Newborn screening for cerebrotendinous xanthomatosis is the solution for early identification and treatment[S]

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Abstract **Cerebrotendinous xanthomatosis (CTX) is a progressive metabolic leukodystrophy. Early identification and treatment from birth onward effectively provides a functional cure, but diagnosis is often delayed. We conducted a pilot study using a two-tier test for CTX to screen archived newborn dried bloodspots (DBSs) or samples collected prospectively from a high-risk Israeli newborn population. All DBS samples were analyzed with flow injection analysis (FIA)-MS/MS, and 5% of samples were analyzed with LC-MS/MS. Consecutively collected samples were analyzed to identify CTX-causing founder genetic variants common among Druze and Moroccan Jewish populations. First-tier analysis with FIA-MS/MS provided 100% sensitivity to detect CTX-positive newborn DBSs, with a low false-positive rate (0.1–0.5%). LC-MS/MS, as a second-tier test, provided 100% sensitivity to detect CTX-positive newborn DBSs with a false-positive rate of 0% (100% specificity). In addition, 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-β-D-glucuronide was identified as the predominant bile-alcohol disease marker present in CTX-positive newborn DBSs. In newborns identifying as Druze, a 1:30 carriership frequency was determined for the c.355delC *CYP27A1* gene variant, providing an estimated disease prevalence of 1:3,600 in this population. These data support the feasibility of two-tier DBS screening for CTX in newborns and set the stage for large-scale prospective pilot studies.—** DeBarber, A. E., L. Kalfon, A. Fedida, V. Fleisher Sheffer, S. Ben Haroush, N. Chasnyk, E. Shuster, H. Mandel, K. Jeffries, E. S. Shinwell, and T. C. Falik-Zaccai. **Newborn screening for cerebrotendinous xanthomatosis is the solution for early identification and treatment. *J. Lipid Res.* 2018.** 59: **2214–2222.**

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Cerebrotendinous xanthomatosis (CTX; OMIM#213700) is an autosomal recessive genetic disorder associated with deficiency in the sterol 27-hydroxylase enzyme important in conversion of cholesterol to the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA).

Although CTX is often not diagnosed until adulthood, with neurological symptoms present in >95% of cases (1, 2), this disorder is becoming recognized as a childhood onset disease (3–9). Cholestatic jaundice can be a presenting symptom of CTX in neonates (6–9). This may be self-limiting or progress to severe liver disease that can result in death from liver failure (8, 9). Chronic diarrhea has been reported as a presenting sign from the neonatal period onward (3, 4). In addition, childhood symptoms can include juvenile cataracts, developmental delay, and cognitive impairment (3, 4). Later onset symptoms may include gait difficulties, ataxia, corticospinal tract abnormalities, seizures, development of tendinous xanthomas, and psychiatric symptoms (1–5, 10, 11). If the disease remains untreated, there is a high probability of neurological decline that can result in severe cognitive impairment, intellectual disability, and dementia (1–5, 10, 11).

Based on the allele frequencies of pathogenic variants in the *CYP27A1* gene, the incidence of CTX is estimated to be from 1:134,970 to 1:416,358 in Europeans, from 1:263,222 to 1:468,624 in Africans, from 1:71,677 to 1:148,914 in Latinos, from 1:64,267 to 1:64,712 in East Asians, and from 1:36,072 to 1:75,601 in South Asians (12). The incidence of CTX is much higher in certain Israeli populations, for example, Moroccan Jews (13, 14) and an isolated Druze

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CDCA-d5-24-glucuronide, chenodeoxycholic acid-d5-24-acyl-β-D-glucuronide; CTGS, 5β-cholestane-tetrol glucuronide isomer species; CTX, cerebrotendinous xanthomatosis; DBS, dried bloodspot; FIA, flow injection analysis; g, glyco; IRB, Institutional Review Board; MRM, multiple reaction monitoring; OHSU, Oregon Health and Science University; PBD-ZSD, peroxisome biogenesis disorders in the Zellweger spectrum; QC, quality control; RSD, relative standard deviation; t, tauro; 7α12αC4, 7α,12α-dihydroxy-4-cholesten-3-one; 5β-cholestane-25-tetrol-glucuronide, 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-β-D-glucuronide.

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community in Galilee (15). In this community a 1:11 carrier frequency was determined for the pathogenic founder c.355delC *CYP27A1* gene variant (15).

A low-risk effective oral therapy for CTX is available in the form of CDCA and CA, the main bile acids deficient in CTX. Treatment with CDCA is considered the standard of care for CTX and has been shown to normalize the biochemical abnormalities and halt progression of the disease (16, 17). Treatment of infants and children affected with CTX has been described, including treatment of presymptomatic cases (6, 18–20). In a limited number of children, treatment with CA was reported as a less hepatotoxic alternative to CDCA (6). Treatment of patients with advanced neurological disease does not appear to reverse the neurological impairment (17). A recent study demonstrated that patients who began CDCA treatment after age 25 were significantly more limited in ambulation and more cognitively impaired compared with those who started treatment earlier (21). Therefore, it is essential to diagnose and treat CTX as early as possible, something that will likely only be achieved through implementation of universal newborn screening for this disorder.

Biochemical approaches to screen newborn dried bloodspots (DBSs) for CTX have recently been described (22–24). The defect in CTX results in the accumulation of bile acid precursors, including the ketosterols, 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one (7α12αC4). DeBarber and colleagues have demonstrated that elevated 7α12αC4 can be measured in CTX newborn DBSs using LC-MS/MS (22, 23). Analysis of this marker can be used to discriminate CTX-positive from CTX-negative newborn DBSs (22, 23), although the necessity for LC and the cost of ketosterol derivatization reagent limits the applicability of this methodology as a first-tier test. The ketosterol, 7α12αC4, is metabolized to form bile alcohol species, such as 5β-cholestane-3α,7α,12α-triol, or is further hydroxylated to form 5β-cholestane-tetrol isomers. The 5β-cholestane-3α,7α,12α,25-tetrol was reported to be the highest concentration tetrol isomer present in CTX blood (25, 26), primarily present in the form of a glucuronide conjugate (25, 26). Recently, in a validation study using 200 premature and term newborn DBSs, Vaz et al. (24) demonstrated that negative mode flow injection analysis (FIA)-MS/MS analysis of elevated 5β-cholestane-tetrol glucuronide isomer species (CTGS) could be used to screen newborn DBSs for CTX. As CTGS can also be elevated in cholestatic liver disease, the metabolite ratio of CTGS over tauro (t)-dihydroxycholanoic acid species (predominantly tCDCA) was used to enhance the sensitivity of testing, a ratio strongly elevated in CTX, but not in cholestasis (24).

Here, we present biochemical and genetic data from a prospective pilot study screening DBSs from a high-risk newborn population for CTX.

MATERIALS AND METHODS

Human subject research considerations and sample collection

Newborn DBSs were obtained from the archives of the Israeli Ministry of Health Central Newborn Screening Laboratory or were prospectively collected from newborns born at two hospitals in the Northern Israel region, the Galilee and Ziv Medical Centers. Informed consent was obtained from all families to test the samples for CTX. In the prospective group, additional DBS samples were collected at the time of routine newborn screening. Families were informed that, in the event of a CTX-positive determination, they would be contacted for diagnostic confirmation, genetic counseling, and medical care to be provided by the Galilee Medical Center Institute for Human Genetics. The study was approved by the Institutional Review Boards (IRBs) of the hospitals and by the central IRB committee of the Israeli Ministry of Health. Non-newborn CTX-positive DBSs were also obtained from participants enrolled in an IRB-approved study at Oregon Health and Science University (OHSU) with informed consent obtained from all study participants. Residual blood samples submitted to the OHSU Sterol Analysis Diagnostic Laboratory for confirmation of CTX were also used with IRB approval.

Chemicals and reagents

The 5β-cholestane-3α,7α,12α,25-tetrol-3-*O*-β-D-glucuronide (5β-cholestane-25-tetrol-glucuronide) was obtained via custom synthesis with identity confirmation by NMR and MS, and >95% purity determined by LC-MS. CDCA-d5-24-acyl-β-D-glucuronide (CDCA-d5-24-glucuronide) and 7α12αC4 were from Toronto Research Chemicals (Toronto, Ontario, Canada). The 7α,12αC4-d9 was obtained via custom synthesis with identity confirmation by NMR and MS, and >95% purity determined by LC-MS. The t-CA, t-CDCA, glyco-CDCA, glyco-CA, t-CDCA-d5 and glyco-CA-d5 were from Toronto Research Chemicals. Whole blood was from the Oregon Clinical and Translational Research Institute. SP1070 charcoal-stripped pooled human serum was from Golden West (Temecula, CA). LC-MS grade solvents and water were from Burdick and Jackson (Muskegon, MI). Formic acid (ACS grade 90%) was J. T. Baker brand (Avantor, Center Valley, PA). Protein Saver 903 filter paper was obtained from Whatman (Miami, FL).

DNA extraction from DBSs and genetic analysis

Genomic DNA was extracted using 3 × 3 mm punches with a QIAamp DNA MicroKit (QIAGEN, Hilden, Germany) following the protocol for isolation of genomic DNA from DBSs.

The genomic DNA samples were genetically screened for the common pathogenic genetic variants found in the Druze or Moroccan Jewish populations as previously described (13, 14, 27). In brief, this consisted of DNA amplification followed by restriction enzyme analyses. Specifically, for the Druze population, CTX allele detection was accomplished by amplification of a 262 bp fragment from genomic DNA containing the c.355delC variant in exon 2 of *CYP27A1*, with sense primer (5′-GCTTGGCCCAGTTATTCAGTTTTG-3′) and antisense primer (5′-GCCCTGTTCCAGTCCCTTCAG-3′). The PCR products were subsequently digested with *Fnu4H*I restriction enzyme (New England Biolabs, Ipswich, MA) and analyzed on 3.5% agarose gels. For Moroccan Jews, all three known pathogenic genetic variants were tested; the c.1016 C>T (pThr339Met) variant by amplification of a 382 bp fragment from exon 5 in *CYP27A1* using sense primer (5′-TGCTTTCACAGGGAAGAAGC-3′) and antisense primer (5′-ACAAAAGGACCATCCCCTGT-3′). The PCR products were digested with *HpyCH4*III restriction enzyme (New England Biolabs) and analyzed on 3.5% agarose gels. To detect the other two Moroccan Jewish genetic variants (14), direct sequencing of the targeted variants in exon 4 was performed using sense primer (5′-GAGGACACCGTGACCTTCGT-3′) and antisense primer (5′-TAGGGAACTGGTTCAGGTTG-3′). Sequences were determined using the BigDye Terminator cycle sequencing kit

(Applied Biosystems, Foster City, CA) and analyzed using the ABI PRISM 3500 XL genetic analyzer (Applied Biosystems).

Preparation of samples for MS/MS analysis

Quality control (QC) and calibrator DBS samples were generated using working dilutions of 5β-cholestane-25-tetrol-glucuronide (from stock solution stored in 85:15 acetonitrile:water) and 7α12αC4 in methanol. The working dilutions were spiked into whole blood that was spotted onto filter paper, dried, and stored desiccated at 80°C. Glycine and taurine conjugates of CDCA and CA were measured using calibrators generated by spiking charcoal-stripped serum with authentic standards spotted onto filter paper and dried. An internal standard solution containing 50 ng of CDCA-d5-24-glucuronide, 1 ng of 7α12αC4-d9, and 0.5 ng each of t-CDCA-d5 and glyco-CA in 300 μl of methanol was added to each DBS. Punches (3.2 mm) from calibrators, QCs, blanks, and unknown samples were extracted in 96-well plates by shaking for 75 min at 45°C. After centrifugation at 3,000 rpm for 5 min, 200 μl of the supernatant were transferred to a new plate and dried using a SpeedVac. The extract was resuspended in 200 μl of a 75:25 mixture of water:acetonitrile before analysis. QC samples were analyzed with each plate for FIA-MS/MS and LC-MS/MS analysis; calibrators were analyzed for LC-MS/MS analysis.

FIA-MS/MS method

FIA-MS/MS analyses were performed using a QTRAP5500 mass spectrometer (Applied Biosystems SCIEX), operating in multiple reaction monitoring (MRM) mode, equipped with a TurboIonSpray ESI source. The ESI interface was operated in the negative mode with the following settings: CUR 35, TEM 750, GS1 40, GS2 20, IS −4,000, and CAD setting medium. Glycine and taurine conjugates of CDCA and CA were detected using modified methodology from Mills et al. (28). Note that signal detected for conjugated CDCA and CA with FIA-MS/MS includes all conjugated dihydroxycholanoic and trihydroxycholanoic acid species present in DBSs. MRM transitions monitored for each analyte were as follows: for 5β-cholestane-25-tetrol-glucuronide, *m/z* 611.4→75 and *m/z* 611.2→85; CDCA-d5-24-glucuronide, *m/z* 572.7→75; tCDCA, *m/z* 498→80.1; tCA, *m/z* 514→80.1; glyco-CDCA, *m/z* 448→74.1; glyco-CA, *m/z* 464→74.1; tCDCA-d5, *m/z* 503.3→80.1 (used as internal standard for tauro-conjugates); and glyco-CA-d5, *m/z* 469.3→74.1 (used as internal standard for glyco-conjugates). The QTRAP5500 was coupled to a Shimadzu UPLC system (Columbia, MD) composed of a SIL-20ACXR autosampler and LC-20ADXR LC pumps. MS/MS analysis was performed with an isocratic 1.2 min method using mobile phase (80:20 acetonitrile:water at 0.1% formic acid) delivered at 0.1 ml/min. At 0.6 min the flow rate was increased to 0.3 ml/min.

LC-MS/MS method

Polarity switching was used for LC-MS/MS analysis. The ESI interface negative mode settings and MRM transitions monitored for quantification were the same as for FIA-MS/MS. The ESI interface was also operated in the positive mode with the following settings: CUR 35, TEM 600, GS1 40, GS2 20, IS 3,000, and CAD medium. Positive mode MRM transitions monitored for quantification of 7α12αC4 were as follows: 7α12αC4, *m/z* 417.3→253.1; and 7α12αC4-d_9, *m/z* 426.3→253.1. LC-MS/MS analysis was performed using a 50 × 2.1 (i.d.) mm, 1.8 μm HSST3 column (Waters, Milford, MA). The 3 min gradient method used mobile phase [solvent A (water) and solvent B (acetonitrile), both at 0.1% formic acid] delivered at 0.5 ml/min. Solvent B was increased from 25% to 90% over 1 min. The column was washed at 90% solvent B for 1.25 min and then equilibrated for 0.75 min. The column temperature was 50°C.

RESULTS

Newborn DBS sample collection

Three CTX-positive DBSs from Druze newborns known to be homozygous for the pathogenic founder c.355delC *CYP27A1* gene variant (27), as well as known carrier newborn DBSs, were retrieved from the Israeli Newborn Screening Laboratory archives. The newborn DBSs had been stored for 3–8 years refrigerated (4°C) and were from children previously identified as being affected with CTX or as carriers through prenatal genetic testing (or testing shortly after birth), performed as part of a community-wide screening program for genetic diseases established and overseen in the region by Falik-Zaccai et al. (15). The archived newborn DBSs were analyzed along with DBSs prospectively collected from a high-risk newborn population born at the Galilee and Ziv Medical Centers.

Genetic analysis

Genetic analysis of CTX-causing founder genetic variants common among the Druze and Moroccan Jewish populations was performed for around 55% of newborn DBS samples consecutively collected in a prospective manner. Among 366 Druze newborn DBS samples tested for the c.355delC *CYP27A1* gene variant, 13 carrier samples were identified, providing a frequency of 1:30 carriership in this sample of Druze-origin population. Based on the Hardy-Weinberg equation, these data predict a disease prevalence of 1:3,600 in the Druze population screened for pilot study.

FIA-MS/MS method performance

QC samples were generated by spiking low tCDCA whole blood with 5β-cholestane-25-tetrol-glucuronide authentic standard at nominal concentrations of 500 and 5,000 ng/ml (low and high QCs). The mean observed GTCS/tCDCA ratio for low QC was close to the lowest metabolite ratio observed for a CTX newborn sample and for high QC ratio was comparable to a mid-range metabolite ratio for a CTX adult sample. The between-run precision data for the GTCS/tCDCA ratio determined for QC samples injected across different FIA-MS/MS batches was <24% relative standard deviation (RSD). Based on the precision data, a reasonable ratio cut-off for first-tier screening using our instrumentation was designated as 0.361 (70% of lowest CTX newborn ratio observed).

LC-MS/MS method performance

To calculate analyte concentrations, calibrators were run with each batch and curves generated using a weighted (1/x) least-squares linear regression for peak area ratio (analyte/internal standard) plotted against nominal concentration. Calibration curves for measurement of 5β-cholestane-25-tetrol-glucuronide in DBSs demonstrated linearity across the range of 250–10,000 ng/ml with correlation coefficients $r^2 > 0.95$. The between-run accuracy was within ±22% and precision <21% for mean 5β-cholestane-25-tetrol-glucuronide concentration measured across LC-MS/MS batches (see supplemental Table S1). This data was generated using CDCA-d5-24-glucuronide as internal

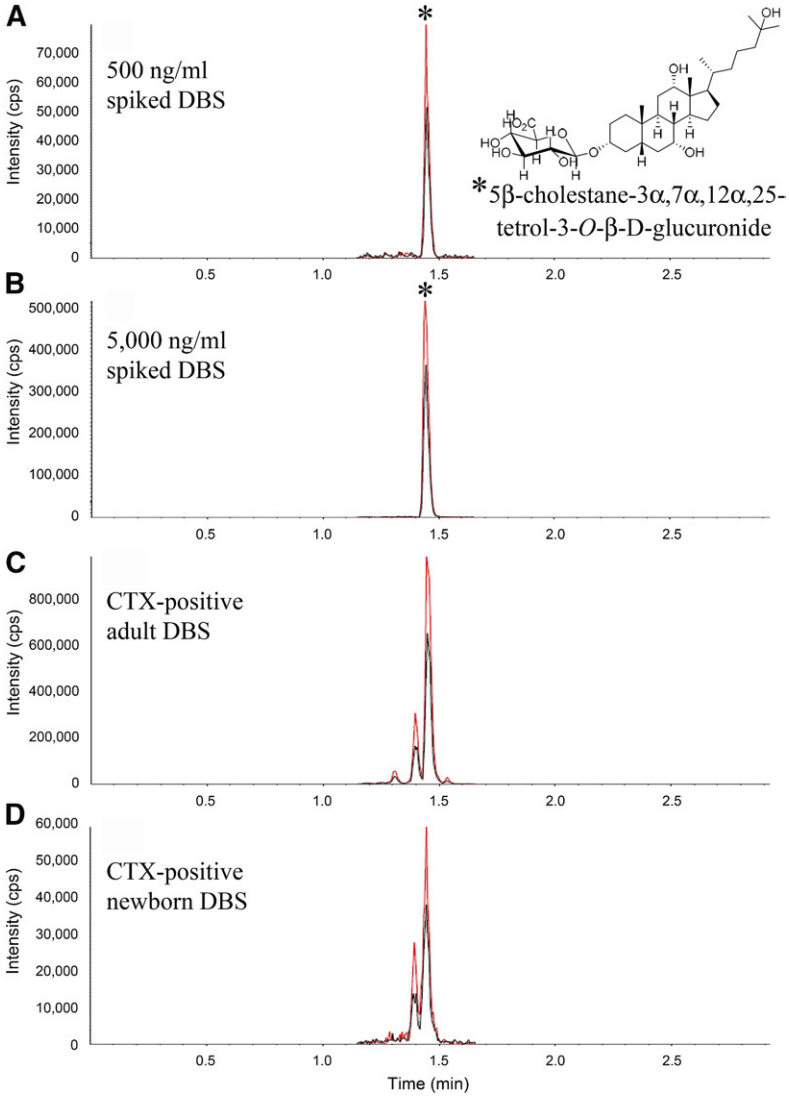

Fig. 1. LC-MS/MS analysis of CTGS present in CTX-positive DBSs. A, B: Extracted ion chromatograms for MRM detection of 5β-cholestane-25-tetrol-glucuronide standard spiked at 500 ng/ml (A) and 5,000 ng/ml (B) into whole blood and spotted onto filter paper [* is the authentic standard at 1.45 min retention time, black and red traces are product ions *m/z* 85 and 75, respectively]. C, D: Extracted ion chromatograms for MRM detection of CTGS in representative adult (C) and newborn (D) CTX-positive DBSs. The primary CTGS peak present co-chromatographs with 5β-cholestane-25-tetrol-glucuronide standard. Additional CTGS peaks elute at retention time 1.32, 1.41, and 1.55 min that are putative 5β-cholestane-3α7α,12α,23- and 24-tetrol glucuronides (25).

standard. Stable isotope-labeled 5β-cholestane-25-tetrol-glucuronide internal standard was not commercially available, but would likely improve the method performance.

Autosampler carryover was acceptable with <20% peak area for 250 ng/ml calibrator present after injection of 10,000 ng/ml calibrator (the highest concentration in untreated CTX-positive DBSs was 10,154 ng/ml).

A stock solution of 5β-cholestane-25-tetrol-glucuronide in acetonitrile:water (85:15) was stable frozen at −80°C for at least 6 months (compared with solid compound stored at −80°C). The 5β-cholestane-25-tetrol-glucuronide spiked into whole blood and spotted onto filter paper was stable frozen at −20°C for up to 6 months. For DBSs stored at 20°C or 40°C, the concentrations for weekly time points were within ±20% of the initial concentration for up to 3 weeks.

Matrix effects for LC-MS/MS quantitation of 5β-cholestane-25-tetrol-glucuronide were determined by comparing the peak area for standard spiked into filter-paper extract to peak area for standard spiked into DBS extracts from unaffected individuals (29). Matrix effects were observed within ±34% with a peak area RSD <15% (n = 4 different individuals). When these samples were analyzed using FIA-MS/MS, increased ion suppression was observed for 5β-cholestane-25-tetrol-glucuronide relative to tCDCA, indicating that a stable isotope-labeled internal standard analog should optimally be used for each compound.

TABLE 1. Distribution of CTGS present in CTX-positive DBS

DBSs from:	GTGS (RT 1.32 min) Peak Area	GTGS (RT 1.41 min) Peak Area	5β-Cholestane-25-tetrol-glucuronide (RT 1.45 min) Peak Area	GTGS (RT 1.55 min) Peak Area
CTX untreated adults (n = 9)	4.9 ± 4.0%	13.1 ± 4.8%	79.3 ± 7.8%	2.7 ± 1.2%
CTX treated adults (n = 8)	2.7 ± 5.8%	11.6 ± 4.5%	80.7 ± 8.6%	5.0 ± 1.9%
CTX untreated newborns (n = 3)	0.0%	17.7 ± 3.1%	79.9 ± 2.7%	2.3 ± 0.8%

Data are expressed as mean concentration ± SD. RT, retention time.

Potential method interference for LC-MS/MS quantitation of 5β-cholestane-25-tetrol-glucuronide was evaluated by examination of the peak shape, shoulder, and area ratio for two MRM transitions acquired [quantifier and qualifier ions (30); *m/z* 75 and 85, respectively]. Data from reversed-phase analysis of CTX-positive DBSs demonstrated that multiple putative CTGS peaks were extracted (with the same precursor ion mass and characteristic *m/z* 75 and 85 glucuronide product ions; see **Fig. 1**). The predominant CTGS isomer present co-chromatographed with authentic 5β-cholestane-25-tetrol-glucuronide standard using a fast 3 min gradient, or shallower gradient performed over 30 min, and possessed similar MS/MS product ion data (see supplemental Fig. S1); nevertheless there is the possibility of other isomeric species with alternate stereochemistries co-eluting.

The percent distribution of CTGS calculated using *m/z* 75 product ion peak area was similar in treated or untreated CTX-positive DBSs, including newborn CTX-positive DBSs (see **Table 1**). As authentic standards were not available for each CTGS, the percent distribution value assumes similar ionization efficiency and *m/z* 75 product ion fragment intensity for each species (in support of this, the ion ratio for *m/z* 75 and 85 product ions was comparable for each CTGS).

FIA-MS/MS analysis of CTGS/tCDCA ratio in DBSs as a test for CTX

FIA-MS/MS analysis was used in a blinded manner to determine the CTGS/tCDCA ratio in all DBS samples (see **Table 2** and **Fig. 2**). CTX-positive newborn DBSs from the Newborn Screening Laboratory archives could be identified with 100% sensitivity using the CTGS/tCDCA ratio, but with a very low false positive rate (0.1–0.5% depending where the cut-off value was placed). The lowest ratio of CTGS/tCDCA determined in CTX-positive newborn DBSs was 0.516 and the highest ratio in unaffected newborn DBSs was 0.634, with overlap between the ratios in CTX-positive and unaffected newborn DBSs. Around 5% of samples, including samples with a ratio >0.361, were subjected to LC-MS/MS analysis.

TABLE 2. FIA-MS/MS analysis of the CTGS to tCDCA ratio in DBSs as a test for CTX

DBS from:	CGTS/tCDCA
CTX untreated adults/children (n = 12)	19.4852 (1.173–41.847)
CTX treated adults/children (n = 8)	3.780 (0.244–20.102)
CTX untreated newborns (n = 3, analyzed in duplicate)	**0.814 (0.516–0.962)**
Identified Druze carrier newborns (n = 9, includes one cholestatic newborn)[a]	**0.110 (0.028–0.271)**
Unaffected newborns (1,216 in total; n = 661 Druze, n = 242 Moroccan Jewish, n = 313 general population)	**0.067 (0.007–0.634)**
Cholestasis newborns[b] (25 in total; n = 11 Druze, n = 1 Moroccan Jewish, n = 13 general population)	**0.015 (0.003–0.041)**

Data are expressed as mean (range of results) for *m/z* 75 product ion. Bold type indicates the 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio values that are provided in Fig. 2A.

[a]Although nine carrier samples overall were identified as part of the MS/MS data analysis (seven prospectively, two previously identified and retrieved from archives), based on the carriership frequency determined for c.355delC *CYP27A1* gene variant, we would estimate at least 16 additional unidentified carrier samples to be present in the Druze DBSs biochemically screened.

[b]Around the highest 2% concentration of total bile acid conjugates (determined by semi-quantitative analysis to be >20,000 ng/ml).

LC-MS/MS analysis of 5β-cholestane-25-tetrol-glucuronide, tCDCA, and 7α12αC4 in DBSs as a test for CTX

Use of LC-MS/MS to determine the 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio, and to quantify 7α12αC4 in DBSs, provided 100% sensitivity to detect the CTX-positive newborn samples with no overlap between the 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio and the 7α12αC4 concentration determined for CTX-positive compared with other newborn DBSs (see **Table 3** and Fig. 2). LC-MS/MS allowed identification as positive 3/3 DBSs from CTX-affected newborns, 0/9 DBSs from identified carrier newborns, 0/15 cholestasis newborn DBSs, and 0/61 unaffected newborn DBSs. Additional test validation data were generated by analyzing DBSs from children/adults affected with CTX (see Tables 2 and 3) and peroxisome biogenesis disorders in the Zellweger spectrum (PBD-ZSDs). DBSs from individuals with PBD-ZSDs may have an elevated CTGS/tCDCA ratio (23). These samples can be discriminated from CTX-positive DBSs using the tauro-trihydroxycholestanoic acid to CTGS ratio (a metabolite ratio decreased in CTX, but not in PBD-ZSDs) (23). Our data indicate that 7α12αC4 was not elevated in these samples and might also be a useful marker to discriminate PBD-ZSDs from CTX-positive DBSs (see Fig. 2C and the numeric data in Table 3).

DISCUSSION

We present here feasibility data from a prospective pilot study screening DBSs from a high-risk newborn population for CTX that supports use of a two-tier screening approach (the proposed screening algorithm is outlined in **Fig. 3**). The approach consists of first-tier negative mode FIA-MS/MS analysis to determine the metabolite ratio CTGS/tCDCA as described by Vaz et al. (24). This method provided 100% sensitivity to detect CTX-positive newborn samples with a false positive rate for unaffected newborn DBSs of 0.1–0.5% depending on the cut-off used. For this validation study, a subset of around 5% of samples was subjected to analysis with LC-MS/MS (including samples with a ratio >0.361). LC-MS/MS analysis provided 100% sensitivity to detect CTX-positive newborn samples with a 0% false positive rate (100% specificity). Any positive samples identified using second-tier LC-MS/MS testing would undergo confirmatory *CYP27A1* gene sequencing (Fig. 3). Adoption of a two-tier approach has proven to be a successful means to reduce false positive results for other newborn screening applications (31) and is considered an acceptable solution to lower false positive rates that could otherwise pose a burden on newborn screening laboratories, clinicians, and families.

Newborn screening programs routinely ascertain, as a quality measure, whether patients diagnosed with disorders later in life escaped detection by newborn screening

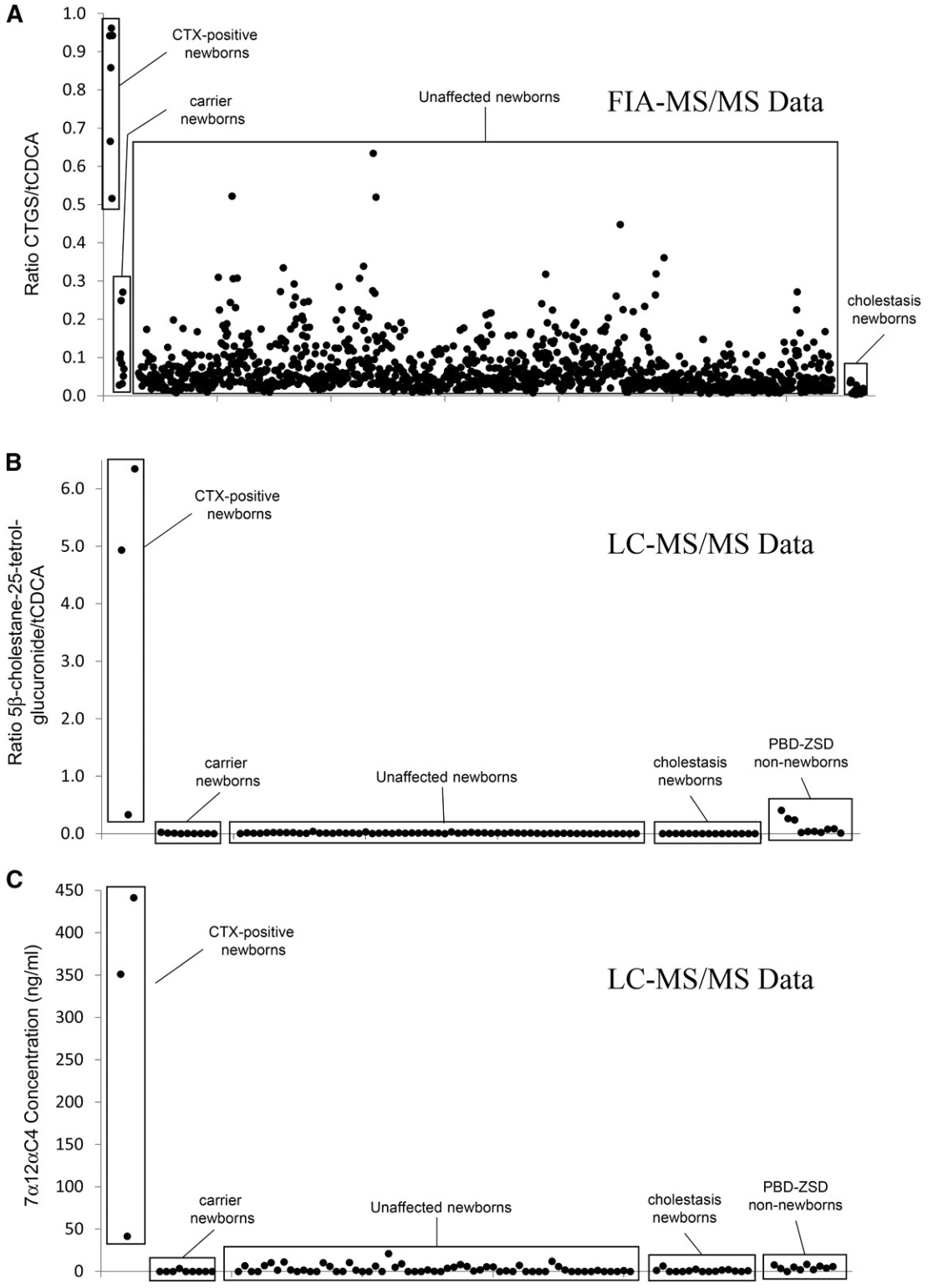

Fig. 2. Determination of disease markers in newborn DBSs as a test for CTX. A: CTGS/tCDCA ratio determined using FIA-MS/MS (all newborn samples, n = 1,250; numeric data is provided in Table 2). Note that the FIA-MS/MS signal detected for tCDCA includes all dihydroxycholanoic acid isomer species present. B, C: The 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio (B) and 7α12αC4 concentration (C) determined using LC-MS/MS (approximately 5% of unaffected newborn samples plus CTX-positive, identified carriers, cholestasis, and non-newborn PBD-ZSD samples, n = 98; numeric data is provided in Table 3).

(false negatives). The Galilee Medical Center is a regional center of expertise for treatment of CTX. To the best of our knowledge, and with the caveat that CTX is not routinely diagnosed in infancy or childhood, no new pediatric CTX cases were identified in the Northern Galilee region since the pilot study started.

Here, we present LC-MS/MS data that identifies 5β-cholestane-25-tetrol-glucuronide as the predominant CTGS

TABLE 3. LC-MS/MS analysis of 5β-cholestane-25-tetrol-glucuronide, tCDCA, and 7α12αC4 in around 5% of total DBSs as a test for CTX

DBS from:	Ratio 5β-Cholestane-25-tetrol-glucuronide/tCDCA	5β-Cholestane-25-tetrol-glucuronide (ng/ml)	tCDCA (ng/ml)	7α12αC4 (ng/ml)
CTX untreated adults/children (n = 12)	202.9312 (1.7446–785.6219)	6,100 (584–10,039)	62 (0–329)	2,423 (268–3,669)
CTX treated adults/children (n = 8)	87.8009 (0.4540–645.3804)	2,017 (146–8,652)	47 (0–129)	515 (18.0–2,075)
CTX untreated newborns (n = 3)	**3.8707[b] (0.3301–6.3481)**	**548[b] (391–720)[a]**	**135[b] (29–333)**	**279.8[b] (41.6–441.5)**
Identified carrier newborns (n = 9)	**0.0064 (0.0009–0.0234)**	**175 (101–728)[a,c]**	**1,462 (202–7,889)**	**0.4 (0–3.5)[a]**
Unaffected newborns (n = 61)	**0.0099 (0.0005–0.0402)**	**43 (29–165)[a]**	**1,573 (137–10,641)**	**2.9 (0–21.0)[a]**
Cholestasis newborns (n = 15)	**0.0013 (0.0004–0.0035)**	**49 (30–82)[a]**	**7,800 (2,203–17,621)**	**6.5 (0–6.5)[a]**
PBD-ZSD adults/children (n = 10)	**0.120 (0.0100–0.4070)**	**Not determined**	**318 (25–1,076)**	**4.6 (0–8.5)[a]**

Data are expressed as mean (range of results) for *m/z* 75. Bold type indicates the 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio and 7α12αC4 that are provided in Fig. 2B and C, respectively.

[a] Outside validated linear analytical measurable range.

[b] For one CTX-positive newborn sample, the mean calculated tCDCA concentration was 262 ng/ml (from n = 3 replicate analyses), with a resultant mean 5β-cholestane-25-tetrol-glucuronide/tCDCA ratio of 0.4546 (RSD 29.9%, n = 3). The mean 5β-cholestane-25-tetrol-glucuronide concentration for this sample was 492 ng/ml (RSD 9.7%, n = 3) and mean 7α12αC4 concentration was 46.5 ng/ml (14.5%, n = 3).

[c] All non-CTX samples were <250 ng/ml 5β-cholestane-25-tetrol-glucuronide except one carrier cholestasis sample with a 5β-cholestane-25-tetrol-glucuronide value of 728 ng/ml.

present in CTX-positive DBSs and provide quantitative data for 5β-cholestane-25-tetrol-glucuronide present in CTX-positive newborn DBSs. Batta et al. (25) previously reported that 5β-cholestane-3α,7α,12α,25-tetrol was the highest concentration bile alcohol isomer in CTX blood. Kosada et al. (26) measured the mean serum concentration of 5β-cholestane-3α,7α,12α,25-tetrol in untreated CTX patients as 3,487 ± 2,129 ng/ml (4.6 ± 1.2 ng/ml in controls and 82.4 ± 55.7 ng/ml in patients with cholestasis). To our knowledge, the major glucuronide isomer of 5β-cholestane-3α,7α,12α,25-tetrol in CTX blood has not been characterized, but 5β-cholestane-25-tetrol-glucuronide was identified as the major CTGS present in CTX bile (32). Identification of 5β-cholestane-25-tetrol-glucuronide as the predominant CTGS and quantitative data for this species present in CTX-positive newborn DBSs will enable production of QC material for future newborn screening endeavors. The 5β-cholestane-25-tetrol-glucuronide is stable in DBSs stored from 20 to 40°C for up to 3 weeks. There are no special requirements to collect, store, or process newborn DBS samples, as this disease marker appears to be chemically stable.

A limitation of this pilot study is that, although eighth-inch (3.2 mm) DBS punches were analyzed, a more sensitive LC-MS/MS instrument than found in many newborn screening laboratories was used for the study. In the Vaz et al. (24) pilot study, a lower sensitivity LC-MS/MS instrument was used, but quarter-inch DBS punches were required.

LC-ESI-MS/MS methodology was previously described utilizing keto derivatization to enable sensitive isotope dilution measurement of 7α12αC4 in DBSs from CTX-positive newborns (22, 23). One advantage of this approach was the ability to synthesize stable isotope-labeled internal standard using labeled derivatization reagent (22). For the pilot study, we used custom-synthesized 7α12αC4-d9 internal standard for isotope dilution measurement of 7α12αC4 without keto derivatization. Including the data we report here, elevated 7α12αC4 has been measured in six CTX-positive newborn DBSs (with a mean value of 222 ng/ml, range 42–431 ng/ml; and a mean control value of 10.2,

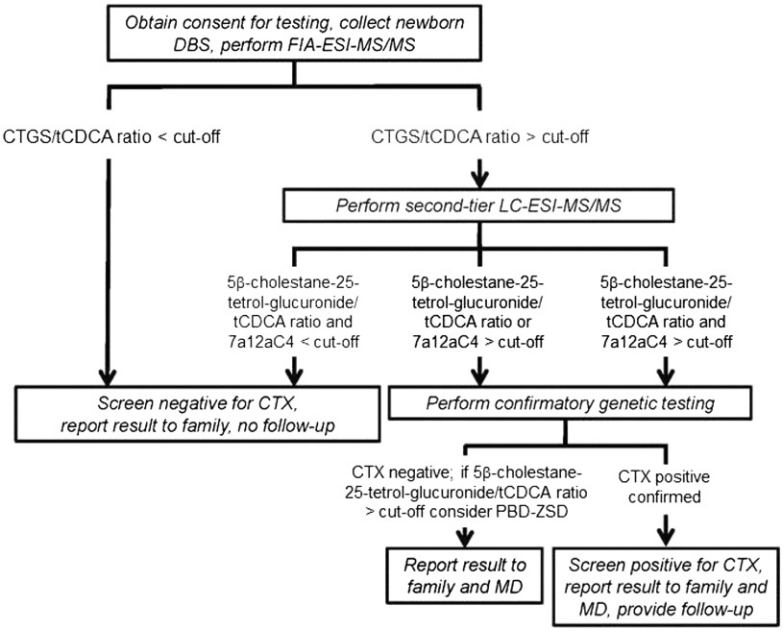

Fig. 3. Two-tier screening approach to screen newborn DBSs for CTX. If the second-tier biochemical positive result is confirmed by genetic testing, or the genetic testing result is ambiguous, families should be contacted for follow-up, which should provide diagnostic and medical evaluation, genetic counseling, and referral to all needed specialists.

range 0–16.5). The laboratory performance metrics for this disease marker thus far are: true positive cases identified, six; false positive cases, zero.

Measurement of 5β-cholestane-25-tetrol-glucuronide and 7α12αC4 in blood may be a useful tool for diagnosis and therapeutic monitoring in CTX (see data for untreated and treated CTX adults/children in Table 3). Measurement of 5α-cholestanol in blood has routinely been used for this purpose and is offered by a number of clinical laboratories worldwide. More recently, measurement of 7α12αC4 has been made available for clinical use as a more specific test for diagnosis of CTX (health care providers seeking information and assistance should contact the authors).

From 2003 onward, Falik-Zaccai et al. (15) at the Galilee Medical Center participated in extensive outreach efforts in nearby Druze-populated regions, using genetic testing to systematically screen communities for severe genetic disorders, including CTX, and implementing the first genetic screening program of its kind in an isolated Druze community in the Galilee. In this community, a 1:11 carrier frequency was determined for the c.355delC *CYP27A1* gene variant (15). For this pilot study, we determined a 1:30 carrier frequency for the c.355delC *CYP27A1* gene variant in Druze newborns born at the Galilee and Ziv Medical Centers. Based on the carrier frequency of 1:30, we have estimated a disease prevalence of 1:3,600 in this Druze newborn population; therefore, we did not necessarily expect to find a true positive during this proof-of-principle pilot newborn screening study. Our goal is to continue screening DBSs collected from newborns born in this region. In moving toward universal newborn screening for CTX, data from stepwise implementation of newborn screening, such as the screening for CTX we describe here, performed in a high-risk newborn population, will be extremely valuable. In addition, to test validation data, data can be generated regarding diagnostic follow-up and treatment of screen positive cases, as well as potential benefits of early detection and treatment.

In conclusion, the prospective pilot study data we present supports the feasibility of newborn screening for CTX; the perfect solution for early identification and timely treatment of a disorder that can otherwise present in the neonatal period as cholestatic jaundice that can progress to severe life-threatening liver disease. Without treatment there is neurodegenerative progression over time in CTX that may not be reversed by treatment. Compared with the current delay in diagnosis and treatment, early identification through newborn screening and treatment from birth onward would greatly benefit those affected with this metabolic disorder, effectively providing a functional cure for CTX.

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