NIEMANN-PICK TYPE C DISEASE

Development of a bile acid–based newborn screen for Niemann-Pick disease type C

Xuntian Jiang,[1] Rohini Sidhu,[1] Laurel Mydock-McGrane,[2] Fong-Fu Hsu,[3] Douglas F. Covey,[2] David E. Scherrer,[1] Brian Earley,[1] Sarah E. Gale,[1] Nicole Y. Farhat,[4] Forbes D. Porter,[4] Dennis J. Dietzen,[5] Joseph J. Orsini,[6] Elizabeth Berry-Kravis,[7] Xiaokui Zhang,[8] Janice Reunert,[9] Thorsten Marquardt,[9] Heiko Runz,[10,11] Roberto Giugliani,[12] Jean E. Schaffer,[1] Daniel S. Ory[1]*

Niemann-Pick disease type C (NPC) is a fatal, neurodegenerative, cholesterol storage disorder. With new therapeutics in clinical trials, it is imperative to improve diagnostics and facilitate early intervention. We used metabolomic profiling to identify potential markers and discovered three unknown bile acids that were increased in plasma from NPC but not control subjects. The bile acids most elevated in the NPC subjects were identified as 3β,5α,6β-trihydroxycholanic acid and its glycine conjugate, which were shown to be metabolites of cholestane-3β,5α,6β-triol, an oxysterol elevated in NPC. A high-throughput mass spectrometry–based method was developed and validated to measure the glycine-conjugated bile acid in dried blood spots. Analysis of dried blood spots from 4992 controls, 134 NPC carriers, and 44 NPC subjects provided 100% sensitivity and specificity in the study samples. Quantification of the bile acid in dried blood spots, therefore, provides the basis for a newborn screen for NPC that is ready for piloting in newborn screening programs.

INTRODUCTION

Niemann-Pick disease type C (NPC) is a rare, progressive, neurodegenerative, cholesterol storage disorder. Mutations in *NPC1* (95% of NPC cases) and *NPC2* are responsible for NPC in humans. NPC1, a multispanning transmembrane protein, and NPC2, a soluble cholesterol-binding protein, both reside in late endosomes/lysosomes (*1*). Loss of function in either protein results in lysosomal cholesterol and sphingolipid accumulation, accompanied by hallmarks of cellular oxidative stress, including increased amounts of reactive oxygen species and a marked increase in cholesterol oxidation products (*2*–*4*). The complex lipid storage in NPC, as in other sphingolipidoses, is associated with alterations in neuronal morphology and neuronal cell loss that underlie the neuroinflammatory and neurodegenerative disease pathology (*5*).

NPC encompasses a broad spectrum of systemic, neurological, and psychiatric signs and symptoms (*1*, *6*). Affected individuals often present in early childhood with ataxia, seizures, and progressive impairment of motor and intellectual function, and usually die in adolescence, although, increasingly, NPC is being recognized among adults with cognitive defects (*7*, *8*). About half of NPC patients initially present with visceral symptoms (enlarged liver or spleen or both) or neonatal cholestasis. Diagnosis of this disorder, however, has been challenging because of the nonspecific symptoms and the lack of a rapid diagnostic assay. As a result, the disease frequently goes unrecognized or misdiagnosed, with diagnostic delays averaging 4 to 5 years, during which time neurological symptoms inexorably progress and opportunities for intervention are lost. Because therapies targeting the lipid storage have been shown to be effective in preclinical models (*9*–*14*) and clinical trials (*15*), there is a pressing need for a sensitive and specific diagnostic test that can be effectively applied to newborn populations.

Current approaches for diagnosing NPC are not well suited for newborn screening. Cholesterol staining of fibroblasts using filipin, which has been the diagnostic standard, requires an invasive biopsy, takes up to 3 months, and is frequently indeterminate (*8*). DNA diagnostics are not practical because of the high preponderance of private mutations causing NPC1 disease and the large number of variants of uncertain functional consequence in the *NPC1* gene (*16*). In contrast to many other lysosomal storage disorders (LSDs), NPC1 is not caused by mutations in a soluble enzyme, but a structural lysosomal protein. Thus, newborn screening based on enzyme activity assays is not feasible. Recently, cholestane-3β,5α,6β-triol—a cholesterol oxidation product—was shown to be a sensitive and specific marker for Niemann-Pick disease (*4*), and a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay that measures the plasma concentration of this metabolite has emerged as a diagnostic tool (*17*). An oxysterol-based screen, however, is limited by the requirement for derivatization and the comigration of an interference peak that derives from the newborn screening cards and cannot be adequately resolved during a short LC run.

To identify a more suitable marker, we performed unbiased metabolomic profiling of plasma from NPC subjects, focusing on bile acids because of the known perturbations in sterol metabolism, the reports of unusual urinary bile acids in NPC (*18*, *19*), and the favorable chemical properties of bile acids for MS detection. Through profiling of bile acid species in the plasma from NPC subjects, we discovered an unusual β-hydroxysteroid bile acid and its glycine and taurine conjugates, which were markedly elevated in the plasma and dried blood spots of NPC1 subjects as compared with controls. Here, we describe elucidation of the structures of these bile acids, development

[1]Diabetic Cardiovascular Disease Center, Washington University School of Medicine, St. Louis, MO 63110, USA. [2]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA. [3]Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA. [4]Section on Molecular Dysmorphology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892, USA. [5]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110, USA. [6]New York State Department of Health, Wadsworth Center, Albany, NY 12201, USA. [7]Rush University Medical Center, Chicago, IL 60612, USA. [8]Genzyme, 500 Kendall Street, Cambridge, MA 02142, USA. [9]Klinik und Poliklinik für Kinder- und Jugendmedizin–Allgemeine Pädiatrie, Universitätsklinikum Münster, Albert-Schweitzer-Campus 1, Gebäude A1, 48149 Münster, Germany. [10]Institute of Human Genetics, University of Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany. [11]Department of Genetics and Pharmacogenomics, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, MA 02115, USA. [12]Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Porto Alegre, Rio Grande do Sul 90035-003, Brazil.
*Corresponding author. Email: dory@wustl.edu

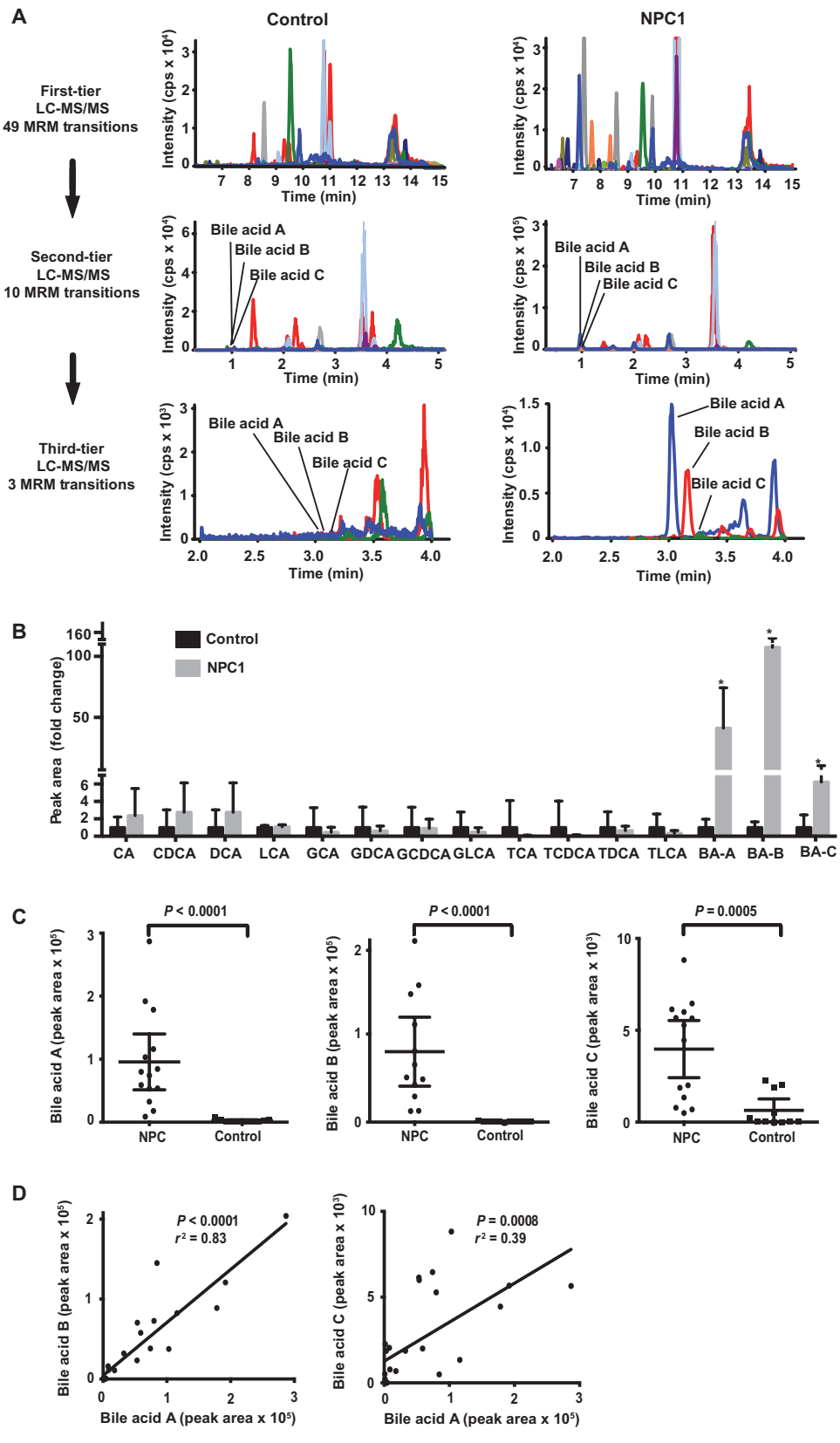


Fig. 1. NPC1 marker screening. (**A**) Three-tier targeted metabolomic strategy for identification of bile acid markers. First-tier screen includes 49 MRM transitions (17-min run time). Second-tier screen includes 10 MRM transitions (7.5-min run time) to characterize peaks with signal-to-noise ratio greater than five. Third-tier screen (6-min run time) quantifies unknown bile acid peaks (A, B, and C) that are elevated in NPC1 compared to control. (**B**) Comparison of bile acid concentration in NPC1 (n = 12) versus control (n = 11) samples obtained from second-tier profiling. Data are presented as mean fold change + SD normalized to control. *P = 0.0005, 0.0003, and 0.0007 for bile acids A, B, and C in NPC1 versus controls, respectively. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GLCA, glycolithocholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TLCA, taurolithocholic acid; BA-A, bile acid A; BA-B, bile acid B; BA-C, bile acid C. (**C**) Comparison of bile acids A, B, and C in NPC1 (n = 12) and control (n = 11) plasma samples obtained from third-tier profiling. Data are presented as means ± 95% confidence interval (CI) peak area. P < 0.0001 for bile acids A and B in NPC1 versus controls. P = 0.0005 for bile acid C in NPC1 versus controls. (**D**) Correlation between bile acids A and B in NPC plasma samples, r^2 = 0.83, P < 0.0001; correlation between bile acids A and C in NPC plasma samples, r^2 = 0.39, P = 0.0008.

of LC-MS/MS methodology for the analysis of bile acids in dried blood spots, and establishment and validation of a newborn screen based on quantification of the conjugated bile acid. This dried blood spot–based assay provides the basis for a screen for NPC that is ready for piloting in newborn screening programs.

RESULTS

Profiling of bile acids in NPC1 and control plasma samples

To identify potential markers, we profiled bile acids in NPC1 and control plasmas using a three-tier targeted metabolomic strategy based on LC-MS/MS operated in multiple reaction monitoring (MRM) mode (Fig. 1A). The first-tier assay included 49 MRM transitions with 17-min run time to broadly detect possible bile acids. For

the second tier, 10 MRM transitions and 7.5-min run time were used to detect only those bile acids with signal-to-noise ratio greater than five. Only three MRM transitions and 6-min run time were used in the third-tier assay to confirm the three-candidate bile acid species (referred to as bile acids A, B, and C) identified by the second-tier assay. In contrast to other bile acids monitored, these three unknown species were markedly elevated in the NPC1 but not control plasma samples (Fig. 1B). The unknown bile acids were detected in the same MRM transitions as CA, GCA, and TCA, respectively, but their retention times differed, suggesting that they might be isomers of these bile acids. Bile acids A and B were increased 41- and 144-fold, respectively, in NPC1 plasma and were able to completely discriminate between NPC1 and control plasma samples (Fig. 1C). Plasma bile acid C was elevated six-fold in NPC1 samples ($P = 0.0005$), but there was partial overlap with control samples. The high correlation between the three bile acids' plasma concentrations suggested that they are related, possibly within the same metabolic pathway (Fig. 1D). Because the bile acid C signal was 20-fold lower than those of bile acids A and B and prone to interferences from plasma, we did not pursue bile acid C further.

Elucidation of the structure of the bile acids

Our strategy for identification of bile acid structures is outlined in Fig. 2A. High-resolution MS analysis of bile acids A and B on LTQ Orbitrap mass spectrometer in the negative mode showed accurate mass/charge ratio (*m/z*) values of 407.2800 and 464.3016, respectively, for $[M-H]^-$ corresponding to formulas $C_{24}H_{39}O_5$ (calculated mass, 407.2803) and $C_{26}H_{42}NO_6$ (calculated mass, 464.3018) with mass errors less than 1 mDa respectively (Fig. 2, B and C). Hydrogen/deuterium (H/D) exchange experiments indicated that there are four (3 OH, COOH) and five (3 OH, NH, COOH) exchangeable hydrogens in bile acids A and B (Fig. 2, D and E), respectively. The higher-energy collisional dissociation (HCD) spectrum of bile acid B contains an abundant ion at *m/z* 74.0256, corresponding to deprotonated glycine ($C_2H_4O_2N$; calculated mass, 74.0248), confirming that bile acid B is a glycine conjugate. However, unambiguous assignment of structures for bile acids A and B was hindered by lack of interpretable fragments from steroid skeletons in HCD spectra (Fig. 2, F and G). Therefore, we converted isolated bile acids A and B into their *N*-(4-aminomethylphenyl) pyridinium (AMPP) amides, which produced informative charge-remote fragmentation for structure identification in HCD and identified the key fragments that can differentiate the positions of hydroxylation (Supplementary Materials and Methods and tables S1 and S2). To aid interpretation of the product ion spectra of derivatized bile acids A and B, we studied the fragmentation patterns of the AMPP derivatives of a series of bile acids and analogs **1** to **10** (figs. S1 to S12 and tables S3 to S5) and identified the key fragments that can differentiate the positions of hydroxylation (Supplementary Materials and Methods and fig. S13). On the basis of these fragmentation patterns, we assigned the hydroxyl groups to bile acids A and B. The structures of bile acids A and B were preliminarily proposed as 5α-cholanic acid-3β,5α,6β-triol and 5α-cholanic acid-3β,5α,6β-triol *N*-(carboxymethyl)-amide, respectively (Supplementary Materials and Methods and fig. S14). These structures were then confirmed by the synthesis of the reference compounds and comparative LC-MS/MS analysis of endogenous and synthesized compounds (Supplementary Materials and Methods and figs. S13 and S15).

Biosynthesis of the bile acids

Although bile acid A has been reported as a major metabolite of cholestane-3β,5α,6β-triol in rat (*20*), the biosynthesis of bile acids A and B in humans has not previously been described. To explore the biosynthetic route of the bile acids, we incubated the human hepatoblastoma-derived cell line Hep G2 with cholestane-3β,5α,6β-triol and 7,7,22,22-d4-cholestane-3β,5α,6β-triol. We found that bile acid A and 7,7,22,22-d4-bile acid A were produced, thus confirming that bile acid A was a product of cholestane-3β,5α,6β-triol metabolism (Fig. 3). No bile acid B was found, consistent with the known defects in synthesis of conjugated bile acids in Hep G2 cells (*21*), presumably due to deficiency of bile acid CoA:amino acid *N*-acyltransferase (BAAT).

Selection of bile acid marker for newborn screening of NPC1 disease

To explore the ability of bile acids A and B to serve as markers for newborn screening of NPC1 disease, we measured these metabolites in 10 NPC and 16 control dried blood spots. Bile acids A and B were elevated 12- and 101-fold, respectively, in NPC1 subjects compared to the control group (Fig. 4). Whereas bile acid A could separate all but one control subject from NPC subjects, bile acid B could unambiguously discriminate NPC1 subjects from controls, indicating that bile acid B may be the more specific marker for purposes of a newborn screening application.

Development and validation of a two-tiered LC-MS/MS assay for bile acid B in dried blood spots

Our goal was to develop a short LC-MS/MS method (~2 min) that would allow sufficient throughput to meet a general requirement for a newborn screening assay. A major challenge for development of a high throughput LC-MS/MS method is separation of interferences from bile acid B within the short LC run time. A long LC run time (7 min) was initially developed, which separated all the interferences from bile acid B. Two interference peaks eluted closely to bile acid B (retention time at 4.05 min; Fig. 5A). Most newborn dried blood spots only showed an interference peak that eluted at 3.85 min, whereas dried blood spots from NPC1 subjects and carriers showed an interference peak that eluted at 4.23 min. We next developed a short LC condition with a sample analysis time of 2.2 min, in which bile acid B (retention time at 1.7 min) was baseline-resolved from the major interference peak (retention time at 1.63 min) in normal newborn dried blood spots. However, under the short LC condition, bile acid B could not be separated from the interference peak in NPC1 and carrier dried blood spots. Therefore, a two-tier assay strategy was adopted, in which short (2.2 min) and long (7 min) LC conditions were used as first- and second-tier assays, respectively (Fig. 5B). Thus, using the first-tier method, more than 500 samples per day can be analyzed. Samples with bile acid B values above the cutoff value due to inability to separate from the second interference peak could then be submitted to the highly selective second-tier assay, permitting adjudication of the false positives from the first-tier assay. Together, this tiered strategy can serve as the basis of a screen for NPC.

Detailed method development is described in the Supplementary Materials. The method was validated according to commonly accepted criteria (*22*, *23*) for sensitivity, selectivity, accuracy, precision, linearity, carryover, recovery, matrix effect, effect of spotting volumes, effect of hematocrit, effect of punch location, and stabilities in whole blood, dried blood spots, processed samples, and stock solutions (Supplementary Materials and Methods and table S8). Bile acid B was stable in dried blood spots in newborn screening cards for up to 66 days at room temperature storage.

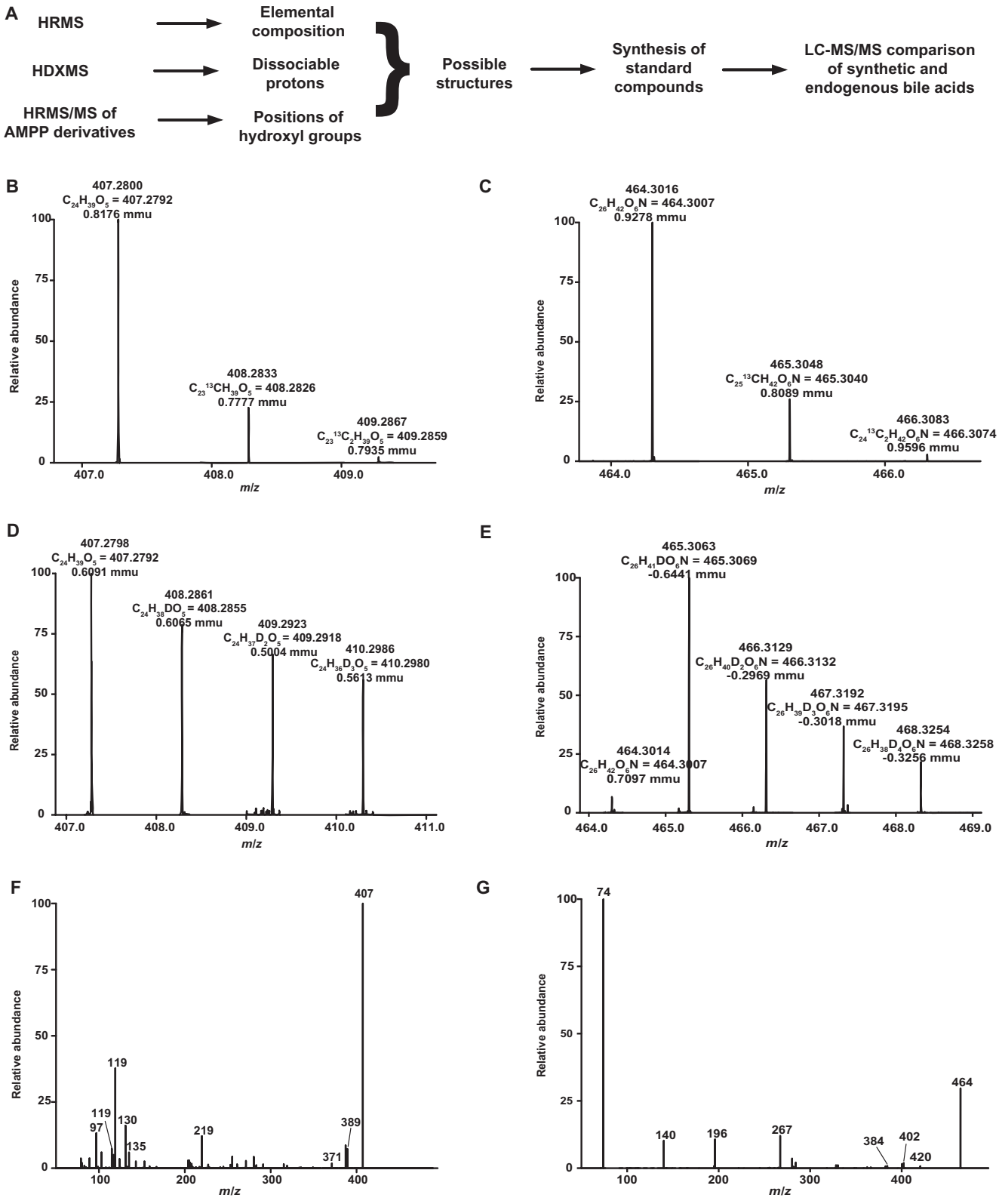


Fig. 2. Identification and confirmation of structure of unknown bile acids. (**A**) Strategy for identification of bile acid structures. HRMS, high-resolution MS; HDXMS, hydrogen/deuterium exchange MS; HRMS/MS, high-resolution MS/MS. (**B** and **C**) High-resolution mass spectra are shown for bile acid A (B) and bile acid B (C). mmu, milli-mass unit. (**D** and **E**) H/D exchange mass spectra are shown for bile acid A (D) and bile acid B (E). (**F** and **G**) HCD mass spectra are shown for bile acid A (F) and bile acid B (G).

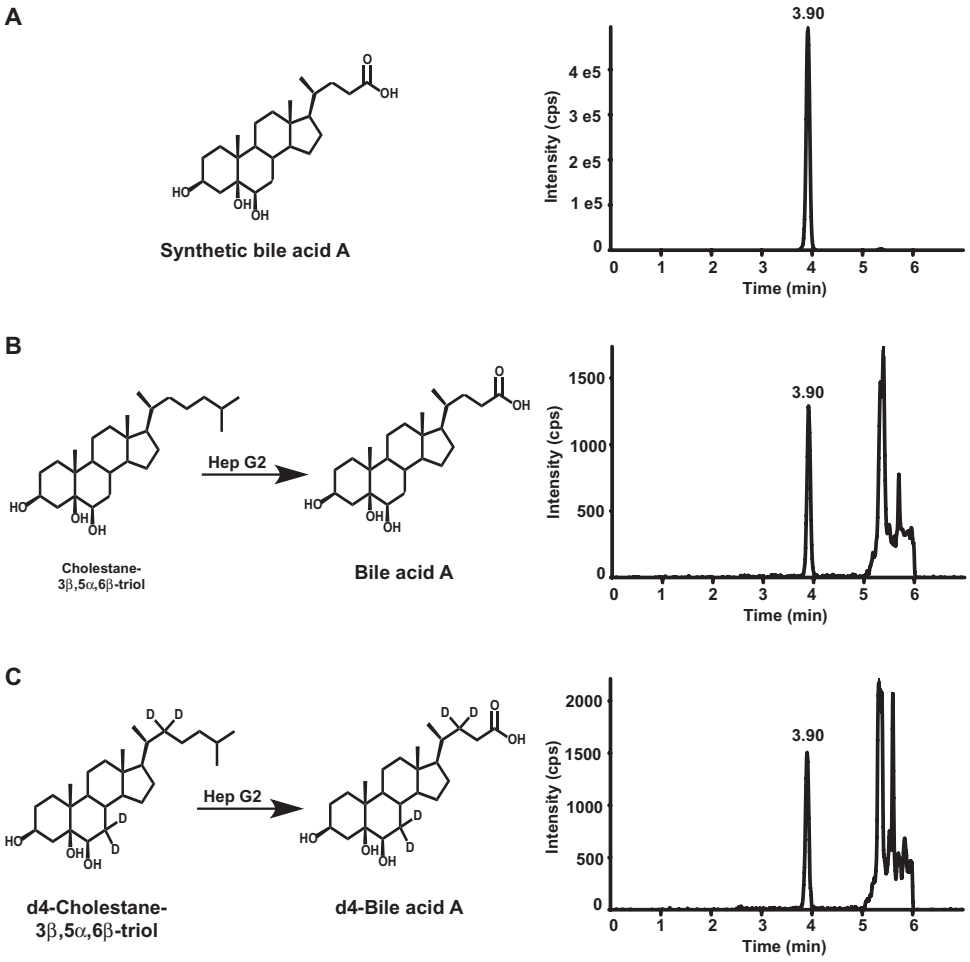


Fig. 3. Biosynthesis of bile acid A from cholestane-3β,5α,6β-triol. (**A**) Synthetic bile acid A detected by MRM transition *m/z* 407→407. cps, counts per second. (**B**) Bile acid A generated from cholestane-3β,5α,6β-triol in Hep G2 cells and detected by MRM transition *m/z* 407→407. (**C**) d4-Bile acid A generated from d4-cholestane-3β,5α,6β-triol in Hep G2 cells and detected by MRM transition *m/z* 411→411.

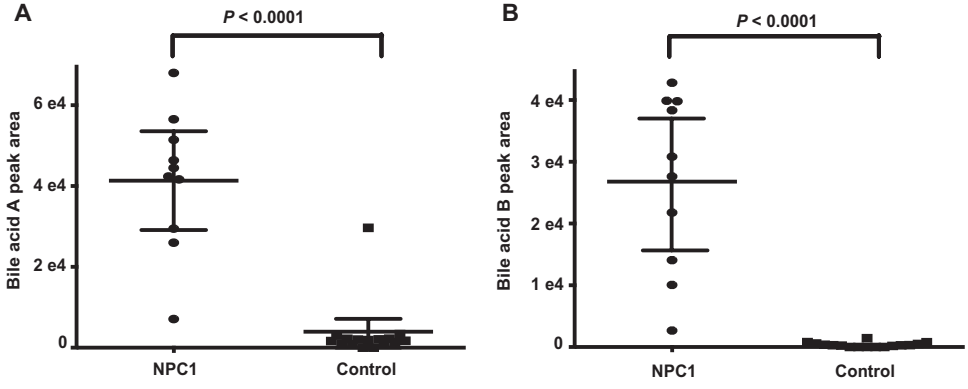


Fig. 4. Detection of bile acid markers in dried blood spots. (**A**) Bile acid A in NPC1 (n = 10) and control (n = 16) dried blood spot samples. Data are presented as means ± 95% CI peak area. P < 0.0001 for NPC1 versus controls. (**B**) Bile acid B in NPC1 (n = 10) and control (n = 16) dried blood spots sample. Data are presented as means ± 95% CI peak area. P < 0.0001 for NPC1 versus controls.

Establishment and validation of cutoff value for newborn screen

We used the validated bile acid B dried blood spot assay to establish the cutoff value for NPC1 newborn screening. For this training set, we analyzed dried blood spot samples from 1013 normal subjects (including 924 newborns and 89 subjects at other ages), 130 NPC1 carriers, and 25 NPC1 subjects. NPC1 and NPC1 carrier dried blood spots were obtained from previously diagnosed patients and obligate heterozygotes, respectively. The reference ranges for control, NPC1 carrier, and NPC1 subjects were <5 to 5.34, <5 to 12.5, and 5.45 to 294 ng/ml, respectively (Fig. 6A). The only NPC1 subject with bile acid B concentration <10 ng/ml was neurologically asymptomatic and was identified because of a sibling with splenomegaly who was diagnosed with NPC1, who carried a high-frequency variant (c.665A>C, p.N222S) (*16*). A cutoff at 13.5 ng/ml allowed nearly complete discrimination of NPC1 subjects from controls and NPC1 carriers.

The cutoff was validated by analysis of a second ("test") set of dried blood spot samples including 4992 normal newborns, 134 NPC1 carriers (130 carriers analyzed in the training set plus 4 new carriers), and 44 NPC1 patients. The range of bile acid B concentrations in normal newborns, NPC1 carriers, and NPC1 patients was <5, <5 to 12.4, and 15.7 to 266 ng/ml, respectively (Fig. 6B). A cutoff of 13.5 ng/ml provided 100% sensitivity and specificity for detection of NPC1 subjects in the test set, yielding a receiver operating characteristic (ROC) area under the curve of 1.0 (Fig. 6C). Only one normal newborn sample (0.017%) needed to be resolved using the second-tier assay. Among NPC1 subjects, there was no significant correlation between bile acid B concentration and age or disease severity (fig. S16). The mean bile acid B level for female NPC1 subjects was 35% lower than male subjects (P < 0.05). All the normal newborns and NPC1 carriers were below the cutoff, whereas all the NPC1 samples were above the cutoff. To assess specificity of the assay, we examined samples from patients with other disorders of sterol metabolism [acid sphingomyelinase deficiency (ASMD), cerebrotendinous xanthomatosis (CTX), lysosomal acid lipase deficiency (LALD), and Smith-Lemli-Opitz syndrome (SLOS)]. Of these, only ASMD subjects

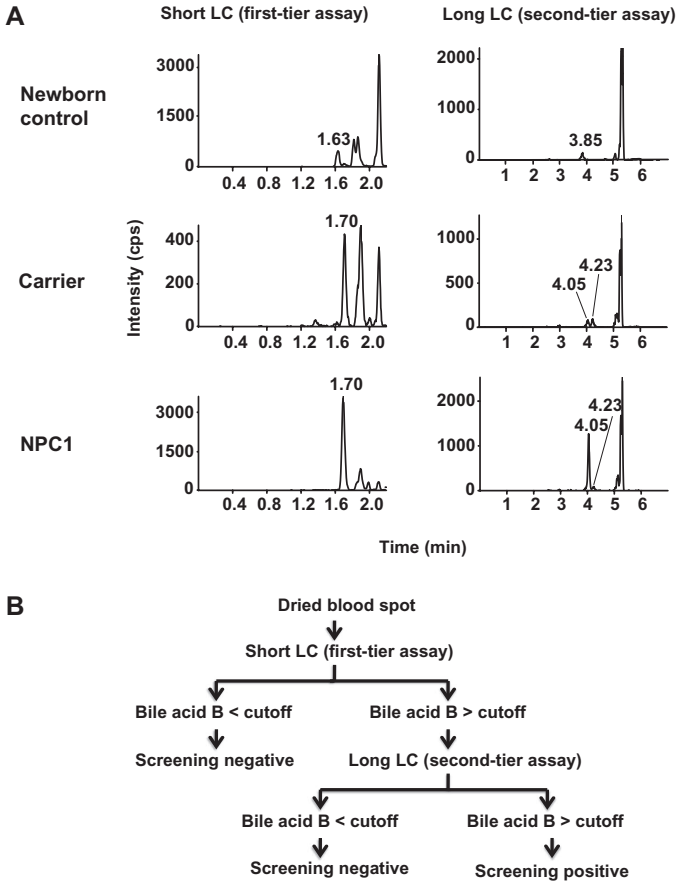


Fig. 5. Two-tier newborn screen for NPC1 disease. (**A**) Chromatograms of bile acid B in dried blood spots from a newborn control, adult NPC1 carrier, and NPC1 patient, as resolved with short LC (first-tier assay) and long LC conditions (second-tier assay). Bile acid B was eluted at 1.7 and 4.05 min under short and long LC conditions, respectively. There are two interferences that eluted close to bile acid B. An interference peak that presents in most newborn dried blood spots was baseline-resolved from bile acid B under both short (1.63 min) and long LC (3.85 min) conditions. The dried blood spots from NPC1 subjects and carriers showed an interference peak that was co-eluted with bile acid B at 1.7 min under the short LC condition, but baseline-separated from bile acid B under the long LC condition at 4.23 min. (**B**) Algorithm for two-tier newborn screening of NPC1 disease.

demonstrated bile acid B concentrations (36 to 92.8 ng/ml) above the cutoff (Fig. 6D). The new screen was "benchmarked" by comparing bile acid B concentrations in dried blood spots with the plasma concentration of cholestane-3β,5α,6β-triol, the current diagnostic standard (Fig. 7). As anticipated, there was significant correlation between the bile acid and its precursor (r = 0.59), and both assays were equally sensitive in detecting NPC1 subjects. However, the bile acid B marker was more specific, completely discriminating NPC1 subjects from NPC1 carriers and permitting proper assignment of non-NPC1 cholestasis.

DISCUSSION

A major barrier to delivery of effective treatment for NPC is the profound diagnostic delay caused by the nonspecific nature of early symptoms, genetic heterogeneity, and lack of a biochemical marker that can be applied to a dried blood spot–based newborn screen. Preclinical studies clearly demonstrate that intervention with effective treatments at this early stage, before the onset of neurological symptoms, can modify the course of the disease and extend life span (*9*, *13*), underscoring the need for new diagnostic algorithms. To address this unmet need, we performed targeted metabolomic profiling, identifying disease markers that were markedly elevated in NPC1 subjects. The metabolite structures were identified by MS techniques and confirmed as 3β,5α,6β-trihydroxycholanic acid and its glycine conjugate. We developed and validated a high-throughput MS-based method to quantify the bile acid glycine conjugate in dried blood spots, which provides the basis for a highly sensitive and specific newborn screen for NPC that is ready for piloting in newborn screening laboratories.

The bile acids identified by our profiling strategy are unusual in that they have not been reported in human plasma and have a 3β-hydroxyl-5α-hydroxyl-cholanic acid rather than the 3α-hydroxyl-5β-cholanic acid characteristic of the common circulating bile acids (*24*). Although low amounts of 3β-hydroxy bile acids have been reported in human fecal contents and urine, their steroid skeletons are different from the bile acids identified in our study (*18*, *19*, *25*). These observations suggest that the 3β-hydroxy bile acids are likely specific for distinguishing Niemann-Pick disease from other causes of cholestasis. Using stable isotope labeling, we demonstrated that 3β,5α,6β-trihydroxycholanic acid (bile acid A) is converted from cholestane-3β,5α,6β-triol in human hepatoma cells, suggesting that 3β,5α,6β-trihydroxycholanic acid may be formed in vivo in humans. Although generation of the glycine conjugate could not be tested in the cell culture model, 3β,5α,6β-trihydroxycholanic acid is likely converted in vivo by bile acid CoA: amino acid acyltransferase into its more polar glycine conjugate (bile acid B). Bile acid C is very likely a taurine conjugate of 3β,5α,6β-trihydroxycholanic acid (fig. S14C). The marked elevation of these unusual bile acids in the plasma of the NPC1 subjects is entirely consistent with the known increases in cholestane-3β,5α,6β-triol, the metabolic precursor of these bile acids, in the plasma of NPC patients. In contrast to cholestane-3β,5α,6β-triol, which is also elevated in other disorders of sterol metabolism (ASMD, CTX, and LALD) (*26*), bile acid B was elevated only in NPC and ASMD subjects. Thus, elevation of these bile acid markers appears to be a downstream consequence of both the tissue oxidative stress that is a prominent feature of inborn errors of sterol metabolism (*2*, *3*, *27*, *28*) and the altered disposal of bile acids in Niemann-Pick disease (*29*).

Detection of the bile acid markers in dried blood spots from newborn screening cards enabled development of a high-throughput newborn screen for NPC. The screen further incorporates a second-tier LC run, a well-accepted strategy in newborn screens to increase specificity without sacrificing sensitivity. The exceptional performance of the screen—100% sensitivity and specificity for discrimination of NPC1 subjects from controls and NPC1 carriers coupled with a 0.017% reassay rate—should minimize false positives caused by the ~1% carrier frequency in the population (*16*). As compared to cholestane-3β,5α,6β-triol, which is the current diagnostic standard, bile acid B eliminates the ~25% overlap of NPC1 carriers that are in the NPC1 range (*17*), as well as false positives among cholestatic neonates caused by interference peaks that cannot be separated in short LC runs (*30*). A potential source of bias in this retrospective study, however, is that nonneonatal NPC1 patients were used as surrogates for assay validation because of the lack of authentic dried blood spots from NPC1 neonates (the

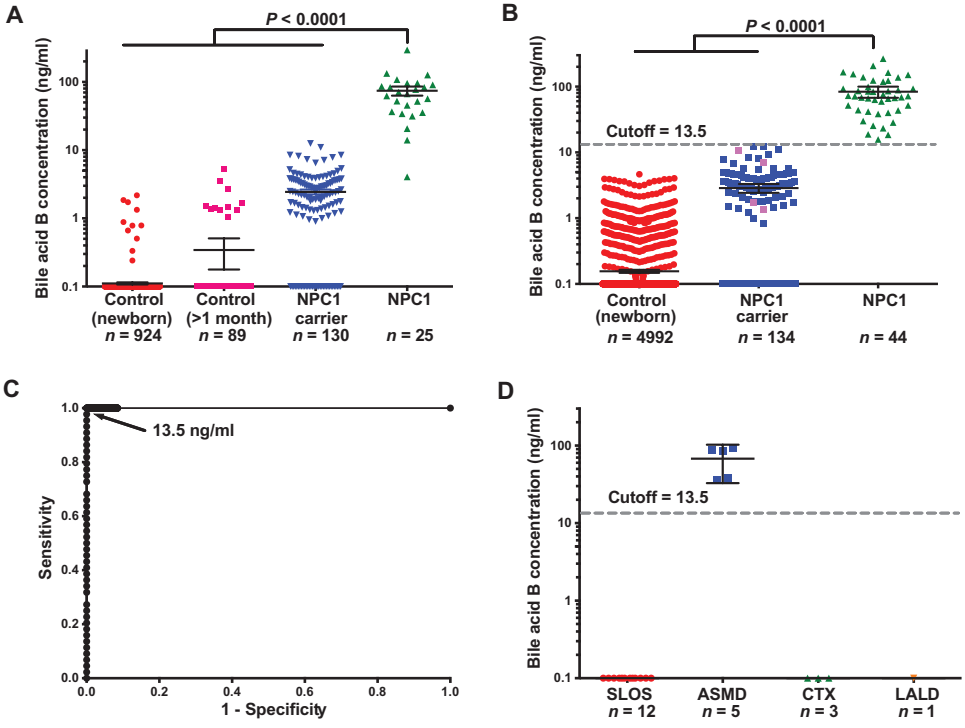
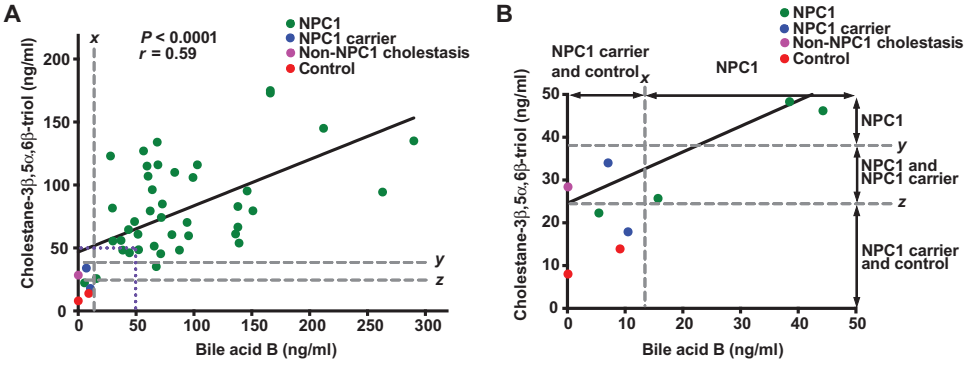


Fig. 6. Establishment and validation of cutoff for NPC1 newborn screen. (**A**) Bile acid B concentrations in dried blood spots from newborn control, control at other age (>1 month old), NPC1 carrier, and NPC1 patients in a training set. Bile acid B concentrations below the LLOQ (5 ng/ml) were quantifiable, although the percent coefficient of variance (%CV) and percent relative error (%RE) for these samples were above acceptance criteria for the validated assay. Data presented on semilog plots are shown as means ± 95% CI. Samples with no detectable bile acid B peak were assigned as 0.1 ng/ml for purposes of plotting. $P < 0.0001$ for NPC1 versus controls and NPC1 carriers. (**B**) Determination of bile acid B concentrations and application of the 13.5 ng/ml cutoff to a test sample set consisting of newborn control, NPC1 carrier, and NPC1 dried blood spots. All newborn control and NPC1 samples are new samples, and carriers denoted by pink symbols are new samples. The NPC1 carriers in blue were analyzed in training set and reanalyzed in validation set. Samples were coded and randomized, and the operator was blinded to group assignment, thus reducing bias and noise/variance in the results and enabling unbiased statistical analysis of the data. Data are presented on semilog plots and are shown as means ± 95% CI. Samples with no detectable bile acid B peak were assigned as 0.1 ng/ml for purposes of plotting. $P < 0.0001$ for NPC1 versus controls and NPC1 carriers. (**C**) Application of cutoff value of 13.5 ng/ml yields sensitivity and specificity of 100%, and ROC area under the curve of 1.0. (**D**) Bile acid B concentrations in SLOS, ASMD, CTX, and LALD dried blood spots from cutoff validation sample set. Data are presented on semilog plots and are shown as means ± 95% CI. Samples with no detectable bile acid B peak were assigned as 0.1 ng/ml for purposes of plotting. $P < 0.0001$ for ASMD versus controls.

youngest patient in the study was 4 months old and neurologically asymptomatic). Nonetheless, in light of case reports demonstrating marked elevation of the bile acid precursor cholestane-3β,5α,6β-triol in NPC1 and NPC2 neonates (*31*), it seems probable that the bile acids will be elevated in NPC patients during the neonatal period. Moreover, bile acid B concentrations were independent of age and severity of disease, suggesting that the screening test will be universally applicable to all NPC1 subjects, including neonates.

Advances in MS have expanded the scope of newborn screening to more than 50 inborn errors of metabolism. MS/MS is considered the method of choice for screening of metabolic disorders, and it is widely used to screen for amino acid, organic acid, and fatty acid disorders. Recently, a multiplex MS/MS assay, which is based on use of a panel of substrates to quantify enzymatic activity, was introduced to screen for five LSDs: Fabry, Gaucher, Krabbe, Niemann-Pick A/B (ASMD), and Pompe diseases (*32*). Newborn screening using this method is mandated to begin in four U.S. states (*33*). There are several reasons to believe that NPC is also an excellent candidate for newborn screening. The frequency of the disorder (1:100,000) (*1*, *6*) is comparable to the five currently screened LSDs (*34*) and at least twice as frequent as many commonly screened metabolic disorders (*35*), and disease-modifying therapies are available (miglustat) or entering into late-stage clinical trials (2-hydroxypropyl-β-cyclodextrin). Moreover, as with other LSDs, it is anticipated that neonatal screening will reduce long-term morbidity, and the screening and treatment will cost less than long-term care and lost productivity

Fig. 7. Comparison of bile acid B in dried blood spots with cholestane-3β,5α,6β-triol in plasma. (**A**) Correlation of bile acid B in dried blood spots with cholestane-3β,5α,6β-triol in plasma ($n = 47$), $r = 0.59$, $P < 0.0001$. *x*, bile acid cutoff for NPC1 carriers and controls; *y*, cholestane-3β,5α,6β-triol cutoff for NPC1 carriers; *z*, cholestane-3β,5α,6β-triol cutoff for controls. (**B**) The inset bounded by the dotted purple line in (A) has been expanded to more clearly compare assay specificity.



(*36*). Finally, coupling of the bile acid assay, which has exceptional ROCs, with a cutoff that will exclude all carriers and controls, could yield a newborn screen capable of detecting the vast majority of NPC1 cases. Determination of the positive predictive value of the assay will await prospective validation. In the future, modifications of this screen, such as elimination of LC or shifting to positive ion mode detection, could facilitate incorporation of the NPC screen into a multiplexed panel.

At present, none of the disease conditions included in the Recommended Universal Screening Panel, which is the newborn screening standard, target a disorder involving an integral membrane (nonluminal enzyme) lysosomal protein or an inborn error of sterol metabolism and trafficking. Thus, this bile acid–based NPC screen represents an important advance in neonatal screening. Broad implementation of newborn screening for NPC would eliminate the diagnostic delay and shift diagnosis of the disease to the newborn period before the onset of neurological symptoms. Drug intervention and nonpharmacological supportive care during this asymptomatic period have the potential to markedly delay disease progression and extend life.

MATERIALS AND METHODS

Study design

The study was a retrospective case-control design. Dried blood spot samples were obtained from four groups: (i) newborn controls, (ii) controls >30 days of age, (iii) NPC1 carriers (obligate heterozygotes who are parents of affected NPC1 subjects), and (iv) previously diagnosed NPC1 subjects. The goal of the study was to evaluate the performance of a dried blood spot–based newborn screen for NPC1. For this study, an assay was developed to measure bile acid B in the dried blood, and this assay met the U.S. Food and Drug Administration (FDA) guidance criteria with respect to precision, accuracy, and replication (*22*). Samples were coded and randomized, and the operator was blinded to group assignment to reduce bias and noise/variance in the results. The assay cutoff was established using a training set of samples. The cutoff was then applied in a second (test) assay using new independent samples except that NPC1 carriers analyzed in the training set were reassayed together with new carriers. Data are presented on semilog plots and are shown as means ± 95% CI. Statistical differences between the groups were determined using Mann-Whitney *U* test. The performance of the assay is presented on an ROC plot.

Chemicals and reagents

DCA, CDCA, CA, α-muricholic acid, β-muricholic acid, GDCA, GCDCA, and GCA were obtained from Steraloids Inc. Bile acid structures are shown in fig. S1. Bile acid A (*37*), 5-cholanic acid-3α,4β,7α-triol (**7**) (*38*), and AMPP (*39*) were synthesized according to the published procedures. *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 4-(dimethylamino)pyridine (DMAP), diethylamine, *N*,*N*-dimethylformamide, trisodium citrate, sodium hydroxide (NaOH), chloroform, Dulbecco's modified Eagle's medium, fetal calf serum, and penicillin G and streptomycin sulfate were obtained from Sigma-Aldrich. High-performance LC (HPLC) solvents (methanol and acetonitrile) were of HPLC grade and were purchased from EMD Chemicals. Milli-Q ultrapure water was prepared in-house with a Milli-Q Integral Water Purification System.

Plasma and dried blood spot sample collection

NPC1 dried blood spot samples were obtained from the National Institutes of Health (NIH), Rush University Medical Center, Universitätsklinikum Münster, and University of Heidelberg. NIH also provided NPC1 plasma, NPC1 carrier, and SLOS dried blood spot samples. All NPC1 and NPC1 carrier dried blood spots were obtained from previously diagnosed patients and obligate heterozygotes, respectively. Normal plasmas and dried blood spots were obtained from anonymized residual samples at St. Louis Children's Hospital and New York State Newborn Screening Program. Genzyme provided ASMD dried blood spot samples. Hospital de Clínicas de Porto Alegre provided ASMD, CTX, and LALD dried blood spot samples. All plasma samples were collected in EDTA-K2–containing tubes. The collection and analysis of deidentified human samples were approved by the Human Studies Committee at Washington University.

Analysis of clinical dried blood spot samples

All the clinical samples were first submitted to first-tier assay. Samples consisting of calibration standards in duplicate, blank, blank with internal standard, quality control (QC) samples (low QC, medium QC, and high QC), and unknown clinical samples were analyzed. The clinical samples with bile acid B above the lower limit of quantification (LLOQ) in the first-tier assay together with calibration standards, blank, blank with internal standard, and QC samples in the same batch were reassayed with the second-tier assay. The LC-MS/MS acceptance criteria were as indicated in FDA recommendations (*22*).

Statistical analysis

The GraphPad Prism version 6.0 (GraphPad Software) was used to perform Mann-Whitney *U* test, ROC analysis, and Pearson correlations. Microsoft Excel was used for calculations of %CV and %RE. Mann-Whitney *U* test was applied to calculate differences between NPC1 and normal (control or control and NPC1 carrier) groups and between ASMD and control groups. All presented *P* values are two-sided, and $P < 0.05$ was considered to be statistically significant. Bile acid A and B correlation was analyzed using Pearson correlations because the data showed a normal distribution.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Structures of bile acids and analogs used to study fragmentation patterns of the AMPP derivatives.

Fig. S2. HCD mass spectra of AMPP-derivatized bile acids **1** (A), **2** (B), **3** (C), **4** (D), **5** (E), **6** (F), **7** (G), **8** (H), **9** (I), and **10** (J).

Fig. S3. Proposed fragmentation pathway to fragments **A** to **H** in AMPP derivatives of unconjugated bile acids **1** to **7**.

Fig. S4. Proposed fragmentation pathway to fragments I to K in AMPP derivatives of unconjugated bile acids **1** to **7**.

Fig. S5. Proposed fragmentation pathway to fragments **L** (**L**′) to **M** (**M**′) in AMPP derivatives of unconjugated bile acids **1** to **7**.

Fig. S6. Proposed fragmentation pathway to fragments **N** to **P** in AMPP derivatives of unconjugated bile acids **1** to **6**.

Fig. S7. Proposed fragmentation pathway to fragments **Q** to **T** in AMPP derivatives of unconjugated bile acids **1** to **7**.

Fig. S8. Proposed fragmentation pathway to fragments **GA** to **GE** and **GH** in AMPP derivatives of glycine-conjugated bile acids 8 to 10.

Fig. S9. Proposed fragmentation pathway to fragments **GF**, **GG**, and **GI** in AMPP derivatives of glycine-conjugated bile acids **8** to **10**.

Fig. S10. Proposed fragmentation pathway to fragments **GJ** to **GM** in AMPP derivatives of glycine-conjugated bile acids **8** to **10**.
Fig. S11. Proposed fragmentation pathway to fragments **GN** to **GW** in AMPP derivatives of glycine-conjugated bile acids **8** to **10**.
Fig. S12. Proposed fragmentation pathway to fragments **GX** to **GZ** and **GAA** to **GAB** in glycine-conjugated bile acid AMPP derivatives.
Fig. S13. Comparison of chromatograms and HCD mass spectra of AMPP derivatives of bile acids A and B in NPC plasma and solutions of synthetic compounds.
Fig. S14. Structures of bile acid markers for NPC.
Fig. S15. Comparison of chromatograms of bile acids A and B in NPC1 plasma and solutions of synthetic compounds.
Fig. S16. Correlation of bile acid B concentrations with patient parameters.
Table S1. Accurate masses and calculated elemental composition of fragment ions of 21,26,27-trinorcholestan-25-oic acid-3β,5α,6β-triol and bile acid A AMPP derivatives.
Table S2. Accurate masses and calculated elemental composition of fragment ions of bile acid B AMPP derivative.
Table S3. Accurate masses and calculated elemental composition of fragment ions of DCA, CDCA, and 5β-cholanic acid-3α,4β,7α-triol AMPP derivatives.
Table S4. Accurate masses and calculated elemental composition of fragment ions of CA, α-muricholic acid, and β-muricholic acid AMPP derivatives.
Table S5. Accurate masses and calculated elemental composition of fragment ions of GCA, GCDCA, and GDCA AMPP derivatives.
Table S6. MRM transitions and MS parameters for bile acids in the first-tier marker screening.
Table S7. MRM transitions and MS parameters for bile acids in the second-tier marker screening.
Table S8. Accuracy and precision of QC samples.
References (*40*–*42*)

REFERENCES AND NOTES

1. M. T. Vanier, Niemann-Pick disease type C. *Orphanet J. Rare Dis.* **5**, 16 (2010).
2. G. Tint, P. Pentchev, G. Xu, A. K. Batta, S. Shefer, G. Salen, A. Honda, Cholesterol and oxygenated cholesterol concentrations are markedly elevated in peripheral tissue but not in brain from mice with Niemann–Pick type C phenotype. *J. Inherited Metab. Dis.* **21**, 853–863 (1998).
3. J. R. Zhang, T. Coleman, S. J. Langmade, D. E. Scherrer, L. Lane, M. H. Lanier, C. Feng, M. S. Sands, J. E. Schaffer, C. F. Semenkovich, D. S. Ory, Niemann-Pick C1 protects against atherosclerosis in mice via regulation of macrophage intracellular cholesterol trafficking. *J. Clin. Invest.* **118**, 2281–2290 (2008).
4. F. D. Porter, D. E. Scherrer, M. H. Lanier, S. J. Langmade, V. Molugu, S. E. Gale, D. Olzeski, R. Sidhu, D. J. Dietzen, R. Fu, C. A. Wassif, N. M. Yanjanin, S. P. Marso, J. House, C. Vite, J. E. Schaffer, D. S. Ory, Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. *Sci. Transl. Med.* **2**, 56ra81 (2010).
5. S. U. Walkley, K. Suzuki, Consequences of NPC1 and NPC2 loss of function in mammalian neurons. *Biochim. Biophys. Acta* **1685**, 48–62 (2004).
6. M. C. Patterson, C. J. Hendriksz, M. Walterfang, F. Sedel, M. T. Vanier, F. Wijburg; NP-C Guidelines Working Group, Recommendations for the diagnosis and management of Niemann–Pick disease type C: An update. *Mol. Genet. Metab.* **106**, 330–344 (2012).
7. P. Bauer, D. J. Balding, H. H. Klünemann, D. E. J. Linden, D. S. Ory, M. Pineda, J. Priller, F. Sedel, A. Muller, H. Chadha-Boreham, R. W. D. Welford, D. S. Strasser, M. C. Patterson, Genetic screening for Niemann–Pick disease type C in adults with neurological and psychiatric symptoms: Findings from the ZOOM study. *Hum. Mol. Genet.* **22**, 4349–4356 (2013).
8. M. Stampfer, S. Theiss, Y. Amraoui, X. Jiang, S. Keller, D. S. Ory, E. Mengel, C. Fischer, H. Runz, Niemann-Pick disease type C clinical database: Cognitive and coordination deficits are early disease indicators. *Orphanet J. Rare Dis.* **8**, 35 (2013).
9. C. D. Davidson, N. F. Ali, M. C. Micsenyi, G. Stephney, S. Renault, K. Dobrenis, D. S. Ory, M. T. Vanier, S. U. Walkley, Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression. *PLOS One* **4**, e6951 (2009).
10. B. Liu, H. Li, J. J. Repa, S. D. Turley, J. M. Dietschy, Genetic variations and treatments that affect the lifespan of the NPC1 mouse. *J. Lipid Res.* **49**, 663–669 (2008).
11. B. Liu, C. M. Ramirez, A. M. Miller, J. J. Repa, S. D. Turley, J. M. Dietschy, Cyclodextrin overcomes the transport defect in nearly every organ of NPC1 mice leading to excretion of sequestered cholesterol as bile acid. *J. Lipid Res.* **51**, 933–944 (2010).
12. B. Liu, S. D. Turley, D. K. Burns, A. M. Miller, J. J. Repa, J. M. Dietschy, Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npc1−/− mouse. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 2377–2382 (2009).
13. C. H. Vite, J. H. Bagel, G. P. Swain, M. Prociuk, T. U. Sikora, V. M. Stein, P. O'Donnell, T. Ruane, S. Ward, A. Crooks, S. Li, E. Mauldin, S. Stellar, M. De Meulder, M. L. Kao, D. S. Ory, C. Davidson, M. T. Vanier, S. U. Walkley, Intracisternal cyclodextrin prevents cerebellar dysfunction and Purkinje cell death in feline Niemann-Pick type C1 disease. *Sci. Transl. Med.* **7**, 276ra26 (2015).
14. M. Zervas, K. L. Somers, M. A. Thrall, S. U. Walkley, Critical role for glycosphingolipids in Niemann-Pick disease type C. *Curr. Biol.* **11**, 1283–1287 (2001).
15. M. C. Patterson, D. Vecchio, H. Prady, L. Abel, J. E. Wraith, Miglustat for treatment of Niemann-Pick C disease: A randomised controlled study. *Lancet Neurol.* **6**, 765–772 (2007).
16. C. A. Wassif, J. L. Cross, J. Iben, L. Sanchez-Pulido, A. Cougnoux, F. M. Platt, D. S. Ory, C. P. Ponting, J. E. Bailey-Wilson, L. G. Biesecker, F. D. Porter, High incidence of unrecognized visceral/neurological late-onset Niemann-Pick disease, type C1, predicted by analysis of massively parallel sequencing data sets. *Genet. Med.* **18**, 41–48 (2016).
17. X. Jiang, R. Sidhu, F. D. Porter, N. M. Yanjanin, A. O. Speak, D. T. te Vruchte, F. M. Platt, H. Fujiwara, D. E. Scherrer, J. Zhang, D. J. Dietzen, J. E. Schaffer, D. S. Ory, A sensitive and specific LC-MS/MS method for rapid diagnosis of Niemann-Pick C1 disease from human plasma. *J. Lipid Res.* **52**, 1435–1445 (2011).
18. G. Alvelius, O. Hjalmarson, W. J. Griffiths, I. Björkhem, J. Sjövall, Identification of unusual 7-oxygenated bile acid sulfates in a patient with Niemann-Pick disease, type C. *J. Lipid Res.* **42**, 1571–1577 (2001).
19. M. Maekawa, Y. Misawa, A. Sotoura, H. Yamaguchi, M. Togawa, K. Ohno, H. Nittono, G. Kakiyama, T. Iida, A. F. Hofmann, J. Goto, M. Shimada, N. Mano, LC/ESI-MS/MS analysis of urinary 3β-sulfooxy-7β-*N*-acetylglucosaminyl-5-cholen-24-oic acid and its amides: New biomarkers for the detection of Niemann–Pick type C disease. *Steroids* **78**, 967–972 (2013).
20. S. Kikuchi, Y. Imai, Z. Suzuoki, T. Matsuo, S. Noguchi, Biologic studies of cholestane-3β,5α,6β-triol and its derivatives. III. The metabolic fate and metabolites of cholestane-3β,5α,6β-triol in animals. *J. Pharmacol. Exp. Ther.* **159**, 399–408 (1968).
21. G. T. Everson, M. A. Polokoff, HepG2. A human hepatoblastoma cell line exhibiting defects in bile acid synthesis and conjugation. *J. Biol. Chem.* **261**, 2197–2201 (1986).
22. *Guidance for Industry: Bioanalytical Method Validation* (May 2001); www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf.
23. N. Spooner, R. Lad, M. Barfield, Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: Considerations for the validation of a quantitative bioanalytical method. *Anal. Chem.* **81**, 1557–1563 (2009).
24. A. F. Hofmann, L. R. Hagey, Key discoveries in bile acid chemistry and biology and their clinical applications: History of the last eight decades. *J. Lipid Res.* **55**, 1553–1595 (2014).
25. A. F. Hofmann, V. Loening-Baucke, J. E. Lavine, L. R. Hagey, J. H. Steinbach, C. A. Packard, T. L. Griffin, D. A. Chatfield, Altered bile acid metabolism in childhood functional constipation: Inactivation of secretory bile acids by sulfation in a subset of patients. *J. Pediatr. Gastroenterol. Nutr.* **47**, 598–606 (2008).
26. S. Pajares, A. Arias, J. García-Villoria, J. Macías-Vidal, E. Ros, J. de las Heras, M. Girós, M. J. Coll, A. Ribes, Cholestane-3β,5α,6β-triol: High levels in Niemann-Pick type C, cerebrotendinous xanthomatosis, and lysosomal acid lipase deficiency. *J. Lipid Res.* **56**, 1926–1935 (2015).
27. L. F. Gonzalez-Cuyar, B. Hunter, P. L. R. Harris, G. Perry, M. A. Smith, R. J. Castellani, Cerebrotendinous xanthomatosis: Case report with evidence of oxidative stress. *Redox Rep.* **12**, 119–124 (2007).
28. Z. Korade, L. Xu, K. Mirnics, N. A. Porter, Lipid biomarkers of oxidative stress in a genetic mouse model of Smith-Lemli-Opitz syndrome. *J. Inherited Metab. Dis.* **36**, 113–122 (2013).
29. L. Amigo, H. Mendoza, J. Castro, V. Quiñones, J. F. Miquel, S. Zanlungo, Relevance of Niemann-Pick type C1 protein expression in controlling plasma cholesterol and biliary lipid secretion in mice. *Hepatology* **36**, 819–828 (2002).
30. X. Jiang, D. S. Ory, Towards a new diagnostic standard for Niemann–Pick C disease. *EBioMedicine* **4**, 18–19 (2016).
31. J. Reunert, A. S. Lotz-Havla, G. Polo, F. Kannenberg, M. Fobker, M. Griese, E. Mengel, A. C. Muntau, P. Schnabel, O. Sommerburg, I. Borggraefe, A. Dardis, A. P. Burlina, M. A. Mall, G. Ciana, B. Bembi, A. B. Burlina, T. Marquardt, Niemann-Pick Type C-2 disease: Identification by analysis of plasma cholestane-3β,5α,6β-triol and further insight into the clinical phenotype. *JIMD Rep.* **23**, 17–26 (2015).
32. Y. Li, C. R. Scott, N. A. Chamoles, A. Ghavami, B. M. Pinto, F. Turecek, M. H. Gelb, Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. *Clin. Chem.* **50**, 1785–1796 (2004).
33. D. Marsden, H. Levy, Newborn screening of lysosomal storage disorders. *Clin. Chem.* **56**, 1071–1079 (2010).
34. R. Y. Wang, O. A. Bodamer, M. S. Watson, W. R. Wilcox; ACMG Work Group on Diagnostic Confirmation of Lysosomal Storage Diseases, Lysosomal storage diseases: Diagnostic confirmation and management of presymptomatic individuals. *Genet. Med.* **13**, 457–484 (2011).
35. W. Burke, B. Tarini, N. A. Press, J. P. Evans, Genetic screening. *Epidemiol. Rev.* **33**, 148–164 (2011).
36. C. D. M. van Karnebeek, T. Mohammadi, N. Tsao, G. Sinclair, S. Sirrs, S. Stockler, C. Marra, Health economic evaluation of plasma oxysterol screening in the diagnosis of Niemann–Pick Type C disease among intellectually disabled using discrete event simulation. *Mol. Genet. Metab.* **114**, 226–232 (2015).
37. J. Kawanami, Bile acids and steroids. XX. Studies on the hog bile acids (Part 4). Synthesis of 3β,6β-dihydroxy-5β-cholanic acid and 3β,6α-dihydroxy-5α-cholanic acid. *Bull. Chem. Soc. Jpn.* **34**, 509–513 (1961).

38. T. Yoshimura, R. Mahara, T. Kurosawa, S. Ikegawa, M. Tohma, An efficient synthesis of 4β- and 6α-hydroxylated bile acids. *Steroids* **58**, 52–58 (1993).
39. M. Wang, R. H. Han, X. Han, Fatty acidomics: Global analysis of lipid species containing a carboxyl group with a charge-remote fragmentation-assisted approach. *Anal. Chem.* **85**, 9312–9320 (2013).
40. P. Ciuffreda, S. Casati, L. Alessandrini, G. Terraneo, E. Santaniello, Synthesis of deuterated isotopomers of 7α- and (25*R,S*)-26-hydroxycholesterol, internal standards for in vivo determination of the two biosynthetic pathways of bile acids. *Steroids* **68**, 733–738 (2003).
41. J. Jopling, E. Henry, S. E. Wiedmeier, R. D. Christensen, Reference ranges for hematocrit and blood hemoglobin concentration during the neonatal period: Data from a multihospital health care system. *Pediatrics* **123**, e333–e337 (2009).
42. K. Yang, B. G. Dilthey, R. W. Gross, Identification and quantitation of fatty acid double bond positional isomers: A shotgun lipidomics approach using charge-switch derivatization. *Anal. Chem.* **85**, 9742–9750 (2013).

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Development of a bile acid–based newborn screen for Niemann-Pick disease type C

Xuntian Jiang, Rohini Sidhu, Laurel Mydock-McGrane, Fong-Fu Hsu, Douglas F. Covey, David E. Scherrer, Brian Earley, Sarah E. Gale, Nicole Y. Farhat, Forbes D. Porter, Dennis J. Dietzen, Joseph J. Orsini, Elizabeth Berry-Kravis, Xiaokui Zhang, Janice Reunert, Thorsten Marquardt, Heiko Runz, Roberto Giugliani, Jean E. Schaffer and Daniel S. Ory

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Expanding the newborn screen

Niemann-Pick disease type C (NPC) is a fatal neurologic disorder caused by the deficiency of an enzyme involved in cholesterol storage. Although this disease was untreatable in the past, new therapeutics are now in clinical trials, but they are most likely to be effective if treatment is started as early as possible, before neurodegeneration has occurred. Jiang *et al*. identified three bile acids that are greatly increased in the blood of patients with NPC compared to healthy controls. The authors also demonstrated that one of these bile acids can be reliably measured in dried blood spots using mass spectrometry, suggesting that this bile acid test should be evaluated for potential addition to neonatal screening programs.