



# Newborn Screening for Vitamin B<sub>12</sub> Deficiency in Germany—Strategies, Results, and Public Health Implications

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**Objective** To evaluate a systematic newborn screening (NBS) strategy for vitamin B<sub>12</sub> deficiency.

**Study design** In a prospective single-center NBS study, a systematic screening strategy for vitamin B<sub>12</sub> deficiency was developed and evaluated. Tandem-mass spectrometry screening was complemented by 2 second-tier strategies, measuring methylmalonic/3-OH-propionic/methylcitric acid, and homocysteine from dried blood spots.

**Results** In a cohort of 176 702 children screened over 27 months, 33 children were detected by NBS in whom (maternal) vitamin B<sub>12</sub> deficiency was confirmed. Homocysteine was the most sensitive marker for vitamin B<sub>12</sub> deficiency, but only combination with a second-tier strategy evaluating methylmalonic acid allowed for detection of all 33 children. Mothers were of various ethnic origins, and 89% adhered to a balanced diet. Treatment in children was performed predominantly by oral vitamin B<sub>12</sub> supplementation (84%), and all children remained without clinical symptoms at short-term follow-up.

**Conclusions** Vitamin B<sub>12</sub> deficiency is a treatable condition but can cause severe neurologic sequelae in infants if untreated. The proposed screening strategy is feasible and effective to identify moderate and severe cases of vitamin B<sub>12</sub> deficiency. With an incidence of 1:5355 newborns, vitamin B<sub>12</sub> deficiency is more frequent than inborn errors of metabolism included in NBS panels. Treatment of vitamin B<sub>12</sub> deficiency is easy, and additional benefits can be achieved for previously undiagnosed affected mothers. This supports inclusion of vitamin B<sub>12</sub> deficiency into NBS but also stresses the need for increased awareness of vitamin B<sub>12</sub> deficiency in caregivers of pregnant women. (*J Pediatr* 2020;216:165-72).

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Newborn screening (NBS) panels differ considerably between countries, ranging from single disorders to up to 60 conditions in some regions of the US.<sup>1</sup> Decisions about the inclusion of additional disorders into NBS programs are commonly based on principles of screening for disease,<sup>2</sup> which include that a condition suitable for screening should be an important health problem, there should be an accepted treatment, a recognizable latent stage, and a suitable test to detect patients.<sup>1</sup> Children with vitamin B<sub>12</sub> deficiency, which is mostly of maternal origin in newborns, are sometimes detected in programs screening for methylmalonic and propionic acidurias using tandem mass-spectrometry (MS).<sup>3-5</sup> These children are detected based on elevated propionylcarnitine (C3), sometimes complemented by second-tier strategies.<sup>4,5</sup> Children with vitamin B<sub>12</sub> deficiency are asymptomatic at birth but may develop severe multisystemic symptoms, including irreversible neurologic and developmental impairment in the second half-year of life.<sup>6-8</sup> Early identification allows for timely treatment by supplementation of vitamin B<sub>12</sub>, which should be more effective than treatment started at the symptomatic stage. Mothers with previously unrecognized vitamin B<sub>12</sub> deficiency, due to nutritional deficiencies or gastrointestinal malabsorption, also would benefit from diagnosis and treatment. Given a suitable screening strategy, vitamin B<sub>12</sub> deficiency is an appropriate candidate for systematic inclusion into NBS panels.<sup>9</sup> Previous reports of clinically diagnosed patients with maternal vitamin B<sub>12</sub> deficiency and retrospective analysis of their NBS samples, however, suggest that C3 levels alone—currently included in many panels to

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Supported by the Dietmar Hopp Foundation, St Leon-Rot, Germany. The study “Long-term outcome of patients with inherited metabolic diseases after diagnosis by expanded newborn screening” (DRKS00013329), providing developmental testing for patients 3 and 9 from this study, is also supported by the Dietmar Hopp Foundation, St Leon-Rot, Germany. The funder of the study had no involvement in the study design, the collection, analysis, and interpretation of data, the writing of the report, and the decision to submit the manuscript for publication. The authors declare no conflicts of interest.

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<https://doi.org/10.1016/j.jpeds.2019.07.052>

3-OH-PA	3-OH-propionic acid	Met	Methionine
C2	Acetylcarnitine	MMA	Methylmalonic acid
C3	Propionylcarnitine	MS	Mass spectrometry
DBS	Dried blood spot	NBS	Newborn screening
HELLP	Hemolysis, elevated liver enzymes, low platelet count	Phe	Phenylalanine
MCA	Methylcitric acid	tHcy	Total homocysteine

detect disorders of the propionate metabolism—are not sensitive enough to detect patients with vitamin B<sub>12</sub> deficiency.<sup>5,9,10</sup>

NBS for inborn disorders of the propionate metabolism based on C3 levels has a high false-positive rate, unwanted in population screening.<sup>11,12</sup> To improve specificity, so-called “second-tier strategies” have been suggested,<sup>12-14</sup> complementing abnormal results for primary screening measures by more specific second-tier metabolites like methylmalonic acid (MMA), 3-OH-propionic acid (3-OH-PA), and methylcitric acid (MCA) from the same first specimen. In NBS for classical homocystinuria and remethylation disorders, second-tier strategies measuring total homocysteine (tHcy) have been evaluated.<sup>15-17</sup>

Our study, Newborn Screening 2020, set out to evaluate a possible extension of the German NBS panel.<sup>18</sup> One aim of this study was to implement and evaluate a systematic screening strategy for vitamin B<sub>12</sub> deficiency, using a combination of 2 second-tier strategies.

## Methods

In a prospective single-center study an extension of the German NBS panel (14 disorders at study initiation, **Table I** [available at [www.jpeds.com](http://www.jpeds.com)]) by an additional 26 conditions (25 metabolic disorders and vitamin B<sub>12</sub> deficiency, **Table II** [available at [www.jpeds.com](http://www.jpeds.com)]) was evaluated at the Heidelberg NBS Center. The aims of the study were to evaluate the technical feasibility of population-based NBS for these disorders and the benefit for detected patients, based on their presymptomatic identification.

The Heidelberg NBS Center performs screening for about 140 000 newborns per year, mainly from South-West Germany, equaling about 17% of children born in Germany. In Germany, participation in NBS is voluntary, but almost all parents choose to participate. NBS samples are taken between the 36th and 72nd hour of life. NBS was performed including electrospray-ionization tandem-MS (Waters Xevo TQD; Waters, Milford, Massachusetts) for determination of amino acids and acylcarnitines.<sup>19</sup> From August 2016 on, all hospitals, physicians, and midwives sending samples for regular NBS to the Heidelberg NBS Center were asked to offer participation in the study (expanded NBS) free of charge to families of newborns born at their institution. Written informed consent was required for screening for the additional conditions, which was performed from the same specimen as regular NBS (Whatman filter paper 903; GE Healthcare, Freiburg, Germany).

The screening algorithms developed for this study including second-tier strategies for vitamin B<sub>12</sub> deficiency and cut-offs for all measures are shown in **Figure 1**. Strategies and cut-offs chosen were based on a previous evaluation of new strategies for NBS for classical homocystinuria, remethylation disorders, and vitamin B<sub>12</sub> deficiency by the NBS Center Heidelberg in cohorts from

Qatar and Germany using retrospective analysis of confirmed patients' NBS results.<sup>15,16</sup>

For 15 of the 26 additional target conditions in the study, abnormal first-tier results are complemented by second-tier testing from the same dried blood spot (DBS). Primary screening measures used in the second-tier algorithms are C3, ratio C3/acetylcarnitine (C3/C2), methionine (Met), and the ratio of methionine/phenylalanine (Met/Phe). Two second-tier methods were used. The first analyzes MMA, 3-OH-PA, and MCA,<sup>14</sup> based on abnormal first-tier results for C3 and C3/C2 (C3 + C3/C2 > cut-off, or C3/C2 > cut-off, or C3 > alarm limit). The second method analyzes tHcy<sup>20</sup> after an abnormal first-tier result for Met (<cut-off low) or Met/Phe (<cut-off low or > cut-off high). Patients with vitamin B<sub>12</sub> deficiency were identified by elevated tHcy, elevated MMA/MCA, or a combination of both.

Depending on the grade of pathology in the first DBS, it was either recommended to send a repeat DBS for tandem-MS screening and analysis of all second-tier measures initially out of range or to also send plasma, serum, and urine samples for additional analyses. Confirmatory work-up was performed in all cases if the second DBS also was abnormal.

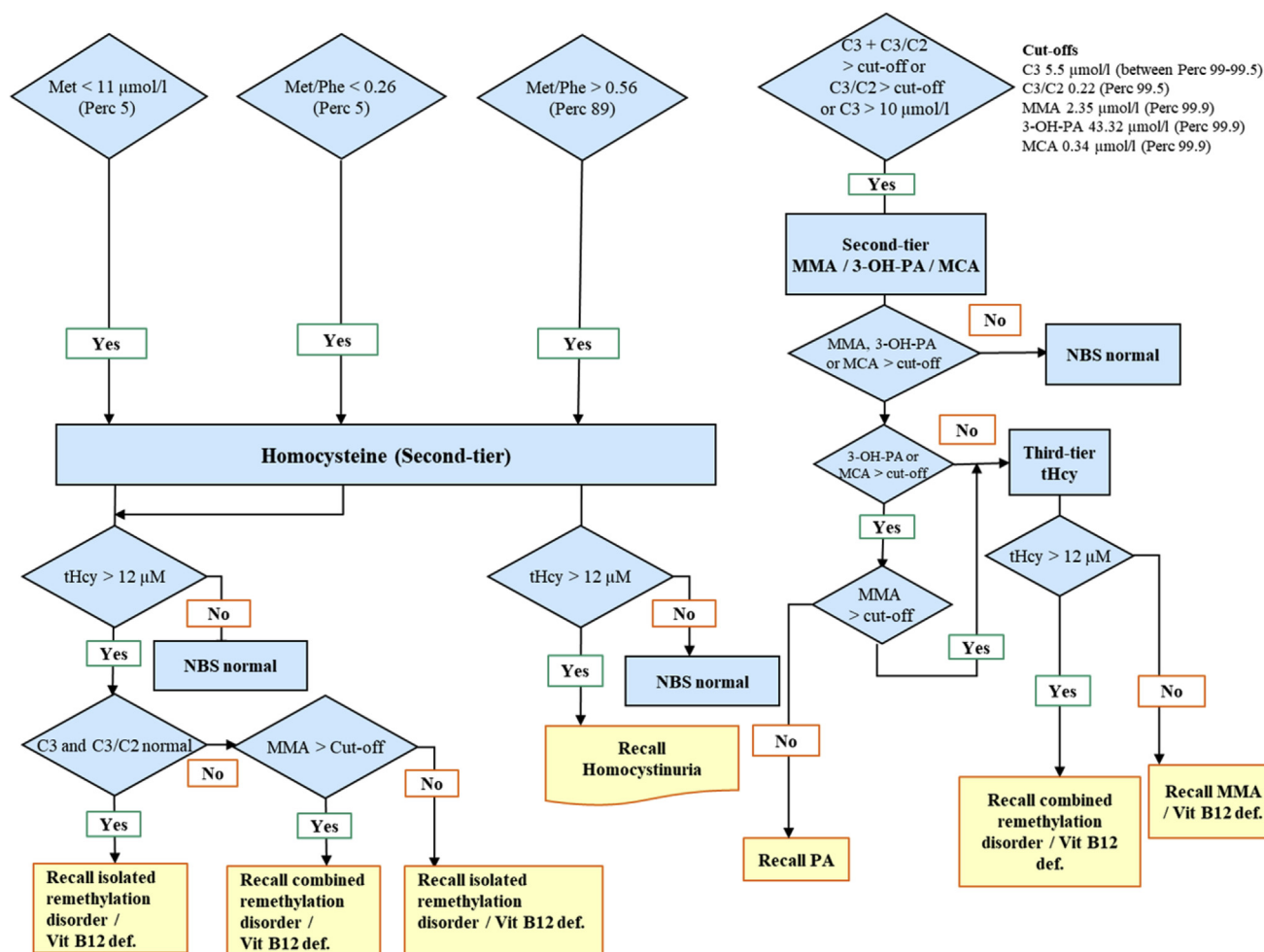
Diagnosis of “vitamin B<sub>12</sub> deficiency” was established in cases with elevation of 1 or more functional markers of vitamin B<sub>12</sub> deficiency (MMA in plasma and/or urine and homocysteine in plasma), in the presence of low vitamin B<sub>12</sub> serum levels, and of “functional vitamin B<sub>12</sub> deficiency” in cases with elevation of 1 or more functional markers of vitamin B<sub>12</sub> deficiency and vitamin B<sub>12</sub> in the low normal range.<sup>21</sup> Normalization of functional markers and of vitamin B<sub>12</sub> serum levels under supplementation was to be tracked. Information on treatment of affected children, feeding modalities, and short-term clinical outcome (presence/absence of clinical symptoms as reported by the treating pediatrician) was documented. For 2 children standardized developmental evaluation (Denver test/Bayley-III screening test) was performed in the context of a study on long-term outcome for patients detected by our NBS Center (DRKS00013329).

In all cases, work-up of the maternal vitamin B<sub>12</sub> status, including functional markers, was recommended. “Maternal vitamin B<sub>12</sub> deficiency” was diagnosed if the mother's vitamin B<sub>12</sub> level was below normal range and/or functional markers were elevated. If maternal vitamin B<sub>12</sub> deficiency was not explained by the dietary regimen, mothers were referred to internal medicine for further work-up and treatment.

The study Newborn Screening 2020 has been approved by the ethics committee of the University Hospital Heidelberg (Number S-533/2015). Written informed consent was obtained from parents before participation of their newborn in the study.

## Results

From August 1, 2016, until October 31, 2018, 176 702 children participated in the study. Participation rate was 66% for children screened at our center by the end of the reported period. Second-tier analyses were performed in 7.4% of



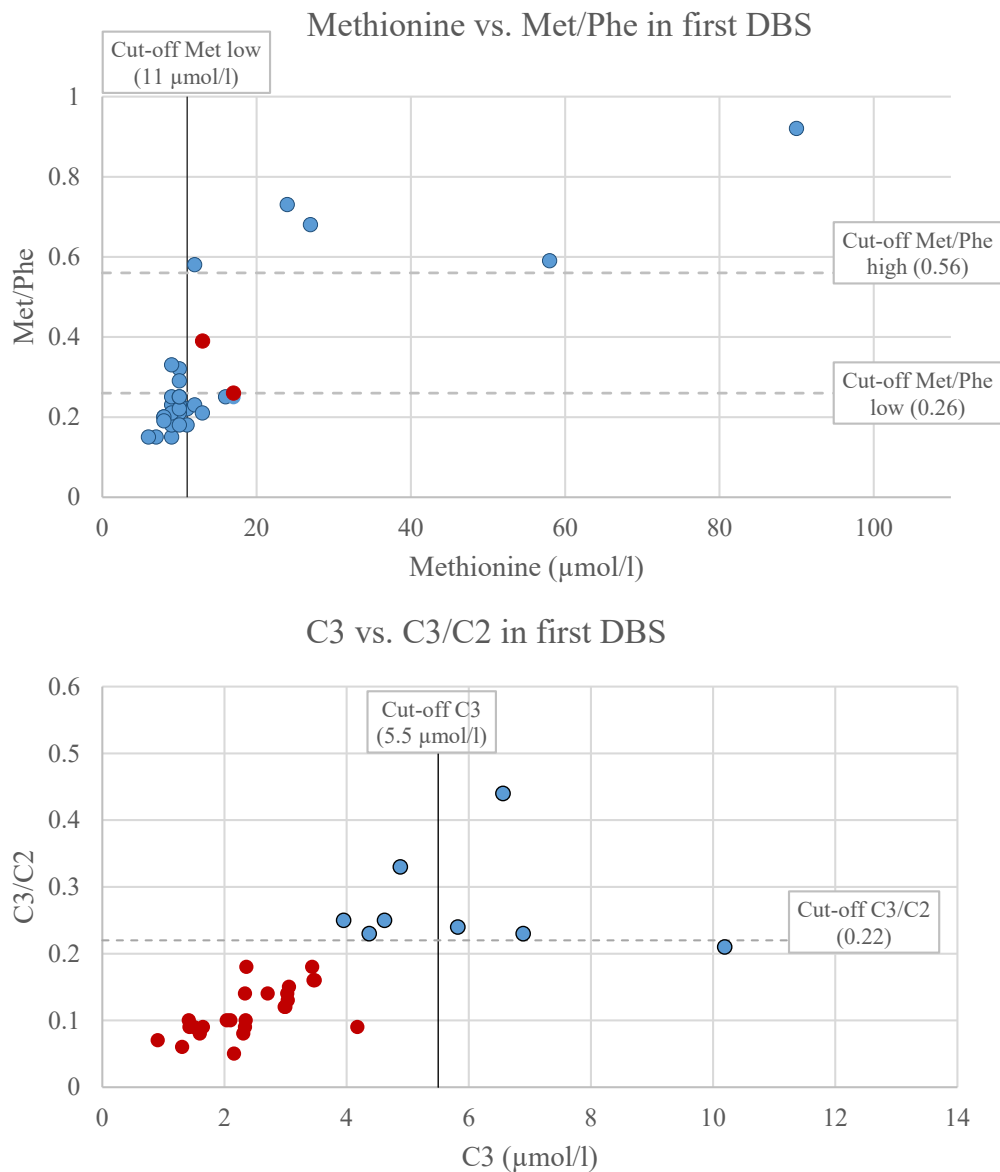
**Figure 1.** Second-tier algorithms used in the study Newborn screening 2020. Recall for vitamin B<sub>12</sub> deficiency if tHcy <50  $\mu\text{mol/L}$ ; recall for isolated or combined remethylation disorder if tHcy  $\geq$ 50  $\mu\text{mol/L}$ . *def.*, deficiency; *Recall MMA*, recall for methylmalonic aciduria; *Recall PA*, recall for propionic aciduria; *Vit*, vitamin; *Perc*, percentile.

samples according to the criteria described previously. After second-tier analysis, 80 participants (0.05%) remained with an abnormal screening result. Of these, 35 cases were confirmed with 1 of the 15 target disorders from the second-tier panel: 33 with vitamin B<sub>12</sub> deficiency, 1 with methylenetetrahydrofolate reductase deficiency, and 1 with propionic aciduria. In 32 children, (functional) vitamin B<sub>12</sub> deficiency was confirmed, and in 1 case work-up revealed only maternal vitamin B<sub>12</sub> deficiency, and the child was not treated apart from formula feeding chosen by the family. Two additional children with a suspicion of vitamin B<sub>12</sub> deficiency were lost to follow-up (samples sent from abroad/family had moved); in 1 case, information about work-up is incomplete. After repeat sampling, 42 cases were classified false-positive, resulting in a positive predictive value of 0.45 for all second-tier based screening strategies. Of the children classified as false-positive, 17 (40%) were born pre-term (before 37 weeks of gestation), 6 of these at 25-31 weeks of gestation, suggesting potential transient elevations of second-tier markers, normalization under enriched formula

feeding, or inadequate cut-offs for second-tier markers in pre-terms. When analyzing only children born after 37 weeks of gestation, positive predictive value was 0.55.

NBS results and confirmatory diagnostics for the 33 children with vitamin B<sub>12</sub> deficiency are shown in [Appendix 1](#) (available at [www.jpeds.com](http://www.jpeds.com)). For these cases, all second-tier analytes have been added retrospectively, even if the respective first-tier analytes were within normal range to allow for complete analysis of the sensitivity of different markers. First-tier markers for the patients identified by the pilot project with respect to cut-offs used are also shown in [Figure 2](#).

According to the algorithms applied, abnormal second-tier markers were prompted by abnormal first-tier markers from the Met pathway only (Met and/or Met/Phe) in 27 cases (2 only Met decreased, 5 only Met/Phe decreased, 4 only Met/Phe increased, 16 Met + Met/Phe decreased), in 3 cases by C3-based markers only (2 only C3/C2 elevated, 1 C3 alarm >10  $\mu\text{mol/L}$ ), and in 3 cases by a combination of abnormal first-tier markers from both the C3 and Met approach.



**Figure 2.** First-tier markers in first DBS of 33 patients with (maternal) vitamin B<sub>12</sub> deficiency with respect to cut-offs used in the pilot project Newborn Screening 2020. Every *circle* depicts 1 patient's results for the respective first-tier markers. Patients in whom the first-tier markers did not fulfill criteria to prompt the respective second-tier analysis according to the algorithms used in the pilot project Newborn Screening 2020 are marked in *red*.

Results of confirmatory analyses in mothers, their dietary history, vitamin supplementation during pregnancy, assumed causes of vitamin B<sub>12</sub> deficiency, and ethnic origin are shown in [Appendix 2](#) (available at [www.jpeds.com](http://www.jpeds.com)).

Of 26 mothers with information on ethnic origin available, 11 were of German, 7 of Arabic, 2 of Turkish, 1 of South-European, and 5 of Eastern-European origin. Vitamin B<sub>12</sub> deficiency was more pronounced in children than in their mothers in 77% of cases (20 of 26 cases with complete simultaneous information for mother and child). Of 27 mothers with information on diet available, 2 adhered to a vegetarian, and none to a vegan diet. One mother had an unbalanced diet with repeated radical fasting. Of 24 mothers who reported to

eat meat at least occasionally (89%), 4 stated to have been poor eaters during pregnancy, for example, due to gastroesophageal reflux. In none of these mothers had vitamin B<sub>12</sub> been evaluated by the gynecologist, and altogether 8 mothers received iron supplementation in pregnancy due to anemia. One mother received iron infusion twice postpartum due to severe anemia. Medical diagnoses in mothers included ulcerative colitis, gastric bypass, HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, and severe pancytopenia due to vitamin B<sub>12</sub> and folic acid deficiency, carbamazepine treatment throughout pregnancy, and previously undiagnosed pernicious anemia detected by work-up in context of the study. Of 17 mothers with

information on vitamin supplementation during pregnancy available, 4 took folic acid and vitamin B<sub>12</sub>, 3 folic acid only, and 10 (59%) received no supplementation at all.

Information on treatment for the children, including feeding modalities (breast milk/formula), follow-up laboratory analyses, and short-term clinical outcome is shown in **Appendix 3** (available at [www.jpeds.com](http://www.jpeds.com)). All patients were reported as asymptomatic at last available time point with clinical information. Most children (84%) were treated with a regimen of exclusively oral vitamin B<sub>12</sub> supplementation established for this study (high initial doses of vitamin B<sub>12</sub> for about 1 week, with reduction to maintenance dosage after normalization of all markers [**Appendix 3**]). For all children, additional follow-up testing of vitamin B<sub>12</sub> status including functional markers was performed/recommended after stopping vitamin B<sub>12</sub> supplementation at a time when regular feeding of meat-containing complimentary foods had been established. If this re-evaluation showed signs of beginning or functional vitamin B<sub>12</sub> deficiency, further diagnostic work-up including hereditary conditions of vitamin B<sub>12</sub> adsorption was recommended. So far, this constellation has not been observed in any of the patients detected by this study.

**Table III** (available at [www.jpeds.com](http://www.jpeds.com)) shows NBS results of 4 patients diagnosed with vitamin B<sub>12</sub> deficiency or functional vitamin B<sub>12</sub> deficiency by selective diagnostics aged 1-8 months, who had normal NBS results and did not fulfill criteria for second-tier analysis in our study. Retrospective second-tier analysis from the initial NBS sample revealed normal concentrations for all markers in 3 of these children. NBS results and retrospective second-tier analysis are also shown for 1 patient born before the study who presented with severe vitamin B<sub>12</sub> deficiency at 8 months. During the reported period, 4 additional children aged 2-11 months were diagnosed symptomatically with vitamin B<sub>12</sub> deficiency by selective diagnostics. These children had undergone NBS in an external screening center not screening for vitamin B<sub>12</sub> deficiency.

Regarding the costs of the proposed screening strategies, the first-tier markers used in the study already are measured in the routine tandem-MS analysis for all samples, but according to regulations have to be blinded in medical validation of routine NBS as respective disorders are not targeted by the current German NBS panel. Therefore, there is no additional cost using these measures as first-tier markers in our approach. At least 1 of the second-tier strategies was performed in 7.4% of all samples. The majority of second-tier measurements was performed for tHcy only, with cost of about 1.50 Euros or about 1.70 USD per sample. The cost for MMA/3-OH-PA/MCA second-tier analysis—which is currently necessary in only 0.2%-0.4% of samples per month—is about 6 Euros or about 6.80 USD per sample. This cost estimation for both methods includes costs for material and reagents but not personnel, energy, machine time, etc. The majority of false-positive cases were confirmed with normal analysis of all second-tiers in a single repeat sample,

resulting in little additional cost for confirmatory testing in these cases.

## Discussion

The NBS strategy for vitamin B<sub>12</sub> deficiency presented here uses 2 second-tier strategies and covers both pathways possibly affected as a consequence of vitamin B<sub>12</sub> deficiency: the pathway involving methionine synthase by measurement of tHcy, and the pathway involving MMA-CoA mutase by measurement of MMA and MCA. The most sensitive measure for vitamin B<sub>12</sub> deficiency was tHcy, which was found as abnormal in 30 of 33 children, while MMA was above the cut-off in only 12 of 33 (**Figure 3**; available at [www.jpeds.com](http://www.jpeds.com)). This is different than previous statements asserting that MMA should be more sensitive for vitamin B<sub>12</sub> deficiency than homocysteine, which were based on the assumption that the pathway of methionine synthase was more preserved than that of MMA-CoA mutase when vitamin B<sub>12</sub> stores are declining.<sup>4,22</sup> Even when applying the 97.5 percentile as a cut-off for MMA in DBS suggested by Schroder et al,<sup>23</sup> only 18 of 33 patients would be detected by elevated MMA. In our study, detection of all 33 children was only possible by combination of both second-tier strategies.

The incidence of patients with vitamin B<sub>12</sub> deficiency detected by NBS differs considerably between different programs. This may reflect true differences between populations but is also dependent on different strategies and cut-offs applied. The Estonian screening program recently reported an incidence of 1 in 3000 newborns identified by elevated C3,<sup>24</sup> and an Italian study reported 1 in 5000 newborns using a second-tier strategy combining C3 and MMA.<sup>4</sup> The Minnesota state NBS reported no cases of vitamin B<sub>12</sub> deficiency detected before the year 2005, followed by a detection rate of 3.02/100 000 after lowering the C3 cut-off and introducing second-tier testing for MMA and tHcy in samples with abnormal C3.<sup>5</sup> A questionnaire based survey of 31 US state NBS programs revealed a reported incidence of vitamin B<sub>12</sub> deficiency detected by C3 based NBS of 0.88/100 000 for the years 2003-2007.<sup>3</sup> As vitamin B<sub>12</sub> deficiency has a high incidence in pregnant women worldwide in different populations and ethnicities,<sup>25-29</sup> we suggest that these low numbers are due to undiagnosed or unreported cases. In line with our findings with C3 elevated in only 4 of 33 patients, previous reports suggested that C3 levels alone are not sensitive enough to detect patients with vitamin B<sub>12</sub> deficiency when applying conventional cut-offs.<sup>5,9,10,30</sup> However, regional differences in prenatal vitamin supplementation also may contribute to regional variation in vitamin B<sub>12</sub> deficiency detected by NBS. Although in our study 59% of affected mothers had not taken any vitamin supplementation before or throughout pregnancy, the rate of prenatal B-vitamin supplementation is much higher in certain countries and was, for example, as high as 93% in early pregnancy in a Canadian survey.<sup>31</sup>

Our strategy found an incidence of vitamin B<sub>12</sub> deficiency (of maternal origin) of 1 in 5355 newborns. All affected children had functional vitamin B<sub>12</sub> deficiency with elevation of homocysteine in plasma. Of the children, 25% had severe vitamin B<sub>12</sub> deficiency with levels <100 pmol/L, 56% 100-200 pmol/L, and 19% > 200 pmol/L (often described as cut-off for subclinical vitamin B<sub>12</sub> deficiency).<sup>21</sup> With regard to the definition by the World Health Organization,<sup>21</sup> 62% of children and 42% of mothers had “vitamin B<sub>12</sub> deficiency” (<148 pmol/L), 19% (children) and 42% (mothers) were in the category of “low vitamin B<sub>12</sub>” (148-221 pmol/L), and 19% (children) and 16% (mothers) showed “vitamin B<sub>12</sub> adequacy” (>221 pmol/L). Vitamin B<sub>12</sub> levels in the low-normal range alone are not sufficient to exclude functional deficiency, and severe functional deficiency has been documented in the presence of normal vitamin B<sub>12</sub> levels.<sup>21</sup>

It cannot be predicted whether children identified with milder functional vitamin B<sub>12</sub> deficiency would have developed symptoms without treatment. However, reported cases diagnosed with severe clinical symptoms of vitamin B<sub>12</sub> deficiency had vitamin B<sub>12</sub> levels at diagnosis of about 100 pmol/L after prolonged episodes of exclusive breast milk feeding.<sup>32</sup> Therefore it can be postulated that even higher neonatal vitamin B<sub>12</sub> levels will result in symptomatic vitamin B<sub>12</sub> deficiency with breast feeding by a vitamin B<sub>12</sub>-deficient mother. Breast feeding was the preferred way of child nutrition for the patients in our study, chosen by 79% of mothers.

Four patients diagnosed in infancy with mild or functional vitamin B<sub>12</sub> deficiency did not meet criteria for second-tier analysis in our study, of whom 3 had normal MMA and tHcy in NBS upon retrospective analysis. Therefore, it can be postulated that vitamin B<sub>12</sub> deficiency may have developed only later on in these fully breastfed children. However, our strategy might not always pick up mild functional vitamin B<sub>12</sub> deficiency, but it has been effective in identifying moderate and severe cases.

In 5 children (15%), confirmatory diagnostics and treatment were only started after repeated tracking-calls by our screening center, demonstrating that also in screening for vitamin B<sub>12</sub> deficiency an efficient tracking strategy is required to assure that all affected children do benefit from NBS.

Patients diagnosed symptomatically with vitamin B<sub>12</sub> deficiency have been reported to show some improvement of symptoms after start of treatment, although their neurologic and intellectual long-term outcome remain poor.<sup>6,8,9,33</sup> Given the negative and partly irreversible health consequences of delayed diagnostics and treatment, the relatively low additional cost of the proposed screening strategy, and the favorable short-term outcome in the patients detected by our study after early treatment, we assume that including vitamin B<sub>12</sub> deficiency into existing NBS programs is in accordance with the Wilson and Jungner criteria<sup>2</sup> and also should be reasonable from a health economic perspective. However, an exact calculation of healthcare costs for cases of vitamin B<sub>12</sub> deficiency left undetected or diagnosed later vs costs for the NBS panel extension including work-up of false-positive cases would require a detailed economic evaluation.<sup>34,35</sup>

Vitamin B<sub>12</sub> deficiency has a high incidence in pregnant women worldwide, ranging between 10% and 50% for different populations and ethnicities.<sup>4,25-29,36</sup> Throughout pregnancy, serum cobalamin levels decline, whereas MMA and tHcy levels increase.<sup>25,37</sup>

All mothers of children with vitamin B<sub>12</sub> deficiency were offered systematic assessment of their vitamin B<sub>12</sub> status and further diagnostics and treatment if indicated. Therefore, an additional benefit of NBS could be achieved for previously undiagnosed mothers. Functional vitamin B<sub>12</sub> deficiency frequently was more pronounced in children than in their mothers, which is consistent with previous reports.<sup>4,8,24</sup> Affected mothers in our study were of various ethnic origins. In contrast to reports by other screening programs,<sup>3,4,30</sup> none of the mothers with vitamin B<sub>12</sub> deficiency in our study adhered to a vegan diet. This could be explained by high awareness for the need of vitamin B<sub>12</sub> supplementation during pregnancy in women following a vegan diet, currently reported for about 0.1%-1% of the German population.<sup>38</sup> Alternatively, it may be that women following restricted diets had more reservations against study participation. Therefore, the true incidence of vitamin B<sub>12</sub> deficiency in our population may be even higher. However, the main reason for non-participation in the study was that some hospitals/doctors were not willing to offer study participation in their institutions due to the requirement of additional informed consent. In hospitals participating in the pilot project, the participation rates were generally high, and only individual families declined participation.

Several mothers reported feeding difficulties in pregnancy and iron supplementation due to anemia, but despite these risk factors or symptoms no diagnostic work-up for vitamin B<sub>12</sub> deficiency had been performed by the gynecologist. This stresses that also caregivers of pregnant women should increase awareness for vitamin B<sub>12</sub> deficiency. In women with anemia, the coexistence of iron, folate, and vitamin B<sub>12</sub> deficiency has been reported,<sup>39</sup> and hematologic changes caused by vitamin B<sub>12</sub> deficiency may be masked by those caused by iron deficiency.

Despite a general recommendation in Germany<sup>40</sup> and many other countries to start folic acid supplementation pre-conceptionally—with many preparations containing also vitamin B<sub>12</sub>—59% of women in our study had not taken any supplementation at all. This poor adherence to recommendations for folic acid supplementation is consistent with a previous German National Health Survey.<sup>41</sup> National German maternity guidelines do not currently include routine evaluation of vitamin B<sub>12</sub> status. However, recommendations by the German Federal Centre for Nutrition include a balanced diet and pre-conceptional start of folate supplementation for all women, dietary counseling for women following vegetarian diets, and pre-conceptional start of micronutrient supplementation including vitamin B<sub>12</sub> plus monitoring of micronutrient status throughout pregnancy for women following vegan diets.<sup>40</sup>

Treatment of vitamin B<sub>12</sub> deficiency in affected children was predominantly (84%) performed by oral supplementation only, which has been reported as an effective alternative to invasive intramuscular applications.<sup>42</sup> All patients remained without clinical symptoms at short-term follow-up. Although most of the patients were still too young at the last time point with clinical information available to draw final conclusions on developmental outcome, at least for the 8 patients who had already reached or passed their second half-year of life clinical symptoms would be expected in untreated vitamin B<sub>12</sub> deficiency. This supports the postulated clinical benefit achieved by NBS and consecutive early and presymptomatic treatment for children with maternal vitamin B<sub>12</sub> deficiency. In 2 patients detected by our study with moderate vitamin B<sub>12</sub> deficiency, standardized developmental testing proved normal development aged 16 and 23 months.

We conclude that vitamin B<sub>12</sub> deficiency is a suitable and important condition for systematic inclusion into the German and other NBS panels. With an incidence in our study of 1 in 5355 newborns, (maternal) vitamin B<sub>12</sub> deficiency is more frequent than inborn errors of metabolism included in NBS panels. The proposed strategy using 2 second-tier algorithms is feasible and effective to identify moderate and severe cases of vitamin B<sub>12</sub> deficiency. However, milder cases or deficiencies developing within the following months of life with prolonged exclusive breast milk feeding might not be detected. In children with risk factors like prolonged exclusive breast-feeding diagnostic work-up including vitamin B<sub>12</sub> status should be initiated by the treating clinicians. The most effective long-term prevention strategy for both mother and child would include screening for maternal vitamin B<sub>12</sub> deficiency throughout the preventive appointments in pregnancy. Given inclusion of vitamin B<sub>12</sub> deficiency in NBS panels, many children and mothers could benefit from early treatment and prevention of long-term sequelae. ■

Submitted for publication Apr 30, 2019; last revision received Jul 14, 2019; accepted Jul 23, 2019.

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**Figure 3.** NBS measures in 33 patients with vitamin B<sub>12</sub> deficiency (first NBS sample). Diagrams show the number of patients with normal (*green*) or abnormal (*red*) results for the respective first- and second-tier measures in the first NBS sample.

**Table I. Current target disorders of the national German NBS program (as of October 2018)**

Disorders	
Endocrine disorders	Congenital hypothyroidism Congenital adrenal hyperplasia
Metabolic disorders	Biotinidase deficiency Galactosemia (classical) Phenylketonuria/hyperphenylalaninemia (including cofactor deficiencies) Tyrosinemia type I* 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*	

\*At the time the study Newborn Screening 2020 started, the German NBS panel included 14 target disorders. Cystic fibrosis was added in September 2016, tyrosinemia type I in March 2018.

**Table II. 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)	Met/Phe (high)	tHcy
MTHFR deficiency, Cbl-D-Hcy, Cbl E-, Cbl-G-defect	Met (low), Met/Phe (low)	tHcy
Cbl C-, D-, F-, J-defect, Transcobalamin II def.	C3, C3/C2, Met (low), Met/Phe (low)	MMA, MCA, tHcy
Methylmalonic acidurias (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, Met (low), Met/Phe (low)	MMA, MCA, tHcy
Propionic aciduria	C3, C3/C2	3-OH-PA, MCA
Urea cycle disorders, e.g. citrullinemia type I, argininosuccinate lyase deficiency	e.g. citrulline, argininosuccinate	None
Multiple acyl-CoA dehydrogenase def.	Multiple acylcarnitines (C4-C18)	None
HMG-CoA lyase def.	3-Hydroxyisovalerylcarnitine (C5-OH), methylglutarylcarnitine (C6-DC)	None
Carnitine transporter deficiency	Free carnitine (C0)	None

**Table III.** NBS results of 4 patients with vitamin B<sub>12</sub> deficiency or functional vitamin B<sub>12</sub> deficiency not identified in the pilot project Newborn Screening 2020 (N1-N4) and 1 patient born before the study diagnosed symptomatically aged 8 months with vitamin B<sub>12</sub> deficiency (S1)

Patients	Newborn screening									Confirmatory testing			
	tHcy, $\mu\text{mol/L}$	Met, $\mu\text{mol/L}$	Met/Phe	C3, $\mu\text{mol/L}$	C3/C2	MMA, $\mu\text{mol/L}$	3-OH-PA, $\mu\text{mol/L}$	MCA, $\mu\text{mol/L}$	Vitamin B <sub>12</sub> (S), pmol/L	Folic acid (S), nmol/L	Hcy (P)	MMA (U) mmol/mol crea If in plasma, stated explicitly, $\mu\text{mol/L}$	
	Cut-off	12 (Perc 99.2)	low 11 (Perc 5) high 35 (Perc 99.5)	low 0.26 (Perc 5) high 0.56 (Perc 89)	5.5 (between Perc 99 and Perc 99.5)	0.22 (Perc 99.5)	2.14 (Perc 99.9)	34.48 (Perc 99.9)	0.08 (Perc 99.9)	160-670*	4.5-21	2-14	<18 (conventional method) <10 <sup>MMA-I</sup>
N1	First DBS	7.6	15	0.41	1.82	0.08	0.93 2.35 (P 99.9) <sup>†</sup>	ND 43.32 (P 99.9) <sup>†</sup>	0.04 0.34 (P 99.9) <sup>†</sup>	<b>144</b>	<b>33</b>	<b>18</b>	<b>78.3</b> <sup>MMA-I</sup>
	Cut-off												
N2	First DBS	9.9	12	0.39	1.09	0.05	<b>2.65</b>	38.68	<b>0.4</b>	<b>126</b>	<b>&gt;45</b>	13	<b>137</b>
N3	First DBS	5	19	0.29	2.50	0.07	ND	ND	0.08	<b>145</b>	<b>&gt;45</b>	10	<b>46</b>
N4	First DBS	2.8	25	0.54	1.63	0.08	ND	ND	0.03	240	mv	<b>29</b>	<b>64</b>
S1	First DBS <sup>‡</sup>	<b>24.1</b>	11	<b>0.25</b>	2.34	0.12	<b>19.7</b>	30.03	<b>0.09</b>				
	DBS (Selective diagnostics)	<b>58.4</b>	<b>7</b>	<b>0.21</b>	2.43	0.12	<b>11.65</b>	26.11	<b>0.1</b>	<b>20</b>	mv	<b>92</b>	Urine <b>1103.8</b> <sup>MMA-I</sup> Plasma <b>16.33</b> <sup>MMA-I</sup> (N < 0.26)

crea, creatinine; Hcy, homocysteine; MMA-I, methylmalonic acid quantification using stable-isotope labeled D3-MMA as internal standard; mv, missing value; ND, not detectable; P, plasma; Perc, percentile; S, serum.

Retrospective analysis of second-tier metabolites from first NBS sample and confirmatory testing were performed age 1 month (patient N1), 2.5 months (patient N2), 8 months (patient N3), 6 months (patient N4), and 8 months (patient S1). Reasons for work-up were abnormal NBS of the twin sibling (patient N1), seizures (patient N2), developmental delay after 7 months exclusive breast-feeding by a vegetarian mother (patient N3), suspicion of seizures in patient N4 exclusively breast-fed for 6 months, and failure to thrive in patient S1.

Out-of-range results with regard to cut-offs in current strategy in the pilot project NBS 2020 are marked in bold

\*Laboratory reference range for vitamin B<sub>12</sub>. Different classifications of severity of vitamin B<sub>12</sub> deficiency are presented in the Discussion section.

<sup>†</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 measures changed in patients N2, N3 and N4. Second-tier measurements for patient S1 were subject to the initial cut-offs stated at the top of Table III.

<sup>‡</sup>Retrospective second-tier determination 9 months after sampling.