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Collaborative evaluation study on 18 candidate diseases for newborn screening in 1.77 million samples

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Abstract

Analytical and therapeutic innovations led to a continuous but variable extension of newborn screening (NBS) programmes worldwide. Every extension requires a careful evaluation of feasibility, diagnostic (process) quality and possible health benefits to balance benefits and limitations. The aim of this study was to evaluate the suitability of 18 candidate diseases for inclusion in NBS programmes. Utilising tandem mass spectrometry as well as establishing specific diagnostic pathways with second-tier analyses, three German NBS centres designed and conducted an evaluation study for 18 candidate diseases, all of

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them inherited metabolic diseases. In total, 1 777 264 NBS samples were analysed. Overall, 441 positive NBS results were reported resulting in 68 confirmed diagnoses, 373 false-positive cases and an estimated cumulative prevalence of approximately 1 in 26 000 newborns. The positive predictive value ranged from 0.07 (carnitine transporter defect) to 0.67 (HMG-CoA lyase deficiency). Three individuals were missed and 14 individuals (21%) developed symptoms before the positive NBS results were reported. The majority of tested candidate diseases were found to be suitable for inclusion in NBS programmes, while multiple acyl-CoA dehydrogenase deficiency, isolated methylmalonic acidurias, propionic acidemia and malonyl-CoA decarboxylase deficiency showed some and carnitine transporter defect significant limitations. Evaluation studies are an important tool to assess the potential benefits and limitations of expanding NBS programmes to new diseases.

KEYWORDS

dried blood spot, evaluation study, Germany, inborn errors of metabolism, newborn screening, public health, tandem mass spectrometry

1 | INTRODUCTION

Newborn screening (NBS) is regarded to be the most successful tool for secondary prevention in medicine, providing the possibility of pre-clinical detection and treatment of individuals with rare diseases and aiming to prevent irreversible harm to affected individuals, their families and the society. Since the initiation of the first NBS programme for phenylketonuria (PKU) about 60 years ago, new analytical techniques such as tandem mass spectrometry (MS/MS) and molecular genetic methods have been introduced enabling a continuous extension of the NBS disease panel. The introduction of screening for new diseases requires the careful assessment of benefits, harms and costs.[1] For instance, the major MS/MS-based extension of the NBS programme in the late 1990s led to the implementation of screening for various inherited metabolic diseases (IMDs) with a life-long risk of acute metabolic decompensation. The evaluation of this extension confirmed that individuals affected with these diseases had a similar overall health benefit from NBS compared to the benchmark condition PKU.[2] However, some shortcomings became evident such as the manifestation of first symptoms prior to NBS results or the identification of novel disease variants with unclear clinical significance and hence the risk of overtreatment.[3]

Despite their reference to the same set of original criteria,[4] NBS programmes worldwide show a substantial variation in the number of conditions included, reflecting national discrepancies in the evaluation of available evidence for the selection of NBS diseases.[5] In the United States for instance, the federally recommended uniform screening panel lists up to 61 conditions (https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp).[6,7] NBS panels and varying approaches within Europe have recently been reviewed.[8] In Germany, the current NBS panel comprises a total of 19 conditions, 13 inherited metabolic diseases, (biotinidase deficiency [MIM 253260], classic galactosemia [MIM 230400], phenylketonuria [MIM 261600], maple syrup urine disease [MIM 248600], tyrosinemia I [MIM 276700]), isovaleric acidemia [MIM 243500], glutaric acidemia I [MIM 231670], deficiencies of medium-chain and very long-chain acyl-CoA dehydrogenase [MIM 201450 and MIM 201475], long-chain 3-hydroxyl-CoA dehydrogenase/mitochondrial trifunctional protein [MIM 609016/609015], carnitine palmitoyltransferase I and II [MIM 255120/600650] and carnitine-acylcarnitine translocase [MIM 212138]), two endocrine disorders (congenital hypothyroidism [MIM 218700], congenital adrenal hyperplasia [MIM 201910]), cystic fibrosis [MIM 219700], severe combined immunodeficiencies (SCID, T cell-negative), sickle cell disease [MIM 603903] and spinal muscular atrophy [MIM 604320].[9]

Evaluation studies have become an important source of evidence for the extension of NBS programmes, evaluating technical feasibility, diagnostic process quality, and the health benefit for individuals with defined candidate diseases.

This evaluation study on 18 IMDs, conducted in the NBS laboratories in Hanover, Heidelberg and Munich aims to evaluate the suitability of these candidate

diseases for inclusion in the national NBS programme. Based on the expert opinion of the NBS laboratories and metabolic physicians involved, the conditions were assigned to three categories in analogy to the three traffic light colours: green (suitable), yellow (possibly suitable) and red (unsuitable).

2 | METHODS

2.1 | Disease panel of the NBS evaluation study

The following diseases were evaluated: galactokinase deficiency [MIM 604313], carnitine transporter defect (CTD) [MIM 212140], citrullinemia type I and II [MIM 215700 and 605 814], 3-hydroxy-3-methylglutaryl (HMG)-CoA lyase deficiency [MIM 246450], multiple acyl-CoA dehydrogenase deficiency [MIM 231680], malonyl-CoA decarboxylase deficiency [MIM 606761], isolated methylmalonic acidurias (methylmalonyl-CoA mutase deficiency [MIM 251000], cblA- and cblB-type MMA [MIM 251100 and 251 110]), propionic acidemia [MIM 606054] and the remethylation disorders methylenetetrahydrofolate reductase (MTHFR) deficiency [MIM 236250] and inherited disorders of the metabolism of vitamin B_{12} (adenosyl- and/or methylcobalamin) resulting in homocystinuria with elevated methylmalonic acid (deficiency of cblC, cblD, cblF and cblJ [MIM 277400, 277 410, 277 380, 614 857]) and normal methylmalonic acid (deficiency of cblE and cblG [MIM 236270 / 250 940]), respectively. The main rationale for the disease panel was to make the best possible use of the MS/MS-based analysis by utilising analytes, which had hitherto not been reported in the German NBS programme. Applying second-tier tests were required to increase the low specificity and sensitivity of the screening parameters propionylcarnitine (C3) and methionine (Met). Adding the determination of galactose enabled NBS for galactokinase deficiency.

2.2 | Analytical methods

Galactokinase deficiency was identified using galactose as the primary screening parameter in combination with the enzymatic activity of galactose-1-phosphate uridyltransferase (GALT) determined to identify individuals with classic galactosemia. Galactose was quantified using a photometric or fluorometric microassay.[10,11] Of note, elevated concentrations of galactose in individuals with normal GALT activity might also identify individuals with the rare genetic galactosemias uridyldiphosphate-galactose epimerase (GALE; [MIM 230350]) and galactose mutarotase (GALM; [MIM 618881]) deficiency (Table 1).

All other disorders were identified by quantitative analysis of acylcarnitines and amino acids in dried blood spots (DBS) by MS/MS as previously described with modifications.[12–14] The cut-offs differed slightly between the three laboratories (Data S1).

Since the study was performed from the same samples as routine NBS, additional pre-analytical or analytical efforts were not required with the exception of the second-tier tests, which were performed in about 8% of all samples. To identify 11 of the 18 conditions, abnormal MS/MS first-tier results for (i) increased concentrations of C3 and (ii) decreased concentrations of Met were supplemented by second-tier strategies reverting to the same filter card. The same second-tier tests were performed in all three laboratories by liquid chromatography-MS/MS (LC–MS/MS) after elution of whole blood from DBS using a modification of the method previously described by la Marca et al.[15]

In samples with elevated C3 concentrations, methylmalonic (MMA), 3-hydroxypropionic (3OH-PA) and methylcitric acid (MCA) were quantitated by LC–MS/MS in the same DBS sample to identify individuals with propionic acidemia as well as isolated methylmalonic acidurias and cobalamin-related remethylation disorders associated with elevated MMA[16] (Table 1).

In samples with decreased Met concentrations, total homocysteine (tHcy) and MMA were quantified to identify individuals with cobalamin-related remethylation disorders with low Met and elevated tHcy associated with or without increased MMA[17] (Table 1).

Data interpretation was performed following algorithms previously described.[15,18–21] Of note, these algorithms also identify newborns with vitamin B_{12} deficiency. The results were reported previously.[20–23]

Whenever one of the second-tier parameters was above cut-off, the NBS result was considered positive and confirmatory analysis was performed either in plasma (Met, tHcy, MMA) or in urine (MMA, 3OH-PA and MCA) using gas chromatography/MS[24,25] (Table 1). Except for CTD and galactokinase deficiency, all candidate disorders can be associated with early metabolic decompensations and/or require a swift start of therapy. Thus, the families were to be contacted on the day of the positive screening result to start confirmatory testing.

2.3 | Study population

According to the national NBS directive in Germany, DBS samples are to be taken from newborns at 36–72 h of life. Participation in the NBS programme is voluntary, and the costs are covered by the health insurance.[9] A cumulative participation rate significantly above 99% is

TABLE 1 Diagnostic pathways and confirmatory testing.

	NBS first-tier		NBS second/third-tier		
Disease	**Parameter(s)**	**Method**	**Parameter(s)**	**Method**	**Confirmatory test**
Carnitine transporter defect	Free carnitine < lower cut-off	MS/MS	–	–	Free carnitine in plasma, tubular reabsorption Genetic testing [*SLC22A5*]
Citrullinemia type I and II	Citrulline > upper cut-off	MS/MS	–	–	Amino acids in plasma Genetic testing [*ASS1; SLC25A13*]
Galactokinase deficiency	Galactose > upper cut-off and normal GALT activity	photo−/fluorometric microassay	–	–	Enzyme activity Genetic testing [*GALK1*] If unremarkable: consider enzyme or genetic testing [*GALE*; *GALM*]
3-Hydroxy-3-methyl-glutaryl (HMG)-CoA lyase deficiency	3-methyl-glutaryl-carnitine and 3-OH-isovaleryl-carnitine > upper cut-off	MS/MS	–	–	Organic acids in urine Genetic testing [*HMGCL*]
Multiple acyl-CoA dehydrogenase deficiency	Short, medium, long chain acylcarnitines > upper cutoff	MS/MS	–	–	Acylcarnitine profile Organic acids in urine Genetic testing [*ETFDH; ETFA; ETFB*]
Malonyl-CoA decarboxylase deficiency	Malonyl-carnitine > upper cut-off	MS/MS	–	–	Acylcarnitine profile, enzyme activity Genetic testing [*MLYCD*]
Isolated methylmalonic acidurias ($mut^{0/-}$, CblA, CblB) and propionic acidemia	C3; C3/C2; C3/C4, C3/C16 > upper cut-off	MS/MS	Methylmalonic acid Methylcitric acid 3-OH-propionic acid	LC–MS/MS	Organic acids in urine Methylmalonic acid in plasma and urine Genetic testing [*MMUT; MMAA; MMAB*] Genetic testing [*PCCB; PCCA*]
MTHFR deficiency and cobalamin related remethylation disorders (CblC, CblD, CblE, CblG, CblF, CblJ)	Methionine (Met) Met/Phenylalanine < lower cut-off	MS/MS	Total Homocysteine Methylmalonic acid Methylcitric acid 3-OH-propionic acid	LC–MS/MS	Methionine, total homocysteine in plasma Methylmalonic acid in urine and plasma Vitamin B_{12} status Genetic testing [*MTHFR; MMACHC; PRDX1; MMADHC; LMBRD1; ABCD4; MTRR; MTR*]

Abbreviations: MS/MS, tandem mass spectrometry; GALT, Galactose-1-phosphate uridyltransferase; GALE, Uridyldiphosphate-galactose epimerase; GALM, Galactose mutarotase; C2, acetyl-carnitine; C3, propionyl-carnitine; C4, butyryl-carnitine (includes isobutyryl-carnitine); C16, palmitoyl-carnitine, MTHFR, methylenetetrahydrofolate reductase.

achieved, highlighting the high level of acceptance of this programme. In addition to the current programme, parents were offered to participate in the collaborative NBS evaluation study, which was conducted in the three largest NBS centres in Germany (Hanover, Heidelberg, Munich) starting at different times. The study was approved by the local ethics committees in Hanover (7771_BO_K_2018), Heidelberg (S-533/2015; DRKS-ID DRKS00025324; S-104/2005, DRKS-ID DRKS00013329) and Munich (RoLAK 09074, 2009).

2.4 | Statistical analysis

Differences in citrulline concentrations both in plasma and DBS samples of patients with early-onset and

attenuated phenotypes of citrullinemia type I were analysed using a Mann–Whitney U test. A p value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Study cohort

The participating NBS centres analysed a total of 1 777 264 NBS samples (Hanover 429 276 [05/2018–12/2020, participation rate 99.9%], Heidelberg 379 557 [08/2016–12/2020, participation rate 67%] and Munich 968 431 [01/2010–12/2020, participation rate 99.9%]). 441 positive NBS results were reported (recall rate 0.02%) resulting in 68 confirmed diagnoses and 373 false positives. Fourteen individuals (21%) were symptomatic when the NBS results became available. The positive predictive value (PPV) ranged from 0.07 (CTD) to 0.67 (HMG-CoA lyase deficiency). Three false negative cases (galactokinase deficiency, cblG, MMA mut$^-$) came to our knowledge. The cumulative prevalence of candidate conditions was approximately 1 in 26 000 newborns. For each candidate condition, details on true and false positives, birth prevalences, process times and ages of the confirmed cases at different time points of the screening process are summarised in Table 2.

3.2 | Disease (group)-specific results

3.2.1 | Carnitine transporter defect

During the study period, 18 individuals with CTD were identified resulting in a birth prevalence of 1 in 98 737 (95% confidence interval [CI] 98 592; 98 882) newborns. Concentrations of free carnitine (C0) were markedly decreased in NBS samples (median 4.1 μmol/L; range 1–6) (reference 7–62) as well as in subsequent confirmatory tests in plasma (median 3.4 μmol/L; range 1.2–8.2) (reference 9–93) or DBS (median 4 μmol/L; range 3–4). Suspected diagnosis of CTD was confirmed by the identification of bi-allelic pathogenic variants in the *SLC22A5* gene in 16 of 18 individuals (Table 3).

3.2.2 | Citrullinemia type I and II

Nine individuals with citrullinemia type I (birth prevalence: 1 in 197 474 newborns; 95% CI 197184; 197 764) but none with citrullinemia type II were identified. Three individuals were asymptomatic at the time of diagnosis, while three presented with hyperammonemic decompensations prior to the report of NBS results. One case was considered asymptomatic but showed plasma hyperammonemia (293 μmol/L) at the time of admission for confirmatory testing. The initial clinical presentation in two individuals with confirmed diagnosis remained unknown. Individuals with citrullinemia type I were identified using elevated citrulline in DBS. Of note, the cut-off applied for citrulline differed among the three NBS centres. A lower cut-off was used by two (Hanover <65 μmol/L, Heidelberg <90 μmol/L) and a higher cut-off by one (Munich <220 μmol/L). In asymptomatic individuals with citrullinemia type I, citrulline concentrations of 216, 358 and 716 μmol/L were found in DBS and 271, 568 and 399 μmol/L at confirmation in plasma. In comparison, symptomatic individuals showed a trend to higher citrulline concentrations in DBS ($n = 4$: 699; 837; 933; 982 μmol/L) ($p = 0.08$) and had higher citrulline concentrations at confirmation in plasma ($n = 4$: 3956; 1756; 2104; 1500 μmol/L) ($p = 0.03$) (Table 4).

3.2.3 | Galactokinase deficiency

We identified ten individuals with galactokinase deficiency. One additional case was missed due to normal galactose concentration at the time of NBS sampling (42 h) despite feeding with breast milk and infant formula. He presented with cataracts at the age of 19 months and a galactokinase activity below the limit of detection. Including this false-negative patient, a birth prevalence of 1 in 127 064 (95% CI 126854; 127 275) newborns was estimated. The median concentration of galactose in DBS was 8770 μmol/L (range 4385-13 266; cut-off <1665) at a median age at blood sampling of 48 h (range 24–63). Diagnosis was confirmed by reduced activities of galactokinase in erythrocytes (median 0.33 nmol/min × g Hb; range 0–2) (reference 6–90) in eight and elevated concentrations of galactitol in urine (8555 mmol/mol creatinine; reference 5–60) in one case. Genetic analysis of the *GALK1* gene was performed in five cases. No cases of GALE or GALM deficiency were detected.

3.2.4 | 3-Hydroxy-3-methylglutaryl (HMG)-CoA lyase deficiency

Two individuals with HMG-CoA lyase deficiency were identified resulting in an estimated birth prevalence of 1 in 888 632 newborns (95% CI 887326; 889 939). Both of them developed metabolic decompensation within the first days of life before NBS results were available. Elevated acylcarnitines in DBS at baseline were confirmed in patients A and B: 3-methyl-glutaryl-carnitine (A: 0.20 to 0.29, B: 0.24 to 0.37 μmol/L; cut-off <0.17) and

TABLE 2 Sample sizes, numbers of true and false positives and performance evaluation of the screened disorders.

	Carnitine transporter defect	Citrullinemia type I	Galactokinase deficiency	3-hydroxy-3-methyl-glutaryl (HMG)-CoA lyase deficiency	Multiple acyl-CoA dehydrogenase deficiency	Malonyl-CoA decarboxylase deficiency	Isolated methylmalonic acidurias ($mut^{0/-}$, CblA, CblB)	Propionic acidemia	MTHFR deficiency and remethylation disorders
Screened samples	1 777 264	1 777 264	1 397 707[a]	1 777 264	1 777 264	1 397 707[a]	1 777 264	1 777 264	1 777 264
Suspected diagnosis	243	21	19	3	15	5	40	48	47
True positives (confirmed cases)	18	9	10	2	3	2	11	5	4 / 4
False positives	225	12	9	1	12	3	29	43	39
False negatives	0	0	1	0	0	0	1	0	1
Birth prevalence 95% CI	1: 98737 [98 592; 98 882]	1: 197474 [197 184; 197 764]	1: 127064[b] [126 854; 127 275]	1: 888632 [887 326; 889 939]	1: 592421 [591 551; 593 293]	1: 698854 [697 695; 700 013]	1: 148105[b] [147 888; 148 323]	1: 355453 [354 930; 355 976]	1: 197474[b] [197 184; 197 764]
Positive predictive value	0.07	0.43	0.53	0.67	0.20	0.40	0.27	0.10	0.09/0.09
Deceased	0	0	0	0	1	0	0	0	0
Consanguinity	Yes (4) No (4) Data missing (10)	Yes (3) No (3) Data missing (3)	Yes (1) No (6) Data missing (3)	Yes (1) No (1)	Yes (1) No (2)	Yes (1) No (1)	Yes (4) No (4) Data missing (3)	Yes (1) No (4)	Yes (2) No (3) Data missing (3)
Ethnicity	Afghanistan Germany/Hungary Kazakhstan (2) Macedonia Pakistan unknown (12)	Bulgaria, Germany Pakistan, Italy Kazakhstan, Turkey unknown (3)	Germany (1) Sinti/Roma (1) unknown (8)	Afghanistan Afghanistan/Guinea	Turkey USA/Bulgaria unknown (1)	Germany unknown	Afghanistan, Egypt Germany (2) Pakistan, Poland Turkey unknown (4)	Germany (3) Tunisia (1) unknown (1)	Germany (3) Italy (1) Turkey (2) unknown (2)
Gestational age [weeks] Median (range)	38 (37–41)	40 (35–41)	38 (33–41)	39;39	37 (35–40)	37;38	39 (38–41)	39 (32–40)	39 (36–41)
Process evaluation									
Age at									
Sampling [hours]	39 (2–72)	58 (39- > 72)	48 (24–63)	40;49	44 (41–44)	64;38	50 (37–77)	48 (36–59)	46 (36–57)
Sample receipt [days]	5 (3–6)	5 (4–90)	4 (4–5)	4;4	6 (5–7)	4;8	5 (2–8)	5 (3–6)	5 (2–8)
Positive screening result [days]	3 (3–8)	6 (4–91)	5 (4–6)	4;7	6 (5–8)	5;9	6 (3–15)	7 (3–7)	7 (3–7)
Start of confirmatory testing [days]	13 (6–44)	5 (2–7) Data missing (2)	6 (3–14) Data missing (2)	3;14	7 (6–9)	7;17	4 (3–15) Data missing (2)	8 (3–9)	11 (5–28)
Start of therapy [days]	19 (4–60) Data missing (8)	6 (2–8) No therapy (1) Data missing (2)	7 (5–10)	2;15	9 (2–60)	7;17	4 (1–16) Data missing (2)	7 (3–10)	11 (5–28)

TABLE 2 (Continued)

	Carnitine transporter defect	Citrullinemia type I	Galactokinase deficiency	3-hydroxy-3-methyl-glutaryl (HMG)-CoA lyase deficiency	Multiple acyl-CoA dehydrogenase deficiency	Malonyl-CoA decarboxylase deficiency	Isolated methylmalonic acidurias ($mut^{0/-}$, CblA, CblB)	Propionic acidemia	MTHFR deficiency and remethylation disorders
Symptoms	Yes (0) No (12) Data missing (6)	Yes (3) No (4) Data missing (2)	Yes (0) No (8) Data missing (2)	Yes (2)	Yes (2) No (1)	No (2)	Yes (5) No (3) Data missing (3)	Yes (2) No (2) Data missing (1)	Yes (0) No (6) Data missing (2)
Onset of symptoms [days]	–	2;2; unknown	-	2;15	2;3	–	3 (1–8)	3;3	–

Abbreviations: MTHFR, Methylenetetrahydrofolate reductase; CI, confidence interval [lower; upper].

[a]Not analysed in one laboratory.

[b]Including one false negative newborn.

3-hydroxy-isovaleryl-carnitine (A: 2.4 to 3.8, B: 1.3 to 2.3 μmol/L, cut-off <0.51). The diagnosis was further verified by increased urinary excretion of 3-hydroxy-3-methyl-glutaric, 3-methyl-glutaconic, 3-methyl-glutaric, 3-hydroxy-isovaleric acid and 3-methyl-crotonyl-glycine. Sequence analysis of the *HMGCL* gene revealed a homozygous mutation in one case (c.286delC;p.Q96Rfs11). Of note, 3-hydroxy-isovaleryl-carnitine and isobaric acylcarnitines also indicate deficiencies of 3-methylcrotonyl-CoA carboxylase [MIM 210200] and ß-keto-thiolase [MIM 203750], which are not part of the screening scope.

3.2.5 | Multiple acyl-CoA dehydrogenase deficiency

We identified three individuals with multiple acyl-CoA dehydrogenase deficiency (MADD) resulting in a prevalence of 1 in 592 421 newborns (95% CI 591551; 593 293). Two of them presented with a severe neonatal phenotype with life-threatening metabolic decompensation at the age of 2 and 3 days, respectively. One of them died at the age of 9 months. The third child remained asymptomatic and demonstrated a less pronounced biochemical phenotype with a normal concentration of C4 acylcarnitines (Table 5).

3.2.6 | Malonyl-CoA decarboxylase deficiency

During the study period, two individuals with malonyl-CoA decarboxylase deficiency (MLYCDD) were identified resulting in an estimated birth prevalence of 1 in 698 854 (95% CI 697695; 700 013) newborns. Diagnosis was confirmed by highly elevated urinary excretion of malonic acid in both newborns combined with elevated methylmalonic acid in one. Sequence analysis of the *MLYCD* gene revealed a homozygous mutation in one case (c.791delA; p.N264Tfs*6).

3.2.7 | Isolated methylmalonic acidurias and propionic acidemia

Eleven individuals with isolated methylmalonic aciduria were identified. One additional case (MMA mut^{-}) was identified at the age of 3 years and 10 months following metabolic decompensation precipitated by adenoviral gastroenteritis. At the time of NBS sampling (53 h), his C3 concentration was slightly elevated (6.2 μmol/L; cut-off 5.9 μmol/L) with normal ratios of C3/C2, C3/C4 and C3/C16. At least one of three ratios (mean value + 4SD)

TABLE 3 Biochemical and genetic data of individuals with carnitine transporter defect.

Confirmed cases	**18**
Newborn screening (dried blood spot)	
Free carnitine, C0 [μmol/l] median (range) Cut-off > 7	4.1 (1–6)
Confirmatory testing	
C0 in plasma [μmol/l] median (range) Cut-off > 9	3.4 (1.2–8.2) data missing (5)
C0 in dried blood spot [μmol/l] median (range) Cut-off > 7	4 (3–4)
Molecular genetics (*SLC22A5* gene)	
cDNA (Allele 1/Allele 2) Protein	
Homozygous variants	Compound heterozygous variants
c.136C > T/c.136C > T (2 patients) p.Pro46Ser/p.Pro46Ser	c.136C > T/c.67_69delTTC p.Pro46Ser/p.Phe23del
c.506G > A/c.506G > A (2 patients) p.Arg169Gln/p.Arg169Gln	c.136C > T/c.522C > T p.Pro46Ser/p.Phe174=
c.641C > T/c.641C > T[a] p.Ala214Val/p.Ala214Val	c.136C > T/c.695C > T p.Pro46Ser/p.Thr232Met
c.729_731delCCT/ c.729_731delCCT p.Leu244del/ p.Leu244del	c.453G > A/c.1364C > G p.Val151=/p.Pro455Arg
c.1139C > T/c.1139C > T p.Ala380Val/p.Ala380Val	c.844C > T/c.-149G > A p.Arg282*/5′UTR initiation codon
c.1319C > T/c.1319C > T p.Thr440Met/p.Thr440Met	
Homozygous variant, but not reported (3) Data missing (2)[a]	

[a]Patients reported previously.[26]

being elevated or an isolated concentration of C3 > 10 μmol/L would have been required to trigger second-tier analysis. Including the false-negative patient, this accounts for a birth prevalence of 1 in 148 105 newborns (95% CI 147888; 148 323). In nine cases, methylmalonic aciduria was caused by bi-allelic pathogenic variants of *MMUT*, resulting in deficiency of the mitochondrial enzyme methylmalonyl-CoA mutase, while in three other individuals, the synthesis of the cofactor adenosylcobalamin was affected due to pathogenic variations in *MMAA* ($n = 2$, cblA type) and *MMAB* ($n = 1$, cblB type), respectively. In five newborns with isolated methylmalonic acidurias, first symptoms already occurred at the time of the first NBS report (Table 6).

Five individuals with propionic acidemia were identified resulting in a birth prevalence of 1 in 355 453 newborns (95% CI 354930; 355 976). Two of them presented with acute metabolic decompensation at the age of 3 days prior to the availability of NBS results (Table 6).

3.2.8 | Methylenetetrahydrofolate reductase (MTHFR) deficiency and cobalamin-related remethylation disorders (cblC, cblG)

Overall, four individuals with MTHFR deficiency and four with cblC deficiency were identified. In addition, one child with cblG with a methionine concentration of 8 μmol/L (cut-off <7) was diagnosed at the age of 4 months presenting with severe anaemia (haemoglobin 2.8 g/dL) and recurrent vomiting due to sinus vein thrombosis. In retrospect, a clearly elevated homocysteine concentration of 107 μmol/L was found in the initial DBS sample. Including the false-negative patient, a birth prevalence of 1 in 197 474 newborn (95% CI 197184; 197 764) was found (Table 7).

4 | DISCUSSION

Grey and coworkers claimed that all screening programmes do harm, while some do good as well or even more good than harm (at a reasonable cost).[1] Therefore, every extension of NBS programmes must be carefully evaluated to minimise harm and maximise benefit. Evaluation of 18 candidate conditions utilising data from the present study elucidated that some of these conditions seem suitable for inclusion in NBS programmes, while some show limitations and one appears unsuitable (Table 8).

4.1 | Carnitine transporter defect

CTD, also referred to as primary carnitine deficiency, is characterised by urinary carnitine wasting, low serum carnitine concentrations and decreased intracellular carnitine supply caused by dysfunction of the OCTN2 transporter. As carnitine is involved in β-oxidation of fatty acids, clinical symptoms of the disorder comprise hypoketotic hypoglycemia, hepatic encephalopathy, skeletal, cardiac myopathy and arrhythmia. Clinical manifestation of CTD can be prevented by treatment with L-carnitine.

TABLE 4 Biochemical and genetic data of individuals with citrullinemia type I.

	Hanover	Heidelberg	Munich
Screened samples	429 276	379 557	968 431
Confirmed cases	2	3	4
False positives	3	9	0
Newborn screening (dried blood spot)			
Citrulline [μmol/l]	180;211 (cut-off <65)	**216**;**716**;837 (cut-off <90)	**358**;699;933;982 (cut-off <220)
Confirmatory testing			
Citrulline in plasma [μmol/l] Reference 9–35	220 Data missing (1)	**271**;**399**;1756	**568**;1500;2104;3956
Arginine in plasma [μmol/l] Reference 6–130	25 Data missing (1)	**26**;**28**;60	**12**;15;28;173
Blood ammonia [μmol/l] Reference <110 (newborn)	107 Data missing (1)	**normal**;**70**;2000	**normal**;886;1160; 293
Orotic acid in urine	Not detectable (1) Data missing (1)	Not detectable (1) Detectable (2)	Not detectable (3) Detectable (1)
Mutational analysis *ASS1* gene cDNA (Allele 1/Allele 2) Protein	Data missing (2)	**c.787G > A/c.787G > A** **p.Val262Met/p.Val262Met** **c.787G > A/c.787G > A** **p.Val262Met/p.Val262Met** c.1030C > T/c.597 + 1021_838 + 1952del p.Arg344*/p.(Asn200Valfs*16)	c.1165A > C/c.1165A > C p.Thr389Pro/p.Thr389Pro c.1168G > A/c.1168G > A p.Gly390Arg/p.Gly390Arg c.40G > A/c.40G > A p.Gly13Ser/p.Gly13Ser **Data missing (1)**

Note: Data of asymptomatic individuals in bold.

Being a potentially life-threatening but readily treatable disorder and its marker metabolite C0 being detected by MS/MS, CTD seemed justified to be included in NBS programmes. However, C0 concentrations in neonates during the first days of life are strongly influenced by maternal C0 concentrations and reflect C0 concentrations of the mothers rather than of the newborns. Hence, NBS programmes for CTD relying on C0 concentrations within the first days of life have been shown to result in low sensitivity and specificity and to identify considerable numbers of asymptomatic carnitine-deficient mothers with unknown clinical relevance.[26] Due to these findings and a low incidence as well as a high prevalence of asymptomatic patients, NBS for CTD was discontinued in New Zealand.[27] The present study confirms high numbers of false positives, resulting in a low PPV of 0.07, in line with previous reports of PPVs between 0.02 and 0.05.[27–30]

Sequence analysis of the *SLC22A5* gene confirmed a low frequency of nonsense, frame-shift and splice-site variants.[31] Bi-allelic pathogenic variants (c.506G > A, c.1319C > T)[31,32] were identified in three of 18 newborns. The majority of variants were missense, inframe or silent variants that have mainly been described in NBS cohorts, i.e. newborns or their asymptomatic mothers. Three of the pathogenic variants (c.136C > T, c.641C > T, c.-149G > A) have been shown to retain substantial residual carnitine transport activity.[32–34] Thus, in addition to the low PPV, individuals with a possibly benign phenotype were identified in a significant proportion of cases. To increase specificity and PPV, it has been proposed using supplemental biomarkers in addition to C0, coupling *SLC22A5* sequencing or second blood sampling at a later age.[35] In the absence of a suitable screening strategy for the reliable detection of clinically relevant CTD within the first days of life, CTD does not seem to be suitable to be included in NBS panels.

4.2 | Citrullinemia type I

Citrullinemia type I is one of the most common urea cycle defects with a heterogeneous clinical manifestation ranging from severe, life-threatening hyperammonemic encephalopathy within the first days of life to more variable attenuated phenotypes presenting after the newborn period or even remaining asymptomatic without need for therapy. This highlights the challenge of including citrullinemia type I in NBS disease panels. A large observational study recently demonstrated that NBS reduced the severity of hyperammonemic crises in newborns, but not

TABLE 5 Clinical, biochemical and genetic data of individuals with multiple acyl-CoA dehydrogenase deficiency.

	Cut-off	Patient 1	Patient 2	Patient 3
Clinical symptoms		Yes	Yes (deceased)	No
Newborn screening (dried blood spot) [μmol/l][a]				
Butyryl-carnitine, C4[b]	<0.91	**8.3**	**3.51**	0.34
Isovaleryl-carnitine, C5	<0.39	**0.74**	**2.0**	0.27
Glutaryl-carnitine, C5DC	<0.34	**2.76**	**0.99**	**0.69**
Hexanoyl-carnitine, C6	<0.19	**0.42**	**0.62**	**0.31**
Octanoyl-carnitine, C8	<0.23	**0.76**	**0.75**	**0.57**
Decanoyl-carnitine, C10	<0.30	**0.95**	**0.64**	**0.69**
Dodecanoyl-carnitine, C12	<0.27	**1.0**	**1.88**	**1.3**
Tetradecenoyl-carnitine, C14:1	<0.48	**1.9**	**1.53**	**1.12**
Palmitoyl-carnitine, C16	<7.46	3.3	**11.75**	4.47
Oleoyl-carnitine, C18:1	<2.29	1.1	**2.29**	1.35
Free carnitine, C0	>7	12	24	19
Confirmatory testing (urine) [mmol/mol Crea]				
Glutaric acid	<8	1113	1067	11
2-hydroxy-glutaric acid	<30	Highly elevated	400	75
Lactic acid	<431	Highly elevated	143	61
Ethylmalonic acid	<19	Highly elevated	300	12
Adipic acid	<30	Highly elevated	248	10
Suberic acid	<10	Highly elevated	74	5
Sebacic acid	<4	Highly elevated	8	1
Isovaleryl-glycine	<17	Not detected	123	1
Hexanoyl-glycine	<2	Not detected	80	1
Molecular genetics				
cDNA (Allele 1/Allele 2) Protein		*ETFA* Homozygous variant consistent with a severe neonatal phenotype (variant not reported)	*ETFDH* c.1142G > C/c.1142G > C p.Gly381Ala/p.Gly381Ala	*ETFDH* c.325A > G/c.1205C > T p.Ile109Val/p.Thr402Ile

[a]Elevated values in bold.
[b](includes isobutyryl-carnitine).

the frequency of subsequent hyperammonemic episodes in individuals receiving dietary treatment and nitrogen scavengers.[36,37] In comparison to the pre-NBS era, individuals with attenuated phenotypes are likely to be overrepresented in NBS cohorts.[36,38] In our cohort, a severe first hyperammonemic event could only be prevented in one of four symptomatic patients, while three individuals remained asymptomatic, two of them with a predicted attenuated phenotype (p.Val262Met; 40% residual activity).[39] Citrulline concentrations in plasma were significantly lower in these three individuals compared to the symptomatic patients and did not exceed 600 μmol/L indicating attenuated phenotypes.[39] In one screening centre, a higher cut-off was set for citrulline (220 μmol/L) to largely avoid identifying asymptomatic variants and thus overtreatment. By this measure, no false positive was found in 968 431 samples. If a cut-off of 220 μmol/L had been applied in all three centres, three individuals (predicted attenuated, $n = 2$; data missing, $n = 1$) would not have been identified and all 12 false positives would have been avoided. More information is required to further optimise the cut-off for citrulline. Despite these limitations, the benefits of NBS for individuals with this disease should be considered significant.

4.3 | Galactokinase deficiency

Galactokinase deficiency affects the phosphorylation of galactose to galactose-1-phosphate and results in the accumulation of galactose and galactitol. If untreated,

TABLE 6 Biochemical and genetic data of individuals with isolated methylmalonic acidurias and propionic acidemia.

		Methylmalonic acidurias ($mut^{0/-}$, cblA, cblB)	Propionic acidemia
Confirmed cases	–	11	5
Newborn screening (dried blood spot)	Cut-off	Median (range)	
C3 [μmol/l]	<5.9	11.4 (8.4–23.2)	12.3 (6.2–23)
C0 [μmol/l]	>7	19 (10.3–42)	18.1 (16.9–24)
C3/C2	<0.2	0.42 (0.26–2.11)	0.42 (0.25–1.06)
C3/C4	<31.1	53 (25–154), available for 8/11 patients	45 (22–84)
C3/C16	<1.81	3.2 (2.1–12.3)	1.92 (1.17–6.7)
Methylmalonic acid [μmol/l] – second-tier	<3	422 (175–1198)	1.0 (0–1.8)
Methylcitric acid [μmol/l] – second-tier	<0.7	14.9 (4.0–24.1), available for 7/11 patients	24.5 (1.8–47.1)
3-hydroxy-propionic acid [μmol/l] – second-tier	<30	131 (53–359), available for 8/11 patients	84.3 (37.4–764)
Confirmatory testing			
Methylcitric acid (urine), qualitative		Detectable	Detectable, available for 7/11 patients
Methylcitric acid (urine) [mmol/mol Crea], quantitative	<9	985 (629–2098), available for 3/11 patients	194;921, available for 2/5 patients
3-hydroxy-propionic acid (urine), qualitative		Detectable, available for 6/11 patients	Detectable
3-hydroxy-propionic acid (urine) [mmol/mol Crea], quantitative	<170	674;5179, available for 2/11 patients	102;5141, available for 2/5 patients
3-hydroxy-valeric acid (urine), qualitative		Detectable, available for 6/11 patients	detectable
3-hydroxy-valeric acid (urine) [mmol/mol Crea], quantitative	<214	511;855, available for 2/11 patients	15;688, available for 2/5 patients
Propionyl-glycine (urine), qualitative		Detectable, available for 6/11 patients	detectable
Propionyl-glycine (urine) [mmol/mol Crea], quantitative	<2	1;2, available for 2/11 patients	2;140, available for 2/5 patients
Methylmalonic acid (urine) [mmol/mol Crea]	<4	11.371 (7–34 886)	
Methylmalonic acid (plasma) [μmol/l]	<0.27	420 (391–1315)	
Methylmalonic acid (dried blood spot)) [μmol/l]	<3	723 (570–1038)	
Homocysteine (plasma) [μmol/l]	<14	3.7 (1.3–10)	

(Continues)

TABLE 6 (Continued)

	Methylmalonic acidurias (mut$^{0/-}$, cblA, cblB)	Propionic acidemia
Molecular genetics/enzymatic testing		
cDNA (Allele 1/Allele 2) Protein	*MMUT* c.360dupT/c.1084-10A > G p.Lys121*/IVS 5 [false negative NBS result] c.1420C > T/c.142C > T[a] p.R474*/p.R474* c.277C > A/c.277C > A p.Arg93Ser/ p.Arg93Ser c.2179C > T/c.2179C > T p.Arg727*/p.Arg727* c.296 T > C/deletion exon 8[a] p.M99T c.1741C > T/c.2194_2197del p.Arg581*/p.Ala732Trpfs*6 c.1420C > T/c.753G > A p. ARG474*/p.Lys251 *MMAA* c.455C > G/deletion exon 1 p.152R *MMAB* c.197-1G > T/c.197–1 G > T splice site variant IVS2 Three patients confirmed, but variants not reported	*PCCA* c.2002G > A/− p.G668R; none Since only one mutation was identified, it was followed by the measurement of propionyl-CoA carboxylase in fibroblasts with a residual activity of 2%. c.1852–1899 + 28del/c.2119-9A > G Deletion >960 bp incl. exon 21/splice site variant IVS24 *c.1456C > T/c.1456C > T* p.A486*/p.A486* *PCCB* c.313G > A/c.1439_1445delTTGCAGT p.D105N/p.I480fs*89 c.313G > A/ c.1218_1231delinsTAGAGCACAGGA p.ASP105Asn/p.Gly407Argfs*14

Note: The gene names (MMUT, MMAA, MMAB, PCCA, PCCB) have been underlined.

Abbreviations: C0, free carnitine; C2, acetyl-carnitine; C3, propionyl-carnitine; C4, butyryl-carnitine (includes isobutyryl-carnitine); C16, palmitoyl-carnitine.

[a]Patients/variants in *MMUT* reported previously.[20]

TABLE 7 Biochemical and genetic data of individuals with methylentetrahydrofolate reductase deficiency and cobalamin-related remethylation disorders.

	MTHFR deficiency	CblC deficiency	CblG deficiency
Confirmed cases	4	4[a]	False negative
Newborn screening (dried blood spot)			Analysed retrospectively
Methionine [μmol/l] Cut-off: Hanover >7 Heidelberg, Munich >11 and methionine/phenylalanine	4,5,6,8	9;9;10;31	8
Methionine/phenylalanine Cut-off: Hanover, Munich >0.18 Heidelberg 0.26–0.56	0.07;0.08;0.13;0.15	0.14;0.23;0.30;0.35	0.19
Homocysteine [μmol/l] Cut-off: <14	75;104;150;221	27;37;150;–	107
Methylmalonic acid [μmol/l] Cut-off <3	Below cut-off (2) not analysed (2)	8;38;60;34	Below cut-off
Propionylcarnitine [μmol/l] Cut-off: <5.9	Below cut-off	8.9;10.4;15.7	Below cut-off
Confirmatory testing			Analysed at diagnosis
Methionine in plasma [μmol/l] Reference >12	2;5;6;7;	6;30;30;–	Not analysed
Homocysteine in plasma [μmol/l] Reference <14	12;205;215;253	32;198;258;–	201
Methylmalonic acid (plasma) [μmol/l] Reference <0.27	Not analysed	264;23420;139500;–	Not analysed
Methylmalonic acid (urine) [mmol/mol Crea] Reference <10	Not analysed	265;283;2576;2791	Not analysed
Molecular genetics			
	MTHFR	*MMACHC*	*MTR*
cDNA (Allele 1/Allele 2) Protein	c.1632 + 2 T > G/c.1632 + 2 T > G splice site variation IVS10 c.1690 T > G/c.1690 T > G[c] p.Phe564Val/p.Phe564Val Compound heterozygosity (variants not reported) Not analysed (1)	c.271dupA/c.271dupA p.Arg91Lysfs*14/p.Arg91Lysfs*14 c.271dupA/c.276G > T p.R91Kfs*14/p.E92D c.271dupA/c.440G > C[b] p.R91Kfs*14/p.G147A c.81 + 1G > T/deletion exon 4[b] splice site variant IVS1	c.382G > C/c.1283 T > G p.Ala128Pro/p.Met428Arg

[a]Three newborns identified by elevated concentrations of propionylcarnitine and methylmalonic acid.
[b]Patients/variants in *MMACHC* reported previously.[20]
[c]Patient reported previously.[23]

galactokinase deficiency leads to the formation of bilateral cataracts in early infancy which conveys a risk of developmental impairment, the need for lensectomy and eventually blindness. Timely treatment with a lactose-free diet prevents visual impairment by averting and resolving cataract formation.

Our data confirm previous studies and case series by showing clearly elevated concentrations of galactose (median 8770 μmol/L; range 4385–13 266 μmol/L) in affected neonates.[40] Applying a cut-off of 1665 μmol/L resulted in a PPV of 0.53. A further reduction of the false-positive rate might be achieved by increasing the cut-off to 2500 μmol/L.[40] Interestingly, our NBS cohort comprised a false-negative individual revealing an inconspicuous galactose concentration at the age of 42 h. So far, unremarkable galactokinase screening results have only been reported in two affected neonates screened before the age of 24 h (9.9 and 16.3 h, respectively), presumably caused

TABLE 8 Assignment of candidate diseases to the categories green (suitable), yellow (possibly suitable) and red (unsuitable).

Disease	Analytical method	PPV	Mode of Confirmation	Benefit of treatment	Comments
Galactokinase deficiency	Photometric assay	0.53	Biochemical, genetic	+	Effective dietary treatment. One false negative patient.
3-Hydroxy-3-Methyl-Glutaryl (HMG)-CoA lyase deficiency	MS/MS	0.67	Biochemical, genetic	+/− d.o.p	Frequent early decompensation before NBS results are available. Long-term outcome favourable despite frequent early decompensation. High positive predictive value.
Citrullinemia type I and II	MS/MS	0.43	Biochemical, genetic	+/− d.o.p.	Frequent early decompensation before NBS results are available. Identification of attenuated phenotypes. Long-term outcome uncertain despite treatment. Optimisation of the citrulline cut-off is to be considered.
Methylenetetrahydrofolate reductase deficiency	MS/MS + 2nd tier	0.09	Biochemical, genetic	+	Favourable outcome of pre-symptomatic treatment.
Remethylation disorders (CblC/CblG)	MS/MS + 2nd tier	0.09	Biochemical, genetic	+	Favourable outcome of pre-symptomatic treatment. One false negative patient (CblG).
Multiple acyl-CoA dehydrogenase deficiency	MS/MS	0.20	Biochemical, genetic	+/− d.o.p.	Potential benefit of pre-symptomatic treatment for late-onset phenotypes. Effective treatment for severe, early-onset phenotypes uncertain.
Isolated methylmalonic acidurias ($mut^{0/-}$, CblA, CblB)	MS/MS + 2nd tier	0.27	Biochemical, genetic	+/−− d.o.p.	Frequent early decompensation before NBS results are available. One false negative patient, long-term outcome uncertain. No effective exclusion of patients with propionic acidemia.
Propionic acidemia	MS/MS + 2nd tier	0.10	Biochemical, genetic	+/−− d.o.p.	Frequent early decompensation before NBS results are available. Long-term outcome poor.
Malonyl-CoA decarboxylase deficiency	MS/MS	0.40	Biochemical, genetic	+/−	Extremely rare, course unpredictable. Effective early treatment of cardiomyopathy.
Carnitine transporter defect	MS/MS	0.07	Biochemical, genetic	+	No reliable detection within the first days of life. High percentage of false positives, identification of asymptomatic mothers.

Abbreviations: MS/MS, tandem mass spectrometry; PPV, positive predictive value; d.o.p., depending on phenotype.

by a still low ingestion of milk.[41] Thus, it needs to be considered that galactokinase deficiency is the only disorder within the current newborn screening panel that requires milk feeding, that is, breastmilk or lactose-based infant formula, to be reliably detected. Affected neonates with feeding difficulties and neonates on parenteral or special nutrition such as hypoallergic formula are at risk to be missed.

Conceivably, the finding of elevated concentrations of galactose with normal GALT activity would also identify individuals with the rare conditions of GALE and GALM deficiency. Both conditions are predominantly associated with mild to no clinical phenotypes and, hence, are prone to overdiagnosis and overtreatment.[42,43]

Given the severe developmental disabilities arising from untreated or late-treated cataract, the straightforward and effective dietary treatment option, and a screening strategy taking advantage of and complementing screening for classical galactosemia (GALT activity), galactokinase deficiency seems suitable for inclusion in NBS programmes.

4.4 | 3-hydroxy-3-methylglutaryl (HMG)-CoA lyase deficiency

HMG-CoA lyase deficiency affects the formation of ketone bodies from fatty acids and leucine resulting in metabolic decompensation with hypoketotic hypoglycemia, lactic acidosis, hepatic dysfunction and hyperammonemia. Clinical manifestation consists of acute decompensation with vomiting, encephalopathy and moderate hepatomegaly. Symptoms occur mostly within the first year of life with a neonatal onset in approximately 40% of patients. The long-term outcome of the disease has been described to be favourable despite the high frequency of early metabolic decompensation.[44]

HMG-CoA lyase deficiency is a rare disorder with only few patients having been identified by NBS so far. Thus, the long-term benefit of early detection by NBS remains to be elucidated. In this study, both individuals with HMG-CoA lyase deficiency sustained metabolic decompensation before NBS results were available. However, NBS enabled swift diagnosis without further delay. In addition, a single false-positive was generated among 1.77 million samples resulting in a high PPV of 0.67 and thus a low burden for the healthy population. Since HMG-CoA lyase deficiency is a potentially life-threatening disorder with favourable long-term outcome and the primary disease biomarkers are routinely available by MS/MS, we recommend the implementation of HMG-CoA lyase deficiency into the NBS disease panel.

4.5 | Multiple acyl-CoA dehydrogenase deficiency

MADD affects dehydrogenases requiring riboflavin as a cofactor and is caused by a defective electron transfer to the respiratory chain. The compromised dehydrogenases are involved in amino acid metabolism and β-oxidation of fatty acids. Early-onset manifestation occurs during neonatal age with severe decompensation comprising hypoglycemia, metabolic acidosis and hyperammonemia, often accompanied by hepatomegaly and cardiomyopathy. The manifestation of late-onset phenotypes is variable ranging from metabolic decompensation to isolated myopathy up to asymptomatic courses. Therapeutic management implies a low-fat and low-protein diet, avoidance of fasting and supplementation of riboflavin, carnitine and ketones. The screening parameters comprise acylcarnitines accumulating from lysine and tryptophan degradation as well as β-oxidation of short-, medium- and long-chain fatty acids. Of note, genetic defects of riboflavin transport as well as maternal vitamin B_{12}- and riboflavin deficiency mimicking biochemical features of MADD have been described and might also be identified by NBS.[45–47]

MADD is a rare disorder with only a few patients identified by NBS so far. The benefit of early detection by NBS remains to be elucidated and can be expected to be low for patients with severe, early-onset phenotypes, except for a clear early diagnosis. Predominantly individuals with late-onset phenotypes might benefit from early diagnosis and treatment, but with a considerable risk of overtreatment.

We identified two individuals with severe, early-onset MADD and markedly elevated concentrations of butyrylcarnitine (C4) (of note: includes also isobutyryl-carnitine), whereas the asymptomatic individual displayed a normal concentration of C4. One might hypothesise that an elevation of C4 might help to discriminate between severe (early onset) and mild (late onset) phenotypes.[48] In addition, acylcarnitine profiles indicative of MADD might be found among positive screening results for glutaric acidemia I or deficiencies of medium-chain and very long-chain acyl-CoA dehydrogenase.

MADD is a potentially life-threatening disorder with readily treatable late-onset phenotypes and diagnostic biomarkers are detectable by MS/MS. Despite the discussed limitations, the inclusion of MADD in NBS programmes might be considered.

4.6 | Malonyl-CoA decarboxylase deficiency

Malonyl-CoA, the substrate for MLYCD plays a role as an intermediate in fatty acid biosynthesis and as a

regulatory effector of fatty acid β-oxidation by inhibition of carnitine palmitoyltransferase I.[49] It has been shown that the accumulation of malonyl-CoA also inhibits fatty acid uptake and β-oxidation in both mitochondria and peroxisomes.[50]

MLYCDD is an extremely rare inherited metabolic disorder with an estimated prevalence of less than 1 in 400 000. Since its first description in 1984, at least 50 patients have been reported. Even after the introduction of NBS,[51] birth prevalence remained very low. Since most individuals were presented as single case reports, little is known about (i) the natural course of the condition, (ii) the significance of the highly variable clinical phenotype with most common manifestations related to neurological involvement and cardiomyopathy and (iii) the benefit of therapy. In two publications, nine patients each were characterised clinically, biochemically and molecularly.[52,53] The course is unpredictable and can significantly differ even in siblings harbouring identical mutations.[54] Although many aspects of the disease are not yet understood, it could be shown, for example, that early treatment can lead to rapid improvement of cardiomyopathy. Due to the low prevalence, there is a low risk of identifying a larger number of (i) healthy and (ii) potentially asymptomatic newborns (in this studies 3 and 2 in 1.4 million, respectively), which would generate a psychological burden for afflicted families. In summary, due to the insufficient level of knowledge at this point in time, MLYCDD should not yet be considered for NBS programmes.

4.7 | Isolated methylmalonic acidurias and propionic acidemia

Isolated methylmalonic acidurias and propionic acidemia are caused by enzyme (methylmalonyl-CoA mutase, propionyl-CoA carboxylase) or cofactor (cblA, cblB) deficiencies involved in the catabolism of propionate. Early-onset phenotypes of the disorders occur within the first days of life and manifest with hyperammonemia and metabolic acidosis. Late-onset phenotypes may occur at any age and show a more variable clinical presentation. Except for vitamin B_{12}-responsive phenotypes of isolated MMA, the overall long-term outcome of the disorders remains poor with the currently available conventional therapeutic strategies comprising nutritional therapy, carnitine supplementation and emergency treatment. The majority of patients develop neurocognitive impairment. Organ affections, most notably cardiomyopathy and chronic kidney disease are frequent. However, organ transplantation, particularly liver transplantation, is increasingly being considered not only in patients with poor metabolic control or to treat the long-term complications of the diseases, but also as a pre-emptive measure in early-diagnosed patients.[55–57]

Isolated methylmalonic acidurias and propionic acidemia have been implemented in NBS programmes with scant evidence for an improvement in long-term outcome so far.[58–60] Our data confirm previous findings that NBS will not prevent sequelae arising from early metabolic decompensation in a significant proportion of patients.[58–61]

The sole determination of C3 and respective ratios was shown to be insufficient to detect inherited disorders of propionate metabolism due to both poor specificity and sensitivity. The implementation of second-tier testing for MMA, MCA and 3OH-PA reduced false positives and bears the potential to lower cut-offs of C3 in order to avoid false negatives.[16,62] The PPVs for isolated methylmalonic acidurias and propionic academia were 0.27 and 0.10, respectively, comparable to previous studies.[62] A lower cut-off for C3 would have identified the false-negative individual with methylmalonyl-CoA mutase deficiency. However, this measure would entail a considerable increase in the number of second-tier tests being required.

Of note, the metabolites C3 and MMA are not specific for genetically determined methylmalonic acidurias but are also sensitive parameters to detect vitamin B_{12} deficiency. Thus, NBS strategies involving C3 and MMA as screening parameters will inevitably also identify newborns with maternally derived vitamin B_{12} deficiency.[63] The results of major parts of our screening cohort regarding vitamin B_{12} deficiency have been previously reported.[20–22] The prevalence of an impaired vitamin B_{12} status in newborns seems to be high.[64] However, the clinical impact of these alterations remains unknown in a considerable number of newborns and their mothers and needs to be elucidated.

Except for a reduced time to diagnosis and the prevention of metabolic decompensation in some individuals with attenuated phenotypes, the beneficial effect of NBS on the long-term outcome of isolated methylmalonic acidurias and propionic acidemia seems to be limited. Due to the discussed limitations, these disorders might not be considered to be included in NBS programmes at this point in time.

4.8 | Methylenetetrahydrofolate reductase (MTHFR) deficiency and cobalamin-related remethylation disorders (cblC, cblG)

Inborn errors of remethylation affect the formation of methionine from homocysteine caused by an impaired enzyme activity of methionine synthase (cblE/cblG) or by

a deficient supply of the cofactors cobalamin (cblC, cblD, cblF and cblJ) or folate (MTHFR). The clinical manifestation varies from early onset within the first weeks of life with acute neurologic deterioration, apnoea and feeding difficulties to later onset during infancy and childhood with progressive neurologic deterioration. Multisystem involvement affecting bone marrow, eye, kidney or gastrointestinal tract is frequent. The biochemical hallmark of remethylation disorders are low Met and elevated tHcy concentrations. An additional elevation of MMA is found in defects affecting transport and processing of methylcobalamin and adenosylcobalamin (cblC, cblD, cblF and cblJ).[65] MTHFR and cblC deficiency are the most prevalent inborn errors of folate and cobalamin metabolism, respectively. Pre-symptomatic diagnosis and early therapeutic intervention have been shown to significantly improve outcome for both disorders and to enable normal cognitive and neurodevelopmental outcome in individuals with MTHFR deficiency.[66–70] The approach of combining low Met concentrations with second-tier determination of tHcy allowed the diagnosis of 4 individuals with MTHFR deficiency and one individual with cblC deficiency, while 3 individuals with cblC deficiency were diagnosed by elevated concentrations of C3 followed by second-tier analysis of MMA and tHcy. One individual with cblG deficiency was missed. Met levels in MTHFR deficiency have been reported to range from 4 to 18 μmol/L and may be in the low reference range in remethylation disorders.[65] Assessment of both the Met concentration and the Met/phenylalanine ratio increases the diagnostic discriminatory power. Severe cognitive and neurodevelopmental disabilities occur in patients treated late and can be prevented by pre-symptomatic treatment. The NBS strategy combines routinely available screening parameters (Met, C3) with feasible second-tier parameters (tHcy and MMA). Thus, MTHFR deficiency and cobalamin-related disorders seem suitable to be included in NBS programmes.

5 | LIMITATIONS AND STRENGTHS

Combining the data from three different NBS laboratories implied slightly different screening strategies. However, except for cobalamin-related remethylation disorders, these differences are mainly applied to cut-offs and reference parameters. Of note, the evaluation of the different cut-off strategies allowed us to suggest valuable cut-off modifications to avoid both false positives in NBS for citrullinemia and false negatives in NBS for MTHFR deficiency and cobalamin-related remethylation disorders. Reporting the results of confirmatory testing to the involved screening laboratories is recommended by the German regulatory authorities, but not mandatory. Although the diagnoses of all identified patients were confirmed biochemically and/or molecularly, some data could not be retrieved.

The important strength of the compiled data of this study is the sample size of 1.77 million newborns. The results give a profound basis to assess both the prevalences of candidate diseases and the diagnostic (process) quality of applied methodologies and screening algorithms. In addition, they disclose the challenges to meet before implementing these disorders into the German NBS panel.

6 | CONCLUSIONS

Expanding the German NBS panel by implementing galactokinase deficiency, HMG-CoA-lyase deficiency, citrullinemia, MTHFR deficiency and remethylation disorders seems feasible and justified. For some disorders, it remains to be elucidated whether benefits of early diagnosis and treatment outweigh possible harms resulting from overtreatment of attenuated or even asymptomatic phenotypes. The carnitine transporter defect is not suitable to be included.

First metabolic decompensation in early-onset intoxication type IMDs cannot be prevented by NBS in many individuals. However, NBS reduces time to diagnosis, allows genetic counselling for family planning and may reduce the number of unclearly deceased newborns. It is crucial to set DBS sampling at the earliest reliable time point (i.e., at about the age of 36 h).

Our data emphasise the need for mandatory and structured reporting of confirmatory testing results and for nationwide prospective outcome studies to estimate the effectiveness and benefits of NBS as a means of public health. Since targeted therapies such as systemic mRNA therapy and gene replacement therapy are under development, the long-term health impact of NBS for individuals with these conditions might require a re-evaluation in the future.

TAKE-HOME MESSAGE

Large evaluation study to assess the suitability of 18 inborn errors of metabolism for newborn screening.

AUTHOR CONTRIBUTIONS

Conceptualization and study design: Esther M. Maier, Wulf Röschinger, Nils Janzen, Ulrike Mütze, Stefan Kölker, Georg F. Hoffmann; Initial manuscript draft: Esther M. Maier, Wulf Röschinger, Ulrike Mütze, Stefan Kölker; Coordination of data collection and data analyses: Esther M. Maier, Wulf Röschinger, Nils Janzen, Ulrike Mütze, Stefan Kölker; Data collection and clinical

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

ETHICS STATEMENT

All participating centres received approval from their local ethics committees before enrolling patients: Hanover (7771_BO_K_2018), Heidelberg (S-533/2015; DRKS-ID DRKS00025324; S-104/2005, DRKS-ID DRKS00013329) and Munich (RoLAK 09074, 2009).

The study was conducted in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all parents/caregivers to participate in the study.

ANIMAL RIGHTS

This article does not contain any studies with animal subjects performed by any of the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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