Molecular Genetics and Metabolism xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Regular Article

Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California

N.M. Gallant [a,b,c], K. Leydiker [d], Y. Wilnai [e], C. Lee [e], F. Lorey [f], L. Feuchtbaum [f], H. Tang [f], J. Carter [f], G.M. Enns [e], S. Packman [g], H.J. Lin [h], W.R. Wilcox [i], S.D. Cederbaum [j,k,l], J.E. Abdenur [b,d,*]

[a] Division of Genetic and Genomic Medicine, University of California, Irvine, Irvine, CA, United States
[b] Department of Pediatrics, University of California, Irvine, Irvine, CA, United States
[c] Stramski Children's Developmental Center, Miller Children's and Women's Hospital, Long Beach, CA, United States
[d] Division of Metabolic Disorders, Children's Hospital of Orange County, Orange, CA, United States
[e] Lucile Packard Children's Hospital, Division of Medical Genetics, Stanford University Medical Center, Stanford, CA, United States
[f] Genetic Disease Screening Program, California Department of Public Health, Richmond, CA, United States
[g] Division of Medical Genetics, Department of Pediatrics, University of California, San Francisco, San Francisco, CA, United States
[h] Division of Medical Genetics, Department of Pediatrics, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA, United States
[i] Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, United States
[j] Department of Psychiatry, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States
[k] Intellectual and Developmental Disabilities Research Center at UCLA, Los Angeles, CA, United States
[l] Semel Institute for Neuroscience, UCLA, Los Angeles, CA, United States

ARTICLE INFO

Article history:
Received 7 June 2017
Received in revised form 29 June 2017
Accepted 30 June 2017
Available online xxxx

ABSTRACT

Carnitine transporter defect (CTD; also known as systemic primary carnitine deficiency; MIM 212140) is due to mutations in the *SLC22A5* gene and leads to extremely low carnitine levels in blood and tissues. Affected individuals may develop early onset cardiomyopathy, weakness, or encephalopathy, which may be serious or even fatal. The disorder can be suggested by newborn screening. However, markedly low newborn carnitine levels can also be caused by conditions unrelated to CTD, such as the low carnitine levels often associated with normal pregnancies and some metabolic disorders occurring in the mother. In order to clarify the biochemical characteristics most useful for identification of CTD in newborns, we examined California Department of Public Health newborn screening data for CTD from 2005 to 12 and performed detailed chart reviews at six metabolic centers in California. The reviews covered 14 cases of newborn CTD, 14 cases of maternal disorders (CTD, 6 cases; glutaric aciduria, type 1, 5; medium-chain acyl CoA dehydrogenase deficiency, 2; and cobalamin C deficiency, 1), and 154 false-positive cases identified by newborn screening. Our results show that newborns with CTD identified by NBS exhibit different biochemical characteristics, compared to individuals ascertained clinically. Newborns with CTD may have NBS dried blood spot free carnitine near the lower cutoff and confirmatory plasma total and free carnitine levels near the normal lower limit, particularly if obtained within two weeks after birth. These findings raise the concern that true cases of CTD may exist that could have been missed by newborn screening. CTD should be considered as a possible diagnosis in cases with suggestive clinical features, even if CTD was thought to be excluded in the newborn period. Maternal plasma total carnitine and newborn urine total carnitine values are the most important predictors of true CTD in newborns. However, biochemical testing alone does not yield a discriminant rule to distinguish true CTD from low carnitine in newborns due to other causes. Because of this biochemical variability and overlap, molecular genetic testing is imperative to confirm CTD in newborns. Additionally, functional testing of fibroblast carnitine uptake remains necessary for cases in which other confirmatory testing is inconclusive. Even with utilization of all available diagnostic testing methods, confirmation of CTD ascertained by NBS remains lengthy and challenging. Incorporation of molecular analysis as a second tier step in NBS for CTD may be beneficial and should be investigated.

Abbreviations: ACMG, American College of Medical Genetics; ARUP, Associated Regional and University Pathologists, Inc.; C0, dried blood spot free carnitine; CHOC Children's, Children's Hospital of Orange County; CTD, carnitine transporter defect; FP, false-positive; GA 1, glutaric aciduria, type 1; MC, maternal condition; MCADD, medium-chain acyl CoA dehydrogenase deficiency; MCTD, maternal CTD; MOC, maternal other condition; NA, not available or not done; NBS, newborn screening; NCTD, newborn CTD; NPTC, newborn plasma total carnitine; NUFC, newborn urine free carnitine; NUTC, newborn urine total carnitine; UCLA, University of California, Los Angeles.

* Corresponding author at: Department of Pediatrics, University of California, Irvine, Irvine, CA, United States.
E-mail address: jabdenur@choc.org (J.E. Abdenur).

http://dx.doi.org/10.1016/j.ymgme.2017.06.015
1096-7192/

1. Introduction

Carnitine transporter defect (CTD; also known as systemic primary carnitine deficiency; MIM 212140) is caused by mutations in the *SLC22A5* gene and produces extremely low circulating carnitine levels. Problems include early-onset cardiomyopathy, weakness, or encephalopathy, which can be life-threatening, and sudden death from cardiac arrhythmia [1,2]. *SLC22A5* [solute carrier family 22 (organic cation/carnitine transporter), member 5; also known as OCTN2; MIM 603377] is a high affinity carnitine transporter expressed most abundantly in the heart, skeletal muscle, proximal renal tubules, and placenta. Prior to expanded newborn screening (NBS), CTD patients were identified by clinical symptoms and diagnosed by biochemical, functional, and/or molecular testing. The classic biochemical feature is an extremely reduced plasma free carnitine level (<5 μmol/L). However, newborn plasma carnitine levels in CTD can be higher, and possibly within the normal range, if obtained too soon after birth, due to placental carnitine transfer from the mother [1].

Because *SLC22A5* is highly expressed in the renal tubule, CTD is also associated with urinary wasting of free carnitine. Excretion of free carnitine in the urine depends on both the level of free carnitine in the plasma and the renal threshold for excretion. However, the renal threshold for carnitine excretion can differ among individuals. For example, Stanley et al. found thresholds for carnitine excretion to be 56 μmol/L in a control child, 13–26 μmol/L in two individuals with medium-chain acyl-CoA dehydrogenase deficiency, and <2 μmol/L in a child with CTD [2].

Other tests, in addition to biochemical assays, are used to establish the presence of CTD. Specifically, reduced carnitine transport in fibroblasts from the patient (<10% of the value for age-matched controls) and/or two germline disease-causing variants in *SLC22A5* confirm a diagnosis [1,3–6].

High-dose L-carnitine treatment is necessary to achieve low-normal plasma carnitine levels, and in some instances even very high doses of L-carnitine cannot normalize plasma carnitine level. Treatment must continue lifelong. Cases have been reported of hypoglycemia or sudden death from arrhythmia in individuals who discontinued L-carnitine against medical recommendations [1]. L-carnitine treatment is generally effective in preventing the major clinical complications. Of concern, however, there is at least one report of sudden death in a patient who was compliant with carnitine therapy [7].

NBS for CTD is achieved with tandem mass spectrometry, by detecting levels of free carnitine below a set cutoff in dried blood spot samples collected soon after birth. However, confirming a diagnosis of CTD after identification by NBS can be challenging. Newborn plasma carnitine levels strongly reflect maternal levels, which may fall drastically during pregnancy [8]. Consequently, NBS for CTD identifies a high frequency of false-positive cases, caused by very low maternal carnitine levels. Moreover, NBS for CTD has unmasked maternal cases of CTD, glutaric aciduria, type I, medium-chain acyl-CoA dehydrogenase deficiency, and cobalamin C deficiency [9–12].

The potential risks of NBS include psychological stress associated with false-positive results and adverse medical outcomes associated with false-negative results [13]. These risks may be heightened in NBS for CTD, because low carnitine is common among normal newborns, whereas true CTD, although rare, is potentially fatal without treatment. Therefore, it is critical to efficiently distinguish true CTD from other causes of low carnitine in newborns.

Currently, the biochemical features that distinguish newborn CTD from other causes of low carnitine are not well-defined. Although the American College of Medical Genetics (ACMG) published a diagnostic algorithm for confirmatory testing [14], there is limited evidence that this approach identifies CTD in asymptomatic infants [15]. The California Newborn Screening Guidelines Committee (an ad hoc committee organized by the Genetic Disease Screening Program) issued a modified version of the ACMG algorithm in February 2009 (Supplementary Fig. 1), but this algorithm is followed inconsistently by different metabolic centers. Post-analytical tools developed by Region 4 Stork have been shown to improve the performance of NBS by tandem mass spectrometry [16]. However, these tools focus exclusively on NBS dried blood spot data and do not address results from confirmatory biochemical testing.

The primary aims of this study are to analyze the biochemical characteristics of newborns with confirmed CTD and to determine if confirmatory plasma and urine biochemical testing in newborns and mothers can distinguish CTD from other causes of low free carnitine in newborns. We examined California NBS data for CTD from the inception of the program, September 2005 through June 2012. Additionally, by use of detailed chart reviews at six metabolic centers throughout California, we retrospectively analyzed confirmatory testing results and clinical information on 14 cases of newborn CTD, 14 cases of maternal conditions (maternal CTD, 6 cases; and other maternal conditions including glutaric aciduria, type 1, 5 cases; medium-chain acyl CoA dehydrogenase deficiency, 2 cases; and cobalamin C deficiency, 1 case), and 154 false-positive cases identified by NBS.

2. Methods

2.1. Patients

CTD was defined by the presence of a NBS dried blood spot free carnitine level below the cutoff value established by the California Department of Public Health NBS laboratory, a confirmatory plasma total carnitine level below the lower limit of normal, and either two variants in *SLC22A5* or decreased fibroblast carnitine uptake (<10% of the value for age-matched controls). Individuals with two known pathogenic variants, one pathogenic variant and one variant of uncertain clinical significance, or two variants of uncertain clinical significance were classified as CTD cases. Confirmed newborn CTD cases were compared with cases of confirmed maternal conditions and false-positive cases (defined as absence of a confirmed or suspected disorder in both the newborn and mother). Cases in which a disorder in the newborn and/or mother was suspected by confirmatory biochemical testing, but for which no genetic or fibroblast carnitine uptake testing was done, were excluded from statistical analysis. Newborns flagged for low free carnitine and a concomitant increase in another marker (or multiple markers), and newborns receiving total parental nutrition were also excluded.

2.2. California Screening Information System

Data were collected using the Screening Information System of the California Department of Public Health Genetic Disease Screening Program, a web-based, secure, computerized data entry system [17]. Information on NBS dried blood spot free carnitine, birth weight, and gender was retrieved for all screened newborns. Available data on confirmatory biochemical and genetic testing, treatment, and clinical outcome were retrieved for cases closed as newborn CTD.

2.3. Medical chart review

Three metabolic centers in California provided detailed biochemical, molecular, and clinical data for all newborns with an initial positive NBS for CTD [Children's Hospital of Orange County (CHOC Children's), University of California, Los Angeles (UCLA), and Cedars-Sinai Medical Center]. Three additional metabolic centers in California (Harbor-UCLA, Stanford University, and University of California, San Francisco) participated by providing detailed biochemical, molecular, and clinical data only for confirmed cases of newborn CTD and maternal conditions. Ethnicity was ascertained by parent self-report on the newborn screening demographic sheet. Some cases of confirmed maternal conditions from the participating centers were published previously [9,10,12,18].

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

2.4. Biochemistry

NBS was performed by the California Department of Public Health Newborn Screening Program, using tandem mass spectrometry. In the vast majority of cases, a single NBS dried blood spot specimen was collected between 24 and 48 h of life. The methodology changed from a derivatized to an underivatized approach in 2009, and the free carnitine cutoff was lowered accordingly. In the vast majority of cases, confirmatory assays for plasma total and free carnitines, qualitative or quantitative urine organic acids, and plasma acylcarnitines were performed at Quest Diagnostics, Inc., Nichols Institute (San Juan Capistrano, CA), using standard methodology. Additional testing was performed at the following accredited laboratories (also using standard methodology): Duke Biochemical Genetics Laboratory (urine carnitines); Mayo Clinic Biochemical Genetics Laboratory (urine carnitines); Kaiser Metabolic Laboratory (plasma carnitines, quantitative urine organic acids, plasma acylcarnitines); CHOC Children's (plasma carnitines, dried blood spot acylcarnitines); and Associated Regional and University Pathologists, Inc. (ARUP) Laboratories (plasma carnitines). Fibroblast carnitine transport analysis was performed at ARUP Laboratories, using their proprietary methods.

In all cases, blood and/or urine specimens were collected prior to initiation of L-carnitine treatment.

2.5. DNA analysis

Molecular sequencing of *SLC22A5* was performed either at ARUP Laboratories or at Baylor Molecular Laboratory, using standard methodology. Novel missense variants were evaluated for the likelihood of being deleterious by use of two separate software programs, Polyphen-2 [19] and SIFT [20]. Effect on splicing was assessed by two programs, NN splice [21] and ESE finder [22]. Maximum allele frequencies were determined, were available, through ExAc [23] and 1000 Genomes Project [24] databases. In addition, the ARUP *SLC22A5* mutation database (http://www.arup.utah.edu/database/octn2/OCTN2_display.php, accessed 3/20/17) and the ClinVar database were searched for updated information on the variant (http://www.ncbi.nlm.nih.gov/clinvar/, accessed 3/20/17).

2.6. Statistical analysis

Means were compared using analysis of variance methods on the appropriate log scale. The post hoc p values under this model were computed using the Fisher criterion for pairwise comparisons. Geometric means and their standard errors are reported on the original scale.

Correlations between two continuous variables were assessed using the parametric Pearson correlation. The log scale was used to calculate Pearson correlations, determined by examining normal quantile plots and computing the Shapiro Wilk statistic to test for a normal distribution.

A multivariate classification tree analysis based on 20 different variables was carried out in attempt to identify a discriminant rule – using confirmatory biochemical testing – to distinguish among cases of confirmed newborn CTD, maternal conditions and false-positives. The variables used in the analysis were: NBS dried blood spot free carnitine; gender; newborn age (days at the time of plasma carnitine collection); maternal days post-delivery (at the time of plasma carnitine collection); plasma and urine carnitine levels (total, free, and esterified, for both newborns and mothers); and esterified:free carnitine ratios (in plasma and urine of both newborns and mothers). The accuracy and ROC area are reported.

All analyses were done using JMP® Statistical Discovery Software. Graphs were created in Excel®.

3. Results

3.1. Prevalence

A total of 3,608,768 newborns were screened in California during the study period (September 2005, through June 2012). There were 1030 screens positive for low free carnitine (roughly 1 in 3500; Fig. 1). Of these, 48 cases were closed as confirmed newborn CTD, giving a positive predictive value of 4.7%. However, the positive predictive value may be overestimated, because only 21 of the 48 cases closed as newborn CTD met our strict criteria for a diagnosis of CTD (based on information in the Screening Information System). For the remaining 27 cases, data were not available, or a diagnosis was based on plasma and/or urine carnitine levels alone, without confirmation by DNA or fibroblast carnitine transport studies. Therefore, the estimated birth prevalence of CTD in California may fall between 1 in 172,000 and 1 in 75,000 (based on either 21 or 48 cases of true CTD, respectively).

To supplement information available in the Screening Information System, detailed chart reviews were performed at participating metabolic centers for a total of 14 cases of newborn CTD, 14 cases of maternal conditions (maternal CTD, 6 cases; and other maternal conditions including glutaric aciduria, type 1, 5 cases; medium-chain acyl CoA dehydrogenase deficiency, 2 cases; and cobalamin C deficiency, 1 case), and 154 false-positive cases. Increased 3-methylglutaconic acid was identified in 12 out of 104 (11%) false-positive cases for which maternal urine organic acid analysis was performed. Additional evaluation was performed in some of these cases, but no underlying disorder was identified. (Supplementary Table 1).

3.2. Biochemical findings

Newborn and maternal plasma and urine carnitine levels were obtained prior to any treatment with L-carnitine (Fig. 2A–C). Newborn plasma total carnitine level was reduced to similar values in newborn CTD (mean 7 μmol/L, range 2–20) and maternal conditions (mean 7 μmol/L, range 2–17). A similar trend was observed for newborn plasma free carnitine. In contrast, newborn urine total carnitine was markedly increased in newborn CTD (mean 670 nmol/mg Cr, range 153–1046), significantly higher compared with maternal condition (mean 104 nmol/mg Cr, range 72–173, $p < 0.0001$) and false-positive cases (mean 185 nmol/mg Cr, range 65–878, $p < 0.0001$). The trend was the same for newborn urine free carnitine.

Biochemical findings differed in newborn CTD compared with maternal CTD, with a tendency for higher carnitine levels in both plasma and urine in newborn versus maternal CTD. For analysis of maternal biochemical findings, the maternal condition group was subdivided into two groups, maternal CTD and other maternal conditions (glutaric aciduria, type 1, medium-chain acyl CoA dehydrogenase deficiency, and cobalamin C deficiency) (Fig. 2D–F). Plasma total carnitine was extremely low in both maternal CTD (mean 6 μmol/L, range 3–9) and other maternal conditions (mean 3 μmol/L, range 2–15). Interestingly, plasma total carnitine was significantly lower in other maternal conditions than in maternal CTD ($p < 0.01$). Similar trends were observed for maternal plasma free carnitine. Urine total carnitine levels were similar most maternal groups, with the exception of a significantly increased level in mothers of newborns with CTD (presumed heterozygotes, mean 174 nmol/mg Cr, range 59–485) compared with the false-positive group (mean 108 nmol/mg Cr, range 33–288). Maternal urine free carnitine was significantly higher in maternal CTD (mean 73 nmol/mg Cr, range 29–104) compared with other maternal conditions (mean 3 nmol/mg Cr, range 1–7, $p < 0.0001$). Mothers with other conditions excreted primarily esterified carnitine, demonstrated by markedly elevated urine esterified to free carnitine ratios (mean 51, range 22–118), significantly higher than the urine esterified to free carnitine ratio in the maternal CTD (mean 0.8, range 0.4–2, $p < 0.0001$), false-positive ($p < 0.0001$) and newborn CTD groups ($p < 0.0001$).

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

Mothers with elevated urinary 3-methylglutaconic acid were excluded from statistical analysis. Biochemical findings were variable in this group. (Supplementary Table 1).

In order to assess whether NBS dried blood spot free carnitine can predict confirmatory plasma carnitine levels, dried blood spot free carnitine levels were compared with follow-up newborn and maternal plasma total and free carnitine levels (Fig. 3). As mentioned above in the Methods section, the assay for NBS dried blood spot free carnitine changed in 2009 from a derivatized to an underivatized procedure, and the cutoff was lowered accordingly. Therefore, for this comparison, NBS dried blood spot free carnitine values are expressed as a percentage of the respective cutoff value, to take into account the lower cutoff value that was applied. Positive, statistically significant correlations were found in the sample as a whole. However, when considering only newborn CTD cases, there was no correlation between NBS dried blood spot free carnitine and newborn or maternal carnitine levels (maternal biochemical data not shown). Importantly, in several newborn CTD cases, the NBS dried blood spot free carnitine level was near the lower cutoff value, but the confirmatory newborn plasma carnitine level was markedly reduced. All false-positive cases had an initial NBS dried blood spot free carnitine >65% of the lower cutoff value, whereas all cases with an initial NBS dried blood spot free carnitine <65% of the lower cutoff value were later classified as newborn CTD or a maternal condition.

To examine the effect of pregnancy on carnitine levels, newborn and maternal plasma carnitine levels were analyzed in relation to newborn age (in days) and maternal days-post-delivery, respectively (Fig. 4). In newborns in the false-positive group, positive correlations were found between age and plasma levels of total ($R = 0.44$, $p < 0.0001$) and free carnitine ($R = 0.5$, $p < 0.0001$). In contrast, no significant trend was identified in the newborn CTD or maternal condition groups. Plasma carnitine levels in newborn CTD and false-positive cases overlapped, particularly if the specimen was collected before 7 days of age. In mothers, maternal days-post-delivery was positively correlated with plasma carnitine levels in false-positive cases (for total carnitine, $R = 0.45$, $p < 0.0001$; for free carnitine, $R = 0.46$, $p < 0.0001$). A similar trend was observed in mothers of newborn CTD babies (presumed heterozygotes), but not for cases of maternal conditions. In false-positive cases, the mean newborn and maternal carnitine levels approached the lower limit of normal by 10–14 days after birth (data not shown). A subset of newborns (27 false-positive cases, 4 newborn CTD cases) underwent repeated plasma carnitine measurements without receiving L-carnitine treatment. Plasma carnitine levels increased over time in the false-positive cases. In contrast, plasma levels either remained essentially the same or decreased in newborn CTD cases, but results were not statistically significant due to the small sample (data not shown).

Multivariate classification tree analysis was performed to determine if NBS and/or confirmatory biochemical testing can distinguish among newborns in the newborn CTD, false-positive, and maternal condition groups (Fig. 5). The two most important predictors of outcomes for the newborn group were maternal plasma total carnitine and newborn urine total carnitine levels. Apart from these two levels, no additional variable, including the newborn plasma carnitine level, increased the chance of correctly predicting outcomes among newborns. This analysis correctly predicted 11 of 14 (79%) newborn CTD cases, 136 of 154 (88%) false-positive cases, and 13 of 14 (93%) maternal condition cases (overall accuracy, 87%).

3.3. Genotype and novel variants

Genotype, biochemical and fibroblast uptake analysis data for the 14 newborn CTD cases are in Table 1. Novel *SLC22A5* variants, characteristics, and in silico predictions are listed in Table 2.

4. Discussion

CTD is a potentially life-threatening but highly treatable disorder. Individuals who are identified clinically have profoundly low plasma carnitine levels, an easily recognized abnormality. CTD therefore appears to be an ideal disorder for NBS. However, NBS for CTD in California is associated with a high false-positive rate and a low positive predictive value (4.7%). The National Taiwan Newborn Screening Center reported a similar positive predictive value, with only 3 CTD cases confirmed out of 101 NBS specimens that were flagged positive for CTD (3%; 2007–09 data) [25].

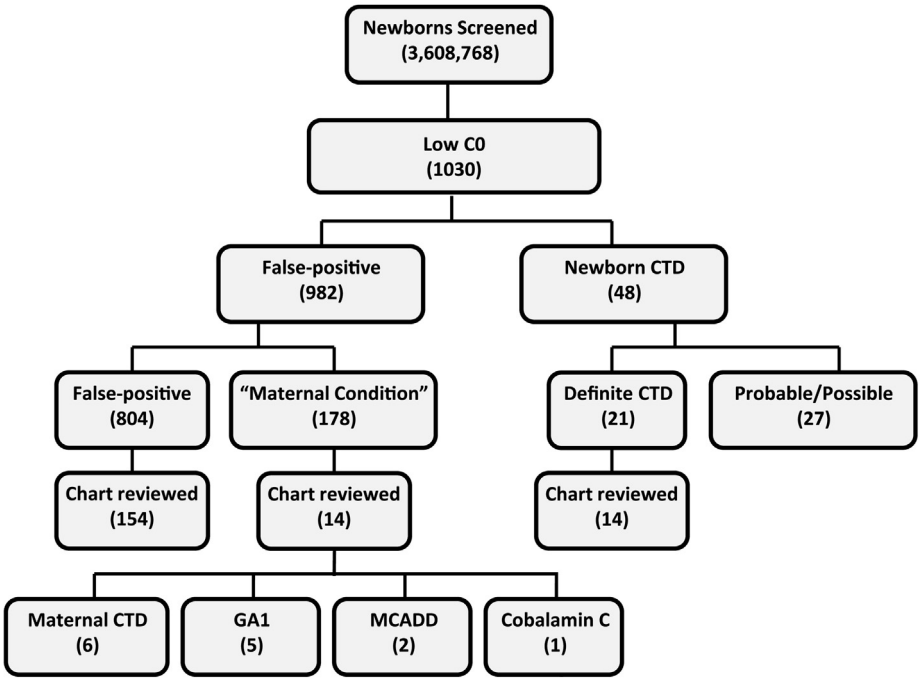


Fig. 1. Study design and birth prevalence of low free carnitine identified by NBS in California (September 2005–June 2012). C0 free carnitine, GA 1 glutaric aciduria, type 1, MCADD medium-chain acyl CoA dehydrogenase deficiency.

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

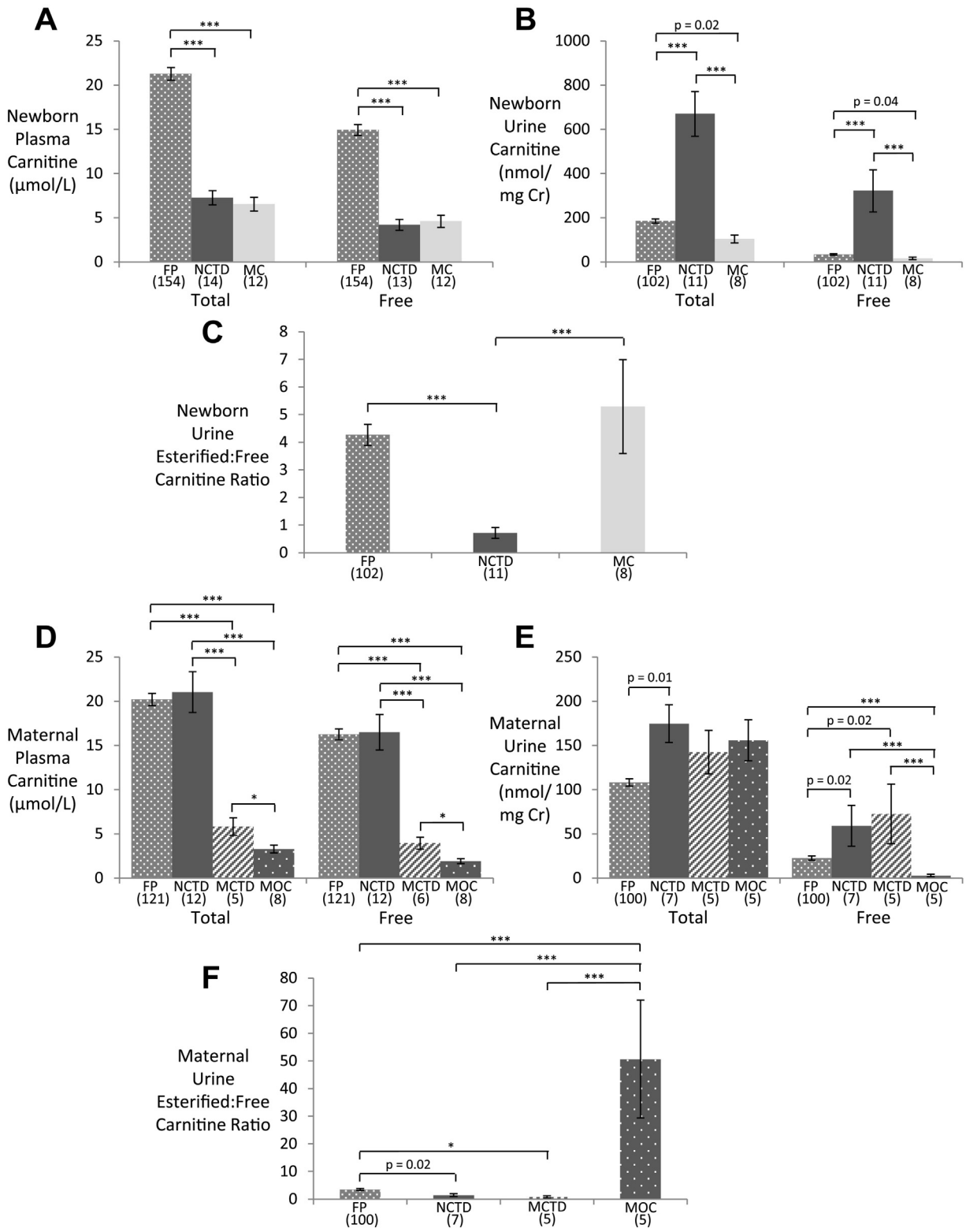


Fig. 2. Confirmatory plasma and urine total and free carnitine in newborns (A–C) and mothers (D–F). Geometric means are shown. Sample sizes are listed in parentheses below group names. Error bars represent standard error of the mean. Statistically significant differences are indicated by p-value or asterisks (*$p < 0.01$, ***$p < 0.0001$) above a bracket connecting two groups. For analysis of maternal biochemical results (D–F), the maternal condition group is subdivided into two groups, maternal CTD and maternal other conditions (glutaric aciduria, type 1, medium-chain acyl CoA dehydrogenase deficiency, and cobalamin C deficiency). FP false positive, NCTD newborn CTD, MC maternal condition, MCTD maternal CTD, MOC maternal other condition.

The high frequency of false-positive screens is not surprising, because newborn carnitine levels reflect maternal levels, which fall drastically during pregnancy [1,8]. Lowering the NBS free carnitine cutoff in order to reduce the number of false-positives could result in missed CTD cases, which is also undesirable. Some NBS programs obtain a repeat dried blood spot specimen at one week of age (or older), in order to improve performance [1]. In addition, post-analytical tools can be considered and were shown retrospectively to improve NBS

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

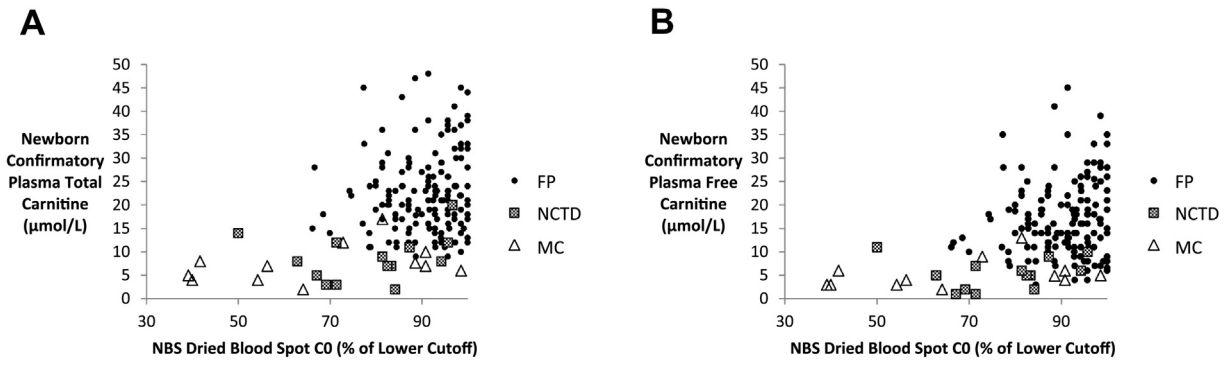


Fig. 3. NBS dried blood spot free carnitine versus newborn confirmatory plasma carnitine values. Free carnitine is expressed as a percentage of the lower control cutoff, to take into account the changes in methodology and the lower cutoff value that was applied halfway during the study period (see the Results section, under Biochemical findings, for more explanation). For the entire sample, a positive, statistically significant correlation was found for dried blood spot free carnitine versus total plasma carnitine ($R = 0.37$, $p < 0.0001$) and dried blood spot free carnitine versus free plasma carnitine ($R = 0.32$, $p < 0.0001$). However, no correlation was found within the newborn CTD or maternal condition groups alone. All cases with dried blood spot free carnitine < 65% of the lower cutoff value were eventually confirmed as newborn CTD or maternal condition. C0 free carnitine, FP false-positive, NCTD newborn CTD, MC maternal condition.

performance in general in California [16]. However, the utility of this approach in NBS for CTD has not been studied prospectively.

Algorithms for confirmatory workup of low free carnitine are available from the ACMG and the California Newborn Screening Program Guidelines Committee. These algorithms, vital to large public health programs like NBS, were based on the best evidence at the time, which consisted mainly of biochemical data on individuals with CTD (who were ascertained clinically). Evidence for the effectiveness of the ACMG algorithm in confirming CTD among newborns is limited [15]. The ACMG algorithm, which is intended to be used by any newborn screening program, does not specify a plasma carnitine level for prompting further workup, whereas the California algorithm uses plasma total carnitine values of <10 μmol/L as a threshold (Supplementary Fig. 1). Both algorithms indicate that urine carnitine should be measured, but neither specifies a level that would indicate a high probability for CTD.

Furthermore, the algorithms may be followed inconsistently by different metabolic centers and individual practitioners. For example, we found that some centers did not obtain urine carnitine levels in all follow-ups, and each participating center used different threshold values (of biochemical results) to decide on further testing. Our results show that 5 out of the 14 California CTD cases would have been misclassified as false-positives, if no further testing was done for newborns with confirmatory plasma total carnitine levels >10 μmol/L. Thus, clinical suspicion for CTD should remain in situations involving infantile-onset cardiomyopathy, weakness, peripheral neuropathy, or recurrent hypoglycemic hypoketotic encephalopathy. On the other hand, some cases were closed as CTD, without obtaining confirmatory molecular or

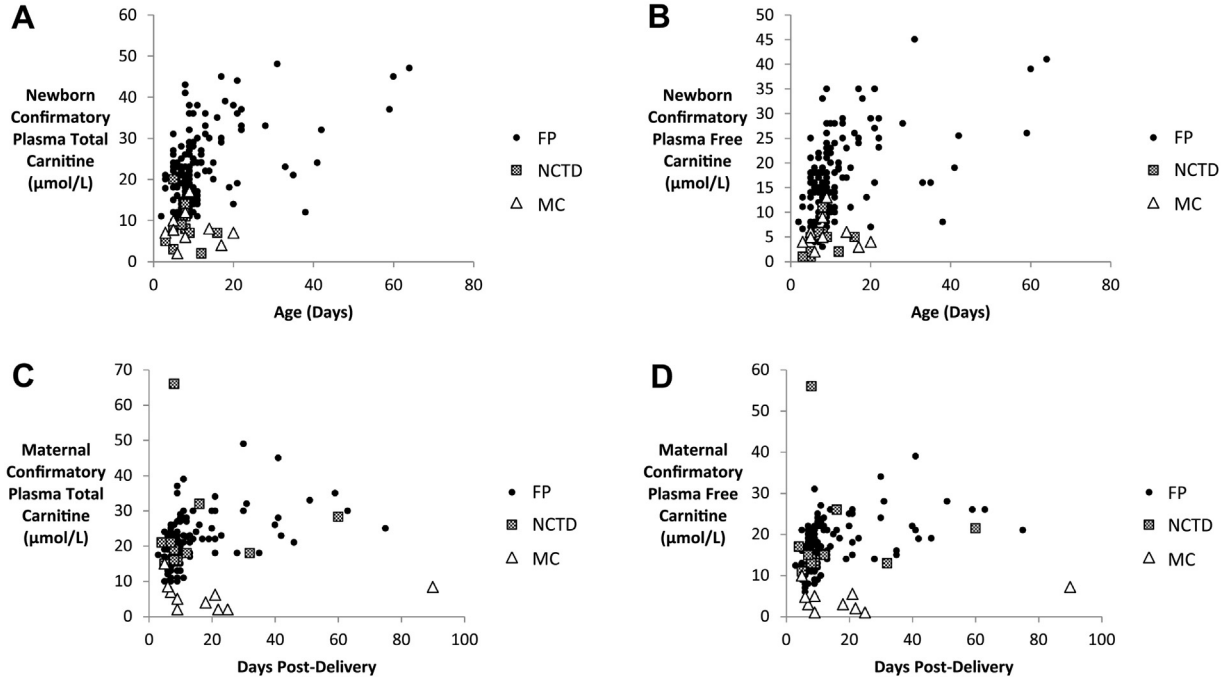


Fig. 4. Confirmatory plasma carnitine levels in relation to time of collection. Newborn (A–B) and maternal (C–D) plasma carnitine levels are plotted against newborn age (days) and maternal days-post-delivery, respectively. Among newborns in the false-positive group, a positive correlation was found between age and plasma total ($R = 0.44$, $p < 0.0001$) and free carnitine ($R = 0.5$, $p < 0.0001$) levels (A–B). In contrast, no significant trend was identified in the newborn CTD or maternal condition groups. Plasma carnitine levels for newborn CTD and false-positive cases overlapped, particularly if the specimen was collected before 7 days of age. Among mothers, maternal days-post-delivery was positively correlated with plasma carnitine levels in false-positive cases (for total carnitine, $R = 0.45$, $p < 0.0001$; for free carnitine, $R = 0.46$, $p < 0.0001$). A similar trend was observed in mothers of newborn CTD babies (presumed heterozygotes), but not in maternal condition cases (C–D). FP false-positive, NCTD newborn CTD, MC maternal condition.

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

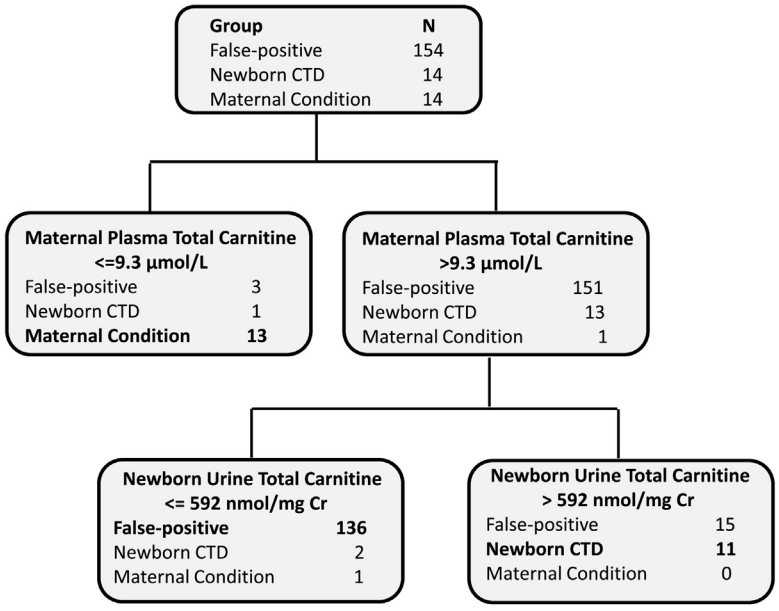
fibroblast uptake analysis. Thus, it is possible that some individuals initially suspected to have CTD (and treated with L-carnitine) based on confirmatory biochemical testing alone may not have had the disorder.

During the follow-up of mothers whose newborns had low NBS free carnitine values, we found a fairly high frequency of mothers with elevated 3-methylglutaconic acid in urine (11% of maternal urine organic acid analyses). A known disorder was not confirmed in any of these individuals, although further testing was not done in all cases (Supplementary Table 1). Increased urinary excretion of 3-methylglutaconic acid in pregnant women has been reported previously [26]. It is possible that the normal range of 3-methylglutaconic acid in urine may be different in adults, or affected by pregnancy. The possibility of an underlying disorder cannot be eliminated, particularly among individuals who had no further workup. However, if a disorder is present in these individuals, it is unlikely to be clinically significant.

Our results show that the biochemical characteristics of newborns with CTD identified by NBS differ from those of individuals ascertained clinically. In the newborn CTD group, confirmatory total plasma carnitine values frequently were >10 μmol/L, and in some cases approached the lower limit of normal. Conversely, false-positive cases showed markedly reduced plasma levels if the blood specimen was collected soon after birth, particularly within the first week of life. Plasma carnitine levels in newborn CTD diverged from the levels found in false-positive cases at 10 to 14 days of life (Fig. 4). Recognizing the usual changes in carnitine levels in normal newborns, performing newborn and maternal biochemical testing after the second week of life could be advantageous for diagnosis, especially when molecular testing is not possible.

Urine carnitine levels also differ in CTD ascertained clinically versus CTD identified through NBS. Clinically ascertained individuals may not have markedly elevated urinary carnitines, especially if the level of free carnitine in the plasma is <2 μmol/L (or below the renal threshold for excretion in CTD [2]). Indeed, in our study, urine carnitine levels were not highly elevated in maternal CTD as expected, because plasma carnitine levels were exceedingly low. Interestingly, mothers in the newborn CTD group, all presumed heterozygotes, had increased urine carnitine compared with mothers in the false-positive group, who likely had varying causes for low plasma carnitine including pregnancy, diet and/or heterozygous carrier for CTD. This finding is consistent with a previous study showing heterozygous carriers for CTD to have a two to three-fold increase in urinary carnitine losses compared with normal controls [27]. In contrast with maternal CTD, urinary carnitine levels were markedly elevated in 12 out of 14 newborns with CTD in our study, none of whom were on L-carnitine treatment at the time of

A



B

		True		
		False-positive	Newborn CTD	Maternal Condition
Predicted	False-positive	**136**	2	1
	Newborn CTD	15	**11**	0
	Maternal Condition	3	1	**13**
	Total	154	14	14
Percent correct		79%	88%	93%

Overall unweighted accuracy (average) 87%

Fig. 5. A multivariate classification tree analysis was performed in order to find a discriminant rule to predict outcome (newborn CTD, false-positive or maternal condition) among newborns with low free carnitine on NBS (A). 20 variables were considered including NBS dried blood spot free carnitine, gender, newborn age (days at the time of plasma carnitine collection), maternal days post-delivery (at the time of plasma carnitine collection), plasma and urine carnitine levels (total, free, and esterified, for both newborns and mothers), and esterified:free carnitine ratios (in plasma and urine of both newborns and mothers). The two most important predictors were, in order of importance, maternal plasma total carnitine and newborn urine total carnitine. Once these two variables were considered, no additional variable further improved the statistical model's ability to accurately predict outcome. Maternal plasma total carnitine less than or equal to 9.3 μmol/L distinguished most cases of maternal condition from the combination of newborn CTD and false-positives, for which maternal plasma total carnitine was typically >9.3 μmol/L. This latter group was further distinguished by newborn urine total carnitine, which was >592 nmol/mg Cr in most cases of newborn CTD and less than or equal to 592 nmol/mg Cr in most false-positive cases. The ability of this classification rule to predict the outcomes of our study cohort is summarized in panel B. The predicted outcomes are arranged as rows and true outcomes are arranged as columns. This discriminant rule correctly predicted 11 of 14 (79%) newborn CTD cases, 136 of 154 (88%) of false-positive cases, and 13 of 14 (93%) of maternal condition cases, for an overall (average) accuracy of 87%.

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

Table 1
Biochemical and genetic characteristics in 14 newborn CTD cases.

Subject	NBS C0	Confirmatory carnitine levels				Genotype			Fibroblast uptake
		NPTC	NPFC	NUTC	NUFC	Variant 1	Variant 2	Variant 3	Analysis Side effects
1	8.3[a]	3	2			c.505C>T (p.R169W)	c.760C>T (p.R254X)		−
2	10[a]	7	5	718	414	c.136C>T (p.P46S)	c.695C>T (p.T232 M)		−
3	11.6[a]	20	NA			c.1195C>T (p.R399W)	***c.761G>A (p.R254Q)***		+
4	9.9[a]	7	5						0.12 ± 0.01, Nl 1.66 ± 0.17 water/h) = 7.2% of control
5	10.1[a]	2	2	681	396	c.424G>T (p.A142S)[c]	c.1462G>A (p.R88H)[c]	c.1196G>A (p.R399Q)	−
6	6.2[b]	11	9	647	397	c.1336G>T (p.V446F)	***c.131C>T (p.A44V)***		+
7	3.5	14	11	1044	608	c.641C>T (p.A214V)	c.1354G>A (p.E452K)		−
8	4.4	8	5	771	405	c.760C>T (p.R254X)	c.51C>G (p.F17L)		−
9	4.7	5	1	602	47	c.43G>T (p.G15 W)	c.95A>G (p.N32S)		−
10	5	12	7	932	638				0.05 ± 0.02, Nl 1.06 ± 0.06 −
11	6.6	8	6	153	45	c.1195C>T (p.R399W)	c.1195C>T (p.R399W)		+
12	6.7	12	10	1047	1030	***c.447C>G (p.F149 L)***	***c.1159T>C (p.Y387H)***		−
13	5	3	1	700	354	c.760C>T (p.R254X)	c.825G>A (p.W275X)		−
14	5.7	9	6	758	484	c.760C>T (p.R254X)	c.51C>G (p.F17L)		−

C0 dried blood spot free carnitine (μmol/L), NPTC newborn plasma total carnitine (μmol/L), NPFC newborn plasma free carnitine (μmol/L), NUTC newborn urine total carnitine (nmol/mg creatinine), NUFC newborn urine free carnitine (nmol/mg creatinine), fibroblast uptake analysis units in (nmol/ml cell water/h), NA not available or not done.
C0 lower cutoff 7 μmol/L unless otherwise noted.
Known disease-causing variants in regular type, novel variants in bold and italic type.
[a] Lower cutoff 12 μmol/L.
[b] Lower cutoff 7.1 μmol/L.
[c] These two variants are pathogenic when in cis. They do not impair carnitine transport when expressed alone [11].

measurement. Plasma free carnitine was reduced, but not <2 μmol/L, in most cases of newborn CTD. Of the two newborns with CTD and normal urine carnitine excretion, one had a plasma free carnitine of 1 μmol/L, so high urine carnitines would not be expected. The renal threshold for carnitine excretion has been shown to be much higher among individuals with carnitine deficiency caused by other disorders, and even higher among healthy individuals [2]. Different thresholds for urinary carnitine excretion in relation to underlying conditions were also observed in our study (Fig. 2).

Multivariate classification tree analysis of biochemical variables did not identify a discriminant rule to distinguish all newborn CTD cases from the two other newborn groups (maternal condition, false-positive) (Fig. 5). Somewhat unexpectedly, maternal plasma total carnitine and newborn urine total carnitine levels were found to be the most important factors for distinguishing among the newborn groups. Moreover, after taking into account these two values, neither the total nor free carnitine level in newborn plasma improved differentiation among the three groups. These findings highlight the importance of obtaining maternal plasma carnitine and newborn urine carnitine levels as part of the confirmatory evaluation. Newborn urine carnitine levels were not checked in all patients or at all centers in our study, and maternal testing is often refused or not feasible. Our finding that biochemical testing alone cannot distinguish all cases of newborn CTD from other causes of low carnitine in newborns supports the recommendation that molecular genetic testing should be an integral part of the confirmatory workup, which has been suggested previously [28]. Unfortunately, molecular genetic testing was not feasible in many cases due to insurance or other barriers. The possibility of incorporating genome sequencing in NBS is being explored [29] and may be particularly beneficial in newborn CTD. However, given the limitations of molecular testing, including imperfect sensitivity and identification of novel variants and/or variants of uncertain clinical significance, biochemical testing is likely to remain an essential component in the diagnosis of newborn CTD.

Our study was limited by the lack of molecular and/or functional testing in the vast majority of false-positive cases, by the lack of determination of phase of *SLC22A5* variants in many newborn CTD cases, and by the inability to confirm pathogenicity of novel *SLC22A5* variants. These limitations, combined with the wide biochemical variability in newborn CTD demonstrated in this study, make it impossible to rule out cases of newborn CTD among cases closed as false-positive or vice versa. We attempted to ameliorate the impact of these limitations by applying strict molecular and/or functional criteria to our definition of newborn CTD and by including a large sample size of false-positive cases.

In this study, we use the term “carnitine transporter defect” to refer to the disease caused by bi-allelic pathogenic variants in *SLC22A5* even though the official OMIM name is “systemic primary carnitine deficiency”, and many alternative names are mentioned in the literature. We propose changing the official name to “carnitine transporter defect”, as this term concisely conveys the biological mechanism of this disease.

In summary, newborns with CTD identified by NBS exhibit different biochemical characteristics, compared with individuals ascertained clinically. Newborns with CTD may have NBS free carnitine levels near

Table 2
Novel variant in-silico analyses.

Subject	Exon	Nucleotide change	Protein change	SIFT	PolyPhen-2 HumVar	Max allele frequency	Splicing prediction	ARUP and/or ClinVar database	Ethnicity
3	4/11	c.761G>A	p.R254Q	0.06 (Tolerated)	Probably damaging (score 0.982)	12/66736 (0.0180%) European (non-Finnish)	No significant effect	ARUP: VUS ClinVar: VUS	White
6	1/11	c.131C>T	p.A44V	0.098	Benign (score 0.271)	1/9724 (0.0103%) Latino	No significant effect	ARUP: VUS ClinVar: no annotation	Mexican, English, German
12	2/11	c.447C>G	p.F149L	0.102	Possibly damaging (score 0.534)	1/66740 (0.0015%) European (non-Finnish)	No significant effect	No annotation	Middle Eastern
12	7/11	c.1159T>C	p.Y387H	0.051	Possibly damaging (score 0.88)	No annotations	No significant effect	No annotation	Middle Eastern

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015

the lower cutoff and confirmatory plasma carnitine levels near the lower normal limit. These findings raise the concern that true cases of CTD may exist that could have been missed by NBS. CTD should be considered as a possible diagnosis in cases with suggestive clinical features, even if NBS for CTD was negative. Moreover, obtaining confirmatory biochemical testing solely within one to two weeks after birth is not ideal due to the overlap of plasma carnitine levels in newborn CTD and false-positive cases during this time frame. Maternal plasma total carnitine and newborn urine total carnitine were shown to be the most important predictors of newborn CTD. However, biochemical testing alone did not yield a discriminant rule to distinguish newborn CTD from maternal conditions and false-positive cases. Because of this biochemical variability and overlap, molecular genetic testing is imperative to distinguish newborn CTD from low carnitine in newborns due to other causes. Additionally, functional testing of fibroblast carnitine uptake remains necessary for cases in which other confirmatory testing is inconclusive. Even with utilization of all available diagnostic testing methods, confirmation of CTD in newborns ascertained by NBS remains a lengthy and arduous process. Inclusion of molecular analysis as a second tier step in NBS for CTD should be investigated, as this may simplify the confirmatory process and reduce the time interval from positive screen to case closure.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2017.06.015.

Acknowledgements

The authors wish to thank Erica Chan and Naghmeh Dorrani at UCLA, and Kara Weisiger at University of California, San Francisco, for assistance with chart review; Jeffrey Gornbein and Daniela Marcovik at UCLA for statistical design and analysis support; and Elizabeth Chao at University of California, Irvine for expertise in characterization of novel genetic variants. Research reported in this publication was supported by the American Academy of Pediatrics under a Resident Research Grant, and the National Institute of General Medical Sciences of the National Institutes of Health under award number 5T32GM008243-27. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

[1] N. Longo, C. Amat di San Filippo, M. Pasquali, Disorders of carnitine transport and the carnitine cycle, Am. J. Med. Genet. C: Semin. Med. Genet. 142C (2006) 77–85.
[2] C.A. Stanley, G.T. Berry, M.J. Bennett, S.M. Willi, W.R. Treem, D.E. Hale, Renal handling of carnitine in secondary carnitine deficiency disorders, Pediatr. Res. 34 (1993) 89–97.
[3] B.O. Eriksson, B. Gustafson, S. Lindstedt, I. Nordin, Transport of carnitine into cells in hereditary carnitine deficiency, J. Inherit. Metab. Dis. 12 (1989) 108–111.
[4] I. Tein, D.C. De Vivo, F. Bierman, P. Pulver, L.J. De Meirleir, L. Cvitanovic-Sojat, R.A. Pagon, E. Bertini, C. Dionisi-Vici, S. Servidei, et al., Impaired skin fibroblast carnitine uptake in primary systemic carnitine deficiency manifested by childhood carnitine-responsive cardiomyopathy, Pediatr. Res. 28 (1990) 247–255.
[5] J. Nezu, I. Tamai, A. Oku, R. Ohashi, H. Yabuuchi, N. Hashimoto, H. Nikaido, Y. Sai, A. Koizumi, Y. Shoji, G. Takada, T. Matsuishi, M. Yoshino, H. Kato, T. Ohura, G. Tsujimoto, J. Hayakawa, M. Shimane, A. Tsuji, Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter, Nat. Genet. 21 (1999) 91–94.
[6] Y. Wang, J. Ye, V. Ganapathy, N. Longo, Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 2356–2360.
[7] S.D. Cederbaum, S. Koo-McCoy, I. Tein, B.Y. Hsu, A. Ganguly, E. Vilain, K. Dipple, L. Cvitanovic-Sojat, C. Stanley, Erratum: carnitine membrane transporter deficiency: a long-term follow up and OCTN2 mutation in the first documented case of primary carnitine deficiency, Mol. Genet. Metab. 78 (2003) 82.
[8] S.C. Winter, L.S. Linn, E. Helton, Plasma carnitine concentrations in pregnancy, cord blood, and neonates and children, Clin. Chim. Acta 243 (1995) 87–93.
[9] E.A. Crombez, S.D. Cederbaum, E. Spector, E. Chan, D. Salazar, J. Neidich, S. Goodman, Maternal glutaric acidemia, type I identified by newborn screening, Mol. Genet. Metab. 94 (2008) 132–134.
[10] L.A. Schimmenti, E.A. Crombez, B.C. Schwahn, B.A. Heese, T.C. Wood, R.J. Schroer, K. Bentler, S. Cederbaum, K. Sarafoglou, M. McCann, P. Rinaldo, D. Matern, C.A. di San Filippo, M. Pasquali, S.A. Berry, N. Longo, Expanded newborn screening identifies maternal primary carnitine deficiency, Mol. Genet. Metab. 90 (2007) 441–445.
[11] A.W. El-Hattab, F.Y. Li, J. Shen, B.R. Powell, E.V. Bawle, D.J. Adams, E. Wahl, J.A. Kobori, B. Graham, F. Scaglia, L.J. Wong, Maternal systemic primary carnitine deficiency uncovered by newborn screening: clinical, biochemical, and molecular aspects, Genet. Med. 12 (2010) 19–24.
[12] H.J. Lin, J.A. Neidich, D. Salazar, E. Thomas-Johnson, B.F. Ferreira, A.M. Kwong, A.M. Lin, A.J. Jonas, S. Levine, F. Lorey, D.S. Rosenblatt, Asymptomatic maternal combined homocystinuria and methylmalonic aciduria (cblC) detected through low carnitine levels on newborn screening, J. Pediatr. 155 (2009) 924–927.
[13] Timmermans, M. Buchbinder, Patients-in-waiting: living between sickness and health in the genomics era, J. Health Soc. Behav. 51 (2010) 408–423.
[14] https://www.acmg.net/StaticContent/ACT/Algorithms/Visio-C0.pdf.
[15] D. Dietzen, P. Rinaldo, R.J. Whitley, W.J. Rhead, W.H. Hannon, U. Garg, S.F. Lo, M.J. Bennett, National Academy of Clinical Biochemistry. Laboratory medicine practice guidelines: follow-up testing for metabolic diseases identified by expanded newborn screening using tandem mass spectrometry; executive summary, Clin. Chem. 55 (2009) 1615–1626.
[16] P.L. Hall, G. Marquardt, D.M. McHugh, R.J. Currier, H. Tang, S.D. Stoway, et al., Postanalytical tools improve performance of newborn screening by tandem mass spectrometry, Genet. Med. 16 (2014) 889–895.
[17] L. Feuchtbaum, S. Dowray, F. Lorey, The context and approach for the California newborn screening short- and long-term follow-up data system: preliminary findings, Genet. Med. 12 (2010) S242–S250.
[18] K.B. Leydiker, J.A. Neidich, F. Lorey, E.M. Barr, R.L. Puckett, R.M. Lobo, J.E. Abdenur, Maternal medium-chain acyl-CoA dehydrogenase deficiency identified by newborn screening, Mol. Genet. Metab. 103 (2011) 92–95.
[19] D.M. Jordan, A. Kiezun, S.M. Baxter, V. Agarwala, R.C. Green, M.F. Murray, et al., Development and validation of a computational method for assessment of missense variants in hypertrophic cardiomyopathy, Am. J. Hum. Genet. 88 (2011) 183–192.
[20] P. Kumar, S. Henikoff, P.C. Ng, Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm, Nat. Protoc. 4 (2009) 1073–1081.
[21] M.G. Reese, F.H. Eeckman, D. Kulp, D. Haussler, Improved splice site detection in Genie, J. Comput. Biol. 4 (1997) 311–323.
[22] P.J. Smith, C. Zhang, J. Wang, S.L. Chew, M.Q. Zhang, A.R. Krainer, An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers, Hum. Mol. Genet. 15 (2006) 2490–2508.
[23] K.J. Karczewski, B. Weisburd, B. Thomas, M. Solomonson, D.M. Ruderfer, D. Kavanagh, et al., The ExAC browser: displaying reference data information from over 60 000 exomes, Nucleic Acids Res. 45 (2017) D840–D845.
[24] A. Auton, L.D. Brooks, R.M. Durbin, E.P. Garrison, H.M. Kang, J.O. Korbel, et al., A global reference for human genetic variation, Nature 526 (2015) 68–74.
[25] N.C. Lee, N.L. Tang, Y.H. Chien, C.A. Chen, S.J. Lin, P.C. Chiu, et al., Diagnoses of newborns and mothers with carnitine uptake defects through newborn screening, Mol. Genet. Metab. 100 (2010) 46–50.
[26] R. Walsh, H. Conway, G. Roche, E. Naughten, P.D. Mayne, 3-Methylglutaconic aciduria in pregnancy, Lancet (Lond. Engl.) 349 (1997) 776.
[27] F. Scaglia, Y. Wang, R.H. Singh, P.P. Dembure, M. Pasquali, P.M. Fernhoff, N. Longo, Defective urinary carnitine transport in heterozygotes for primary carnitine deficiency, Genet. Med. 1 (1998) 34–39.
[28] T.J. Urban, R.C. Gallagher, C. Brown, R.A. Castro, L.L. Lagpacan, C.M. Brett, et al., Functional genetic diversity in the high-affinity carnitine transporter OCTN2 (*SLC22A5*), Mol. Pharmacol. 70 (2006) 1602–1611.
[29] J.S. Berg, P.B. Agrawal, D.B. Bailey Jr., A.H. Beggs, S.E. Brenner, A.M. Brower, et al., Newborn sequencing in genomic medicine and public health, Pediatrics 139 (2017).

Please cite this article as: N.M. Gallant, et al., Biochemical characteristics of newborns with carnitine transporter defect identified by newborn screening in California, Mol. Genet. Metab. (2017), http://dx.doi.org/10.1016/j.ymgme.2017.06.015