NEWBORN SCREENING FOR INBORN ERRORS OF METABOLISM

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NEWBORN SCREENING FOR INBORN ERRORS OF METABOLISM

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EDITORIAL
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Editorial: Newborn Screening for Inborn Errors of Metabolism: Is It Time for a Globalized Perspective Based on Genetic Screening?

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Keywords: newborn screening (NBS), inborn errors of metabolism (IEMs), next generation sequencing - NGS, tandem mass spectrometry, pathogenic variants, dried blood spots, clinical outcome

Editorial on the Research Topic

Newborn Screening for Inborn Errors of Metabolism

Inborn errors of metabolism (IEMs) are a large group of debilitating hereditary disorders, commonly manifesting during infancy and early childhood (Mak et al., 2013). They are categorized mainly according to the chemical nature of the characteristic metabolites accumulating in each disease. Major categories include aminoacidopathies, organic acid disorders, lysosomal storage disorders, fatty acid oxidation defects, and many others. Collectively, they constitute over 1,000 individual genetic disorders resulting in substantial societal and financial burdens overloading families, communities, and health care authorities worldwide (Péntek et al., 2016; Zeltner et al., 2017). These burdens are only expected to go higher under the current pandemic situation necessitating better planning for a better care of such disorders in the future (Elmonem et al., 2020).

Historically, the concept of newborn screening (NBS) was first applied to the amino acid metabolic disorder phenylketonuria using a simple bacterial inhibition assay to detect high phenylalanine concentrations in dried blood spots almost 60 years ago (Guthrie and Susi, 1963). Since then, the technologies used, and the metabolites discovered for the newborn screening of various IEMs have significantly advanced. Furthermore, with the recent discovery of novel therapeutic modalities for many inborn errors of metabolism, such as enzyme replacement therapies and substrate reduction therapies, the importance of newborn screening for these disorders is gaining more momentum. Especially that for most inborn errors, the earlier the specific therapy starts, the better the prognosis will be (Selim et al., 2014). Currently, hundreds of regional and national health care authorities all over the world have running programmes for the newborn screening of IEMs, which usually vary widely depending on the disease spectrum and financial limitations of each country.

Similar to the beginnings of NBS, metabolite-based screening is still the dominant form in all running programmes worldwide for the detection of various IEMs today. Almost two decades ago, tandem mass spectrometry (TMS) based techniques have revolutionized the metabolite screening for IEM allowing the multiplex screening of various metabolites and various diseases in the same run using the same sample with the added benefits of cost-effectiveness, high throughput, and low false positive and false negative rates (Pollitt et al., 1997; Rashed et al., 1997). This allowed the widespread use of TMS in the newborn screening for IEMs even in many developing countries in spite of their financial limitations (Hassan et al., 2016; Borrajo, 2021). However, the numbers

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of disorders that can be evaluated simultaneously by the same mass spectrometric methodology are limited. Furthermore, the sensitivity and specificity of the multiplex method toward various metabolites and the stability of these metabolites are not equal favoring the diagnostic outcomes of certain diseases over others.

Next generation sequencing in the form of whole exome and whole genome analysis is now being proposed strongly as a potential alternative for mass spectrometric newborn screening for IEMs (Narravula et al., 2017; Adhikari et al., 2020; Wojcik et al., 2021). These techniques have the advantages of high throughput, high accuracy and potentially being able to detect all types of genetic disorders even beyond the scope of inborn errors of metabolism with almost identical sensitivity and specificity. The transition to genetic diagnosis in newborn screening requires major logistical and ethical hurdles to be overcome. However, the major limiting factors include data interpretation dilemmas, which will be required to handle variants of uncertain significance in each individual, and the current relatively high cost of such techniques. Interestingly, with gaining experience through an actual newborn screening program, it is expected that variants of uncertain significance will be mostly reclassified into either benign/likely benign or pathogenic/likely pathogenic in the span of a few years. Furthermore, the running costs of genetic diagnostics are getting lower and lower by the day giving hope to the application of a universal screening technology for all hereditary disorders and leaving the role of metabolite analysis and biochemical assays for the second-tier and confirmatory tests. It seems like a second revolution in newborn screening for the detection of inborn errors of metabolism is approaching.

This Research Topic collection includes 13 articles from several countries applying newborn screening and genetic diagnosis for the detection of IEMs. Wang Y. et al. have discussed an important but commonly overlooked cause of sample contamination in newborn screening by TMS, which is the injection syringe. They summarized their experience in the Nanjing NBS programme in eastern China and the steps they performed to reach the actual cause of contamination. They further described how to treat and guard against the contamination recurrence in the future. Similarly, Peng et al. have provided valuable data about the important role the timing of sampling could play in impacting screening performances of mass spectrometric NBS programmes. They compared three sampling times: early (12–23 h), standard (24–48 h), and late (49–168 h) in a large population-based newborn cohort from California, USA. They found a significantly higher false positive rate for phenylketonuria testing when age at blood collection was between 12 and 23 h. Other analytes were also impacted in the early and late sampling groups.

Several studies included in this article collection have summarized the outcomes of different running NBS programmes in different countries. Maguolo et al. described their experience in the diagnosis, management and follow up of biotinidase deficiency patients showing key strategies and unsolved questions of the management of their patients in Verona, Italy. The study concluded that NBS introduction for biotinidase deficiency in Verona had a dramatic impact on its diagnosis and the incidence has increased significantly compared to other areas in Italy. Remec, Urh Groselj et al. described patients with very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) diagnosed mainly through a recent expanded NBS programme by TMS that started in Slovenia in 2018. The study further investigated the genetic background of all screen positive newborns and reported four novel pathogenic variants in the *ACADVL* gene. Wang X. et al. reported on the diagnostic outcomes of their NBS programme of congenital adrenal hyperplasia in Nanjing, China over the period 2000–2019. During this period, over one million newborns were screened, and 62 patients were diagnosed with 21-hydroxylase deficiency with an incidence of 1/19,858 live birth. The study further reported 18 different pathogenic variants in the *CYP21A2* gene and commented on their genotype-phenotype correlations. A broader scope of the NBS programme for all detectable IEMs by TMS in Liuzhou, Southern China was reported by Tan et al. Hotspot mutations in *PAH* gene (Phenylketonuria), *IVD* gene (Isovaleric academia), *ACADS* gene (short chain Acyl-CoA dehydrogenase deficiency), *SLC22A5* gene (Creatine deficiency) and *GCDH* gene (Glutaric aciduria type-I) were reported.

Stinton et al. conducted a systematic review of test accuracy of acylcarnitines measurement in dried blood spots by TMS for the newborn screening of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD) and mitochondrial trifunctional protein deficiency (MTP). They showed that the positive predictive value of the test in the literature varied considerably, while sensitivity, specificity, and negative predictive value could not be calculated as the negatively screened newborns were not followed-up. Koracin et al. further evaluated the status of newborn screening for IEMs in South-Eastern-Europe through conducting detailed surveys about the services provided for the screening of IEMs in each of the 12 participating countries. Two consecutive surveys were conducted; the first was in 2013/2014, while the second was in 2020. The study concluded that the current status of NBS programs in South-Eastern-Europe is very variable and is still underdeveloped.

Pure genetic studies reporting novel phenotypes and novel genetic variants in families complaining of D-Bifunctional protein deficiency and sulfite oxidase deficiency were reported by Chen et al. and Zhao et al., respectively. Zeng et al. further evaluated the screening of *SLC25A13* genetic variants for the detection of citrin deficiency. In their study, a real-time PCR-based multicolor melting curve analysis was developed to detect the four most prevalent mutations in *SLC25A13* in the Chinese Han population in one closed-tube reaction. The melting curve analysis identified previously diagnosed patients accurately and determined the carrier frequency of the four common pathogenic variants in *SLC25A13* in 5,332 healthy newborns, which was surprisingly high.

Finally, the promise of replacing the metabolite-based newborn screening approach with the genetic-based approach has been extensively discussed in two reviews: Remec, Trebusak Podkrajsek et al. and Woerner et al. The first review focused on the technical feasibility, economic considerations, and clinical and ethical issues of the sequencing-based techniques, while the second review focused on the comparison between historical and current biochemical screening methods and current and future

genetic methods of newborn screening, as well as the practical aspects and challenges for the use of genomic testing for NBS.

In conclusion, there is still a huge gap in most countries between the current applied technologies for the newborn screening of IEMs and the ideal situation, which gives every potentially diseased newborn the right to be diagnosed as early and as accurately as possible. We hope that with the application of the genetic NBS methods, we will be able to address the identified gap in the near future.

AUTHOR CONTRIBUTIONS

MAE drafted the manuscript that was reviewed and edited by LvH. Both authors co-edited the Research Topic.

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BRIEF RESEARCH REPORT
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Timing of Newborn Blood Collection Alters Metabolic Disease Screening Performance

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Blood collection for newborn genetic disease screening is preferably performed within 24–48 h after birth. We used population-level newborn screening (NBS) data to study early postnatal metabolic changes and whether timing of blood collection could impact screening performance. Newborns were grouped based on their reported age at blood collection (AaBC) into early (12–23 h), standard (24–48 h), and late (49–168 h) collection groups. Metabolic marker levels were compared between the groups using effect size analysis, which controlled for group size differences and influence from the clinical variables of birth weight and gestational age. Metabolite level differences identified between groups were correlated to NBS data from false-positive cases for inborn metabolic disorders including carnitine transport defect (CTD), isovaleric acidemia (IVA), methylmalonic acidemia (MMA), and phenylketonuria (PKU). Our results showed that 56% of the metabolites had AaBC-related differences, which included metabolites with either decreasing or increasing levels after birth. Compared to the standard group, the early-collection group had elevated marker levels for PKU (phenylalanine, Cohen's $d = 0.55$), IVA (C5, Cohen's $d = 0.24$), MMA (C3, Cohen's $d = 0.23$), and CTD (C0, Cohen's $d = 0.23$). These findings correlated with higher false-positive rates for PKU ($P < 0.05$), IVA ($P < 0.05$), and MMA ($P < 0.001$), and lower false-positive rate for CTD ($P < 0.001$) in the early-collection group. Blood collection before 24 h could affect screening performance for some metabolic disorders. We have developed web-based tools integrating AaBC and other variables for interpretive analysis of screening data.

Keywords: newborn screening, inborn errors of metabolism, age at blood collection, gestational age, sex, race and ethnicity

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INTRODUCTION

The timing of blood sampling and postnatal age are important parameters for accurately interpreting test results for newborn screening. The Clinical and Laboratory Standards Institute (CLSI) recommends blood spot collection on filter paper for genetic disease screening between 24 and 48 h of age (1). NBS programs have implemented different cutoff values for some metabolic disorders detectable by tandem mass spectrometry (MS/MS) depending on the infant's age (in hours) at blood collection (AaBC). Blood spots drawn too early may impair the detection of some metabolic disorders due to the infant's biochemical transition

from a mother-dependent to an autonomous state, while collection after 48 h of age could delay diagnosis and initiation of treatment for some infants (2, 3). Under some circumstances such as birth stress, prematurity, low birth weight or infant disease, blood sampling could be delayed. Several studies examining the association between AaBC and MS/MS-based screening have focused on a single or a few metabolic analytes or groups of metabolic disorders (4–9). In addition to AaBC, metabolic changes have also been associated with other confounding clinical variables such as gestational age (GA), birth weight (BW), sex, season of birth and race/ethnicity status reported by the parents (10–17).

In this study, we used population-level data reported by the California NBS program to study early postnatal metabolic changes and whether AaBC could impact screening performance for inborn metabolic disorders on the Recommended Universal Screening Panel (RUSP) (18). Since both GA and BW are known to influence metabolic marker levels (13, 14, 16), we controlled for the influence of these covariates in the analysis of metabolite levels between AaBC groups. We also studied the influence of race/ethnicity status and total parenteral nutrition (TPN) on metabolic analyte levels across different AaBC timepoints ranging from 12 to 168 h after birth. Finally, the influence of the AaBC on false-positive newborn screens was investigated. The identified AaBC-related differences in metabolite levels were correlated to false-positive cases for eleven inborn metabolic disorders. Based on these findings, web-based tools were developed to aid the interpretation of NBS data in relation to AaBC (http://rusptools.shinyapps.io/AaBC/), and to support development of algorithms that incorporate information on a variety of clinical variables in genetic disease screening.

MATERIALS AND METHODS

Data Summary

NBS data from 503,935 screen-negative singleton babies born between 2013-2017 were analyzed. The cohort was selected at random by the California NBS program. The data included 41 metabolic analytes measured by MS/MS (19) and six clinical variables of birth weight (BW), gestational age (GA), sex, race/ethnicity, total parenteral nutrition (TPN), and age at blood collection (AaBC). Infants with unknown AaBC or blood collection before 12 or after 168 h were removed from the analysis as were infants with BW smaller than 1,000 g or larger than 5,000 g, or with GA smaller than 28 weeks or larger than 42 weeks, which resulted in 500,539 newborns remaining for downstream analysis (**Supplementary Table 1**). In addition, we analyzed data from screen-positive newborns for eleven inborn metabolic disorders reported by the California NBS program. This cohort consisted of confirmed true-positive cases and of first-tier false-positive cases for argininosuccinic aciduria (ASA), citrullinemia type 1 (CIT-I), citrullinemia type 2 (CIT-II), carnitine transporter deficiency (CTD), homocystinuria (HCY), isovaleric acidemia (IVA), methylmalonic acidemia (MMA), propionic acidemia (PA), phenylketonuria (PKU), ornithine transcarbamylase deficiency (OTCD), and very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) (**Table 1**). All screen-positive newborns were collected between 2013 to 2017 except for MMA, OTCD, and VLCADD collected between 2005 to 2015. This study was overseen by the institutional review boards at Yale University (protocol #1505015917), Stanford University (protocol #30618), and the State of California Committee for the Protection of Human Subjects (protocol #13-05-1236).

Analysis of AaBC

To reduce the influence from the covariates of GA and BW on metabolite levels (13, 14, 16), our AaBC analysis included 414,869 screen-negative term infants [37–41 weeks] with BW range of 2,500 g to 4,000 g. Infants with positive or unknown TPN status were removed from analysis (21). We first investigated metabolite changes across different AaBC timepoints between 12 to 72 h after birth ($n = 410{,}918$). Infants were divided into nine AaBC groups of 6 h collection windows except for the last group (66–72 h). AaBC data after 72 h were excluded from analysis due to small sample size. Metabolite levels of 41 MS/MS metabolites in the 12–17 h AaBC group were used as the standard. This choice was made to explore the gradual changes in metabolite levels shortly after birth. We performed effect size analysis using Cohen's d (22) to calculate marker level differences for each of the nine remaining AaBC groups in comparison to the 12–17 h standard group. Cohen's *d* values calculated for each AaBC group were recorded in a data matrix and hierarchical clustering was used to compare AaBC-related profiles between the metabolites (**Figure 1**).

Analysis of AaBC in Relation to Other Variables

Two metabolites with decreasing (phenylalanine and free carnitine, C0) and two metabolites with increasing (leucine-isoleucine and C18:1) levels between 18 to 72 h after birth were selected to study the influence of clinical variables on metabolite levels (**Figure 2**). These four primary NBS markers were among the metabolites found with the largest changes related to AaBC. Firstly, for GA, the changes in metabolite levels related to AaBC were compared between preterm (28–36 weeks) and term (37–41 weeks) newborns using a generalized additive model (23). Because BW and GA are highly correlated and GA is a stronger predictive covariate compared to BW (24), we did not control for birth weight in this analysis. Infants with positive or unknown TPN status were removed from analysis. We did not study post-term (>41 weeks) infants due to small samples size ($n = 159$) amongst infants with AaBC between 49 to 168 h (**Supplementary Table 2**). Secondly, for sex, the changes in metabolite levels related to AaBC were compared between female and male newborns using a generalized additive model (23). In this analysis, we only included infants born at term (37–41 weeks) and with BW between 2,500 g and 4,000 g, while infants with positive or unknown TPN status were removed. Thirdly, for race/ethnicity, the changes in metabolite levels related to AaBC were compared between four major race/ethnicity groups (Asian, Black, Hispanic, and White) using the same data and methods as described above. The race/ethnicity status of the newborn

TABLE 1 | Correlation of metabolite levels between screen-negatives and false-positives at early, standard and late AaBC.

		Effect Size^		Number of False Positives (%)		
Disorder	**Marker (20)**	**Early**	**Late**	**Early**	**Standard**	**Late**
CTD	C0↓	**0.23**$^{+}$	−0.015	44 (9.3%) E: 102***	381 (80.9%) E: 347	46 (9.8%) E: 22***
PA	C3↑	**0.23**$^{\#}$	−0.11	29 (29.6%) E: 21	65 (66.3%) E: 72	4 (4.1%) E: 5
MMA	C3↑	**0.23**$^{+}$	−0.11	66 (34.7%) E: 41***	97 (51.1%) E: 140	27 (14.2%) E: 9***
IVA	C5↑	**0.24**$^{+}$	**0.31**$^{\&}$	12 (41.4%) E: 6*	16 (55.2%) E: 21	1 (3.5%) E: 1
VLCADD	C14:1↑	−0.04	**−0.26**$^{\&}$	20 (15.2%) E: 29	97 (73.5%) E: 97	15 (11.4%) E: 6**
CITR$^{\$}$	Citrulline↑	0.06	**−0.28**$^{\&}$	19 (19.6%) E: 21	68 (70.1%) E: 71	10 (10.3%) E: 4*
OTCD	Citrulline↓	0.06	**−0.28**$^{+}$	27 (11.0%) E: 53	129 (52.7%) E: 181	89 (36.3%) E: 11***
HCY	Methionine↑	**0.50**$^{\#}$	**−0.34**$^{\#}$	3 (25.0%) E: 3	9 (75.0%) E: 9	0 (0.0%) E: 1
PKU	Phenylalanine↑	**0.55**$^{+}$	**−0.41**$^{+}$	51 (31.3%) E: 35*	112 (68.7%) E: 120	0 (0.0%) E: 7*

$^{\$}$*CITR includes three disorders (ASA, CIT-I, and CIT-II) detected through elevated citrulline levels.*

**P < 0.05; **P < 0.01; ***P < 0.001. The percentage difference between false positive and screen negative infants in early and late collection groups was compared using Chi-squared test. There are 90,060 (21.7%), 305,674 (73.7%), and 19,135 (4.6%) screen negative newborns in the early, standard and late collection groups, respectively. E indicates expected number of false positives.*

$^{\#}$*Sample size of false positive cases is relatively small.*

$^{+}$*Consistent and* $^{\&}$*Inconsistent between Cohen's d and number of false-positives. See for definition in* ***Supplementary Table 3****.*

^*Effect size analysis using Cohen's d for marker level differences between early (12–23 h) and late (49–168 h) collection-groups compared to the standard group (24–48 h). Cohen's d with absolute value larger than 0.2 in* ***bold****.*

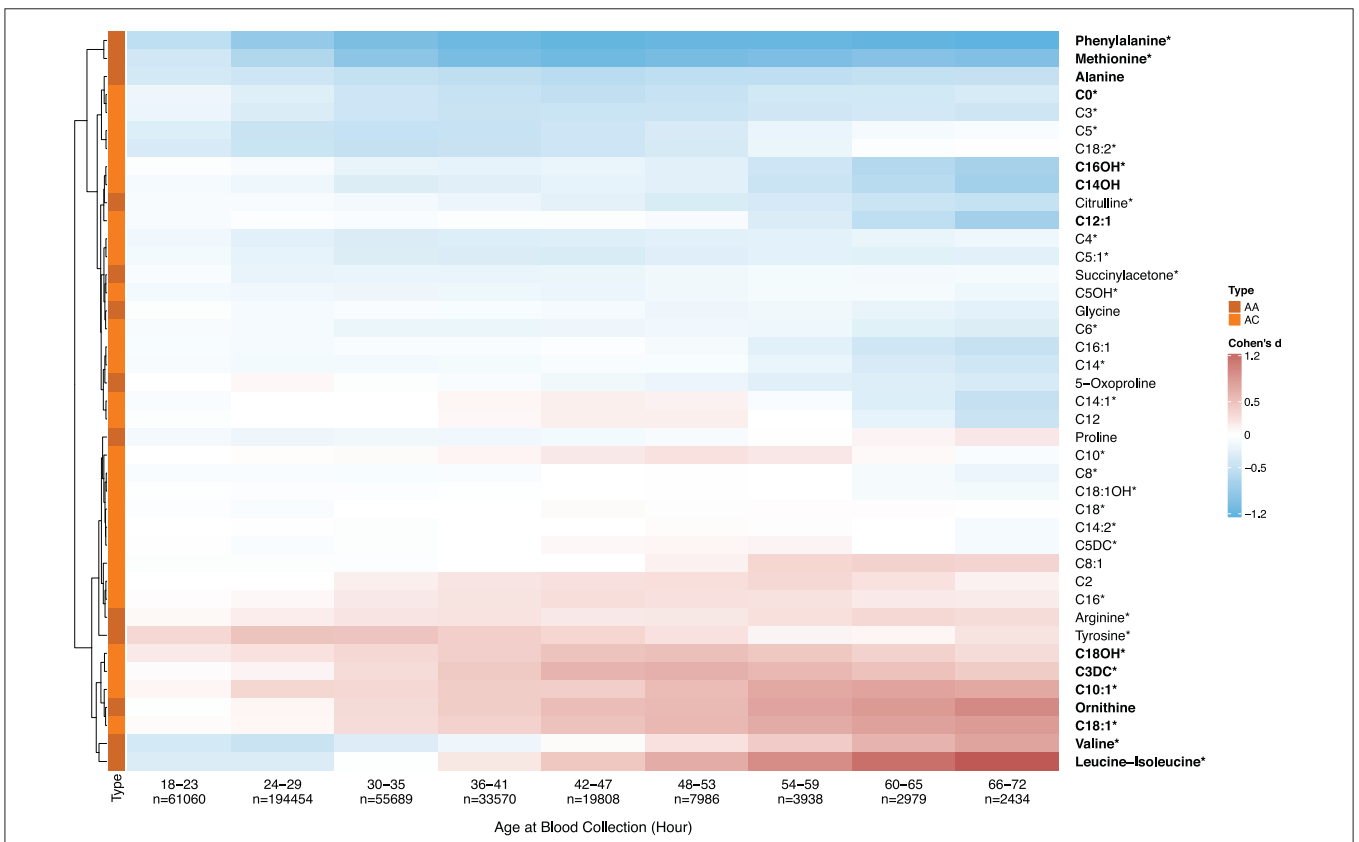

FIGURE 1 | Metabolic analyte levels and age at blood collection. To explore early postnatal metabolic changes, we selected ten infant groups based on AaBC differences with the first group (12–17 h, $n = 29{,}000$) being defined as a standard for each metabolite. Effect size differences (Cohen's *d*) for all 41 metabolites between each of nine AaBC groups (18–72 h) and the standard (12–17 h) was calculated. Positive Cohen's *d* (in red) indicates elevated metabolite levels, negative Cohen's *d* (in blue) indicates decreased levels compared to the standard. Hierarchically clustering was used to group metabolites into two cluster of either decreasing (on top) or increasing (at bottom) levels after birth. Thirty four percent of the markers showed significant differences between at least one of the nine AaBC groups and the standard group (Cohen's $d \geq 0.5$), including 30 primary NBS markers of metabolic disorders on the RUSP (18) (labeled with*). AA, amino acid; AC, acylcarnitine.

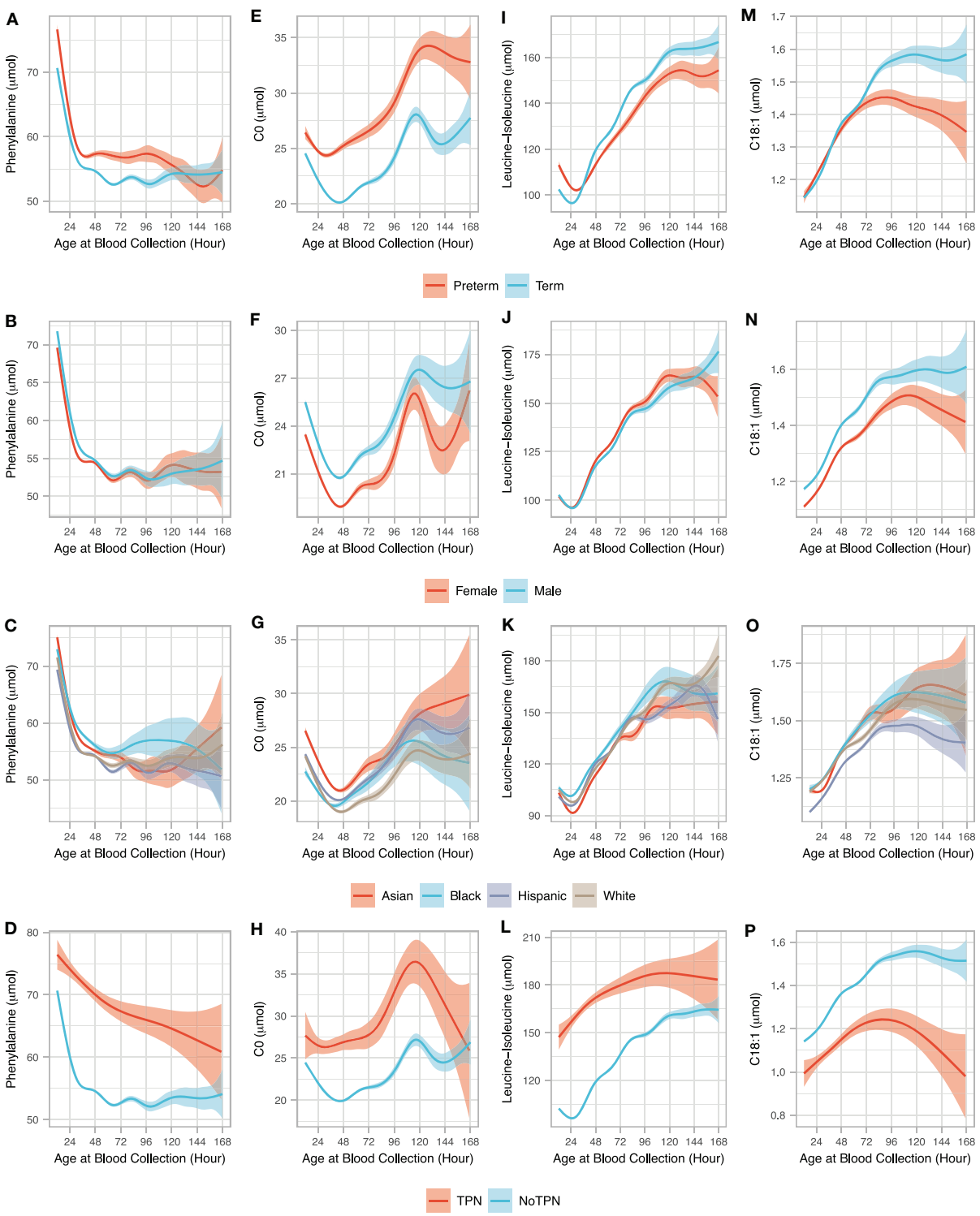

FIGURE 2 | Age at blood collection and clinical variables. The association between AaBC (12–168 h) and four clinical variables (gestational age, sex, race/ethnicity, and total parenteral nutrition) are shown for four metabolites including phenylalanine **(A–D)**, C0 **(E–H)**, leucine-isoleucine **(I–L)**, and C18:1 **(M–P)**. For each of the 4 metabolites, relationship between the different AaBC timepoints are shown for preterm (n = 24,447) and term (n = 462,806) infants without TPN **(A,E,I,M)**; for male (n = 209,855) and female (n = 203,799) infants born at term and normal BW and without TPN **(B,F,J,N)**; for infants from different race/ethnicity groupings born at term and normal BW and without TPN including Asian (n = 63,528), Black (n = 27,301), Hispanic (n = 207,499), and White (107,561) **(C,G,K,O)**; and for term infants with normal BW and with (n = 1,686) and without TPN (n = 414,869). The solid smoothed lines are the mean estimated from a generalized additive model with the shading showing the 95% confidence interval of the mean estimation.

was self-reported by the parents. Of the 503,935 infants studied, 80.1% (n = 403,425) were reported as being of Asian, Black, Hispanic, or White origin. Newborns recorded with more than one race/ethnicity (17.8%, n = 89,765) were classified according to NBS program guidelines (25) as follows: (a) Hispanic, if reported Hispanic and any other race/ethnicity; (b) Black, if reported Black and any other race/ethnicity except Hispanic; (c) Asian, if reported Asian and any other race/ethnicity except Hispanic and Black; (d) White, if reported White only. All other ethnicities and unknown race/ethnicity were recorded as Other/Unknown (2.1%, n = 10,745). Lastly, for TPN, the changes in metabolite levels related to AaBC were compared between newborns without and with TPN.

Analysis of AaBC-Related Differences and False-Positive Results

The eleven metabolic diseases studied were detected in NBS by elevated (ASA, CIT-I, CIT-II, HCY, IVA, MMA, PA, PKU, and VLCADD) or by decreased (CTD and OTCD) marker levels (**Table 1**). Here we studied whether timing of blood collection could impact NBS performance for detecting these diseases. Screen-negative newborns were grouped based on their reported AaBC into early (12–23 h), standard (24–48 h), and late (49–168 h) collection groups. We only used data from infants born at term (37 to 41 weeks), birth weight between 2,500 g and 4,000 g, and without TPN to control for the confounding effects of GA, BW, and TPN on marker levels. Data from 414,869 newborns (82.9% of the total) was used in the analysis including 90,060 (21.7%) in the early, 305,674 (73.7%) in the standard, and 19,135 (4.6%) in the late collection groups, respectively. Effect-size analysis using Cohen's d (22) for all 41 metabolites was used to compare the early and the late collection-group to the standard group. Metabolites identified in the early or late collection-groups with absolute Cohen's d larger than 0.2 were matched to metabolic markers for 11 diseases studied (**Figure 3**). Only false-positive cases with the same ranges for GA and BW and without TPN as the screen-negatives were selected for analysis. For each disease, the proportion of false positive cases was compared to the proportion of screen negative infants for each of the three AaBC categories using Chi-squared test.

Statistical Analysis and Online Tool

Statistical analyses, graphs, design of the research and the online tool were performed in R software 3.6.122 with the following R packages: effsize (26), ggplot2 (27), ggsci (28), ggpubr (29), ComplexHeatmap (30), and shiny (31). Effect size analysis using Cohen's d (22), which is defined as the difference between two group means divided by the pooled standard deviation, was performed to compare metabolite levels between AaBC groups. Cohen's d, which is not influenced by sample size, allowed for direct comparison of metabolite levels between groups with different sample sizes. A novel web-based tool was developed (http://rusptools.shinyapps.io/AaBC/) using the R shiny package (31) for analysis and interpretation of all 41 metabolic analytes and their ratios from 500,539 newborns in relation to AaBC and other covariates (**Supplementary Figure 1**). A detailed description of the online tool and user guide is provided under **Supplementary Material**. The source code for the new software is available at GitHub (https://github.com/peng-gang/AaCShiny).

RESULTS

Identification of Metabolic Differences Related to AaBC

Population-level MS/MS and timing of blood collection data reported by the California NBS program were used to identify metabolic changes during the first days of life. The 41 MS/MS metabolites were found to cluster into two major groups according to their changing profiles in relation to AaBC (**Figure 1**). The two clusters showed either decreasing or increasing metabolite levels, while additional changes were observed within each cluster. For example, phenylalanine levels decreased sharply in the first 36 h after birth with very small changes after 48 h, while C16OH decreased steadily from 18 to 72 h after birth. In contrast, leucine-isoleucine levels decreased during the first 30 h and then increased.

We selected four NBS primary markers (two amino acids and two acylcarnitines) that were among the top metabolites identified in **Figure 1** to showcase the dynamic metabolic changes associated with AaBC and other clinical variables including GA, sex, race/ethnicity, and TPN (**Figure 2**). For phenylalanine, preterm infants had higher levels than term infants in the first 108 h after birth. Sex and race/ethnicity status did not have a major effect on phenylalanine levels, while infants under TPN had much higher levels than those without TPN (**Figures 2A–D**). For free carnitine (C0), its level decreased during the first 48 h except for newborns with TPN, and then increased between 48 and 120 h. GA, sex, race/ethnicity and TPN status had relatively large influence on C0 levels with preterm, male, Asian and newborns with TPN having higher C0 compared to term, female, Black, and infants without TPN (**Figures 2E–H**). For leucine-Isoleucine, levels initially decreased except for newborns with TPN, and then increased after 30 h. Compared to term infants, preterm infants had higher leucine-isoleucine level in the first 30 h (P <0.001) and lower levels after 48 h (P = 0.01). Newborns with TPN had higher leucine-isoleucine than newborns without TPN in the first 120 h (**Figures 2I–L**). For C18:1, preterm and term newborns had similarly increasing levels until 72 h, after which levels continued to increase and then plateaued for term newborns, while levels for preterm newborns plateaued and then slightly decreased after 96 h. Females and infants with TPN had lower C18:1 levels compared to males and infants without TPN (**Figures 2M–P**).

Correlation of AaBC-Related Differences to False-Positive Results

We identified AaBC-related differences for 56% (23 of 41, Cohen's d > 0.2) of the metabolites when comparing levels between the early or late collection-group to the standard group (**Figure 3**). Seven of the 23 metabolites are primary NBS markers for detection of 11 metabolic disorders for which we had information on false-positive cases (**Table 1**). We reasoned that

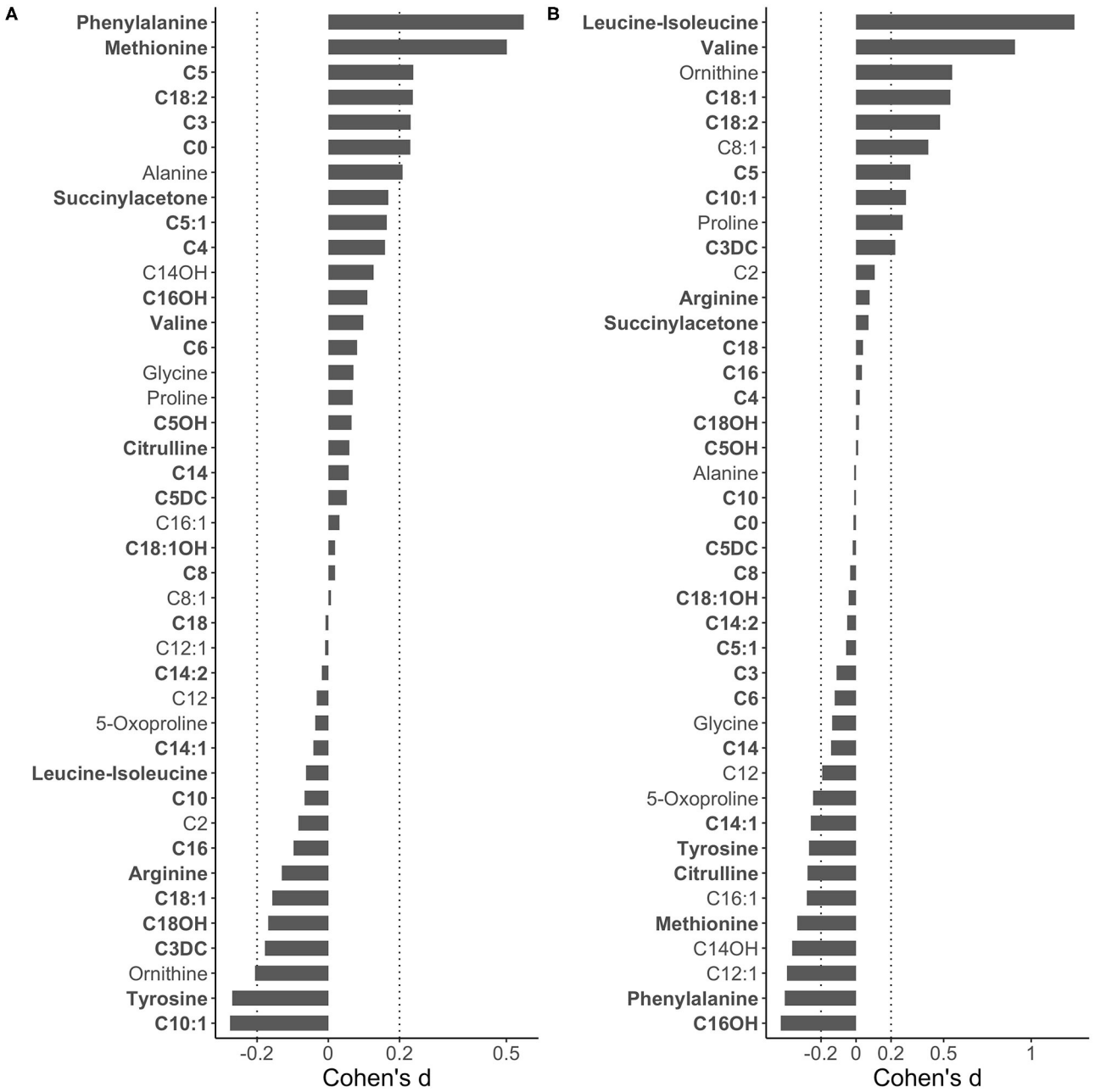

FIGURE 3 | Metabolic difference between early and late blood collection. To identify metabolite level difference related to AaBC, 414,869 screen-negative term infants (37–41 weeks) with BW range of 2,500 g to 4,000 g and without TPN were grouped into early (12–23 h), late (49–168 h) and standard (24–48 h) collection groups. Effect size differences (Cohen's *d*) for each of 41 MS/MS metabolites were calculated between the early and the standard group **(A)** and between the late and standard group **(B)**. In comparison to the standard, positive Cohen's *d* values indicate relatively higher metabolite levels in the early and the late collection groups, respectively. Metabolites are ranked from top to bottom based on Cohen's *d* values with primary NBS disease markers in bold.

a disease marker elevated in the early-collection group could also lead to a higher number of false-positives in this group. In turn, a marker with significantly lower physiological levels at early AaBC could lead to a relatively lower number of false-positives compared to later collection-time groups. We defined this correlation as consistent with this hypothesis, or as inconsistent if this correlation was not found (**Supplementary Table 3**). Compared to the standard group, the early-collection group had elevated levels of PKU marker phenylalanine (Cohen's $d = 0.55$), IVA marker C5 (Cohen's $d = 0.24$), MMA marker C3 (Cohen's $d = 0.23$), and CTD marker C0 (Cohen's $d = 0.23$). These findings correlated with higher false-positive rates for PKU ($P < 0.05$), IVA ($P < 0.05$), and MMA ($P < 0.001$), and lower false-positive rate for CTD ($P < 0.001$, decreased C0

level in CTD patients) in the early-collection group. The late-collection group had decreased levels of phenylalanine (Cohen's d = −0.41) and citrulline (Cohen's $d = -0.28$), which was associated with fewer false-positive cases for PKU ($P < 0.05$) and more false-positives for OTCD ($P < 0.001$, decreased citrulline level in OTCD patients). There were also inconsistent results including an unexpectedly lower false-positive rate for IVA, and a higher false-positive rate for CITR and VLCADD in the late-collection groups despite the elevated C5, decreased citrulline and decreased C14:1 levels in this group.

DISCUSSION

Here we used population-level mass spectrometry screening data to systematically examine postnatal metabolic changes and whether AaBC could impact the performance of newborn screening for selected metabolic diseases on the RUSP (18). We followed a stringent study design by controlling for the influence from the important covariates of birth weight and gestational age in the analysis of metabolite levels across different AaBC timepoints. A cluster analysis of 41 metabolites reported for 410,918 screen-negative infants in relation to their AaBC revealed two large metabolite groups characterized by either decreasing or increasing levels after birth (**Figure 1**). While largely exploratory, this analysis could shed new light on early postnatal metabolism and the dynamic changes of individual screening markers. For example, phenylalanine levels markedly decreased within 48 h after birth, which may require different cutoff values in PKU screening based on the infant's AaBC. For the C3/C2 ratio, a screening marker for MMA and PA, levels decreased after 120 h (**Supplementary Figure 2**). This finding could explain the discrepancy in testing of two babies affected with PA and MMA, respectively. In each case, the initial NBS test showed a positive result while a second confirmatory test several days later was found negative. At the time it was not known that the first test was a true-positive while the second was a false-negative (32).

We next studied the influence on metabolite levels for several clinical variables (GA, sex, ethnicity and TPN status) and their relationship with AaBC (**Figure 2**). At AaBC under 72 h, the four selected metabolites displayed similar patterns in relation to differences in GA, sex and ethnicity, while patterns changed at AaBC after 96 h. A potential cause for these changes could be limitations in sample size, which decreased with increasing AaBC (97–120 h: $n = 1657$; 121–144 h: $n = 627$; 145–168 h: $n = 326$) leading to increased variance of the estimated mean. Other reasons for the metabolic pattern changes related to later AaBC may be the postnatal advance and increasing environmental changes, or differences related to race/ethnicity status (17, 25). We found that White infants had a tendency for later blood collection (26.0% between 24–48 h, 32.6% between 49–168 h, $P < 0.001$), which could lead to differences in metabolic patterns in later AaBC groups. A larger sample size is required to explore these questions and to control for the influence of the different variables. In contrast to the other covariates studied, TPN was associated with different metabolic patterns in relation to AaBC, and particularly for blood sampling before 24 h (**Figures 2D,H,L,P**). While these differences could be caused directly by TPN, there may also be other confounding factors related to TPN such as preterm birth or an unknown disease status (33). We only included term infants (37–41 weeks) with a normal birth weight (2,500 g to 4,000 g) in the TPN analysis based on our finding of a smaller fraction of newborns with TPN amongst term infants (0.45%) compared to preterm infants (12.13%).

We reasoned that the AaBC-related differences identified for 56% of the metabolites (**Figure 3**) could lead to false-positive screens. We selected 11 diseases with frequent false-positive screening results (**Table 1**). Analysis of false-positives for some of these diseases indicated AaBC-related differences, which correlated with differences in marker levels discovered in the respective collection groups. For example, infants in the early-collection group (12–23 h) were more likely false-positive in PKU, MMA and IVA screening, which correlated with the elevated phenylalanine, C3 and C5 levels in screen-negatives in this group. Similarly, higher levels of C0 in the early-collection group correlated with fewer false-positives for CTD (decreased C0 marker) in this group. C0 levels were found to be lower in the standard group (24–48 h) potentially contributing to a relatively higher false-positive rate for CTD in this group (**Supplementary Figure 3**). In the late-collection group (49–168 h), infants were more likely false-positives for OTCD, which correlated with the lower citrulline levels in screen-negatives in this group. In contrast, we did not find significantly more false positives for HCY and PA in the early-collection groups which was likely due to the smaller sample size of false-positives for these two disorders. Another unexplained result was the high false-positive rate for CITR and VLCADD, and the low false-positive rate for IVA in the late-collection group despite the decreased citrulline and C14:1 levels, and increased C5 levels amongst screen-negatives in this group. It is possible that marker levels may have been adjusted at late AaBC for these diseases; infants could have received blood transfusions, particularly in the late-collection group; or the infant's condition, which contributed to false-positive results, had precluded collection of the first newborn screening specimen before 48 h of life.

Here we identified an association between MS/MS disease markers and timing of blood collection, and showed that these differences could lead to false-positive screens for some disorders (**Table 1**). Previous studies suggested different cutoff values according to AaBC for hypothyroidism screening (7), or polynomial regression models to adjust metabolite levels and ratios for age at collection and birth weight in order to reduce false-positive results for lysosomal disorders (16). As shown in **Figure 2**, AaBC did not have a linear relationship with metabolite levels and different patterns were found for different metabolites. The association between metabolite levels and AaBC was also dependent on other confounding variables, of which GA and TPN had the largest influence. For example, both AaBC and GA are associated with differences in tyrosine levels. While tyrosine levels were higher at standard AaBC (24–48 h) compared to late AaBC (49–72 h) for both preterm ($P < 0.001$) and for term infants ($P < 0.001$), preterm infants

had significantly higher tyrosine levels than term infants at both AaBC timepoints (**Supplementary Figure 4**). Relationships between AaBC and other covariates can be explored for all metabolites using an online tool accompanying this study (http://rusptools.shinyapps.io/AaBC/). Our results indicate that relying on cutoff values or regression model adjustment for metabolite levels based on AaBC could have limitations. Development of novel data mining models that incorporate all screening metabolites and clinical variables could further our understanding of complex metabolite-covariate relationships and improve prediction of metabolic disease status (34). Implementing these new tools and approaches is challenging and relies on collaborative efforts between NBS programs worldwide (35).

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data used in this study were obtained from the California Biobank Program (CBP) under SIS request 886. The data can be obtained by others after submitting a new request to the CBP coordinator. Requests to access these datasets should be directed to CaliforniaBiobank@cdph.ca.gov.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review boards at Yale University, Stanford University, and the State of California Committee for the Protection of Human Subjects. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GP and CS designed the study and wrote the manuscript. GP and YT performed the statistical analysis. TC, HZ, and CS provided input on data analysis and interpretation. All authors edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.623184/full#supplementary-material

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ORIGINAL RESEARCH
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An Easily Overlooked Contamination of Syringes in Newborn Screening by Tandem Mass Spectrometry

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Background: Tandem mass spectrometry becomes a common and important test in newborn screening, but potential contamination of the equipment has largely been ignored.

Methods: The source of contamination through Biosan quality control samples was examined prospectively, and further confirmed by retrospective analysis of patient samples.

Results: We found that the source of contamination came from a syringe in the Biosan quality control samples. Furthermore, we found that a large number of indicators in the patient sample were interfered by syringe contamination in our center, and also in two other newborn screening centers, but the affected indicators were different in different screening centers.

Conclusion: Syringe contamination will affect the detection of patient samples by tandem mass spectrometry and should be monitored carefully and immediately.

Keywords: syringe, contamination, tandem mass spectrometry (MS/MS), quality control (QC), newborn screening

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In recent years, newborn screening has definitely become one of the most successful applications of tandem mass spectrometry (MS/MS) in the clinic (1). The measurement of amino acids (AA) and acyl carnitines (AC) by MS/MS enables the identification of over 20 inherited metabolism diseases (IMD) only a few days after birth, in one test (2). MS/MS has been under development at the Nanjing Newborn Screening Center since 2013, including two sets of MS/MS TQD and Xevo-TQD (XTQD).

MS/MS DETECTION PLATFORM IN JIANGSU PROVINCE AND ZHEJIANG PROVINCE

The Nanjing Newborn Screening Center is one of 13 screening centers in Jiangsu Province. Newborn screening centers in Jiangsu Province and Zhejiang Province both use the Tandem Mass Spectrometry (MS/MS) platform of Waters Corporation. The platform consists of four parts: instrument control and a data processing system (computer), a 1,525 μ high performance liquid pump, 2,777C samples manager and tandem mass spectrometry (**Figure 1**).

Generally speaking, the 2,777C samples manager has three components: injection needle, a valve, and the peek tube. One syringe (injection needle) in the samples manager is used to complete the injection of the samples. The main body of the syringe is made of glass and stainless steel which are anticorrosion materials, and means therefore, that the syringe will not be replaced until it is

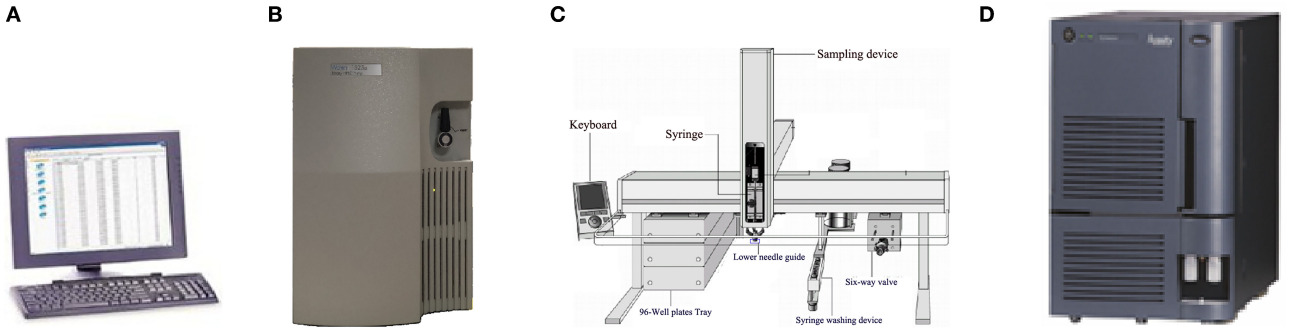

FIGURE 1 | Exploded view of MS/MS detection platform [**(A)**: Computer provided by Waters corporation; **(B)**: 1,525 μ high performance liquid pump of Waters corporation; **(C)**: 2,777C samples manager; **(D)**: Mass spectrometer of Waters Corporation].

Plate 1

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A
CDC-A	BS-L	PE-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H
BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	PE-L	BS-H	BS-H	Blank	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H

Plate 2

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D
CDC-D	BS-H	PE-L	BS-H	BS-H	BS-H	BS-H	BS-H	Blank	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-M
BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M
BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H	PE-L	BS-H	BS-H	BS-H	BS-H
BS-H	BS-H	BS-H	BS-H	BS-H	Blank	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-M	PE-H	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	Blank

Plate 3

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D
CDC-D	BS-L	PE-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H
BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	PE-L	BS-H	BS-H	Blank	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H

Plate4

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D
CDC-D	BS-H	PE-L	BS-H	BS-H	BS-H	BS-H	BS-H	Blank	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-M
BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M
BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H	PE-L	BS-H	BS-H	BS-H	BS-H
BS-H	BS-H	BS-H	BS-H	BS-H	Blank	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-M	PE-H	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	Blank

Plate 5

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D
CDC-D	BS-L	PE-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-L	BS-L	BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H
BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	PE-L	BS-H	BS-H	Blank	BS-L
BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L	BS-L
BS-L	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	BS-M	PE-H	BS-M	BS-M
BS-M	BS-M	BS-M	BS-M	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H	BS-H

Plate 6

1	2	3	4	5	6	7	8	9	10	11	12
Blank	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-A	CDC-D	CDC-D	CDC-D	CDC-D	CDC-D
CDC-D	BS-H	PE-L	BS-H	BS-H	BS-H	BS-H	BS-H	Blank			

FIGURE 2 | Schematic diagram of Biosan QC samples in 96-well plates.

damaged (**Supplementary Figure 1**). The injection needle is a consumable material in the sample management and is made up of two components: a glass barrel and a plunger. The plunger has to be regularly replaced, without needing to replace the glass barrel. Therefore, the glass barrel could be contaminated with the samples, but is not noticed or reported.

A range of different interferences in newborn screening has been reported. For example, the impact of punch location (3), sample volume (4), EDTA in dry blood spots (DBS) (5), and contamination of the DBS (6). However, contamination of the syringe has never been reported. The purpose of this study was to determine whether syringe contamination interferes with MS/MS detection and to establish a model for regular maintenance of syringes.

THE PROCESS OF ELIMINATING POSSIBLE CONTAMINATION

Samples were taken from 96-well plates by a syringe, mixed with flow solvent in the valves, delivered to mass spectrometry via the peek tube, and analyzed by mass spectrometry ionizing through the ESI-SS capillary (**Supplementary Figure 2**). Accordingly, the possible contaminated parts before MS/MS analysis were as follows: syringe, lower needle guide, valves, peek tube, and ESI-SS capillary.

METHODS

1. Amino acids and acyl carnitines were detected by commercial non-derivatized MS/MS kits (PerkinElmer, PE company) as described previously (7). Quality control (QC) relied on measurements of PE and American CDC control samples. The third internal QC sample was provided by the Biosan company which was divided into three concentration groups as low (L), middle (M) and high (H). Detection indexes in Biosan's samples contained 11 kinds of amino acids (Ala, Arg, Cit, Leu, Met, Orn, Phe, Tyr, Val, Gly, and Pro), 16 kinds of acyl carnitine (C0, C2, C3, C4, C5, C5DC+C6OH, C4DC+C5OH, C6, C8, C10, C12, C14, C16, C18) and succinylacetone (SA). Each set of the Biosan sample contains two "big" dry blood spots of L, M, and H concentration. The diameter of each "big" blood spot is not $<$ 13.0 mm. In testing, a "small" blood spot with a diameter of 3.2 mm needs to be cut from the "big" blood spot as a test sample by P9 (panthera-puncherTM 9TM, PE company). The samples were processed by P9 as shown in **Supplementary Figure 3**. Ten sets of Biosan samples were taken randomly with the same batch

number, and the layout of a total of 140 blood spots are shown in **Figure 2**. Each 96-well plate included internal QC samples from the American CDC and PE to ensure the accuracy and comparability of the data. Samples of Biosan were distributed in the order of 14 low QC (BS-L) - 14 middle QC (BS-M) - 14 high QC (BS-H).

2. There are five parts that need to be checked step by step. The troubleshooting was established step by step following 2.1–2.5 (**Figure 3**). For each step, we arranged two blank samples and two Biosan low QC samples with a PE high QC sample (**Table 1**).

2.1 Just replacing the old syringe with a new syringe.

2.2 Using the old syringe, cleaning the lower needle guide and valves ultrasonically several times, and changing the plunger, peek tube, and ESI-SS capillary.

2.3 Using the old syringe and washing it two to four times after the injection of the PE high QC sample.

2.4 Using the old syringe and cleaning the old syringe with anhydrous formic acid (Aladdin, 64-18-6) for 3 min before drawing samples.

2.5 Using the old syringe and cleaning the old syringe with anhydrous formic acid for 10 min before drawing samples.

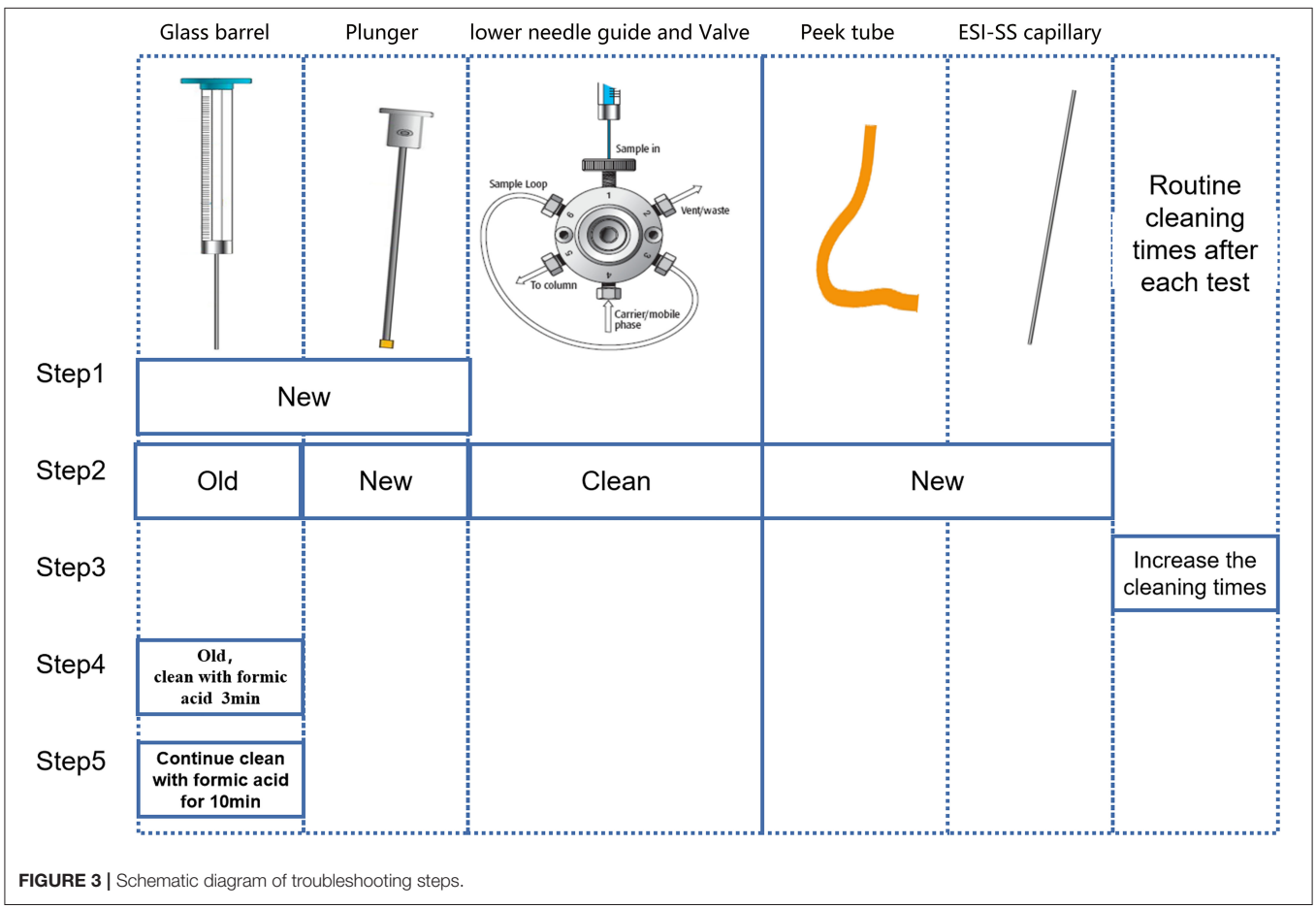

FIGURE 3 | Schematic diagram of troubleshooting steps.

TABLE 1 | Schematic diagram of samples arrangement position in troubleshooting experiments.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank 1	Blank 2	Blank 3	PE-H 1	Blank 4	Blank 5	PE-H 2	Blank 6	Blank 7	PE-H 3	BS-L 1	BS-L 2
	PE-H 4	BS-L 3	BS-L 4									
B	PE-H 5	Blank 8	Blank 9	PE-H 6	Blank 10	Blank 11	PE-H 7	BS-L 5	BS-L 6	PE-H 8	BS-L 7	BS-L 8
C	PE-H 9	Blank 12	Blank 13	PE-H 10	Blank 14	Blank 15	PE-H 11	BS-L 9	BS-L 10	PE-H 12	BS-L 11	BS-L 12
D	PE-H 13	Blank 16	Blank 17	PE-H 14	Blank 18	Blank 19	PE-H 15	BS-L 13	BS-L 14	PE-H 16	BS-L 15	BS-L 16
E	PE-H 17	Blank 20	Blank 21	PE-H 18	Blank 22	Blank 23	PE-H 19	BS-L 17	BS-L 18	PE-H 20	BS-L 19	BS-L 20
F												
G												

PE-H, PE-high-QC sample; BS-L, BS-low-QC samples.

TABLE 2 | Basic values of each index.

Index	L-X	L-SD	L-CV (%)	M-X	M-SD	M-CV	H-X	H-SD	H-CV
ALA	436.68	24.8	5.68	1,099.76	63.23	5.75%	1,554.76	87.20	5.61%
ARG	8.36	0.54	6.44	67.04	3.47	5.17%	174.57	9.42	5.39%
CIT	38.31	2.15	5.62	137.33	7.35	5.35%	727.30	40.70	5.60%
LEU	202.62	10.57	5.22	372.54	19.93	5.35%	994.36	59.81	6.02%
MET	15.85	0.96	6.08	43.63	2.46	5.64%	264.45	13.59	5.14%
ORN	144.57	8.33	5.76	444.58	24.06	5.41%	1,594.27	102.32	6.42%
PHE	56.72	3	5.3	105.85	5.57	5.27%	536.74	28.74	5.36%
TYR	141.54	7.13	5.04	325.28	17.66	5.43%	825.28	46.51	5.64%
VAL	204.11	10.71	5.25	356.77	19.61	5.50%	786.00	48.84	6.21%
GLY	299.57	17.99	6	1,014.02	59.51	5.87%	1,586.02	95.94	6.05%
PRO	134.42	7.29	5.42	540.44	29.12	5.39%	1,444.08	78.61	5.44%
C0	13.37	0.74	5.55	57.44	3.10	5.39%	126.33	6.36	5.04%
C2	41	2.17	5.29	74.21	4.09	5.52%	152.02	8.87	5.83%
C3	1.97	0.12	6.17	6.21	0.37	5.88%	10.91	0.63	5.80%
C4	0.38	0.03	7.17	1.00	0.06	6.02%	3.41	0.21	6.29%
C5	0.32	0.02	6.7	1.06	0.05	4.79%	5.88	0.30	5.17%
C5DC+C6OH	0.24	0.02	9.06	0.88	0.06	6.58%	4.09	0.23	5.75%
C4DC+C5OH	0.32	0.01	6.25	0.95	0.04	4.54%	4.96	0.25	4.98%
C6	0.14	0.01	9.06	0.42	0.03	7.35%	1.02	0.06	5.61%
C8	0.54	0.03	6.18	1.39	0.08	5.94%	2.21	0.14	6.37%
C10	0.33	0.02	7.08	0.86	0.05	6.30%	1.39	0.08	6.10%
C12	0.26	0.02	6.36	0.67	0.04	6.25%	1.14	0.08	6.72%
C14	0.52	0.04	7.61	1.72	0.13	7.54%	4.60	0.33	7.28%
C16	0.73	0.07	**9.72**	2.96	0.28	**9.45%**	10.78	0.76	7.09%
C16OH	0.14	0.01	7.9	0.66	0.04	6.30%	2.18	0.15	7.01%
C18	0.61	0.05	8.95	1.41	0.10	7.23%	3.69	0.28	7.49%
SA	1.1	0.09	8.35	2.56	0.18	7.05%	9.19	0.58	6.33%

L-X, Mean of BS-low-QC samples; L-SD, Standard Deviation of BS-low-QC samples; L-CV, Coefficient of Variation of BS-low-QC samples; M-X, Mean of BS-medium-QC samples; M-SD, Standard Deviation of BS-medium-QC samples; M-CV, Coefficient of Variation of BS-medium-QC samples; H-X, Mean of BS-high-QC samples; H-SD, Standard Deviation of BS-high-QC samples; H-CV, Coefficient of Variation of BS-high-QC samples. C16 had the highest L-CV and M-CV values which were marked in bold.

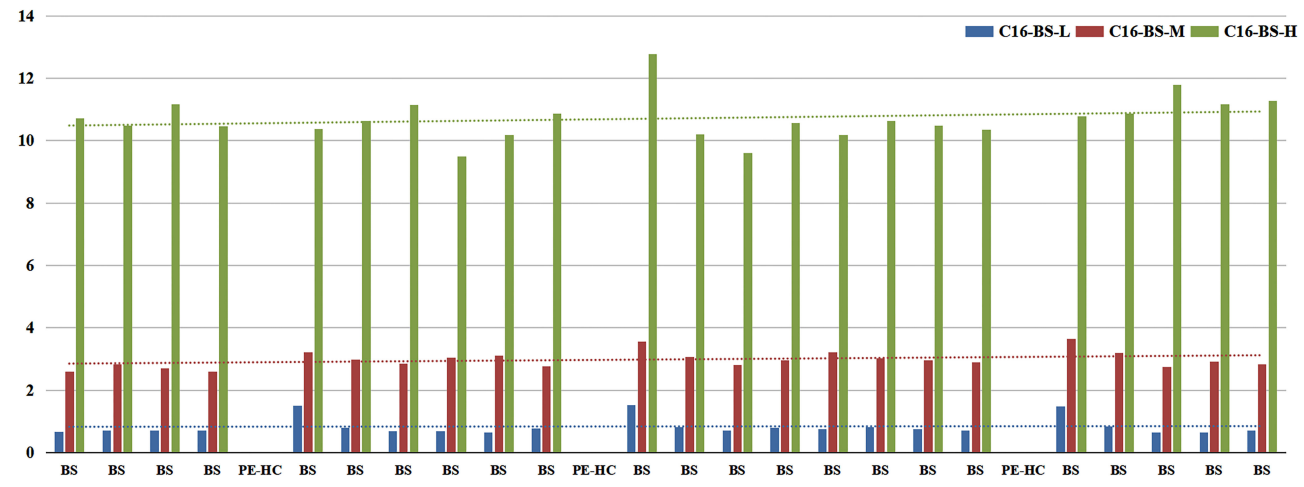

FIGURE 4 | Column chart of C16 value detected before and after the PE-high-QC samples.

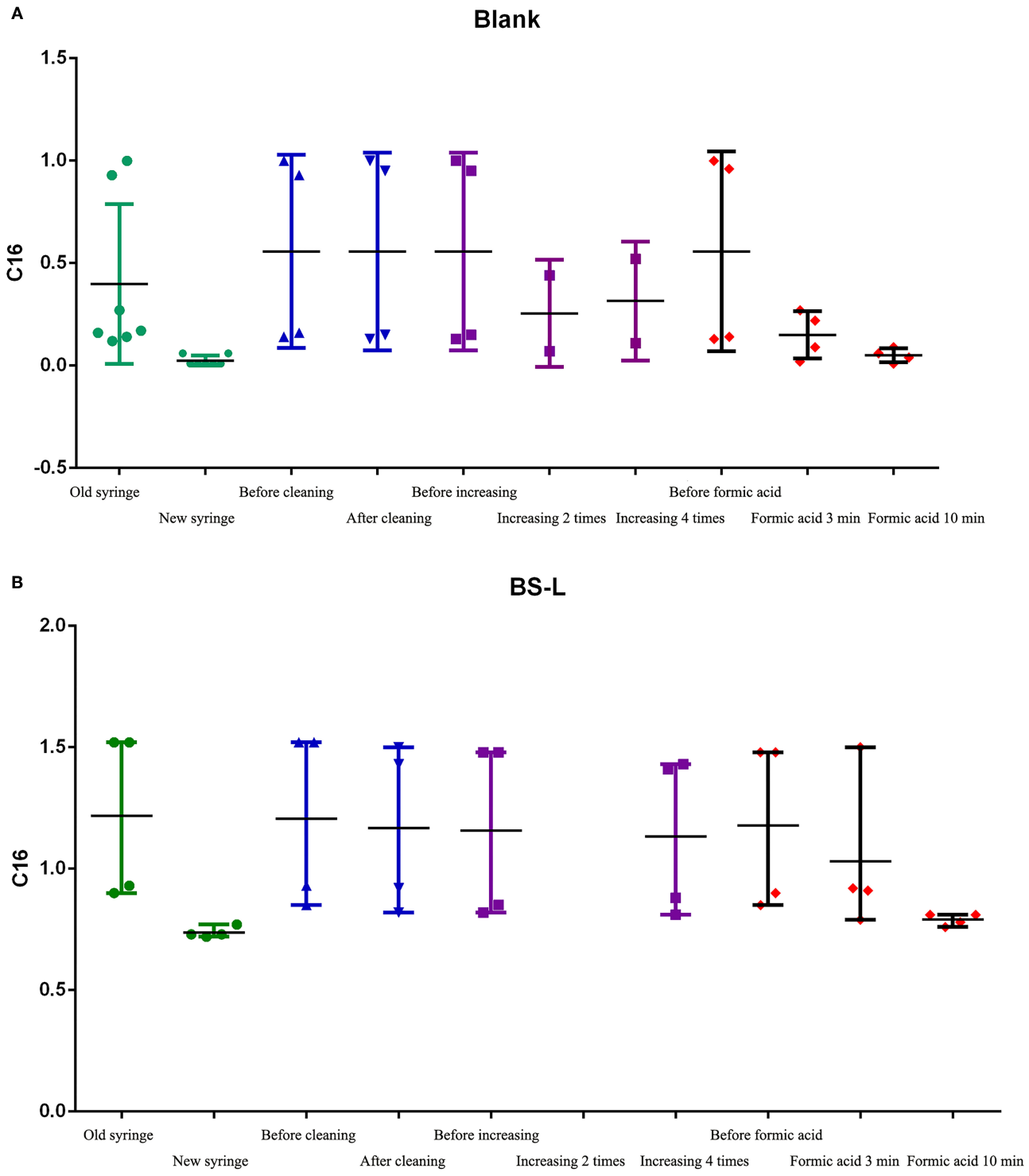

FIGURE 5 | The C16 results of steps 2.1-2.5. **(A)** The results of C16 in Blank samples. **(B)** The results of C16 in BS-L-QC samples.

3. This study was approved by the Ethical committee of Nanjing Maternity and Child Health Care Hospital, and 50,000 consecutive cases of newborn screening in 2018 were recruited.

RESULTS

1. Basic values of each index are shown in **Table 2**. In all indicators, the coefficient of variation (CV) in C16 was the

TABLE 3 | The concentration of the first sample after PE-high-QC and other samples.

Index	Median				
	A: Total (n = 53,111)	**B: The first sample after PE-high-QC ?? (n = 1,146)**	**C: Other sample (n = 51,965)**	**Multiple**	**Bias (B-C)/C%**
ALA	316.63	320.86	316.52	1.01	1.37%
ARG	9.05	8.96	9.06	0.99	−1.10%
CIT	12.94	13.63	12.92	1.05	5.46%
GLY	470.8	481.05	470.52	1.02	2.24%
LEU+ILE+PRO-OH	134.37	134.99	134.35	1	0.47%
MET	20.55	21.68	20.52	1.06	5.63%
ORN	104.07	104.7	104.07	1.01	0.60%
PHE	48.06	49.84	48.03	1.04	3.76%
PRO	193.36	197.81	193.26	1.02	2.35%
SA	0.77	0.81	0.77	1.05	5.19%
TYR	93.72	95.73	93.68	1.02	2.18%
VAL	126.15	127.6	126.1	1.01	1.19%
C0	19.83	20.27	19.82	1.02	2.25%
C2	18.32	18.76	18.31	1.02	2.46%
C3	1.51	1.59	1.51	1.05	4.97%
C3DC+C4OH	0.1	0.1	0.1	1	0.00%
C4	0.2	0.21	0.2	1.05	5.00%
C4DC+C5OH	0.2	0.2	0.2	1	0.00%
C5	0.1	0.1	0.1	1	0.00%
C5:1	0.01	0.01	0.01	1	0.00%
C5DC+C6OH	0.1	0.1	0.1	1	0.00%
C6	0.04	0.04	0.04	1	0.00%
C6DC	0.09	0.09	0.09	1	0.00%
C8	0.05	0.05	0.05	1	0.00%
C8:1	0.09	0.1	0.09	1.11	**11.11%**
C10	0.07	0.08	0.07	1.14	**14.29%**
C10:1	0.06	0.06	0.06	1	0.00%
C10:2	0.01	0.01	0.01	1	0.00%
C12	0.07	0.09	0.07	1.29	**28.57%**
C12:1	0.04	0.05	0.04	1.25	**25.00%**
C14	0.17	0.22	0.17	1.29	**29.41%**
C14:1	0.08	0.09	0.08	1.13	**12.50%**
C14:2	0.02	0.02	0.02	1	0.00%
C14OH	0.01	0.01	0.01	1	0.00%
C16	2.87	3.67	2.85	1.29	**28.77%**
C16:1	0.16	0.16	0.16	1	0.00%
C16:1OH	0.03	0.03	0.03	1	0.00%
C16OH	0.02	0.02	0.02	1	0.00%
C18	0.82	1.02	0.82	1.24	**24.39%**
C18:1	1.34	1.33	1.34	0.99	−0.75%
C18:1OH	0.02	0.04	0.02	2	**100.00%**
C18:2	0.2	0.19	0.2	0.95	−5.00%
C18OH	0.01	0.01	0.01	1	0.00%

(B−C)/C values over 10% were marked in bold.

highest. Furthermore, CV of BS-middle-QC and BS-low-QC samples was higher than BS-high-QC. The data were sorted in the original order in 96-well plates. It is worth noting that C16 in the first sample after the PE-high-QC sample was in particular higher than others, especially in BS-low-QC samples (**Figure 4**). This was the reason the CV in C16 was so high.

2. Therefore, in subsequent steps, to identify the source of contamination, we mainly analyzed C16 in BS-low-QC samples. The results of steps 2.1–2.5 are shown in **Figure 5**.

2.1 Test results of replacing the syringe: after syringe replacement, the detection of C16 in Blank-samples and BS-L samples was significantly lower than before syringe replacement and was close to the mean of C16 in **Table 2** (0.73).

2.2 Test results of C16 with the old syringe, after cleaning the lower needle guide and valves, changing the plunger, peek tube, and ESI-SS capillary: Before and after cleaning, C16 in Blank-samples and BS-L samples had no obvious difference, suggesting that the above parts were not contaminated.

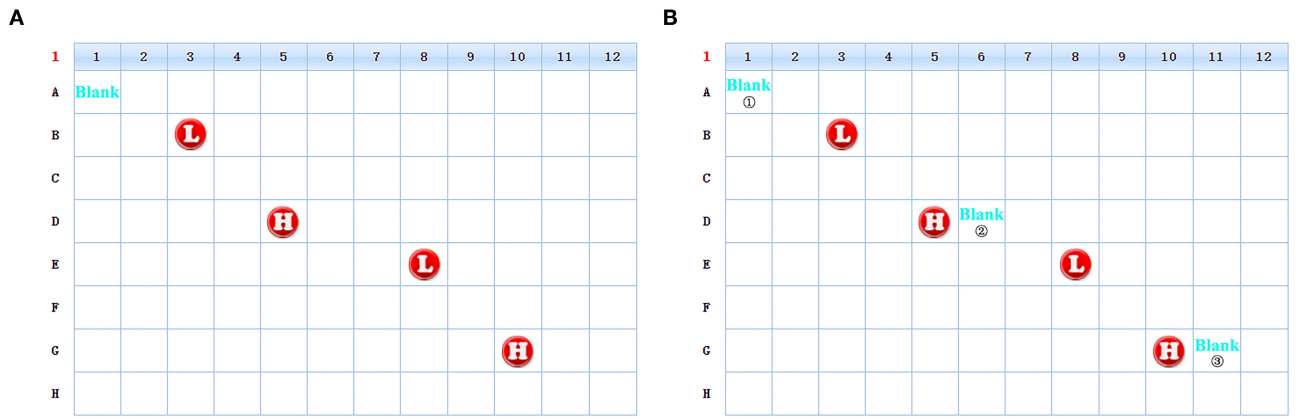

FIGURE 6 | The schematic diagram of the change of the sample position in 96-well plate. [**(A)** Schematic diagram of old version scheme. **(B)** Schematic diagram of new version scheme. The unmarked locations in the figures are all screening samples].

2.3 Test results of C16 with the old syringe, after increasing washing to between two and four times: The value of C16 had changed slightly, but it failed to reach normal levels as shown in **Table 2**. The results suggest that the number of times washed is not the primary factor in contamination.

2.4 and 2.5 Test results of C16 with the old syringe, after cleaning the old syringe by formic acid before drawing samples: After repeated cleaning by formic acid, the color of formic acid solution changed from colorless to pink (**Supplementary Figure 4**). The value of C16 changed significantly after cleaning by formic acid for 3 min but returned to normal after cleaning by formic acid for 10 min. These results suggest that the syringe is the primary factor of carrying contamination, especially the glass barrel.

It is also worth noting that the value of the second sample after the PE-high-QC sample was close to the normal value.

3. Finally, 53,111 cases were included in the retrospective analysis. The concentrations of the first sample after PE-high-QC were significantly higher than other samples, especially C8:1, C10, C12, C12:1, C14, C14:1, C16, C18, and C18:1OH with a bias over 10% (**Table 3**).

In order to confirm our findings, we contacted two other Newborn Screening Centers. We found similar results, but the indexes with a bias over 10% were different. CIT, C4, C5, C5DC+C6OH, C8, C10, C12, C14, C14:1, and C18:1OH were found in one Newborn Screening Centre, but ARG, CIT, SA, TYR, C2, C3DC+C4OH, C4, C4DC+C5OH, C5DC+C6OH, C6DC, C12, C14, C16, C16:1, C16:1OH, C18, C18:1, C18:1OH, and C18:2 were found in the other Newborn Screening Centre.

4 Applying the research results to the clinic.

4.1 All indicators in the PE-high-QC are several times higher than the screening sample. The first well sample after the PE-high-QC might be contaminated by the syringe. In order to avoid this interference, the Blank well is set between PE-high-QC and the screening sample (**Figure 6**).

4.2 Appling the research results to Routine maintenance. After each experiment, Blank ①, ②, and ③ in **Figure 6** were compared in order to monitor the changes. When Blank ② or ③ was significantly higher than Blank ①, the Syringe was cleaned with formic acid and the cleaning time was 10 min each time (**Supplementary Figure 5**). The Syringe is replaced once a year.

DISCUSSION

In this study, the contamination of syringes was identified by BS-QC samples and patient data. The syringe is generally cleaned with 50% methanol-water mixtures in ordinary maintenance. However, as the sample size increases, the sample residue would remain in the internal part of the glass barrel, especially in the fixation of the needle to the barrel. In addition, as the plunger ages, the tightness of the syringe is reduced, which could exacerbate the sample residue, especially after injecting the PE-high-QC. Therefore, it is necessary to pay attention to the cleaning, maintenance, and replacement of syringe in clinical practice.

Based on the differences in concentrations of patient samples and PE-QC, especially PE-high-QC, we found that the carrying effect of PE-high-QC mainly affected the first patient sample while the subsequent samples were not affected. Therefore, we suggest adding one blank sample after the PE-high-QC. The mode of detection could change from "PE-high-QC→patient sample" to "PE-high-QC→Blank→patient sample." In addition, we should examine the fluctuation of indexes in the Blank sample, such as C16. If the concentration of some indexes in the Blank sample increase significantly after the PE-high-QC, we should clean the syringe with formic acid immediately.

Since the fore-end of the plunger is a rubber plug, it wears out and the tightness of syringe reduces after long-term use. Therefore, we should pay attention to the wear condition of plungers and replace it in time. We recommend replacing the glass barrel every 1–2 years.

CONSENT FOR PUBLICATION

The study did not involve human participants. The research subject is focused around the quality control of materials which came from the United States CDC, Pthe ekinElmer company, and the Biosan company.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was approved by Ethical committee of Nanjing Maternity and Child Health Care Hospital, and 50,000 consecutive cases of newborn screening in 2018 were recruited.

AUTHOR CONTRIBUTIONS

YW led the review process, drafted the initial manuscript, and extracted data. YS reviewed the draft. TJ designed the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.596321/full#supplementary-material

Supplementary Figure 1 | Schematic diagram of the syringe which is composed of glass barrel and plunger.

Supplementary Figure 2 | The possible contamination parts before MS/MS analysis.

Supplementary Figure 3 | Schematic diagram of punch position in dry blood spot of Biosan QC sample (6 "small" blood spot samples were drilled for each "big" blood spot to avoid the center position).

Supplementary Figure 4 | Color change of solution before and after cleaning by formic acid. **(A)** Manual cleaning. The formic acid before cleaning was colorless and transparent. **(B)** The solution after cleaning turned pink.

Supplementary Figure 5 | Schematic diagram of changes of ALA and C16 in Blank before and after cleaning with formic acid.

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Neonatal Screening and Genotype-Phenotype Correlation of 21-Hydroxylase Deficiency in the Chinese Population

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Background: Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders encompassing enzyme deficiencies in the adrenal steroidogenesis pathway that leads to impaired cortisol biosynthesis. 21-hydroxylase deficiency (21-OHD) is the most common type of CAH. Severe cases of 21-OHD may result in death during the neonatal or infancy periods or sterility in later life. The early detection and timely treatment of 21-OHD are essential. This study aimed to summarize the clinical and genotype characteristics of 21-OHD patients detected by neonatal screening in Nanjing, Jiangsu province of China from 2000 to 2019.

Methods: Through a retrospective analysis of medical records, the clinical presentations, laboratory data, and molecular characteristics of 21-OHD patients detected by neonatal screening were evaluated.

Results: Of the 1,211,322 newborns who were screened, 62 cases were diagnosed with 21-OHD with an incidence of 1:19858. 58 patients were identified with the classical salt-wasting type (SW) 21-OHD and four patients were identified with simple virilizing type (SV) 21-OHD. Amongst these patients, 19 cases patients accepted genetic analysis, and another 40 cases were received from other cities in Eastern China. Eighteen different variants were found in the *CYP21A2* gene. The most frequent variants was c.293-13A/C>G (36.29%). The most severe clinical manifestations were caused by large deletions or conversions of *CYP21A2*.

Conclusions: This study suggested that neonatal screening effectively leads to the early diagnosis of 21-OHD and reduces fatal adrenal crisis. Our data provide additional information on the occurrence and genotype-phenotype correlation of 21-OHD in the Chinese population which can be used to better inform treatment and improve prognosis.

Keywords: congenital adrenal hyperplasia, 21-hydroxylase deficiency, neonatal screening, genotype-phenotype, CYP21A2

INTRODUCTION

Congenital adrenal hyperplasia (CAH) (OMIM# 201910) comprises a family of autosomal recessive disorders that are characterized by a group of enzymatic defects in cortisol biosynthesis due to defects in the steroid 21-hydroxylase gene (*CYP21A2*, OMIM* 613815) (Nimkarn et al., 1993; Baumgartner-Parzer et al., 2020). Impaired cortisol production removes negative feedback control on the hypothalamus and pituitary glands that acts to increase the secretion of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) resulting in hyperplasia of the adrenal cortex (Merke and Bornstein, 2005). Steroid 21-hydroxylase deficiency (21-OHD OMIM: 201910) accounts for over 95% of CAH cases (Speiser et al., 2010, 2018; El-Maouche et al., 2017). The impairment of mineral corticoid synthesis causes adrenal crises and electrolyte disorders in infants (Parsa and New, 2017).

21-OHD can be classified as salt-wasting (SW), simple virilizing (SV), and non-classical (NC) types in neonates. SW type is the most severe form of 21-OHD that is responsible for ∼75% of 21-OHD cases. SV type disease accounts for around 25% of cases, whilst NC type is rare in the clinic and accounts for <1% of cases (Parsa and New, 2017). The major clinical manifestations of 21-OHD include hyperpigmentation, ambiguous reproductive organs in female neonates, precocious puberty in male neonates, premature pubarche in children, and short stature in adulthood (Witchel, 2017). In addition to the above symptoms, patients with SW type disease who do not receive treatment may experience electrolyte disorders, hypovolemia and shock due to aldosterone deficiency. Severe cases may even result in death during the neonatal or infancy periods (White, 2009; Gidlöf et al., 2014). As patients show no initial symptoms, NC type disease can be manifested as hyperandrogenism that results in sterility in later life (Parsa and New, 2017).

Clinical evidence has shown ambiguous genitalia mainly in female patients at birth or SW symptoms such as feeding intolerance, vomiting, diarrhea, and skin pigmentation during the neonatal period can lead to the suspicion of 21-OHD. In male patients, particularly those with atypical SW symptoms, 21-OHD can be easily misdiagnosed and so elevated 17-hydroxyprogesterone (17-OHP) levels remain the best biomarker for early diagnosis (Jailer et al., 1955; Miller, 2019). Early diagnosis and long-term standardized treatments can reduce mortality and greatly improve the prognosis of 21-OHD patients (Grosse and Van Vliet, 2007).

This study aimed to characterize the clinical phenotypes and genotype data of 21-OHD patients. We analyzed and summarized the clinical data from the neonatal screening of 17-OHP in China over 20 years to gain insight into the occurrence and genetics of 21-OHD to better inform treatment and improve prognosis.

MATERIALS AND METHODS

Patients

We screened 1211322 neonates born in Nanjing, Jiangsu province of China from January 2000 to December 2019, among the screen-positive newborns, 62 were diagnosed as 21-OHD. They all came from unrelated families.

This study was approved by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital affiliated with Nanjing Medical University. All the parents of participating neonates provided written informed consent.

Neonatal Screening of 17-OHP

When the neonates were 48–72 h after birth with full lactation, 200 μL of heel blood was collected to create a dry blood filter paper. The concentrations of 17-OHP were measured using time-resolved immunofluorescence with a cut-off level of 30 nmol/L from 2000 to 2018 (Wallac 1420: Januar 2000 to December 2013; AutoDEFIA Wallac1235: January 2014 to Octobe 2018, Turku, Finland). From October 2018 to the present day, the cut-off value of 12 nmol/L was established by our laboratory obtained through the analysis of a percentile method combined with ROC curve (Wallac 2021-0010: October 2018 to now). The assay kits were purchased from Perkin Elmer (B015:2000–2013; B016: 2014–2018; B024: 2018 to now, Turku, Finland).

Clinical Diagnosis

Cases with 17-OHP levels greater than the cut-off values (30 nmol/L from January 2000 to October 2018; 12 nmol/L from October 2018 to the present day) twice were suspicious 21-OHD (Cases with low birth weight or premature delivery. If the recall review was still higher than the normal range, sample were retested for the third time when the correct gestational ages were close to 40 weeks or the weights were more than 2500 g). For suspicious cases, blood biochemical criteria (electrolysis quality, ACTH, cortisol, testosterone) were determined and genetic analysis performed. Definitive diagnosis was made base on abnormal biochemical criteria, gene analysis, and clinical manifestation such as hyperpigmentation, vomiting, dehydration, hypotension, hyponatremia, hyperkalemia, a lack of weight gain, shock, and ambiguous genitalia according to the consensus statement on diagnosis and treatment of congenital adrenal hyperplasia due to 21-OHD (Subspecialty Group of Endocrinologic, 2016).

Treatment and Follow-Up

Once the clinical diagnosis is clear, hydrocortisone acetate 15–20 mg/m^2/day should be given immediately, and 9α flurocortisone 0.05–0.15mg/day should be added to SW. Hydrocortisone 8–10mg/ kg should be given intravenously to patients with cortical functional crisis (It is mainly neonatal period) 2–3 times a day and they should be taken orally after the crisis is relieved. During the follow-up, Blood electrolyte, 17-OHP, plasma electrolysis quality and adrenocorticotropic hormone were reexamined every 3 months, bone age was reexamined every 12 months to adjust the treatment dose. The dosage of hydrocortisone acetate increased by 2–3 times when the cases had a fever, diarrhea and other infections. Female patients with hermaphroditism usually complete clitoral orthopedics before 2 years old. Some cases especially with SW may use growth hormone or Tamiflu before puberty.

Locus-Specific PCR

QIAamp DNA blood kits (Qiagen, Venlo, The Netherlands) were used to extract genomic DNA from 3 to 5ml of anticoagulated peripheral blood samples. *CYP21A2* rearrangement products were confirmed by locus-specific PCR. Four primers were designed which specific located upstream and downstream of either *CYP21A2* (ME0008 and ME0066) or CYP21A1P (ME0059 and ME0067) (Lee et al., 1996; Keen-Kim et al., 2005), and the specific PCR products obtained were shown in **Supplementary Table 2**. The 50 μL reaction mixture contained 100 ng DNA template, 1× GC Buffer I, 0.4 mM dNTP, 0.4 μM of each primer, and 2.5 U LA Taq DNA polymerase (TaKaRa, Dalian, China). The PCR amplification conditions were: 94°C for 1 min, then 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 10 min. To ensure the specificity of each reaction, we performed restriction endonuclease analysis of the four PCR amplicons. After PCR, each of the four products was digested with EcoRI (TaKaRa, Dalian, China) for 3 h at 37°C and the digested products were analyzed by electrophoresis on a 1.0% agarose gel. The *CYP21A2* gene contains one EcoRI site in intron 4, while the CYP21A1P pseudogene has two EcoRI sites in intron 2 and exon 4. The EcoRI digestion pattern of amplicons 3 and 4 depended on the location of the recombination breakpoint relative to the EcoRI site in intron 2.

MLPA Analysis

Large gene deletions and conversions in the *CYP21A2* were identified by MLPA analysis using the SALSA MLPA kit (P050-B2 CAH, Amsterdam, Netherlands). This CAH probe mix contains 33 probes, including five probes for *CYP21A2* (exons 1, 3, 4, 6, and 8), three probes for CYP21A1P (exon 1, intron 2, and exon 10), 3 TNXB probes, 1 C4A probe, 1 C4B probe, 1 CREBL1 probe, 2 probes for chromosome 6p21.3, 1 UTY probe, and 16 reference probes. Hybridization, ligation, and amplification were performed according to the manufacturer's protocol. Amplification products were detected using an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA) with LIZ500 (Applied Biosystems) as an internal size standard. The raw data were analyzed by using Coffalyser MLPA data analysis software (MRC Holland) (Ma et al., 2014). The *CYP21A2* mutations were named following Human Genome Variation Society nomenclature guideline (http://www.hgvs.org/mutnomen) by using RefSeq sequence (accession number: NM_000500.7).

Statistical Analyses

Data are expressed as the median and range or the median ± standard deviation. An unpaired, two-way ANOVA test was used for between-group comparisons. Differences were considered significant when $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

Neonatal Screening of 21-OHD During 2000–2019

1,211,322 neonates were screened for 21-OHD who were born in Nanjing, Jiangsu province of China from January 2000 to December 2019 (**Figures 1**, **2**). Sixty-two cases of screen-positive newborns were diagnosed as 21-OHD including 58 SW cases (93.55%) (40 males, 18 females) and 4 SV cases (6.45%) (All males). NC cases were not found in this study. The incidence was one in 19,858 (**Table 1**) and the 17-OHP concentrations and other related indices are summarized in **Tables 2**, **3**. The initial treatment ages of the neonates were 15 ± 6 days, and clinical follow-up after treatment showed that 17-OHP and other biochemical indicators were in the normal range.

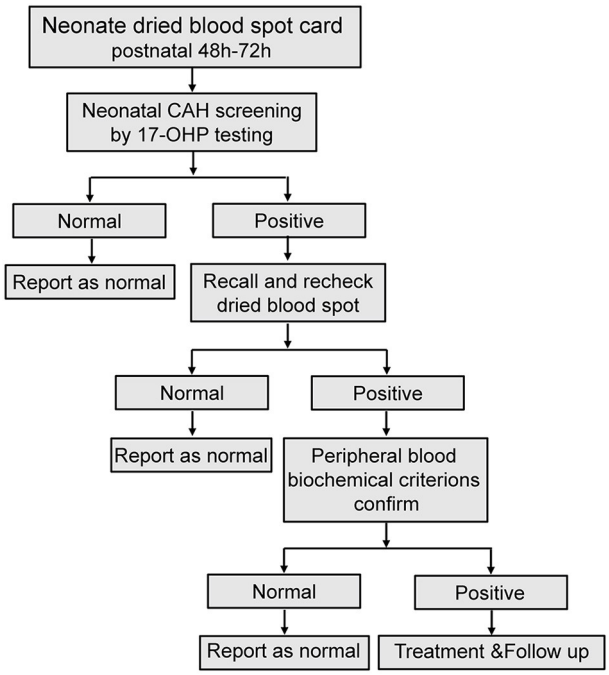

FIGURE 1 | Flowchart for neonatal CAH screening. Recall and recheck dried blood spot recheck, re-analysis with another new dried blood spot from the recalled patient; Peripheral blood biochemical criterions confirm, analysis peripheral blood sample of biochemical criterions from recalled patient.

CYP21A2 Mutation Spectrum Analysis

Since 2014, genetic analysis has been widely used in neonatal screening, particularly for the diagnosis of 21-OHD patients who are suspected as CAH cases based on the presentation of clinical symptoms and biochemical analysis. Nineteen families with 21-OHD patients were born in Nanjing and 40 cases who had newborn screening in other cities in Eastern China and had then come to our hospital for further diagnosis and treatment. These patients accepted genetic analysis and came from unrelated families which none of the parents were consanguineous.

Variant analysis was performed after clinical diagnosis at 1–3 months after birth. A total of 18 variants were found in the 59 patients. The most frequent variant was c.293-13A/C>G (36.29%). Rare pathogenic variants were also found that included c.549+1G>A and c.499G>C. 17 of the variants were reported before (Bidet et al., 2009; New et al., 2013; Hong et al., 2015; Wang et al., 2016; Concolino and Costella, 2018;

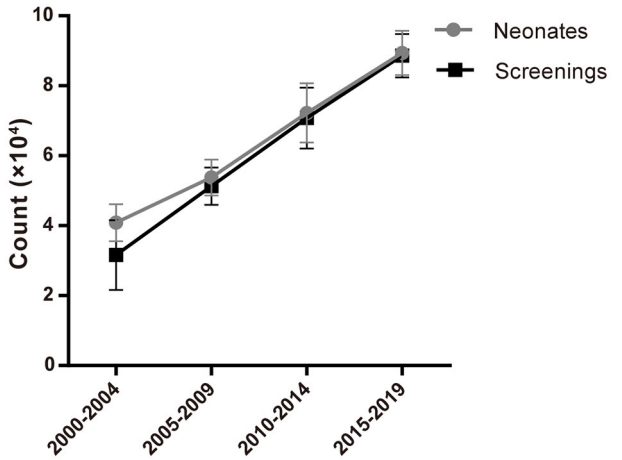

FIGURE 2 | Number of neonates and screenings from 2000 to 2019.

TABLE 1 | Statistical summary of 21-OHD incidence detected by neonatal screening.

Time frame	Neonate	Screening	Positive	Incidence
2000–2004	204275	157973 (77.33%)	4	1:39493 (0.00265%)
2005–2009	268834	256564 (95.44%)	15	1:17104 (0.00598%)
2010–2014	361188	353770 (97.95%)	19	1:18619 (0.00540%)
2015–2019	446762	443015 (99.16%)	24	1:18459 (0.00531%)
2000–2019	**1281059**	**1211322 (94.56%)**	**62**	**1:19537 (0.00512%)**

Bold value indicates total analysis of data from 2000 to 2019.

TABLE 2 | Summary of 17-OHP concentrations from initial screening.

Type	Cases (Males/Females)	Concentration nmol/L	Normal range nmol/L
Salt wasting type	54 (36/18)	386.26 (45.70–1000)	0∼30
	4 (4/0)	198.62 (102.47–350.60)	0∼12
Simple virilizing type	4 (4/0)	52.7 (35.2–83.8)	0∼30

Concentration data are presented as the median (range).

Dundar et al., 2019). One novel mutation of small deletion was detected, g.732_897del166bp, which leads to partial deletion of exon 3 and intron 3 and suspected pathogenic mutation while the specific functional impact is unknown yet (**Figure 3**; **Supplementary Figure 1**).

Relationship Between Genotype and Clinical Phenotype

Enzyme activity is highly correlated with clinical severity of 21-OHD and variants in the *CYP21A2* have differential impacts on enzyme activity (New et al., 2013). In our study, we combined the pattern, degree, and location of mutations with clinical test indicators and clinical manifestations to explore the relationship between different genotypes and clinical phenotypes of 21-OHD. We found that SW 21-OHD patients (28 males and 22 females) with different variants showed different degrees of clinical symptoms. Patients (six males) with large deletions or conversions of *CYP21A2* showed severe clinical manifestations including hyperpigmentation, vomiting, hypotension, hyponatremia, hyperkalemia, and shock, with a high level of 17-OHP (**Table 4**; **Figure 4**). Patients (11 males and 10 females) with partial deletion or conversion and point mutation, showed dehydration, hyperpigmentation, and some of the females had obvious ambiguous genitalia. Patients (three males and seven females) with frameshift and point mutations showed mild dehydration and hyperpigmentation. Females with these variants had different degrees of clitoromegaly. The coding impact of SV 21-OHD patients (six males and three females) was shown to be largely due to point mutations and the most frequent mutation detected as c.515T>A (p. I172N). Patients with SV 21-OHD showed mild hyperpigmentation, without vomiting, diarrhea or obvious genitals malformation with no significant increase in 17-OHP concentration.

TABLE 3 | Laboratory findings for the 17-OHD patients at diagnosis.

Hormone	Units	SW	SV	Normal range
ACTH	pg/ml	185.46 ± 134.80	36.53 ± 23.28	0∼46
Cortisol	ug/dl	4.76 ± 3.58	3.27 ± 2.13	4.26∼24.86(am.) 2.9∼17.3(pm.)
Testosterone	ng/ml	9.12 ± 3.00	7.34 ± 2.42	1.42∼9.23
K	mmol/L	5.98 ± 0.44	-	3.5∼5.5
Na	mmol/L	126.03 ± 1.41	-	135∼145
Cl	mmol/L	101.46 ± 8.31	-	96∼108

Data are presented as the median ± standard deviation.

According to the clinical severity of 21-OHD, the corresponding variants were sorted from the most to least severe and ranked accordingly from large gene deletions and conversions, frameshift variants, point mutations (c.293-13A/C>G; c.1066C>T; c.952C>T; c.518T>A) and c.515T>A (only found in SV patients).

Clinical Follow-Up

In our study, glucocorticoid and mineralocorticoid replacement therapy were used for continuous treatment of 21-OHD patients who were followed up to allow the medication to be adjusted. In these patients, females with more severe 21-OHD also received genital reconstruction and had a good prognosis. The follow-up results showed that the condition of patients had significantly improved.

DISCUSSION

Neonatal screening for CAH has been successfully implemented in China and demonstrated to effectively prevent adrenal crisis, reduce mortality and reduce the harm associated with hyperandrogenism. Our research found that from 2000 to 2019, the number of newborns increased year by year along with an increased proportion of neonatal screening over the same period

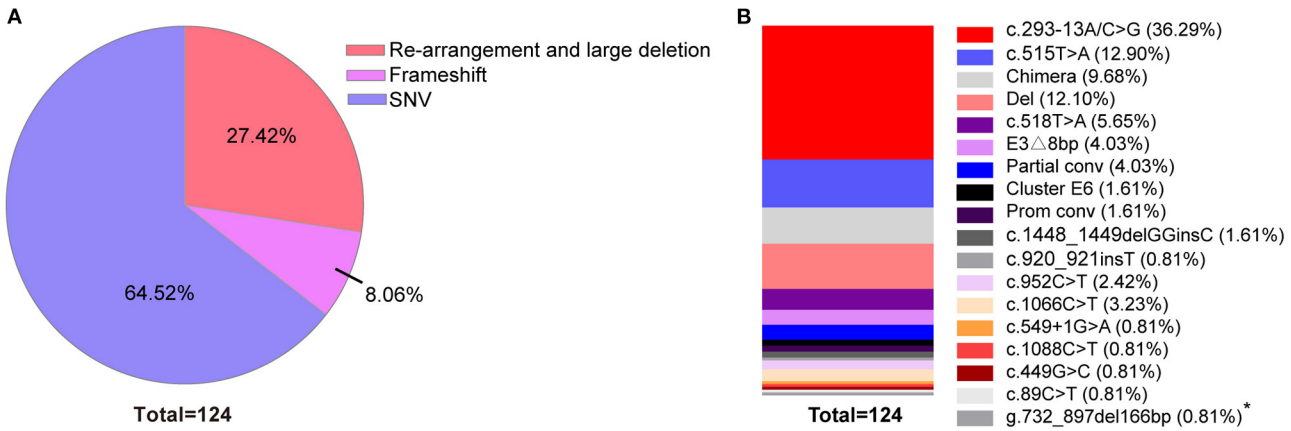

FIGURE 3 | Percentage of variants across 59 classic 21-OHD patients. **(A)** The percentage of variants classified by re-arrangement and large deletion, frameshift and SNV. **(B)** The percentage of each variants shown by vertical slice-chart. (n = 124). *Variant site which not been reported to date.

(**Figure 1**; **Table 1**). These observed trends further highlight the importance of neonatal screening and its increased use in providing the timely detection and treatment of newborn diseases. Also, improvements in newborn screening procedures will provide further information in the improved diagnosis and treatment of more newborn diseases.

Our data show that the incidence of CAH had obvious differences according to race (Khalid et al., 2012; New et al., 2013; Heather et al., 2015; Tsuji et al., 2015; Kopacek et al., 2017; Güran et al., 2019; Hou et al., 2019). For instance, the incidence of CAH in Tokyo, Japan has been reported as one in 19,859 (Tsuji et al., 2015) which is similar to the incidence in Nanjing, Jiangsu province of China (**Table 1**). This compares to levels in Great Britain of one in 166,667 and Turkey of one in 7787 (Khalid et al., 2012; Güran et al., 2019).

The 21-OHD enzyme is encoded by the *CYP21A2* gene (OMIM: 613815) located on chromosome 6p21.33 (Concolino et al., 2017; Simonetti et al., 2018). The cDNA is 2 kb long and the encoded protein is predicted to contain 494 amino acids. Approximately 30 kb from the *CYP21A2* there is a non-functional pseudogene, *CYP21A1P*. Both the functional gene and the pseudogene share a high level of nucleotide sequence homology of 98% in the exon regions and 96% homology in the introns (Gidlöf et al., 2014). To date, more than 1,300 genetic variants of *CYP21A2* have been reported (http://www.hgmd.cf.ac.uk) but only 230 have been shown to affect human health.

By analyzing the 17-OHP concentrations corresponding to 21-OHD patients of different severity, we found that the concentration of 17-OHP was significantly different between SW and SV patients (**Tables 2**, **4**; **Figure 4**). Furthermore, in SW patients, we found that patients with large deletions and conversions of *CYP21A2* were correlated with severe clinical manifestations. These patients also had higher levels of 17-OHP compared to SW patients with other variants (**Table 4**; **Figure 4**). These data suggest the detection of 17-OHP can be used to infer the severity of clinical manifestations in SW patients.

Genotypes are closely related to clinical phenotypes (Khalid et al., 2012; Neocleous et al., 2018; Xu et al., 2019). In a report from Southern China, the most frequent mutation was c.293-13A/C>G (41.1%) (Hou et al., 2019) which is in agreement with our data on neonatal CAH screening (**Figure 3B**; **Supplementary Figures 1B,D**). However, European data show that the most frequent mutation was c.841G>T (23.9%), followed by c.293-13A/C>G (New et al., 2013) indicating that mutations of *CYP21A2* may also have different prevalence across different races.

In our study, we found that in SW cases, the most frequent variants were c.293-13A/C>G and deletions. In SV cases the most frequent variants were c.515T>A. Given the complexity of genetic mutations and the existence of pseudogenes, mutations cannot be accurately detected using a single technology and so we established a combined approach using three different technologies. Gene sequencing was used to detect small mutations, whilst locus-specific PCR and MLPA analysis were used to detect large deletions or gene conversion mutations.

The safety of long-term use of prednisone (PD) vs. hydrocortisone (HC) in the treatment of children with 21-OHD of CAH remains controversial (Riepe and Sippell, 2007; Ahmed et al., 2019). Several issues related to patient growth and the final height of children remain to be fully resolved (Bonfig, 2017). The increased risk of developing obesity is another possible consequence of hypercortisolism in children with CAH (Völkl et al., 2006). Our treatment was mainly based on the administration of intravenous hydrocortisone during adrenal crisis (mainly in the neonatal period with high potassium and low sodium) at a dose of 100 mg/kg twice a day and oral administration of 9α-flurocortisone. Hydrocortisone acetate (10–15 mg/m2, every 8 h) and 9α-flurocortisone were given orally after the condition had stabilized. Based on our clinical experience, the dose of hydrocortisone acetate should be kept at a low level and the normal high limit of 17-OHP should be maintained. Also, careful monitoring of blood hormone levels, growth rate and bone age should be performed.

TABLE 4 | Variant distributions in 21-OHD patients.

Index	Coding impact	Genotype	Variant location	17-OHP (nmol/L)	ACTH (pg/ml)	Cortisol (ug/dl)	T (ng/ml)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	CAH phenotype	Severity	Sex (Male/ Female)	Patients proportion
1	Large gene deletions and conversions	Del/Del		647.68 ± 169.62	124.49 ± 0.36	35.99 ± 62.07	27.79 ± 13.04	127.50 ± 124.85	6.45 ± 5.81	97.93 ± 97.21	SW	++++	2/0	2/59
2		Del/Chimera									SW	++++	2/0	2/59
3		Chimera/Cluster E6	Exon 6								SW	++++	1/0	1/59
4		Chimera/c.549+1G>A	Exon 4								SW	++++	1/0	1/59
5	Gene deletions or conversions and point mutations	Del/c.293-13A/C>G	Intron 2	303.97 ± 223.33	339.38 ± 239.19	16.47 ± 11.91	11.34 ± 16.75	124.60 ± 7.76	5.86 ± 1.08	97.28 ± 6.76	SW	+++	3/3	6/59
6		Del/c.952C>T	Exon 8								SW	+++	0/1	1/59
7		Del/c.1088C>T	Exon 8								SW	+++	0/1	1/59
8		Del/c.515T>A	Exon 4								SW	+++	1/0	1/59
9		Chimera/c.518T>A	Exon 4								SW	+++	3/0	3/59
10		Chimera/c.293-13A/C>G	Intron 2								SW	+++	1/2	3/59
11		Chimera/c. [293-13A/C>G; 952C>T]	Intron 2; Exon8								SW	+++	0/1	1/59
12		Chimera/c.515T>A	Exon 4								SW	+++	0/1	1/59
13		Partial conv/c.293-13A/C>G	Intron 2								SW	+++	3/0	3/59
14		Partial conv/ Prom conv; c. 293-13A/C>G	Promoter; Intron 2								SW	+++	0/1	1/59
15	Frameshift mutation and point mutation	g.732_897del166bp/ c.515T>A	Exon 3- Intron 3; Exon 4	288.70 ± 199.92	185.76 ± 135.10	8.63 ± 4.45	3.3 ± 2.52	132.84 ± 3.25	5.06 ± 0.65	104.5 ± 6.18	SW	++	1/0	1/59
16		c.1448_1449delGGinsC/c.293-13A/C>G	Exon10; Intron 2								SW	++	0/1	1/59
17		c.1448_1449delGGinsC/ c.515T>A	Exon10; Exon 4								SW	++	1/0	1/59
18		c.293-13A/C>G/c.293-13A/C>G; E3Δ8nt	Intron 2; Exon3								SW	++	1/0	1/59
19		E3Δ8nt/c.293-13A/C>G	Exon3; Intron 2								SW	++	0/3	3/59
20		c.920_921insT/c.515T>A	Exon7/4								SW	++	0/1	1/59
21		Prom conv; c.89C>T / c.293-13A/C>G; E3Δ8nt	Promoter; Intron 2; Exon 1/3								SW	++	0/1	1/59
22		Cluster E6/c.518T>A	Exon 4/6								SW	++	1/0	1/59
23	Point mutations	c.293-13A/C>G/c.293-13A/C>G	Intron 2	327.37 ± 154.88	202.57 ± 163.15	11.75 ± 8.60	6.29 ± 5.59	125.71 ± 12.21	5.70 ± 1.13	97.47 ± 8.09	SW	++	6/2	8/59
24														
25		c.293-13A/C>G/c.1066C>T	Intron 2; Exon 8								SW	++	0/2	2/59
26		c.293-13A/C>G/ c.952C>T	Intron 2; Exon 8								SW	++	1/0	1/59

(Continued)

TABLE 4 | Continued

Index	Coding impact	Genotype	Variant location	17-OHP (nmol/L)	ACTH (pg/ml)	Cortisol (ug/dl)	T (ng/ml)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	CAH phenotype	Severity	Sex (Male/ Female)	Patients proportion
27		c.518T>A/c.518T>A	Exon 4								SW	++	1/1	2/59
28		c.293-13A/C>G/c.515T>A	Intron 2; Exon 4	132.22 ± 105.79	61.38 ± 86.63	12.16 ± 1.03	2.94 ± 1.48	-	-	-	SV	+	4/2	6/59
29		c.515T>A/c.1066C>T	Exon 4/8								SV	+	2/0	2/59
30		c.515T>A/c.515T>A	Exon 4								SV	+	0/1	1/59

Data are presented as the median ± standard deviation. Del, entire CYP21A2 gene deletion; Chimera, CYP21A1P/CYP21A2 chimeric gene, exon 1- exon 3; Partial conv, partial conversion; Prom conv, promoter conversion (g. [−126C>T;−113G>A;−110T>C;−103A>G]); E3Δ8nt, c.329_336delGAGACTAC; Cluster E6, c. [707T>A; 710T>A; 716T>A]. Severity of clinical manifestations is scaled (+ to + + ++). These were defined as follows; Obvious hyperpigmentation of the skin over the entire body, vomiting, diarrhea, hypotension, hyponatremia, hyperkalemia, and shock (+ + ++); Dehydration, hyperpigmentation and some of the females showing obvious ambiguous genitalia (+ + +); Mild dehydration and hyperpigmentation, some of the females showing different degrees clitoromegaly (++); Mild hyperpigmentation only around the perineum and areola, without vomiting, diarrhea or obvious genitals malformation (+).

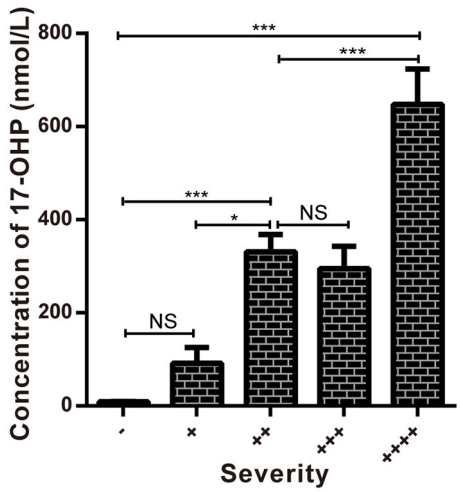

FIGURE 4 | Comparison of the concentrations of 17-OHP in different severity groups. *$P < 0.05$ ***$P < 0.001$; NS, no significance.

A major issue in neonatal screening programs for 21-OHD with the detection of 17-OHP is the high rate of false-positives (Cavarzere et al., 2009; Fingerhut, 2009), particularly in preterm neonates (van der Kamp et al., 2005). Urinary steroid metabolite analysis using gas chromatography-mass spectrometry (GC-MS) is a suitable diagnostic tool to determine 17-hydroxyprogesterone levels (Speiser et al., 2018), however, this approach is not suitable for the rapid, high throughput analysis of large numbers of samples. Therefore, we explored the potential of adding more specific markers for joint detection based on 17-OHP, such as 21-Deoxycortisol (21-deoxy) (Jailer, 1953; Jailer et al., 1955; Miller, 2019). This method could dramatically reduce the rate of false-positives and could be a robust approach for large scale analysis in the future.

Another important issue concerning neonatal screening programs for 21-OHD is that the detection of 17-OHP cannot detect NC-CAH newborns. Individuals with the NC form may be compound heterozygous with one severe and one mild pathogenic variant. They may also be homozygous with two mild pathogenic variants in which 20–60% residual enzymatic activity can be preserved (Gidlöf et al., 2013). Enzyme activity is closely correlated with clinical severity (New et al., 2013). Patients with NC-CAH exhibit a mild phenotype and are rarely accompanied by an increase in 17-OHP during the neonatal period. In these cases, the clinical symptoms are also not obvious in newborns but are observed in adolescents and adults due to androgen excess including premature pubarche, acne, hirsutism, polycystic ovary syndrome, and subfertility (Marino et al., 2011). Further determination of NC-CAH requires investigation using ACTH-stimulation tests (Livadas et al., 2015). Due to a large number of newborns, it is difficult to achieve stimulation tests during newborn screening and this approach is yet to be widely implemented. Currently, there is no NC-CAH newborn screening program.

In contrast to classical CAH patients, adrenal replacement is not required as a therapy for NC-CAH patients. The management of excess androgens uses antiandrogens and oral contraceptives to improve long-term outcomes. Glucocorticosteroid (GCS) therapy can be used to obtain special outcomes such as restoration of fertility (Auchus and Arlt, 2013). To effectively treat children with CAH including patients with NC-CAH, the conditions should be detected as early as possible. Secondary screening using technologies such as next-generation sequencing should be carried out as early as possible to ensure more comprehensive and effective screening in neonatal diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Nanjing Maternity and Child Health Care Hospital affiliated with Nanjing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YS and TJ designed the research. XW analyzed data and wrote the manuscript with contributions from all of the authors. YW, DM, and ZZ carried out the screening tests. YL and PY contributed to the follow-up. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.623125/full#supplementary-material

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frontiers in Pediatrics

SYSTEMATIC REVIEW
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Newborn Screening for Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase and Mitochondrial Trifunctional Protein Deficiencies Using Acylcarnitines Measurement in Dried Blood Spots—A Systematic Review of Test Accuracy

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Background: Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies are rare autosomal recessive fatty acid β-oxidation disorders. Their clinical presentations are variable, and premature death is common. They are included in newborn blood spot screening programs in many countries around the world. The current process of screening, through the measurement of acylcarnitines (a metabolic by-product) in dried blood spots with tandem mass spectrometry, is subject to uncertainty regarding test accuracy.

Methods: We conducted a systematic review of literature published up to 19th June 2018. We included studies that investigated newborn screening for LCHAD or MTP deficiencies by tandem mass spectrometry of acylcarnitines in dried blood spots. The reference standards were urine organic acids, blood acylcarnitine profiles, enzyme analysis in cultured fibroblasts or lymphocytes, mutation analysis, or at least 10-year follow-up. The outcomes of interest were sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Assessment of titles, abstracts, and full-text papers and quality appraisal were carried out independently by two reviewers. One reviewer extracted study data. This was checked by a second reviewer.

Results: Ten studies provided data on test accuracy. LCHAD or MTP deficiencies were identified in 23 babies. No cases of LCHAD/MTP deficiencies were identified in four studies. PPV ranged from 0% (zero true positives and 28 false positives from 276,565 babies screened) to 100% (13 true positives and zero false positives from 2,037,824 babies screened). Sensitivity, specificity, and NPV could not be calculated as there was no systematic follow-up of babies who screened negative.

Conclusions: Test accuracy estimates of screening for LCHAD and MTP deficiencies with tandem mass spectrometry measurement of acylcarnitines in dried blood were variable in terms of PPVs. Screening methods (including markers and thresholds) varied between studies, and sensitivity, specificity, and NPVs are unknown.

Keywords: systematic review, test accuracy, LCHAD deficiency, MTP deficiency, newborn blood spot screening

INTRODUCTION

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies (Enzyme Commission Number 1.1.1.211) are recessive autosomal fatty acid β-oxidation disorders. They are caused by mutations in the genes coding for MTP. LCHAD deficiency arises as a result of mutations in the HADHA gene; MTP deficiency arises from mutations in HADHA and HADHB genes (1). The conditions are characterized by lethargy, hypoglycemia, hypotonia, cardiomyopathy, and acute metabolic crisis (2, 3). Long-term complications include liver disease, peripheral neuropathy, and retinopathy (3, 4). Signs and symptoms may present immediately after birth or later in life (5). Three main forms of LCHAD/MTP deficiencies have been reported: an early-onset form, which is associated with cardiomyopathy, hypoglycemia, and sudden infant death; an infant-onset form, which is characterized by recurrent hypoketotic hypoglycemia and lethargy during illness or fasting; and a milder, late-onset form that is triggered by exercise, fasting, or infections and is associated with progressive peripheral neuropathy and recurrent rhabdomyolysis (6, 7). There is no cure for LCHAD or MTP deficiencies, and premature death is common. Approximately 38% of infants die before, or within 3 months of, diagnosis (5). A number of management strategies are available, namely a high-carbohydrate and fat-modified/decreased diet that is low in long-chain fatty acids, supplements (L-carnitine, docosahexaenoic acid, and medium-chain triglyceride oil, such as triheptanoin), and avoidance of fasting (1). There is some evidence that these treatments are associated with improved clinical outcomes (e.g., reduced mortality, delayed visual complications), but the effects are variable, study sample sizes are small, and few data are available from long-term follow-up studies (1, 8–10). The incidence of LCHAD and MTP deficiencies varies widely around the world. A recent estimate from the USA gives an incidence of 1:363,738 for LCHAD deficiency and 1:1,240,467 for MTP deficiency (11).

It has been proposed that earlier recognition and treatment of LCHAD/MTP deficiencies may be critical for improving health outcomes (5), and the two conditions are included in newborn screening programs in many countries. Screening is conducted through the measurement of acylcarnitines (primarily C16OH, C16:1 OH, C18OH) in dried blood spots using tandem mass spectrometry (TMS). These markers are not specific to LCHAD/MTP deficiencies, however, and levels can be raised in other conditions (e.g., carnitine palmitoyltransferase II deficiency, very long-chain acyl-CoA dehydrogenase deficiency) and in babies of very low birth weight/being treated in neonatal intensive care (12). The results of a recent report from the UK suggest that screening for LCHAD/MTP deficiencies would not lead to the identification of additional cases as compared with the current practice of clinical detection (13). In contrast, data from other countries have suggested that screening does lead to earlier detection of LCHAD/MTP deficiencies (14, 15).

To date, there has been one systematic review examining test accuracy of screening for LCHAD/MTP deficiencies (16). Searching up to 2012, Einoder-Moreno et al. (16) identified six studies and concluded that sensitivity, specificity, and negative predictive value (NPV) of acylcarnitine measurement in dried blood spots are close to 100%, and that the positive predictive value (PPV) ranges from 9 to 100%. However, three relevant papers were missed by their search (12, 17, 18), and the calculation of sensitivity, specificity, and negative predictive value were based on an assumption about the disease status of babies who screened negative, as no follow-up of these babies was conducted in the included studies. This approach can lead to overestimation of sensitivity and underestimation of specificity (19). The aim of the current paper, therefore, is to conduct a systematic review of test accuracy metrics (sensitivity, specificity, positive and negative predictive values) of acylcarnitine measurement in newborn screening dried blood spots (DBS) for LCHAD/MTP deficiencies using tandem mass spectrometry using a broader search than the previous review and taking into consideration whether or not babies who screen negative received follow-up assessment.

Abbreviations: C14OH, 3-hydroxytetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; C16OH, 3-hydroxypalmitoylcarnitine; C16OH/C16, 3-hydroxypalmitoylcarnitine/palmitoylcarnitine; C16:1OH, 3-hydroxypalmi toleylcarnitine; C18OH, 3-hydroxystearoylcarnitine; C18:1OH, 3-hydroxyoleoylcarnitine; C18:1, oleoylcarnitine; DBS, dried blood spot; FP, false positive; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; MTP, mitochondrial trifunctional protein; NPV, negative predictive value; PPV, positive predictive value; QUADAS-2, Quality Assessment Tool for Diagnostic Accuracy Studies 2; TMS, tandem mass spectrometry; TP, true positive.

MATERIALS AND METHODS

The review protocol is registered at PROSPERO (registration number CRD42018094356).

Search Strategy

We conducted a search of the following electronic databases: MEDLINE, MEDLINE In-Process, MEDLINE Daily, MEDLINE ePub Ahead of Print, the Cochrane Library, Web of Science, and Embase. Search terms (free text and subject headings)

related to the disease area (e.g., "mitochondrial trifunctional protein," "long-chain-3-hydroxyacyl-CoA dehydrogenase," "fatty acid oxidation disorder") and screening (e.g., "newborn screening," "dried blood spot," "tandem mass spectrometry"). Full details of the search are provided in **Supplement 1**. The reference lists of included articles and relevant systematic reviews were also examined. The search was conducted on 19th June 2018, with no restrictions on the publication date or language of articles.

Eligibility Criteria

We included journal articles and reports that investigated newborn screening for LCHAD or MTP deficiencies by TMS analysis of acylcarnitines in dried blood spots. The reference standards were urine organic acids, blood acylcarnitine profiles, enzyme analysis in cultured fibroblasts or lymphocytes, mutation analysis, or at least 10-year follow-up. These could be on their own or in any combination. Appropriate study designs were cross-sectional test accuracy studies, case-control studies, and cohort studies. The outcomes of interest were sensitivity, specificity, PPV, and NPV (or sufficient data to allow us to calculate these). We excluded non-human studies, letters, editorials, communications, conference abstracts, gray literature, studies of fatty acid β-oxidation disorders where data for LCHAD/MTP deficiencies could not be separated from data for other conditions, studies with no extractable data, and studies where more than 10% of the study sample did not meet our inclusion criteria.

Screening and Data Extraction

Titles, abstracts, and full-text papers were independently screened by two reviewers. Data extraction was conducted by a single reviewer and checked by a second reviewer. At each stage of the review, disagreements were resolved through discussion between the reviewers, with the involvement of a third reviewer if consensus could not be achieved.

Quality Appraisal

Two reviewers independently assessed risk of study bias and applicability concerns using the Quality Assessment Tool for Diagnostic Accuracy Studies (QUADAS-2) (20), which was tailored to the research question. Tailoring comprised defining cut-offs for exclusions, identifying appropriate reference standards, selecting a suitable interval between index tests and reference standards, and producing guidance notes. Disagreements were resolved through discussion by the two reviewers, leading to a consensus on assessment of risk of bias and applicability concerns for all studies. The QUADAS-2 tool is presented in **Supplement 2** and the guidance notes in **Supplement 3**.

Data Summary and Synthesis

Due to incomplete 2×2 tables and heterogeneity between study designs, a narrative summary of the evidence is provided. We calculated confidence intervals for test accuracy metrics using the Wilson score method with continuity correction (21).

RESULTS

Searching, Sifting, and Sorting

Full details of the flow of studies through the review are outlined in **Figure 1**. One thousand one hundred and ninety-four unique records were identified through searching electronic databases. After examination of titles and abstracts, 39 papers were retained for full-text assessment. Eleven of these papers met the review's inclusion criteria (12–15, 17, 18, 22–26). Two papers included overlapping cohorts (17, 22). Only the data from the larger, more recent paper by Lindner et al. (17) [which included all of the data from Schulze et al. (22)] are reported here. A list of excluded studies [with reasons for exclusion] is provided in **Supplement 4**.

Quality Appraisal

A summary of the risks of bias and applicability concerns of the included papers is provided in **Figure 2**. Ratings of risks of biases and applicability concerns for each individual study are provided in **Supplement 5**.

Risk of bias was considered to be high in two or more domains for nine of the 10 studies (90%) (12–14, 17, 18, 23–26) and in one domain for the remaining study (10%) (15). In the patient selection domain, risk of bias was rated as unclear in seven (70%) studies due to unclear/incomplete reporting (12, 15, 17, 23–26), and low in three (30%) studies (13, 14, 18). There were considerable concerns regarding applicability to the UK context in seven (70%) studies as blood samples were taken before day 5 in six studies (12, 15, 17, 18, 23, 25), and the incidence of MTPD was lower than expected in one study (1:300,000 compared with 1:149,254 in the UK) (24). Applicability concerns were unclear in two studies (14, 26) and low in one study (13).

In the index test domain, five studies (50%) were rated as having high risk of bias as the cut-off for "screen positive" was altered during the study period (13) or was not pre-specified (12, 23, 24, 26). Of the remaining studies, two were at unclear risk of bias (18, 25) and three were at low risk of bias (14, 15, 17). Two (20%) studies had high applicability concerns as one included additional markers (C14:1, C14-OH) (15) and one included both blood and urine samples (18). Applicability concerns were unclear in two studies (17, 25) and low in six studies (12–14, 23, 24, 26).

In the reference standard domain, risk of bias was rated as unclear in all 10 studies because it was not possible to tell the reference standard results were interpreted without knowledge of the results of the index test (12–15, 17, 18, 23–26). Applicability concerns were unclear in six (60%) studies (12, 15, 23–26) and low in the remaining four (40%) (13, 14, 17, 18).

Finally, all studies were judged to be at high risk of bias in the flow and timing domain (12–15, 17, 18, 23–26). The reasons for this were that the reference standards used to confirm disease status for screen positives and screen negatives were not the same, follow-up of those children who screened negative was not defined or not conducted, and losses to follow-up were not reported.

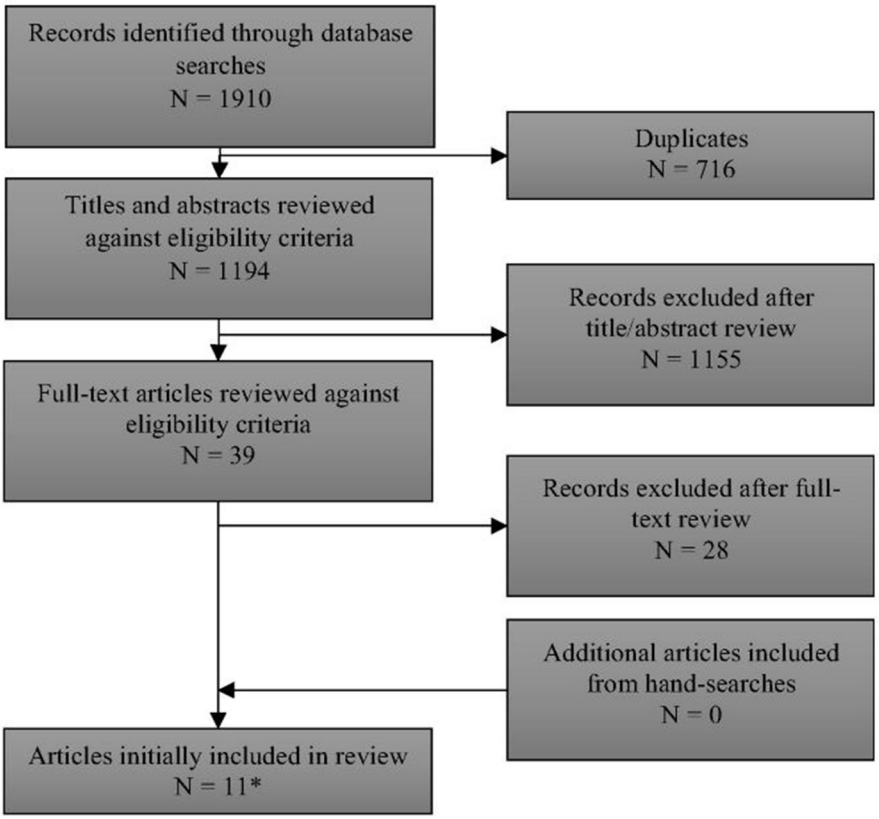

FIGURE 1 | PRISMA flow diagram of records through the systematic review. *See **Supplement 4** for list of excluded studies with reasons.

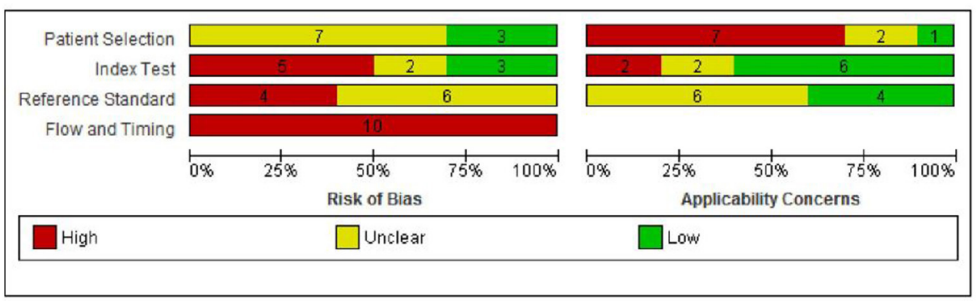

FIGURE 2 | Risk of bias and applicability concern graph: review authors' judgments about each domain presented as percentages across included studies.

Characteristics of Included Studies

Details of the included studies are provided in **Table 1** and **Supplement 6**. Ten studies were included, four pilot programs (12, 13, 23, 25), three mixed pilot and national/regional screening programs (14, 17, 24), and three national/regional screening programs (15, 18, 26). Six studies took place in Europe [Denmark, Faroe Islands, and Greenland (14), Germany (15, 17), Slovenia (23), Spain (18), UK (13)], two in Asia (China (26), Hong Kong (25)), and two in North America [USA (12, 24)]. Sample sizes ranged from 2,440 (25) to 1,200,000 (15). In total, the 10 studies screened 3,951,358 newborns. Twenty-three true positives (TP) and 40 false positives (FP) were identified. The 23 true positives comprised 11 babies with LCHAD deficiency, two with MTP deficiency, and 10 babies for whom LCHAD and MTP deficiencies were not differentiated.

Description of Screening and Diagnostic Tests

Details of the screening methodology and diagnostic tests used in each study are provided in **Supplement 6**.

TABLE 1 | Accuracy of newborn screening tests for LCHADD/MTPD using TMS measurement of acylcarnitines.

References	Number screened	Marker(s)	Cut-off(s) (μmol/L)	2 × 2 table				Test accuracy[a]			
				TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)*	NPV (95% CI)
Couce et al. (18)	210,165	C16OH, C18:1OH, C18OH	NR	2[b]	0	NR	NR	NA	NA	1 (0.20, 1)	NA
Frazier et al. (24)	239,415	C16OH, C18:1, C18:1OH	>0.18, >4.08, >0.14	2[b]	0	NR	NR	NA	NA	1 (0.20, 1)	NA
Lindner et al. (17)	1,084,195	C14OH (and/or): C16:1OH; C16OH; C18:1OH; C18OH	>0.12, >0.22, >0.20, >0.12, >0.11	6 (5 NBS, 1 cascade testing)[b]	0	NR	NR	NA	NA	1 (0.52, 1)	NA
Lund et al. (14)	504,049	Primary: C16OH; secondary: C18:1OH	>0.12U, >0.1U	3[c]	0	NR	NR	NA	NA	1 (0.31, 1)	NA
Mak et al. (25)	2,440	Unclear	Unclear	0	2	NR	NR	NA	NA	0 (0, 0.80)	NA
Sander et al. (15)	1,200,000	C16OH, C18:1OH, C14:1, C14OH	>0.08, >0.06, >0.35, >0.2	9 (7 LCHADD, 2 MTPD)	10	NR	NR	NA	NA	0.47 (0.25, 0.71)	NA
Smon et al. (23)	10,048	C16:1OH, C16OH, C18:1OH, C18 OH, C16OH/C16	0.042[d] , 0.028[d], 0.014[d], 0.009[d], 0.015[d]	0	8	NR	NR	NA	NA	0 (0, 0.40)	NA
UK NSC (13)	436,969	Primary: C16OH; secondary: C16-OH; C16:1-OH; C18-OH	>0.12, >0.15–lowered from 0.2	1[c]	2	NR	NR	NA	NA	0.33 (0.02, 0.87)	NA
Yang et al. (26)	100,077	LCHADD: C16OH; MTPD: C18OH, C18:1OH	>0.04, >0.03, >0.05	0	13 (4 LCHADD, 9 MTPD)	NR	NR	NA	NA	0 (0, 0.28)	NA
Zytkovicz et al. (12)	164,000	C16OH/d-C16	0.1[e]	0	5	NR	NR	NA	NA	0 (0, 0.54)	NA

[a]*Confidence intervals calculated using Wilson score method with continuity correction.* [b]*LCHAD deficiency.* [c]*LCHAD/MTP deficiencies.* [d]*Data provided by study authors.* [e]*Determined during the study to ensure that no more than 0.02% of population would be flagged. CI, confidence interval; FN, false negative; FP, false positive; LCHADD, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; MTPD, mitochondrial trifunctional protein deficiency; NA, not applicable; NBS, newborn bloodspot; NPV, negative predictive value; NR, not reported; PPV, positive predictive value; TN, true negative; TP, true positive; UK NSC, UK National Screening Committee. *The values were calculated by the review authors.*

In brief, eight different analytes or ratios were used as markers to screen for LCHAD/MTP deficiencies: 3-hydroxytetradecanoylcarnitine (C14OH), tetradecenoylcarnitine (C14:1), 3-hydroxypalmitoylcarnitine (C16OH), 3-hydroxypalmitoleylcarnitine (C16:1OH), 3-hydroxystearoylcarnitine (C18OH), 3-hydroxyoleoylcarnitine (C18:1OH), oleoylcarnitine (C18:1), and 3-hydroxypalmitoylcarnitine/palmitoylcarnitine (C16OH/C16), with each study employing a unique combination. The cut-offs used varied between studies (e.g., cut-offs ranged from >0.04 to >0.20 μmol/L for C16OH, >0.03 to >0.15 μmol/L for C18OH, and >0.05 to >0.14 μmol/L for C18:1OH), with no two studies using the same combination of markers and thresholds. Screening samples were collected between 24 h and day 37 of life (25, 26).

The reference standards used varied between and within studies. For screen-positive babies, reference standards were blood acylcarnitines, urinary organic acids, and DNA analysis

(13, 24, 25); enzyme and/or molecular studies (18); enzyme activity in fibroblasts/lymphocytes and mutation analysis (15); acylcarnitine profile in plasma/DBS and/or genotype and/or enzyme activity (17); urine organic acids, plasma acylcarnitines, and molecular-genetic analyses (14); organic acid in urine, next-generation sequencing, and an additional acylcarnitine profile in DBS (23); and urinary organic acids or DNA analysis (26). Lastly, one study used "standard metabolic criteria" (12). No systematic follow-up of babies who screened negative was conducted in any of the studies.

Accuracy of Screening Tests

The cut-offs used to classify a positive case of LCHAD/MTP deficiencies and the diagnostic tests used to confirm this varied between studies. Therefore, we report positive screening results as those that met/exceeded the cut-off and were diagnostically confirmed as presented in the individual study. Test accuracy data are show in **Table 1**.

Positive and Negative Predictive Values

PPV varied considerably between studies. It was 0% in four studies, with zero true positives and 28 false positives from 276,565 babies screened (12, 23, 25, 26), 33% in one study, with one true positive and two false positives from 436,969 babies screened (13), 47% in one study, with 9 true positives and 10 false positives from 1,200,000 babies screened (15), and 100% in four studies, with 13 true positives from 2,037,824 babies screened (14, 17, 18, 24). In the UK study, the single case reported as a true positive was being treated for LCHADD at the point of screening, as they had already been detected clinically (13). Confidence intervals were wide due to the small number of cases of LCHAD/MTP deficiencies detected (23 in total, zero to nine per study). It was not possible to calculate NPV as newborns who screened negative were not systematically followed up.

Sensitivity and Specificity

We were not able to determine sensitivity or specificity due to a lack of information on babies who screened negative.

DISCUSSION

We assessed the test accuracy of acylcarnitine measurement in newborn DBS using TMS for LCHAD/MTP deficiencies. Ten relevant studies were identified. All studies had a high risk of bias in at least one domain, and 9/10 (90%) studies had a high risk of bias in at least two domains. Across the 10 studies, ∼4,000,000 babies were screened and 23 cases of LCHAD/MTP deficiencies were identified; 11 babies had LCHAD deficiency, two had MTP deficiency, and 10 had undifferentiated LCHAD/MTP deficiencies. One of the cases reported as a true positive had already been detected clinically at the point at which screening took place (13). Arguably, the PPV for this study should be 0% rather than 33%, as reported in the study. Forty additional babies screened positive but were subsequently found not to have LCHAD or MTP deficiency. In four studies, no cases of LCHAD/MTP deficiencies were identified (12, 23, 25, 26). However, in three of these studies, the sample sizes were too small to be likely to detect such rare diseases [screening population sizes were 2,440 (25), 10,048 (23), and 100,077 (26)]. The fourth study included a larger sample ($n = 164{,}000$) but only included one marker (C16OH), which might have made the screening process less accurate (12).

The only measure of test accuracy that was consistently reported (or where sufficient data were present to allow us to calculate it) was PPV. PPV in the 10 studies ranged from 0% (zero true positives and 28 false positives from 276,565 babies screened) to 100% (13 true positives from 2,037,824 babies screened). It was not possible to calculate sensitivity, specificity, or NPV as there was no systematic follow-up of babies who had screened negative. In a pilot or national screening program for a rare disease using a "promising" test, negative tests will inevitably represent the vast majority of test results. While some studies provided very high PPV, PPV is not intrinsic to the test itself, and at any particular values of sensitivity and specificity, the estimates of PPV (and also NPV) are strongly dependent on disease prevalence. This relationship is illustrated in **Supplement 7** over a range of prevalence values similar to those in the included studies and over a range of specificity values. In order to provide a complete assessment of test accuracy, all four metrics (sensitivity, specificity, NPV, PPV) are required.

Whether newborn screening for LCHAD/MTP deficiencies with acylcarnitines measurement in dried blood spots using TMS is appropriate is currently unclear due to a lack of data on babies who screen negative and a lack of consistency between screening test methods. Partial verification bias is a key issue in the included studies; from nearly 4,000,000 babies screened, only 63 (those who screened positive) received a reference standard. Therefore, we cannot know the true disease state of the babies who screened negative. Partial verification bias is common in studies of test accuracy because it is often impractical, unethical, and not cost-effective to follow-up every participant. Alternative approaches to whole population follow-up include statistical methods to attempt to correct for the bias, follow-up of samples of participants who screen negative, and searching disease registers to find false negatives. Statistical methods may introduce other forms of bias (27, 28).

There were substantial differences between screening test methods. For example, there were differences in the extraction and calibration methods (in-house or commercially available test kits); analysis as acylcarnitine butyl esters or free acids (underivatised); screening markers used (e.g., C14OH, C14:1, C16OH, C16:1OH, C18OH, C18:1, C18:1OH, C16OH/C16 ratio); whether markers were employed in isolation or in combination with each other [two studies did not report which marker(s) were used (18, 25)] and variability in the cut-offs between studies, with cut-offs not specified in three studies (18, 23, 25). The majority of FP were found in studies which used lower thresholds for C16OH (>0.03 to >0.10 μmol/L), C16:1OH (>0.04 to 0.15 μmol/L), and C18:1OH (>0.01 to 0.06 μmol/L).

While screening for LCHADD/MTP is conducted in the newborn bloodspot programs of a number of countries, there is little published data on the benefits and harms of these. Taylor-Phillips et al. (29) reviewed the evidence on national

policy recommendations on screening newborn babies for rare diseases. They highlight three elements that might determine the balance of benefits and harms from screening programs: test accuracy, the benefit of early detection and treatment, and overdiagnosis (the detection and subsequent treatment of disease that would never have caused symptoms within a person's lifetime). Many of the national policy recommendations (including for LCHADD) did not assess all of these three elements. In relation to screening for LCHADD/MTP, the current review suggests that the evidence on test accuracy is uncertain. A recent systematic review has examined the potential benefit of early detection and treatment, comparing the health outcomes of people with LCHADD/MTP who were treated with pre-symptomatic dietary management following screen detection of the conditions compared with people detected following symptomatic presentation (30). There was some evidence of an association between timing of intervention and outcomes, such as mortality, heart problems, liver problems, visual problems, motor/muscular problems, and hypoglycemia. However, the majority of included studies found no statistically significant differences in outcomes between the two groups. Furthermore, the review identified few studies from which to draw conclusions and high risks of bias in included studies. There is no published evidence on overdiagnosis. Overall, the paucity of data and variability between studies lead to considerable uncertainty regarding the benefits and harms of screening for LCHADD/MTP.

Our review has a number of limitations. First, we were not able to synthesize (meta-analyze) our results numerically due to a lack of data on FN and TN and because of variability between screening test methods. Second, we tailored the QUADAS-2 to reflect newborn screening in the UK; this resulted in high concerns regarding applicability in the patient selection domain, as screening is often conducted sooner after birth in other countries. The definition of a high applicability concern is likely to differ in other countries.

There is currently insufficient evidence to clearly judge test accuracy. This is driven, in part, by a wide range of markers and thresholds being used in the included studies: PPV estimates differed greatly by study, with some suggesting good PPV, albeit on small numbers of cases. It was not possible for us to combine data from different studies or determine which combination of markers and thresholds may yield good accuracy as results were not presented by marker. Future research could involve collaboration between researchers to report scores on a range of relevant markers for cases of LCHAD, cases of MTP, and in the general population using consistent units. There is a precedent for this approach in the form of the Region 4 Stork (R4S) project and accompanying multivariate pattern recognition software (subsequently developed into the interactive web tool Collaborative Laboratory Integrated Reports (CLIR), https://clir.mayo.edu/). The R4S project aimed "(a) to achieve uniformity of testing panels by MS/MS to maximize detection of affected newborns within the region; (b) to improve overall analytical performance; and (c) to set and sustain the lowest achievable rates of false positive and false negative results" (31). Reference and disease ranges for LCHAD/MTP markers were reported for the R4S project (31). To date, the CLIR tool has been used in a small number of research projects (32–35). An additional piece of work should aim to clarify the disease states of babies who screen negative. This could be achieved in a number of ways, such as searching hospital/primary care records or disease registers, or following up samples of babies who have screened negative.

CONCLUSIONS

Measurement of acylcarnitines in newborn dried blood spots using TMS may prove to be a useful way to screen for LCHAD/MTP deficiencies, but currently, there are significant concerns regarding the high number of false positives in some of the studies, risks of bias in the studies, heterogeneity in the methods used, and a lack of data on sensitivity, specificity, or negative predictive values. Clinicians interested in the identification of LCHAD/MTP may consider partnership development across clinical and research networks to address the knowledge gaps identified from this study, including data available for long-term follow-up studies and alignment of diagnostic methodologies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CS undertook project planning and research design, coordinated the review process, conducted all aspects of the review, and co-wrote the paper with HF. HF undertook project planning, undertook all sifting, sorting, data extraction, and quality appraisal, and co-wrote the paper with CS. JG contributed to sifting and sorting and commented on draft and final versions of the paper. RJ contributed to sifting and sorting and quality appraisal and commented on first and final drafts of the paper. MC undertook project planning, and commented on first and final drafts of the paper. SJ developed and conducted the literature searches, managed references, and helped obtain full-text references. AC undertook project planning, oversight of search strategies and methods, and commented on first and final drafts of the paper. ST-P undertook project planning and research design and commented on first and final drafts of the paper. All members of the team contributed to the development of the protocol. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.606194/full#supplementary-material

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Very Long-Chain Acyl-CoA Dehydrogenase Deficiency: High Incidence of Detected Patients With Expanded Newborn Screening Program

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Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is a rare autosomal recessive disorder of fatty acid metabolism with a variable presentation. The aim of this study was to describe five patients with VLCADD diagnosed through the pilot study and expanded newborn screening (NBS) program that started in 2018 in Slovenia. Four patients were diagnosed through the expanded NBS program with tandem mass spectrometry; one patient was previously diagnosed in a pilot study preceding the NBS implementation. Confirmatory testing consisted of acylcarnitines analysis in dried blood spots, organic acids profiling in urine, genetic analysis of *ACADVL* gene, and enzyme activity determination in lymphocytes or fibroblasts. Four newborns with specific elevation of acylcarnitines diagnostic for VLCADD and disease-specific acylcarnitines ratios (C14:1, C14, C14:2, C14:1/C2, C14:1/C16) were confirmed with genetic testing: all were compound heterozygotes, two of them had one previously unreported *ACDVL* gene variant each (NM_000018.3) c.1538C > G; (NP_000009) p.(Ala513Gly) and c.661A > G; p.(Ser221Gly), respectively. In addition, one patient diagnosed in the pilot study also had a specific elevation of acylcarnitines. Subsequent *ACDVL* genetic analysis confirmed compound heterozygosity. In agreement with the diagnosis, enzyme activity was reduced in five patients tested. In seven other newborns with positive screening results, only single allele variants were found in the *ACDVL* gene, so the diagnosis was not confirmed. Among these, two variants were novel, c.416T > C and c.1046C > A, respectively (p.Leu139Pro and p.Ala349Glu). In the first 2 years of the expanded NBS program in Slovenia altogether 30,000 newborns were screened. We diagnosed four cases of VLCADD. The estimated VLCADD incidence was 1:7,500

which was much higher than that of the medium-chain acyl-CoA dehydrogenase deficiency (MCADD) cases in the same period. Our study also provided one of the first descriptions of *ACADVL* variants in Central-Southeastern Europe and reported on 4 novel variants.

Keywords: VLCAD deficiency, VLCADD, neonatal screening, NBS, MS/MS, NGS, *ACADVL* gene, acylcarnitines

INTRODUCTION

Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD, OMIM 201475) is the second most common disorder of inborn errors of fatty acid metabolism; its incidence varies between 1:30,000 and 1:400,000 live births, with some outliers such as Saudi Arabia with reported incidence of 1:3200 and Taiwan with 1:1,400,000. In Europe incidence range from 1:77,000 in Germany and Netherlands to 1:400,000 in Czech Republic (Arnold et al., 2009; Spiekerkoetter et al., 2009; Lindner et al., 2010; Marsden et al., 2021). Incidence of VLCADD increased after the introduction of an expanded newborn screening program with the use of tandem mass spectrometry (MS/MS) allowing early detection of patients (Boneh et al., 2006; Merritt et al., 2014). VLCADD is caused by pathogenic variants in the *ACADVL* gene and is inherited in an autosomal recessive manner, resulting in deficient enzyme in the mitochondrial β-oxidation of long-chain fatty acids. Fatty acids are an important source of energy during prolonged fasting, physical exercise, and febrile infections when the body requires more energy. In VLCADD long-chain fatty acids with chain lengths of 14–20 carbons are not metabolized, which can lead to metabolic crises due to inadequate energy supply. This lack of energy may result in symptoms such as lethargy and hypoglycemia. Fats that are not properly broken down can also build-up and damage tissues in the heart, liver, and skeletal muscles, which can cause the other clinical features observed in people with VLCADD [Leslie et al., 1993; Very Long Chain Acyl CoA Dehydrogenase Deficiency (VLCADD) - NORD (National Organization for Rare Disorders)].

Clinical presentations in patients are very heterogeneous, with three major phenotypes. Severe infantile VLCADD has an early onset, usually within the first months of life. It has high mortality and a high incidence of hypoketotic hypoglycemia, liver disease, cardiac arrhythmias, cardiomyopathy, and pericardial effusion. The moderate VLCADD has a later onset (late infancy to early childhood) and usually presents with lower mortality, hypoketotic hypoglycemia, hepatomegaly, and rarely cardiomyopathy. The mild or late-onset VLCADD presents in older children and young adults (usually > 10 years of age) with isolated skeletal muscle involvement, exercise intolerance, myalgia, rhabdomyolysis, and myoglobinuria usually triggered by exercise, fasting, or stress, but viral infection can also precipitate this presentation. In rare cases, it can lead to renal failure and can be fatal. Some patients presenting with myopathic disease may have a history of hypoglycemia in infancy or childhood (Leslie et al., 1993; Vianey-Saban et al., 1998; Very Long Chain Acyl CoA Dehydrogenase Deficiency (LCAD) – National Organization for Rare Disorders [NORD], 2020).

Optimal VLCADD management requires ongoing assessment of clinical and nutritional status. Since nutritional intervention is a cornerstone in treating VLCADD, close collaboration with metabolic clinical dietitian is crucial to improve patients' clinical outcomes. The goals of nutrition therapy are to minimize the production of abnormal fatty acid metabolites with long-chain triglycerides (LCT) restriction and prevention of metabolic crises due to a lack of an adequate energy supply. Nutrition therapy is tailored individually, dependent on the severity of the disorder and patients' age and typically includes restriction of dietary intake of LCT together with supplementation with medium-chain triglycerides (MCT) (Van Calcar et al., 2020).

VLCADD can be detected with the accumulation of characteristic acylcarnitines in blood only a few days after birth. The specific acylcarnitine profile can be accurately measured in newborn dried blood spots (DBS) by MS/MS (Wilcken et al., 2003). The primary marker is elevated C14:1 acylcarnitine (tetradecenoylcarnitine), together with other long-chain acylcarnitines and disease-specific acylcarnitines ratios that can be calculated (C14, C14:2, C14:1/C2, C14:1/C16). As previously reported, MS/MS has a high false-positive rate and it is often difficult to differentiate between true positives, heterozygous carriers, and false positives (Spiekerkoetter, 2010; Bo et al., 2020). False positives can also occur due to insufficient breastfeeding, which might cause an elevation of C14:1 and C14:1/C2. That's why it is suggested that in suspected VLCADD, attending doctors should pay attention to body weight changes recorded during newborn body mass screening (Bo et al., 2020).

On the other hand, the second DBS sample for acylcarnitine measurement could give normal results even if disease is present, due to the switch to anabolic condition at the time of the second sampling (Boneh et al., 2006; Spiekerkoetter, 2010; Hesse et al., 2018). NGS could be of great help in confirmatory diagnostics; however, it is inconclusive in cases of novel genetic variants, and variants of unknown significance (VUS) (Hoffmann et al., 2012). Despite the abovementioned limitations, we decided to introduce next-generation sequencing (NGS) for confirmatory testing of positive NBS results (Smon et al., 2018; Lampret et al., 2020). In the case of positive NGS, enzyme testing in fibroblasts and lymphocytes as well as flux studies in fibroblasts have to be performed (Diekman et al., 2015; Hesse et al., 2018; Bleeker et al., 2019; Rovelli et al., 2019).

This study aimed to describe five patients with VLCADD diagnosed through the pilot and expanded newborn screening (NBS) program in Slovenia (from 2018).

MATERIALS AND METHODS

Dried blood samples were taken 48–72 h after the birth. Acylcarnitines were analyzed from 3 mm disk of DBS with MS/MS (Xevo TQD, Waters, Milford, Massachusetts, United States) using NeoBase® 2 Non-derivatized MSMS kit (Perkin Elmer, Waltham, Massachusetts, United States) which quantifies the tested metabolites with stable-isotope-labeled internal standards and allows the detection of numerous metabolites simultaneously in a single run. The sample preparation was based on extraction without derivatization. Positive electrospray ionization and multiple reaction monitoring (MRM) mode were used for MS/MS.

For NGS confirmatory testing, an in-house panel of 72 genes was developed (Supplement 1; Lampret et al., 2020). The NGS panel was designed around the core of causative genes for the 18 diseases included in the current NBS program. Additional genes for the conditions that present with the increase of the same metabolites as the targeted disease were added, to aid differential diagnostics (Šmon et al., 2015; Lampret et al., 2020). Variants detected with NGS with vertical coverage below 100× were additionally confirmed by Sanger sequencing. For genetic analysis, whole blood samples were used for isolation of genomic DNA with established laboratory protocols based on FlexiGene DNA isolation kit (Qiagen, Hilden, Germany). For genetic analysis, NGS was performed using MiSeq desktop sequencer coupled with MiSeq Reagent kit v3 (both Illumina, San Diego, United States). The regions of interest were enriched using Agilent SureselectXT Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, United States) following the manufacturer's instructions. We reached at least 10× coverage for 99.9% of regions of interest for each patient. Genetic testing of *ACADVL* gene of Patient 1 was performed at Laboratory Genetic Metabolic Diseases, Emma Chirdren's Hospital AMC.

Urine organic acids were measured with an in-house method (Lampret et al., 2015). Briefly, to 1 mL of urine, we added O-ethylhydroxylamine hydrochloride (Sigma-Aldrich, Munich, Germany) and incubated the solution for 15 min at room temperature. 2-phenylbutyric acid (Sigma-Aldrich, Munich, Germany) was added as an internal standard in the concentration of 100 mmol per mol of creatinine. Urine was acidified and then the solution was saturated with NaCl (Sigma-Aldrich, Munich, Germany). After the addition of ethylacetate (Sigma-Aldrich, Munich, Germany) the solution was vortexed and centrifuged. The organic layer was transferred to a clean glass tube, and the ethylacetate evaporated under a stream of nitrogen. Finally, pyridine (Fluka, Buchs, Switzerland) and N,O-bis(trimethylsilyl)trifluoroacetamine (Sigma-Aldrich, Munich, Germany) were added, the solution mixed and analyzed with Agilent 5975C Series GC/MSD (Agilent Technologies, Santa Clara, CA, United States) on Agilent Ultra2 column (Agilent Technologies, Santa Clara, CA, United States).

If the second DBS card was collected before 10 days after birth, the same method as for the first sample was used (NeoBaseTM 2). When the second sample was collected later than that, confirmatory testing was performed with the Chromsystems method and different reference ranges were applied. Acylcarnitines for confirmatory testing were analyzed from dried blood spots and derivatized with Chromsystems kit MassChrom^{TM} Amino Acids and Acylcarnitines from Dried Blood (Chromsystems Instruments & Chemicals GmbH, Grüfelfing/Munich, Germany). The sample preparation was based on extraction from a 3 mm disk of DBS followed by derivatization to butyric esters. Positive electrospray ionization and multiple reaction monitoring (MRM) mode were used. They were quantified by PerkinElmer 200 HPLC system (Perkin Elmer, Waltham, Massachusetts, United States) coupled to AB Sciex 3200 QTRAP (AB SCIEX, Singapore).

Enzymatic activity of VLCAD was determined using the UPLC-UV method at AMC Amsterdam UMC, Amsterdam on lymphocytes from whole blood samples and in one case from cultured fibroblasts (Amsterdam UMC and Locatie AMC, 2021 - Very long-chain acyl-CoA dehydrogenase (VLCAD)).

To assess the adequacy of the patient's diet, their caregivers were asked to record the intake of all foods, drinks, and food supplements consumed over 3 consecutive days. The average values of recording for all 3 days together were calculated and compared with recommendations for energy, total fat, long-chain fat, medium-chain fat, linoleic acid, and alpha-linolenic acid intake (**Table 1**) by an experienced clinical dietitian. For the nutritional analysis, we used the Prodi 6.10 Expert plus software (Nutri-Science, Stuttgart, Germany, 2020), which contains the database of approximately 15,000 foods from the Bundeslebensmittelschlüssel 3.01 (BLS 3.01) database, Fachmann-Kraut-Nährwerttabellen (FKN, Stuttgart, 2005) database, and industrial products and dietetic foods.

Informed consent for genetic testing and anonymous presentation of clinical data was obtained from parents of all patients that underwent confirmatory NGS, and all analyses were performed as a part of a diagnostic procedure according to the principles of the Helsinki Declaration (WMA, 2020 WMA Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects—WMA—The World Medical Association). The studies involving human participants were reviewed and approved by The National Medical Ethics Committee (KME: 56/01/14).

Case Series

Patient 1 is a 7-year-old girl, who was first seen by our NBS team at 9.5 months after screened positive on a pilot metabolic screening project. She is the first child of non-consanguineous parents. The paternal grandfather had hypertrophic cardiomyopathy. She was born at 41-weeks of gestation, with appropriate birth measures. In the asymptomatic state, ammonia was up to 90 μmol/L (10–47 μmol/L), and creatine kinase (CK) 2.52 μkat/L (<2.50 μkat/L). VLCADD was confirmed by low enzyme activity (**Table 2**) and analysis of the *ACADVL* gene (**Table 3**). She has had no metabolic crisis to date. She had a single episode of leukocytopenia and thrombocytopenia, which resolved spontaneously. Echocardiogram (ECHO) of the heart was performed every 2 years and revealed no pathological findings. There has been no organomegaly on the abdominal ultrasound (US). Growth parameters and developmental assessment have

TABLE 1 | Characteristics of nutritional management of VLCADD patients.

Patient*	Type	Calculated actual intake+							Recommended intake					
		Energy		Total fat	Long-chain fat	Medium-chain fat	Linoleic acid	alpha-Linolenic acid	Energy#		Total fat$	Long-chain fat$	Medium-chain fat$	Linoleic acid$	Alpha-Linolenic acid$
		kcal/day	kcal/kg	% of total energy					kcal/day	kcal/kg	% of total energy				
1	Moderate	1267	51	25	9	16	3	0.5	1530	67	25–35	15–25	10–20	3–6	0.5–1.2
2	Moderate	958	88	31	16	15	4	0.6	770	80	30–40	20–30	10–20	3–6	0.5–1.2
3	Moderate	994	83	33	20	13	4	0.6	720	80	30–40	20–30	10–20	3–6	0.5–1.2
4	Severe	997	110	31	10	21	5	0.5	770	80	30–40	10–15	10–30	3–6	0.5–1.2

**Actual intake is based on dietary records of 3 consecutive days calculated by Prodi 6.10 Expert plus Nutri-Science software (Stuttgart, Germany, 2020). *Patient 5 is not included in the table as she does not yet have special dietary restrictions except for fasting precautions. #Average daily energy requirements in clinical practice adapted from: chapter 1, table 1.12 page 11 (Shaw, 2020). $Recommended fat (total, long-chain and medium chain) intake for individuals with VLCAD when well adopted from Van Calcar et al. (2020).*

been in the normal range. The patient was exclusively breastfed for 3 weeks and then, due to an insufficient weight gain, breastfeeding was supplemented with the regular milk formula. A LCT restricted diet was introduced at the age of 2 years. The intake of LCT was limited to 20–35% of total energy (**Table 1**), according to the recommendations (Van Calcar et al., 2020). Dietary requirements for essential fatty acids are being met with walnut oil and docosahexaenoic acid (DHA) supplementation.

Patient 2 is a 20-months old girl who was first evaluated at 5 days due to a positive NBS result. She is the third child of non-consanguineous parents. Her 6 and 3-years older siblings are healthy. She was born with C-section at 39 weeks of gestation due to the breech presentation. Birth measurements were normal. At the first evaluation, liver transaminases and CK were in the normal range, and the acylcarnitine profile was consistent with VLCADD; the diagnosis was confirmed by low VLCAD enzyme activity (**Table 2**) and analysis of the *ACADVL* gene (**Table 3**). She required parenteral hydration with glucose during acute bronchiolitis at 5 months of age and influenza B at 9 months but without overt hypoglycemia. Laboratory tests were mildly elevated aspartate aminotransferase (AST) 2.25 μkat/L (<1.22 μkat/L); alanine aminotransferase (ALT) 1.08 μkat/L (<0.98 μkat/L); CK 24.39 μkat/L (<4.92 μkat/L); myoglobin 116.2 μg/L (<110.0 μg/L). Yearly checkup showed no alterations in structure or function on ECHO of the heart; and normal electrocardiogram (ECG). The US of the abdomen showed no abnormalities. Growth and developmental milestones have been appropriate. The patient was exclusively breastfed for the first month of life. After the first month fat-free milk formula (Basic f, Nutricia, Amsterdam, Netherlands) enriched with 2% MCT oil (2 mL of 100% MCT oil per 100 mL of prepared milk formula), alternately with breastfeeding, was introduced. At the age of 6 months, complementary foods as part of the LCT restricted diet were included. The intake of LCT was limited to 15–30% of total energy intake and at the age of 1 year to 20–30% of total energy intake (**Table 1**), according to the recommendations (Van Calcar et al., 2020). Dietary requirements for essential fatty acids are being met with walnut oil supplementation. DHA is supplemented when required.

Patient 3 is a 14-months old boy who was first evaluated at 9 days after screened positive on NBS. After the first and uneventful pregnancy of non-consanguineous parents, he was born at 39 weeks with appropriate birth measurements. On examination, repetitive movements of the head were noticed. The further neurologic evaluation showed axial hypotonia, ataxia, obstructive hypopnea, symmetrical mild hypermetropia, and pale optic papillae were present. The electroencephalogram was normal. Cystic dysplasia of the cerebellum and vermis and hypoplasia of pons were noticed at magnetic resonance scan. A Poretti-Boltshauser syndrome was confirmed. AST, ALT, gamma-glutamyl transferase (GGT), CK, ammonia, and lactate were in the normal range. Acylcarnitine profile was consistent with VLCADD and was confirmed by low enzyme activity (**Table 2**) and analysis of the *ACADVL* gene (**Table 3**). The patient did not experience any metabolic crisis to date. ECHO of the heart at the age of 3-months showed no cardiomyopathy. The

TABLE 2 | Comparison of biochemical characteristics of confirmed VLCADD patients, heterozygous *ACADVL* variant carriers and those who were negative at re-testing at NBS.

	Subject*		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18
			VLCADD positive patients					VLCADD negative subjects												
								Single variant on one allele									No variant			
	NBS (NB2)$^{+}$	Ref. Range (μmol/L)																		
	C14	0–0.42	–	1.41	1.07	4.28	0.54	0.54	0.63	0.36	0.46	0.57	0.48	0.39	0.50	0.37	0.45	0.53	0.72	0.52
	C14:1	0–0.32	–	3.41	1.42	6.35	1.01	0.52	0.63	0.45	0.43	0.49	0.46	0.42	0.48	0.42	0.49	0.43	0.76	0.52
	C14:2	0–0.05	–	0.44	0.16	0.74	0.16	0.06	0.05	0.08	0.03	0.04	0.06	0.05	0.06	0.05	0.06	0.07	0.10	0.04
	C14:1/C2	0–0.014	–	0.200	0.070	0.761	0.039	0.01	0.023	0.018	0.015	0.016	0.017	0.025	0.021	0.026	0.017	0.009	0.020	0.021
	C14:1/C16	0–0.08	–	0.84	0.30	0.50	0.25	0.09	0.10	0.12	0.09	0.07	0.10	0.09	0.09	0.11	0.09	0.06	0.11	0.12
	Recall (NB2)$^{+}$	Ref. range (μmol/L)																		
	C14	0–0.42	–	0.41	0.29	1.43	–	0.14	–	0.10	–	0.17	–	–	0.06	–	–	–	–	–
	C14:1	0–0.32	–	0.84	0.39	3.39	–	0.05	–	0.07	–	0.07	–	–	0.04	–	–	–	–	–
	C14:2	0–0.05	–	0.18	0.18	0.62	–	0.01	–	0.04	–	0.02	–	–	0.01	–	–	–	–	–
	C14:1/C2	0–0.014	–	0.090	0.062	1.101	–	0.01	–	0.005	–	0.004	–	–	0.004	–	–	–	–	–
	C14:1/C16	0–0.08	–	0.31	0.28	0.92	–	0.04	–	0.06	–	0.02	–	–	0.07	–	–	–	–	–
Confirmatory testing	**Acylcarnitines (Chromsys.)$^{\#}$**	**Ref. range (μmol/L)**																		
	C14:1	0–0.210	0.52	–	–	–	0.236	–	0.048	–	0.038	–	0.058	0.020	–	0.021	0.028	0.033	0.043	0.020
	C14	0.08–0.5	0.49	–	–	–	0.206	–	0.146	–	0.138	–	0.125	0.058	–	0.092	0.092	0.123	0.194	0.092
	C14:1/C2	0–0.011	0.026	–	–	–	0.020	–	0.0038	–	0.003	–	0.006	0.004	–	0.0023	0.003	0.002	0.003	0.002
	C14:1/C16	0–0.082	0.08	–	–	–	0.054	–	0.0247	–	0.041	–	0.027	0.027	–	0.0364	0.027	0.031	0.031	0.027
	Organic acids		–	–	Elevated	Normal	Normal	–	–	–	Normal	–	–	–	–	–	Normal	Normal	–	–
	NGS (*ACADVL*)																			
			pat.	lik. pat	lik. pat.	pat.	lik.pat.	–	–	–	–	–	–	–	–	–	–	–	–	–
			lik.pat.	pat.	pat.	pat.	pat.	pat.	pat.	VUS	lik. pat.	pat.	pat.	lik. pat.	–	–	–	–	–	–
	Enzyme activity																			
	Lymphocytes†		1.26	0.30	0.25	<0.18	0.36	–	–	–	–	1.34	0.91	1.01	–	–	–	–	–	–
	Fibroblasts‡		0.27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*All DBSs were collected 48–72 h after birth during a routine newborn screening procedure. *Patient 1 discovered in pilot study prior to start of expanded newborn screening. $^{+}$Acylcarnitines measured using Perkin Elmer NeoBase2 non-derivatized kit. $^{\#}$Acylcarnitines measured using Chromsystems MassChrom® Amino Acids and Acylcarnitines from Dried Blood derivatized kit; pat., pathogenic, lik. pat., likely pathogenic, VUS, variant of unknown significance (Richards et al., 2015). †For enzyme activity in lymphocytes the reference values are as follows: P1 (1.84–4.80); P2–P4 (2.15–3.79) [nmol min^{-1}mgprot^{-1}]. ‡ or enzyme activity in cultured fibroblasts the reference values are: P1 (1.48–5.24) [nmol min^{-1}mgprot^{-1}].*

TABLE 3 | Overview of all the *ACADVL* gene variants found.

PATIENT/CARRIER	Nucleotide change (NM_000018.4)	Amino acid change (NP_000009)	ACMG classification*	ACMG criteria met	Reported by
P1	c.1837C > T	p.Arg613Trp	Pathogenic	PS3, PP5, PM1, PP2, PM2, PM5, PP3	Strauss et al., 1995
	c.205-8_205-7delCTinsGC	/	Likely pathogenic	PS3, PM2	Hesse et al., 2018
P2	c.773T > C	p.Ile258Thr	Likely pathogenic	PS3, PM2, PM1, PP2, PP3	Hesse et al., 2018
	c.1358G > A	p.Arg453Gln	Pathogenic	PS3, PP5, PM1, PP2, PM2, PP3	Andresen et al., 1999
P3	c.1077 + 2T > C	/	Pathogenic	PS3, PVS1, PM2, PP5	Andresen et al., 1999
	c.1538C > G	p.Ala513Gly	Likely pathogenic	PS3, PM2, PP2	**Novel**
P4	c.1077 + 2T > C	/	Pathogenic	PS3, PVS1, PM2, PP5	Andresen et al., 1999
	c.1358G > A	p.Arg453Gln	Pathogenic	PS3, PP5, PM1, PP2, PM2, PP3	Andresen et al., 1999
P5	c.661A > G	p.Ser221Gly	Likely pathogenic	PM2, PP3, PP2	**Novel**
	c.848T > C	p.Val283Ala	Pathogenic	PP5, PM2, PM1, PP2, PP3	Andresen et al., 1996
P6	c.1358G > A	p.Arg453Gln	Pathogenic	PS3, PP5, PM1, PP2, PM2, PP3	Andresen et al., 1999
	Not found	–	/	/	/
P7	c.848T > C	p.Val283Ala	Pathogenic	PP5, PM2, PM1, PP2, PP3	Andresen et al., 1996
	Not found	–	/	/	/
P8	c.416T > C	p.Leu139Pro	VUS	PM2, PP3, PP2	**Novel**
	Not found	–	/	/	/
P9	c.1046C > A	p.Ala349Glu	Likely pathogenic	PM1, PP2, PM2, PP3, PP5	**Novel**
	Not found	–	/	/	/
P10	c.848T > C	p.Val283Ala	Pathogenic	PP5, PM2, PM1, PP2, PP3	Andresen et al., 1996
	Not found	–	/	/	/
P11	c.1837C > T	p.Arg613Trp	Pathogenic	PS3, PP5, PM1, PP2, PM2, PM5, PP3	Strauss et al., 1995
	Not found	–	/	/	/
P12	c.1242G > C	p.Glu414Asp	Likely pathogenic	PM2, PM5, PP3, PP2	Hisahara et al., 2015
	Not found	–	/	/	/

*Altogether, four novel variants were found. Patients P1–P5 are all compound heterozygous; patients P6–P12 only one heterozygous variant was found. *ACMG classification according to Richards et al. (2015).*

liver structure was hyperechogenic, without organomegaly. He has a moderate developmental delay; he crawled and sat stably. Growth parameters were normal. Nutritional management of the patient was the same as for patient 2 (**Table 1**); complementary foods were introduced at 5 months of age. Dietary requirements for essential fatty acids are being met with walnut oil and DHA supplementation.

Patient 4 is a 1-year-old boy who was first evaluated at 25-days, after the second invitation at a positive NBS. He was born after the first and uneventful pregnancy; delivery was stimulated at 37 weeks of gestation due to detected IUGR (BW 2.3 kg, BL 45 cm). Laboratory tests at first evaluation were mildly elevated AST 1.93 μkat/L (1.22 μkat/L); ALT 0.81 μkat/L (0.98 μkat/L); GGT 2.43 μkat/L (<2.05 μkat/L); CK 1.35 μkat/L (<4.92 μkat/L); ammonia 43 μmol/L (<75 μmol/L); lactate 4.32 mmol/L (1.1–3.5 mmol/L). Acylcarnitine profile was consistent with VLCADD. The diagnosis was later confirmed by non-detectable VLCAD enzyme activity (**Table 2**) and analysis of the *ACADVL* gene (**Table 3**). He had no acidosis or hypoglycemia episodes to date; however, he was admitted during respiratory infection with lactate elevation (5.49 mmol/L) at the age of 6 weeks and was given glucose supplementation. US showed enlarged liver (65 mm in MCL) with hyperechogenicity of parenchyma, elastography measured 5.6–6.8 kPa. The developmental assessment showed no delay, and catch-up growth occurred after 2.5 months with the now normal growth rate. Nutritional management of the patient was the same as for patient 3 with the addition of walnut oil to the milk formula (**Table 1**). The walnut oil and DHA supplementation were later continued.

Patient 5 is a 5-months old girl who was first evaluated by our NBS metabolic team at 10-days. She had a 5 years old brother, who was followed due to dysplastic kidneys. The mother has arrhythmia. The girl is the second child of non-consanguineous parents. The girl was born at term with normal birth measurements. On examination, poor weight-gain was noticed; the mother reported that she needed to wake the newborn up for breastfeeding. Diet advice was given and weight normalized. AST, ALT, CK, and ammonia were in the normal range. Lactate was mildly elevated [3.84 mmol/L (1.1–3.5 mmol/L)]. Acylcarnitine profile was consistent with VLCADD. The diagnosis was later confirmed by variant analysis of the *ACADVL* gene (**Table 3**) and by low VLCAD enzyme activity (**Table 2**). Further evaluation of the heart, and US of the abdomen are still required; but unfortunately, parents' compliance was poor. The patient was exclusively breastfed for the first 3 weeks of life and then, due to an insufficient weight gain, breastfeeding was supplemented with the regular milk formula. Complementary feeding without mandatory dietary restrictions except for fasting avoidance was introduced at the age of 5 months.

RESULTS

Results of the first 30,000 newborns screened with extended NBS program revealed 18 subjects with elevated acylcarnitines and acylcarnitine ratios characteristic for VLCADD. Confirmatory testing was done with acylcarnitine analysis from the second DBS card, organic acids analysis from urine, NGS testing, and enzyme activity measurement. Five patients were subsequently confirmed with NGS and enzyme activity testing. Patient 1 was discovered during a pilot study with a different analytical method so no results with the NeoBase2 method are available and patient 1 is therefore omitted from the graphical comparison. For patient 5 confirmatory testing was performed after 10 days after birth and was therefore performed with the Chromsystems method, so there are no results with the NeoBase2 method for recall. NGS confirmatory testing revealed 4 novel *ACADVL* gene variants, 7 heterozygous carriers, and 6 false-positive subjects. Results are presented in **Table 2**.

Acylcarnitines Results From the First DBS

Acylcarnitine concentrations and disease-specific acylcarnitines ratios of patients were compared to those of 17,000 healthy newborns. Patients' (P2–P5) values for all the chosen acylcarnitines and ratios were notably higher compared to values of healthy controls. A box represents the 25th and 75th percentile, the line in the box is the median, whiskers are 1st and 99th percentile (**Figure 1**). Values of C14:1 in subjects P6–P18 were higher than the highest measured value in healthy newborns. For C14 only P8, P12 and P14 had values below the 99th percentile. For ratio C14:1/C2 only P6 and P16 had values below 99th percentile and for ratio C14:1/C16 only P16 had values below 99th percentile. For C14:2 subjects P7, P9, P10, P12, P14, and P18 had values below the 99th percentile. Patient 1 was analyzed with the Chromsystems method because that was the method of choice in the pilot study.

Organic Acids

Organic acids in urine were abnormal only in patient 3. Octenedioic, decadienedioic, and hydroxydecanedioic acids were slightly elevated.

Acylcarnitines Results From Second DBS as Follow-Up

Follow-up DBS samples for patients 2, 3, and 4 came within the first 10 days after birth, and for patient 5 after 10 days. P5 was therefore analyzed with the Chromsystems method. All 4 patients had elevated acylcarnitines and ratios on follow-up. Patient 5 had only elevated C14:1 and C14:1/C2. Among subjects P6–P18, four follow-up DBS samples were analyzed within 10 days after birth, and nine were analyzed later than that. Subjects 6 through 18 showed normal acylcarnitine profile on follow-up from the second card (**Table 2**).

Next Generation Sequencing

Patients P1–P5 are compound heterozygotes. In subjects P6–P12 only one heterozygous variant was found and in subjects P13–P18 no variants were found in the *ACADVL* gene. Four novel missense *ACADVL* variants were found. Patients P1 and P2 had one known pathogenic and one known likely pathogenic ACADVL variant. Patient P4 had two known pathogenic *ACADVL* variants. The novel variant in patient P3

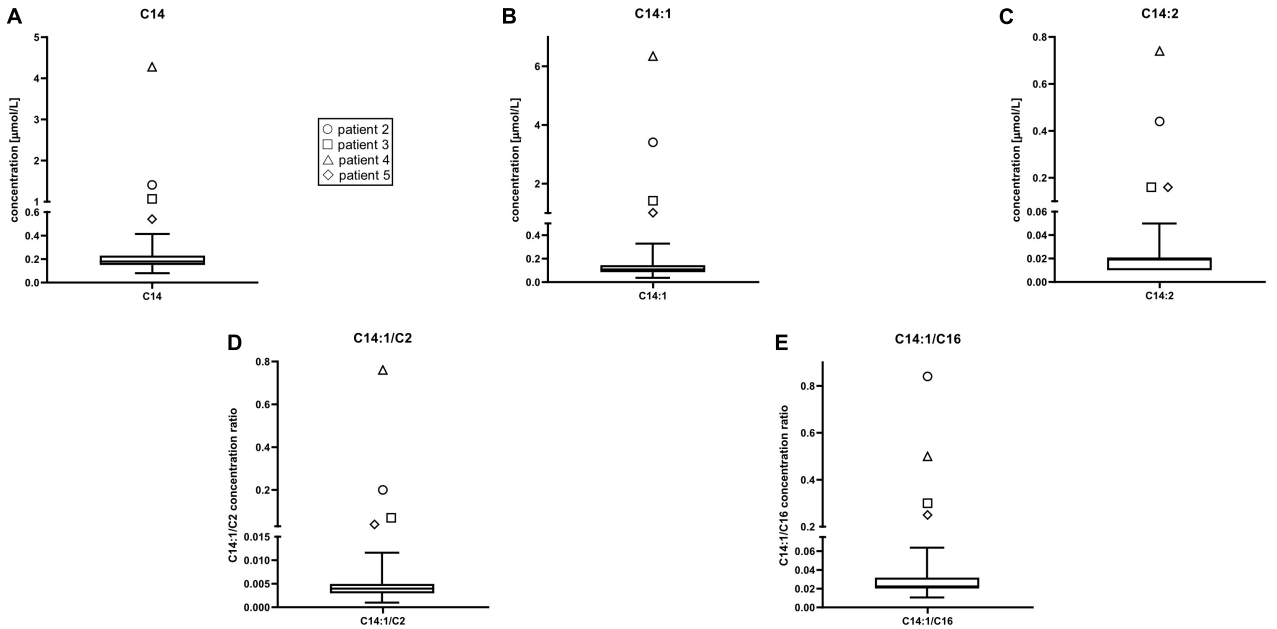

FIGURE 1 | Acylcarnitine concentrations in healthy newborns were compared to those in patients 2 through 5. Patient 1 was not included in the representation because acylcarnitines analysis was performed with different method with different reference values. Patients' values for all the chosen acylcarnitines were higher than in healthy controls. Box plot and whiskers from data of 17,000 healthy controls. A box represents the 25th and 75th percentile, the line in the box is the median, whiskers are 1st and 99th percentile. C14, tetradecanoylcarnitine (myristoylcarnitine); C14:1, tetradecenoylcarnitine; C14:2, tetradecadienoylcarnitine; C2, acetylcarnitine; C16, hexadecanoylcarnitine. **(A)** C14 concentration (µmol/L), **(B)** C14:1 concentration (µmol/L), **(C)** C14:2 concentration (µmol/L), **(D)** C14:1/C2 concentrations ratio, **(E)** C14:1/C16 concentrations ratio.

(NM_000018.3): c.1538C > G results in p.Ala513Gly substitution (**Table 3**), with conflicting classification with different prediction algorithms (SIFT, Polyphen 2, MutationTaster, Provean, and CADD). Considering reduced enzyme activity, the variant meets the ACMG criteria for likely pathogenic variant (Richards et al., 2015). The second novel variant in patient P5 c.661A > G results in p.Ala349Glu substitution (**Table 3**), is classified by most of the prediction algorithms as damaging (SIFT, Polyphen 2, MutationTaster, Provean, and CADD). Considering reduced enzyme activity, the variant meets the ACMG criteria for likely pathogenic variant (Richards et al., 2015). In seven unaffected newborns (P6–P12), only single allele *ACADVL* variants were found in heterozygous state; among these, two were previously unreported. The first one detected in patient P8 c.416T > C results in p.Ala513Gly. Prediction algorithms classify it as pathogenic or likely pathogenic (SIFT, Polyphen 2, MutationTaster, Provean, and CADD) the variant meets ACMG criteria for VUS (Richards et al., 2015). The second variant c.1046C > A was detected in patient P9 and results in p.Ser221Gly. Prediction algorithms classify it as pathogenic or likely pathogenic (SIFT, Polyphen 2, MutationTaster, Provean, and CADD) the variant meets ACMG criteria for likely pathogenic (Richards et al., 2015; **Table 3**). Enzyme activity.

Enzyme testing on lymphocytes was performed for all patients (P1–P5) with two *ACADVL* variants found with NGS analysis. Results of enzyme activity testing are presented in **Table 2**. All patients had markedly reduced enzyme activity consistent with genetic findings and confirmatory of the disease. Patient 4 which was the only one with two known pathogenic *ACADVL* variants had the lowest enzyme activity. Patient 1 had enzyme activity measured in both lymphocytes and fibroblasts because the result of enzyme activity in lymphocytes was inconclusive. Enzyme testing was also performed for subjects P10, P11, and P12 which also showed partly reduced enzyme activity in the range of heterozygous carriers.

DISCUSSION

We reported our initial experiences with expanded NBS using MS/MS and confirmatory NGS for detecting patients with VLCADD. Newborn screening for VLCADD was included in the Slovenian NBS program in 2018. For confirmatory testing of positive results with NGS an in-house panel of causative genes for the diseases included in the NBS program was designed. Additionally, the second DBS card was also analyzed and organic acids measured in the urine of suspected patients.

All acylcarnitines and ratios indicative of VLCADD have been substantially increased in all five confirmed patients (P1–P5). All patients had increased values of C14:1 and other acylcarnitines and ratios on confirmatory test from second DBS card, whereas subjects (P6–P18) in which we did not confirm VLCADD had normal acylcarnitine profile from second card. Organic acids in urine were abnormal only in patient P3 and were as such not a suitable as confirmatory test for VLCADD (Boneh et al., 2006).

Four newborns with specific elevation of acylcarnitines and disease-specific acylcarnitines ratios for VLCADD were confirmed with genetic testing (P2–P5). All patients were compound heterozygotes, with two of them having one novel variant each (c.1538C > G and c.661A > G). One patient was detected through a pilot study before the NBS program was introduced, with specific elevation of acylcarnitines (Smon et al., 2018). Samples in the pilot study were analyzed retrospectively, the average age of the samples at the time of analysis was 8.5 months (from 6 to 11 months). Subsequent NGS *ACADVL* genetic analysis confirmed the diagnosis, the patient was a compound heterozygote. In seven other newborns with positive initial screening results, only single allele variants were found, so the diagnosis was not confirmed. Among these two novel *ACADVL* variants were confirmed. Six newborns with positive screening results were shown to be false positive. These results give us 76.5% of healthy carriers and false positives. Acylcarnitines concentrations of first DBS were higher at four newborns with confirmed disease (C14:1 > 1 μmol/L in all cases) compared to unaffected carriers and false positives. Concentrations at second DBS remained above the reference range for patients 2–4, though lower than the first sample. For newborns where disease was not confirmed results of second testing were not elevated. Still, it is recommended that in the case of positive NBS results suspicious for VLCADD confirmatory testing should be done, as results of second testing can be normal (Hesse et al., 2018). Some authors report that 57% of newborns with positive NBS results for VLCADD are healthy carriers, with one variant in the *ACADVL* gene (Miller et al., 2015). Hesse and coworkers published that 85% of NBS positive cases did not show impaired enzyme function.

There are many causative variants associated with VLCADD; the Human Gene Mutation Database[1] lists 348 different variants. There is no common variant described as is the case for MCADD, where 54–91% of the alleles carry a common variant (Matsubara et al., 1990; Rhead, 2006). All patients reported in the study were compound heterozygotes. In literature, the variant c.848T > C was described as the most frequent variant causing mild phenotype (Boneh et al., 2006; Miller et al., 2015; Pena et al., 2016; Hesse et al., 2018). In our cohort, it is present on three alleles, in patient 5 in compound heterozygous state together with likely pathogenic variant and in two newborns in heterozygous state. Patient 5 had the lowest acylcarnitine concentrations on NBS of confirmed cases. Variant c.1358G > A was also detected on three alleles. The same variant was described in a homozygous state in two adult patients from Serbia (Fatehi et al., 2020). Enzyme activity was grossly reduced in all five patients (P1–P5) and reduced to about 50% of the lower limit in three carriers (P10–P12) tested, which is in agreement with claims in literature (Leslie et al., 1993; Tajima et al., 2008). Patient 1 discovered in the pilot study, had VLACD activity tested in lymphocytes first, and the result was similar to that of heterozygous carriers. Then sequencing of the *ACADVL* gene was performed in AMC Amsterdam, and two variants were found. The first was a known pathogenic variant and the second was a previously unreported variant with unknown significance. Because of VUS, the genetic result was inconclusive and enzyme testing in fibroblasts was performed to confirm the diagnosis. The result of enzyme activity testing in fibroblasts was markedly reduced enzyme activity and the diagnosis of VLCADD was confirmed. On the three of the heterozygous subjects (P10–P12), we also performed enzyme activity testing. Because there remains a small possibility, that with NGS we would miss a variant on the second allele, and since there is no reliable biochemical testing for VLCADD we decided in 2020 that we would perform enzyme testing on all heterozygous subjects uncovered with NGS.

The estimated VLCADD incidence for the past 2 years was 1:7,500. That is unusually high compared to the literature data with an estimated incidence between 1:30,000 and 1:400,000 live births, and comparable only to the incidence in Saudi Arabia which is known to have a high level of consanguinity. In our families, no consanguinity was reported, and all patients are compound heterozygous. There are large differences in incidence of VLCADD between different populations, which are difficult to compare because of different size and characteristics of populations, details of screening methods, cutoffs, and use of different confirmatory methods (Arnold et al., 2009; Spiekerkoetter et al., 2009; Lindner et al., 2010; Marsden et al., 2021). It is known, that with expanded screening more patients are detected, which is in agreement with our study. There were three MCADD diagnosed cases in the same period, which gives the incidence of 1:10,000. MCADD is the most common inborn error of fatty acid metabolism, with an incidence estimated at 1 in 15,000 live births (Orphanet, 2020: Medium-chain acyl CoA dehydrogenase deficiency; Grosse et al., 2006; Lindner et al., 2010), and our results are in agreement with that. In Southeastern Europe there is currently no NBS for VLCADD in a majority of the countries with exception of Slovenia and Croatia (Groselj et al., 2014a,b, Šmon et al., 2015; Bilandžija et al., 2018; Smon et al., 2018; Lampret et al., 2020; Loeber et al., 2021), which would detect more patients than are diagnosed clinically. To the best of our knowledge, only two adult patients with VLCADD were described so far in Southeastern Europe (Fatehi et al., 2020). Our study presents one of the first descriptions of *ACADVL* variants in Central-Southeastern Europe, detected after implementation of an expanded NBS, showing potentially higher incidence throughout the region as compared to the expected incidence from the literature.

As the treatment strategies for VLCAD include preventing catabolic episodes by providing sufficient energy and nutrient intake, avoiding excessive fasting (especially during illness), and restricting the LCT intake, regular collaboration with an experienced clinical dietitian is required. Medical nutritional therapy for individuals with VLCAD is tailored to the severity of the disorder. Therefore, optimal VLCAD management requires ongoing assessment of clinical as well as nutritional status. Nutrition assessment typically consists of anthropometrics, calculated dietary records with analysis, feeding schedule, and food preferences of the patient. The requirement of special nutrients supplementation (i.e., vitamins A, D, E, DHA, and

[1] https://portal.biobase-international.com

others) is also assessed. When the patients' dietary intake does not comply with the recommendations, the patients and their caregivers are invited to an additional dietary consultation with the purpose of dietary adjustment. It is important to monitor the patients regularly and refer them to the hospital when ill, as they can be in danger even with a milder form of VLCADD (Boneh et al., 2006).

The limitation of our study is a relatively small number of NBS results due to only 2 years of expanded NBS. The strength of the study is that all NBS tests and confirmatory diagnostics except enzyme activity measurement are performed in our laboratory. As our laboratory performs routine genetic diagnostics, we can utilize NGS in combination with an in-house panel of genes for confirmatory testing. We have an excellent overview of the age of newborns at the time of sampling, as all the data are automatically transferred from hospitals to laboratory information system (Lampret et al., 2020).

CONCLUSION

In the first 2 years of the expanded NBS program in Slovenia altogether about 30,000 newborns were screened. We diagnosed four cases of VLCADD (with the fifth patient diagnosed previously during the pilot study). The estimated VLCADD incidence was 1:7,500; which is much higher than that of MCADD cases in the same period. Our study also provided one of the first descriptions of *ACADVL* variants in Central-Southeastern Europe.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Medical Ethics Committee (KME: 56/01/14). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

UG and BR conceptualized and designed the study. UG, MZ, and AD performed the clinical work. NL supervised nutritional treatment and collected and analyzed nutritional data. ZR, DP, and MD performed genetic analyses. ZR, VC, DP, BU, and BR carried out the initial NBS analyses and interpreted the results. ZR coordinated and supervised data collection and analysis. BR drafted the initial manuscript. ZR, NL, and AD helped in writing. UG, BR, MD, JK, MZ, TB, and ZR critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Rapid Genetic Diagnosis of Citrin Deficiency by Multicolor Melting Curve Analysis

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Citrin deficiency caused by *SLC25A13* genetic mutations is an autosomal recessive disease, and four prevalent mutations including c.851_854del, c.1638_1660dup, IVS6+5G>A, and IVS16ins3kb make up >80% of total pathogenic mutations within the Chinese population. However, suitable assays for detection of these mutations have not yet been developed for use in routine clinical practice. In the current study, a real-time PCR-based multicolor melting curve analysis (MMCA) was developed to detect the four prevalent mutations in one closed-tube reaction. The analytical and clinical performances were evaluated using artificial templates and clinical samples. All four mutations in the test samples were accurately genotyped *via* their labeling fluorophores and *Tm* values, and the standard deviations of *Tm* values were indicated to be <0.2°C. The limit of detection was estimated to be 500 diploid human genomes per reaction. The MMCA assay of 5,332 healthy newborns from southern China identified a total of 107 *SLC25A13*-mutation carriers, indicating a carrier rate of 2%. The genotypes of 107 carriers and 112 random non-carriers were validated using direct sequencing and Long-range PCR with 100% concordance. In conclusion, the assay developed in this study may potentially serve as a rapid genetic diagnostic tool for citrin deficiency.

Keywords: citrin deficiency, SLC25A13, multicolor melting curve analysis, rapid genetic diagnosis, newborn screening

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INTRODUCTION

The *SLC25A13* gene is localized on chromosome 7q21.3 and encodes citrin, which is a calcium-stimulated mitochondrial aspartate/glutamate carrier (1). Citrin is expressed abundantly in the liver and serves important roles in urea metabolism, aerobic glycolysis, and gluconeogenesis (2, 3). Citrin deficiency that is caused by biallelic *SLC25A13* mutations is an autosomal recessive disease and can present as neonatal intrahepatic cholestasis (NICCD; OMIM, 605814) in infants or as adult-onset citrullinemia type II (CTLN2; OMIM, 603471) in adolescents and adults (4, 5). Infants with NICCD are characterized by intrahepatic cholestasis, hepatomegaly, diffuse fatty liver, variable liver dysfunction, and hyperammonemia (6, 7). In early infancy, these symptoms overlap with those of other cholestatic liver diseases, such as neonatal hepatitis and biliary atresia, making it difficult for clinicians to obtain a prompt, and accurate diagnosis. Patients with CTLN2 are characterized by exhibiting adult-onset symptoms, hyperammonemia, and a number of

neuropsychiatric manifestations (4, 8), and may be misdiagnosed with other neurological diseases. Recently, an additional phenotype, which is characterized by the failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD), has been described (9). The phenotypic features of patients with citrin deficiency are complex and highly variable. Although, a series of clinical manifestations and biochemical findings have been observed and described in patients with citrin deficiency, none of these features are pathognomonic, and *SLC25A13* genetic analysis has been proposed as an accurate diagnostic tool for citrin deficiency (10–12).

Citrin deficiency was first reported and described in Japan, but later was recognized to be a pan ethnic disease with high prevalence among the East Asian population (1, 11, 13, 14). The overall pathogenic variant carrier rate has been reported to be as high as 2% in southern China, which is the highest carrier frequency currently reported (13, 15). A total of >11,000 individuals are estimated to be homozygous or compound heterozygous for *SLC25A13* pathogenic variants in the Guangdong province in southern China (15). Currently, >80 *SLC25A13* pathogenic variants have been identified, but among them, four prevalent mutations c.851_854del (p.R284fs286X), c.1638_1660dup (p.A554fs570X), IVS6+5G>A (p.A206fs212X), and IVS16ins3kb (p.A584fs585X) account for >80% of the Chinese population who exhibit citrin deficiency (16–18). Therefore, it is important to develop a rapid and cost-effective screening method for these common *SLC25A13* mutations in areas with prevalent citrin deficiency.

A number of methods have been suggested for use in *SLC25A13* genetic analysis, including PCR-restriction fragment length polymorphism, multiplex ligation-dependent probe amplification, mass spectrometry and direct sequencing (10, 19–21). However, the long operational time, low throughput or the requirement of specialized equipment limit the application of these methods in clinical practice. Real-time-PCR-based multicolor melting curve analysis (MMCA) is a recently described detection method that allows the detection of multiple mutations in a single reaction, and has the benefits of speed, ease-of-use and cross-platform compatibility (22). The successful use of MMCA has been reported previously for the detection of genetic disease and pathogen typing (23–25). In this study, a novel MMCA method was developed that allows rapid screening of the four prevalent *SLC25A13* mutations in one closed-tube reaction. The present study also determined this method analytical performance and evaluated its clinical performance.

MATERIALS AND METHODS

DNA Templates and Clinical Samples

For the development and optimization of the MMCA assay, 29 peripheral blood samples with known genotypes (**Supplementary Table 1**), and eight artificial plasmid DNA templates containing the individual mutations or wild-types of c.851_854del, c.1638_1660dup, IVS6+5G>A, and IVS16ins3kb of *SLC25A13,* were used. The artificial plasmids were prepared by cloning the chemically synthesized gene fragment (the size and sequence of cloned fragment in each plasmid is listed in **Supplementary Table 2**) into a pUC57 vector, and their sequences were confirmed using Sanger sequencing (Sangon Biotech Co. Ltd). The mixture of c.851_854 wild-type plasmid, c.1638_1660 wild-type plasmid, IVS6+5G>A wild-type plasmid, and IVS16ins3kb wild-type plasmid at a copy-number ratio of 1:1:1:1 was used as an artificial DNA template of the *SLC25A13* wild-type, as well as the other *SLC25A13* genotype plasmid mixtures, respectively (**Supplementary Table 2**).

For the clinical evaluation and the estimation of the heterozygous carrier frequency, a total of 5,332 healthy newborn blood samples (including 4,574 cord blood, 227 peripheral blood and 531 dried blood spot) were tested. Genomic DNA (gDNA) was extracted from the blood sample using a commercial kit (Tiangen Biotech Co., Ltd.), and the gDNA concentration was determined using a Nanodrop OneC spectrophotometer (Thermo Fisher Scientific, Inc.).

All the clinical samples (29 peripheral blood samples with known genotypes and 5,332 healthy newborn blood samples) were collected from Jiangmen Maternity and Child Health Care Hospital. This study was approved by the Research Ethics Committee of Jiangmen Maternity and Child Health Care Hospital and informed consent was obtained from the participants' legal guardian/next of kin.

Primers and Probes

DNA sequences of *SLC25A13* (NCBI reference sequence: NC_000007.14) were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/). To detect the four prevalent *SLC25A13* mutations in the Chinese population, four sets of primers as well as the labeled self-quenched probes targeting the mutation regions were designed using Primer 5.0 software according to the principles of MMCA (**Table 1**). All DNA oligonucleotides were synthesized and purified by Sangon Biotech Co. Ltd.

Real-Time PCR and Melting Curve Analysis

The MMCA assay was performed in a closed tube with 20 μl reaction mixture using a SLAN-48P real-time PCR system (Shanghai Hongshi Medical Technology Co., Ltd.). Each 20 μl PCR reaction mixture contained 1× PCR buffer (10 mM Tris-HCl; pH 8.9; 50 mM KCl), 0.3 mM dNTPs, 3.75 mM $MgCl_2$, 0.5 U Taq HS DNA polymerase (Takara Bio, Inc.), 0.01–0.05 μM limiting primers, 0.07–0.75 μM excess primers, 0.01–0.05 μM probes and 1 μl extracted gDNA or artificial DNA template. The temperature protocol for PCR amplification and subsequent melting curve analysis was as follows: 95°C for 5 min; 10 touchdown PCR cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s (–0.5°C/cycle), and extension at 72°C for 30 s; 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; denaturation at 95°C for 3 min, hybridization at 45°C for 3 min, and a continuous temperature rise from 45 to 80°C at a ramp rate of 0.06°C/s.

Abbreviations: *SLC25A13*, solute carrier family 25 member 13; MMCA, multicolor melting curve analysis; Tm, melting temperature; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; CTLN2, adult-onset citrullinemia type II; FTTDCD, failure to thrive and dyslipidemia caused by citrin deficiency; gDNA, genomic DNA; –dF/dT, the negative derivative of fluorescence over temperature; CNY, Chinese Yuan.

TABLE 1 | The primer-probe sets used in this study.

Genotype	Primer-probe set	Name	Sequence	Amplicon size
c.851_854	1	1F	5′-GCCAAACTGAAGGCTATACTG-3′	164 bp
		1R	5′-CTCTTCCAGAGGAGCAAT-3′	
		1P	5′-FAM-TTTGTTTTTCCCCTACAGACGTATGACCTTAGCA-BHQ1-3′	
c.1638_1660dup	2	2F	5′-GACTGAGATGGTGTTGTGT-3′	124 bp
		2R	5′-ACTCCGCTGTAAGTGGTT-3′	
		2P	5′-CY5-ACGAGATTACTGGTGGCTGCCCGGGGAGATTACAG-BHQ2-3′	
IVS6+5G>A	3	3F	5′-CGACTTCCGAGACATCAT-3′	278 bp
		3R	5′-ATTCCGTATTACCCAGACAA-3′	
		3P	5′-CY5-AGTAGCTGTAAGTTGTAAC-BHQ2-3′	
IVS16ins3kb	4	4F	5′-ATACTGCGTGAAGAAGGAC-3′	291 bp (Wild) 447 bp (Mutation)
		4R1	5′-CTACGACAACAGAGCATTAG-3′	
		4R2	5′-CCCTCACTGCTGATTCTTAGATAG-3′	
		4P1	5′-ROX-CAGATTTAGCATGATACTTACACTCCTC-BHQ2-3′	
		4P2	5′-ROX-TTCTTTATATTTGATAGACTGC-BHQ2-3′	

Fluorescence data from FAM, ROX and CY5 channels were collected at the annealing step during the second 40 cycles and at each step of the continuous temperature rise during the melting stage. Melting curves and fluorescence melting peaks were obtained by plotting the negative derivative of fluorescence over temperature (–dF/dT) vs. temperature. Additionally, graphics output with melting temperature (*Tm*) values were automatically generated in the SLAN-48P real-time PCR system software.

Analytical Study

Following the establishment of the MMCA assay for detecting the four prevalent mutations, its accuracy, reproducibility and sensitivity were evaluated. To confirm its accuracy, a total of 29 gDNA samples with known genotypes, including 14 wild-type, 11 heterozygous mutant, three homozygous mutant and one compound heterozygous mutant were detected using the MMCA assay in a blinded manner (**Supplementary Table 1**). To study the reproducibility of *Tm*, a total of five gDNA samples, and six artificial plasmid DNA templates with known genotypes were analyzed by two technicians on 5 non-consecutive days (**Supplementary Table 3**). The *Tm* values of each genotype were measured and the differences in *Tm* (ΔTm) between the wild-type and mutant were calculated using their mean values. The mean and standard deviation (SD) values of *Tm* values for each genotype were determined using SPSS 22.0 software. To examine the analytical sensitivity of the MMCA assay, all plasmid mixtures for the 15 *SLC25A13* genotypes were serially diluted 10-fold with TE buffer (10 mM Tris-HCl; 1 mM Ethylenediaminetetraacetic acid; PH 8.0) to generate artificial DNA templates containing gene copy numbers ranging from 10^2 to 10^5 copies/μl. Each dilution was analyzed in triplicate, and TE buffer was used as a no-template control.

Clinical Study

For further evaluation of the clinical performance of the assay and for the estimation of the heterozygous carrier frequency in Jiangmen, which is a city in southern China, 5,332 gDNA samples from healthy newborns were screened for the four prevalent *SLC25A13* mutations using the MMCA assay. All detected mutations and 112 randomly selected negative samples were confirmed using direct sequencing and Long-range PCR.

RESULTS

Development of the *SLC25A13* Mutation Detection System

The developed *SLC25A13* mutation detection system is a real-time PCR-based multicolor (three color) melting curve assay that detects the four prevalent mutations for Citrin deficiency in one closed-tube reaction using four primer-probe sets. The working principle of this system is illustrated in **Figure 1**, and the primer-probe sets are presented in **Table 1**. To genotype the mutations of c.851_854del and IVS6+5G>A, the probes were designed to span the mutation regions and be fully complementary to the wild-type allele in the primer-probe set 1 and 3, respectively. The presence of a four-nucleotide deletion or single-nucleotide substitution would result in a lower *Tm* value compared with that of wild-type allele (**Figures 2A,C**). To genotype the c.1638_1660dup mutation, the probe in primer-probe set 2 was designed to span the repeat region and be highly complementary to the mutant allele, generating a higher *Tm* value compared with the wild-type allele (**Figure 2B**). To genotype the IVS16ins3kb mutation, in the primer-probe set 4, a common forward primer and two reverse primers specific to the wild-type and the mutant alleles were used for the amplification of wild-type and insertion mutant alleles, and two probes with different melting temperatures were used for the identification of the amplified allele (**Figure 2D**). The artificial templates of 15 *SLC25A13* genotypes were detected using the MMCA assay, and the mutations were individually identified by their labeling fluorophores and *Tm* values. All the mutant alleles generated distinct melting peaks corresponding to the wild-type alleles, and the representative results are displayed in **Figure 2**.

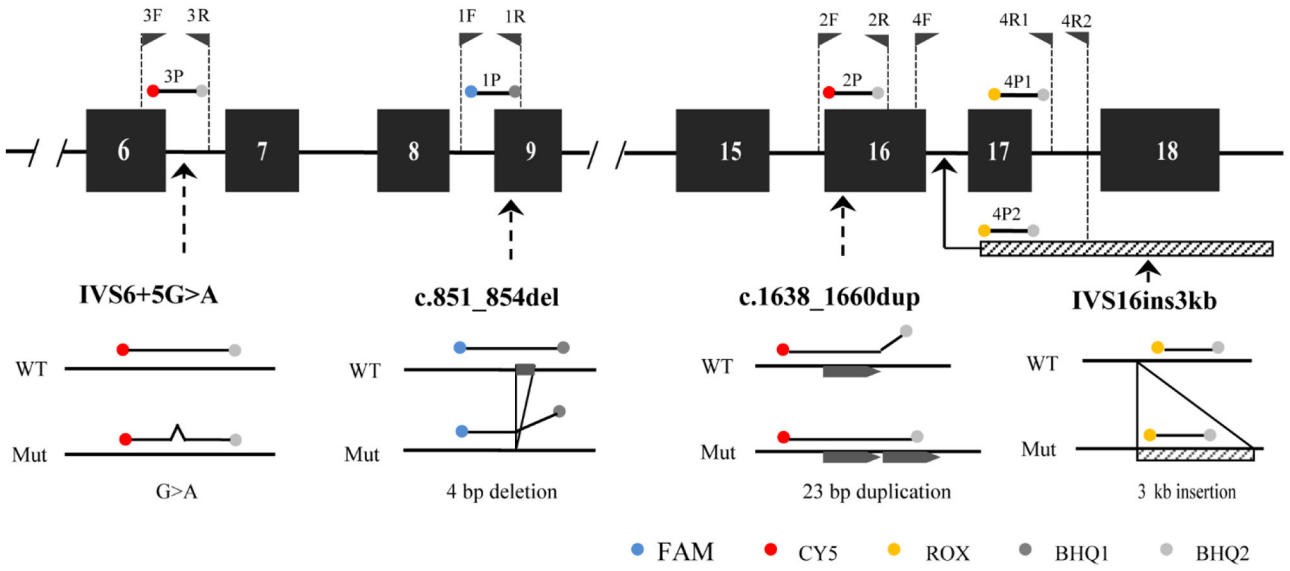

FIGURE 1 | The principle of MMCA assay for the detection of the four prevalent *SLC25A13* mutations. The relative positions of the four designed primer-probe sets within the *SLC25A13* gene are marked. MMCA, multicolor melting curve analysis.

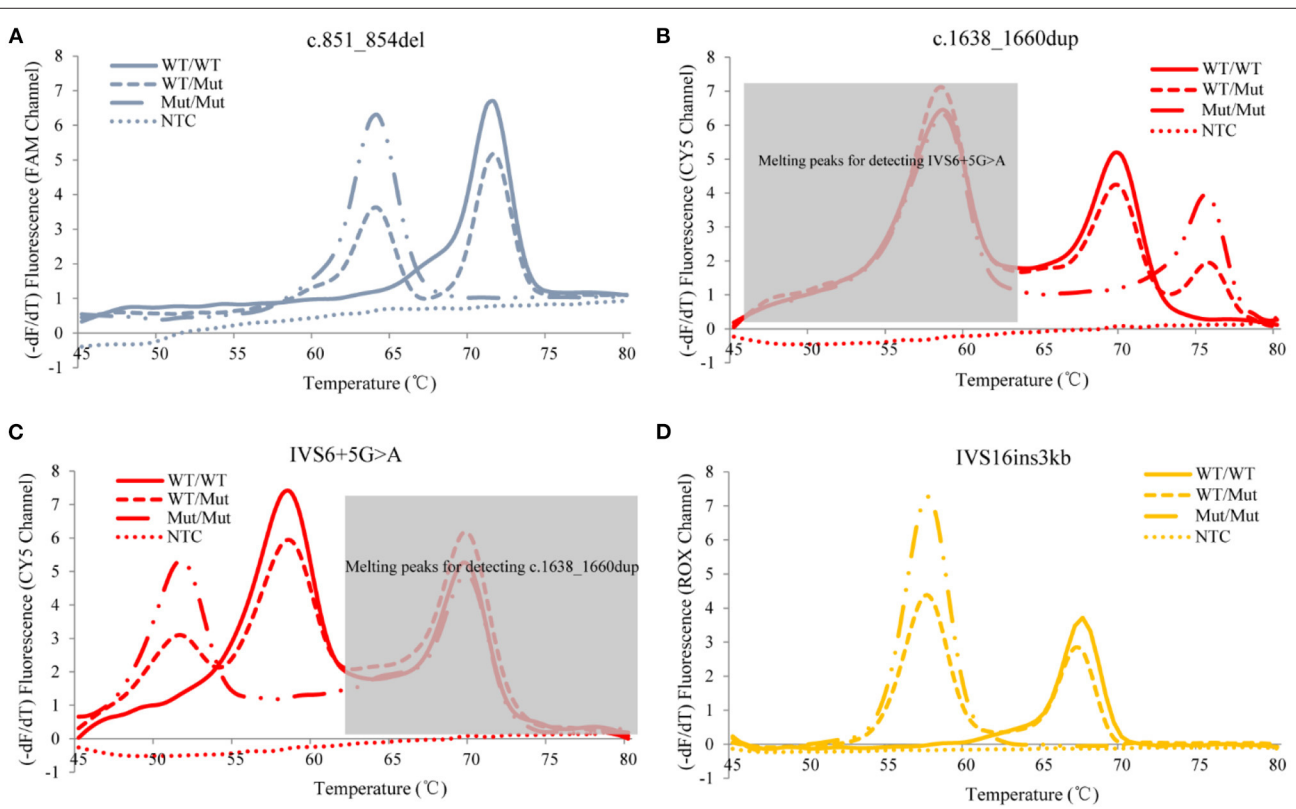

FIGURE 2 | Representative genotyping results of the four prevalent *SLC25A13* mutations using the MMCA assay. Melting curves and fluorescence melting peaks, as well as corresponding genotypes, are provided according to the detection mutations above the panel [**(A)** c.851_854del; **(B)** c.1638_1660dup; **(C)** IVS6+5G>A; **(D)** IVS16ins3kb]. In each panel, melting curves are displayed in only one channel that is related to the detection mutation, and marked on the y-axis. The mutations are individually identified by their labeling fluorophores and *Tm* values, and all mutant alleles generate distinct melting peaks corresponding to the wild-type alleles. –dF/dT, the negative derivative of fluorescence over temperature; WT, wild-type; Mut, mutant; NTC, no DNA template control; MMCA, multicolor melting curve analysis.

Analytical Studies

Firstly, we evaluated the accuracy of the developed MMCA assay using 29 reference samples with known genotypes, and all the samples gave expected *Tm* values in accordance with the respective genotypes of the four prevalent mutations (**Supplementary Table 1**). In order to further validate the assays accuracy, the genotypes of 107 *SLC25A13*-mutation carriers and 112 randomly selected non-carriers in 5,332 newborn screenings, which had already been screened using the MMCA assay, were subsequently detected using direct sequencing and Long-range PCR. The genotypes determined using both methods were identical for all 219 gDNA samples, and the representative comparison results from 4 *SLC25A13*-mutation carriers are presented in **Figure 3**. The MMCA assay accurately detected all the genotypes from the 219 samples, indicating its high sensitivity (100%) and specificity (100%) for the four prevalent *SLC25A13*-mutations. The reproducibility study indicated that the SDs of *Tm* values were $<0.2^{\circ}$C in the replicate analysis and all four mutations could be resolved with $\Delta Tm > 5^{\circ}$C (**Table 2** and **Supplementary Table 3**). The analytical sensitivity study indicated that all 15 artificial DNA templates revealed reproducible positive results at gene copy numbers ranging from 10^3 to 10^5 copies/μl. Therefore, it may be concluded that the MMCA assay exhibited an overall detection limit of 1,000 gene copies per reaction, corresponding to 500 diploid human genomes which is close to 3.5 ng gDNA.

Clinical Evaluation and Frequency of Four Prevalent Mutations

To further evaluate the clinical performance of the MMCA assay and investigate its potential for screening newborns for citrin deficiency, 5,332 healthy newborns born in Jiangmen were recruited and screened using blood samples remaining from previous clinical testing. The results indicated that 107 newborns were heterozygous for one of the four prevalent mutations, including c.851_854del that was identified in 77 newborns, c.1638_1660dup that was identified in 16 newborns, IVS6+5G>A that was identified in seven newborns and IVS16ins3kb that was identified in seven newborns (**Table 3** and **Supplementary Table 4**). As aforementioned, the parallel Sanger sequencing and Long-range PCR analysis in this cohort indicated fully consistent results. Therefore, the carrier rate of the four prevalent *SLC25A13* mutations was ∼2% (107/5,332) in the Jiangmen population. Unexpectedly, MMCA genotyping of the c.1638_1660dup mutation displayed abnormal melting peaks in three screening samples, and Sanger sequencing subsequently uncovered one sample carrying heterozygous c.1656C>T mutation and two samples carrying heterozygous c.1658G>A mutation (**Figure 4**). Collectively, the MMCA assay can accurately detect the four prevalent *SLC25A13* mutations and simultaneously indicate other possible mutations located in the probe regions.

DISCUSSION

Citrin deficiency is an autosomal recessive disease that exhibits a number of different clinical manifestations (11, 26). Diagnosis of citrin deficiency requires a high level of clinical suspicion and is confirmed by identifying pathogenic mutations in the *SLC25A13* gene. The *SLC25A13* pathogenic mutation spectrums and the carrier rates present significant geographic differences (13, 27). In southern China, the frequency of *SLC25A13*-mutation carriers was estimated to be 1/48, and the variations c.851_854del, c.1638_1660dup, IVS6+5G>A, and IVS16ins3kb were identified as being the four high-frequency mutations and accounted for >80% of the total mutations in the population (13, 16, 17). In the current study, a novel MMCA assay was successfully developed to detect the four prevalent mutations in one closed-tube reaction. The analytical results indicated that each mutation could be reliably detected from all 29 reference samples and 15 artificial DNA templates. The clinical studies of 107 carrier samples and 112 random negative samples demonstrated that the MMCA assay was as accurate at detecting mutations as direct Sanger sequencing and Long-range PCR.

In the present study, the MMCA assay was performed in a closed-tube PCR reaction, which avoided complex post-PCR manipulations and decreased the risk of PCR contamination. After the gDNA is collected, the entire procedure only requires the addition of gDNA into the reaction tube. In the current study, the cost and turnaround time of this assay was compared with Sanger sequencing and Long-range PCR. The material cost of the MMCA assay for each sample is ∼15 CNY and the entire assay can be accomplished within 3.5 h. In comparison, for Sanger sequencing and Long-range PCR, the cost is estimated to be 50 CNY per sample, and the time taken to perform the reaction is doubled. In addition, the high analytical sensitivity of the MMCA assay (3.5 ng/μl gDNA) is beneficial for DNA screening in newborns where dried blood spot samples with limited amounts of extracted DNA are commonly used. In the current study, the minimum concentration of gDNA extraction from 531 dried blood spot samples was 5.9 ng/μl, and all dried blood spot samples were successfully screened for the four prevalent *SLC25A13* mutations. Kikuchi et al. (28) established and reported a real-time PCR-based melting-curve analysis system with adjacent hybridization probes for 11 common *SLC25A13* mutations in the Japanese population. However, in their analysis system, the prevalent mutation IVS6+5G>A was not included, and the other three prevalent mutations were detected in three PCR reactions. In other previous studies, the real-time PCR-high resolution melting analysis was reported to be suitable for screening the presence of *SLC25A13* mutations, but required further sequencing to identify the mutation types (25, 26). Tokuhara et al. (29) reported that citrin was deficient in lymphocytes among patients with citrin deficiency and Western blot analysis of citrin protein in lymphocytes isolated from peripheral blood was established as an alternative diagnostic method for citrin deficiency even in patients without known genetic mutations. Our method, in combination with protein analysis, may have various benefits (e.g., improving the diagnostic yield and clarifying the aetiology) for the diagnosis of citrin deficiency.

A molecular survey on citrin deficiency epidemiology in Guangdong province, China, reported that the carrier rate of the four prevalent *SLC25A13* mutations was ∼2.06% (50/2,428), theoretically, with the number of citrin-deficiency patients

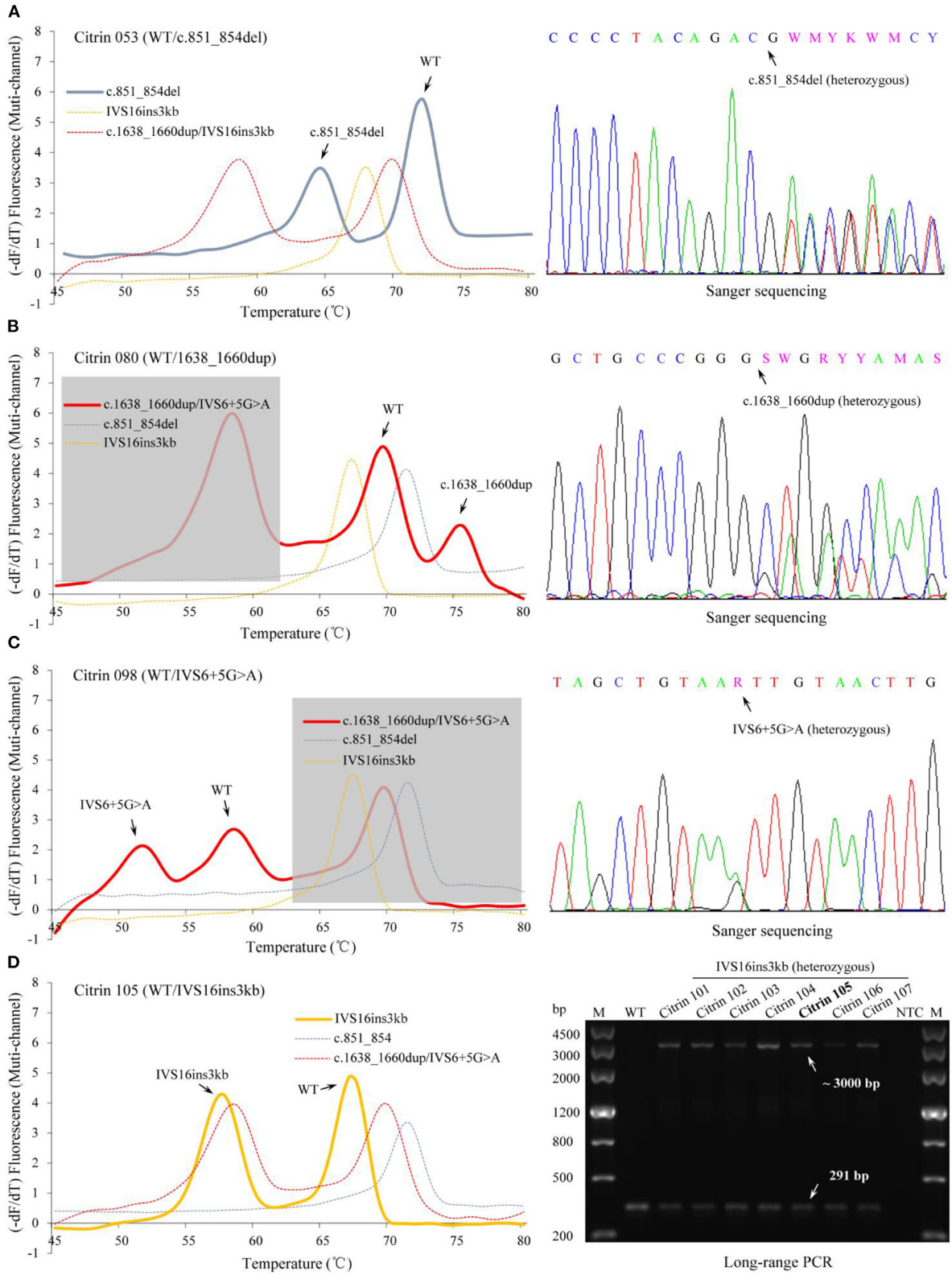

FIGURE 3 | Identification of *SLC25A13* genotypes in the newborn cohort using the MMCA assay. Representative melting curves and fluorescence melting peaks of 4 *SLC25A13*-mutation carriers are presented in the left panels, and the specimen number and genotype of each mutation carrier are marked above the panels

(Continued)

FIGURE 3 | [**(A)** Citrin 053, c.851_854del heterozygous carrier; **(B)** Citrin 080, c.1638_1660dup heterozygous carrier; **(C)** Citrin 098, IVS6+5G>A heterozygous carrier; **(D)** Citrin 105, IVS16ins3kb heterozygous carrier]. In each panel, melting curves are displayed in the multi-channel, and the channel revealing the occurrence of the mutation is presented as a solid line. The results of the MMCA assay were further detected by Sanger sequencing and Long-range PCR, obtaining the fully consistent results, which are presented in the right-hand panels. –dF/dT, the negative derivative of fluorescence over temperature; M, marker; WT, wild-type; Citrin 101 to 107, IVS16ins3kb heterozygous carriers; NTC, no DNA template control; MMCA, multicolor melting curve analysis.

TABLE 2 | The *Tm* value of the four prevalent *SLC25A13* mutations in the MMCA Assay.

Channel	Genotype	Tm_1 °C (Mutant) (mean ± SD)	Tm_2 °C (Wild-type) (mean ± SD)	Δ*Tm* (Tm_2-Tm_1) (mean)
FAM	c.851_854del	63.86 ± 0.10	71.25 ± 0.07	7.39
CY5	c.1638_1660dup	75.52 ± 0.10	69.70 ± 0.08	–5.82
CY5	IVS6+5G>A	51.29 ± 0.15	58.30 ± 0.09	7.01
ROX	IVS16ins3kb	57.45 ± 0.08	67.21 ± 0.08	9.75

TABLE 3 | The four prevalent *SLC25A13* mutations detected in Jiangmen.

Genotype	Carriers	Allele frequency
c.851_854del	77	1.44% (77/5,332)
c.1638_1660dup	16	0.30% (16/5,332)
IVS6+5G>A	7	0.13% (7/5,332)
IVS16ins3kb	7	0.13% (7/5,332)
In total	107	2.01% (107/5,332)

>11,000 in this population (15). These survey samples were collected from different cities in Guangdong province, but did not include Jiangmen city. The current study collected 5,332 samples from Jiangmen and screened the four prevalent mutations, showing the similar carrier rate of 2.01% (107/5,332). The present study has expanded and detailed the epidemiologic data for the evaluation of citrin deficiency effect in the Guangdong population. The *SLC25A13* mutation spectrum in a large citrin deficiency cohort (274 cases involved 264 Chinese families) indicated that the four prevalent mutations had a relative combined frequency of ∼85% (18). These data suggest that the detection of the four prevalent mutations using the MMCA assay could be initially performed for the rapid genetic diagnosis of patients with citrin deficiency in China, and especially in Guangdong. Furthermore, although a diversity of *SLC25A13* pathogenic variants was discovered in the East Asian populations with significant geographic differences, three of the four mutations included in this MMCA assay (c.851_854del, c.1638_1660dup, and IVS16ins3kb) were also common in other East Asian populations, including in Japan and Korea (13, 27, 30). Therefore, the developed MMCA assay may also be adapted and used in other East Asian populations.

In the newborn screening procedure, the heterozygous c.1656C>T mutation and heterozygous c.1658G>A mutation were observed using the MMCA assay and the results were further confirmed by Sanger sequencing. The variant of c.1658G>A causes the change of the amino acid sequence R553Q, which is considered to be a damaging variant and may result in a deleterious effect on protein function as reported in a previous study (31). The variant of c.1656C>T is a synonymous mutation and is not located in the splice site, suggesting that the novel mutation is likely to be a rare polymorphism. Therefore, other possible mutations that are located in the probe regions could also be indicated by the MMCA assay. In addition, to the best of our knowledge, the MMCA assay may be able to detect ≥8 mutations in a one-tube reaction using the real-time PCR instrument with four-color channels. This means that other prevalent *SLC25A13* mutations can be added to the assay to further improve its diagnostic performance for citrin deficiency.

Tandem mass spectrometry (MS/MS) technology provides an opportunity to identify several inherited metabolic diseases in a single test and is widely applied in newborn screening (32, 33). In Italy, expanded newborn screening using MS/MS is currently carried out by law for about 40 inherited metabolic diseases, including citrin deficiency (32, 34). Elevated citrulline values and ratios of citrulline to total amino acids are the primary screening markers for citrin deficiency (21, 35). Because of the instability of citrulline level after birth and the uncertainty about the relation of citrulline level and genotype, it is still challenging to accurately detect citrin deficiency in newborn metabolic screening using MS/MS (21, 35). The combination of MS/MS with genetic approach could aid in improving the detection performance of newborn screening and elucidating the genetic background of citrin deficiency. In addition, the MMCA assay could efficiently identify prevalent *SLC25A13* mutations, which would be a good test for preconception carrier screening and contribute to the prenatal counseling of citrin deficiency.

In conclusion, a closed one-tube MMCA assay for the detection of the four prevalent *SLC25A13* mutations, including c.851_854del, c.1638_1660dup, IVS6+5G>A, and IVS16ins3kb was developed in this study. While the detected mutations are limited at current stage, these mutations account for the majority of the Chinese population with citrin deficiency, and the detection performances were validated in the analytical and clinical studies. This accurate, rapid and cost-effective citrin deficiency genotyping assay could be useful for pre-conception carrier screening and improve the performance of newborn screening by combining metabolic and genetic approach.

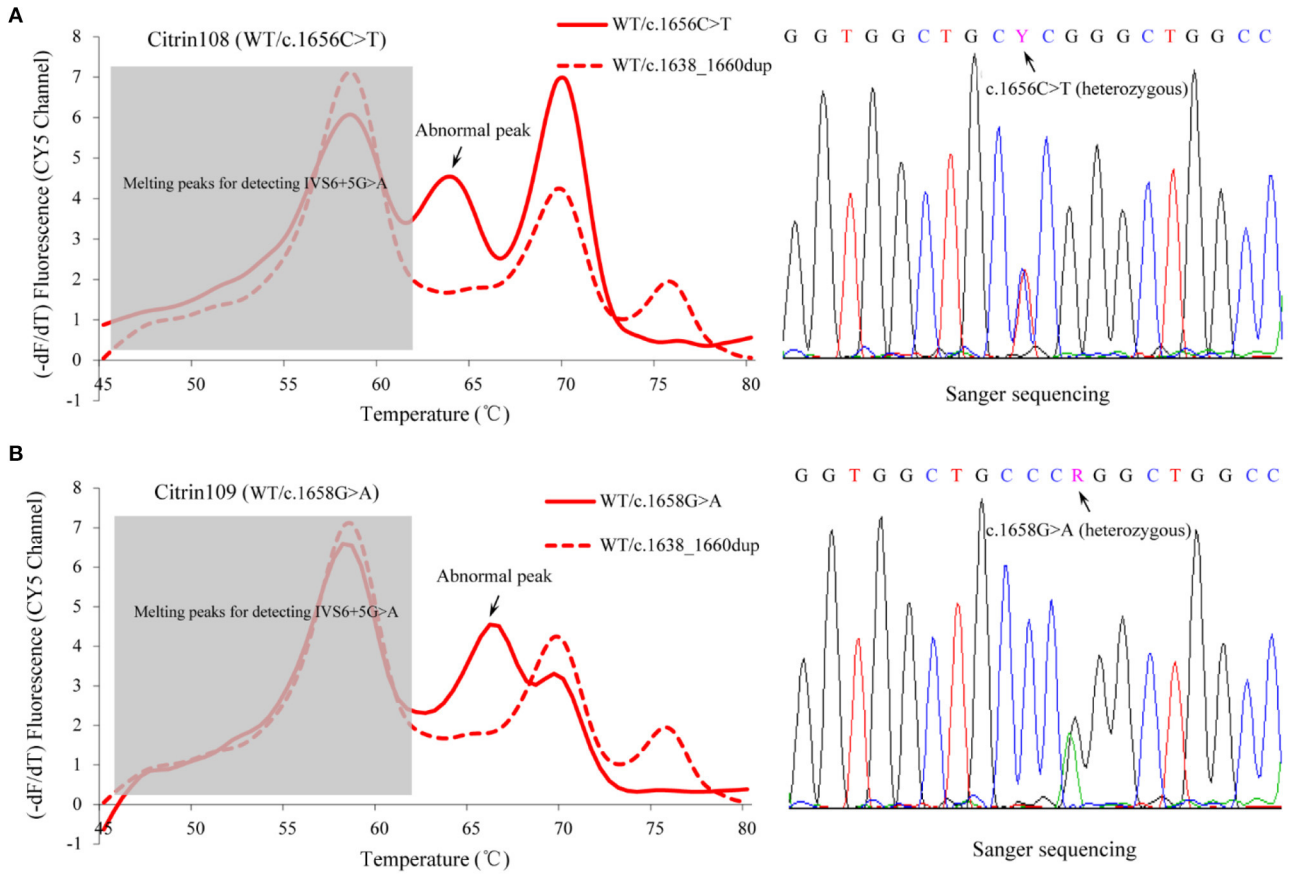

FIGURE 4 | Abnormal fluorescence melting peaks in the MMCA assay. MMCA genotyping of the *SLC25A13* c.1638_1660dup mutation displayed abnormal melting peaks with unexpected melting temperature values in three screening samples, and Sanger sequencing of the probe region uncovered one sample carrying **(A)** heterozygous c.1656C>T mutation and **(B)** two others carrying heterozygous c.1658G>A mutation (one representative is shown). Melting curves are only displayed in the CY5 channel, and the abnormal melting peaks are marked in the solid lines that correspond to the melting curves of the screened samples. The dashed line represents the melting curve of WT/ c.1638_1660dup genotype, indicating the positions of normal melting peaks. –dF/dT, the negative derivative of fluorescence over temperature; WT, wild-type; MMCA, multicolor melting curve analysis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Jiangmen Maternity and Child Health Care Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QZ and TO conceived the study and mainly contributed to data analysis and drafting of the manuscript. YL, CD, and YuY provided clinical samples and relevant information. QZ, JX, ST, and SS kept and pretreated samples. QZ, YiY, and JL performed experiments. TO and YL contributed scientific insights and refined the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.654527/full#supplementary-material

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Novel Compound Heterozygous Pathogenic Variants in *SUOX* Cause Isolated Sulfite Oxidase Deficiency in a Chinese Han Family

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Aim: To explore the clinical imaging, laboratory and genetic characteristics of a newborn boy with isolated sulfite oxidase deficiency (ISOD) in a Chinese mainland cohort.

Methods: Homocysteine and uric acid in plasma and cysteine and total homocysteine in the blood spot were assessed in a Chinese newborn patient with progressive encephalopathy, tonic seizures, abnormal muscle tone, and feeding difficulties. Whole exome sequencing and Sanger sequencing facilitated an accurate diagnosis. The pathogenicity predictions and conservation analysis of the identified mutations were conducted by bioinformatics tools.

Results: Low total homocysteine was detected in the blood spot, while homocysteine and uric acid levels were normal in the plasma. *S*-sulfocysteine was abnormally elevated in urine. A follow-up examination revealed several progressive neuropathological findings. Also, intermittent convulsions and axial dystonia were observed. However, the coordination of sucking and swallowing was slightly improved. A novel paternal nonsense variant c.475G > T (p.Glu159*) and a novel maternal missense variant c.1201A > G (p.Lys401Glu) in *SUOX* were identified in this case by co-segregation verification.

Conclusion: This is the second report of early-onset ISOD case in a non-consanguineous Chinese mainland family. Combined with the clinical characteristics and biochemical indexes, we speculated that these two novel pathogenic variants of the *SUOX* gene underlie the cause of the disease in this patient. Next-generation sequencing (NGS) and Sanger sequencing provided reliable basis for clinical and prenatal diagnoses of this family, it also enriched the mutation spectrum of the *SUOX* gene.

Keywords: isolated sulfite oxidase deficiency, *SUOX*, molecular diagnosis, infant, neurometabolic disease, genetic counseling

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INTRODUCTION

Isolated sulfite oxidase deficiency (ISOD, OMIM: 272300) is an autosomal recessive inherited neurometabolic disease caused by deficient activity of sulfite oxidase. It is characterized by some severe neurological symptoms, including seizures, often non-effective to anticonvulsant medications, and rapidly progressive encephalopathy resulting in a similar condition of neonatal

hypoxic ischemia. The majority of the patients developed microcephaly, feeding difficulties, and dislocated ocular lenses. Tissue accumulation and high urinary excretion of sulfite, thiosulfate, and *S*-sulfocysteine were the main biochemical features of the disease (Tan et al., 2005). The time of onset is neonatal or early infantile period. The incidence of ISOD has not been reported epidemiologically. To date, < 50 cases have been reported worldwide (van der Klei-van Moorsel et al., 1991; Rupar et al., 1996; Garrett et al., 1998; Johnson et al., 2002a; Lee et al., 2002; Seidahmed et al., 2005; Claerhout et al., 2018; Chen et al., 2014; Rocha et al., 2014; Zaki et al., 2016; Brumaru et al., 2017; Lee et al., 2017; Mhanni et al., 2020; Sharawat et al., 2020; Du et al., 2021). Recently, four early-onset ISOD patients have been reported in Hong Kong and Taiwan, China (Chan et al., 2002; Lee et al., 2002, 2017; Chen et al., 2014), one early-onset patient in Chinese mainland (Du et al., 2021), and one late-onset ISOD pedigree including three patients have been reported in Chinese mainland (Tian et al., 2019).

Oxidation of sulfite is catalyzed by sulfite oxidase (SO) to sulfate, which constitutes the terminal reaction in the oxidative degradation of sulfur-containing amino acids, methionine, and cysteine. SO is a molybdo hemoprotein comprising of 545 amino acids. The gene encoding SO (*SUOX*, OMIM 606887) maps to chromosome 12q13.2-12q13.3, and the coding sequence contains three exons and two introns (Johnson et al., 2002a). To date, only 29 *SUOX* variants were reported in HGMD database, including missense, nonsense, and deletion, or insertion mutations, which have been identified in unrelated individuals with ISOD worldwide. However, only 5/29 mutations were reported in Taiwan patients (Chen et al., 2014). In this study, we presented the clinical, imaging, and biochemical characteristics of an 18-day-old newborn boy with SO deficiency in the mainland Chinese cohort and two previously unreported pathogenic variants in the *SUOX* gene. The patient was diagnosed based on the clinical features and genetic analysis.

MATERIALS AND METHODS

Clinical Features and Biochemical Findings of the Patient

The proband was a male child born to non-consanguineous chinese parents with a full-term gestation and a vaginal delivery. He had a normal weight (3,020 g) and head circumference (34 cm) at birth. The family history was unremarkable. All members of his family participated in this study after providing written informed consent. The Ethics Committee of the Xi'an Children's Hospital reviewed and approved our study protocol that was in compliance with the Helsinki declaration.

The proband had projectile vomitting at the age of 16 days, accompanied by irritable crying, fever, and diarrhea. After 2 days, he was admitted for further treatment, wherein cardiovascular, abdominal, genitourinary, electrolytes, hepatic, and renal functions were found to be normal except abundant leukocytes detected in the urine routine. The results of blood tandem mass spectrometry analysis were normal. Urine organic acidemia screening showed slightly elevated 3-hydroxypropionic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenyl-lactic acid. The day after the admission, he presented enophthalmos in the crying or quiet state. Brain magnetic resonance imaging (MRI) did not show any significant abnormality (**Figure 1Aa**). The visual evoked potential showed decreased binocular amplitude and prolonged latency. The physicians suspected diarrhea and urinary tract infections, which could be treated before discharge from the hospital.

At the age of 33 days, he was readmitted for fever and diarrhea, which rapidly progressed to encephalopathy, including tonic seizures, unconsciousness, dyspnea, and lethargy. Physical examination did not reveal dysmorphia. The birth weight increased only 230 g in 1 month. Moreover, the patient was irritable, hypertonic, and his coordination of sucking and swallowing was severely impaired (**Table 1**). Blood and cerebrospinal fluid cultures yielded negative results. Also, the serum ammonia and lactic acid level were significantly elevated. Electro encephalogram (EEG) showed moderately abnormal neonatal data: multifocal sharp waves and frequent discharge. The seizures were partially controlled by phenobarbital. Craniocerebral ultrasound showed cerebral edema. Brain MRI showed diffuse signal abnormalities in bilateral cerebral hemispheres, basal ganglia, and thalamus (**Figure 1Ab**), and hence, a neurometabolic disorder was suspected. Fundus examination showed ischemic changes in the optic nerve in both eyes. Plasma amino acid and urinary organic acid profiles did not reveal any obvious abnormality. The following treatment measures were adopted for the patients: (1) Anti-infection treatment of ceftazidime, and the fluid volume was limited to 80–100 mL/kg/d; (2) Mannitol and furosemide were used to reduce intracranial pressure and brain edema; (3) Phenobarbital was used to control seizures in the early stages, following which, levetiracetam was applied. (4) Either oxygen or passive inhalation of oxygen was supplied; (5) L-carnitine and sodium bicarbonate infusion were given to correct acidosis; (6) Fasting was initiated, and then the low-protein milk powder was fed through the gastrointestinal tract. After 18 days of treatment, despite the difficulty in feeding (a tiny spoon feeding was necessary) and abnormal muscle tension, the infant showed the following symptoms: normal body temperature, steady breathing, flat bregma, seizure reduction, correction of acidosis, and decreased blood ammonia and lactate. Subsequently, it was instructed to continue feeding the patient with low-protein milk powder with oral administration of levetiracetam and levocanidin.

A follow-up examination at the age of 5 months, he was presented with slow increase of body weight and progressive microcephaly. His weight was 5,500 g, and the head circumference was 40 cm which is 2 SD below the mean. Sucking and swallowing were significantly improved, but he also presented intermittent convulsions and axial dystonia. A repeated MRI on the same day showed polycystic encephalomalacia and atrophy with bilateral subdural effusion (**Figure 1Ac**). Serum ammonia and lactic acid levels returned to normal. Moreover, based on the genetic test results, we detected the level of *S*-sulfocysteine in patient's urine and the level of t-homocysteine in patient's dry blood spots by liquid chromatography-mass spectrometry (Shimadzu, Tokyo, Japan)

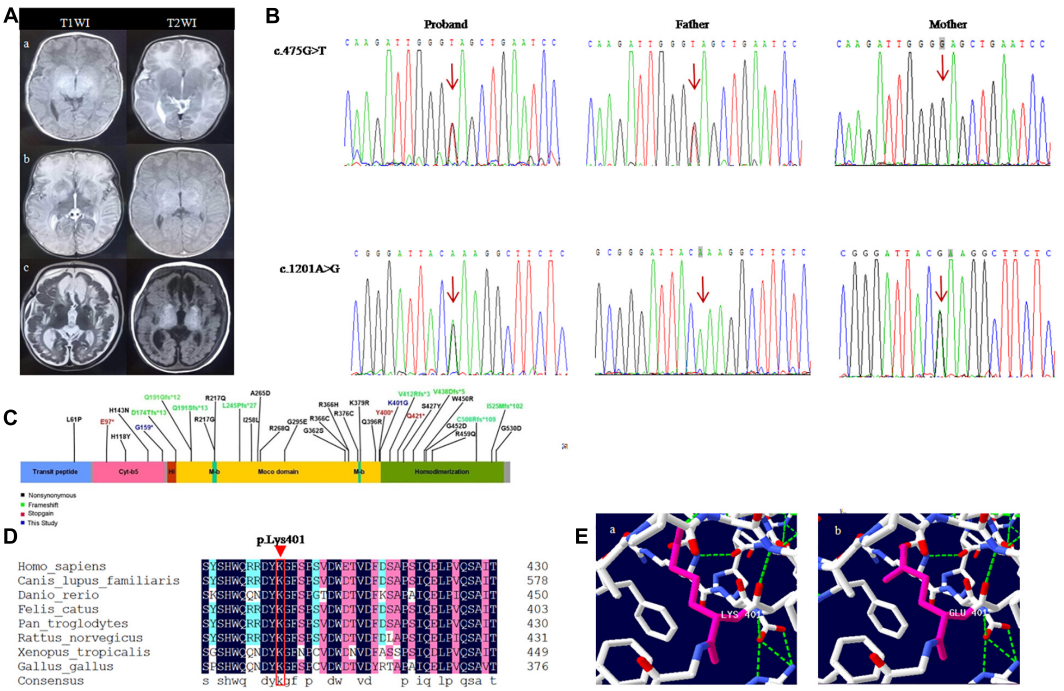

FIGURE 1 | Shows the neuroradiological features and genetic results of the patient. **(A)** Brain MRI of the follow-up of a child with sulfite deficiency enzyme. **(Aa)** MRI findings were normal at the age of 18 days; **(Ab)** 1 month and 3 days after birth, MRI showed high signal on T2WI and low signal onT1WI in bilateral cerebral hemispheres, basal ganglia, and thalamus, DWI showed high signal, and ADC showed low signal; **(Ac)** Follow-up to 5 months, MRI showed polycystic encephalomalacia and atrophy with bilateral subdural effusion. **(B)** Sanger sequencing analysis of *SUOX* gene exon 6 in genomic DNA from the family. **(C)** Linear map of the mutations in *SUOX*. **(D)** Conservation of the p.Lys401Glu variant found in this study. **(E)** Amino acid and conformation changes of the p.Lys401Glu polypeptide wild-type **(Ea)** and mutant type **(Eb)**.

(Fu et al., 2013; Sass et al., 2004). We also detected the level of uric acid in patient's serum by uric acid method (Maccura, Chengdu, China), and the level of homocysteine in patient's serum by cycling method (Gcell, Beijing, China) (Roberts and Roberts, 2004). The data showed that low total homocysteine was found in blood spot, while homocysteine and uric acid levels were normal in the plasma. *S*-sulfocysteine was abnormally elevated in urine (**Table 2**). The patient was treated with low methionine, low protein diet and low cysteine, and rehabilitation (dysphagia and sports) training was given. At 9 months, the patient died of worsened condition due to renewed fever and convulsions.

Genetic Analysis

Genomic DNA was extracted from 3 mL of peripheral blood leucocytes using the QIAamp Blood Midi Kit (Qiagen, Valencia, CA, United States), according to the manufacturer's instructions. Whole exomes were captured (MyGenostics Inc., Beijing, China) and sequenced on Illumina HiSeq 2000 sequencer. Alignment and variant calling were performed by applying an in-house bioinformatics pipeline (MyGenostics). The variants with a minor allele frequency of <0.05 in population databases, such as 1,000 genome, ESP6500, dbSNP, EXAC, and in-house database (MyGenostics), expected to affect protein coding/splicing or present in the Human Gene Mutation Database (HGMD), were included in the analysis. The identified mutation was verified among the remaining family members by Sanger sequencing. The pathogenicity of candidate variants was deduced according to the American College of Medical Genetics and Genomics (ACMG) guidelines. The effect of missense variation on the three-dimensional (3D) structure of SUOX protein was analyzed by Swiss-PDB viewer (PDB: 1MJ4).

RESULTS

Genetic Analysis and Co-segregation in the Family

Two novel variants were identified in the patient by NGS and bioinformatics analysis: a missense variant c.1201A > G(p.Lys401Glu) and a nonsense variant c.475G > T(p.Glu159*) on exon 6 of the *SUOX* gene (NM_000456.3). Subsequently, the co-segregation verification of these two variants was demonstrated by Sanger sequencing. The father carried c.475G > T (p.Glu159*) variant and the mother carried c.1201A > G (p.Lys401Glu) variant (**Figure 1B**). So the patient is compound heterozygous. None of these variants were reported previously. Both variants were absent in gnomAD (**Supplementary Table 1**) (PM2-Supporting). c.475G > T(p.Glu159*) variant leads to premature termination of protein translation, which might damage gene function

TABLE 1 | Clinical features of the present cases and the reported in the literature.

Case	Sex	Onset age	Feeding problem	Seizures	Microcephaly	Lens subluxation	Extrapyramidal sign	Developmental delay	Dystonia	Outcome	Variants	References
1	M	16 days	+	+	+	–	+	+	+	9 months	c.475G > T(p.Glu159*) and c.1201A > G(p.Lys401Glu)	Present case
2	m	36 hours	+	+	+	+	+	+	+	32 months	c.571delC(p.Gln191Serfs*13), hom	Rupar et al., 1996
3	M	53 hours	+	+	NR	NR	NR	+	+	50 days	c.794C > A(p.Ala265Asp) and c.1280C > A(p.Ser427Tyr)	Edwards et al., 1999
4	F	24 hours	+	+	+	NR	NR	NR	+	10 weeks	c.571_574del CAGC (p.Gln191Glyfs*12), hom	Johnson et al., 2002b
5	M	21 days	+	+	NR	+	+	+	+	N/S	c.650G > A(p.Arg217Gln) and c.1200C > G(p.Tyr400*)	Lee et al., 2002
6	M	2 days	NR	+	+	–	NR	+	+	N/S	c.520delG (p.Asp174Thrfs*13), hom	Seidahmed et al., 2005
7	F	2 days	NR	+	+	+	NR	NR	+	14 months	c.1234_1235delGT (p.Val412Argfs*3), hom	Salih et al., 2013
8	M	3 days	+	+	+	NR	NR	+	+	Alive		
9	F	3 days	+	+	NR	NR	NR	NR	+	N/S	c.1200C > G (p.Tyr400*) + UPD (12) pat	Cho et al., 2013
10	M	1 day	NR	+	+	NR	NR	+	+	Alive	c.1136A > G (p.Lys379Arg), hom	Holder et al., 2014
11	F	14 hours	NR	+	NR	NR	NR	NR	NR	N/S	c.1200C > G (p.Tyr400*) and c.1355G > A (p.Gly452Asp)	Chen et al., 2014
12	F	1 days	+	+	+	–	+	+	+	N/S	c.1200C > G (p.Tyr400*), hom	Lee et al., 2017
13	M	3 weeks	+	+	+	NR	NR	NR	+	N/S	c.352C > T(p.His118Tyr) and c.649C > G (p.Arg217Gly)	Brumaru et al., 2017
14	M	3 days	+	+	+	NR	+	+	+	Alive	c.1084G > A(p.Gly362Ser), hom	Bender et al., 2019
15	M	15 days	+	+	+	–	+	+	+	N/S	c.884G > A(p.Gly295Glu), hom	Zaki et al., 2016
16	F	40 days	+	+	+	–	+	+	+	N/S		
17	F	24 hours	+	+	+	+	+	+	+	N/S	c.1313_1316delTAGA (p.Val438Aspfs*5), hom	Tan et al., 2005
18	F	2 days	NR	+	+	NR	+	+	+	Alive	c.1200C > G (p.Tyr400*) and c.1549_1574dup (p.Ile525Metfs*102)	Du et al., 2021
19	F	1 week	NR	+	NR	NR	+	+	+	9 years	c.1521_1524del TTGT(p.Cys508Argfs*109), hom	Mhanni et al., 2020
20	M	5 days	NR	+	NR	NR	+	+	+	15 months		
21*	F	5 months	+	+	NR	+	+	+	+	N/S	c.650G > A(p.Arg217Gln), hom	Garrett et al., 1998
22*	F	1 months	NR	NR	NR	NR	NR	+	+	Alive	c.427C > A (p.His143Asn), hom	Del Rizzo et al., 2013
23*	F	12 months	NR	NR	NR	+	+	+	+	Alive	c.182T > C (p.Leu61Pro) hom	Rocha et al., 2014
24*	M	2 years	NR	–	–	NR	NR	+	+	Alive	c.1096C > T (p.Arg366Cys) and c.1376G > A(p.Arg459Gln)	Tian et al., 2019
25*	F	14 months	NR	+	–	NR	+	+	+	Alive		
26*	M	16 months	NR	–	–	NR	+	+	+	Alive		
27*	M	9 months	NR	+	NR	–	+	+	+	Alive	c.1382A > T(p.Asp461Val), hom	Sharawat et al., 2020
28*	M	24 months	NR	+	–	NR	+	+	+	Alive		
29*	F	5 months	NR	+	+	+	NR	+	+	N/S	c.650G > A(p.Arg217Gln), hom	Lam et al., 2002

*NR, not report; +, present; -, absence; *, late-onset patients or mild patients; N/S, not specified in case report.*

TABLE 2 | Biochemical finding of the case.

Biochemical finding	Results	Reference value
S-sulfocysteine (urine)	112.786 μmol/mmolCrn	[Frame1]0.1–10 μmol/mmolCrn
Total-homocysteine (blood spot)	0.87 μmol/L	5–20 μmol/L
Homocysteine (plasma)	3.24 μmol/L	0–15 μmol/L
Uric acid (plasma)	227 μmol/L	210–430 μmol/L

(PVS1); c.1201A > G(p.Lys401Glu) variant was detected in the *trans* position of the c.475G > T variant (PM3); c.1201A > G is predicted to be deleterious using multiple algorithm for missense mutation annotation (SIFT, PolyPhen-2, and MutationTaster) (PP3). According to the ACMG guidelines, c.475G > T(p.Glu159*) is defined as "likely pathogenic" (PVS1 + PM2-Supporting) and c.1201A > G(p.Lys401Glu) as "uncertain significance" (PM3 + PP3 + PM2-Supporting) (Richards et al., 2015).

Structure-Function Correlations of *SUOX* Variants

Sulfite oxidase is a homodimeric protein in the intermembrane space of mitochondria. It plays a vital role in the metabolic pathway of sulfur amino acids that are involved in the last step reaction in the oxidative degradation of the sulfur-containing amino acids, cysteine and methionine (Macleod et al., 1961; Feng et al., 2007). The SO deficiency prevents the sulfites from being oxidized to sulfates. The natural enzyme is a homodimer with a molecular mass of approximately 110 kDa. Each monomer include three different domains: a smaller N-terminal cytochrome b5 heme-binding domain, a central domain harboring the molybdenum cofactor (Moco), and a larger C-terminal dimerization domain with crucial residues at the dimer interface (**Figure 1C**) (Kisker et al., 1997). The nonsense variant p.Glu159* is harbored on the C-terminus of the cytochrome b5 heme-binding domain and near the beginning of the molybdopterin-binding domain of the SO, which might produce a truncated protein containing 159 amino acids, lacking a crucial molybdopterin-binding domain. The missense variant p.Lys401Glu is present in the last residue of the molybdopterin-binding domain, leading to the glutamic acid instead of lysine acid at position 401 in the SO protein. Reportedly, other missense variants (R160Q) in this domain can reduce enzyme activity (Garrett et al., 1998). Moreover, lysine 401 is conserved across evolution of SO (**Figure 1D**). SWISS-MODEL[1] simulates the prominent amino acid and conformational changes in the influenced polypeptide (**Figure 1E**). Consequently, the length of the side chain was altered after the substitution of lysine by glutamic acid.

[1]https://swissmodel.expasy.org

DISCUSSION

Moco is a core component of the sulfite oxidase maturation process. On the other hand, the synthesis of Moco requires several steps, the related enzymes are encoded by the genes *MOCS1*, *MOCS2*, *MOCS3*, and *GEPH*. Hence, the defect of Moco synthesis results in combined deficiencies of the enzymes SO, xanthine dehydrogenase, and aldehyde oxidase (Atwal and Scaglia, 2016). The two forms of SO deficiencies are regarded as Moco deficiency (MoCD) and ISOD, respectively. Nonetheless, these deficiencies are difficult to distinguish based on clinical manifestations. Biochemically, the affected individuals with ISOD and MoCD show the accumulation of sulfite, thiosulfate, and *S*-sulfocysteine in the tissues and body (Zaki et al., 2016). However, individuals with MoCD also display elevated urinary xanthine and hypoxanthine levels (Schwarz, 2005). In addition, urinary urothione, a breakdown product of the molybdenum cofactor, is absent in MoCD but present in ISOD (Sass et al., 2010). Therefore, genetic analysis is vital for the definite diagnosis of ISOD.

Most of the ISOD patients see a doctor in the neonatal period and the clinical manifestation is usually severe, including a progressive course with spasticity, intellectual deficit, microcephaly, and possible development of lens dislocation. In addition, ISOD is an incurable disease without an effective long-term therapy. Also, late-onset and mild forms of the illness have been described (Barbot et al., 1995; Touati et al., 2000; Del Rizzo et al., 2013; Rocha et al., 2014; Tian et al., 2019). The neuropathological characteristic of ISOD is significant but non-specific. The neuroimaging by computed tomography (CT) or MRI showed progressive neuropathological results, including cerebellar and cerebral atrophy, white matter changes, ventriculomegaly, and cystic leukomalacia (Claerhout et al., 2018). The clinical phenotype of our patient with ISOD was similar to that reported in the literature except for the absence of lens dislocation. Moreover, the results of the brain MRI showed progressive development; the MRI at the 5 months of age showed gradual polycystic encephalomalacia and atrophy with bilateral subdural effusion compared to that in the newborn. The natural history of ectopia lentis is difficult to describe because not all patients present lens subluxation in the first year of life (Lueder and Steiner, 1995). Our patient did not display ectopic lens but only ischemic changes in the optic nerve in both eyes, and the phenotype may or not appear with the age, thereby necessitating a regular follow-up. Biochemically, the patient presented low total homocysteine in the blood spot, while homocysteine and uric acid in plasma were normal. *S*-sulfocysteine presented an abnormally elevated level in urine. These clinical manifestations and laboratory results were in accordance with the diagnosis of ISOD.

Sulfite oxidase is a molybdo hemoprotein with a homodimer structure. Each monomer of SO contains three identical domains. Presently, the potential functionality of SO is not clear, but the dimerization of SO is crucial for a functional enzyme. Thus, mutations around the dimerization interface of SO result in the inactivation of the enzyme (Karakas and Kisker, 2005).

In the central molybdenum domain, the pterin-based Moco forms the catalytic site of SO. Moreover, Moco is a vital constitute of the SO maturation process and a primary factor for heme integration and dimerization, further necessitating mitochondrial localization of SO (Atwal and Scaglia, 2016). The patient carried the heterozygous variant p.Lys401Glu, which is localized in the last residue of the molybdenum domain and adjacent to the dimer interface. Hence, we speculated that p.Lys401Glu affects the interaction between molybdenum and dimerization domains, which might disturb the structural stability of the protein. Thus, it is speculated that the positive charge lysine is replaced by the negative charge of glutamic acid, which might affect the binding of the enzyme active site. In addition, the lysine guanidino group might attract the divalent sulfite anion. The second novel variant p.Glu159* in the first domain of SO introduced stop codons and led to the premature termination of protein translation. Therefore, this variant led to a severe form of SO deficiency in our patient. Herein, we conducted genetic analysis on the family and identified that the variants, c.475G > T(p.Glu159*) and c.1201A > G(p.Lys401Glu) derived from the father and mother, respectively. Genetic counseling is indispensable for the family which has a ISOD proband because the situation is often lethal in the neonatal period. Although the patient beyond the neonatal period, severe sequelae are unavoidable. In view of this situation, amniocentesis should be carried out between 15 and 23 weeks of the subsequent pregnancy in this couple for prenatal diagnosis (Özcan et al., 2017). The analysis of *SUOX* exon 6 is recommended to deduce whether the fetus carries any of the pathogenic variants from his parents.

The correlation between genotype and phenotype of ISOD has not yet been well elucidated. Reportedly, the clinical manifestations of patients with *SUOX* missense mutations were milder than those with null mutations (Claerhout et al., 2018), because these missense mutations of the *SUOX* gene only resulted in reduced enzyme synthesis, while null mutations abolished SUOX biosynthesis (Rocha et al., 2014). Herein, we reviewed 29 ISOD patients who reported genotypic and phenotypic features with integrity (**Table 1**); 20/29 patients were early-onset and 9/29 were late-onset or mild presentation. Interestingly, all the late-onset patients carried the missense variants that were distributed in the three structural domains of the SUOX protein. Conversely, among the 40 alleles carried by early-onset patients, nonsense variants accounted for 26/40 (65%) and missense variants accounted for 14/40 (35%) (**Figure 1C**). The age of onset ranged from 14h to 40 days in patients with early-onset or severe phenotypes, while it ranged from 1 month to 2 years in patients with late-onset or mild phenotypes and even in patients who spontaneously recovered without treatment. Therefore, the age at onset of ISOD patients may be related to the type of genetic variation. This conclusion provides a reasonable explanation for the clinical severity of our case.

To date, the treatment for neonatal ISOD is not promising. Typically, symptomatic treatment is primarily used to control seizures but with little success. However, dietary restriction intake of methionine, cysteine, and taurine has been found to be effective for mild patients with ISOD (Barbot et al., 1995; Touati et al., 2000; Del Rizzo et al., 2013; Rocha et al., 2014; Tian et al., 2019). In some circumstances, spontaneously recovery of late-onset mild ISOD has been reported (Tian et al., 2019). Belaidi et al. (2015) reported that the oxygen reactivity of mammalian SO provides a novel therapeutic route for the treatment of ISOD and MoCD. According to a recent study, oxidative stress and mitochondrial dysfunction underlie the pathophysiology of the brain damage of ISOD, providing novel viewpoints for the potential therapeutic strategies for this condition (Wyse et al., 2019). Thus, we tried low sulfur amino acid diet and oral levetiracetam, which improved the feeding difficulties; however, epilepsy did not improve significantly.

In conclusion, ISOD is a rare neurometabolic disorder that is difficult to diagnose by clinical symptoms alone. The two novel potentially pathogenic variants in *SUOX* were found in a Chinese mainland newborn patient with ISOD, and the clinical features were described comprehensively. Thus, the patients with suspected ISOD maybe more effectively diagnosed by genetic analysis, which would further improve the mutation spectrum of *SUOX*. In addition, genetic counseling is crucial because severe neurodegeneration develops, especially in the early neonatal period that prevents birth defects.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Committee of the Xi'an Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JZ: conceptualization, writing manuscript, and editing. YA: data curation and sample sequencing. HJ: data curation, software, and methodology. HW: funding acquisition. FC: writing manuscript, editing, and manuscript review. YY: funding acquisition, project administration, and manuscript review. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Current Status of Newborn Screening in Southeastern Europe

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Significant part of Southeastern Europe (with a population of 76 million) has newborn screening (NBS) programs non-harmonized with developed European countries. Initial survey was conducted in 2013/2014 among 11 countries from the region (Albania, Bulgaria, Bosnia and Herzegovina (BIH), Croatia, Kosovo, Macedonia, Moldova, Montenegro, Romania, Serbia, and Slovenia) to assess the main characteristics of their NBS programs and their future plans. Their cumulative population at that time was ∼52,5 million. At that time, none of the countries had an expanded NBS program, while phenylketonuria screening was not introduced in four and congenital hypothyroidism in three of 11 countries. We repeated the survey in 2020 inviting the same 11 countries, adding Cyprus, Greece, Hungary, and Malta (due to their geographical position in the wider region). The aims were to assess the current state, to evaluate the change in the period, and to identify the main obstacles impacting the implementation of expanded NBS and/or reaching a wider population. Responses were collected from 12 countries (BIH—Federation of BIH, BIH—Republic of Srpska, Bulgaria, Croatia, Greece, Hungary, Kosovo, North Macedonia, Malta, Montenegro, Romania, Serbia, Slovenia) with a population of 68.5 million. The results of the survey showed that the regional situation regarding NBS only modestly improved in this period. All of the surveyed countries except Kosovo screened for at least congenital hypothyroidism, while phenylketonuria was not screened in four of 12 countries. Croatia and Slovenia implemented an expanded NBS program using tandem mass spectrometry from the time of last survey. In conclusion,

the current status of NBS programs in Southeastern Europe is very variable and is still underdeveloped (or even non-existent) in some of the countries. We suggest establishing an international task-force to assist with implementation and harmonization of basic NBS services where needed.

Keywords: newborn screening, NBS, southeastern Europe, survey, expanded NBS program, neonatal screening, dried blood sample

INTRODUCTION

Newborn screening (NBS) programs include an important set of tests conducted in the early newborn's life aimed at the pre-symptomatic discovery of various rare inborn diseases, where an early detection and treatment is crucial for preventing severe health damage or even death (1). (Available at: https://ec.europa.eu/eurostat/web/population-demography-migration-projections/data). (Available at: https://data.worldbank.org/indicator/NY.GDP.PCAP.CD).

NBS started in the 1960's with Guthrie's test for phenylketonuria (PKU) and gradually expanded to over 50 different diseases in some of the developed countries (2, 3). A tandem mass spectrometry (MS/MS) method has been successfully implemented in the last two decades in many countries allowing fast expansion and simultaneous screening for many diseases from a dried blood spot (DBS) sample (4). New generation sequencing (NGS) is another promising method that can be used for second tier testing and discovery of responsible pathological genetic variants (5). Some of the developed countries are now adding NBS for severe combined immunodeficiency based on T-cell receptor excision circles (TRECs), cystic fibrosis (CF), lysosomal storage disorders (LSD) and others to their NBS (3, 6–8).

Wilson and Jungner described the principles to guide screening decisions, which include available tests, accepted treatments and the cost-effectiveness of the screening, but also emphasize the importance of available facilities for diagnosis and treatment (9). The last-mentioned could be problematic in developing countries, where the lack of financial resources often impedes or even prevents the establishment of screening facilities and employment of appropriately educated staff.

Southeastern Europe (SE Europe) is a heterogeneous region, comprising of developed and developing countries with ∼76 million inhabitants. The state of NBS varies significantly between the individual countries. The results of the last study conducted in 2013/2014 showed that four out of 11 countries in the region did not screen for PKU and three of them did not screen for congenital hypothyroidism (CH). At that point, Albania and Kosovo did not have a screening programe. Screening for both PKU and CH existed in Bosnia and Herzgovina (BIH), Bulgaria, Croatia, Moldova, Romania, Serbia and Slovenia, while Macedonia and Montenegro screened for CH only. Screening for congenital adrenal hyperplasia (CAH) was introduced in Bulgaria. At that time none of them used MS/MS for NBS and three of the countries reported plans to implement the MS/MS in planned expansions of NBS (10).

In order to assess the current state of NBS in SE Europe a repeated survey was conducted in 2020 (this time including Cyprus, Greece, Hungary and Malta due to their geographical position in the wider region). Our primary aim was to assess the current state and to evaluate the changes in the NBS in this region in years 2014–2020, and to possibly identify the main obstacles impacting the implementation of expanding the NBS and its outreach.

METHODS

Survey was conducted inviting the identified professionals from 15 countries from SE Europe: Albania, BIH, Bulgaria, Croatia, Cyprus, Greece, Hungary, Kosovo, North Macedonia, Malta, Moldova, Montenegro, Romania, Serbia and Slovenia. Among participants were pediatricians, laboratory geneticists and biochemists responsible for their national NBS programs.

A questionnaire (in **Supplementary Material**) was designed to assess the main characteristics of NBS of each country, the changes in programs made between 2014 and 2020 and their plans for the future. It consisted of altogether 20 questions, 11 of them asked about the current state of the NBS in the country and eight of them about the possible expansion in the last seven years and in the future. The last question was to provide existing references about the NBS program in each country. The demographics data was obtained using Eurostat website (1) and GDP per capita (in USD) of each country from the World Bank data (2).

The questionnaires were created with the SurveyMonkey® survey platform (SVMK Inc., San Mateo, CA) and distributed to the participants by e-mail. The responses were collected through the same platform and through e-mail. The distribution and collection of the questionnaires took place in November and December 2020 along with final clarifications and data authorization by e-mail. A single response was obtained from each country, except from BIH, where due to organizational aspects, separate responses were collected from the entity of Federation of BIH and the entity of Republic of Srpska (but no response was obtained for Sarajevo). No responses were received from three countries invited (Albania, Cyprus and Moldova). All the responsible participants of the survey were invited as coauthors of the study and have authorized the data provided on behalf of their countries.

RESULTS

Responses from 12 (out of 15 invited) countries were received. Their cumulative population in 2019 was approximately 68.5

FIGURE 1 | The map of southeastern Europe with countries represented in the survey (in blue) and their populations.

million (**Figure 1**). The GDP per capita ranged from 29,820 USD (Malta) to 4,420 USD (Kosovo). The number of newborns in 2019 ranged from 188,135 (Romania) to 4,376 (Malta). The number of screening centers in the country ranged from five in Romania to zero in Kosovo (**Table 1**).

All of the countries except Kosovo screened for CH. Mandatory screening for PKU was not introduced in Kosovo, North Macedonia, Malta and Montenegro. However, North Macedonia reported selective screening for PKU in six bigger nurseries since 2011. Screening for CF was included in the NBS of North Macedonia, Bulgaria screened for congenital adrenal hyperplasia (CAH), Greece screened for glucose-6-phosphate dehydrogenase (G6PD) deficiency and classic galactosemia (GALT). Malta was the only country screening for haemoglobinopathies (sickle cell disease). Expanded NBS (increasing the screening panel of disorders by the use of MS/MS were implemented in Croatia (in 2017, total of eight screened diseases), Hungary (already in 2007, total of 16 diseases) and Slovenia (in 2018, total of 18 diseases) (**Table 2**).

The age of screened newborns ranged from 24 to 120 h, the majority (six) countries started the screening at the age of 48–72 h. In at least three countries with established NBS (Bulgaria, Malta and Romania) more than 10% of the newborns were reportedly not screened (**Table 1**).

The Delfia method was used in eight countries and the fluorimetric method in four. High-performance liquid chromatography (HPLC) was used for screening for haemoglobinopathy in Malta. Five countries reported the use of MS/MS as a screening method (Croatia, Hungary, North Macedonia, Romania and Slovenia). Croatia used genetic testing for confirming a common mutation in MCADD (985A→ G), Greece used genetic screening processor (Perkin Elmer) and Slovenia used NGS as a follow-up test. The cost of screening per newborn ranged from three EUR in Montenegro to 22 EUR in BIH—Republic of Srpska. Furthermore, the cost of screening in North Macedonia if performed by a private hospital reached 26 EUR (**Table 1**).

Most of the countries reported country wide organization of NBS, while BIH reported regionally organized NBS programs (organized by its three constitutive entities). NBS programs were financed by the Ministry of health in five countries, by the national health insurance schemes in two and by the combination of both in three of them. Five of the countries participated in an international cooperation program on NBS (**Table 1**).

Five other countries were planning an expansion of the NBS between 2013 and 2019 but could not accomplish it. The main obstacles in expanding the NBS in that period were lack of financial resources, organization and lack of political will (**Table 2**).

Seven countries plan the expansion of the NBS in the future, six of them are going to conduct a pilot study before the expansion. The urgency to expand the program ranged from three to five (five being the highest urgency and one the lowest), with a median of four. Lack of financial resources, organization and political will continued to be perceived as the main obstacles for expansion (**Table 2**).

TABLE 1 | Demographics and newborn screening programs characteristics in southeastern Europe.

Country	Total pop. in 2019 (Mil.)	GDP per cap. in 2019 (USD)	Screened/all Nb in 2019	No. of screening centers	Diseases mandatory screened (year of introduction)	Age when screened (h)	Lab. methods in NBSP	NBS cost (per Nb)	Organization of NBS	How is the NBS financed	International cooperation program on NBS
BIH—Federation of BIH (without Sarajevo)	3,503[a]	6,110	13,071/13,680	2	CH (2000, 2005)[b], PKU (2001, 2005)[c]	48–96 h	D, F	6 EUR	RO	NHIS	Yes
BIH—Republic of Srpska	3,503[a]	6,110	9,274/10,180 (all Nb for IEM + PKU controls)	1	CH (2007), PKU (2007)	48–72 h	D, F	22 EUR	EL	NHIS	No
Bulgaria	7,0	9,830	55,315/61,882	2	CAH, CH, PKU (1978–1979),	24 h	F	5 EUR	CW	MH	No
Croatia	4,08	14,930	36,248/36,296	1	CH (1985), CUD (2017), GAI (2017), IVA (2017), VLCADD (2017), LCHADD (2017), MCADD (2017), PKU (1978)	48–72 h	D, TMS (MS/MS), GT for confirmation[d]	19 EUR	CW	NHIS	No
Greece	10,72	19,580	Approx. 83,000/83,763	1	CH (1979), GALT (2006), PKU (1974), G6PD def. (1977)	72–120 h	Genetic Screening Processor (Perkin Elmer), Home (G6PD)	4–5 EUR	CW	MH	Yes
Hungary	9,78	16,730	90,000/90,000	2	CH (1980), CUD (2007), GALT (1975), PA/MMA (2007), GAI (2007), GAII (2007), IVA (2007), VLCADD (2007), LCHADD (2007), MCADD (2007), MSUD (2007), FAH (2007), 3MCC (2007), PKU (1975), BTD (1980), CTNI (2007)	48–72 h	D, F, TMS (MS/MS)	18 EUR	CW	NHIS	No
Kosovo	1,79	4,420	0/26,263	0	/	/	/	/	/	/	Yes
North Macedonia	2,08	6,020	19,408/19,845	1	CH (2007), CF (2018)[e]	48h	D, TMS (MS/MS)	16 - 26 EUR[f] hospital	CW[g]	NHIS, MH[h]	Yes
Malta	0,49	29,820	3,394/4,376	1	CH (1989), HBP (1989)	72–120 h	D, HPLC	Nd	CW	MH	No
Montenegro	0,62	8,910	7,220/7,223	1	CH (2007)	48–72 h	D	3 EUR	CW	NHIS, MH	No
Romania	19,41	12,920	157,226/188,135	5	CH (2010), PKU (2010)	24–72 h	F, TMS (MS/MS)	4,5 EUR	CW	MH	Yes
Serbia	6,96	7,410	Nd/64,399	1	CH (1983); PKU (1983)	48–72 h	D, F	4 EUR	CW	/	/
Slovenia	2,08	25,940	Approx. 19,000/19,328	1	CH (1981), PKU (1979), CUD (2018), GAI (2018), GAII (2018), PA/MMA (2018), IVA (2018), VLCADD (2018), MCADD (2018), LCHADD (2018), MSUD (2018), FAH (2018), 3MCC (2018), CPDI (2018), CPDII (2018), 3HMGA (2018), HSD (2018), BKT (2018)	48–72 h	D, F, TMS (MS/MS), NGS	9,24 EUR	CW	MH	Yes

ARG, arginase deficiency; BIH, Bosnia and Herzegovina; BKT, β-ketothiolase deficiency; BTD, biotinidase deficiency; CAH, congenital adrenal hyperplasia; cap., capita; CF, cystic fibrosis; CH, congenital hypothyroidism; CITI, citrullinemia type 1; CITII, citrullinemia type 2; CPDI, carnitine palmitoyltransferase deficiency type 1; CPDII, carnitine palmitoyltransferase deficiency type 2; CTNI, cardiac troponin I; CUD, carnitine uptake defect; CW, country wide; D, Delfia method; EL, entity level; F, fluorimetric method; FAH, tyrosinemia type 1; GAI, glutaric acidaemia type I; GAII, glutaric acidaemia type II; GALT, classic galactosemia; GDP, gross domestic product; G6PD def., glucose-6-phosphate dehydrogenase deficiency; GT, genetic testing; HBP, haemoglobinopathy; HCY, homocystinuria; 3HMGA, 3-hydroxy-3-methylglutaric aciduria; H-PHE, hyperphenylalaninemia; HPLC, high-performance liquid chromatography; HSD, holocarboxylase synthethase deficiency; IEM, inborn errors of metabolism; IVA, isovaleric acidaemia/2-methylbutyrylglycinuria; Lab., laboratory; LCHADD, long-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency/trifunctional protein deficiency; MAL, malonic acidemia; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; 3MCC, 3-hydroxy-methylglutaric aciduria; MET, hypermethioninemia; MSUD, maple syrup urine disease; MH, Ministry of health; Mil., millions; Nb, newborn; NBS, newborn screening program; Nd, no data; NGS, next generation sequencing; NHIS, national health insurance schemes; NKH, non-ketotic hyperglycinemia; No., number; PA/MMA, propionic/methylmalonic aciduria; PKU, phenylketonuria; pop., population; RO, regional organization; TMS (MS/MS), tandem mass spectrometry; TYR, tyrosinemia; VLCADD, very long-chain acyl-CoA dehydrogenase deficiency.

[a] Total population of Bosnia.

[b] 2000 in Tuzla Canton, 2005 in Federation of Bosnia and Herzegovina (except Sarajevo).

[c] 2001 in Tuzla Canton, 2005 in Federation of Bosnia and Herzegovina (except Sarajevo).

[d] Genetic testing is done in Zagreb only for MCADD—common mutation 985A→ G. Other genetic tests for confirmatory purposes are done in laboratories abroad. Organic acids done on GC/MS are used in evaluation of patients positive for GAI, IVA, CUD, MCADD, LCHADD/TFP deficiency and VLCADD (or their mothers).

[e] Selective screening (of 4,001 out of 19,845 newborns in 2019) for PKU, H-PHE, MSUD, CITI, CITII, MET, HCY, ORNT2 mutation, ARG, TYR-I, TYR-II, TYR-III, 3HMGA, NKH, GAI, IVA, PA/MMA, MAL, IBC, BKT, HSD, 3MCC, S-MGAI, TFP (from 2013).

[f] 16 EUR in public hospitals, 26 EUR if performed by a private hospital.

[g] CH screening is organized on a state level (coverage of 98%), IEM is covering larger hospitals and covers ∼ 1/3 of all newborns in the country, private hospitals additionally send samples abroad (∼1,500 per year).

[h] CH screening is completely covered by the MH, other IEM are covered by the MH for public nurseries, NHIS are involved in private nurseries.

TABLE 2 | Past developments and future plans in newborn screening programs in southeastern Europe.

Country	Expansion of NBS between 2013 and 2019	Diseases included in expanded NBS program (year)	Diseases planned but unrealised	Main obstacles in expanding NBS 2013–2019	Plans for further expansion (year)	Diseases for further expansion plan	Pilot study before further expansion	Main obstacles for further expansion	Perceived urgency for expanding NBS (1- lowest urgency, 5—highest urgency)
BIH—Federation of BIH (without Sarajevo)	No	/	/	FR	No	/	/	FR	3
BIH—Republic of Srpska	No	/	CF, CAH, GALT	FR	Yes	CF, GAI, CAH, GALT	Yes	FR	4
Bulgaria	No	/	CF	FR, S, PW	Yes	CF	Yes	FR, S, O,PW	3
Croatia	Yes	MCADD, VLCADD, LCHADD/TFPD, GAI, IVA, CUD (2017)	/	FR, S, O, L	Yes	PA/MMA, HCY, SMA	Yes[a]	FR, S, O, L, incomplete e-Newborn service	4
Greece	No	/	CF	O, L, PW	Yes (2021)	CF, CAH, BTD, expanding the use of TMS (MS/MS)	No	O, L, PW	5
Hungary	No	/	CF	FR, PW	No	/	Yes (CF)	FR	4
Kosovo	No	/	/	FR, PW, country after the war and in process of development	/	/	/	/	5
North Macedonia	Yes	CF (2018), PKU, H-PHE, MSUD, CITI, CITII, MET, HCY, ORNT2, ARG, TYR-I, II, III, 3HMGA, NKH, GAI, IVA, PA/MMA, MAL, IBC, BKT, HSD, 3MCC, S-MGAI, TFP (2013)	Expansion of screening for IEMs to the whole country.	FR, PW	Yes	To first cover the entire country with a screening for IEMs, CAH after that.	/	FR, PW	4
Malta	No	/	PKU	O, L	Yes	CF	Yes	S, O, L	3
Montenegro	No	/	/	FR, S, O, L, SI, PW	No	/	/	FR, S, O, L, SI, PW	3
Romania	No	/	CAH, GAL, CF	FR, O, PW	Yes (2022)	CAH, MSUD, CF, FAH, ASA, CITI, ARG, HPTI, GAI, IVA, 3MCC, PA/MMA, MCADD, LCHADD, TFP, VLCADD, CUD, SCAD, GALT	Yes (2021)	FR, O, PW	5
Serbia	No	/	/	FR, S, O	Nd	/	/	FR, S, O	4
Slovenia	Yes	CUD, GAI, GAII, PA/MMA, IVA, VLCADD, MCADD, LCHADD, MSUD, FAH, 3MCC, CPDI, CPDII, 3HMGA, HSD, BKT (2018)	/	/	Yes (2021)	SMA, SCID, CF, CAH	No	S	4

ARG, arginase deficinecy; ASA, argininosuccinic aciduria; BIH, Bosnia and Herzegovina; BKT – β-ketothiolase deficiency; BTD, biotinidase deficiency; CAH, congenital adrenal hyperplasia; cap., capita; CF, cystic fibrosis; CH, congenital hypothyroidism; CITI, citrullinemia; CITII, citrullinemia type 2; CPDI, carnitine palmitoyltransferase deficiency type 1; CPDII, carnitine palmitoyltransferase deficiency type 2; CTNI, cardiac troponin I; CUD, carnitine uptake defect; D, Delfia method; EL, entity level; F, Fluorimetric method; FAH, tyrosinemia type 1; FR, lack of financial resources; GAI, glutaric acidaemia type I; GAII, glutaric acidaemia type II; GALT, classic galactosemia; GDP, gross domestic product; G6PD def., glucose-6-phosphate dehydrogenase deficiency; GT, genetic testing; HBP, haemoglobinopathy; HCY, homocystinuria; 3HMGA, 3-hydroxy-3-methylglutaric aciduria; H-PHE, hyperphenylalaninemia; HPLC, high-performance liquid chromatography; HPTI, hypoxantine-guanine phosphoribosyltransferase deficiency; HSD, holocarboxylase synthethase deficiency; IEM, inborn errors of metabolism; IVA, isovaleric acidaemia/2-methylbutyrylglycinuria; L, later management; LCHADD, long-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency/trifunctional protein deficiency; MAL, malonic acidemia; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; 3MCC, 3-hydroxy-methylglutaric aciduria; MET, hypermethioninemia; MSUD, maple syrup urine disease; NBS, newborn screening program; Nd, no data; NKH, non-ketotic hyperglycinemia; O, organization; PA/MMA, propionic/methylmalonic aciduria; PKU, phenylketonuria; PW, lack of political will; S, lack of staff; SCAD, short chain acyl-CoA dehydrogenase deficiency; SCID, severe combined immunodeficinecy; SI, small incidences; SMA, spinal muscular atrophy; TFP, trifunctional protein deficiency; TMS (MS/MS), tandem mass spectrometry; TYR, tyrosinemia; VLCADD, very long-chain acyl-CoA dehydrogenase deficiency.

[a] *Expanded NBS still in pilot phase.*

DISCUSSION

The study assessed the current status of NBS in SE Europe, focused on the characteristics of NBS in each country. The progress from the time of the previous survey done in 2013/2014 was also evaluated (10). The results showed even greater heterogeneity of the NBS in the region than before, considering that Croatia and Slovenia managed to expand the NBS by the use of MS/MS with high coverage (percentage of newborns included), while the basics – for example PKU screening remained sub-optimally implemented in the region, as some of the countries (Montenegro) still did not have a mandatory screening for it (10–12). On the other hand, mandatory screening for CH was successfully implemented in North Macedonia, Montenegro and Romania, where the national registry for CH (MEDILOG) was established in the same year (13–15). The circumstances in Kosovo were worrying, as the NBS was non-existent. The coverage in the region was still not ideal, as more than 10% of newborns were not screened in Bulgaria and Romania. Similar coverage was reached in Malta, where the GDP is approximately three times higher (**Table 1**). A phenomenon where screening for PKU and some other inborn errors of metabolism (IEMs) by the use of MS/MS was otherwise available in the country but only to newborns in six large nurseries was observed in North Macedonia (10, 16, 17). The main reason for not achieving the goals of expanding the NBS reported in the 2013/2014 survey was for most of the countries' lack of financial resources.

Historically, the NBS in Europe was initiated with smaller programs for screening for PKU during the 1960s, the screening for CH followed a few years later (18, 19). The majority of the SE European countries introduced screening for PKU and CH between 1970s and 1980s, but in BIH, North Macedonia, Montenegro and Romania, the screening was first introduced in the 2000s (**Table 1**). The only European countries without screening for PKU (Montenegro) and screening for CH (Moldova) are a part of SE Europe. NBS in some form is now present in every European country, except in Albania, Kosovo and Tajikistan (20).

The introduction of MS/MS allowed simultaneous screening for multiple disorders from one DBS and increased the number of amino acidemias, organic acidemias and fatty acid oxidation disorders in the screening panels in the 1990s and first decade of the 21st century (21–23). The first country of SE Europe to expand the NBS with the use of MS/MS was Hungary in 2007, Greece between 2007 and 2009, followed by Croatia (2017) and Slovenia (2018) (5, 10, 24–26) (**Table 1**). Approximately 50% of the European countries screen for CF and CAH, which were mostly implemented between 2005 and 2010. In SE Europe, CAH is a part of NBS only in Bulgaria. While several SE European countries reported plans for implementing CF in NBS, it was only available as a part of selective screening in some hospitals in North Macedonia at the time of our study (**Table 2**). Some regions in Italy and the Netherlands started screening for LSD, but they are not a regular part of other NBS programs in Europe (20). Screening for multiple LSD using MS/MS was considered economically justifiable in Hungary in 2012 due to cumulative frequency of LSD similar to acylcarnitine and amino acid IEMs (27).

Modern technologies, such as NGS, were already implemented for CF screening and as a second-tier test in Norway, while the UK conducted a trial of its use as a part of screening algorithm for CF (20, 28, 29). Croatia reported plans for introducing the method as a second-tier as well and Slovenia used it in the pilot study before expanding the NBS in 2018 (5, 20). A survey conducted in 2017 in Bulgaria on potential use of whole-genome sequencing (WGS) in conjunction with the traditional NBS showed that Bulgarian pediatricians and geneticists believed that selective WGS could strengthen their current NBS programs while non-selective WGS for all newborns was not perceived as feasible at that time (30).

Molecular technologies enabled most recent additions to the NBS in some European countries, such as screening for CF, spinal muscular atrophy (SMA) and severe combined immunodeficiency (SCID) (20). Expanding the screening panel with SMA is planned in Croatia and Slovenia, and additionally with SCID in Slovenia (**Table 2**). To sum up, the screening panels of some countries of SE Europe are already comparable to developed parts of Europe and most of the countries plan on further expansion (25, 26, 31–35).

Secondly, the reported coverage in most of the European countries between 2010 and 2020 was higher than 90%, while the initial coverage in Kyrgyzstan and Turkmenistan, where the NBS was recently established, was 30% (20). In SE Europe over 10% of the newborns are not screened in Bulgaria, Romania and Malta, while the coverage in other countries is over 90% (**Table 1**). Most of the countries with 100–20,000 newborns per year have one screening laboratory, the number varies due to politico-geographical and socio-economic reasons. Countries from SE Europe with higher-than-necessary number of screening laboratories by that definition are Bulgaria, BIH, Hungary, Romania and Serbia (**Table 1**) (20). Some of the smaller European countries send the samples to neighboring countries for analysis (e.g., Liechtenstein is covered by Switzerland), which is also done in some parts of Kosovo, where the samples are sent to Serbia (20).

Finally, the decisions on diseases included in NBS are made independently in each European country, as there are currently no policy recommendations or direct oversight at the European level or within the EU (36). Health care has not been included in topics to be governed or overseen by the European Commission, as the member states of the EU consider it to be their own responsibility (20). Therefore, the circumstances regarding NBS in the wider region remain heterogeneous.

The obstacles in comparable regions of the world that lack total NBS coverage are usually poor economies, insufficient health education, lack of government support, early hospital discharge, and large numbers of out-of-hospital births (37, 38).

Similar to countries in SE Europe, parts of Latin America introduced national NBS in the 1990s and the first decade of the 21st century and are working on expanding NBS with MS/MS. The coverage ranges from as low as 1% in Guatemala to 99% in countries with higher socio-economic standards (e.g., Uruguay) but also in Cuba, where NBS is decentralized through

more than 175 laboratories (3). Several countries in the Middle East and North Africa (MENA) region have a coverage of screening for CH higher than 90%, while expanded NBS with the MS/MS is often limited or available as a part of selective screening. Nevertheless, it reaches over 90% of the newborns in Israel, that is already considering including SCID in the screening panel, and 100% in Qatar, where the samples are sent to screening laboratory in Heidelberg, Germany (3, 39, 40). In India the challenges are similar, the unsatisfactory state of NBS in one of the countries with largest screening populations has been reviewed and the authors made suggestions to the government for screening implementation, such as convening a central advisory committee to plan for program development, conditions recommended for immediate introduction in urban hospitals, and screening with MS/MS, once a firm infrastructure is in place (37, 38, 41). A model for developing programs in South Asia is the NBS in the Philippines, with 65% coverage, implementation of expanded NBS with MS/MS and even screening for CF (3).

Government prioritization, full or partial government financing, public education and acceptance, health practitioner cooperation/involvement and government participation in program institutionalization were identified as crucial to success for sustainable NBS programs (37, 38).

Despite the small geographical distance, there is a great inequality in the region concerning the level of development of the NBS programs in each country. While some of them still struggle to establish a sustainable screening for PKU and CH, for example Albania and Kosovo, others reach the level of Western Europe and already make plans for including more diseases in already expanded NBS by the use of MS/MS and for introducing NGS as a second tier test (20). Consequently, this could create an even greater divergence between the countries with higher GDP, member states of the EU, and the post-war countries, countries with lower GDP, lack of educated staff and political conditions that do not prioritize good health policies.

The strength of our study was that it included professionals responsible for the NBS in each country and is therefore presenting the first-hand data and experience. As a limitation, we failed to include all the countries in the region, despite making several attempts to reach all the representatives. In addition, the study provides only a partial insight of the state of NBS programs in each country, since we investigated the analytical part of the screening (e.g., screening panels and diagnostic methods used), and omitted the characteristics of the pre-analytical (e.g., taking and derivation of the sample) and post-analytical aspects (confirmation, follow-up and treatment of patients) that are also essential parts of the NBS when it is considered a public health policy.

The current status of NBS in the region of SE Europe is very variable and is still underdeveloped or even non-existent in some of the countries. Furthermore, the situation has not changed very much in the past seven years. A few countries introduced an expanded NBS, while a greater part of them still screen for the CH and PKU only and one of the surveyed countries still does not have a NBS at all. Very recent surveys confirmed a persisting lack of harmonization of NBS programs among European countries, emphasizing the need for more comprehensive guidelines at the European level (20, 42). The urge to put further effort and support into harmonization of the state of NBS in SE Europe through international cooperation and sharing of practical and theoretical knowledge persists. We suggest possibly establishing an international task-force to assist with implementation and harmonization of basic NBS services everywhere needed. Firstly, a careful assessment of the current situation is needed and has to be included in relevant state-of-the art documents and international initiatives. Following from that, more active support in implementing basic standards should be provided, perhaps starting and/or continuing with initiatives to introduce the newborn screening programs where necessary. In addition, a minimal set of disorders to be screened in any specific region could be defined. These efforts could be even more eagerly supported especially by the relevant professional forums, international organizations but also by industry and charities (11).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All the participants agreed to participate in the survey.

AUTHOR CONTRIBUTIONS

UG and VK conceptualized and designed the study. UG, VK, and MM carried out the survey and interpreted the results. UG coordinated and supervised data collection and analysis. VK drafted the initial manuscript. UG helped in writing. UG, MM, VK, and TB critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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REVIEW
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Next-Generation Sequencing in Newborn Screening: A Review of Current State

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Newborn screening was first introduced at the beginning of the 1960s with the successful implementation of the first phenylketonuria screening programs. Early expansion of the included disorders was slow because each additional disorder screened required a separate test. Subsequently, the technological advancements of biochemical methodology enabled the scaling-up of newborn screening, most notably with the implementation of tandem mass spectrometry. In recent years, we have witnessed a remarkable progression of high-throughput sequencing technologies, which has resulted in a continuous decrease of both cost and time required for genetic analysis. This has enabled more widespread use of the massive multiparallel sequencing. Genomic sequencing is now frequently used in clinical applications, and its implementation in newborn screening has been intensively advocated. The expansion of newborn screening has raised many clinical, ethical, legal, psychological, sociological, and technological concerns over time. This review provides an overview of the current state of next-generation sequencing regarding newborn screening including current recommendations and potential challenges for the use of such technologies in newborn screening.

Keywords: newborn screening, NBS, neonatal screening, next generation sequencing, NGS, expanded NBS program, DNA sequencing, high-throughput sequencing

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INTRODUCTION

Newborn screening (NBS) began with the invention by Dr. Robert Guthrie of a relatively simple and rapid test for the detection of elevated levels of phenylalanine in the blood, combined with an ingenious method of sampling, which later proved to be useful in a multitude of different tests (Guthrie, 1961). The success of screening for phenylketonuria (PKU) spurred the expansion of the NBS program (Guthrie and Whitney, 1965; Brosco and Paul, 2013; Groselj et al., 2014a). In the 1970s, the screening began for congenital hypothyroidism (CH), and in the next two decades, a few other disorders like congenital adrenal hyperplasia (CAH), hemoglobinopathies, biotinidase deficiency, cystic fibrosis (CF), and tyrosinemia type I (HT1) were added sporadically to the different NBS programs in different states and countries (Dussault et al., 1975; Wilcken and Wiley, 2015). With the development and the accessibility of electrospray ionization (ESI) tandem mass

spectrometry (TMS) in the 1990s, the ability to quantitate multiple metabolites simultaneously and, thus, the simultaneous detection of multiple inborn errors of metabolism (IEM) facilitated the first big expansion of the NBS programs around the globe, though the less developed countries did generally not share the progress (Sweetman, 1996; Levy, 1998; Bennett and Rinaldo, 2001; Groselj et al., 2014a,b; Bouvier and Giguère, 2019). Different tests are continuously developed spurred by the development of clinical treatments for conditions such as severe combined immunodeficiencies (SCID), spinal muscular dystrophy (SMA), and lysosomal storage diseases (Spacil et al., 2013; Chien et al., 2017; Puck, 2019; Czibere et al., 2020). With the constant growth of conditions added to the NBS programs, the number of different analytical methods used is also increasing. It soon became apparent that the use of different methods results in a much heavier workload for the laboratories. At the same time, the progress of genetics was accelerated with the development of next-generation sequencing (NGS) methods (Metzker, 2010; Morey et al., 2013; Reuter et al., 2015; Levy and Myers, 2016). NGS is an epitome of multiparallel platforms because it enables simultaneous processing of a large number of samples, and it is easily expandable from a couple of genes to a whole genome. Many authors therefore see it as the method that could enable the next big expansion and methodological unification of NBS programs (Department of Health, 2003; Collins, 2009; Drmanac, 2012; Smon et al., 2018; Lampret et al., 2020). As the cost of sequencing steadily decreases, the feasibility of NBS with the use of NGS is becoming more and more possible (Wetterstrand, 2021).

However, despite growing technical possibilities, most of the human disorders are not suitable to be included in the NBS program. Wilson and Jungner proposed 10 criteria that should be met for the disease to be included in screening programs, which were later further revised (Wilson et al., 1968; Andermann, 2008). Since the first idea of using NGS in the framework of NBS, several concerns have been raised, among them technical, medical, legal, economical, ethical, psychological, and sociological (Friedman, 2015; Howard et al., 2015; Reinstein, 2015; Berg et al., 2017; Murray et al., 2018; Bouvier and Giguère, 2019; de Wert et al., 2020). In this review, we will discuss the recent advances in the use of NGS in the context of NBS, the remaining obstacles in its implementation in NBS, and the wider implications of its use in the NBS program. A brief outline of this review is demonstrated in **Figure 1**.

TECHNICAL FEASIBILITY

Since the first idea of using genetic methods in the NBS setting, numerous authors presented several concerns regarding the technical feasibility of using genomic technologies for NBS: possibility to achieve appropriate turnaround time, accuracy of the obtained results, ability to correctly interpret the results, confirmation of the results with an independent method, and safe storage of sequenced data (Friedman, 2015; Howard et al., 2015; Reinstein, 2015; Berg et al., 2017; Bouvier and Giguère, 2019).

The first technical concern is the ability to prepare, analyze, interpret, and report the results fast enough to fulfill the requirements of the NBS. Standard protocols for NGS library preparation are time-consuming and could not be used in an NBS time frame. The second time-consuming step of the NGS approach is the interpretation of the results. One of the largest obstacles would be the interpretation of the sheer bulk of data containing many variants for which their clinical significance is still unknown or ambiguous (Berg et al., 2017). There is still not enough ethnic-specific data available for an accurate evaluation of the possible causality of many such variants (Friedman, 2015). However, there are new optimized protocols reported, enabling the possibility of the results to be reported on day 4 from sample booking in the laboratory (van Campen et al., 2019).

Different NGS approaches yield different technical problems regarding raw sequencing data. Roughly, there are three main approaches to NGS: sequencing of the panels of selected genes, sequencing of coding regions of all known genes or whole-exome sequencing (WES), and sequencing of the whole genome (WGS). When using WES or other targeted approaches (panels of genes), we have to capture the selected regions of DNA and then use enrichment of them for further processing. Here, we risk that we would fail to capture certain parts of specific targeted genes, which could lead to false-negative results. With WGS, there is no need for capture or enrichment, so there is less possibility for certain regions to have poor sequencing coverage (Reinstein, 2015). WGS enables better detection of the copy number variants (CNV), which could be missed using a targeted approach. Some software and technical solutions have already been developed, to address this problem in targeted methods (Belkadi et al., 2015; Kerkhof et al., 2017; Mu et al., 2019). Often overlooked is also the problem of sequencing of genes with known pseudogenes with a high level of homology, which could result in false results of sequencing in all the abovementioned methods, including WGS (García-García et al., 2016). Performing NBS with WGS would also require numerous high-throughput next-generation sequencers (including backup machines) to facilitate uninterrupted NBS, which still have a very high price, which is possibly beyond many countries' healthcare systems capacity (Schwarze et al., 2020).

Regardless of the approach, WGS or targeted, there are some properties of the human genome being uncovered, that neither could be detected without complex technical modifications or a special type of human genome analysis. The role of epigenetics and non-coding RNAs on the expression of genes is just starting to be discovered (Tesovnik et al., 2020). The role of topologically associated domains and interchromosomal interactions and the influence of the 3D structure of the genome in the expression of genes and potential cause of the disease are still not fully understood (Maass et al., 2018, 2019). Oligogenic or multifactorial conditions are only starting to be understood, but could in the future be a very important part of preemptive medicine. Presently, it is difficult to predict which kind of methodological approach would be better for the detection of oligogenic conditions, but it seems that WGS would be more appropriate (Tada et al., 2018; Kim et al., 2019). As it seems that all of the abovementioned conditions and diagnostic methods have

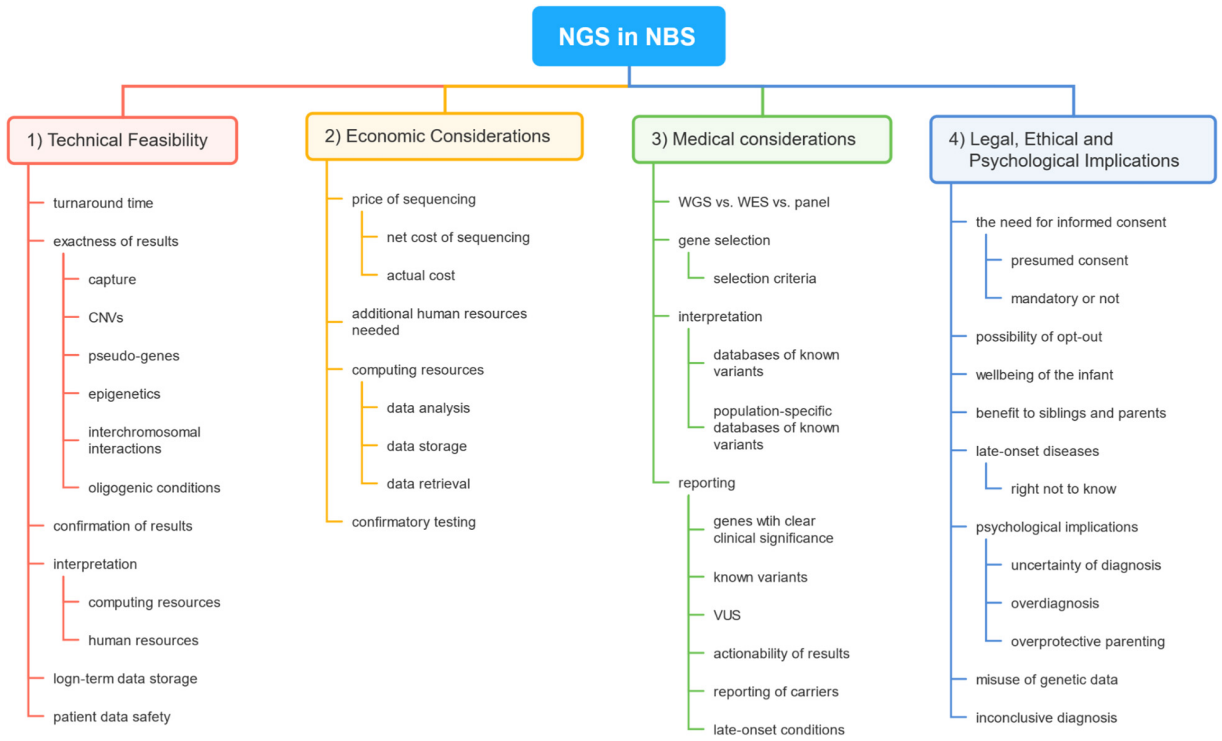

FIGURE 1 | A brief outline of topics covered in this review. The first section covers concerns regarding the basic technical feasibility of the use of next-generation sequencing (NGS) technology in newborn screening (NBS). The second section reveals the main factors impacting the cost of the use of such technology in NBS. The third section deals with medical problems the use of NGS would raise in NBS. The final sections engage in discussing legal, ethical, and psychological problems concerning NGS in the setting of NBS.

the potential, with the maturation of the analytical technology and increasing knowledge of their impact on human health, they become part of the NBS in the future.

Sequencing of the whole population would produce an enormous amount of computer data that would have to be stored safely for a long time depending on the local regulation of medical data storage, handling, and distribution. Some authors propose that newborns would be screened/sequenced once in a lifetime, then their genomic data would have to be stored securely for an entire lifespan or even more. In such cases, data would have to be accessible for subsequent reinterpretation as new knowledge regarding genes and related diseases becomes available (Drmanac, 2012; Friedman, 2015; Howard et al., 2015).

Integration of obtained data and results is another challenge for informatics technologies. Because of the sensitivity of genetic data, permissions for access to the results and the ability to interpret the data would have to be thoughtfully assigned. Currently, the task of interpreting genetic data is exclusively in the domain of clinical and laboratory geneticists (CLGs), which is a well-recognized profession worldwide. Some authors propose that some part of the increased analytical burden in NBS could in the future be taken over by medical doctors (MDs) (Friedman, 2015; Howard et al., 2015; Berg et al., 2017). If we consider the part MDs play in healthcare systems and their assignments, we usually conclude that they are already overburdened. Although it is conceivable that MDs could interpret some results of the simpler genetic tests, the majority of analytical burden will and should remain on specialists of clinical and laboratory genetics (Liehr et al., 2017, 2019).

Regardless of the abovementioned technical hurdles, NGS simply could not provide a unified platform for NBS, as some authors suggest, because some of the diseases presently screened have a very weak genetic background or very variable penetrance. A couple of such examples are CH for which only 10–15% of patients have a genetic background (Rastogi and LaFranchi, 2010), some conditions of fatty-acid metabolism, and Pompe disease, which does not have a clear genotype–phenotype correlation (Matern and Rinaldo, 1993; Kroos et al., 2012). Another obstacle of using NGS as a first-tier method in some disorders is the sensitivity of sequencing analysis, especially when analyzing a selected panel of known genetic variants such as in cystic fibrosis (Castellani and Massie, 2014). For many of the known genetic diseases, there is currently no valid clinical evaluation by which to validate genomic prediction (Berg et al., 2017).

ECONOMIC CONSIDERATIONS

With the advancement of NGS methods, the price of sequencing per one million bases (1 Mb) dropped significantly and continues to drop even more (Wetterstrand). Nonetheless, the aspirational

price of the whole human genome sequencing is still advertised around a $1,000 mark, but depending on the country and equipment used, it can be increased by multiple factors including the costs of accreditation, validation, maintenance, and external quality control, and specialists of different fields (genetics, bioinformatics, etc.) can raise the cost of a single human genome much higher (Liehr, 2017; Schwarze et al., 2020). With or without additional costs, the cost of NGS is still significantly higher than the current cost per sample in all of the existing NBS programs. For example, the reported cost per newborn in Europe ranged from €1 (Moldova; screening for 2 conditions) to €43.24 (the Netherlands; screening for 17 conditions) (Groselj et al., 2014b; Loeber et al., 2021). In the United States, most states fund the NBS program through a "kit fee" which is paid to the birthing facility which is currently roughly $100 per newborn to screen for over 30 conditions (Botkin and Rothwell, 2016). In Israel, they screen for 12 diseases and the cost per sample is estimated around $45 (Friedman, 2015).

Another important aspect of cost is the computing and human power required to process the data obtained with NGS. After data are processed, there would be the need to interpret the clinical significance of obtained variants. With the increase in the number of sequenced genes, there would also be an increase in the number of variants found that would need interpretation. Many software tools are already available to ease the process of interpretation of results, but in the end, there is still the need for highly trained geneticists to make a final decision. With these much data, interpretation would require a great increase in manpower compared with the current workflow (Howard et al., 2015). To address this problem, some authors propose automated software pipelines that would report only on currently known pathogenic and likely pathogenic variants (Berg et al., 2017; van Campen et al., 2019).

The need to confirm the results obtained through genetic screening would possibly increase the cost of confirmatory testing because of the greater number of screened conditions. There are several possibilities of confirming NGS results. For known genes, the golden standard is still Sanger sequencing of the variant in question. If we consider WGS, the use of the so-called "trio" approach, comparing WGS results of a child to the results of parents, could be useful for diagnosing *de novo* autosomal dominant intellectual disabilities and autosomal recessive disorders, but the price, time frame, and workload will be beyond the scope of NBS for some considerable time (Mu et al., 2016; Rossi et al., 2020). Genetic NBS would, depending on the design, possibly need informed consent (parental permission) that would require counseling before testing and again when returning the results, which would again increase the need for CLGs' time and, therefore, require more manpower (Frank et al., 2013). As it was always the case in NBS, every screening for a new condition invariably increases the workload in the clinic where patients get treated.

The quantity of data produced by NGS can be enormous. The size of a human genome on a computer disk represents around 60 gigabytes (GB), which would significantly increase data storage requirements. Long-term digital data persistence and safety would have to be guaranteed. The average lifespan of data on a hard drive is currently between 5 and 6 years, which does not provide long-term data storage, so other solutions would have to be developed (Rahmanto and Riasetiawan, 2018). Many authors predict that genetic data obtained at NBS would be useful throughout the person's life, especially when considering WGS, where a lot of information is yet to be uncovered.

CLINICAL ISSUES

The current debate regarding the use of NGS in the setting of NBS revolves around the question which sequencing approach is better, WGS or targeted. The authors advocating the WGS approach claim that having the information of the whole genome at the clinician's disposal would enable not only NBS analysis but further analysis of the genes and regulatory regions of DNA later in a patient's life when further knowledge on causative variants and genome regions would be available. The authors also claim that the study of data acquired during NBS would enable research of gene variants on a population scale, which would have a substantial benefit on public healthcare in the near future (Drmanac, 2012; Howard et al., 2015; Reinstein, 2015). Another emerging field that would benefit greatly from the data obtained with WGS is personalized medicine, namely pharmacogenomic, which could use genomic data from NBS later in life, to determine patients' specific drug-metabolizing traits. According to the working group of the personalized laboratory medicine of the European Federation of Laboratory Medicine, there are some recognized organizational shortcomings that impede the progress of pharmacogenomics, among them technological and methodological deficits (Malentacchi et al., 2015; Karas Kuželički et al., 2019). WGS also enables research of non-coding regions that we know can harbor deleterious variants (Meienberg et al., 2016) but are more challenging to properly interpret. Another option would be WGS and analysis of only selected regions/genes. Such an approach would facilitate easier expansion of core NBS diseases and interpretation of genes for new conditions (Di Resta et al., 2018).

Whole-exome sequencing, on the other hand, covers exclusively the coding regions of most of the known genes and is therefore only 1.5% the size of the whole genome, which means faster and cheaper sequencing, but still with a relatively large amount of genomic data. A more "traditional" approach would be a gradual implementation of NGS technology, and several authors suggest a selection of genes for which gene–disease correlation is well known (Evans et al., 2013; Howard et al., 2015; Berg et al., 2017). NGS with a targeted approach, with the use of capture probes for selected panels of genes or with the use of software filters in the case of WGS, has proven its utility in routine diagnostics, so many laboratories already have experience with such methods (Di Resta et al., 2018). The targeted approach would adopt the same inclusion process as was used until now for biochemical screening but now focused on the NGS methodology. Conditions would be added gradually after careful consideration. One important advantage of the targeted NGS approach is its scalability. Conditions can be added with little or no changes or additional cost to the established

workflow at all. With this approach, the interpretation of the variants would be less time-consuming and would better fit into the strict NBD time frame, while at the same time the whole process would allow a much greater level of automation, including bioinformatics analysis.

Selection of Gene–Disease Pairs

Considering the targeted approach, the main challenge would be the selection process of the included genes associated with the targeted disease. Several documents relevant to the use of genetic technologies in NBS have been published over the years. Some of them were written in the field of NBS and others written in the field of medical genetics. Because of the lack of specific guidelines for the use of NGS in NBS, the use of a combination of different documents would be required. At the same time, an expert panel to establish specific guidelines and recommendations for the introduction of NGS as a first-tier screening technology is well overdue, although some international projects are aiming to fill that void in the next couple of years.

The oldest document for screening programs is the WHO criteria, which would have to be met and at the same time considered from an NGS point of view (Wilson et al., 1968; Andermann, 2008). The suitable candidate conditions would have to have a clear Mendelian inheritance pattern and clear genotype–phenotype correlation. There should be ample knowledge of known variants present in the gene, high penetrance, and effective presymptomatic intervention (Howard et al., 2015; Berg et al., 2017; Bouvier and Giguère, 2019). If we consider the current set of core conditions included in NBS programs, some of them do not meet the above criteria, so could not be included in NGS screening, but would have to be screened with existing methods (Green et al., 2006; Rastogi and LaFranchi, 2010; Howard et al., 2015).

In the United States recently, the Discretionary Advisory Committee on Heritable Disorders in Newborns and Children updated the panel of recommended disorders for NBS. The RUSP includes 57 conditions including 31 core disorders and 26 secondary disorders (Health Resources and Services Administration, 2020). The authors suggest that this panel could serve as a template for the selection process of conditions that would be screened using NGS (Evans et al., 2013; Berg et al., 2017; Di Resta et al., 2018; Bouvier and Giguère, 2019). Others argue that it would be difficult to acquire the same specificity and sensitivity for conditions currently screened with the TMS method (Castellani and Massie, 2014; Howard et al., 2015). That is why some propose to include only the disorders for which currently there is no valid method for screening (Berg et al., 2017). The American College of Medical Genetics and Genomics (ACMG) issued recommendations for reporting of incidental findings in clinical exome and genome sequencing in 2013. They list several conditions and genes for which they conclude that known pathological variants in those genes should be reported (Green et al., 2013). Evans et al. (2013) propose only a few of the genes from the ACMG list, for example, genes associated with Lynch syndrome, some highly penetrant cancer predisposition genes (e.g., *APC*, *BRCA1*, *BRCA2*, *MYH*, *PTEN*, and *VHL*), genes associated with high risk for preventable vascular catastrophe (e.g., *FBN1*, *COL3A1*, and *MYH11*), and possibly genes for familial hypercholesterolemia (Evans et al., 2013; Klančar et al., 2015; Groselj et al., 2018). The European Society of Human Genetics (ESHG) published recommendations on opportunistic genomic screening, where for minors they approve reporting of variants only for genes of conditions that are actionable early in life (such as MEN type 2A and hereditary arrhythmias such as long QT and Brugada syndrome) (de Wert et al., 2020). Recently, Milko et al. (2019) published a novel method to assess the potential actionability of genomic sequencing for certain conditions based on age. Such semiquantitative methods could pave the way to a more evidence-based and uniform decision-making process regarding the inclusion of disorders and related genes into an NGS-based NBS program (Berg et al., 2016; Strande et al., 2017).

Interpretation and Reporting of Genetic Variants

Many authors expressed concerns regarding the clinical interpretation of acquired data. In WGS/WES, there is an enormous bulk of obtained information that is far from easy to analyze even with powerful computing power. If we disregard the time it takes to process the data, there is still the question of reliable analysis and interpretation of obtained data. Depending on the software and databases used, interpretation will inevitably vary between laboratories (Levy, 2014). The cause of this variation is the current lack of knowledge about genetic variants, many of which are presently classified as variants of unknown significance (VUS) (Cooper and Shendure, 2011; O'Daniel et al., 2017). New national databases of variants should be developed, to decrease VUS and improve population-specific knowledge on variants. Besides incomplete knowledge about genetic variants, classification of the same variant with different databases or predictive algorithms is often conflicting, which would make reliable interpretation even more difficult. With the inception of genomic NBS, many more variants of unknown significance will be uncovered because of the sheer volume of samples sequenced (Lyon and Wang, 2012; Rabbani et al., 2012).

With the genome or at least parts of it at our disposal, it remains to be determined which variants would be included in the report and returned to the parents in the end. Considering information obtained with WGS, the data will contain many very different types of genetic variants, from known variants in genes for monogenic conditions to polygenic conditions, childhood-onset to late-onset disorders, and autosomal dominant or recessive (homozygous or carrier) to X-linked or mitochondrial; variants for certain predispositions with variable penetrance and pharmacogenomic data; and last but not least, VUS (Wade et al., 2013; Malentacchi et al., 2015). In the case of WGS or WES, we must take into account that the acquired information will include all of the genes even the ones whose clinical significance is not yet understood. Some sort of scrutiny regarding actionability and gene–disease association will be required when reporting genomic or exomic data (Friedman, 2015). In most of the current conditions included in the NBS, the heterozygous carriers never develop clinical signs or symptoms, and in very

few actionable conditions, heterozygous carriers develop them already in childhood. This raises the question how and when, if at all, should this information be reported. We learned from the experience of the NBS program for sickle cell disease that reporting of genetic carriers can do more harm than good (Rutkow and Lipton, 1974; Tarini and Goldenberg, 2012). On the other hand, in Fabry disease, some heterozygous females can develop full clinical presentation and should be identified to issue enzyme replacement treatment (Ortiz et al., 2018).

There is also the dilemma of late-onset conditions for which reporting to asymptomatic minors has been, until recently, only recommended when established prevention or treatment that may alter the course of the condition is available at the time of testing (Borry et al., 2009; van El et al., 2013; Howard et al., 2015; Bondio, 2017). A revised version of ACMG recommendations for reporting of secondary findings in clinical exome and genome sequencing is the first document that recommends reporting of information on select few actionable late-onset conditions in minors, with the explanation that this information could prove invaluable for the health of the parents (Kalia et al., 2017). The ESHG published recommendations for opportunistic genomic screening in which they do not object to reporting of population pharmacogenomics (PGx) variants and variants leading to early onset actionable conditions (de Wert et al., 2020). Despite professional guidelines deferring asymptomatic testing, carrier testing, or testing for late-onset diseases in minors, the public opinion may be in favor, as studies suggest (Shkedi-Rafid et al., 2015; Waisbren et al., 2015).

THE NEED FOR INFORMED CONSENT

Several current NBS programs are mandatory with most of them offering an opt-out option (Tarini and Goldenberg, 2012; Lampret et al., 2020; Loeber et al., 2021). Many of them are free of charge and do not require parental permission because in most NBS programs the participation is still considered in the best interest of the child's health. The model of presumed consent is still considered to be the most suitable in the context of NBS for conditions for which clear benefit to the child has been proven (Howard et al., 2015). When we consider some form of genomic screening, the consent and counseling of parents seem requisite. Currently, all genetic testing requires informed consent to be obtained from the patient, parents, or legal guardian during a genetic counseling session performed by a medical geneticist or similar, before the testing (ACMG Board of Directors, 2013). Many authors argue that if NBS will be based on genetic testing, some form of informed consent will be necessary (Friedman, 2015; Howard et al., 2015; Berg et al., 2017; de Wert et al., 2020). Others propose solutions such as a panel of core conditions with proven benefits for the child for which no consent would be necessary (Tarini and Goldenberg, 2012; Friedman, 2015). One study researched the possibility of using an electronic decision aid to assist parents in decision-making regarding genetic NBS which would guide them to informed consent they would understand (Berg et al., 2017). A multistep or dynamic informed consent has been proposed in case of reporting secondary findings in cancer patients (Pujol et al., 2018). Some authors fear that the need for explicit consent could lead to reduced participation in the NBS program and would pose an additional burden to healthcare workers involved (Jepson et al., 2001; Feuchtbaum et al., 2007; Tarini and Goldenberg, 2012).

ETHICAL AND PSYCHOLOGICAL IMPLICATIONS

The WHO principles for screening programs have been the center of every NBS program. Emanating from those principles, the focus of NBS has always been on the well-being of the infant to the degree that in many countries the opportunity to intervene and dramatically improve an infant's course of life supersedes parental autonomy (Wilson et al., 1968; Tarini and Goldenberg, 2012; Howard et al., 2015).

With the prospect of genetic testing of newborns, various authors see several different benefits of such testing, not pertaining solely to the child (Bombard et al., 2010; Drmanac, 2012; Best et al., 2018; Lantos, 2019). The ACMG proposal recommends the reporting of variants in the selected 59 highly penetrant and actionable genes, no matter what the indication for which clinical sequencing was requested and regardless of patient's age. The rationalization for such recommendation is that for these genes the benefit of other family members outweighs the child's right not to know. The proposed benefits of testing a minor or in the case of NBS a newborn for an adult-onset disease would be a benefit of the parent or sibling who could become ill with the disease in question and could benefit from the NBS results of the child. The result could also enable parents to make informed decisions about future pregnancies (McGuire et al., 2013; Wilfond et al., 2015; de Wert et al., 2020). Reporting of such variants would inevitably put enormous strain on the health system, with entire families requiring some form of management. The enormous pool of data that would be obtained through WGS NBS could serve as a powerful research tool, which could lead to a novel understanding of genetic mechanisms (Drmanac, 2012; Howard et al., 2015; Berg et al., 2017; de Wert et al., 2020). The question is, are the benefits of genomic screening such that we should shift the focus of NBS from the infant to the whole family or even the society at large. This is the question that not only scientists and clinicians but also experts from other fields and policymakers still need to answer.

If we assume that benefits for the family or society outweigh the benefits for the child, that raises another ethical issue. Testing the newborns for adult-onset diseases for the benefit of their parents would deprive them of their autonomy about decision-making regarding the results of their genetic NBS. Various international and national legal acts recognize the "right not to know" as one of the core patient's rights. The right not to know emerged with the progress of the field of genetics. With genetic results, it is easy to imagine that a person might not want to know if he or she is going to fall ill with a late-onset disease for which there is no cure or any preventive measure. So, the right not to know was integrated in the UNESCO Universal Declaration on the Human Genome and Human Rights and also in the European

Convention on Human Rights and Biomedicine (UNESCO, 1997; J Med Philos, 2000).

There is currently little known about the psychological impact of such knowledge on parents and children. Some early studies implied that there is a lingering effect of false-positive results from NBS, which makes parents of such children utilize the medical system more, but the following studies did not confirm that: Rothenberg and Sills (1968); Lipstein et al. (2009), and Tu et al. (2012). There is certainly some psychological impact on the parents, but it seems that the matter is more complex than just receiving a false-positive NBS result (Lipstein et al., 2009). With the expansion of NBS and screening for Krabbe disease, there appeared a new type of dilemma. Because of difficulties in predicting, if positive patients will develop clinical symptoms, there are children with positive results of NBS that are waiting if the disease will manifest itself. Many parents developed depression or were severely upset upon getting positive NBS results for Krabbe disease, which meant that their child might develop a devastating neurodegenerative illness. All of these symptoms were exacerbated by the fact that families were told that nothing could be done to ascertain if the disease will progress or not (Salveson, 2011; Dees and Kwon, 2013).

Many authors fear that tampering with the right not to know of neonates could lead to overdiagnosis and misdiagnosis, which could lead to mental health issues of the parents, overprotective parenting, and low self-esteem of such children. Misinterpretation and mishandling of genetic data could lead to discrimination in several areas of social life including education, employment, and health insurance (Tarini and Goldenberg, 2012; Wade et al., 2013; Frebourg, 2014; Berg et al., 2017; Murray et al., 2018). The situation of the detection of patients with a positive NBS test but an inconclusive diagnosis (CFSPID) is known in cystic fibrosis since 2005 (Cystic Fibrosis Foundation et al., 2009) and is a significant problem, since the ratio of infants with CF compared with CFSPID ranged from 1.2:1 (Poland) to 32:1 (Ireland) (Barben et al., 2017; Munck, 2020), but little is known regarding the psychological implication of such inconclusive diagnosis in families (Hayeems et al., 2017).

DISCUSSION

Before the NGS methodology could be implemented in NBS setting, the abovementioned obstacles and concerns have to be addressed. We need to make sure to obtain the best possible technical and methodological execution of NGS but in reasonable time regarding NBS. The choice of breadth of NGS (WGS, WES, or smaller panel) will have an impact on several aspects of NBS. Sequencing of larger parts of the genome will have as a consequence reduced capacity for multiplexing of samples, more acquired data, more complex interpretation, and higher cumulative cost. Each of the abovementioned types of approaches to NGS has its benefits and drawbacks.

Regarding present knowledge, a smaller targeted approach appears to be the better option to start the NBS. Despite slightly longer library preparation than WGS, smaller panels of genes could be sequenced faster and many more at the same time. New automated methods were already described to speed up DNA isolation and library preparation (Saavedra-Matiz et al., 2013; van Campen et al., 2019; Hendrix et al., 2020). Sequencing a smaller panel of genes would yield less data, which would require less computing power for basic data processing and subsequent analysis. Bioinformatics software pipelines for data management and variant calling should ease the load of the interpretation, but could not so far be used to automate reporting. Less data would require less disk space for storage and would be easier to integrate into existent information systems for interpretation and reporting. Such integration would enable later reinterpretation if additional knowledge about a certain gene or variant becomes available. Storage of gVCF files only would additionally reduce the size of data that required storage, so long-term data storage and data integrity could be instrumented (Berg et al., 2017; Nadeau et al., 2019; van Campen et al., 2019).

The subject of reporting of the detected genetic variants is unavoidably linked to the ethical dilemmas and psychological consequences of NBS. Knowledge about genetic idiosyncrasies of oneself or one's child is not always good, welcomed, or easy to understand, especially when it is not unequivocal (Dees and Kwon, 2013; Howard et al., 2015; Bouvier and Giguère, 2019). So, the conditions for NGS screening should be chosen carefully to avoid unwanted consequences. Best results of NGS screening would be achieved for conditions with clear Mendelian inheritance and with good genotype–phenotype correlation, which is highly penetrant, and there is a substantial amount of knowledge on variants. To the best of our knowledge, there is yet no condition screened neonatally using NGS as a first-tier method, but cases of cascade childhood screening for familial hypercholesterolemia using NGS have been reported (Klančar et al., 2015; Knowles et al., 2017; Groselj et al., 2018). Also, some NBS centers already use NGS as second-tier confirmatory testing, and their experience could provide some of the answers (Smon et al., 2018; Lampret et al., 2020; Tangeraas et al., 2020). When we would tackle neonatal screening using NGS, we would ultimately have to decide how to report on variants found for each condition individually. Ample knowledge of incidence, prevailing genotype (like in the cases of CF and SMA) if such exists, population-specific data on gene variants, and detailed natural history for the condition would be necessary for objective decision-making regarding reporting of variants (Chien et al., 2017; Czibere et al., 2020). We propose that a unified methodology be used for the assessment of the actionability of the conditions and variants (Milko et al., 2019; Hayeems et al., 2020).

Another problem is the reporting of variants in late-onset conditions, which clashes with the right of the child not to know his or her genetic result. As much as such knowledge would benefit parents or siblings, there is not enough insight into the psychological and sociological consequences of such information on the child or parents. Too much or non-actionable information would put an unnecessary burden on parents and could cause them psychological stress; we therefore recommend that such data should not be reported until more studies are performed (Howard et al., 2015; Reinstein, 2015; Berg et al., 2017; Murray et al., 2018; de Wert et al., 2020).

We propose that the fundamental design of genetic screening remains the same as biochemical NBS, where a panel of disorders would be selected following a scrupulous selection process. The conditions included in NGS NBS should have clear actionability early in life, strong genotype–phenotype correlation, sufficient population-specific variant data, well-defined criteria for reporting, and adequate specificity and sensitivity. The reporting of the results would essentially remain the same as the results from methods currently in use in NBS. Focus would remain solely on the newborn, and such screening would possibly not need additional informed consent or counseling before testing. One option would be the use of a stepwise software decision aid that would be used to either inform parents about genetic NBS or even obtain an informed consent if needed. Nonetheless, great care should be taken on how to implement genetics into newborn screening.

In conclusion, despite several remaining obstacles, NGS will likely enter or has already entered many NBS programs in the near future. NGS has great potential to improve and expand NBS and with it our understanding of genetic mechanisms, which in turn will enable us to better diagnose conditions and offer personalized treatments. Therefore, it is necessary that we set to this task with great care and attention to ethical standards and evidence-based decision-making, to ensure a reliable and beneficial program that will continue to improve the lives of newborns and their parents.

AUTHOR CONTRIBUTIONS

KT and ZR conceived the project. ZR drafted the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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frontiers in Pediatrics

BRIEF RESEARCH REPORT
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Newborn Screening for Biotinidase Deficiency. The Experience of a Regional Center in Italy

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Introduction: Biotinidase deficiency (BD) is an autosomal recessive disease causing a defect in the biotin-releasing enzyme. Newborn screening (NBS) allows early diagnosis and treatment, ensuring excellent prognosis. The aim of this study was to describe our experience in the diagnosis, treatment, and follow-up showing key strategies and unsolved questions of the management of BD patients.

Methods: We analyzed data of patients identified by the Regional Centre for Newborn Screening of Verona and followed by the Inherited Metabolic Disease Unit of Verona and Neonatal Intensive Care Unit of Bolzano, Italy, from 2014 to 2020.

Results: Thirty-seven patients were diagnosed by NBS (five profound and 32 partial BD), with a total incidence of 1:5,996. All were started on biotin at diagnosis and presented no symptoms at follow-up. Analysis of parents and siblings led to identification of five asymptomatic patients with partial BD: one asymptomatic parent and four young siblings. Genetic analysis of the *BTD* gene identified 17 different genotypes and one mutation not previously known.

Discussion: Our data confirm that NBS introduction had a dramatic impact on BD diagnosis, and the incidence has increased significantly compared to other areas. Partial defects are more common than profound and have a distinctive genotype. Partial BD treatment is still controversial even at what dose of biotin and for how long. At the end, BD treatment is very easy and inexpensive and prevents severe neurological damage. Sharing experiences is essential to achieving guidelines for treatment and follow-up and a better genotype–phenotype correlation.

Keywords: biotinidase enzymatic activity, genotype-phenotype correlation analysis, biotinidase deficiency incidence, biotinidase deficiency, newborn screening, biotinidase deficiency disorder gene

BACKGROUND

Biotinidase deficiency (BD, OMIM: 253260) is an autosomal recessive disease caused by an altered activity of the enzyme biotinidase. Biotinidase releases biotin from biocytin or small biotinylated peptides downstream the proteolytic turnover of holocarboxylases and other biotinylated proteins. In particular, biotin acts as cofactor for four carboxylation enzymes in the body: 3-methylcrotonyl-CoA carboxylase (MCC), pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), and propionyl-CoA carboxylase (PCC).

Based on the residual serum enzyme activity, the defect is distinguished in profound when the residual enzyme activity is $< 10\%$ and partial when enzyme activity is between 10 and 30% of mean serum activity calculated in the general population. The incidence reported in literature is about 1:60,000 (1), even if recent authors reported case studies with a significantly higher incidence (2, 3). The clinical presentation is heterogeneous and varies from neurological manifestations such as hypotonia, developmental delay, ataxia, seizures, sensorineural hearing loss, and visual problems, including optic atrophy, to dermatological manifestations such as alopecia, skin rash, and conjunctivitis (4, 5). Patients may present metabolic complications as well, like lactic acidosis, ketoacidosis, and hyperammonemia, and less frequently organic aciduria (5). Some patients may also present respiratory problems, such as hyperventilation, laryngeal stridor, and apnea (4).

Treatment with biotin may not be able to reverse neurological complications as moderate or severe developmental delay, hearing loss, and optic atrophy, especially if a long period has elapsed between their onset and the initiation of treatment (1, 4). On the contrary, dermatological manifestation usually responds favorably to biotin treatment.

As early treatment with oral biotin prevents the onset of clinical symptoms, BD has been successfully included in newborn screening (NBS) programs since 1984 (Commonwealth of Virginia in the United States), allowing for early diagnosis and treatment and ensuring an excellent prognosis of this inborn error of metabolism (5).

While for profound BD the efficacy of NBS and lifelong treatment is a consolidated practice, for partial BD there is a lack of clear indications on the treatment, dosage, and follow-up, resulting in a great heterogeneity between Centers.

The purpose of our study was to describe our experience in the diagnosis, treatment, and follow-up of BD, trying to show the key strategies and unsolved questions of the management of this disease.

METHODS

We carried out a retrospective analysis of patients affected by BD identified from January 2014 to December 2020 by the Metabolic Hereditary Diseases Center of Verona.

Abbreviations: BD, Biotinidase deficiency; NBS, Newborn screening; DBS, Dried blood spot.

NBS for BD have been performed since 1986 at the Regional Center for Neonatal Screening, Diagnosis and Treatment of Inherited Congenital Metabolic and Endocrinological Diseases of Verona covering North East of Italy while, from 2014, the Inherited Metabolic Diseases Unit is actively involved in treatment and follow-up of children diagnosed with total and partial biotinidase deficiency. The newborn dried blood spots (DBS) were collected at 36–72 h of life. If the first DBS value was below cutoff, a second sample was requested and possibly a third one. Until December 2014, the enzyme activity on DBS was analyzed with a non-quantitative colorimetric method and afterward was semi-quantitatively measured with the GSP Neonatal Biotinidase kit (Perkin Elmer, Wallac Oy), an assay combining an enzyme reaction with a solid phase time-resolved immunofluorescence assay.

The diagnostic confirmation was performed by the analysis of serum activity (6) and by molecular analysis of the *BTD* gene in all probands and parents.

Patient serum has been separated from whole blood within 2 h and sent to the laboratory in dry ice, together with the parents' samples and a non-family-related serum specimen collected and sent for transport condition control purposes. Upon lab arrival, all of them were stored at $-80°C$ for a maximum of 2 weeks. Serum biotinidase activity has been measured by a colorimetric assay using the artificial substrate N(+)-biotinyl-4-aminobenzoic acid (B-PABA): the enzyme cleaves the amide bond of B-PABA, freeing biotin and p-aminobenzoic acid (PABA). PABA is then converted to a purple compound, easily spectrophotometrically quantitated. No color develops when biotinidase is either missing or completely inactive.

Quality check of the batch has been performed by including a serum sample of an individual with normal biotinidase activity. Such a sample has been stored at $-70°C$ in aliquots and thawed just once. Furthermore, a positive control has been prepared and included in each batch as well, by heating a serum sample for 1 h at $60°C$.

Genomic DNA was extracted from patients' peripheral venous blood on EDTA by means of the QIAmp DNA Blood Mini kit (QIAGEN S.p.A, Milan, Italy), following the manufacturer's instructions. All exons and part of the flanking intron regions of the *BTD* gene (NM_000060.4) were amplified by polymerase chain reactions and sequenced for molecular analysis (primers available upon request). For each variant identified, databases available online have been used to verify if mutations were already reported in literature, such as Pubmed, dbSNP, ClinVar, and Human Genetic Variation Database (HGVD). If not previously reported, *in silico* prediction analyses were performed for missense variants using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and Mutation Taster (http://www.mutationtaster.org/) tools.

Therapy with biotin was started at a dosage of 10 mg/day for profound or 5–10 mg/day for partial BD in all patients with the suggestion to eliminate raw eggs, containing avidin from the diet, a protein interacting with biotin and decreasing its bioavailability (4).

The patients underwent the following biochemical and clinical follow-up: blood count with formula, biochemical profile

with lactate, ammonia, blood gas analysis, audiometry, eye examination, and pediatric evaluation to assess growth and neurological development and look for skin problems. All were carried out every year for profound BD and every 2 years for partial BD (4). Furthermore, patients with profound BD underwent urinary organic acids evaluation every year to monitor efficacy and compliance to therapy (1, 4).

RESULTS

Patients

Among 293,784 newborns screened by the Regional Screening Centre of Verona since 2014 until the end of 2020, 287 were recalled to repeat DBS in cases of suspected BD and 49 were diagnosed with BD, with a total incidence of 1:5,996 newborns. In particular, an incidence of 1:58,757 for profound BD and 1:6,677 for partial BD were found. A total of five (10.2%) patients with profound BD was detected. Thirty-four patients were followed up in Verona (four patients with profound and 30 with partial BD) and three patients (one profound and two partial BD) in Bolzano, and 12 remaining patients were followed up in another center. Eighteen patients were male (48.6%), and 19 patients were female (51.4%). All of them, identified by NBS, started biotin supplementation at diagnosis at the dosage of 10 mg/day for profound BD and 5–10 mg/day for partial deficiencies.

None of the patients, profound BD included, presented signs or symptoms related to BD at diagnosis or at clinical follow-up (**Table 1**). Psychomotor development and auditory and visual functions were normal, and no relevant dermatological problems attributable to the deficiency were detected. Only patient 3 with partial BD presented language delay at 2.5 years, despite proper therapy, not related to BD. The mean follow-up period was 43.3 months +/– 23.0 standard deviation.

Five patients with partial BD were identified through family study: one asymptomatic parent of a proband (35 years of age at diagnosis) and four siblings of patients (**Table 1**). They promptly started therapy with oral biotin at the dosage of 10 mg a day, except for the adult parent that remained asymptomatic throughout her life. Two siblings of patient 3, 10, and 6 years at diagnosis, presented with a history of mild language delay with learning disability and a reported hearing impairment ongoing assessment, respectively.

A third familiar case, sibling of patient 22 identified at 6 years of age, presented at the diagnosis a dermatitis, not previously investigated which improved with moisturizing lotions.

Molecular Analysis

Genetic analysis of the gene *BTD* was completed in all 42 patients (**Table 1**).

We identified 17 different genotypes in the entire group, which are presented in **Figure 1**. The most frequent genotype was found in nine patients (21.4%) with partial BD and is characterized by the frameshift mutation c.98_104delGCGGCTGinsTCC (p.Cys33Phefs) and the c.1330G>C (p.Asp444His) variant on the *BTD* gene present in compound heterozygosity, both already reported in literature (7, 8). The second two most frequent genotypes, identified in eight patients with partial BD each (19.0%), were characterized by c.1368A>C (p.Gln456His) and c.1330G>C (p.Asp444His) on the *BTD* gene present in compound heterozygosity, both already reported in literature (8, 9); while the second one was characterized by the already known mutation c.511G>A (p.Ala171Thr) (11) and the c.1330G>C (p.Asp444His) variant in cis and c.1330G>C (p.Asp444His) present in the other allele.

The known mutation c.1330G>C (p.Asp444His) has been identified in all patients with partial BD, with a pathogenic variant in compound heterozygosity. This is a mutation with high prevalence in the European population (about 4%) (19), which produces an enzyme with about 50% of residual activity (20).

Two patients affected by profound BD are homozygous for common mutations c.1612C>T (p.Arg538Cys) and c.98_104delGCGGCTinsTCC (p. Cys33Phefs), known in literature to be found in about 30 and 50% of patients with profound deficiency, respectively (7). Pt 34 is homozygous for the mutation c.1489C> T (p.Pro497Ser), already reported in literature associated with profound BD (17). In this case, genetic analysis helped us to diagnose this patient as profound deficiency despite a borderline enzymatic activity of 1.1 nmol/pABA/min/mL at the diagnosis. Pt 36 carries the known c.184G>A (p.Val62Met) variant (18) in compound heterozygosity with the c.511G>A (p.Ala171Thr) and c.1330G>C (p.Asp444His) variants in cis, and patient 33 is homozygous for the unknown substitution c.508G>A (p.Val170Met). Both patients 33 and 34 have certain degrees of consanguinity of the parents in their medical history.

Hence, a mutation never previously reported in literature has been identified in our study: c.508G>A (p.Val170Met) in homozygosity in patient 33 with profound BD (enzymatic activity 0.7 nmol/pABA/min/mL) predicted likely pathogenic by prediction tools. In contrast, patient 2 presented the c.1613G>T (p.Arg538Leu) missense variant that has been reported in dbSNP rs397514429 with uncertain clinical significance but in our study is clearly associated with partial BD (enzymatic activity 2.7 nmol/pABA/min/mL) when in compound heterozygosity with the common variant c.1330G>C (p.Asp444His).

DISCUSSION

From our data, a remarkable overall incidence of 1:5,996 newborns with BD, combining partial and total deficiencies, emerged. Our incidence is significantly higher than previously reported in literature (5), even if recently some authors reported case studies identified by NBS with comparable incidences in different countries (2, 3, 13, 21).

All patients identified by NBS, thanks to the timely initiation of therapy and follow-up, did not present any clinical signs and symptoms related to the spectrum of BD. Although our data come from a fairly short follow-up, a recent study confirms this finding by reporting adolescents and adults, ages 16–32 years old, with profound biotinidase deficiency ascertained by NBS with excellent outcomes (22).

While the fundamental usefulness of NBS in ensuring early diagnosis and good prognosis for profound BD is unquestionable, the management of patients with partial BD diagnosed thanks to NSB is still a matter of discussion. Although they may show milder symptoms, most patients with partial BD

TABLE 1 | Diagnosis, clinical and genetic characterization of patients affected by biotinidase deficiency.

Id	Duration of follow up (months)	Age (years) at follow-up	M/F	Diagnosis	Biotinidase enzimatic activity on DBS (U/dL)	Biotinidase enzymatic activity on serum (nmol/pABA/min/mL)	Allele 1	Molecular consequence	Reported citation or pathogenic classification	Allele 2	Molecular consequence	Reported citation or pathogenic classification	Type of deficit	Free biotin treatment	Audio metric evaluation	Ophthal mologic evaluation	Neurological development assessment	Dermato logical evaluation	Degree of kinship
pt 1	73	6.1	F	NBS	52	2.7	c.98_104 delGCG GCTGin sTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Atopic dermatitis	N/A
pt 2	26	2.1	M	NBS	74	2.7	c.1613G>T p.Arg538Leu	Missense	Uncertain clinical significance	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 3	35	2.9	M	NBS	55.7	2.7	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	N/A	Language regression	Normal	Sibling of pt 38-39
pt 4	33	2.7	F	NBS	44	2.9	c.454A>C p.Thr152Pro	Missense	Milánkovics et al. (10)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 5	23	1.8	M	NBS	66	2.9	c.98_104 delGCGGC TGinsTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	N/A	Normal	Normal	N/A
pt 6	70	5.8	F	NBS	56	1.3	c.[470G>A; 1330G>C] p.(Arg157His; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 7	53	4.4	F	NBS	39	2.2	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Transmissive hypoacusia	Hyper metropia	Normal	Atopic dermatitis	N/A
pt 8	27	2.2	F	NBS	51	2.3	c.341G>T p.Gly114Val	Missense	Wolf et al. (12)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 9	38	3.1	M	NBS	44.5	2.1	c.[470G>A; 1330G>C] p.(Arg157His; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Eczema	N/A
pt 10	65	5.4	M	NBS	42	2.4	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 1/die	Left trans missive deafness	Normal	Normal	Normal	N/A
pt 11	64	5.3	M	NBS	47.5	2.9	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 1 /die	Normal	Normal	Normal	Normal	N/A
pt 12	17	1.4	M	NBS	42	2.5	c.98_104del GCGGCT GinsTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Normal	N/A
pt 13	41	3.4	M	NBS	52.3	2.2	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 14	67	5.5	M	NBS	53.5	2.7	c.218C>T p.Pro73Leu	Missense	Canda et al. (13)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Exophoria	Normal	Normal	N/A
pt 15	63	5.2	F	NBS	46.5	2.6	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 1/die	Normal	Normal	Normal	Normal	N/A
pt 16	66	5.5	F	NBS	45	1.7	c.1595C>T p.Thr532Met	Missense	Seker Yilmaz et al. (14)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Normal	N/A

(Continued)

TABLE 1 | Continued

Id	Duration of follow up (months)	Age (years) at follow-up	M/F	Diagnosis	Biotinidase enzimatic activity on DBS (U/dL)	Biotinidase enzymatic activity on serum (nmol/ pABA/ min/mL)	Allele 1	Molecular consequence	Reported citation or pathogenic classification	Allele 2	Molecular consequence	Reported citation or pathogenic classification	Type of deficit	Free biotin treatment	Audio metric evaluation	Ophthal mologic evaluation	Neurological development assessment	Dermato logical evaluation	Degree of kinship
pt 17	33	2.7	F	NBS	55	2.8	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Mild dermatitis	Daughter of pt 40
pt 18	68	5.6	M	NBS	43	2.9	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 19	16	1.3	F	NBS	62	2.5	c.278A>Gp. Tyr93Cys	Missense	Wolf et al. (15)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	N/A	Normal	Normal	N/A
pt 20	21	1.7	M	NBS	58	2.8	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 1/die	Normal	N/A	Normal	Normal	Sibling of pt 32
pt 21	20	1.6	M	NBS	42.5	2.7	c.1595C>T p.Thr532Met	Missense	Seker Yilmaz et al. (14)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	normal	Normal	Normal	N/A
pt 22	73	6.1	M	NBS	N/A	1.3	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Normal	Sibling of pt 41
pt 23	56	4.7	F	NBS	36.5	2.2	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 24	20	1.6	M	NBS	53.3	1.7	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	N/A	Normal	Normal	N/A
pt 25	19	1.6	M	NBS	53	2.7	c.631C>T p.Arg211Cys	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	Normal	Normal	Normal	Sibling of pt 42
pt 26	66	5.5	F	NBS	36.5	2.3	c.334G>A p.Glu112Lys	Missense	Laszlo et al. (16)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 27	31	2.6	M	NBS	44.5	2.5	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 28	31	2.5	F	NBS	53.7	2.2	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 29	5	0.3	F	NBS	49.5	2.1	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/a	N/A	Normal	Normal	N/A
pt 30	4	0.2	F	NBS	60	1.2	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/a	N/A	Normal	Normal	N/A

(Continued)

TABLE 1 | Continued

Id	Duration of follow up (months)	Age (years) at follow-up	M/F	Diagnosis	Biotinidase enzimatic activity on DBS (U/dL)	Biotinidase enzymatic activity on serum (nmol/pABA/min/mL)	Allele 1	Molecular consequence	Reported citation or pathogenic classification	Allele 2	Molecular consequence	Reported citation or pathogenic classification	Type of deficit	Free biotin treatment	Audio metric evaluation	Ophthal mologic evaluation	Neurological development assessment	Dermato logical evaluation	Degree of kinship
pt 31	90	7.6	F	NBS	N/A	2.4	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Normal	N/A
pt 32	65	5.4	M	NBS	42.5	2.7	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 1/die	Normal	N/A	Normal	Normal	Sibling of pt 20
pt 33	74	6.1	M	NBS	8.5	0.7	c.508G>A p.Val170Met	Missense	Likely pathogenic by prediction tools	c.508G>A p.Val170Met	Missense	Likely pathogenic by prediction tools	Profound	10 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 34	43	3.5	F	NBS	15	1.1	c.1489C>T p.Pro497Ser	Missense	Sarafoglou et al. (17)	c.1489C>T p.Pro497Ser	Missense	Swango et al. (8)	Profound	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 35	45	3.7	F	NBS	9	0.7	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	c.98_104del GCGGCTG insTCC p.Cys33Phefs	Frameshift	Pomponio et al. (7)	Profound	5 mg × 2/die	Normal	N/A	Normal	Normal	N/A
pt 36	20	1.6	F	NBS	8	0.5	c.184G>A p.Val62Met	Missense	Muhl et al. (18)	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Swango et al. (8)	Profound	5 mg × 2/die	Normal	N/A	Normal	Normal	N/A
pt 37	71	5.9	F	NBS	7	0.2	c.1612C>T p.Arg538Cys	Missense	Pomponio et al. (7)	c.1612C>T p.Arg538Cys	Missense	Swango et al. (8)	Profound	5 mg × 2/die	Normal	Normal	Normal	Normal	N/A
pt 38	35	13.0	M	Familial screening	N/A	1.7	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Mild language delay and learning disability	Normal	Sibling of pt 3
pt 39	35	8.5	M	Familial screening	N/A	2.9	c.1368A>C p.Gln456His	Missense	Norrgard et al. (9)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A hypoacusia?	N/A	Normal	Normal	Sibling of pt 3
pt 40	32	37.8	F	Familial screening	N/A	2.9	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	No treatment	Normal	Normal	Normal	Normal	Mother of pt 17
pt 41	72	11.6	M	Familial screening	N/A	2.1	c.[511G>A; 1330G>C] p.(Ala171Thr; Asp444His)	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	Normal	N/A	Normal	Dermatitis	Sibling of pt 22
pt 42	7	11.1	F	Familial screening	N/A	2.6	c.631C>T p.Arg211Cys	Missense	Norrgard et al. (11)	c.1330G>C p.Asp444His	Missense	Swango et al. (8)	Partial	5 mg × 2/die	N/A	N/A	Normal	Normal	Sibling of pt 25

In column of DBS is reported the average value of repeated analysis. DBS, dried blood spot; NBS, newborn screening; N/A, not applicable or not available; M, male; F, female. Reference sequences: NM_000060.4; NP_000051.1.

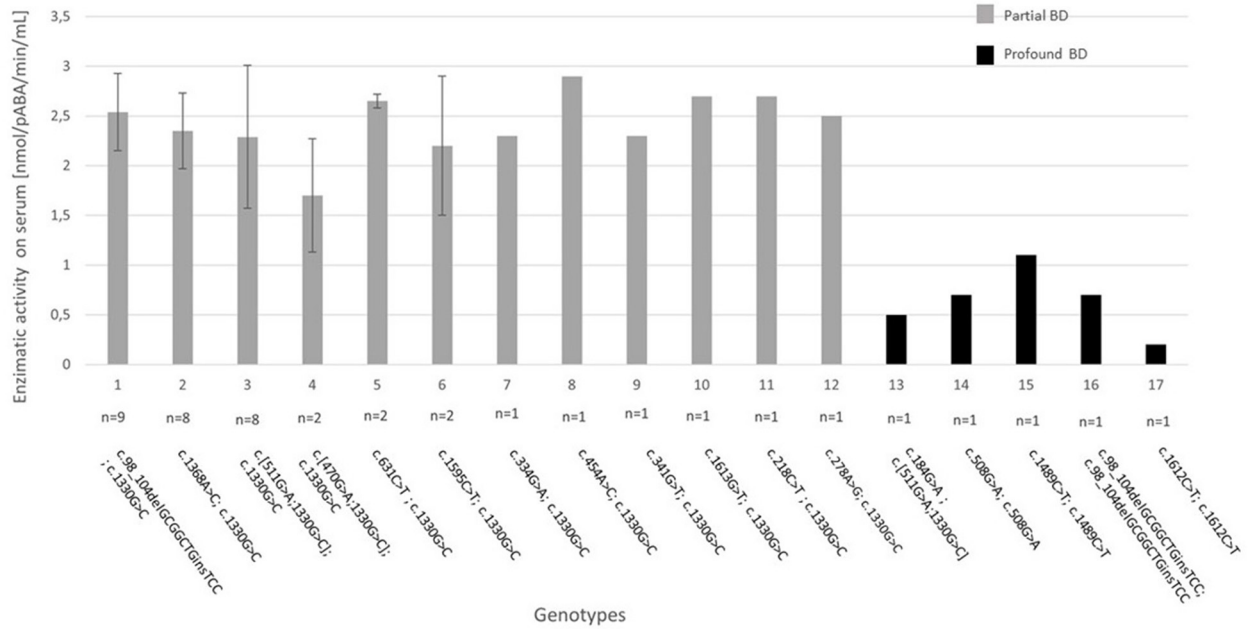

FIGURE 1 | Genotype–biochemical phenotype correlation for the 17 genotypes identified in our study. The enzymatic activity on serum is expressed as absolute value when $n = 1$ or mean +/– standard deviation when $n > 1$. BD, biotinidase deficiency. Reference sequence for variants: NM_000060.4.

remain asymptomatic throughout life (23). Additionally, milder symptoms are usually characterized mainly by cutaneous signs such as rash and alopecia, which are usually reversible with the initiation of therapy (24).

However, there have been reported in literature patients with partial BD not taking biotin with more severe clinical pictures, identified either by screening or clinically (20). Wolf and colleagues in 2015 reported several patients with partial BD with a clinical onset characterized by a severe neurological symptomatology with seizures, psychomotor development delayed, hearing loss, and ataxia (20).

Therefore, patients with partial BD could develop even severe clinical symptoms, if not treated, especially during stressful events, like severe infections (13, 20). It is precisely for this reason, and given the absence of side effects associated with biotin supplementation at commonly used doses, that there is currently consensus on starting oral biotin supplementation even in patients with partial BD. Nonetheless, there are still no precise guidelines on the proper dosage of oral biotin supplementation, the duration of the therapy, and the need and timing of clinical and biochemical follow-up.

In some case series, patients with profound BD were treated with up to 15–20 mg of biotin per day, with the indication to continue lifelong. Although there are no clear indications, particularly for partial deficits, it seems reasonable to start with a dosage of 10 mg per day in a single administration or divided in two administration for profound BD and with a dosage ranging between 1 and 10 mg per day for partial deficiencies, possibly to increase according to the appearance of symptomatology (1, 22).

All our partial BD patients identified by NBS were persistently asymptomatic during follow-up, with normal psychomotor development, except for patient 3, belonging to a familiar case discussed thoroughly later. Auditory and visual problems, likewise dermatological problems, revealed at the follow-up evaluations of our NBS patients, were not attributable to BD such as transmissive hypoacusia, orthoptics problems, atopic dermatitis, and transient eczema, as reported in **Table 1**.

Instead, partial BD identified by familiar screening at diagnosis presented some phenotypes, but it is difficult to assert if it was caused by BD. The two siblings identified at the age of 10 and 6 years, after the diagnosis of patient 3 by NBS, have presented a history of mild language delay with learning disability and hypoacusia, ongoing assessments. They have started biotin therapy after NBS result of patient 3, without any symptomatology improvement. Also, patient 3 developed delayed speech at the age of 2.5 years, similarly to one brother, despite good compliance in therapy with biotin from birth. It is therefore plausible that the familial phenotype was not caused by BD, and further diagnostic investigations are required to define the etiology.

Four patients among those detected in the family analysis were not identified by NBS, although they were born after its introduction in our region. This is likely due to the old non-quantitative colorimetric method which has been discontinued since December 2014 in favor of the enzymatic and immunofluorescence reaction. In fact, comparison studies suggested that the fluorescence method was slightly more specific and sensitive than the colorimetric assay (25).

It is therefore important to underline the relevance that the clinical diagnosis of BD still has nowadays. In fact, BD must be recognized and considered in the differential diagnosis in case of symptomatic patients with late onset who not only may not have been identified at screening, but who may belong to the pre-NBS era, since in most cases these patients respond to biotin treatment (4).

Our genetic data confirmed that partial BD are more frequent than profound (88.1%) and are characterized by a distinctive genotype with a high prevalence of the c.1330G>C (p.Asp444His) variant (8, 19). Essentially, all individuals with partial BD have the mutation c.1330G>C (p.Asp444His) in one or both alleles of the *BTD* gene in combination with a mutation associated with profound BD in heterozygosity, and this is in agreement with other studies already reported in the literature (10, 14, 16, 21).

The high frequency of c.1330G>C (p.Asp444His) is characteristic of European populations, with existing geographical differences in frequencies observed in other countries (19).

Genetic analysis is useful for the diagnostic confirmation of these defects; particularly, it can be decisive in the case of borderline enzymatic activity (4). Our patient 34 with a borderline serum enzymatic activity of 1.1 nmol/pABA/min/mL at diagnosis has eventually been defined as profound BD, thanks to the finding of a genotype characterized by the c.1489C>T (p.Pro497Ser) homozygous mutation, already associated in literature with profound BD (17). This genotype–phenotype correlation was further confirmed by a repeated enzymatic analysis on serum, which resulted < 10% of the mean calculated in the general population.

Similarly, genetic analysis may be useful if the serum enzymatic activity is approximately 30% of normal activity to discriminate between heterozygous and partial BD and decide accordingly whether to start treatment (19, 21).

Although genetic analysis confirmed and supported the diagnosis, particularly in cases of borderline enzymatic activity, further investigations are needed to elucidate a clear genotype–phenotype correlation for BD. In fact, in literature the association between the *BTD* genotype and biotinidase activity is not always consistent (2, 3, 19) and decisions regarding treatment should be based primarily on enzymatic activity (4). This disagreement may be due to factors that influence the biotinidase activity assay, as well as to genetic factors that currently remain unknown such as the presence of variants in non-coding regions of the *BTD* gene (19). Anyway, in our case study, genotype–biochemical phenotype association was quite reliable for patients identified by NBS. In fact, the three most numerically represented genotypes all have overlap serum enzymatic activity values, as it is possible to see from the small standard deviations reported in **Figure 1**.

From our case series, a high molecular heterogeneity emerged: on 42 patients, genetic analysis of the *BTD* gene identified 17 different genotypes and one mutation not previously reported in the literature and predicted as likely pathogenic by prediction tools.

CONCLUSION

BD can be properly diagnosed and treated through NBS Programs. Treatment is inexpensive and easy to go. On the other hand, neurological consequences of a missed diagnosis may be dramatic for children and families.

This should prompt all Countries all around the world to implement in their screening policies BD identification through enzyme activity on DBS. However, it is of paramount importance to consider BD in the differential diagnosis of patients with late onset, particularly with neurological symptoms, as early treatment with biotin can reverse the clinical picture.

After the inclusion of BD in the NBS program, the number of diagnosed patients has increased significantly, especially for partial BD. Currently, there are no European or international management guidelines so there is great heterogeneity in the dose used for treatment and in the follow-up for these patients. Furthermore, given the relatively recent introduction of NBS, there are still few studies with long follow-ups that allow us to understand the clinical course of these patients, especially for what concern adult ages.

Although the measurement of enzymatic activity remains the major tool for the diagnosis of BD, our experience confirms that genetic analysis is useful for genotype–phenotype correlation study and for the diagnostic confirmation, particularly in cases of borderline enzymatic activity. Nonetheless, further studies are needed to improve our knowledge about genotype–phenotype correlations for BD. On the other end, only by bringing together the experience of multiple centers and sharing databases by combining clinical information, enzymatic activity, and DNA sequence will it be possible to develop guidelines and improving the management of BD patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants [legal guardian/next of kin] provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors are responsible for reported research. All authors have participated in the concept and design, analysis and interpretation of data, and drafting or revising of the manuscript, and they have approved the manuscript as submitted.

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REVIEW
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The Use of Whole Genome and Exome Sequencing for Newborn Screening: Challenges and Opportunities for Population Health

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Newborn screening (NBS) is a population-based program with a goal of reducing the burden of disease for conditions with significant clinical impact on neonates. Screening tests were originally developed and implemented one at a time, but newer methods have allowed the use of multiplex technologies to expand additions more rapidly to standard panels. Recent improvements in next-generation sequencing are also evolving rapidly from first focusing on individual genes, then panels, and finally all genes as encompassed by whole exome and genome sequencing. The intersection of these two technologies brings the revolutionary possibility of identifying all genetic disorders in newborns, allowing implementation of therapies at the optimum time regardless of symptoms. This article reviews the history of newborn screening and early studies examining the use of whole genome and exome sequencing as a screening tool. Lessons learned from these studies are discussed, along with technical, ethical, and societal challenges to broad implementation.

Keywords: newborn screening, whole genome sequencing, whole exome sequencing, next-generation sequencing, recommended uniform screening panel

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INTRODUCTION

Medical screening is an important part of health care and public health initiatives. The purpose of a medical screening test is to identify a medical condition early, ideally in the pre-symptomatic phase, so that appropriate treatment can be initiated to decrease morbidity and mortality. Screening is particularly indicated for medical conditions in which early treatment is more effective than treatment in later stages of the condition. Population screening adds a requirement of broader societal benefit in addition to benefit for the individual. Newborn screening (NBS) was cited by the United States Centers for Disease Control and Prevention as one of the most impactful public health initiatives of the twentieth century and in the twenty first century has undergone significant expansion through improved techniques of high-throughput biochemical analysis of targeted analytes, enzymatic activities, and specific molecular defects. It now stands on the verge of incorporating increasingly large-scale molecular sequencing to detect a growing number of disorders. This article will provide a history of NBS, specifically focusing on the elements pertinent to implementation of whole exome and whole genome sequencing (WES and WGS, respectively), review efforts to date on the

use of sequencing for NBS, and discuss the challenges for the future for larger incorporation in NBS programs.

HISTORY OF NEWBORN SCREENING

Inborn Errors of Metabolism

In 1935, phenylpyruvic acid was reported in the urine of a subset of individuals with intellectual disability and later termed phenylketonuria (PKU) (1). Subsequently, the possibility of treatment for PKU with restriction of dietary phenylalanine was postulated, and in 1954, the first report was published of a child with PKU treated with a diet restricted in phenylalanine who showed significant clinical improvement and elimination of phenylpyruvate in the urine (2). While the detection of urine phenylpyruvate could be used to diagnose PKU, it was not until Robert Guthrie developed a bacterial inhibition assay in 1958 that early, rapid, and accurate testing of phenylalanine levels for diagnosis and management of PKU became a reality (3). The final piece fell into place in 1961 when a filter paper method for collecting samples as a dried blood spot (DBS) was developed that is used in NBS to this day (4). Screening for PKU began in New York in 1961, and in 1963, Massachusetts became the first state to mandate NBS for PKU (3, 5). The introduction of NBS for PKU was clearly a turning point for the disease, as it allowed normal development in identified babies who otherwise would have suffered devastating neurodevelopmental symptoms. Over time, it was determined that NBS for PKU not only identifies classic PKU due to phenylalanine hydroxylase deficiency but also non-PKU hyperphenylalaninemia and tetrahydrobiopterin deficiency, all of which are characterized by elevation of a single metabolite, phenylalanine.

The success of NBS for PKU allowing early identification and intervention that prevented intellectual disability led to the hope that expansion of NBS would translate to similar improvement for other appropriate conditions (6, 7).

The Wilson and Jungner Principles

Given a growing interest in expanding NBS to more disorders, a meeting sponsored by the World Health Organization (WHO) in 1968 led to the publication of the Wilson and Jungner guidelines outlining 10 principles to guide development and implementation of a screening test and provided the framework for the further development of NBS programs (**Table 1**) (8). Necessary characteristics of a screening test include the availability of an economical method of identification of important health problems for which treatment is available, as well as the infrastructure to provide such treatment. These guidelines clearly went beyond the principle that a disease should be added to NBS based on technical ability and formed the basis of NBS expansion for several decades.

Expansion of NBS Beyond Inborn Errors of Metabolism

While inborn errors of metabolism (IEM) were the initial focus of NBS, the scope of NBS quickly began to expand. In 1973, a method for identifying hemoglobinopathies from DBS was developed, and the first NBS for hemoglobinopathies began in New York in 1975 (10). Yet another milestone in NBS was achieved in 1987 when a process for extracting DNA from DBS was developed, and its use as a second-tier test to confirm the diagnosis of sickle cell disease after an initial positive NBS with isoelectric focusing (IEF) or high-performance liquid chromatography (HPLC) was reported (11, 12). Second-tier DNA testing is necessary to identify sickle cell disease, as multiple hemoglobinopathies are identified with IEF and HPLC (10).

Newborn screening for congenital hypothyroidism was first introduced in 1974 and represents another triumph in NBS akin to that of PKU, allowing early identification, treatment, and prevention of intellectual disability using a new technology, immunodetection (13). Shortly thereafter, NBS for a second endocrinopathy, congenital adrenal hyperplasia (CAH), was introduced using an immunoassay to identify the most common type of CAH, 21-hydroxylase deficiency (14). Newborn screening for CAH prevented neonatal death in the salt-wasting forms through early diagnosis and appropriate treatment, though CAH screening via immunoassay alone had a high false positive rate, particularly in premature infants (14).

Newborn screening for cystic fibrosis (CF) was introduced in 1988 and, similar to the two-tiered approach for hemoglobinopathy screening, utilized DNA extraction from DBS and assessment for common pathogenic variants in the *CFTR* gene after a positive first-tier screen, in this case with immunoreactive trypsinogen (IRT) as the first step in screening (15).

NEW TECHNOLOGIES AND EXPANDED NEWBORN SCREENING TARGETS

Tandem Mass Spectrometry

The early successes of NBS were all based on identifying one disease at a time, though as noted for PKU and as is true for other disorders, such as galactosemia, sometimes more than one disease can be diagnosed. This paradigm required the development and implementation of a new test for every proposed disorder added to screening. The next major advance in NBS came in the 1990's with the introduction of tandem mass spectrometry (MS/MS), which allows rapid simultaneous screening for multiple IEM using a single test (16–18). The concept of multiple markers for multiple disorders not only broadened the scope of initial screening tests but also significantly increased the differential diagnosis of a positive newborn screen (**Figure 1**). However, with this advancement in technology also came additional challenges. The technology was beyond that in use by most public NBS programs. Moreover, while MS/MS identified IEM that conformed to Wilson and Jungner principles for NBS disorders, it also identified others that do not meet these criteria (8, 16). For example, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a disorder of fatty acid oxidation first identified in the late 1970s in a subset of children presenting with Reye syndrome-like features and abnormal urinary metabolites (19, 20). Prior to newborn screening for MCAD deficiency, affected individuals presented with acute crises characterized by hypoglycemia,

TABLE 1 | Wilson and Jungner principles and 2006 ACMG criteria.

Wilson and Jungner 10 principles of a screening test (8)

1. The condition sought should be an important health problem.
2. There should be an accepted treatment for patients with recognized disease.
3. Facilities for diagnosis and treatment should be available.
4. There should be a recognizable latent or early symptomatic stage.
5. There should be a suitable test or examination.
6. The test should be acceptable to the population
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8. There should be an agreed policy on whom to treat as patients.
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
10. Case-finding should be a continuing process and not a "once and for all" project.

2006 ACMG criteria (9)

1. The clinical characteristics of the condition
   a. Incidence of the condition
   b. Signs and symptoms were clinically apparent in the first 48 h of life
   c. Burden of disease if untreated
   d. Individual benefit of early intervention
   e. Family and societal benefits of early intervention
   f. Prevention of mortality through early diagnosis and treatment
2. The analytical characteristics of the test
   a. Sensitive and specific screening test algorithm
   b. Test performed on DBS or a simple point-of-care method
   c. High throughput (more than 200 per day)
   d. Cost <$1 per test per condition
   e. Multiple analytes relevant to a single condition detected in the same run
   f. Other conditions detected by the same analytes
   g. Multiple conditions can be detected by the same test
3. Diagnosis, follow-up, treatment, and management of the condition
   a. Cost and availability of treatment
   b. Potential of existing treatment to prevent negative consequences of the condition
   c. Availability of diagnostic confirmation and acute management
   d. Simplicity of therapy

lethargy, vomiting, seizures, hepatomegaly, and liver dysfunction, progressing to coma and death with a reported mortality rate of 20–30% in individuals diagnosed on a clinical basis (21, 22). In those who survived, an additional 40% showed evidence of significant neurological damage as a sequela of hypoglycemic crises (21, 23). The specific enzymatic defect was identified in 1982, and NBS for MCAD deficiency became a reality in 1990 when MS/MS on DBS became available (19, 20). The treatment for MCAD deficiency is avoidance of prolonged fasting and administration of IV dextrose if oral intake cannot be maintained due to intercurrent illness. After NBS for MCAD deficiency was introduced, morbidity and mortality decreased significantly, with a recent study reporting a mortality rate of 3.5% (20, 24). Clearly, the advent of NBS by MS/MS has saved lives and has a substantial beneficial effect on the natural history of MCAD deficiency. However, MS/MS also identifies short-chain acyl-CoA dehydrogenase deficiency (SCAD), another defect of fatty acid oxidation, which is now considered an asymptomatic biochemical condition and does not meet the Wilson and Jungner criteria (25). Other examples of disorders with significant clinical impact such as isovaleric acidemia were also balanced by disorders ultimately shown to have a low risk of clinical symptoms (3-methylcrotonyl-CoA carboxylase deficiency). Furthermore, other identified diseases were less amendable to early intervention (for example, mitochondrial trifunctional protein deficiency), though screening led to earlier diagnosis and avoidance of an extended diagnostic odyssey, providing an important benefit to families and allowing for genetic counseling for the couple and other family members.

Tiered Testing Strategies

Tiered testing strategies, including utilization of MS/MS, have led to improved positive predictive value of abnormal screens (9, 26). For example, steroid profiling via MS/MS as a second-tier test after a positive NBS for CAH has been shown to decrease the false positive rate of the initial test (15). Screening for congenital hypothyroidism with thyroxine (T4) is highly sensitive but not particularly specific; the addition of thyrotropin (TSH) as a second-tier test in those neonates with low T4 improves the specificity and decreases the false positive rate (9, 27). Other examples of tiered testing strategies include DNA testing on DBS after positive screens for CF, hemoglobinopathies, and MCAD deficiency (9, 10, 15). It is important to distinguish tiered screening testing strategies from diagnostic testing. A positive NBS, with or without second-tier testing, requires follow-up diagnostic testing. In some cases, the technology may be the same used in the initial screening test (e.g., MS/MS for fatty acid oxidation disorders); however, confirmatory diagnostic testing is still indicated as the cut-off values for screening tests are different than those of diagnostic testing (9). In addition, confirmatory diagnostic testing often utilizes additional methodologies not included in the initial NBS test (e.g., urine organic acid analysis, enzyme analysis, and/or DNA sequencing).

THE RECOMMENDED UNIFORM SCREENING PANEL

As more states began utilizing MS/MS for NBS, it became possible to screen for a larger number of disorders, and the variability in NBS among states increased (9). Recognizing the increasing complexity of the NBS landscape, in the late 1990's, the US Health Resource and Services Administration (HRSA) requested that the American Academy of Pediatrics (AAP) review the current state of affairs of NBS in the United States and provide recommendations for improvement. The AAP Newborn Screening Task Force concluded that there was a need for consistency and equity among NBS programs in the United States (28). In response, HRSA charged the American College of Medical Genetics and Genomics (ACMG) with developing a national framework for NBS and, specifically, with the development of a uniform panel of conditions as well as model policies, procedures, minimum standards, methods for expansion of NBS programs, as well as quality and oversight on a national level (9).

The ACMG developed a scoring system with which to evaluate each condition to be included on a recommended uniform screening panel, based on the following categories (9):

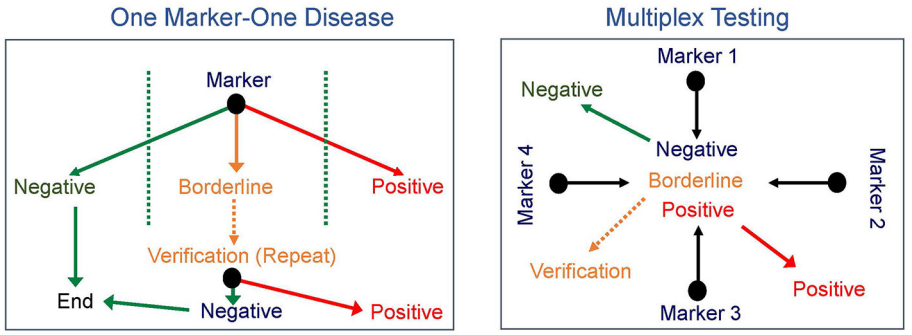

FIGURE 1 | Newborn screening paradigms. Newborn screening progressed from one marker for each disease (left) to multiplex testing with multiple markers identified from a single test (right) broadening the scope of initial screening tests and significantly increasing the differential diagnosis of a positive newborn screen.

1. The clinical characteristics of the condition;
2. The analytical characteristics of the test; and
3. Diagnosis, follow-up, treatment, and management of the condition.

Each category included specific criteria upon which the tests were scored, outlined in **Table 1**. Based on the sum of scores from each category, a condition was then assigned to one of three groups, namely, the core panel, the secondary targets (differential diagnosis of core panel disorders), and conditions not appropriate for NBS (9). The ACMG also recommended standardization of reporting language, reporting standards, as well as improved oversight, long-term data collection, quality improvement, follow-up, and funding (9). With the inclusion of secondary targets, the ACMG broadened the original Wilson and Jungner criteria definition of benefit to include not only direct benefit for the individual being screened in the form of a specific treatment but also benefit to the individual screened, the family, and society derived from identification and management of disorders for which a specific treatment may not exist (9, 29, 30).

In 2008, the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC), established by the Department of Health and Human Services (DHHS) in 2003, was given the responsibility to complete regular evidence-based systematic review of and recommendations for conditions to be included on the Recommended Universal Newborn Screening Panel (RUSP), which is based on the 2006 ACMG expert report outlining principles and processes for uniform NBS as well as the original Wilson and Jungner criteria (8, 9, 29, 31). Newborn screening in all states is mandatory, with limited ability for parents to opt out of testing (32). The current core and secondary targets can be found on the ACHDNC website at Recommended Uniform Screening Panel | Official web site of the U.S. Health Resources & Services Administration (hrsa.gov) (33).

Point-of-Care NBS

Additional methods for NBS fall outside the DBS paradigm for testing. The first point-of-care NBS began with neonatal early hearing detection and intervention screening in Hawaii in 1990 and is now part of the RUSP (34). Identification of critical congenital heart disease by pulse oximetry screening, another point-of-care NBS test, was added in 2011 (35).

Primary DNA NBS

Newborn screening for severe combined immunodeficiency (SCID) began in the United States in 2008, was added to the RUSP in 2010, and ushered in a new era of primary DNA screening (36). DNA extracted from DBS had been used for second-tier testing for sickle cell disease (SCD) utilizing PCR and allele-specific oligonucleotide hybridization, as SCD is due to a single specific beta-globin gene point mutation (12). NBS for SCID utilizes DNA extracted from DBS as the primary screen; quantitative PCR is used to assess DNA copy number (10, 15, 36). Severe combined immunodeficiency is characterized by decreased amounts of T- and/or B-cells and leads to the inability of the body to effectively fight infections (36). Without appropriate treatment, such as hematopoietic stem cell transplant or enzyme replacement, death due to overwhelming infections inevitably occurs. Severe combined immunodeficiency can be effectively screened for with the identification of decreased T-cell receptor excision circles (TRECs), which are small pieces of leftover DNA formed through DNA recombination in the process of T-cell maturation (36). Low numbers of TRECs identified on NBS indicate inadequate T-cells and trigger secondary testing with flow cytometry to identify the specific deficiency, as NBS for SCID also identifies other non-SCID T-cell deficiencies (36).

A more recent example of NBS utilizing DNA extracted from DBS as a primary target is *SMN1*-related spinal muscular atrophy (SMA), which was added to the RUSP in 2018 (31, 37). *SMN1*-related SMA is a progressive disorder characterized by muscle weakness due to loss of anterior horn cells and has a broad phenotypic spectrum ranging from a severe neonatal presentation with weakness, hypotonia, respiratory failure, and death in early infancy to an adult-onset presentation of muscle weakness without respiratory insufficiency and with a normal lifespan (31). Treatment for *SMN1*-related SMA has recently become available and slows or prevents the progression of the disorder once therapy is initiated, thus making it an ideal candidate for NBS (31). Screening identifies a common gene deletion, and as for SCID, the primary screen is a quantitative

PCR technique; combining SCID and SMA in one newborn screen assay has been proposed (37). For both of these conditions, DNA is used for the primary screen but neither employs DNA sequencing. However, as a result of the introduction of SCID and SMA newborn screening, NBS programs now routinely extract DNA from DBS, allowing the introduction of other DNA-based assays, such as DNA sequencing. This and improvements that allow high-throughput DNA sequencing with a rapid turnaround time have led to an increased interest in the use of DNA sequencing as a primary NBS test.

WHOLE EXOME AND WHOLE GENOME SEQUENCING IN DISEASE DIAGNOSIS

The production of the map of the human genome promised a new era in medicine, one in which genetic and genomic information would be used routinely in health care (38). With an available map, advanced DNA sequencing techniques that are high throughput and low cost offered the promise of the personalized genome, that is, sequencing of an individual's genome, in order to provide a personal health benefit (39). In the two decades since the completion of the human genome, the utility of DNA sequencing for health benefit has been demonstrated multiple times and is now routine in some areas of medicine. The first use of individual genome information for disease diagnosis occurred in 2009 at the University of Wisconsin for a child with severe inflammatory bowel disease. Sequencing of his entire genome and DNA variant analysis yielded an answer, immunodeficiency due to a pathogenic variant in the *XIAP* gene (40). This test costs $75,000 and required 4 months of analysis (41). It resulted in life-saving treatment; based on this genetic diagnosis, the child was successfully treated for his debilitating disease with a cord blood transplant. Additional confirmation of the power of next-generation sequencing (NGS) technology came from a proof-of-principle paper in which exome sequencing identified the correct genetic diagnosis for four individuals with the rare disorder Freeman–Sheldon syndrome (42). Shortly thereafter, next-generation sequencing and DNA variant analysis of four individuals with Miller syndrome, which lacked a causative disease gene at that time, resulted in the identification of that gene, *DHODH* (43). Exome sequencing remains a method for disease diagnosis and for disease gene discovery (44, 45).

WHOLE EXOME AND WHOLE GENOME SEQUENCING IN DISEASE SCREENING

Newborn Sequencing in Genomic Medicine and Public Health

The power of exome sequencing and computational analysis to identify new disease genes and diagnose individuals with rare disorders suggested other uses for this technology including pre-conception carrier testing and NBS (46). The ability to extract DNA from dried blood spots (11) and the development of high-throughput technologies for DNA sequencing have led to the use of next-generation sequencing in NBS in follow-up testing after an abnormal enzyme or analyte primary screen and have held out the promise of the use of this as a primary screen, especially for those early-onset treatable disorders that lack a current NBS test (11, 47–52). Recognizing the need for research in the area of DNA sequencing and newborn health, the NIH funded the Newborn Sequencing in Genomic Medicine and Public Health (NSIGHT) network in the early 2010's (53). The three key research questions to be addressed were as follows: (1) For disorders currently screened in newborns, how can genomic sequencing replicate or augment known newborn screening results? Can sequencing replace current modalities? (2) What knowledge could genomic sequencing provide about conditions not currently screened for in newborns? (3) What additional clinical information could be learned from genomic sequencing relevant to the clinical care of newborns (53)? The four funded projects addressed these questions through separate study designs and patient populations (53). Two projects included cases with abnormal newborn screen results. In the North Carolina Newborn Exome Sequencing for Universal Screening (NC NEXUS) project, healthy newborns (61 subjects) and infants and children <5 years of age with known abnormal newborn screening results (17 subjects) or with hearing loss (28 subjects) were enrolled for exome sequencing. Analysis was blinded to phenotype, and analyzed genes included those with childhood-onset medically actionable disorders (NBS-NGS, 466 genes, all subjects) and those with diagnostic findings (affected subjects, additional indication-based genes analyzed). This project included randomization to an arm in which parents could decide whether to learn additional information from the genomic analysis including low or no actionability childhood-onset conditions, high actionability adult-onset conditions, and carrier status for recessive disorders (54). The NBSeq project performed exome sequencing retrospectively on DNA obtained from dried blood spots of cases with known IEM diagnosed through conventional newborn screening in California. These were de-identified samples and included cases with false positive newborn screens. The role of exome sequencing as a primary or secondary test for IEM was assessed (55). The two additional NSIGHT projects addressed the utility of WGS in sick newborns and the role of WES in sick and healthy newborns (BabySeq) and did not have a primary newborn screening aim (53).

The NC NEXUS project found that NBS-NGS was 88% sensitive for cases with an abnormal newborn screen for an IEM and 18% sensitive for the hearing loss cohort (54). Four individuals had abnormal NBS-NGS results not identified by other methods, including female heterozygote status for OTC deficiency in a child with PKU and heterozygous status for a known pathogenic variant in *LDLR* causing autosomal dominant familial hypercholesterolemia in a child with a known family history of this. Two children in the hearing loss cohort had additional clinically relevant findings, one with a variant in *DSC2* causing autosomal dominant arrhythmogenic right ventricular dysplasia and one with two variants in the gene associated with factor XI deficiency (54). These findings addressed the second aim of the NSIGHT consortium.

The NBSeq project found that DNA could not substitute as a primary newborn screen for disorders currently screened for by analyte, due to insufficient sensitivity (88%) and specificity (94%), but that DNA testing could be beneficial in follow-up

testing in reducing follow-up of false positive results and in identifying disease diagnosis, which address the first aim of the NSIGHT consortium and demonstrate that DNA sequencing cannot substitute for current conventional screening but can augment it (55).

In the BabySeq project, which did not have a primary NBS component, three cases were found through WES to have newborn screening-related disorders missed on conventional screening. They were partial biotinidase deficiency (not associated with symptoms in most cases), non-classical congenital adrenal hyperplasia, and post-lingual *KCNQ4* hearing loss (56). These findings addressed the third aim of the NSIGHT consortium, by demonstrating that non-classical forms of some disorders can be identified, though there is no current information regarding sensitivity for these.

The NSIGHT projects have demonstrated the current capabilities and the gaps to be addressed to improve the performance of NGS in NBS. Importantly, despite technological advances, the Ethics and Policy Board of NSIGHT recommended against genomic sequencing of all babies at birth and called for a nuanced use of genomic technologies, taking into account the contexts of screening vs. diagnosis and the contexts of clinical care, public health, and direct to consumer testing (57).

Practical Aspects of Genomic Testing for NBS

A crucial issue in genomic medicine is the large number of DNA variants in each individual. Depending on the context, narrowing the number of genes to be evaluated can be critical for improving test interpretation. Therefore, targeted testing has been proposed in carrier screening and NBS. This can also affect coverage of the individual genes assessed. In 2015, Naylor and colleagues reported the performance of two targeted next-generation sequencing (TNGS) platforms in two clinical contexts. They used targeted DNA sequencing either through WES with an *in silico* gene filter for 126 genes or through a next-generation sequencing panel, NBDx, which included a DNA capture step for exons of the 126-NBDx gene panel. These TNGS platforms were used both after an abnormal newborn screen and for ill infants in the neonatal intensive care unit (NICU). The authors compared these two platforms in the retrospective evaluation of 36 individuals with known IEM from the Amish and Mennonite communities. While the NBDx gene panel had advantages over WES, an important result from this study in a homogeneous population is that only 27 of 36 disorders were correctly identified in the experimental cohort in the absence of clinical information (50). This paper not only demonstrated the technical feasibility of obtaining DNA for NGS from dried blood spots and of developing a rapid and cost-effective test applicable to newborns with abnormal NBS and ill infants in the NICU, but also highlighted the limitation of DNA sequence information alone for disease diagnosis and the importance of clinical information to aid DNA variant interpretation. The authors suggest the possible use of NGS as a primary NBS in the future.

In 2017, the utility of NBS by WGS, rather than WES, was shown taking advantage of two DNA sequencing studies in newborns in the Inova Health System in Virginia (58). This study used two different WGS platforms on which 163 genes were analyzed. In contrast to the WES retrospective proof-of-principle study in a small number of affected individuals from the Amish and Mennonite communities, this study was conducted in an ancestrally diverse population of almost 1,700 newborns. Importantly, and unrealistically for a NBS public health program, parents were also sequenced, allowing phasing of variants for autosomal recessive disorders. Variants were classified using the ACMG criteria of pathogenic, likely pathogenic, benign, likely benign, and variant of uncertain significance (59). Only pathogenic and likely pathogenic variants were called. Importantly, exon coverage differed by gene and sequencing technology. While WGS did identify two cases missed on Virginia state NBS (hemoglobin SC disease and Duarte variant galactosemia, the latter a non-disease), the conclusion of this paper was that conventional NBS could not be replaced by WGS as NBS identified 4/5 affected neonates in the cohort and WGS identified only 2/5. This team suggested periodically reassessing NBS and WGS and using a larger cohort in later studies. The benefits of WGS in NBS were lower false positives than conventional NBS, resolution of inconclusive NBS results, distinguishing the correct disorder in cases of ambiguity with NBS results, and decreased numbers of follow-up samples required for preterm infants.

Next-generation DNA sequencing has been assessed as a primary newborn screen in Korea, where the use of a 307-gene panel for 159 disorders, including 60 neonatal IEM, was assessed. The study also addressed turnaround time (TAT), cost, and variant interpretation. They sequenced 103 subjects, 81 affected individuals and 22 controls. Remarkably, for the affected individuals, only 12% of causal variants were annotated in databases. Crucially, for the 307 genes, each subject had 8.6 variants, for 3.4 diseases; thus, manual curation and clinical information were required for each subject. Eighty-eight percent of the variants were non-disease causing (60). Variant interpretation was identified as a critical limiting factor in the use of TNGS as a primary screen in this work.

The UK is investing significantly in genomic sequencing, and the role of NGS in NBS in their National Health Service has been reported (52). They demonstrated that screening for five genes (*ACADM, PAH, TSHR, CFTR*, and *HBB*) in the National Health Service in the UK is feasible. The genes chosen for the study corresponded to disorders already on the NBS panel, specifically MCAD deficiency, PKU, congenital hypothyroidism, and sickle cell disease, respectively. They achieved a TAT of 4 days and could process 1,000 samples per week (a typical NBS lab processes 50,000 samples per year) but suggested that cost might be prohibitive as the cost of the current NBS test is 25 pounds, and the NGS test would cost 62 pounds or more. A consideration of adding diseases based on merit and not technologies was also recommended.

These papers and those of the NSIGHT network, discussed here and outlined in **Table 2**, demonstrate that while technically feasible, perhaps even with sufficient TAT to be appropriate for

TABLE 2 | Summary of studies on next-generation sequencing for newborn screening.

References	Study type	Platform	Number of genes assessed	Samples	Study population	Sensitivity	Specificity
Bhattacharjee et al. (50)	Retrospective	NGS gene panel – NBDx; and WES	126	36 subjects with known IEM – proband only	Amish and Mennonite	75% without clinical information; 94% with clinical information	Not addressed
Bodian et al. (58)	Retrospective	WGS – Illumina or complete genomics	163	1,696 neonates – trios	Family trios enrolled at Inova Fairfax Hospital	88.6% concordance of NBS and WGS	Not addressed – for recessive disorders 2.9% with uncertain WGS results compared to 0.013% for NBS
Cho et al. (60)	Retrospective	WES	307 total, 65 related to NBS	103 patients 81 known patients 10 carriers 12 negative controls	Patients at Yonsei Severance Hospital, Republic of Korea	92.5% – with clinical information	Not addressed
van Campen et al. (52)	Proof of principle to address feasibility including cost and TAT	NGS gene panel – NBS2	5	Healthy adults	Adults in the UK	Analytic sensitivity 100%, disease samples not assessed	Analytic specificity 99.96%, disease samples not assessed
Roman et al. (54)	Prospective for the healthy cohort; retrospective for the affected cohorts	WES	466	106 newborns	61 healthy 17 with an IEM 28 with hearing loss	88% for IEM	Not addressed
Adhikari et al. (55)	Retrospective	WES	78	1,012 individuals in the test set: 674 affected with an IEM and 338 unaffected and false positive on MS/MS NBS	IEM-affected individuals from a birth cohort of 4.5 million newborns over 8.5 years in California	88–93.7% after clinical review of cases	98.4%

NBS, the high cost and limitations of variant detection (intronic variants, regulatory regions, copy number variants, structural variants, and trinucleotide repeats), variant interpretation and of annotated disease databases remain a significant barrier to the use of DNA as a primary screen. The contrast between the success of DNA in diagnosis and the difficulties in the use of DNA in screening highlights that the lack of a phenotype to guide variant interpretation remains the chief limitation of WES and WGS in NBS. In the next sections, we will examine technical aspects of NGS interpretation, especially as it relates to NBS.

CURRENT TECHNOLOGIES TO DETECT GENETIC VARIANTS

To effectively identify disease from DNA, the relevant disease-causing genetic variants should be both technologically detectable and clinically interpretable (**Figure 2**, **Table 3**). Historically, genetic tests have been limited in scope to a single or few genes associated with specific genetic conditions and performed using conventional technologies like Sanger sequencing of PCR-amplified coding regions of the gene(s) of interest. In recent years, high-throughput (next-generation) sequencing has greatly expanded the scope of genetic loci that can be technologically assayed in a single individual's genome. In particular, as noted in the previous section, WES is now a commonly used tool in diagnosis of various rare genetic disorders (61–66). However, challenges remain in applying WES or WGS to NBS, including the fact that capture and read coverage may be non-uniform and some disease-causing variants may be missed due to poor coverage. Recent simulations indicate that read mapping and variant calling in some NBS genes could be affected by homologous genomic regions (*CYP21A2*, *SMN1*, *CBS*, and *CORO1A*) (67). Whole genome sequencing expands the scope of detectable variants beyond coding regions. Besides revealing the ∼98% of the non-coding genomic regions not visible to WES, WGS can provide a better view of the coding regions as well as more uniform coverage. Indeed, in some IEM, WGS revealed large deletions in IEM genes not observed by WES alone (55).

Still, there are regions of the genome that are difficult to sequence using both conventional WES and WGS that rely on short-read sequencing (e.g., large insertions and

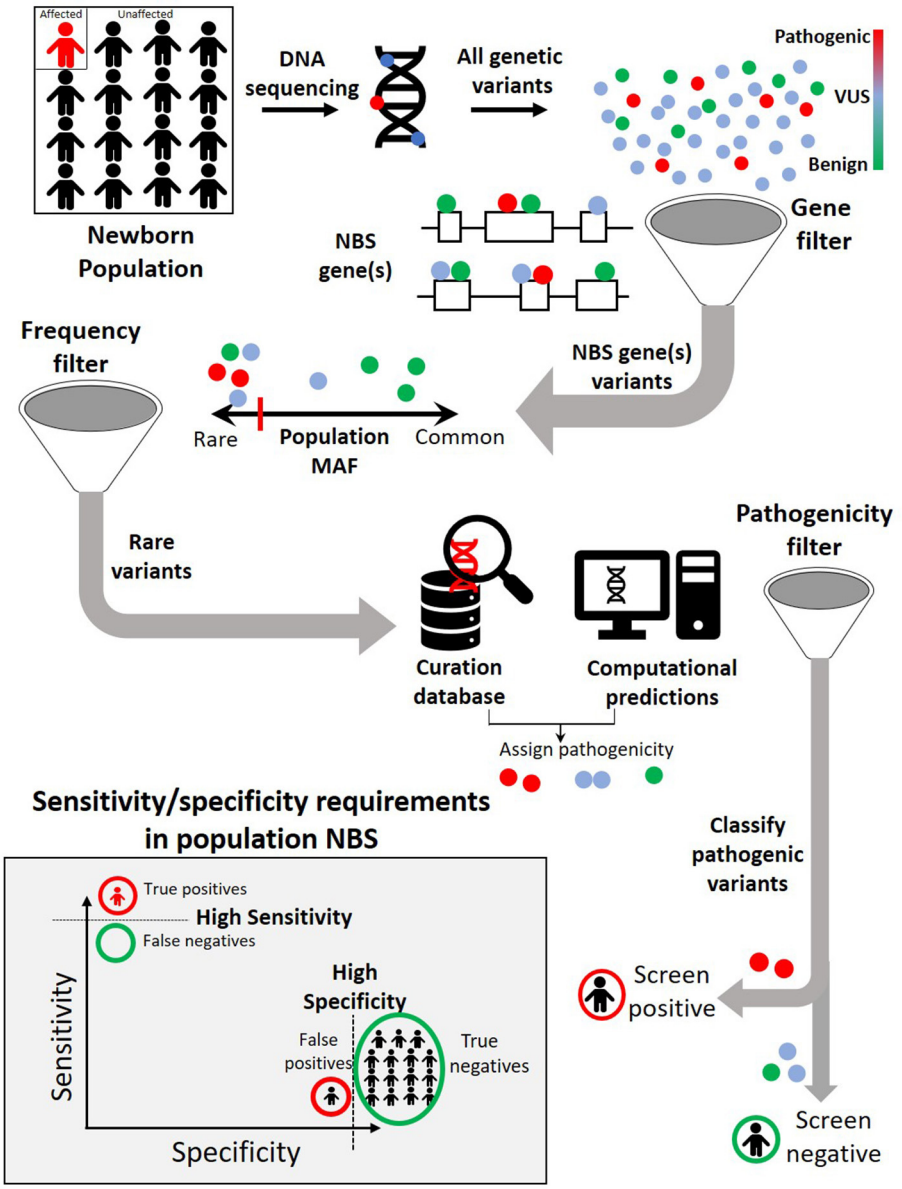

FIGURE 2 | A typical genomic analysis pipeline in the context of newborn screening (NBS). Among all the variants observed in the newborn DNA sequence, only those occurring in previously identified NBS genes are considered further. Within NBS genes, rare variants are prioritized over common variants. A combination of curated pathogenic variant databases and computational prediction tools is utilized to assign variant pathogenicity and screen for individuals who carry such variants. For NBS, the pipeline will need to demonstrate both a high sensitivity (screen positive almost all newborns with disease) and a high specificity (screen negative almost all newborns without disease).

deletions, tandem-repeat expansion, and complex chromosomal structural aberrations). Recent advances in long-read sequencing technologies could reveal such classes of variations to expand the repertoire for rare disease diagnosis. Even though such technologies are costly and data analysis methods are in early stages, there have been promising recent studies that have leveraged long-read sequencing to pathogenic variants in rare disease not detectable by conventional WES/WGS (68).

VARIANT INTERPRETATION AND CURRENT CHALLENGES

Assuming the causative genetic variants are confidently detected, another major challenge for integrating genomics in NBS will be the clinical interpretation of genetic variants. To implicate pathogenic variants, the field leverages both curation of expert knowledge as well as prediction from computational tools.

TABLE 3 | Current primary technologies for detecting human genetic variants.

	Scope	Advantages	Limitations
Targeted gene panel	Captures variants within a few target genes (10s to 100s of genes)	- Disease-specific focus - High degree of customizability - Low cost and turnaround time	- List of all genes relevant to a disease needs to be explicitly defined beforehand - Needs to be updated as new disease genes are discovered
Whole exome sequencing	Captures variants within all exonic regions from the entire genome (~20,000 genes)	- Designed to capture all coding variants - Ideal for novel gene discovery in idiopathic conditions	- Misses non-coding and structural variants - Coverage and data quality can vary across genes
Whole genome sequencing	Captures variants from the entire genome	- More uniform coverage - Reveals non-coding and structural variants	- Lack of reliable tools to interpret non-coding variants - High cost, turnaround time and data storage requirements

Over the last few years, extensive clinical guidelines for variant interpretation have been developed, converging to ordinal five-tier "pathogenic" to "benign" labels for genetic variants (59). However, these guidelines currently do not accommodate the possibility that the same variant may need to be considered differently for specific diseases under different clinical contexts. In the context of NBS, similar to global collaborative efforts for harmonization of cutoff values in MS/MS data, collaborative efforts are needed to standardize and share variant classification and evidence across laboratories (69). In a recent study evaluating concordance of variant classification across nine laboratories (eight CLIA-accredited and one research laboratory), 54% of variants reached complete concordance, whereas 11% had a discordance that could affect clinical recommendation (70). After subsequent review of the discordant variants, the concordance increased to 84%. Even after review and data sharing, a significant proportion of existing variants remains of uncertain significance, labeled VUS, where additional evidence in the form of functional or computational studies are needed to resolve pathogenicity.

Several computational tools have been developed to interpret human genetic variants and predict their consequence on clinical phenotypes. Most of these tools aim to distinguish disease-causing pathogenic variants from those that are benign based on various existing information. Most tools predominantly leverage evolutionary conservation information, while others additionally incorporate physiochemical properties and protein structural data (71–74). Besides directly predicting clinical pathogenicity, specialized tools also exist for predicting various intermediate biochemical phenotypes including protein stability, RNA splicing, subcellular protein localization, protein interactions, and mechanism of action (75–79). Recently, meta-predictors have also emerged, which combine scores from several prediction tools using machine learning methods and report an integrated pathogenicity score (80, 81).

While tools can leverage the information about the knowledge of gene and protein function for coding variants, computational predictions of pathogenicity for noncoding variants can be even more challenging. Several tools have been developed to annotate non-coding regulatory regions distal (enhancers) or local (in promoters, untranslated regions or introns) to genes based on methods that integrate information from histone marks, transcription binding, gene expression, phylogenetic analyses, and chromatin accessibility (82–85). Other tools have emerged in this area that leverage machine learning techniques to integrate such annotations into pathogenicity scores (86–89). Besides single-nucleotide changes, several computational tools have also emerged to detect pathogenic structural variants from genomic data (90–92).

Despite over a hundred computational tools currently available for variant interpretation [recent catalog in ref (93)], challenges remain. Most computational tools train their algorithms in a supervised fashion across large databases of previously characterized human disease-associated and neutral variants. These tools typically use similar underlying training datasets, model features, and design principles, which leads to confounding issues of circularity and overfitting (94). Most tools assume that the properties that determine a variant's deleteriousness are generalizable across all genes. Yet, to make more accurate predictions of variant impact in a particular gene of interest, computational tools of the future may need to incorporate properties that capture the biological context specific to that gene. A recent work focused on computational tools specialized in predictions for a gene or gene family of interest has found that incorporating the context of an individual gene, biological pathway, and disease can improve quality of predictions (95, 96). The main challenge for gene-specific computational approaches, however, is the lack of sizable variant datasets for training on individual genes, particularly for rare diseases. A potential solution is emerging in the form of high-throughput functional assays that measure the variety of molecular and cellular consequences of all possible variants, in particular disease-relevant loci (97–99).

Another limitation of current variant interpretation tools is that they ignore the diplotypic context of an individual's genetic variant during both training and inference. The impact of a single pathogenic variant on the eventual clinical phenotypes is often modulated by other variants in the individual's genome, sometimes even within the same gene (100, 101). For example, in recessive disorders, the combination of pathogenic variants in both the paternal and maternal copies of a gene determines the clinical phenotypes and disease severity. To improve clinical utility of DNA analysis pipelines, the next

generation of variant interpretation tools will need to incorporate the full diplotypic context in an individual's genome to predict both the likelihood as well as severity of diseases. For now, pathogenicity assertions about genetic variants using computational tools alone are not sufficient, and human review is still required for proper variant interpretation and return of genetic results.

SENSITIVITY/SPECIFICITY TRADEOFFS IN DIAGNOSIS VS. SCREENING

Even though most genomic analysis pipelines for rare disease use similar sets of parameters, the optimal design and thresholds depend on the clinical context and application (102). Unlike diagnostic settings where DNA analysis is guided by phenotypic data to identify genetic variants that could explain an individual's clinical features, NBS is performed on asymptomatic newborns with no *a priori* phenotypes. The trade-offs of sensitivity and specificity can be different in these two contexts. Typically, diagnostic DNA pipelines report true positive rates (sensitivity) of around 25–60%, but the true negative rates (specificity) are often not reported. For population-scale NBS, both the sensitivity and specificity requirements are much stricter. Because most of the population will be unaffected in rare diseases typically screened for in the newborn period, even a 1% false positive rate for a screening test can translate to a large burden of false positive cases that require follow-up. Therefore, systematic exploration of parameters in analysis pipelines is necessary to achieve a balance between sensitivity and specificity that is best suited for screening (103).

As noted above, the NBSeq project, a retrospective study evaluating WES as a primary NBS test in an 8.5-year population-scale cohort for 48 IEM in California, achieved 88% overall sensitivity with a specificity of 98.4% (55). In comparison, current MS/MS analyte-based screening has a sensitivity and specificity of 99.0 and 99.8%, respectively, in the same cohort (69, 104–106). WES was therefore concluded to be insufficiently sensitive or specific as a general primary NBS test for IEM. The NC NEXUS study was performed in a smaller cohort and found similar results (54).

The appeal of genomic sequencing as a promising single test for all genetic diseases in the future should be reconciled with the possibility that the analytical performance of sequencing as a screening test may vary widely across different individual disorders. Such differences could arise from a range of factors, including limited prior genetic and clinical data, particularly in very rare conditions, as well as incomplete biological characterization of some genetic diseases. When grouped by prevalence, indeed the most common IEM (>2.5 per 10,000) had higher WES sensitivity of 91% and 78%, respectively, compared to the rarest (<0.04 per 10,000) (55).

An additional complexity arises from the fact that current databases of disease-associated genetic variants are largely Eurocentric. The larger proportion of previously uncharacterized variants in underrepresented populations could result in poorer performance of a sequencing-based screening test in such populations. Indeed, previous studies have demonstrated that variant misclassification in understudied populations can lead to genetic misdiagnoses with potential for exacerbating health disparities (107).

Besides analytical performance, bioinformatics analysis screening poses implementation challenges that may differ from a diagnostic scenario. Whereas, genomic analysis in diagnostic settings typically involves *ad hoc* rule-based pipelines with expert review of individual cases along with additional genomic data from other family members, NBS has to be performed at a population-level scale, requiring the analysis pipelines to be largely automated and streamlined.

ETHICAL AND SOCIETAL CONSIDERATIONS

Finally, even if all of the technical issues surrounding the application of next-generation sequencing to NBS are solved, social and ethical barriers to universal implementation will remain. Based largely on the ACMG criteria, many (perhaps most) genetic disorders would not qualify as candidates for NBS since early detection will not lead to specific therapy that will change the course of the disease and thus provide a broad societal benefit. This limitation, of course, does not address the potential non-specific benefits of early identification such as implementation of early intervention programs, appropriate prospective disease monitoring, and even family planning, as well as circumventing the prolonged diagnostic odyssey that individuals with rare diseases often face. The cost, not only of screening but also of follow-up, for affected individuals by government-funded programs must also be considered. In essence, WES/WGS screening would mandate a transfer of considerable health care resources from later-onset diagnostic expenses to neonatal screening and follow-up efforts. Additional ethical concerns also abound, most prominently the loss of the right to self-determination for the neonate identified with a later onset of disease.

CONCLUSIONS

The evolution of NBS from testing one disease at a time to potentially identifying all possible diseases brings both great promise and challenges to modern health care systems. It is possible, perhaps even likely, that the overall cost to society will be reduced as NGS becomes more economical. However, the decision to adopt such a program must address a much more expansive set of issues that are likely to limit implementation in the near future, including identification of late-onset diseases for which no immediate therapy is available, improvement of turnaround time, and development of larger and multi-ethnic data sets of curated pathogenic variants that allow movement beyond variants of unknown significance. Societal concerns of genetic discrimination, especially as it relates to health insurance, must also be resolved. Rather, the use of NGS technology is more likely to continue to find its optimum use in diagnostic and follow-up settings, though moving to a primary role rather

than as a tool of last resort as is now common. Instead, NGS panels and/or analysis focused on disorders with a significant neonatal, or even broader pediatric, footprint may play a role as a bridge to true WES/WGS NBS. Regardless, one of the key goals of traditional newborn screening, better care at lower cost, will ultimately be realized, translating next-generation sequencing to next-generation care.

AUTHOR CONTRIBUTIONS

AW, RG, JV, and AA contributed to conception and design of the article and wrote sections of the manuscript. AW compiled **Table 1**. RG compiled **Table 2**. JV designed **Figure 1**. AA designed **Figure 2**. All authors contributed to manuscript revision, read, and approved the submitted version.

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CASE REPORT
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Two Novel *HSD17B4* Heterozygous Mutations in Association With D-Bifunctional Protein Deficiency: A Case Report and Literature Review

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Background: D-Bifunctional protein deficiency (D-BPD) is an autosomal recessive disorder caused by peroxisomal β-oxidation defects. According to the different activities of 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase protein units, D-bifunctional protein defects can be divided into four types. The typical symptoms include hypotonia and seizures. The gene that encodes D-BP was *HSD17B4*, which is located in chromosome 5q23.1.

Case Presentation: We report the first case of D-BPD in a Chinese patient with neonatal onset. Cosmetic malformations, severe hypotonia and seizures are prominent. The blood bile acid profile showed increased taurocholic acid, glycocholic acid, and taurochenodeoxycholic acid. Very-long-chain fatty acids (VLCFAs) revealed significant increases in hexacosanoic acid (C26:0), tetracosanoic acid/docosanoic acid (C24:0/C22:0), and hexacosanoic acid/docosanoic acid (C26:0/C22:0). Cranial MRI revealed bilateral hemispheric and callosal dysplasia, with schizencephaly in the right hemisphere. EEG showed loss of sleep–wake cycle and epileptiform discharge. Other examinations include abnormal brainstem auditory evoked potentials (BAEPs) and temporal pigmented spots on the optic disc in the right eye. After analysis by whole-exome sequencing, heterozygous c.972+1G>T in the paternal allele and c.727T>A (p.W243R) in the maternal allele were discovered. He was treated with respiratory support, formula nasogastric feeding, and antiepileptic therapy during hospitalization and died at home due to food refusal and respiratory failure at the age of 5 months.

Conclusions: Whole-exome sequencing should be performed in time to confirm the diagnosis when the newborn presents hypotonia, seizures, and associated cosmetic malformations. There is still a lack of effective radical treatment. Supportive care is the main treatment, aiming at controlling symptoms of central nervous system like seizures and improving nutrition and growth. The disease has a poor outcome, and infants often die of respiratory failure within 2 years of age. In addition, heterozygous deletion variant c.972+1G>T and missense mutations c.727T>A (p.W243R) are newly discovered pathogenic variants that deserve further study.

Keywords: D-bifunctional protein deficiency, neonatal seizures, *HSD17B4*, peroxisomal disease, hypotonia

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INTRODUCTION

Peroxisomal diseases are divided into two categories: peroxisome biogenesis diseases and single peroxisomal enzyme/transporter defects. D-Bifunctional protein deficiency (D-BPD) (OMIM261515) is an autosomal recessive disorder caused by peroxisomal β-oxidation defects (1–3). The prevalence of peroxisomal defects has been roughly estimated at 1:30,000 and of D-BPD at 1:100,000 (4). The first D-BPD patient was reported in 1989 by Watkins et al. (5), and it was found that the true defect in this patient is the level of the D-BP but not the level of the L-BP in 1999 (6), since D-BP was discovered in 1996 (7). D-Bifunctional protein (D-BP) is a steroid metabolizing enzyme situated only in mammalian peroxisomes and is widely distributed in various organs throughout the body. D-BP contains three functional units: a 2-enoyl-CoA hydratase unit, a 3-hydroxyacyl-CoA dehydrogenase unit, and a sterol carrier protein 2 unit. The three functional units of D-BP are essential for the decomposition of very-long-chain fatty acids (VLCFAs), α-methyl branched-chain fatty acids, and bile acid intermediates such as dihydroxycholanic acid (DHCA) and trihydroxycholanic acid (THCA) (7–11). D-BP participates in peroxisomal β-oxidation reactions, specifically catalyzing the second (dehydration) and third (dehydrogenation) reactions of the peroxisomal β-oxidation of D-3-hydroxyacyl-CoA.

D-BPD has been classified into three types: type I, deficiency of 2-enoyl-CoA hydratase unit, and 3-hydroxyacyl-CoA dehydrogenase unit; type II, isolated hydratase deficiency; and type III, isolated dehydrogenase deficiency (2). The three profiles had similar clinical characteristics but different severities. The Kaplan–Meier survival analysis shows that type I deficient patients had the most severe symptoms, with 6.9 months as a mean age of death, while type II deficient patients and type III deficient patients had longer mean age of death, which was 10.7 and 17.6 months, respectively. And type I deficient patients would die within the first 14 months of life and had a poorer prognosis than patients with type II or III D-BPD (4). A type IV phenotype has been proposed based on the presence of missense mutations in each enzyme domain, and this mutation results in significantly reduced but detectable hydratase and dehydratase activities of D-BP, termed juvenile-type D-BPD (12). Absence of one or both of these enzymes (hydratase and dehydrogenase) invariably leads to impaired catabolism of VLCFA, DHCA, THCA, and pristanic acid. So accumulation of VLCFA, DHCA, and THCA is a prominent manifestation of D-BPD and can be confirmed by functional analysis and mutational analysis of enzyme activity in patient cells, usually skin fibroblasts (4).

D-BPD may develop in neonates, adolescents, or adults, but the onset of symptoms usually occurs in the neonatal period. Hypotonia (98%) and seizures (93%) usually occur during the first month of life, and patients usually die within 2 years after birth (4).

The gene that encodes D-BP was *HSD17B4* (13), which is located in chromosome 5q23.1 and was found to be more than 100 kbp in length. The gene consists of 24 exons and 23 introns. Homozygous or compound heterozygous mutations in *HSD17B4* gene cause D-BPD. In addition, *HSD17B4* is also one of the genes responsible for Perrault syndrome (PRLTS), manifesting with sensorineural hearing loss in both sexes, primary ovarian insufficiency in females, and neurological feature. Chen et al. (14) gave a report of a PRLTS family in China and found an *HSD17B4* mutation c298G>T (p.A100S) to confirm the relationship. Here, we report the first case of a Chinese neonatal-onset D-BPD patient with novel compound heterozygous mutations of *HSD17B4* (OMIM601860), including a splicing mutation and a missense mutation, detected by exome sequencing. And we have also summarized the clinical and genetic characteristics of the patient.

FIGURE 1 | The patient with severe hypotonia and facial dysmorphism.

CASE PRESENTATION

A 1-day-old male proband was hospitalized in the Department of Neonatology, Yuying Children's Hospital Affiliated to Wenzhou Medical University, in August 2020 due to "shortness of breath and hypotonia for 1 day, convulsions for 8 h." The child was G3P2, born at the 39 weeks of gestation, singleton, by cesarean section due to "decreased fetal movement." There were no placental, umbilical cord, or amniotic fluid abnormalities, and the Apgar scores were all 8 at 1, 5, and 10 min (−1 each for respiration and muscle tone). The patient's birth weight was 2,900 g (25th percentile), and length was 50 cm (25th−50th percentile). The patient has non-consanguineous parents, a healthy 11-year-old sister, and no history of familial genetic diseases. Convulsions and hypotonia (**Figure 1**) were found on the first day of life. Convulsions were characterized by fist clenching, eye gazing, and cyanosis of lips, which lasted for 10 s and resolved spontaneously. The infant was conscious during the interictal period but had poor responses including no spontaneous activity, no eyes pursuit, or normal sucking and swallowing. On examination, the infant was found to have craniofacial deformities, which showed a long head deformity (158 mm), high forehead, wide eye distance, and high arch of the palate, in addition to varus of both feet and left cryptorchidism. Basic reflexes (swallowing, sucking, and cough) were also depressed.

After admission, the patient was given oxygen inhalation with hood, formula nasogastric feeding, phenobarbital injection

for stopping convulsion, intravenous fluid support, and other treatments. On the 1st day after admission, the patient had a convulsion, manifested as described before. The frequency of convulsive seizures gradually increased, and the infant's reaction did not improve. On the 9th day after birth, apnea was caused by sputum blockage, and the patient was given tracheal intubation and mechanical ventilation support. On the 11th day after birth, the patient developed fever with elevated C-reactive protein (CRP), considering sepsis, and was given anti-infection treatment. At 14 days after birth, the infant had frequent convulsive seizures, and levetiracetam oral solution was administered and gradually increased, but convulsions could not be controlled. At 21 days after birth, a midazolam injection was maintained for 1 week, and the dose was gradually increased, but the convulsion still could not be controlled. At 22 days after birth, levetiracetam tablets were increased to 60 mg/kg/day. On day 28, the endotracheal tube was withdrawn and changed to hood oxygen support again. After 35 days of life, he was treated with sodium valproate oral solution. Oxygen was withdrawn at 40 days after birth. Antiepileptic therapy was adjusted during hospitalization with the assistance of a pediatric neurologist. At 42 days after birth, the infant still had convulsive seizures more than 10 times a day under antiepileptic treatment with levetiracetam, topiramate tablets, and sodium valproate oral solution. The patient was discharged with drugs after the family learned nasogastric feeding. At the age of 5 months, the child died at home due to food refusal and respiratory failure.

Laboratory Tests

Laboratory tests after birth showed no significant abnormalities in blood routine and CRP (transient increase due to infection), liver and kidney function, electrolytes, infectious disease screening, TORCH, coagulation function, blood glucose, blood ammonia, blood lactate, and blood gas analysis. The cerebrospinal fluid routine was normal with negative culture. Peripheral blood chromosome was 46XY. Blood lipids and cholesterol showed no abnormalities; blood tandem mass spectrometry showed no abnormalities in the measured amino acids and acylcarnitines. Urine tandem mass spectrometry reported increased methylcrotonylglycine. The blood bile acid profile showed that taurocholic acid 6.490 μmol/L (reference range $\leq$ 0.31 μmol/L), glycocholic acid 6.180 μmol/L (reference range $\leq$ 4.96 μmol/L), and taurochenodeoxycholic acid 2.09 μmol/L (reference range $\leq$ 0.8 μmol/L) were significantly increased, with the normal remaining bile acids and total bile acids. Peroxisome parameters, i.e., VLCFA, revealed significant increases in hexacosanoic acid (C26:0) 10.58 nmol/ml (normal range $\leq$ 1.30 nmol/ml), tetracosanoic acid/docosanoic acid (C24:0/C22:0) 2.07 (normal range $\leq$ 1.39), and hexacosanoic acid/docosanoic acid (C26:0/C22:0) 0.291 (normal range $\leq$ 0.023).

Imaging and Electroencephalogram Examination

The first cranial magnetic resonance imaging (MRI) examination showed no abnormalities at 7 days after birth. The second cranial MRI revealed bilateral hemispheric and callosal dysplasia at 31 days after birth, with schizencephaly in the right hemisphere. On the 2nd day after birth, the background activity of amplitude-integrated electroencephalogram (aEEG) was continuous normal voltage, but no sleep–wake cycle was observed. Examination of aEEG on the 3rd day after birth showed recovered sleep–wake cycle, but subclinical convulsive seizures were observed three times. On postnatal day 7, clinical convulsive seizures were observed on aEEG, and a single seizure showed sharp slow wave firing on the right fronto-central region and spreading to peripheral brain regions for 1–2 min. EEG at 21 days after birth showed abnormal background, and occasionally isolated asynchronous sharp waves predominantly bilateral on the fronto-central region, with the seizure type of peak dysrhythmia. The onset of convulsive seizures was more frequent than before, 2.75 times/h, with a single seizure time of 30 s to 1 min. Examination of aEEG and EEG at 1 month after birth showed no significant improvement. Other examinations discovered abnormal brainstem auditory evoked potentials (BAEPs) and temporal pigmented spots on the optic disc in the right eye. Cardiac ultrasound showed no abnormality; abdominal ultrasound revealed mild separation of the collecting systems of both kidneys; no denervated potential was observed in the muscles examined by electromyography (EMG), and the number of muscle units (MUs) decreased during re-contraction, showing a small amount—simple phase or simple phase.

Mutation Analysis of *HSD17B4* Gene

Genomic DNA samples were prepared from peripheral blood leukocytes of patients and subjected to whole-exome-generation sequencing analysis. Two heterozygous mutations were monitored in the subject on gene *HSD17B4* in 5q23.1. They are c.972+1G>T heterozygous mutation and c.727T>A heterozygous mutation (**Figure 2**). The splicing mutation c.972+1G>T is located in intron 12 and may lead to functional defects in hydratase unit; the non-synonymous single-nucleotide variant (SNV) mutation c.727T>A (p.W243R) is located in exon 10, resulting in changes in the short-chain dehydrogenase unit. The above two variants were not found in the Chinese population-specific database "Shenzhou Genome Database," human exon database (ExAC), reference population 1,000 Genomes (1000G), and population genome mutation frequency database (gnomAD).

Peripheral venous blood was collected from the proband's parents and sister for validation and source analysis by whole-exome sequencing. The results suggested that his father was a carrier of the c.972+1G>T mutation and his mother was a carrier of the c.727T>A (p.W243R) mutation (**Figure 2**). Thus, the patient contained a mixed heterozygous mutation in *HSD17B4*, including c.972+1G>T in the paternal allele and c.727T>A (p.W243R) in the maternal allele. According to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines and the application recommendations of ClinGen Sequence Variant Interpretation (SVI) expert group for the guidelines (15–17), it is suggested that these two variants are pathogenic variants. Combined with VLCFA, bile acid profile, and cranial MRI results, this variant was considered as an extremely pathogenic variant.

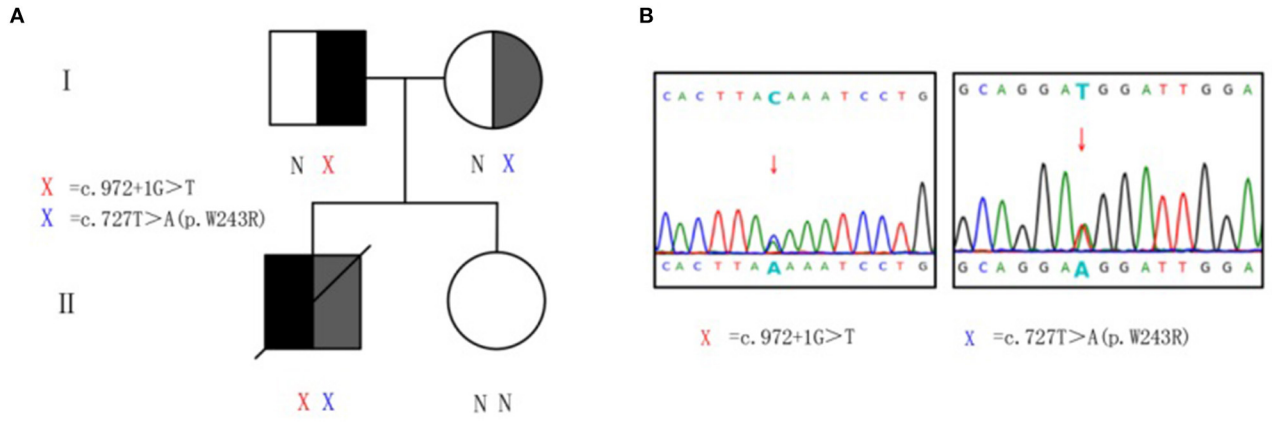

FIGURE 2 | Heterozygous mutations of *HSD17B4* in the family. **(A)** Pedigree of family with D-bifunctional protein (DBP) deficiency. X, variant; N, normal. **(B)** Sanger sequencing validation of *HSD17B4* variants identified by exome sequencing. For the mutated gene c.972+1G>T, the sequencing validation is reversed, and the complementary chain of G>T is C>A. Genomic DNA was amplified for sequencing with primers flanking intron 12 and exon 10. Arrow indicates heterozygous mutations. Red X, c.972+1G>T at chr5:119496647–119496647. Blue X, c.727T>A (p.W243R) at chr5:119492112–119492112.

DISCUSSION

In this case report, the patient showed typical clinical abnormalities including cosmetic deformities (long head deformity, high forehead, wide eye distance, high palatal arch, and talipes varus). It is consistent with the literature reports that most patients with early onset had craniofacial deformities (1, 18–20). The infant had severe neonatal hypotonia and convulsions, without primitive reflex elicited on the 1st day after birth, which was consistent with the literature reports that convulsive seizures occurred within a few days after birth, generally starting on the 2nd day after birth (1, 3, 18–21). After the patient's family signed the informed consent form for antiepileptic drugs, a combination of antiepileptic drugs, including levetiracetam tablets, topiramate tablets, and sodium valproate oral solution, was administered successively and at the maximum dose within the safety range, but the patient still had more frequent convulsive seizures of more than 10 times a day. It is consistent with most literatures reporting that the antiepileptic effect of this triple drugs still cannot control convulsive seizures (20, 22). In addition, McMillan et al. (12) and Khan et al. (22) reported the presence of retinitis pigmentosa in patients with D-BPD. We performed two fundus examinations on the patient, both of which revealed temporal pigment spots on the optic disc in the right eye, and may progress to retinitis pigmentosa, which needs further follow-up. This may be because long-chain polyunsaturated fatty acids are important substrates for DHA biosynthesis, and their β-oxidation requires D-BP involvement. Therefore, the lack of D-BP will indirectly lead to DHA deficiency, ultimately affecting brain and retinal development. Bae et al. (18) reported that although DHA supplementation in patients could increase DHA levels, it still could not improve clinical outcomes in patients with DHA deficiency. And patients showed progressive visual impairment and brain deterioration despite early DHA supplementation (within a month) (18). In addition, the disease often causes abnormal BAEP and even leads to deafness. Children with infantile onset have a higher mortality rate according to the above case presentations in the literature. In our case, we performed BAEP for two times in the patient, both of which suggested that the patient had binaural hearing impairment.

It has been reported in the literature that cranial MRI in adulthood revealed cerebellar atrophy and ataxia (23), while neonatal cranial MRI revealed no significant brain atrophy (24). Our patient's cranial MRI showed shallow sulci, local widening and deepening of the lateral fissure cistern of the right cerebral hemisphere, extensive hyperintense white matter changes in the cerebral hemisphere on T2WI, and dysplasia of the corpus callosum, which were similar to the clinical report that MRI in children with D-BDP showed different severities of lateral fissure, peripheral multiple microgyria, and delayed myelination (3, 18, 21).

The proband in this study was examined for plasma bile acid profile at 32 days after birth; and it was found that taurocholic acid, glycocholic acid, and taurochenodeoxycholic acid levels were significantly increased, which was consistent with the manifestations of bile acid metabolism disorders in D-BPD as reported in the literature (25).

The biochemical diagnosis of D-BPD is based on the accumulation of VLCFA, DHCA, THCA, and pristanic acid in plasma. Biochemical analysis requires the supplementation of erythrocyte acetal phospholipids, phytanic acid, and bile acid intermediates in plasma in order to make a preliminary distinction for possible diagnosis (19). There was a good correlation between patient survival and the level of C26:0 in fibroblasts. Patients who survived had more residual enzyme activity and lower 26:0 levels. In patients surviving more than

4 years, no abnormal plasma fibrinogen was found (3). In recent years, it has become increasingly clear that, despite the presence of peroxisomal disease, there are conditions in which very-low-density lipoprotein cholesterol and/or other peroxisomal metabolites are normal. Landau et al. (21) reported that the levels of VLCFA (including phytic acid) were within the normal range in two of three patients. It is a pity that we did not test the patient's C26:0 level of fibroblasts, but examination of serum VLCFA levels 33 days after birth suggested that the C26:0, C26:0/C22:0, and C24:0/C22:0 ratios were higher than normal levels, which was consistent with those reported in the literature (18, 19, 26). However, peroxisome metabolism is often at normal levels in the neonatal period, and common hematuria metabolic screening did not detect VLCFA metabolism abnormalities, so the diagnosis of similar cases will be easily missed. In that case, whole-exome sequencing is recommended for suspicious cases. A report of Lines et al. (23) states that all reported diagnoses of D-BPD in adolescents are done by whole-exome sequencing rather than by traditional clinical means.

To date, all D-BPD patients reported in the literature are homozygous or compound heterozygous for *HSD17B4* mutations. Our patient had a heterozygous deletion variant c.972+1G>T and missense mutations c.727T>A (p.W243R). These two mutations have never been reported before.

D-BPD type I defects are often associated with nonsense mutations, frameshift mutations, or in-frame deletions of 20 or more residues in the dehydrogenase domain; and in patients with type I deficiency, *HSD17B4* protein is almost always undetectable in fibroblasts. Defects are associated with missense mutations or in-frame deletions in the hydratase domain, and type III defects are associated with missense mutations or single amino acid deletions in the dehydrogenase domain (27, 28). The expression of mutant *HSD17B4* protein was severely reduced in compound heterozygotes. D-BPD types includes type I, type II, type III, and type IV, among which type III is the most common D-BPD type in infantile onset (<2 years) patients. In our case, the exon mutation c.727T>A (p.W243R) of *HSD17B4* leads to a disturbance in SDR domain of dehydrogenase unit, and intron mutation c.972+1G>T may lead to splicing abnormal in hydratase unit. Regarding intron mutation pathogenesis, it has been shown that fetus with homozygous mutations in intron IVS5+1G>C of *HSD17B4* has increased VLCFA levels (29). In our case, although we did not detect the activity of the two units of D-BP, we suggest the patient may have had type I D-BPD.

We report the first Chinese patient with bifunctional protein deficiency, analysis of this variant according to the ACMG guidelines suggests an extremely pathogenic variant, and the child's clinical presentation is compatible with D-BPD. The mutations found in our case have not been previously reported worldwide. The mutation type was compound heterozygous and may result in D-BPD type I, although we did not measure *HSD17B4* protein activity and could not identify the specific D-BP type, which is a limitation of this report. Given the early onset and severe disease of this patient, it is estimated that there is a high possibility of almost complete lack of *HSD17B4* protein activity. In terms of treatment, the disease is based on symptomatic and supportive treatment. There is still a lack of effective radical treatment, and children often die of respiratory failure within 2 years of age.

CONCLUSIONS

Our case provides clinical features of a rare D-BPD and a new type of *HSD17B4* gene mutation to provide a reference for early diagnosis. The presence of hypotonia and intractable epilepsy in infancy, associated with cosmetic deformities, especially if cranial MRI is associated with polymicrogyria, severe developmental delay, hearing loss, or primary adrenal insufficiency, regardless of their VLCFA condition, should be considered for this disease. The disease has a poor outcome, and infants often die of respiratory failure within 2 years of age. In addition, heterozygous deletion variant c.972+1G>T and missense mutation c.727T>A (p.W243R) are newly discovered pathogenic variants that deserve further study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by 2021-K-12-01. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SiC, LD, YiL, YuL, ShC, and YaL drafted the manuscript or revised it critically for important intellectual content, provided the final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.679597/full#supplementary-material

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Tandem Mass Spectrometry Screening for Inborn Errors of Metabolism in Newborns and High-Risk Infants in Southern China: Disease Spectrum and Genetic Characteristics in a Chinese Population

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Inborn errors of metabolism (IEMs) often causing progressive and irreversible neurological damage, physical and intellectual development lag or even death, and serious harm to the family and society. The screening of neonatal IEMs by tandem mass spectrometry (MS/MS) is an effective method for early diagnosis and presymptomatic treatment to prevent severe permanent sequelae and death. A total of 111,986 healthy newborns and 7,461 hospitalized high-risk infants were screened for IEMs using MS/MS to understand the characteristics of IEMs and related gene mutations in newborns and high-risk infants in Liuzhou. Positive samples were analyzed by Sanger sequencing or next-generation sequencing. The results showed that the incidence of IEMs in newborns in the Liuzhou area was 1/3,733, and the incidence of IEMs in high-risk infants was 1/393. Primary carnitine deficiency (1/9,332), phenylketonuria (1/18,664), and isovaleric acidemia (1/37,329) ranked the highest in neonates, while citrullinemia type II ranked the highest in high-risk infants (1/1,865). Further, 56 mutations of 17 IEMs-related genes were found in 49 diagnosed children. Among these, *HPD* c.941T > C, *CBS* c.1465C > T, *ACADS* c.337G > A, c.1195C > T, *ETFA* c.737G > T, *MMACHC* 1076bp deletion, *PCCB* c.132-134delGACinsAT, *IVD* c.548C > T, c.757A > G, *GCDH* c.1060G > T, and *HMGCL* c.501C > G were all unreported variants. Some related hotspot mutations were found, including *SLC22A5* c.51C > G, *PAH* c.1223G > A, *IVD* c.1208A > G, *ACADS* c.625G > A, and *GCDH* c.532G > A. These results show that the overall incidence of IEMs in the Liuzhou area is high. Hence, the scope of IEMs screening and publicity and education should be expanded for a clear diagnosis in the early stage of the disease.

Keywords: disease spectrum, genetic characteristics, genetic mutation, incidence of IEMs, inborn errors of metabolism, tandem mass spectrometry

INTRODUCTION

Inborn errors of metabolism (IEMs), also known as inherited metabolic diseases, is a group of hereditary diseases caused by genetic defects, which lead to the functional defects of some enzymes, carriers, receptors, and other proteins needed to maintain normal metabolism. IEMs result in the disorder of biochemical metabolic pathways, accumulation of intermediate or bypass metabolites, or a lack of terminal metabolites. There are several forms of IEMs, including amino acid metabolism disorders (including urea cycle disorders, branched-chain amino acid metabolism abnormalities, and sulfur amino acid metabolism disorders), organic acidemia, fatty acid oxidation metabolism disorders, glucose metabolism disorders, and other diseases. Dozens of genetic metabolic diseases, such as amino acid metabolic disorders, organic acid metabolic disorders, and fatty acid oxidation disorders, can be screened for simultaneously with high flux and high accuracy by determining the amino acid and acylcarnitine content in neonatal dried blood on filter paper. This method has gradually become popular in China in recent years. A large number of genetic metabolic diseases can be detected at an early stage in newborns due to the wide popularization of tandem mass spectrometry in China. The incidence rate of IEMs varies among different countries and populations worldwide (Webster et al., 2003; Yoon et al., 2005; Sanderson et al., 2006; Dhondt, 2010; Lindner et al., 2011; Lim et al., 2014; Hassan et al., 2016; Karaceper et al., 2016; Yunus et al., 2016; Alfadhel et al., 2017; Shibata et al., 2018).

The incidence of IEMs also varies greatly among the different cities and regions within China (Guo et al., 2018; Wang et al., 2019). Significant differences in IEMs spectra, prevalence, and genetic characteristics exist among the different regions and populations in China. The Liuzhou area has 48 ethnic minorities, and the custom of intermarriage among these ethnic groups is widespread. In Liuzhou, newborn screening for congenital hypothyroidism and phenylketonuria (PKU) began 1994, screening for glucose-6-phosphate dehydrogenase deficiency, thalassemia, and congenital adrenocortical hyperplasia began in 2000, and tandem mass spectrometry screening for genetic metabolic diseases began in 2012. At present, the screening coverage rate of genetic metabolic diseases is about 50% of newborns in Liuzhou, and it is expected that the tandem mass spectrometry screening of IEMs in Liuzhou area will reach full coverage in newborns in 2025. In this study, the amino acid and acylcarnitine profiles of 111,986 newborns born in Liuzhou from December 2012 to June 2020 and 7,461 hospitalized children with suspected genetic metabolic diseases were screened, and then diagnoses were confirmed by first-generation sequencing or high-throughput sequencing. Finally, 30 cases of genetic metabolic diseases were diagnosed in 111,986 newborns, at an incidence rate of 1/3,733; 19 cases were diagnosed in high-risk infants, at an incidence rate of 1/393. Further, 56 mutations of 17 IEMs-related genes were found in 49 diagnosed children. What is noteworthy is that the incidence of primary carnitine deficiency (PCD) is the highest in Liuzhou, about 1/9,332, which is higher than that reported in other areas of China. *SLC22A5* gene c.51C > G may be a hotspot mutation in Liuzhou, but whether it is related to ethnic groups in this area needs to be further explored in the future.

Abbreviations: CPS1, Carbamyl phosphate synthase 1 deficiency; CPT2, carnitine palmitoyltransferase II deficiency; CTLN2, citrullinemia; GA-I, glutaric aciduria type I; HCY, homocystinuria; HMGCLD, 3-hydroxy-3-methylglutaryl-coenzyme A lyase deficiency; H-TYR, tyrosinemia; IVA, isovaleric acidemia; MADD, multiple acyl-CoA dehydrogenase deficiency; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; MMA, methylmalonic acidemia; OTC, ornithine transcarbamylase deficiency; PA, propionic acidemia; PCD, primary carnitine deficiency, PKU, phenylketonuria, SCADD, short-chain acyl-CoA dehydrogenase deficiency.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Ethical Committee of Liuzhou Maternity and Child Health Hospital, and informed consent of the parents.

Study Cohort and Specimen Collection

From December 2012 to June 2020, The heel blood of newborns 3 days after birth and high-risk infants was collected, dripped on filter paper (Waterman Company, S&S903#, Britain), and dried naturally at room temperature. Dried blood samples from 111,986 newborns and 7,461 hospitalized children with suspected genetic metabolic diseases were collected. Among the 111,986 newborns, there were 53,753 Han, 40,763 Zhuang, 10,394 Dong, 6,217 Miao and 859 Yao. There were 5,596 Han, 4,594 Zhuang, 2,096 Zhuang, 543 Dong, 130 Miao and 98 Yao among the 7,461 high risk infants.

Tandem Mass Spectrometry Screening

Dried blood spots were pretreated using a non-derivatized MS/MS kit according to the manufacturer instructions (FengHua, China) and then analyzed using a tandem mass spectrometry system (ABI3200, United States). The screening indicators included: free carnitine (C0); 11 amino acids and 33 acylcarnitines (**Table 1**). Repeated MS/MS tests were performed on the recalled positive samples, and biochemical tests or genetic analysis were performed on the re-examined positive cases.

Genetic Analysis

For all positive samples, 2 mL of whole-blood samples were collected from the newborns and their parents after obtaining the informed consent of the guardian and the approval of the hospital ethics committee. Genomic DNA was extracted to amplify all exons and part of the intron region at the exon boundary. The amplified products were sequenced by Sanger sequencing and compared with the human genome gene sequence. Some genetic metabolic diseases, such as PKU and methylmalonic acidemia (MMA), which are caused by multiple genes, were screened by high-throughput sequencing by Jiajian Medicine (Guangzhou, China) and then verified by Sanger sequencing.

TABLE 1 | Blood markers and cutting values of amino acid and acyl carnitine spectrum.

Indicators	Minimum value (μmol/L)	Maximum value (μmol/L)
Ala	120	600
Gly	170	1,150
Pro	110	400
Leu	51	295
Val	45	270
Met	5	38
Phe	24	116
Tyr	45	260
Cit	5	41
Orn	42	390
Arg	1	52
C0	9.00	55.00
C2	8.00	50.00
C3	0.35	4.20
C3DC	0.02	0.20
C4	0.06	0.50
C4-OH	0.03	0.40
C4DC	0.05	0.50
C5	0.04	0.50
C5:1	0.00	0.10
C5-OH	0.05	0.60
C5DC	0.01	0.20
C6	0.02	0.20
C6:1	0.01	0.10
C6DC	0.00	0.10
C8	0.01	0.24
C8:1	0.04	0.45
C8DC	0.01	0.10
C10	0.02	0.25
C10:1	0.02	0.25
C12	0.03	0.60
C12:1	0.01	0.30
C14	0.07	0.50
C14:1	0.02	0.40
C14-OH	0.00	0.10
C16	0.30	5.70
C16:1	0.03	0.45
C16-OH	0.00	0.10
C18	0.14	1.86
C18:1	0.40	3.00
C18-OH	0.00	0.05
C3/C0	0.03	0.25
C3/C2	0.04	0.25
C4/C2	0.00	0.03
C5/C2	0.00	0.03
C5-OH/C3	0.02	0.40
C5DC/C8	0.20	3.00
C8/C2	0.00	0.10
C14:1/C8:1	0.10	4.00
C0/(C16 + C18)	2.00	30.00
(C16 + C18:1)/C2	0.07	0.40

Bioinformatics Analysis

The Human Gene Mutation Database,[1] single-nucleotide polymorphism,[2] ExAC,[3] and 1,000 Genomes Project[4] were queried for the newly discovered variation sites, and protein functions were predicted using Sorting Intolerant from Tolerant and PolyPhen-2 bioinformatics software. At the same time, the pathogenicity was interpreted according to the standards and guidelines for the interpretation of gene variation issued by the American Society of Medical Genetics and Genomics in 2015.

Statistical Analysis

Statistical analysis was performed using SPSS17.0 version. The difference of categorical data was compared using Chi-square test. The difference of measurement data was compared by analysis of variance. $p < 0.05$ was considered to be statistical significance. Primary screening positive rate = number of positive cases at primary screening/number of children screened × 100%; positive predictive value = number of confirmed cases/number of positive recalls × 100%; incidence = number of confirmed cases/number of children screened × 100%.

RESULTS

Screening Results of Amino Acids and Acylcarnitines in Newborns

Among the 111,986 newborns, 2,464 tested positive at the primary screening, with a positive rate of 2.2% (2,464/111,986), and 2,275 suspected positive cases were recalled, with a positive recall rate of 92.3% (2,275/2,464). Finally, 30 cases of genetic metabolic diseases were clinically diagnosed (the disease spectrum is shown in **Table 2**, and the MS/MS results are shown in **Table 3**), and the positive predictive value was 1.3%. Preliminary statistics showed that the incidence of

[1]http://www.hgmd.cf.ac.uk/ac/
[2]http://www.ncbi.nlm.nih.gov/
[3]http://exac.broadinstitute.org/
[4]https://www.internationalgenome.org/

TABLE 2 | Disease spectrum of 30 cases of genetic metabolic diseases screened from 111,986 newborns.

Categories	Total	Incidence rate	IEM	Cases	Incidence rate
AAMD	8	1/13,998	PKU	6	1/18,664
			CTLN2	1	1/111,986
			HCY	1	1/111,986
FAMD	15	1/7,466	PCD	12	1/9,332
			SCADD	2	1/55,993
			MCADD	1	1/111,986
OAMD	7	1/15,998	IVA	3	1/37,329
			MMA	1	1/111,986
			PA	1	1/111,986
			GA-1	1	1/111,986
			HMGCLD	1	1/111,986

TABLE 3 | Results of tandem mass spectrometry in 49 children with genetic metabolic diseases (μmol/L).

IEM	Cases	Reference result	Reference value	Preliminary screening results *M* (min∼max)	Re-examination results *M* (min∼max)
PKU	6	Phe	24.0–116.0	378.3 (201–1000.5)	376.9 (230.5–789.3)
		Phe/Tyr	0.2–2.0	10.24 (3.02–25.6)	14.64 (4.77–20.12)
CTLN2	5	Cit	5–41	236.2 (128.3–365.7)	240.12 (134.8–345.7)
		Met	5–38	129.0 (94.0–181.5)	145.5 (124.5–163.2)
OTC	2	Cit	5–41	1.85 (1.6–2.1)	2.05 (1.8–2.3)
		Cit/Phe	0.14–0.72	0.095 (0.09–0.10)	0.075 (0.06–0.09)
CPS1	1	Cit	5–41	2.4	2.8
HCY	1	Met	5–38	152.6	480
		Met/Phe	0.25–1.2	2.65	3.20
H-TYR	1	Tyr	45–260	526.0	498.2
		Phe/Tyr	0.2–2.0	0.12	0.14
PCD	14	C0	9.0–55.0	2.83 (0.74–5.45)	3.95 (1.07–5.82)
SCADD	4	C4	0.06–0.50	1.99 (1.69–2.49)	2.06 (1.02–2.84)
		C4/C2	0–0.03	0.09 (0.069–0.114)	0.11 (0.062–0.169)
MCADD	1	C8	0.01–0.24	1.89	3.53
		C8/C2	0–0.1	0.19	0.42
CPT2	1	C16	0.3–5.7	24.2	28.6
		C18	0.14–1.86	4.35	5.02
		C18:1	0.30–2.9	4.65	4.36
		(C16 + C18:1)/C2	0.07–0.40	14.0	13.8
MADD	1	C4	0.06–0.50	2.59	2.64
		C5	0.04–0.50	6.98	7.32
		C6	0.01–0.10	0.62	0.56
		C8	0.01–0.24	1.24	1.12
		C10	0.01–0.30	1.70	1.58
		C12	0.02–0.30	1.92	1.56
		C14	0.05–0.40	2.44	2.68
IVA	4	C5	0.04–0.50	7.5 (5.6–11.6)	5.90 (4.32–8.96)
		C5/C2	0–0.03	0.57 (0.35–0.74)	0.66 (0.48–0.83)
GA-1	3	C5DC	0.01–0.2	1.75 (0.98–2.70)	2.34 (1.58–3.78)
		C5DC/C8	0.2–3.0	65.9 (17.5–139.9)	69.8 (26.5–120.0)
MMA	2	C3	0.35–4.2	8.83 (8.70–8.96)	11.1 (8.32–13.87)
		C3/C2	0.04–0.25	0.94 (0.56–1.32)	0.69 (0.62–0.76)
		C3/C0	0.03–0.25	1.33 (0.46–2.2)	0.56 (0.51–0.61)
PA	2	C3	0.35–4.2	9.67 (9.61–9.73)	8.30 (7.82–8.78)
		C3/C2	0.04–0.25	2.24 (1.53–2.94)	1.16 (0.85–1.46)
		C3/C0	0.03–0.25	1.57 (1.06–2.08)	1.58 (1.46–1.69)
HMGCLD	1	C5-OH	0.05–0.6	2.38	2.51
		C5-OH/C3	0.02–0.4	4.65	1.02

genetic metabolic diseases in the Liuzhou area was 1/3,733. The total incidence of amino acid metabolic diseases was 1/13,998, of which there were six cases of phenylalanine hydroxylase deficiency (PHD) (1/18,664; highest), one case of hyperhomocysteinemia (1/111,986), and one case of citrullinemia type I (CTLN2; 1/111,986).

Seven cases of organic acidemia (1/15,998) were reported, of which isovaleric acidemia (IVA) was the highest (1/37,329); besides, there were one case of MMA (1/111,986), one case of glutaric acidemia type I (1/111,986), and one case of 3-hydroxy-3-methyl glutaric acidemia (1/111,986). Fifteen cases of fatty acid metabolism disorder (1/7,466) were found, of which PCD was the highest (1/9,332); besides, there were two cases of short-chain acyl-CoA dehydrogenase deficiency (SCADD; 1/55,993) and one case of medium-chain acyl-CoA dehydrogenase deficiency (MCADD; 1/111,986) (**Table 2** and **Figure 1**).

Diagnosis of IEMs in High-Risk Infants

Among 7,461 hospitalized high-risk infants with suspected genetic metabolic diseases, 19 cases were diagnosed as genetic metabolic diseases, including eight cases of amino acid metabolic diseases (1/933), of which there were four cases of CTLN2 (1/1,865), two cases of ornithine carbamoyltransferase deficiency (1/3,731), one case of tyrosinemia (H-TYR; 1/7,461), and one case of carbamyl phosphate synthase I deficiency (CPS1; 1/7,461). Further, six cases of fatty acid metabolism disorder (1/1,244) were found, including two cases of PCD (1/3,731), two cases of SCADD (1/3,731), one case of carnitine palmitoyltransferase II

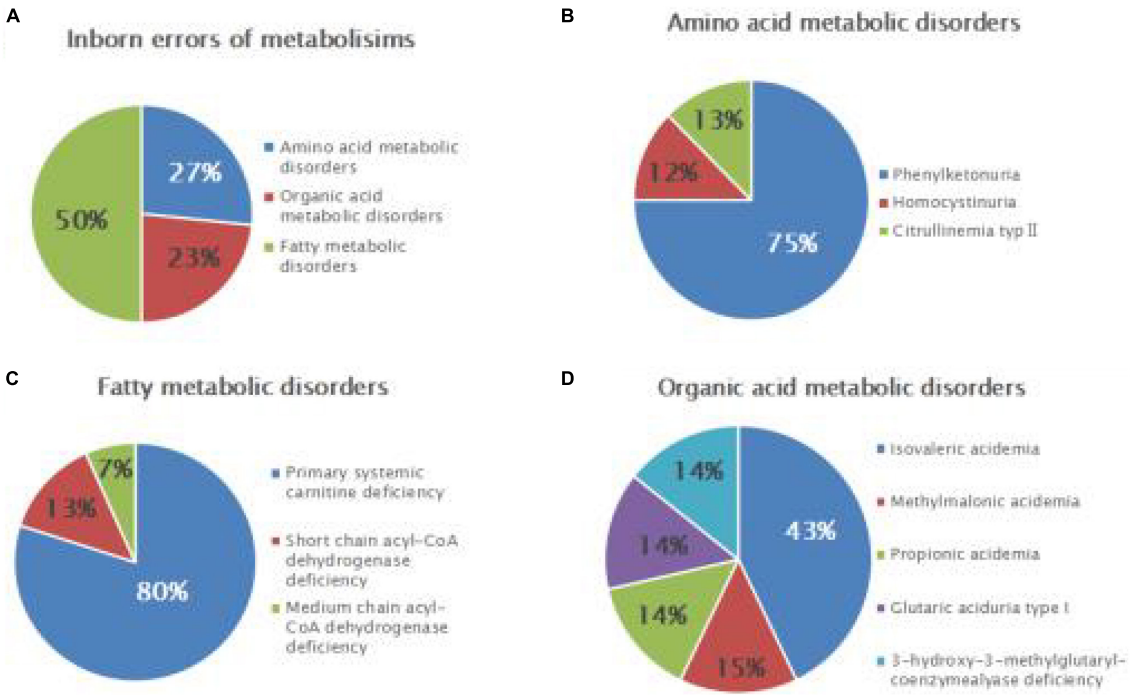

FIGURE 1 | Disease spectrum and distribution of inborn errors of metabolism (IEMs) in newborns. Relative proportions of different categories of IEMs **(A)**, amino acid metabolic disorders **(B)**, fatty acid metabolic disorders **(C)**, and organic acid metabolic disorders **(D)**.

TABLE 4 | Disease spectrum of 19 children with genetic metabolic diseases screened from 7,461 high-risk infants.

Categories	Total	Incidence rate	IEM	Cases	Incidence rate
AAMD	8	1/933	CTLN2	4	1/1,865
			OTC	2	1/3,731
			CPS1	1	1/7,461
			H-TYR	1	1/7,461
FAMD	6	1/1,244	PCD	2	1/3,731
			SCADD	2	1/3,731
			CPT2	1	1/7,461
			MADD	1	1/7,461
OAMD	5	1/1,492	GA-1	2	1/3,731
			MMA	1	1/7,461
			PA	1	1/7,461
			IVA	1	1/7,461

deficiency (CPT2; 1/7,461), and one case of glutaric acidemia type II (1//7,461). Moreover, five cases of organic acidemia (1/1,492) were reported, including two cases of glutaric acidemia type I (1/3,731), one case of propionic acidemia (PA; 1/7,461), one case of IVA (1/7,461), and one case of MMA (1/,7461) (**Table 4** and **Figure 2**).

Gene Detection Results

The gene detection results revealed 56 mutations of 17 IEMs-related genes in 49 diagnosed children. A total of 11 kinds of 26 mutation sites were detected in the *SLC22A5* gene: c.51C > G, c.976C > T, c.1411C > T, c.1195C > T, c.760C > T, c.919delG, c.1400C > G, c.517delC, c.839C > T, c.505C > T, and c.338G > A. A total of eight kinds of 12 mutation sites were detected in the *PAH* gene: c.1174T > A, c.1223G > A, c.208_210del, c.611A > G, c.331C > T, c.28G > A, c.320A > G, and c.721C > T. Also, five mutation types and eight mutation sites were found in the *IVD* gene: c.158G > A, c.214G > A, c.548C > T, c.757A > G, and 1208A > G. The *ACADS* gene had six kinds of eight mutation sites: c.268G > A, c.320G > A, c.337G > A, c.625G > A, c.1031A > G, and c.1148G > A. A total of three kinds and six mutation sites were detected in the *GCDH* gene: c.532G > A, c.655G > A, and c.1060G > T. The detection of other disease-related genes is shown in **Tables 5**, **6**. Among these, *HPD* gene c.941T > C, *CBS* gene c.1465C > T, *ACADS* gene c.337G > A and c.1195C > T, *ETFA* gene c.737G > T, *MMACHC* gene 1076-bp deletion, *PCCB* gene c.132-134delGACinsAT, *IVD* gene c.548C > T and c.757A > G, *GCDH* gene c.1060G > T, and *HMGCL* gene c.501C > G were all unreported variants.

DISCUSSION

The incidence of genetic metabolic diseases varies across different countries and regions worldwide. In this study, the overall incidence of genetic metabolic diseases in Liuzhou, Guangxi, was about 1/3,733, which was similar to that reported in other domestic studies. PCD had the highest incidence of all genetic metabolic diseases in newborns in Liuzhou, and ranked first

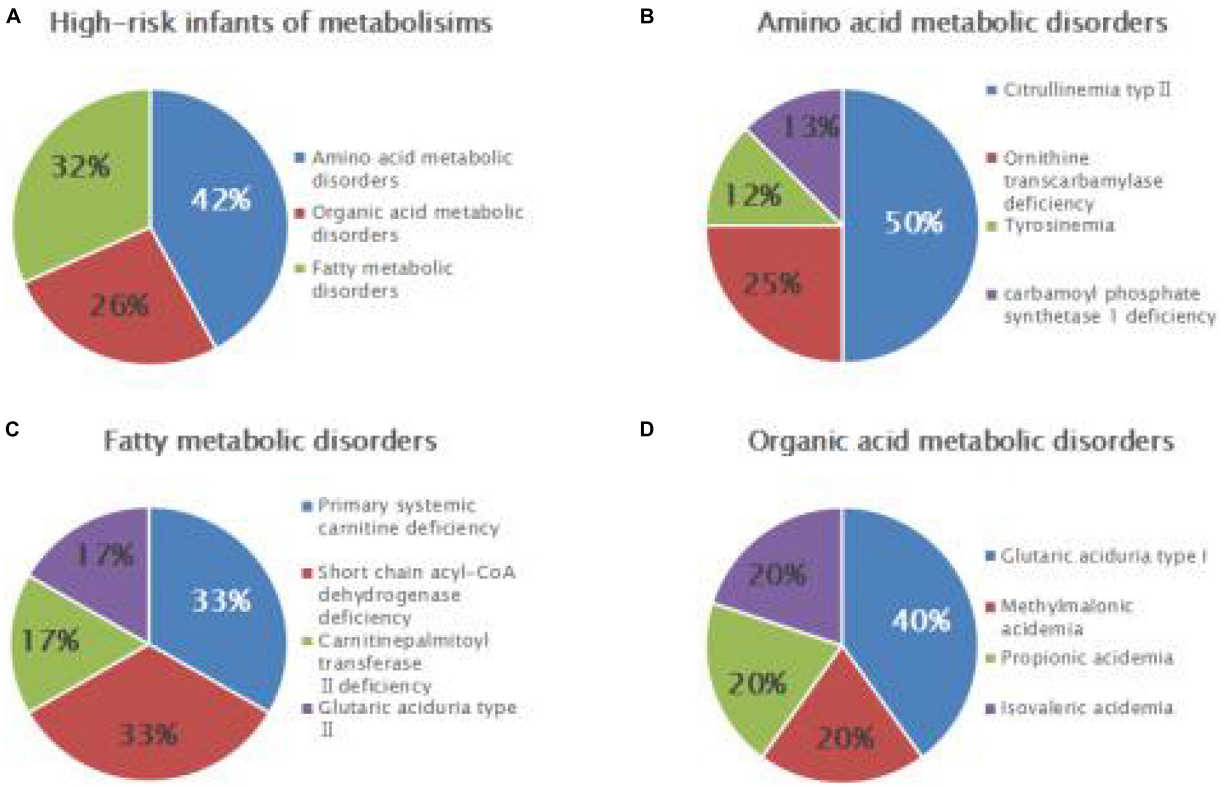

FIGURE 2 | Disease spectrum and distribution of IEMs in high-risk infants. Relative proportions of different categories of IEMs **(A)**, amino acid metabolic disorders **(B)**, fatty acid metabolic disorders **(C)**, and organic acid metabolic disorders **(D)** in high-risk infants.

among the fatty acid metabolic disorders, at 1/9,332, which is higher than that reported in other parts of China. The global prevalence rate of PCD is about 1/20,000–1/120,000 (di San Filippo et al., 2008). At present, more than 180 kinds of *SLC22A5* gene mutations have been reported, most of which are missense mutations, followed by non-sense mutations and frameshift mutations, while splice site mutations are relatively rare; the most frequent mutation is exon 1 (Li et al., 2010). The hotspot mutations of the *SLC22A5* gene vary among different races and regions worldwide. R282X mutations are common in Caucasians (Burwinkel et al., 1999; Vaz et al., 1999; Wang et al., 1999), and W132X and W283C mutations are most common in East Asian populations (Koizumi et al., 1999; Nezu et al., 1999; Tang et al., 1999). R254X mutations were found in children with PCD in Taiwan and Shanghai (Tang et al., 2002; Lee et al., 2010). In this study, 11 kinds of 26 mutation sites were detected in newborns and high-risk infants: c.51C > G, c.976C > T, c.1411C > T, c.1195C > T, c.760C > T, c.919delG, c.1400C > G, c.517delC, c.839C > T, c.505C > T, and c.338G > A. Inconsistent with previously reported hotspots of mutations, c.51C > G mutations occurred as often as eight times in 14 children in this study, with a frequency of about 28.6%. At the same time, 2,093 newborns were screened for the *SLC22A5* gene mutation, and 24 cases of reported mutation carriers were detected, of which the c.51C > G mutation occurred as often as 13 times, accounting for 54% of the carriers. The c.51C > G mutation had a high carrying rate in the population of Liuzhou and was the most frequent mutation of the *SLC22A5* gene reported in this study. Primary screening of 119,986 newborns resulted in 2,464 positive tests, from these newborns 2,275 were examined for a second time and 30 positive cases were established. The main reasons for the high false positive rate of tandem mass spectrometry are as follows: The first is the quality of the blood tablets to be tested. For example, whether the blood collection time is standardized, whether the blood collection process is polluted, and the transportation time of blood filter paper is too long. The second is the quality control of the experimental process. Such as reagent preparation, quality control material selection. Finally, the quality audit and interpretation of the experimental results, there may be subjective factors in the interpretation of laboratory results.

PHD had the second highest incidence of all genetic metabolic diseases and ranked first among the amino acid metabolic disorders. In this study, PHD was detected in six newborns, at a rate of 1/18,664. Studies have shown that the incidence of PHD in China is 1/3,000–1/16,000 and incidences are higher in northern China than in southern China. The Liuzhou area of Guangxi is located in the south of China, and the detection rate of PKU is significantly lower than that in other areas in the north of China. So far, more than 800 *PAH* gene mutations have been found in patients with *PAH* deficiency. The *PAH* gene has some hotspot mutations, which vary among different populations. c.728G > A is the most common in China and Korea (Lee et al., 2008;

TABLE 5 | Spectrum of IEMs and variants in confirmed cases of newborns.

IEM	Gender	Ethnicity	Gene	Variant 1	Variant 2
PKU			*PAH*		
1	M	Zhuang		c.1174T > A (p.F392I)	c.1223G > A (p.R408Q)
2	M	Han		c.1223G > A (p.R408Q)	c.208_210del (p.70del)
3	F	Han		c.611A > G (p.Tyr204Cys)	c.611A > G (p.Tyr204Cys)
4	F	Han		c.331C > T (p.Arg111Ter)	c.728G > A (p.Arg243Gln)
5	M	Han		c.320A > G (p.His107Arg)	c.1223G > A (p.Arg408Gln)
6	M	Han		c.721C > T (p.Arg241Cys)	c.728G > A (p.Arg243Gln)
CTLN2			*SLC25A13*		
1	M	Yao		c.851_854delGTAT	c.1638_1660dup23
HCY			*CBS*		
1	F	Han		c.1465C > T (p.Gln489Ter)#	c.1465C > T (p.Gln489Ter)#
PCD			*SLC22A5*		
1	M	Han		c.51C > G (p.Phe17Leu)	c.760C > T (p. Arg254Ter)
2	M	Han		c.760C > T (p. Arg254Ter)	c.919delG (p.Val307LeufsX14)#
3	F	Han		c.1400C > G (p.Ser467Cys)	c.1400C > G (p.Ser467Cys)
4	F	Han		c.1195C > T (p.Arg399Try)	c.517delC (p.Leu173CysfsX3)
5	F	Zhuang		c.51C > G (p.Phe17Leu)	c.51C > G (p.Phe17Leu)
6	F	Han		c.51C > G (p.Phe17Leu)	c.338G > A (p.Cys113Tyr)
7	F	Han		c.1400C > G (p.Ser467Cys)	c.839C > T (p.Ser280Phe)
8	F	Miao		c.976C > T (p.Gln326X)#	c.505C > T (p.Arg169Trp)
9	M	Han		c.760C > T (p. Arg254Ter)	c.760C > T (p. Arg254Ter)
10	M	Han		c.51C > G (p.Phe17Leu)	c.51C > G (p.Phe17Leu)
11	M	Zhuang		c.51C > G (p.Phe17Leu)	c.51C > G (p.Phe17Leu)
12	M	Han		c.338G > A (p.Cys113Tyr)	c.338G > A (p.Cys113Tyr)
SCADD			*ACADS*		
1	F	Zhuang		c.625G > A (p.Gly209Ser)	c.625G > A (p.Gly209Ser)
2	F	Han		c.268G > A (p.Gly90Ser)	c.337G > A (p.Gly113Arg)#
MCADD			*ACADM*		
1	M	Zhuang		c.580A > (p.Asn194Asp)	c.580A > (p.Asn194Asp)
IVA			*IVD*		
1	F	Zhuang		c.214G > A (p.Asp72Asn)	c.1208A > G (p.Tyr403Cys)
2	M	Han		c.548C > T (p.Ala183Val)#	c.757A > G (Thr253Ala)#
3	M	Zhuang		c.149G > A (p.Arg50His)	c.1199A > G (p.Tyr400Cys)
GA- I			*GCDH*		
1	M	Zhuang		c.532G > A (p.Gly178Arg)	c.532G > A (p.Gly178Arg)
MMA			*MMACHC*		
1	F	Han		c.315C > G (p.Tyr105Ter)	1076bp del#
PA			*PCCB*		
1	F	Zhuang		c.1559-4_1559-3insAGAAGCA	c.1594C > T(p.Arg532Cys)
HMGCLD			*HMGCL*		
1	F	Dong		c.252 + 1G > A	c.501C > G (pTyr.167Ter)#

The symbol "#" in superscript represents unreported, and "–" represents no mutation or not checked out.

Zhang et al., 2018), the c.1222C > T mutation is the most common in the United States (Kaul et al., 1994), c.1238G > C is common in Japan (Okano et al., 2011), and IVS10-11G > A is the most common in Iran and Spain (Bueno et al., 2013; Rastegar Moghadam et al., 2018). In the present study, eight kinds of 12 mutation sites were found in the PHA gene: c.1174T > A, c.1223G > A, c.208_210del, c.611A > G, c.331C > T, c.728G > A, c.320A > G, and c.721C > T. Further, c.1223G > A was the most common *PAH* mutation site, accounting for 25% of all mutation types, potentially representing a hot spot mutation in this region.

IVA had the third highest incidence of all genetic metabolic diseases and ranked first among organic acidemia disorders, with a detection rate of 1/37,329, which was higher than that reported in previous studies. IVA occurs due to the loss of the functional activity of isovaleryl-CoA dehydrogenase caused by *IVD* gene mutation, resulting in leucine metabolism disorder. The disease was first reported by Tanaka et al. (1966). Significant differences in the incidence of IVA exist among different regions and populations: about 1/250,000 in the United States (Frazier et al., 2006), about 1/365,000 in Taiwan (Lin et al., 2007), and about

TABLE 6 | Spectrum of IEMs and variants in confirmed cases of high-risk infants.

IEM	Gender	Ethnicity	Gene	Variant 1	Variant 2
CTLN2			*SLC25A13*		
1	F	Han		c.851_854delGTAT	c.851_854delGTAT
2	M	Han		c.851_854delGTAT	c.851_854delGTAT
3	F	Zhuang		c.851_854delGTAT	c.851_854delGTAT
4	M	Zhuang		c.851_854delGTAT	c.851_854delGTAT
OTC			*OTC*		
1	F	Zhuang		IVS7-1G > A	–
2	M	Han		2-7exon del	–
H-TYR			*HPD*		
1	F	Yao		c.93 + 1delG#	c.941T > C (p.Ile314Thr)
CPS1			*CPS1*		
1	M	Han		c.1649C > T (p.Thr550Met)	c.858G > C (p.Lys286Asn)
PCD			*SLC22A5*		
1	F	Dong		c.976C > T (p.Gln326Ter)#	c.976C > T (p.Gln326Ter)#
2	M	Zhuang		c.1411C > T (p.Arg471Cys)	c.1195C > T (p.Arg399Try)
SCADD			*ACADS*		
1	M	Han		c.1148G > A (p.Arg383His)	c.1031A > G (p.Glu344Gly)
2	F	Zhuang		c.320G > A (p.Arg107His)	c.1195C > T (p.Arg399Trp)#
CPT2			*CPT2*		
1	F	Han		c.886C > T (p.Arg296Ter)	c.1148T > A (p.Phe383Tyr)
MADD			*ETFA*		
1	M	Han		c.494T > C (Val165Ala)	c.737G > T (p.Gly246Val)#
IVA			*IVD*		
1	M	Han		c.1208A > G (p.Tyr403Cys)	c.1208A > G (p.Tyr403Cys)
GA- I			*GCDH*		
1	M	Han		c.532G > A (p.Gly178Arg)	c.655G > A (p.Ala219Thr)
2	M	Zhuang		c.532G > A (p.Gly178Arg)	c.1060G > T (p.Gly354Cys)#
MMA			*MMUT*		
1	F	Han		c.103C > T (p.Gln35Ter)	c.346G > A (p.Val116Met)
PA			*PCCB*		
1	F	Han		c.132-134delGACinsAT#	–

The symbol "#" in superscript represents unreported, and "–" represents no mutation or not checked out.

1/67,000 in Germany (Ensenauer et al., 2011). Some thermal mutations were found in the *IVD* gene in different regions. The c.932C > T mutation is the main mutation in Germany and the United States (Vockley and Ensenauer, 2006). The c.457-3_2CA > GG mutation is common in Korea (Lee et al., 2007), and the c.149G > C and c.1208A > G mutations are common in Taiwan (Lin et al., 2007). The Chinese scholar Wenjuan Qiu reported a case of IVA with compound heterozygous mutations of the *IVD* gene, c.149G > A and c.466G > C (Qiu et al., 2008). Shiyue Mei reported two cases of IVA and detected compound heterozygous mutations of the *IVD* gene, c.1195G > C and c.466-3_466-2delinsGG, and the homozygous mutation c.1208A > G (Mei et al., 2018). Xiyuan Li reported three cases of children with atypical IVA (Li et al., 2014) in which six mutation types were detected: c.157C > T, c.214G > A, c. 1183C > G, c.1208A > G, c.1039G > A, and c.1076A > G. In this study, five mutation types and eight mutation sites of the *IVD* gene were detected in four children with IVA: c.158G > A, c.214G > A, c.548C > T, c.757A > G, and 1208A > G, of which c.548C > T and c.757A > G were not included in the database. The frequency of the 1208A > G mutation was the highest (appearing four times in this study). In a previous study, high-throughput sequencing was carried out in 2,095 normal newborns in this area, and 10 cases of *IVD* gene carriers were detected, of which as many as five cases were c.1208A > G mutation carriers. At present, no reports are available on the mutation spectrum and hot spots of patients with IVA in China. However, the c.1208A > G mutation has a high frequency in China, according to the *IVD* gene mutation types detected in four children with IVA in this study and other domestic studies. Whether the mutation is a hotspot mutation in the Chinese population or local population needs to be further verified by expanding the sample size.

In this study, the detection rate of SCADD in newborns was about 1/55,993, and the rate in Suzhou, China, was about 1/28,690 (Wang et al., 2019). Zytkovicz reported that the global incidence of SCADD was about 1/25,000–1/45,000 (Zytkovicz et al., 2001). More than 70 types of gene mutations have been reported in ACADS. Gregersen et al. (2008) and Jethva et al. (2008) found that c.625G > A and c.511C > T mutations were dominant in Europe and the United States. The two mutation sites were screened in 694 newborns in the United States, revealing that the c.625G > A mutation accounted for 22% of all detected alleles.

C.511C > T accounted for 3%. The incidence of SCADD was significantly lower in the Asian population than in Caucasians, while the carrying rate of the c.625G > A mutation in Hispanics was as high as 30%. In this study, six mutation sites of the ACADS gene were detected in newborns and high-risk infants: c.268G > A, c.320G > A, c.337G > A, c.625G > A, c.1031A > G, c.1148G > A, and c.1195C > T, of which c.337G > A and c.1195C > T were unreported mutation sites; the c.625G > A mutation accounted for 25%, which was consistent with reports from Gregersen et al. (2008) and Jethva et al. (2008). At present, there is still controversy about whether to screen SCADD and some variants of ACADS gene. At the same time, whether the impact of SCADD on the population is the same in different countries or different regions needs to be further observed in the later stage.

One case of glutaric acidemia type I was detected in newborns, and the detection rate was 1/111,986. Two cases were detected in high-risk infants, and the detection rate was 1/3,731. The incidence of glutaric acidemia type I in newborns is about 1/100,000 (Kölker et al., 2011), with ethnic and regional differences. The incidence of glutaric acidemia type I was about 1/130,000 in the United States (Basinger et al., 2006). Of the 129,415 newborns screened, only 2 cases were diagnosed with glutaric acidemia type I in Zhejiang province (Yang et al., 2011). At present, more than 200 *GCDH* gene variants have been reported, with obvious heterogeneity in different regions and populations. The common variation in Pennsylvania is c.1296C > T (Korman et al., 2007), while the hotspot of variation in Taiwan and Hong Kong is IVS10-2A > C (Tang et al., 2000; Shu et al., 2003; Hsieh et al., 2008). At present, no hotspots of variation exist in the Chinese mainland area. Lin et al. (2018) analyzed gene variations in five cases of glutaric aciduria type I (GA-I); c.1244-2A > C mutation frequency was the highest. Three variants of the *GCDH* gene were found by Sanger sequencing: c.532G > A, c. 655G > A, and c.1060G > T, of which c.532G > A appeared four times, which might be a hotspot variation in this area; c.1060G > T variation has not been reported.

In addition, other types of genetic metabolic diseases were also detected, such as hyperhomocysteinemia, MCADD, MMA, propionemia, 3-hydroxy-3-methylglutaric acidemia, ornithine carbamoyltransferase deficiency, H-TYR, CPS1, CPT2, and glutaric acidemia type II. Due to the small number of cases, these diseases were not discussed in this study. In this study, only one mutation was detected in 2 cases of OTC gene and 1 case of PCCB gene, which may be caused by large deletion, deep intronic mutations or promoter mutations. To sum up, after screening newborns and some high-risk infants in this area for genetic metabolic diseases, the detection rate and disease spectrum of some diseases were preliminarily confirmed and some mutation hotspots and new mutations were detected. These mutation hotspots may be potential candidate sites for gene screening and may expand the gene mutation spectrum of genetic metabolic diseases. The findings of this study may have a certain reference value for genetic counseling and prenatal diagnosis of genetic metabolic diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Liuzhou Maternal and Child Health Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JT formulated the study, analyzed the data, wrote the manuscript, and designed the tables. DC, RoC, LP, JY, DY, LH, TY, HN, and JW performed screening by MS/MS and collected the data. ReC provided intellectual thoughts, revised the manuscript, and led the project. All authors contributed to the article and approved the submitted version.

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