



# High incidence of maternal vitamin B<sub>12</sub> deficiency detected by newborn screening: first results from a study for the evaluation of 26 additional target disorders for the German newborn screening panel

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## Abstract

**Background** Newborn screening (NBS) in Germany currently includes 15 target disorders. Recent diagnostic improvements suggest an extension of the screening panel.

**Methods** Since August 2016, a prospective study evaluating 26 additional target disorders (25 metabolic disorders and vitamin B<sub>12</sub>-deficiency) in addition to the German screening panel is performed at the Newborn Screening Center Heidelberg. First-tier results from tandem-MS screening are complemented by second-tier strategies for 15 of the additional target disorders. NBS results of seven patients diagnosed symptomatically with one of the additional target disorders by selective screening since August 2016 are retrospectively evaluated.

**Results** Over a 13-month period, 68,418 children participated in the study. Second-tier analyses were performed in 5.4% of samples. Only 59 (0.1%) of study participants had abnormal screening results for one of the additional target disorders. Target disorders from the study panel were confirmed in 12 children: 1 3-hydroxy-3-methylglutaryl coenzyme A (CoA)-lyase deficiency, 1 citrullinemia type I, 1 multiple acyl-CoA dehydrogenase-deficiency, 1 methylenetetrahydrofolate reductase-deficiency, and 8 children with maternal vitamin B<sub>12</sub>-deficiency. In addition, six of seven patients diagnosed symptomatically outside the study with one of the target disorders would have been identified by the study strategy in their NBS sample.

**Conclusions** Within 13 months, the study “Newborn Screening 2020” identified additional 12 children with treatable conditions while only marginally increasing the recall rate by 0.1%. Maternal vitamin B<sub>12</sub>-deficiency was the most frequent finding. Even more children could benefit from screening for the additional target disorders by extending the NBS panel for Germany and/or other countries.

**Keywords** Metabolic disorders · Newborn screening · Second-tier · Vitamin B<sub>12</sub> deficiency

## Introduction

Newborn screening (NBS) is a prerequisite for early diagnosis and successful treatment of many severe conditions presenting in the neonatal period or infancy. The German NBS panel currently includes 15 target disorders, including 12 metabolic disorders, 2 endocrine disorders, and cystic

fibrosis. Recent improvements in diagnostics and therapeutics suggest an extension of the existing screening panel [1]. For example, there is conclusive evidence for the benefit of early identification by NBS and consecutive early treatment for patients with homocystinuria due to cystathionine β-synthase (CBS) deficiency, remethylation disorders, especially severe methylenetetrahydrofolate reductase (MTHFR) deficiency, and some disorders of intracellular cobalamin (Cbl) metabolism [2–4]. In addition, for severe maternal vitamin B<sub>12</sub> deficiency, a clear benefit of early detection and treatment is evident for both child and mother [5–7]. NBS for propionic aciduria and methylmalonic acidurias is recommended in some countries, e.g., Austria, Spain, and the United States [8, 9], while others have decided not to include

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these disorders in their screening panel [10, 11]. NBS for these disorders as well as for combined remethylation disorders based on results for propionylcarnitine (C3) and its respective ratios with other acylcarnitines is, however, associated with a high false-positive rate [10, 12]. Second-tier strategies have been proposed to reduce the false-positive rate [12–15].

Second-tier strategies are a promising tool for NBS. In this approach, by analysis of additional, more specific second-tier metabolites for the respective target disorder from the same first specimen in case of abnormal results for primary parameters, the false-positive rate in NBS may be significantly reduced. We previously evaluated and presented new strategies for NBS for classical homocystinuria, remethylation disorders and vitamin B<sub>12</sub> deficiency in cohorts from Qatar and Germany [16, 17], and suggested a second-tier strategy for screening for these disorders in Germany using methionine (Met), ratio Met/phenylalanine (Met/Phe), C3 and ratio C3/acetylcarnitine (C3/C2) as first-tiers, and total homocysteine (tHcy) as second-tier if at least one of the first-tier parameters was out of range [16]. In August 2016, a study named “Newborn Screening (NBS) 2020” was started at the Newborn Screening Center, Heidelberg. In this project, an extension of the German NBS panel of 15 disorders (Table 1) by 26 additional conditions (25 metabolic disorders and vitamin B<sub>12</sub> deficiency, Table 2) is evaluated including also remethylation disorders, propionic and methylmalonic acidurias, and vitamin B<sub>12</sub> deficiency, utilizing several second-tier strategies [18]. The objective of the study is to evaluate: 1)

technical feasibility of NBS for these disorders in a population-based NBS programme in Germany, and 2) the benefit for detected patients based on whether patients were successfully identified presymptomatically. The rationale for now presenting findings of the first 13 months of this study is that awareness of these findings is important from a public health point of view. Providing proof for the feasibility of the methods used in a population-based screening programme and of the benefit for patients identified will hopefully lead to timely inclusion of these additional disorders into the NBS panel for Germany as part of regular health insurance.

The target disorders were chosen according to: 1) the assumed feasibility of NBS using either first-tier parameters from tandem-MS screening only or additional second-tier strategies from dried blood spots (DBS), and 2) the assumed benefit of early detection and early treatment. For some disorders, solely existing parameters from tandem-MS screening were used as first-tiers, which are so far already measured in all samples, but not evaluated in NBS according to the German national screening panel, e.g., carnitine transporter deficiency, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-lyase deficiency, citrullinemia type I, argininosuccinate lyase deficiency, and multiple acyl-CoA dehydrogenase deficiency (MADD). For 15 of the target disorders, results from tandem-MS screening as first-tier results were complemented by second-tier testing from DBS for methylmalonic acid (MMA), 3-OH-propionic acid (3-OH-PA), methylcitric acid (MCA), and/or tHcy (compare Table 2). In addition, screening for tyrosinemia type I was performed using succinylacetone from DBS as first-tier parameter. Disorders regarded as non-diseases such as e.g., short chain acyl-CoA dehydrogenase deficiency or 3-methylcrotonyl-CoA carboxylase deficiency or disorders without sufficient evidence for benefit of early treatment such as non-ketotic hyperglycinemia were not included in the study.

**Table 1** Current target disorders of the national German newborn screening programme

Disorders	
Endocrine disorders	Congenital hypothyroidism
	Congenital adrenal hyperplasia
Metabolic disorders	Biotinidase deficiency
	Galactosaemia (classical)
	Phenylketonuria/hyperphenylalaninemia (including cofactor deficiencies)
	Maple syrup urine disease
	Glutaric aciduria type I
	Isovaleric aciduria
	Medium-chain acyl-CoA dehydrogenase deficiency
	Long-chain-3-OH-acyl-CoA-dehydrogenase deficiency
	Very long-chain acyl-CoA-dehydrogenase deficiency
	Carnitine palmitoyltransferase I deficiency
	Carnitine palmitoyltransferase II deficiency
	Carnitine acylcarnitine translocase deficiency
	Cystic fibrosis

## Methods

### Newborn screening in the study NBS 2020

The national German NBS panel currently includes 15 disorders (Table 1). Samples for NBS in Germany are to be taken between the 36th and 72nd hour of life onto NBS cards (Neonatal Screening Card; Whatman filter paper 903, GE Healthcare Europe GmbH, Freiburg, Germany) and dried for 3–4 hours at room temperature. Samples are then transported to the Heidelberg NBS laboratory by mail. At this screening center, samples from about 130,000–140,000 newborns, mainly from southwest Germany, are screened every year. This equals about 17% of children born annually in Germany. The population screened by this laboratory should be

**Table 2** Additional target disorders and diagnostic algorithms in the study Newborn Screening 2020

Disorders	First tier	Second tier
Tyrosinemia type I	Succinylacetone	None
Classical homocystinuria (CBS deficiency)	Methionine/phenylalanine (high)	Homocysteine
MTHFR deficiency, Cbl-D-Hcy, Cbl E-, G-defect	Methionine (low), methionine/phenylalanine (low)	Homocysteine
Cbl C-, D-, F-, J-defect, transcobalamin II deficiency	Propionylcarnitine (C3), C3/acetylcarnitine (C2)	MMA, MCA, homocysteine
Methylmalonic aciduria (mut <sup>0</sup> , mut <sup>-</sup> , Cbl A- and Cbl B-defect)	C3, C3/C2	MMA, MCA
Vitamin B <sub>12</sub> deficiency	C3, C3/C2, methionine (low), methionine/phenylalanine (low)	MMA, MCA, homocysteine
Propionic aciduria	C3, C3/C2	3-OH propionic acid, MCA
Urea cycle disorders, e.g., citrullinemia type I, argininosuccinate lyase deficiency	Citrulline, argininosuccinate	None
Multiple acyl-CoA dehydrogenase deficiency	Multiple acylcarnitines (C4-C18)	None
HMG-CoA lyase deficiency	3-hydroxyisovalerylcarnitine (C5-OH), methylglutaryl-carnitine (C6-DC)	None
Carnitine transporter deficiency	Free carnitine (C0)	None

*CBS* cystathionine β-synthase, *MTHFR* methylenetetrahydrofolate reductase, *Cbl* cobalamin, *Hcy* homocysteine, *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *MMA* methylmalonic acid, *MCA* methylcitric acid

representative for the rest of Germany without any known major differences in the incidence of certain target disorders of NBS [19] or differences concerning nutritional deficiencies. NBS including electrospray ionization tandem-MS (Micromass Ultima, Waters Xevo TQD, Waters, Milford, MA, USA) for determination of amino acids and acylcarnitines was performed in all samples as previously described [20]. In addition to the regular NBS panel, all senders (hospitals, paediatricians, midwives, etc.) were asked to inform parents about the study NBS 2020 since August 2016. This study screens for 25 additional metabolic disorders and for vitamin B<sub>12</sub>-deficiency (Table 2) from the same specimen as the regular NBS. By German legislation written informed consent of a least one legal guardian is required as a prerequisite for NBS. If parents decided to participate in the “NBS 2020” study, written informed consent for the study had to be sent with the sample to the Heidelberg Newborn Screening Center. The study was approved by the Institutional Review Board of the University Hospital Heidelberg.

Metabolites already available from tandem-MS screening were used as primary markers for 25 of the target disorders in the study (Table 2). These parameters had already been measured for all samples before the study, but according to regulation results had to be blinded in medical validation as respective disorders are not targeted by the current German NBS panel. Screening for tyrosinemia type I was performed by measurement of succinylacetone from DBS as primary marker [21]. For 15 of the target disorders first-tier results from tandem-MS screening were complemented by second-tier testing from the same DBS for MMA, 3-OH-PA, MCA, and/or tHcy (compare Table 2). The algorithms to

proceed to second-tier analysis were developed in analogy to the strategy previously proposed by us for samples from Germany based on a retrospective evaluation of confirmed patients with remethylation disorders and vitamin B<sub>12</sub> deficiency [16]. In this strategy, second-tier measurements of tHcy from DBS are performed based on abnormal first-tier results for Met (< cut-off low) and Met/Phe ratio (< cut-off low or > cut-off high). tHcy from DBS was analysed as described previously [22]. In addition to Hcy MMA, 3-OH-PA, and MCA were analysed as second-tier parameters from the same DBS using a newly established method [23], based on abnormal first-tier results for C3 + ratio C3/C2 (> cut-off), or ratio C3/C2 (> cut-off), or C3 above alarm limit. The respective first and second-tier parameters per target disorder are shown in Table 2. The cut-offs currently used in our study are shown for the relevant first-tier and all second-tier parameters per patient in Tables 3 and 4.

Depending on the grade of pathology in the first DBS analysis, recommendations given are either to send a repeat sample for analysis of acylcarnitines and amino acids, tHcy, MMA, 3-OH-PA, and MCA in DBS, or to send also plasma and urine samples for additional analyses.

Diagnosis of vitamin B<sub>12</sub> deficiency in the child was established in cases with elevation of one or more functional markers of vitamin B<sub>12</sub> deficiency (MMA in plasma and/or urine, Hcy in plasma) in conformational analysis in the presence of vitamin B<sub>12</sub> serum levels below the norm or in the lower normal range. In addition, normalization of all functional markers of vitamin B<sub>12</sub> deficiency and of vitamin B<sub>12</sub> serum levels after supplementation had to be documented to establish this diagnosis. In all of these cases, work up of the

mother's vitamin B<sub>12</sub> status was recommended, including functional markers (MMA in plasma and/or urine, and Hcy in plasma). "Maternal vitamin B<sub>12</sub> deficiency" was stated if vitamin B<sub>12</sub> levels in the mother were below the normal range and/or functional markers of vitamin B<sub>12</sub> deficiency were elevated. This does not yet include any implications on the cause of the deficiency (nutritional, malabsorption, etc.). Dietary history in mothers was taken and in cases of "maternal vitamin B<sub>12</sub> deficiency" which could not be explained by the mother's dietary regimen, work-up by a specialist in internal medicine was recommended including evaluation of vitamin B<sub>12</sub> malabsorption. If results for vitamin B<sub>12</sub> were provided by an external laboratory in the unit pg/mL, results and reference ranges of the respective laboratory were converted to pmol/L for better comparability and are presented in addition to the original result in the results section.

Measurement of MMA in urine and/or plasma for conformational diagnostics in the metabolic laboratory in Heidelberg was performed using stable isotope-labelled D3-MMA as internal standard in analogy to [24].

### Retrospective evaluation of NBS results and the strategy of NBS 2020 in patients identified by selective screening

During the course of the study, seven symptomatic patients with one of the target disorders of the study were diagnosed by selective metabolic investigation. Six of these patients were either born before the study had started (patients S2, S3, and S7) or did not participate in the study (patients S1, S5, and S6). Patient S4 participated in the study, but first-tier results in the first DBS did not fulfil criteria for second-tier analyses. The applied cut-offs of our study were retrospectively evaluated in all these patients, in whom NBS results were available from our center. C3, C3/C2, Met, Met/Phe are being measured in all children from Germany screened by tandem-MS screening at our center before and outside the study, but according to regulation results have to be blinded in medical validation as respective disorders are not targeted by the current German NBS panel. The results for these metabolites from tandem-MS screening are, however, available in the NBS database and were retrieved for this study. If the original NBS DBS was still available, second-tier analyses (tHcy, MMA, 3-OH-PA, and MCA) were performed retrospectively in the samples of these patients. If several dried blood spots had been measured in NBS for these patients (e.g., due to prematurity, etc.) the first sample taken after the age of 36 hours of life was evaluated. Retrospective evaluation of screening data of these patients has also been approved by the Institutional Review Board of the University Hospital Heidelberg.

## Results

### Newborn screening in the study NBS 2020

From 1st of August 2016 to 31st of August 2017, 68,418 children participated in the study. Second-tier analyses were performed in 5.4% of these samples based on out-of-range first-tier results according to the decision criteria described above. After second-tier analysis, 59 (0.1%) of study participants remained with abnormal screening results for one of the target disorders of the study, with need for a second DBS or additional work-up. Of these 59 cases, 33 were classified abnormal after use of a second-tier strategy, 26 were recalled for a target disorder without a second-tier strategy in place [9 carnitine transporter deficiency, 1 HMG-CoA-lyase deficiency, 2 citrullinemia type I, 1 MADD, 13 tyrosinemia type I (the high number of recalls for tyrosinemia type I being explained in the course of adaptation of a newly established cut-off for succinylacetone in DBS)]. In two of the 59 cases, the transfer of the final result of confirmation is still pending (two cases with suspicion of vitamin B<sub>12</sub> deficiency or isolated remethylation disorder). A target disorder from the study panel was confirmed in altogether 12 children. In four of these, an inherited metabolic disorder was confirmed: 1 HMG-CoA-lyase deficiency, 1 citrullinemia type I, 1 MADD, 1 MTHFR deficiency. In eight children and one additional sibling (twin) maternal vitamin B<sub>12</sub> deficiency was diagnosed. The screening results including results of the second-tier analyses for the patients with maternal vitamin B<sub>12</sub> deficiency and isolated remethylation disorder (patients N1-N10) are shown in detail in Table 3. All patients were referred for further work-up to a metabolic center and all except one (MADD) were clinically asymptomatic at time of diagnosis. The study participation rate at the end of the reported time period for the month of August 2017 was 61.3%.

Patient N1 showed slightly elevated C3 and ratio C3/C2, as well as slightly decreased Met in the first screening card. Second-tier analysis from this sample showed increased MMA, MCA and tHcy (for detailed results compare Table 3). Work-up in patient N1 revealed elevated Hcy of 27.9 µmol/L in plasma, elevated methylmalonic acid in urine (280 mmol/mol Krea), and very low vitamin B<sub>12</sub> in serum (66 pg/mL; equivalent to 49 pmol/L). Also in the mother vitamin B<sub>12</sub> was low (185 pg/mL, *N* 191–663) (equivalent to 137 pmol/L, *N* 141–489), methylmalonic acid in plasma was slightly elevated, while Hcy in plasma was normal. The mother did not adhere to any restrictive (vegan/vegetarian) diet during the pregnancy. The final diagnosis in the mother is pending, work-up has been planned including gastrointestinal malabsorption of vitamin B<sub>12</sub>. The child was



**Table 4** Newborn screening (NBS) results and DBS results at time of selective diagnostics of seven patients with one of the new target disorders of the study, who were diagnosed by selective diagnostics due to clinical symptoms

Patient No.	Diagnosis	Hcy (μmol/L)	Met (μmol/L)	Met/Phe	C3 (μmol/L)	C3/C2	C3/C0	C3/Met	MMA (μmol/L)	3-OH-PA (μmol/L)	MCA (μmol/L)	Remarks	
S1	Combined remethylation disorder (Cbl C, D, F, or J) <sup>a</sup>	Cut-off	Low 11 (P 5) High 35 (P 99.5)	Low 0.26 (P 5) High 0.56 (P 89)	5.5 (between P 99 and P 99.5)	0.22 (P 99.5)	0.28 (P 99.5)	0.37	2.14 (P 99.9)	34.48 (P 99.9)	0.08 (P 99.9)	Retrospective tandem-MS measurement and second tier determination 3 mon after sampling	
		First DBS (NBS, 3rd d of life)	54.5	5	0.15	4.21	0.30	0.21	0.84	26.29	13.34	0.21	Selective diagnostics at age 8 d
S2	Mild propionic aciduria	Second DBS (8th d of life)	111.5	5	0.14	9.70	0.75	0.54	61.87	41.46	0.78	Retrospective second tier determination 11 mon after sampling	
		First DBS (NBS)	2.1	17	0.53	9.39	0.55	0.78	3.24	73.81	1.24	Selective diagnostics at age 11 mon	
S3	Vitamin B <sub>12</sub> deficiency	Second DBS	ND	14	0.50	5.03	0.56	0.36	2.72	49.20	0.36	Retrospective second tier determination 9 mon after sampling	
		First DBS (NBS)	24.1	11	0.25	2.34	0.12	0.11	19.70	30.03	0.09	Selective diagnostics at age 8 mon	
S4	Vitamin B <sub>12</sub> deficiency	Second DBS	58.4	7	0.21	2.43	0.12	0.12	11.65	26.11	0.10	Retrospective second tier determination 2.5 mon after sampling	
		First DBS (NBS)	9.9	12	0.39	1.09	0.05	0.08	2.35 (P 99.9) <sup>b</sup>	43.32 (P 99.9) <sup>b</sup>	0.34	Selective diagnostics at age 2.5 mon	
S5	Methylmalonic aciduria	Second DBS	9.9	23	0.55	2.69	0.11	0.07	9.24	33.41	0.34	Retrospective second tier determination 2.5 mon after sampling	
		First DBS (NBS)	7.1	14	0.12	14.19	0.51	0.59	1.01	2266.84	373.92	35.31	Selective diagnostics at age 4 d
S6	Cbl C defect	Second DBS	94.0	5	0.12	7.02	0.78	0.64	1.40	229.11	68.69	6.08	Retrospective second tier determination 2.5 wk after sampling
		First DBS (NBS)	85.6	5	0.14	7.89	1.97	1.13	1.58	326.41	63.90	7.55	Selective diagnostics at age 3 wk
S7	Methylmalonic aciduria	Second DBS	2.3	16	0.32	6.30	0.26	0.37	0.39	1018.64	297.04	5.42	Retrospective second tier determination 10.5 mon after sampling
		First DBS (NBS)	2.8	14	0.39	4.67	0.31	0.58	0.33	279.76	141.32	1.30	Selective diagnostics at age 11 mon

Three of these children did not participate in the study (S1, S5, and S6), three were born before the study was started (S2, S3, and S7). Patient S4 participated in the study, but did not fulfil criteria for second-tier analysis in the NBS sample. Out-of-range results with regard to cut-offs in the current strategy of the pilot project NBS 2020 are marked in bold. DBS dried blood spot, NBS newborn screening, Cbl cobalamin, Hcy homocysteine, Met methionine, Phe phenylalanine, C3 propionylcarnitine, C2 acetylcarnitine, C0 free carnitine, MMA methylmalonic acid, 3-OH-PA 3-OH propionic acid, MCA methylcitric acid. <sup>a</sup>In patient S1, final subspecification of the combined remethylation disorder was still pending at time of publication, therefore, diagnosis is stated as “combined remethylation disorder” (includes Cbl-C, D-MMA-Hcy, F, J); <sup>b</sup>In the course of the study, the method for second-tier measurement MMA, 3-OH-PA, and MCA was adapted to new reagents, therefore, the cut-off for the parameters had changed at the time patients S4-7 were evaluated



treated with parenteral vitamin B<sub>12</sub>, followed by oral supplementation. This led to rapid normalization of all parameters (Hcy in plasma, methylmalonic acid, vitamin B<sub>12</sub>). In addition, the mother has been supplemented with vitamin B<sub>12</sub> parenterally.

Patient N2 showed decreased Met and ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed increased tHcy. Work-up in patient N2 showed elevated Hcy in plasma of 36 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA 101.8 mmol/mol creatinine,  $N < 10$ ), and low normal vitamin B<sub>12</sub> in serum 258 pg/mL ( $N$  211–911) (equivalent to 190 pmol/L,  $N$  156–672). At the time of work-up in the child vitamin B<sub>12</sub> was also low in the mother (167 pmol/L,  $N$  160–670), without any signs of functional vitamin B<sub>12</sub> deficiency (methylmalonic acid in urine and Hcy in plasma normal). The mother did not adhere to any restrictive diet during the pregnancy, but showed iron deficiency during pregnancy and was reported to have been a poor eater. The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of all parameters (Hcy in plasma, methylmalonic acid in urine, and vitamin B<sub>12</sub>). Therefore, the diagnosis of functional vitamin B<sub>12</sub> deficiency in the child due to low maternal vitamin B<sub>12</sub> level was established.

Patient N3 showed elevated ratio Met/Phe with low normal Met in the first screening card. Second-tier analysis from this sample showed increased tHcy. Work-up in patient N3 revealed elevated Hcy in plasma of 20 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA 38.8 mmol/mol creatinine,  $N < 10$ ), and low vitamin B<sub>12</sub> in serum (151 pmol/L,  $N$  160–670). Vitamin B<sub>12</sub> was also low in the mother (159 pmol/L,  $N$  160–670). The mother showed a slight elevation of Hcy in plasma (19 µmol/L,  $N < 15$ ), and normal methylmalonic acid in urine. The mother did not adhere to any restrictive diet during pregnancy, but reported pronounced feeding difficulties in pregnancy due to massive heartburn. She showed severe iron deficiency during pregnancy which required supplementation. Patient N4, twin sibling to patient N3, was also diagnosed with vitamin B<sub>12</sub> deficiency in the course of selective work-up due to the sibling's (N3) abnormal NBS result. The child N4 himself had not fulfilled criteria for second-tier analysis in the first NBS sample (compare Table 3) and retrospective analysis of the second-tier analytes in the original NBS sample also revealed normal concentrations of tHcy and MMA. At the time of selective diagnostics aged 22 days he showed slight elevation of markers for functional vitamin B<sub>12</sub> deficiency [methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA) 78.3 ( $N < 10$ ); Hcy in plasma 18 µmol/L ( $N < 15$ ); vitamin B<sub>12</sub> in plasma 144 pmol/L ( $N$  160–670)] under feeding

with infant formula. Both children (N3 and N4) were supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of all parameters (Hcy, MMA, vitamin B<sub>12</sub>). In both children also iron deficiency was detected and treated consecutively. The mother received counselling on food sources of vitamin B<sub>12</sub> and will be followed by a specialist in internal medicine.

Patient N5 showed elevated ratio C3/C2 in the first screening card, second-tier analysis from this card revealed slightly increased MMA, which was also confirmed in the second card. Work-up in this fully breast-fed child at age 3.5 weeks revealed slightly elevated Hcy in plasma of 17 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA 53.8 mmol/mol creatinine,  $N < 10$ ), and low vitamin B<sub>12</sub> in serum (79 pmol/L,  $N$  160–670). Vitamin B<sub>12</sub> status in the mother is not known, as she refused further work-up for herself. The mother did not adhere to any restrictive diet during pregnancy. The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of MMA in urine and vitamin B<sub>12</sub>. Therefore, the diagnosis of maternal vitamin B<sub>12</sub> deficiency as a cause of vitamin B<sub>12</sub> deficiency in this child was assumed.

Patient N6 showed decreased ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed increased tHcy. Work-up at age 10 days in this fully breast-fed child revealed elevated Hcy in plasma of 20.7 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA 39 mmol/mol creatinine,  $N < 10$ ) and plasma (1.72 µmol/L,  $N$  0.06–0.64), and low vitamin B<sub>12</sub> in serum (115 pmol/L,  $N$  160–670). Vitamin B<sub>12</sub> was also low in the mother (131 pmol/L,  $N$  160–670). The mother showed a slight elevation of Hcy in plasma (15.2 µmol/L,  $N < 15$ ), and normal methylmalonic acid in urine and plasma. She did not adhere to any restrictive diet during pregnancy. The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid decrease of MMA in urine. For the mother work-up and treatment by a specialist in internal medicine has been recommended.

Patient N7 showed decreased Met and ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed increased tHcy and MMA. This was confirmed in the second card, in which tHcy remained elevated and MMA had increased further. Work-up in patient N7 showed elevated Hcy in plasma of 21 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (171 mmol/mol creatinine,  $N < 18$ ), and vitamin B<sub>12</sub> in serum in the low normal range with 374 pg/mL ( $N$  210–910) (equivalent to 276 pmol/L,  $N$  155–672). The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of Hcy in plasma and MMA in urine, while vitamin B<sub>12</sub> level had risen to the upper normal range. Therefore, the diagnosis of maternal

vitamin B<sub>12</sub> deficiency as a cause of functional vitamin B<sub>12</sub> deficiency in this child was assumed. For the mother work-up and treatment by a specialist in internal medicine has been recommended.

Patient N8 showed decreased Met and ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed increased tHcy and MMA. This was confirmed in the second card, in which tHcy and MMA had increased further. Work-up in patient N8 showed elevated Hcy in plasma of 28 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (124 mmol/mol creatinine,  $N < 18$ ). Vitamin B<sub>12</sub> in serum determined in the treating hospital was reported as decreased. The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of Hcy in plasma and MMA in urine. For the mother work-up and treatment by a specialist in internal medicine has been recommended.

Patient N9 showed clearly decreased Met and ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed markedly increased tHcy. Work-up in patient N9 showed elevated Hcy in plasma of 135 µmol/L ( $N 5–12$ ) and decreased Met in plasma of 6 µmol/L ( $N 10–40$ ). Methylmalonic acid in urine and vitamin B<sub>12</sub> in serum were normal. Therefore, the diagnosis of an isolated remethylation disorder was established, final subspecification confirmed MTHFR deficiency. Treatment was started with betain, hydroxocobalamin and folinic acid.

Patient N10 showed decreased Met and ratio Met/Phe in the first screening card. Second-tier analysis from this sample showed increased tHcy. This was confirmed in the second card with elevated tHcy and now also elevated MCA. Work-up in patient N10 showed elevated Hcy in plasma of 25 µmol/L ( $N < 15$ ), elevated methylmalonic acid in urine (quantification using stable isotope-labelled D3-MMA 23.8 mmol/mol creatinine,  $N < 10$ ), and decreased vitamin B<sub>12</sub> in serum (104 pmol/L,  $N 160–670$ ). Vitamin B<sub>12</sub> was also low in the mother (141 pmol/L,  $N 160–670$ ), without any signs of functional vitamin B<sub>12</sub> deficiency (methylmalonic acid in urine and Hcy in plasma normal). The mother did not adhere to any restrictive diet during pregnancy. However, also in the father vitamin B<sub>12</sub> deficiency was known. For the mother work-up and treatment by a specialist in internal medicine has been recommended. The child was supplemented with vitamin B<sub>12</sub> orally, which led to rapid normalization of vitamin B<sub>12</sub> in serum, Hcy in plasma and MMA in urine.

### Retrospective evaluation of NBS results and the strategy of NBS 2020 in patients identified by selective screening

During the course of the study, seven symptomatic patients with one of the target disorders of the study were diagnosed by selective metabolic investigation: two children with a

combined remethylation disorder (patients S1 and S6), one child with mild propionic aciduria (patient S2), two children with vitamin B<sub>12</sub> deficiency (patients S3 and S4), and two children with methylmalonic aciduria (patients S5 and S7). The results of the tandem-MS analysis in the initial NBS sample and the sample taken at time of selective diagnostics, results of the (retrospective) second-tier analyses from DBS, as well as the time of storage before second-tier analysis are summarized in Table 4.

Patient S1 with combined remethylation disorder showed seizures on day 8 of life. The final subspecification of the combined remethylation disorder as confirmed by molecular genetic analysis is pending. This child would have been detected by our algorithm in the sample taken on day 8 of life, and also in the very first NBS sample, which was sent to us from an external NBS center after consent of parents and was reanalysed at our laboratory 3 months after sampling.

Patient S2 with mild propionic aciduria was diagnosed in the course of a first metabolic decompensation with metabolic acidosis, hypoglycemia, ketosis and hyperammonemia triggered by an infection aged 11 months. Patient S3 with vitamin B<sub>12</sub> deficiency was diagnosed aged 8 months due to failure to thrive. Patient S5 with methylmalonic aciduria showed metabolic decompensation on day 4 of life with hyperammonemia. At that time, the initial NBS sample, sent to an external NBS center, had not been analysed yet. Patient S6 with combined remethylation disorder (Cbl C defect) underwent selective diagnostics aged 3 weeks due to failure to thrive and muscular hypotonia. Patient S7 with methylmalonic aciduria showed metabolic decompensation aged 11 months. All these children would have been detected by our algorithms in the initial NBS sample.

Patient S4 was admitted aged 10 weeks with suspicion of seizures. Laboratory work-up revealed slight functional vitamin B<sub>12</sub> deficiency (vitamin B<sub>12</sub> 126 pmol/L,  $N 160–670$ , methylmalonic acid in urine 137 mmol/mol creatinine, but normal Hcy in plasma with 13 µmol/L,  $N < 15$ ). Vitamin B<sub>12</sub> was at that time also low in the mother, who did not adhere to a restricted diet. The child was fully breastfed. The child had participated in the pilot project NBS 2020 but did not fulfil criteria for second-tier analysis in the NBS sample. Retrospective analysis from the initial NBS sample showed a normal tHcy concentration and only slightly increased methylmalonic acid in this sample.

## Discussion

Within 13 months, the strategies used in a cohort of 68,418 study participants in our project “Newborn Screening 2020” led to identification of additional 12 children with metabolic disorders or maternal vitamin B<sub>12</sub> deficiency while only



marginally increasing the recall rate by 0.1%. This means that in this cohort, about one in 5700 children was affected by one of the additional disorders, while about one in 1300 children—equalling about 50 children in this cohort—is affected by one of the metabolic or endocrine disorders from the current German screening panel (compare Table 1) [19]. Eleven of 12 children identified so far with one of the target disorders from the study panel were diagnosed before the onset of clinical symptoms.

Maternal vitamin B<sub>12</sub> deficiency was the most frequent finding with about 1 in 8500 newborns in the screened population. It is a particular strength of our pilot study that all mothers are referred for systematic assessment of vitamin B<sub>12</sub> status and evaluation of dietary history. None of the mothers who were evaluated adhered to a vegan or vegetarian diet. Vitamin B<sub>12</sub> deficiency in these mothers was caused by nutritional problems during pregnancy or suspected undetected dysfunctions of gastrointestinal absorption. While in all children in the NBS group, markers for functional vitamin B<sub>12</sub> deficiency were elevated at time of conformational diagnosis, in their mothers markers of functional vitamin B<sub>12</sub> deficiency were only slightly abnormal or even normal despite low vitamin B<sub>12</sub> levels, which is in line with other reports [25].

As the current participation rate in our study is about 60%, the true frequency of maternal vitamin B<sub>12</sub> deficiency in the population screened by our laboratory—about 130,000–140,000 newborns per year—may be even higher. Other programmes reported vitamin B<sub>12</sub> deficiency in children mainly as a consequence of a maternal vegan or vegetarian diet [25–27]. The percentage of people following a vegan diet in Germany is estimated to be 0.1–1% of the population [28]. Based on the current participation rate, we cannot draw final conclusions at this point, whether this issue is not of relevance in the population screened by our laboratory, whether mothers with vegan/vegetarian diets are especially considerate during pregnancy concerning supplementation of vitamin B<sub>12</sub>, or if mothers with vegan/vegetarian diets have so far been more reluctant to participate in the study.

A screening programme in Estonia revealed an incidence of vitamin B<sub>12</sub> deficiency as high as 1 in 2500 newborns [29]. A further study in Italy using C3 as first, and MMA as second-tier test in a NBS cohort of 35,000 children found vitamin B<sub>12</sub> deficiency in about 1 in 5000 newborns in a 6-year period [25]. This study also evaluated vitamin B<sub>12</sub> status in a cohort of unselected healthy females admitted to hospital for delivery and found low vitamin B<sub>12</sub> levels in about 48% of examined women [25]. It has to be considered in this context that serum Cbl levels decline throughout gestation [30, 31]. In accordance with this MMA and Hcy levels have been reported to increase by late pregnancy [32, 33]. A Canadian study found that 10% of women are vitamin

B<sub>12</sub> deficient in early pregnancy [34], and several consecutive studies have confirmed similar findings or even higher frequencies for vitamin B<sub>12</sub> deficiency in pregnancy of up to 30% in different populations and ethnicities [31, 35–38]. This underscores the relevance of this condition for NBS programmes and stresses that also in caregivers of pregnant women awareness for B<sub>12</sub> deficiency should be increased.

Other programmes reported lower incidences of patients with vitamin B<sub>12</sub> deficiency detected by NBS [27, 39, 40]. Different strategies and cut-offs were used in different programmes [25, 27, 40, 41].

In addition, six of seven patients diagnosed symptomatically with one of the target disorders outside the study would have been identified by our strategy in the first NBS sample. These patients were born just before the study had started or did not participate in the study. The combination of first tier cut-offs and second-tier strategies would have detected these patients. Most of these children could presumably have benefitted from early detection by NBS—especially those with late-onset symptoms and with vitamin B<sub>12</sub> deficiency [6, 16, 26]. For severe vitamin B<sub>12</sub> deficiency, several reports document that children diagnosed symptomatically may die or neurological deficits persist despite treatment [5]. This underscores the enormous benefit of early detection. For Cbl C deficiency, there is a clear benefit of early detection and treatment in patients with late-onset forms [2, 42]. However, it has to be noted, that although early treatment seems to have a beneficial effect on non-neurological symptoms and mortality in Cbl C deficiency, long-term neurological and ophthalmological symptoms may not be significantly influenced [43, 44]. A screening approach for Cbl C deficiency based on low Met as secondary metabolite in cases with elevated C3 has been suggested by Weisfeld-Adams and colleagues [45]. However, in our collective, one of two patients with a combined remethylation disorder showed C3 in the normal range in the first DBS sample.

Patient S5 with methylmalonic aciduria diagnosed symptomatically showed a neonatal decompensation in the first days of life and could most probably not have been identified presymptomatically by NBS.

It has to be noted that in patient S3 with vitamin B<sub>12</sub> deficiency, the first tier analyte Met/Phe was only slightly below our cut-off (low, P5) which triggers second-tier tHcy measurement. Patient N4, twin sibling to patient N3, was diagnosed with maternal vitamin B<sub>12</sub> deficiency in the course of selective work-up due to the sibling's abnormal NBS result. The child N4 himself did not fulfil criteria for second-tier analysis in the first NBS sample and also retrospective analysis of the second-tier analytes in the original NBS sample revealed normal concentrations of tHcy and MMA. At the time of selective diagnostics aged 22 days, however, the child showed slight elevation of markers for functional vitamin B<sub>12</sub> deficiency under feeding with infant formula. An

additional patient with slight vitamin B<sub>12</sub> deficiency (patient S4) diagnosed aged 10 weeks following laboratory work-up in the context of seizures had participated in the pilot project NBS 2020 and did not fulfil criteria for second-tier analysis in the NBS sample. As can be assumed from the normal tHcy concentration and the only slightly increased MMA in the retrospective analysis from the initial NBS sample, mild vitamin B<sub>12</sub> deficiency seems to have developed in the following months in this fully breastfed child. The clinical symptoms with seizures are, however, most probably unrelated to the mild vitamin B<sub>12</sub> deficiency. Of note, second-tier measurements in the initial NBS sample were performed retrospectively after 2.5 months of storage at 4 °C. For storage of DBS at room temperature, an increase of MMA over time has been described [46], while MMA in DBS has been reported as stable for 8 weeks at 2–4 C° [47].

### Different strategies in NBS for vitamin B<sub>12</sub> deficiency

The frequency of vitamin B<sub>12</sub> deficiency detected in NBS greatly depends on different strategies and cut-offs used in different programmes [25, 27, 40, 41]. Compared to our current strategy, the study by Scolamieri and colleagues used a considerably lower cut-off for C3 (3.16 µmol/L, corresponding to the 90th percentile of the laboratory) as criterion for second-tier analysis of MMA [25]. In the patients detected by NBS in Estonia, all C3 levels have been reported to have been > 4.31 µmol/L (percentile of the laboratory not stated) [29]. Concerning C3 our approach is rather conservative (cut-off between 99 and 99.5 P.), and also isolated elevation of C3/C2 in the presence of normal C3 leads to second-tier analysis of MMA in our strategy. Only one of our altogether 11 patients with vitamin B<sub>12</sub> deficiency (NBS or selective screening) showed a C3 above our cut-off in the first NBS sample. This proportion rises only slightly to 4 of 11 patients even when applying the much lower cut-off for C3 used by Scolamiero and colleagues [25]. Also other studies have come to the conclusion, that C3 is an insensitive marker for vitamin B<sub>12</sub> deficiency [26].

In our patients with vitamin B<sub>12</sub> deficiency ratio Met/Phe was decreased in 6, elevated in 1 and normal in 4 patients in the initial NBS sample. It has previously been stated that MMA should be more sensitive for vitamin B<sub>12</sub> deficiency than Hcy, as the pathway of Met synthase is supposed to be more preserved than that of MMA-CoA mutase when vitamin B<sub>12</sub> stores are declining [25, 48]. In our collective MMA was elevated above the 99.9th percentile (cut-off for MMA and MCA in our study) in 6 of 11 children, and MCA in 3 of 11 children in the initial NBS sample. Therefore, the most sensitive marker in DBS seems to be Hcy, which was elevated in the first DBS in eight patients. It was initially normal in three children, including the two children who

were not identified by our screening strategy in the first DBS and later on diagnosed with mild vitamin B<sub>12</sub> deficiency—the twin sibling diagnosed aged 22 days in the course of selective diagnostics of patient N3 and patient S4 diagnosed aged 10 weeks. As discussed above it seems likely that in both children, mild vitamin B<sub>12</sub> deficiency has developed in the first weeks of life. Comparing MMA results in our patients with a publication by Schroder and colleagues presenting a reference interval for MMA in DBS in full-term infants of 0.45–1.33 µmol/L [46] 9 of 11 children with vitamin B<sub>12</sub> deficiency from our study had MMA results in their first NBS sample above this reference range, the upper limit equalling the 97.5th percentile. This comparison is, however, limited because of the use of different methods [23, 46]. The 97.5th percentile for MMA using our method is currently 1.85 µmol/L (and was 1.66 µmol/L with the reagents used by the time patient samples N1–N4 and S3 were analysed). With regard to these cut-offs, 8 of 11 children with vitamin B<sub>12</sub> deficiency showed elevated MMA in their first NBS sample.

From our current stage of evaluation we conclude that the proposed second-tier strategy in our study is very effective for identification of maternal vitamin B<sub>12</sub> deficiency for moderate and severe cases, but that mild vitamin B<sub>12</sub> deficiency might not always be picked up. The results of the first 13 months of this study are presented, as awareness of these findings is important from a public health point of view. Provided proof for the feasibility of the methods used in a population-based screening programme in more than 68,000 samples, and of the benefit for patients identified will hopefully lead to timely inclusion of these additional disorders into the NBS panel for Germany as part of regular health insurance.

In conclusion, within the first 13 months, the strategies currently used in our study “Newborn Screening 2020” identified additional 12 children with metabolic disorders or maternal vitamin B<sub>12</sub> deficiency in a cohort of 68,418 study participants, while the recall rate was only marginally increased by 0.1%. Except one child, all patients were identified presymptomatically. Maternal vitamin B<sub>12</sub> deficiency was the most frequent finding. This project will allow to evaluate the false-positive rate and positive predictive value of the proposed second-tier strategy over the following years. It can be expected that numerous children will benefit from screening for the additional target disorders in the further course of the study and even more in case of a future extension of the NBS panels in Germany and other countries.

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## Compliance with ethical standards

**Ethical approval** The study “Newborn Screening 2020” as well as retrospective evaluation of NBS results of patients diagnosed by selective screening has been approved by the Ethics Committee of the University Hospital Heidelberg (no. S-533/2015). Written informed consent was obtained from parents before participation of children in the study “Newborn Screening 2020”.

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