed at NIH and Rush University Medical Center, and the detailed protocol was described in a recent paper (35). Plasma samples were collected at pre-dose, 8, 24, 30, 48, and 72 h post-dose after either saline or HPβCD infusion. CSF (1 mL) from treated NPC1 study participants was collected via lumbar puncture into polypropylene tubes with 20 mg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). NPC1 CSF samples from untreated patients were also collected at Boston Children's Hospital. Control CSF samples were obtained from pediatric patients with other clinical indications at St. Louis Children's Hospital.

Collection of plasma samples from IV HPβCD treatment of NPC disease

Under FDA-approved individual patient investigational new drug applications, two female NPC1 patients (patients 1 and 2) and a male NPC1 patient started IV HPβCD at 21 months, 10 years, and 13 years old, respectively. The doses for patients 1, 2, and 3 were 500, 2000, and 2000 mg/kg/week, respectively. Pre-dose plasma samples were collected from patient 1 at Children's Hospital of Orange County, and from patients 2 and 3 at Asante Pediatric Hematology and Oncology - Medford.

Animal studies

Cats were raised in the animal colony of the School of Veterinary Medicine at the University of Pennsylvania under NIH and US Department of Agriculture guidelines for the care and use of animals in research. Six groups of cats were evaluated, including 1) normal control cats; 2) untreated NPC1 cats; 3) NPC1 cats receiving intracisternal (IC) saline every 14 days beginning at 3 weeks of age; 4) NPC1 cats receiving 120 mg IC HPβCD every 14 days beginning at 3 weeks of age; 5) NPC1 cats receiving a combination of 1000 mg/kg subcutaneous (SC) HPβCD every 7 days and 120 mg IC HPβCD every 14 days beginning at 3 weeks of age; 6) NPC1 cats receiving 120 mg IC HPβCD every 14 days beginning at 16 weeks of age (34). The serum or plasma and CSF were collected at pre-dose. NPC1 cats were sacrificed when they were no longer able to maintain sternal recumbancy. The liver and and brain samples were collected from NPC1, heterozygous NPC1 and normal cats. Experimental procedures were approved by the Washington University and University of Pennsylvania Animal Studies Committees and were conducted in accordance with the US Department of Agriculture Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

LC-MS/MS methods and method validation

Detailed sample preparation, LC-MS/MS conditions, method validation are described in the Supplementary Information.

Analysis of human plasma and CSF samples

Samples consisted of calibration standards in duplicate, a blank, a blank with internal standard, quality control (QC) samples (low QC, medium QC and high QC), and unknown clinical samples were analyzed. The standard curve covered the expected unknown sample

concentration range, and samples that exceeded the highest standard were diluted and re-assayed. In the dilution sample re-assay, a diluted QC in triplicate would be also included in the analytical run. The results of the QC samples provided the basis of accepting or rejecting the run according to FDA guidelines (39).

Analysis of cat plasma, serum, liver and brain samples

The analyzed samples consisted of calibration standards in duplicate, a blank, a blank with internal standard, and unknown study samples. The standard curve covered the expected unknown sample concentration range, and samples that exceeded the highest standard were diluted and re-assayed.

Statistics

For group comparisons, and the statistical significance of differences in mean values was determined by a two-tailed Student's t test and one-way ANOVA test with Dunnett test as follow-up test. A p value of 0.05 or less was considered significant.

RESULTS

LC–MS/MS method development and validation

Detailed method development and validation are described in the Supplementary Information. The methods were validated for sensitivity, selectivity, accuracy, precision, linearity, carryover, recovery, matrix effect, and stabilities of PPCS in plasma, CSF, processed samples, and stock solutions, and commonly accepted criteria (39) were met (Supplementary Materials and Methods, and Table S1, Figure S1).

Blood diagnostic test for NPC based on PPCS

PPCS concentrations in plasma samples from 179 NPC1 subjects (0 – 55 years; mean age 9 years), 130 controls (0 – 27 years; mean age 9 years) and 48 obligate heterozygotes (parents of NPC1 subjects) or known sibling carriers (4–66 years; mean age 44 years) were determined (Figure 1A). PPCS plasma concentrations were significantly elevated in the NPC1 subjects (mean 2492 ng/mL; range 254–18200 ng/mL; $P < 0.001$), compared to controls (mean 19.1 ng/ mL, range 1.3–542 ng/mL) and NPC1 heterozygotes (mean 125 ng/ mL, range 14.8–680 ng/mL), consistent with previous reports. PPCS was also significantly elevated in NPC1 heterozygotes, as compared with controls (Figure 1A). The receiver-operator characteristic (ROC) analysis demonstrated that the area under the curve (AUC) was 0.9992 (Figure 1B). A cut-off value of 248 ng/mL yielded a sensitivity of 100% and specificity of 96.63% to discriminate NPC1 subjects from controls and NPC1 carriers.

To assess specificity of the assay, we examined samples from patient with other disorders, including Fabry, FH, Gaucher, Krabbe, CTX, GM1, GM2, SPG5, MPS I, II, IIIA, IIIB, IIIC, IVA, VI, VII, CLN1-, CLN2-, and CLN3-Batten, ML II/III, SYNGAP1, lysosomal acid lipase deficiency (LALD) including CESD and Wolman, ASMD, and ASMD carriers. Only ASMD subjects demonstrated PPCS levels above the cutoff for NPC1; PPCS levels in other disorders and ASMD carriers were below the cutoff (Figure 1C and 1D).

The plasma PPCS levels were not significantly correlated with NPC neurological disease severity scores (r^2 = 0.03338, p = 0.0962) (Figure 1E), but significantly correlated with NPC annual severity increment score that is the ratio of NPC neurological disease severity score to age (40) (r^2 =0.04802, p = 0.0233) (Figure 1F). The change of PPCS in the plasma over time collected from the natural history study varied heterogeneously (Figure 1G). There was no significant difference in plasma PPCS levels between male and female study participants (Figure 1H).

Application of PPCS to assessment of treatment with HPβCD in NPC1 disease

Response of PPCS to HPβCD treatment in NPC1 study participants—The plasma samples collected from NPC1 study participants in Phase 1/2a trial of IT HPβCD at pre-dosed time point in each visit were used to evaluate the long-term effect of IT HPβCD on PPCS. The plasma PPCS levels during 72 hours in baseline visit (saline treatment) and in the first visit (IT 900 mg HPβCD) varied less than 12% and 15%, respectively (Figure 2A), suggesting that the short-term pre- and post-treatment variation of PPCS were less than 15%. Thus, the difference in pre-dosed PPCS between baseline visit and later visits larger than 15% was considered as significant. PPCS was decreased, increased, and unchanged in 44% (Figure 2B), 17% (Figure 2C), 33% of subjects (Figure 2D), respectively. Only plasma samples were collected in an IV HPβCD trial, PPCS in 3 patients was significantly reduced, and in 1 patient was close to normal level (Figure 2E).

PPCS levels in NPC1 CSF are significantly elevated (Figure 3A) and significantly correlated with NPC neurological disease severity scores (r^2 = 0.3416, p = 0.0043) (Figure 3B) but not annual severity increment scores (r^2 = 0.08675, p = 0.1949) (Figure 3C). However, CSF PPCS levels were also highly correlated with plasma levels (r^2 = 0.6708, p < 0.0001) (Figure 3D), suggesting that CSF PPCS was largely derived from blood, and its ability to evaluate the change of PPCS in central nervous system is confounded. In the IT HPβCD trial, the pre-treatment CSF samples supplied with 2% CHAPS that prevented absorption loss of PPCS were collected from only 2 study participants. CSF PPCS did not change after HPβCD treatment in these 2 individuals (Figure 3E).

Response of PPCS to HPβCD treatment in NPC1 cat model—The cat plasma and serum PPCS values were very close ($PPCS_{plasma}$ = 0.9058*$PPCS_{serum}$ - 4.453, r^2 = 0.9566, p < 0.0001; Figure 4A), suggesting that the plasma values can be used at time points at which serum samples were not collected, and *vice versa*. Compared to normal (Figure 4B) and heterozygous cats (Figure 4C), PPCS in NPC1 cat serum (Figure 4D) was significantly elevated at all the ages analyzed, including the asymptomatic 3 weeks time point. While the serum PPCS in normal and heterozygous cats showed no significant difference and did not change with the age (Figure 4B and 4C), PPCS levels in untreated and saline-treated NPC1 cats rapidly increased between 3 and 6 – 10 weeks and reached the maximum at 15 – 25 weeks. There is inconsistency between 15 – 30 weeks in which PPCS was reduced in majority of NPC1 cats, though increase in a few of NPC1 cats was also observed (Figure 4D and 4E). Whereas 3 out of 4 NPC1 cats treated with IC 120 mg/kg HPβCD beginning at 3 weeks showed post-treatment increase of PPCS, PPCS in 1 cat was not changed over 192 weeks (Figure 4F). Serum PPCS in NPC1 cats treated with IC 120 mg/kg HPβCD beginning

at 16 weeks was reduced after receiving the treatment (Figure 4G). The serum PPCS levels were not significantly increased in all 9 NPC1 cats treated with IC 120 mg/kg HPβCD and SC 1000 mg/kg HPβCD beginning at 3 weeks for at least 47 weeks, and increased 6-fold in only 1 cat at 130 weeks (Figure 4H).

PPCS in livers ($p = 0.0002$) and brains ($p = 0.0001$) of untreated NPC1 cats was significantly elevated compared to normal and heterozygous cats (Figure 5A and 5B). The serum PPCS values significantly correlated with liver values ($r^2 = 0.465$, $p = 0.021$; Figure 5C), but not brain values ($r^2 = 0.05618$, $p = 0.4858$; Figure 5D). Of the cat CSF samples were not collected in the tubes with CHAPS, significant absorption loss of PPCS was found, and many of them showed undetectable PPCS. The cat CSF data were not used for further study. Treatments with saline and HPβCD did not significantly change PPCS in liver and brain (Figure 5A and 5B).

DISCUSSION

Currently biomarker profiling and genetic test are first line diagnostics for NPC disease (6). Among the biomarkers used, cholestane-3β,5α,6β-triol and 7-ketocholesterol are generated from nonenzymatic cholesterol oxidation due to oxidative stress in NPC cells (8, 41, 42), and 3β,5α,6β-trihydroxycholanoyl-glycine is a metabolite from cholestane-3β,5α,6β-triol (28). PPCS and lysosphingomyelin have a phosphocholine group, and their accumulation in NPC disease may be related to reduced acid sphingomyelinase in NPC disease (43). While plasma lysosphingomyelin was only moderately elevated in NPC disease, PPCS was dramatically elevated and more sensitive for identification of NPC1 patients (21–27). Profiling cholesterol oxidation products and phosphocholine metabolites such as PPCS provides complementary testing results for diagnosis of NPC1 disease. Previous diagnostic assays based on PPCS were developed without the standard curves prepared from authentic compound, and assay accuracy was impaired; thus, accurate reference ranges for control, heterozygotes, and NPC1 patients could not be obtained (21–26). Recently we identified the structure of PPCS, and synthesized the standard compound and its deuterated internal standard (38). In this study, we developed a reliable PPCS-based diagnostic assay for NPC using authentic compound, established reference ranges for diagnosis and for direct inter-laboratory data comparison. Our assay can serve as a prototype test and is ready for dissemination to other clinical laboratories.

PPCS showed excellent performance for diagnosis of NPC1 disease: 100% sensitivity and 96.6% specificity for discrimination of NPC1 affected individuals from controls and NPC1 carriers with AUC of 0.9992. PPCS also showed high specificity in differentiation of NPC1 disease from other diseases including Fabry, FH, Gaucher, Krabbe, CTX, GM1, GM2, SPG5, MPSI, MPSII, MPSIIIA, MPSIIIB, MPSIIIC, MPSIVA, MPSVI, MPSVII, CLN1-, CLN2- and CLN3-Batten, MLII/III, SYNGAP1, LALD (CESD and Wolman). In contrast to the current diagnostic standard cholestane-3β,5α,6β-triol, which was also elevated in other lysosomal lipidosis such as CTX and LALD (11), PPCS was not elevated in these disorders. PPCS was elevated in patients with ASMD, in which cholestane-3β,5α,6β-triol was also elevated and overlapped with NPC1 disease (14). Due to the overlap between NPC1 and ASMD in PPCS, additional tests such as acid sphingomyelinase assay and molecular test for

SMPD-1 are required for the final diagnosis. Although no samples from NPC2 patients have been assayed, PPCS may also have utility for diagnosis in NPC2 patients as well.

PPCS was elevated in plasma/serum, liver, and brain from the NPC cat model, and plasma and CSF from NPC1 patients, suggesting that it is a peripheral and central nervous system biomarker in NPC1 disease. The serum and liver PPCS levels in cat model were significantly correlated. The rapid increase of PPCS in NPC1 cat serum until 16 weeks of age may reflect disease progress in the peripheral organs including liver and blood cells during this period, even though the clinical signs had not yet appeared. Plasma PPCS levels in NPC1 patients were significantly correlated with NPC annual severity increment scores but not NPC neurological disease severity scores, which was in agreement with previous report (21), though the correlation with annual severity increment scores was not very strong. We have found that PPCS was generated in whole blood, plasma, red blood cells, and white blood cells when they were drying on newborn screening card (38). Interestingly, the annual severity increment score was also correlated with B cell lysosomal volume (40), suggesting that there is an association between the annual severity increment score and storage in the hematopoietic system. The NPC neurological disease severity score represents the NPC neurological disease progression, and thus is not associated with PPCS generated in peripheral blood. Despite being confounded by contribution of PPCS from blood, CSF PPCS levels showed significant correlation with NPC neurological disease severity scores, suggesting that it was associated with central nervous system pathology and might be useful to predict central nervous system progression. The lack of correlation between CSF PPCS and annual severity increment score may be explained by non-linear relationship of increase in the NPC neurological disease severity score and age (44).

The therapeutic efficacy of HPβCD in slowing progression of neurological signs and death has been demonstrated in NPC1 mice and cats (31–34). The phase 1/2a intrathecal HPβCD trial indicated acceptable safety profile, improved neuronal cholesterol homoeostasis, decreased neuronal damage, and decreased neurological progression in HPβCD-treated participants (35–37). Besides its efficacy in central nervous system, HPβCD has also been used to treat peripheral disease in NPC1. In the present study, we evaluated the response of PPCS to HPβCD treatment in NPC1 patients and cat model. IV HPβCD treatment in 3 NPC1 patients significantly lowered the plasma PPCS levels. The responses of plasma PPCS in NPC1 patients were heterogeneous after IT administration of HPβCD that was excreted from central nervous system into blood, and the degrees of reduction in those responders were much smaller than IV treatment. Similar to NPC1 patients, only SC HPβCD treatment prevented elevation of serum PPCS in most NPC1 cats. The significant correlation between serum and liver PPCS levels suggests that the serum levels may be useful to monitor PPCS change in liver. There was no significant difference in liver PPCS between SC HPβCD treated and untreated NPC1 cats; however, this comparison was obscured by large within-group variation in small number of treated animals and different age between 2 groups. Progressive elevation of serum PPCS in individual NPC1 cats before 16 weeks followed by a subsequent greater degree of variability suggested that using pre-treatment samples as their own historical controls may be most appropriate. PPCS in CSF of NPC1 patients and in brains of NPC cats did not decrease upon IT/IC HPβCD treatment, suggesting that the effect of HPβCD on metabolism of PPCS in central nervous system differs from that in peripheral

organs. There was no reduction of PPCS in central nervous system by IT HPβCD treatment, which redistributed lysosomal cholesterol and improved neuronal cholesterol homoeostasis, implying that the lysosomal accumulation of cholesterol may not be a prerequisite for elevation of PPCS in NPC disease.

In summary, we developed a plasma NPC diagnostic assay using authentic PPCS that allowed establishment of accurate reference ranges for controls, NPC1 carriers, and NPC1 patients. This diagnostic marker showed high sensitivity and specificity. There was no overlap between NPC1 and a number of lysosomal storage diseases, except for ASMD. This assay is valuable for confirmation of the diagnosis of NPC1 disease. Significant elevation of PPCS in brains from NPC1 cat model and correlation of CSF PPCS in NPC1 patients with NPC neurological disease severity scores indicate that PPCS may play a role in central nervous system disease. The IT HPβCD treatment reduced lysosomal cholesterol accumulation in neuron but did not affect PPCS levels, suggesting that PPCS elevation in NPC1 disease was caused by pathophysiologic mechanisms other than lysosomal cholesterol accumulation. Further investigation into the precise role of PPCS in NPC1 disease is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ASMD	acid-sphingomyelinase deficiency
AUC	area under the curve
CESD	cholesteryl ester storage disease
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CSF	cerebrospinal fluid
CTX	cerebrotendineous xanthomathosis
%CV	percent coefficient of variance

FH	familial hypercholesterolemia
GM1	GM1 gangliosidosis
GM2	GM2 gangliosidosis
HPβCD	2-hydroxypropyl-β-cyclodextrin
IC	intracisternal
IT	intrathecal
IV	intravenous
LALD	lysosomal acid lipase deficiency
LC-MS/MS	liquid chromatography-tandem mass spectrometry
ML	mucolipidosis
MPS	mucopolysaccharidosis
NPC	Niemann-Pick type C
PPCS	N-palmitoyl-O-phosphocholineserine
QC	quality control
ROC	receiver-operator characteristic
SC	subcutaneous
SPG5	spastic paraplegia type 5

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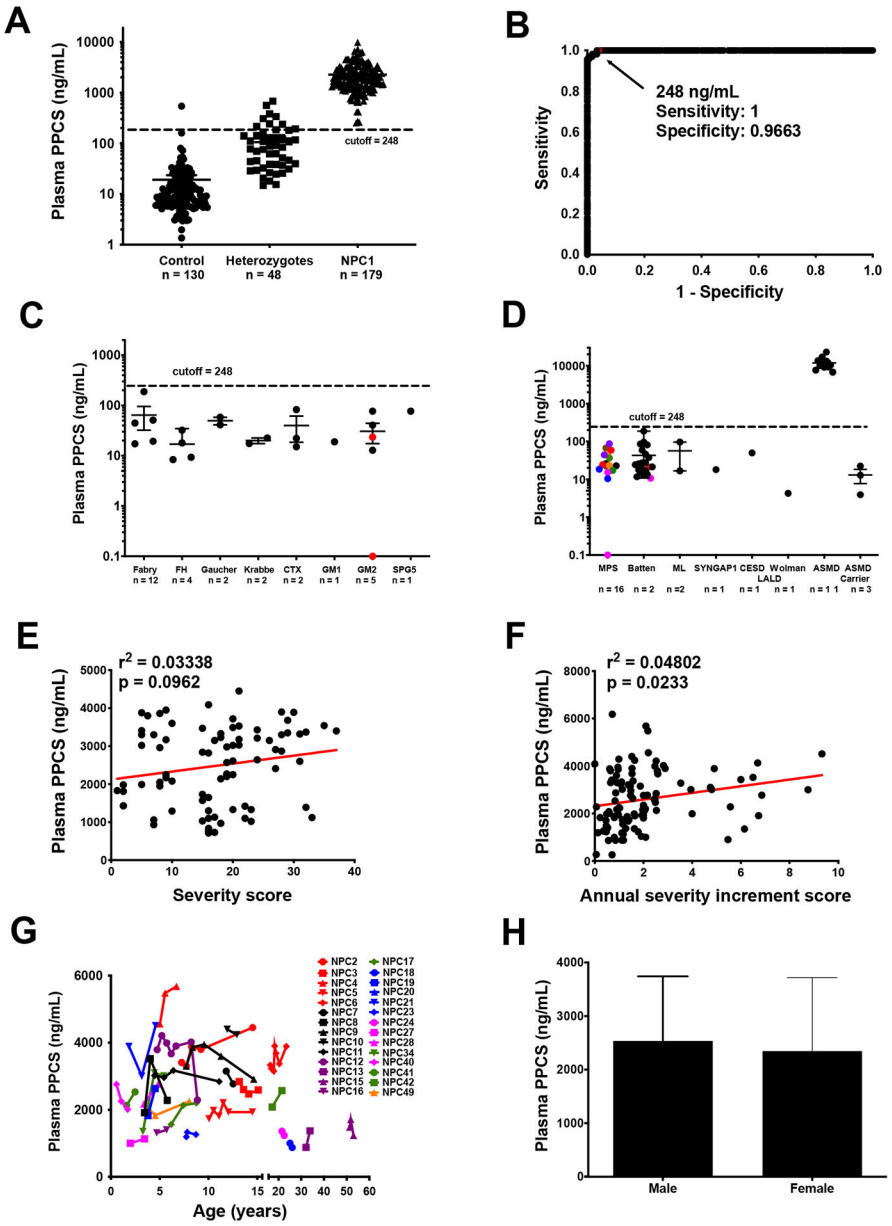

Figure 1.

Plasma PPCS in patients affected with lysosomal storage diseases. (**A**) PPCS in plasma samples from control (n = 130), NPC1 heterozygote (n = 48), and NPC1 affected individuals. (n = 179). Data are presented as mean ± SEM. P < 0.0001 for heterozygotes versus controls and for NPC1 versus controls. (**B**) ROC curve demonstrates 0.9992 area under the curve for PPCS. A cut-off value of 248 ng/mL yields a sensitivity of 100% and specificity of 96.63% to discriminate NPC1 affected individuals from controls and NPC1 heterozygotes. (**C**) PPCS in plasma samples from patients with Fabry (n = 12), FH (n = 4), Gaucher (n = 2), Krabbe (n = 2), CTX (n = 2), GM1 (n = 1), GM2 including Tay-Sachs (black, n = 3) and Sandhoff (red, n = 2), and SPG5 (n = 1) diseases. Data are presented as mean ± SEM. (**D**) PPCS in plasma samples from patients with MPS I (black, n = 2), II

(brown, n = 2), IIIA (orange, n = 2), IIIB (red, n = 2), IIIC (green, n = 2), IVA (pink, n = 2), VI (purple, n =2), VII (blue, n =2), Batten (CLN1, red, n = 1; CLN2, pink, n = 1; CNL3, black, n = 20), MLII/III (n = 2), SYNGAP1 (n = 1), CESD (n = 1), Wolman (n = 1), ASMD (n = 11) diseases, and ASMD carriers (n = 3). Data are presented as mean ± SEM. (**E**) Correlation of plasma PPCS levels with NPC neurological disease severity scores. (**F**) correlation of plasma PPCS levels with NPC annual severity increment scores. (**G**) PPCS in the plasma over time collected from the natural history study. (**H**) Comparison of plasma PPCS levels in male and female NPC1 patients.

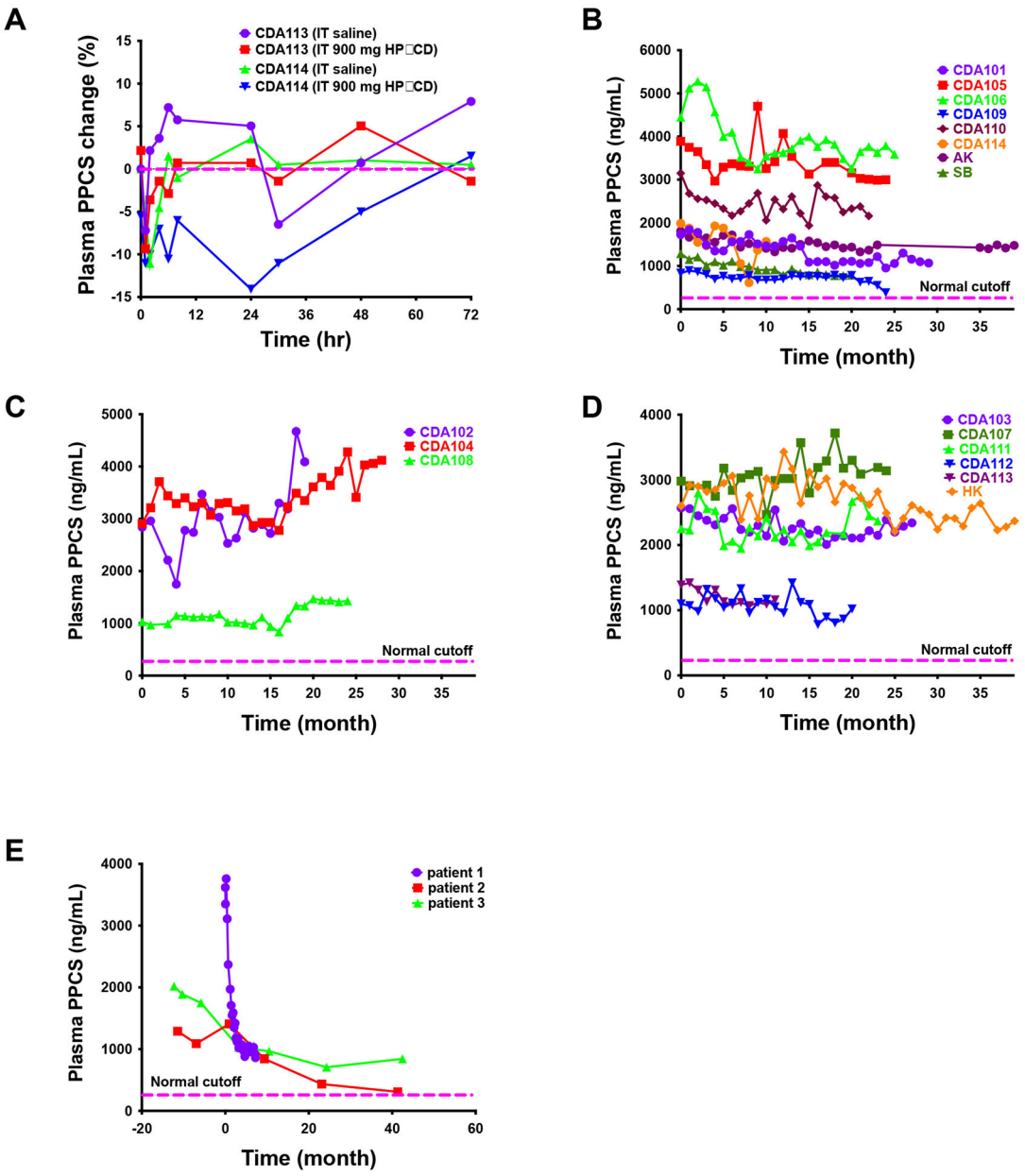

Figure 2.

Response of plasma PPCS levels to HPβCD treatment in NPC1 study participants. (**A**) Variation of plasma PPCS during 72 hours in IT saline and 900 mg HPβCD treatments. (**B**) NPC1 study participants with reduced plasma PPCS in response to monthly IT HPβCD treatment. (**C**) NPC1 study participants with increased plasma PPCS in response to monthly IT HPβCD treatment. (**D**) NPC1 study participants with unchanged plasma PPCS in response to monthly IT HPβCD treatment. (**E**) Response of plasma PPCS to weekly IV HPβCD treatment.

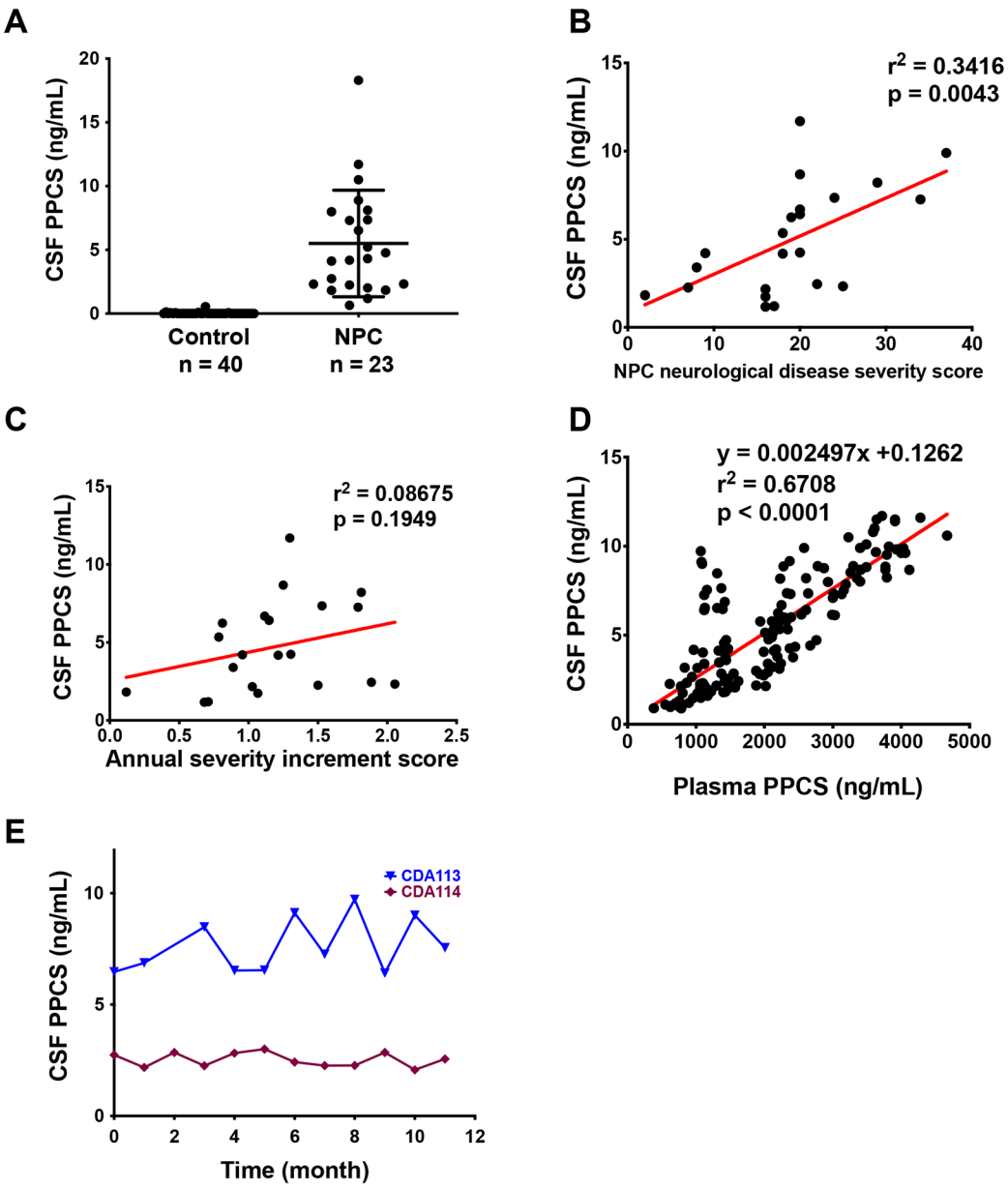

Figure 3.

PPCS levels in human CSF. (**A**) PPCS in CSF samples from control (n = 40) and NPC1 affected individuals. (n = 23). Data are presented as mean ± SEM. P < 0.0001 for NPC1 versus controls. (**B**) Correlation of CSF PPCS levels with NPC neurological disease severity scores. (**C**) Correlation of CSF PPCS levels with NPC disease annual severity increment scores. (**D**) Correlation in PPCS levels between CSF and plasma. (**E**) Response of CSF PPCS to monthly IT HPβCD treatment.

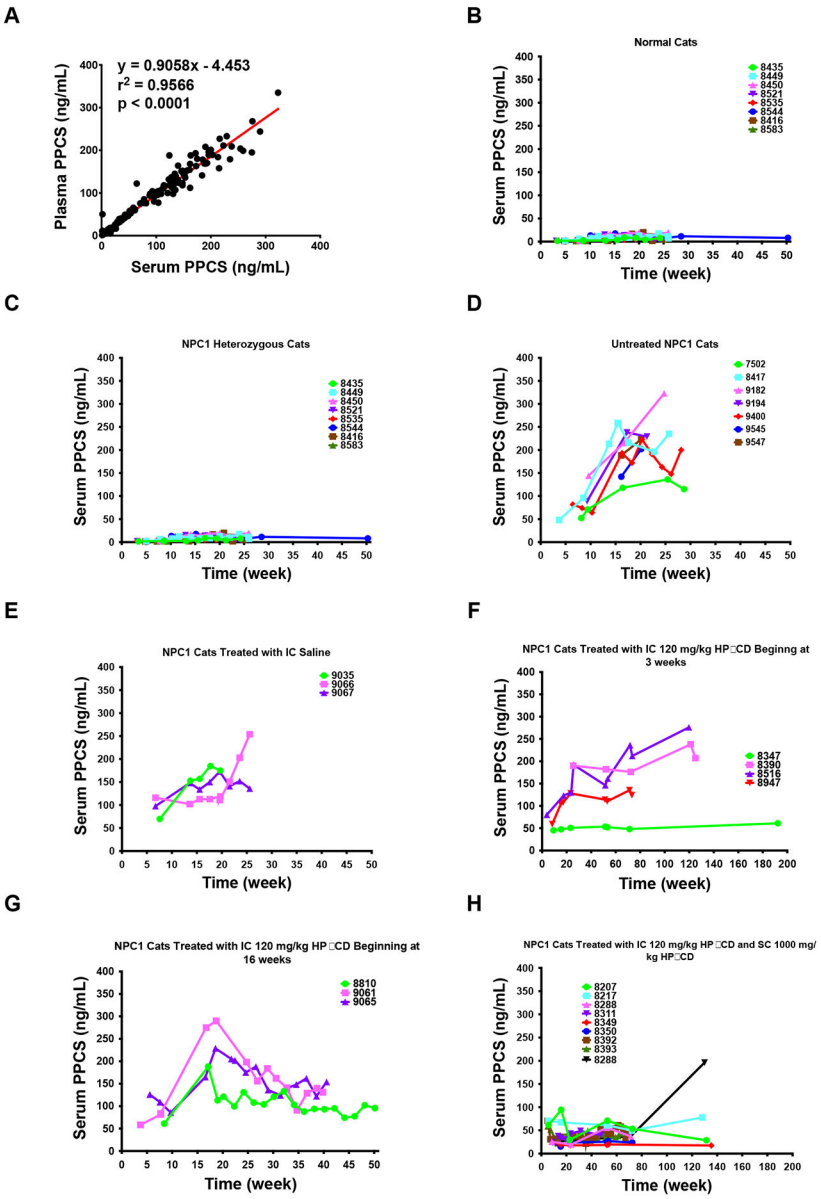

Figure 4.

PPCS in cat serum. (**A**) Correlation in PPCS levels between cat plasma and serum. (**B**) Serum PPCS in normal cats. (**C**) Serum PPCS in NPC1 heterozygous cats. (**D**) Serum PPCS in untreated NPC1 cats. (**E**) Serum PPCS in NPC1 cats treated with saline every other week. (**F**) Serum PPCS in NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 3 weeks. (**G**) Serum PPCS in NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 16 weeks. (**H**) Serum PPCS in NPC1 cats treated with IC 120 mg/kg HPβCD and SC 1000 mg/kg HPβCD every other week beginning at 3 weeks.

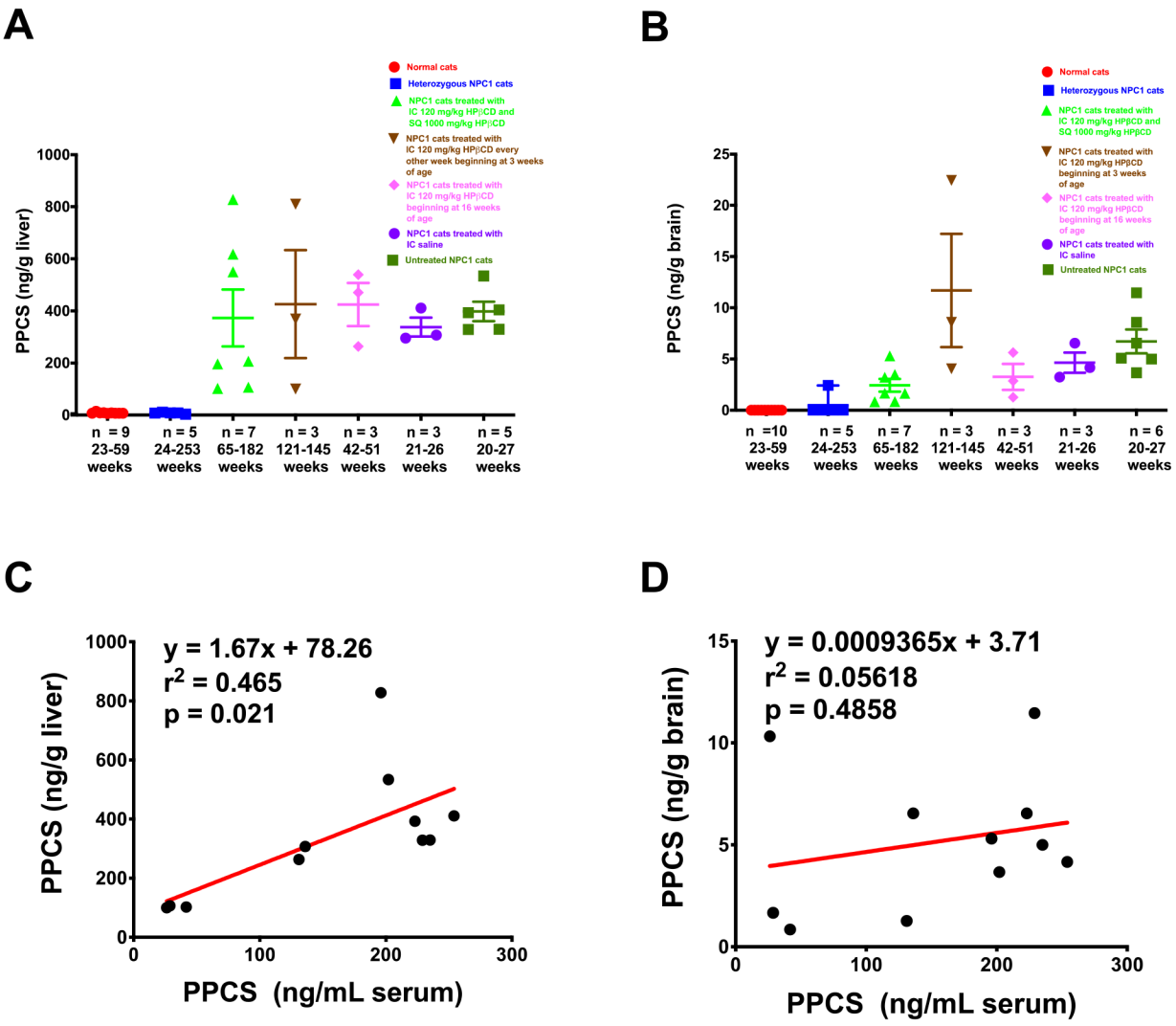

Figure 5.

PPCS in cat liver and brain tissues. (**A**) PPCS in liver of normal cats (n = 9, 23–59 weeks), heterozygous cats (n = 5, 24 – 253 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD and SC 1000 mg/kg HPβCD every other week beginning at 3 weeks (n = 7, 65 – 182 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 3 weeks (n = 3, 121 – 145 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 16 weeks (n = 3, 42 – 51 weeks), NPC1 cats treated with IC saline every other week beginning at 3 weeks (n = 3, 21 – 26 weeks), and untreated NPC1 cats (n = 5, 20 – 27 weeks). Data are presented as mean ± SEM. P = 0.0002 for untreated NPC1 cats versus normal cats. (**B**) PPCS in cat brains of normal cats (n = 10, 23 – 59 weeks), heterozygous cats (n = 5, 24 – 253 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD and SC 1000 mg/kg HPβCD every other week beginning at 3 weeks (n = 7, 65 – 182 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 3 weeks (n = 3, 121 – 145 weeks), NPC1 cats treated with IC 120 mg/kg HPβCD every other week beginning at 16 weeks (n = 3, 42 – 51 weeks), NPC1 cats treated with IC saline every other week beginning

at 3 weeks (n = 3, 21 – 26 weeks), and untreated NPC1 cats (n = 6, 20 – 27 weeks). Data are presented as mean ± SEM. P = 0.0001 for untreated NPC1 cats versus normal cats. (**C**) Correlation in PPCS levels between serum and liver. (**D**) Correlation in PPCS levels between serum and brain.