Original article

Newborn screening for remethylation disorders and vitamin B_{12} deficiency-evaluation of new strategies in cohorts from Qatar and Germany

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Background: **Newborn screening is a precondition for early diagnosis and successful treatment of remethylation disorders and classical homocystinuria (cystathionine-*ß*-synthase deficiency). Newborn screening for classical homocystinuria using total homocysteine measurement in dried blood spots has been very successfully performed for many years for newborns from Qatar.**

Methods: **A new optimized newborn screening strategy for remethylation disorders and homocystinuria was developed and evaluated for newborns from Qatar using total homocysteine measurement as first-tier and methionine, methionine-phenylalanine-ratio and propionylcarnitine as second-tiers. Proposed cut-offs were also retrospectively evaluated in newborn screening samples of 12 patients with remethylation disorders and vitamin B_{12} deficiency from Qatar and Germany.**

Results: **Over a 12 months period, the proposed strategy led to a decrease in the recall rate in homocysteine screening for Qatar from 1.09% to 0.68%, while allowing for additional systematic inclusion of remethylation disorders and vitamin B_{12} deficiency into the screening panel for Qatar. In the evaluated period the applied strategy would have detected all patients with classical homocystinuria identified by the previous strategy and in addition 5 children with maternal nutritional vitamin B_{12} deficiency and one patient with an isolated remethylation disorder. Additional retrospective evaluation of newborn screening samples of 12 patients from Germany and Qatar with remethlyation disorders or vitamin B_{12} deficiency showed that all of these patients would have been detected by the cut-offs used in the proposed new strategy. In addition, an adapted strategy for Germany using methionine, methionine-phenylalanine-ratio and propionylcarnitine as first-tier, and homocysteine as a second-tier test was also positively evaluated retrospectively.**

Conclusions: **The proposed strategy for samples from Qatar allows inclusion of remethylation disorders and vitamin B_{12} deficiency in the screening panel, while lowering the recall rate. An adapted second-tier strategy is presented for screening in Germany and will be prospectively evaluated over the next years in a pilot project named "Newborn Screening 2020".**

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Key words: **classical homocystinuria; newborn screening; remethylation disorders; second-tier; vitamin B_{12} deficiency**

Introduction

Worldwide experience with newborn screening for homocystinuria and remethylation disorders is limited. However, there is conclusive evidence for the benefit of early identification by newborn screening and consecutive early treatment for patients with homocystinuria due to cystathionine *ß*-synthase (CBS) deficiency and certain remethylation disorders, especially severe methylenetetrahydrofolate reductase (MTHFR) deficiency and some disorders of intracellular cobalamin metabolism.[1-3] Also for severe maternal vitamin B_{12} deficiency a clear benefit of early detection and treatment can be assumed for both child and mother.[4] In principle, these conditions can be detected by newborn screening using markers

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like methionine (Met), methionine/phenylalanine (Met/Phe) ratio and total homocysteine (Hcy), and in case of combined remethylation disorders also propionylcarnitine (C3) and its respective ratios to other acylcarnitines. In our screening center, newborn screening for CBS deficiency has been performed for newborns from Qatar since 2006 using total Hcy in dried blood spots (DBS) as the primary marker. Results from this screening project and methods applied have been previously published.[5] After analysis of homocystinuria screening results from 2006 to 2013, an optimized second-tier strategy in screening for CBS deficiency has been recently established and presented.[6] In addition, we now developed and evaluated an improved strategy to systematically include also all remethylation disorders in the newborn screening panel for Qatar. This strategy uses total Hcy as a primary marker, and other metabolites from tandem-mass spectrometry (tandem-MS) screening as secondary markers. The proposed cut-offs used in this approach were in addition retrospectively evaluated in newborn screening samples of patients with confirmed remethylation disorders and vitamin B_{12} deficiency from Qatar and Germany.

Methods

Current newborn screening for Qatar

Blood spots taken between the 36th and 72nd hour of life were sampled on newborn screening cards (Neonatal Screening Card; Whatman filter paper 903, GE Healthcare Europe GmbH, Freiburg, Germany) and dried at room temperature. Samples were then transported to the Heidelberg Newborn Screening Laboratory by airplane. Newborn screening using electrospray ionization tandem-MS (Micromass Ultima) for determination of amino acids and acylcarnitines was performed in all samples as previously described.[7] In addition, measurement of total Hcy from DBS was performed for all newborn screening samples from Qatar as described previously.[5] The primary target of Hcy newborn screening so far was detection of CBS deficiency. So far, if total Hcy in DBS was above the cut-off (12 μmol/L) in the first screening sample, analysis of Hcy and Met in plasma was requested to be performed in Qatar and a second DBS card was requested for analysis of total Hcy in our laboratory. Plasma samples for analysis in Qatar were drawn at the same time as the second DBS card in the context of an outpatient visit to the metabolic center in Doha or -if required due to the clinical situation of the child- in the context of a hospital admission in Doha. The second DBS card was again transported to the Heidelberg Newborn Screening Laboratory by airplane.

New approach for Qatar including newborn screening for remethylation disorders

A new approach was established to additionally include remethylation disorders in newborn screening for Qatar, by evaluation of the second-tier markers Met, Met/Phe ratio, C3 and the propionylcarnitine/acetylcarnitine (C3/C2) ratio in samples with elevated Hcy concentrations. The logistics of sampling and transportation, and the methods of analysis remained unchanged to the old approach. Concerning interpretation of results, the proposed new algorithm for the first newborn screening (NBS) sample is depicted in Fig. 1. This algorithm also includes a strategy for samples where no valid result for Hcy

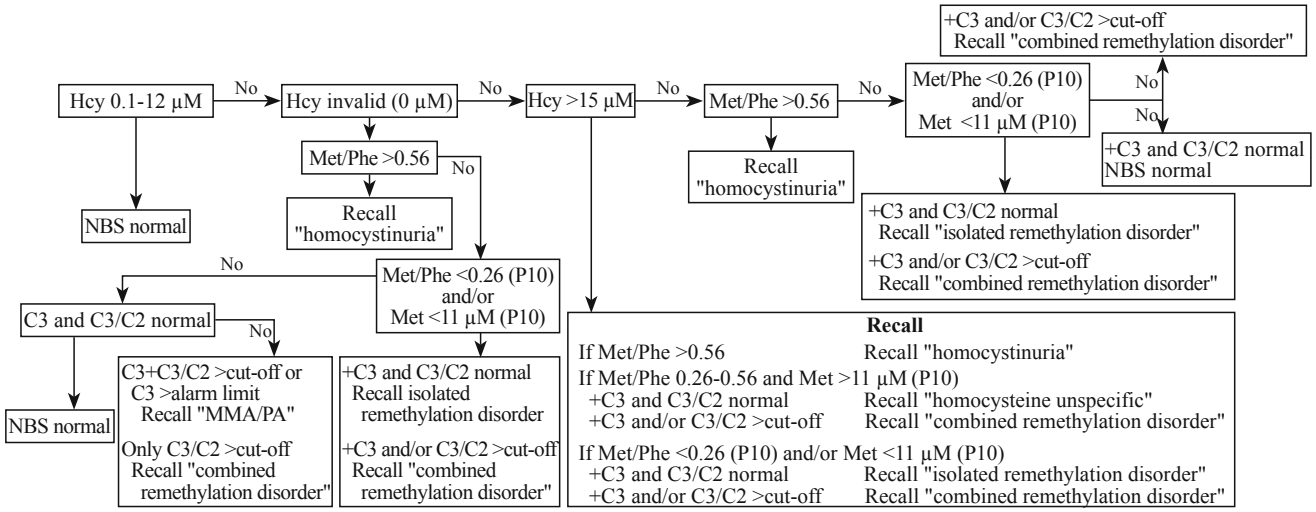

Fig. 1. Proposed new algorithm for the first blood spot in newborn screening for Qatar. Hcy: homocysteine; Met: methionine; Phe: phenylalanine; NBS: newborn screening; C3: propionylcarnitine; C2: acetylcarnitine; MMA: methylmalonic aciduria; PA: propionic aciduria.

Table 1. Newborn screening results of five patients from Qatar with classical homocystinuria due to cystathionine ß-synthase deficiency, five patients with maternal vitamin B_{12} deficiency and one patient with isolated remethylation disorder detected by newborn screening between January 2015 and February 2016

Patient number	Diagnosis	Cut-offs Hcy (µmol/L) 12 (P99.2)	Met (µmol/L) Low 11 (P10) High 35 (P99.5)	Met/Phe Low 0.26 (P10) High 0.56 (P89)	C3 (µmol/L) 5.5 (between P99 and P99.5)	C3/C2 0.22 (P99.5)	C3/C0 0.28 (P99.5)
N1	CBS deficiency						
	First DBS	**44.4**	**46**	**1.18**	2.19	0.13	0.09
	Second DBS	**46.4**	**155**	**4.84**	0.88	0.10	0.04
N2	CBS deficiency						
	First DBS	**56.5**	**82**	**1.28**	1.65	0.09	0.09
	Second DBS	**67.1**	**111**	**3.83**	0.47	0.06	0.03
N3	CBS deficiency						
	First DBS	**48.8**	**34**	**0.77**	1.71	0.08	0.11
	Second measurement from plasma (performed in Qatar)	**100.0**	**500**	-	-	-	-
N4	CBS deficiency						
	First DBS	**62.5**	**48**	**0.89**	2.88	0.13	0.14
	Second measurement from plasma (performed in Qatar)	**99.6**	**70**	-	-	-	-
N5	CBS deficiency						
	First DBS	**59.2**	**85**	**2.30**	2.59	0.14	0.13
	Second DBS	**77.9**	**194**	**8.43**	0.72	0.10	0.04
N6	Maternal vitamin B_{12} deficiency*						
	First DBS	**32.9**	12	**0.15**	**6.74**	**0.67**	**1.12**
	Second DBS (taken under supplementation with vitamin B_{12})	**12.2**	21	0.38	1.01	0.20	0.20
N7	Maternal vitamin B_{12} deficiency						
	First DBS	**15.6**	31	0.40	**10.96**	**1.00**	**0.91**
	Second DBS	**47.9**	33	**0.23**	**7.13**	**0.71**	**0.89**
N8	Maternal vitamin B_{12} deficiency						
	First DBS	**65.8**	**7**	**0.18**	**5.58**	0.19	0.23
	Second DBS	**48.9**	12	0.34	4.40	**0.29**	**0.31**
N9	Maternal vitamin B_{12} deficiency						
	First DBS	**50.1**	17	**0.25**	2.27	0.09	0.09
	Second DBS	**18.6**	12	0.40	3.41	**0.24**	0.16
N10	Maternal vitamin B_{12} deficiency						
	First DBS	**26.6**	11	**0.23**	**8.09**	**0.24**	**0.29**
	Second measurement from plasma (performed in Qatar)	**24.2**	24	-	-	-	-
N11	Isolated remethylation disorder (cbl D-Hcy, E, G, or MTHFR)†						
	First DBS	**194.7**	**5**	**0.09**	1.47	0.10	0.08
	Second measurement from plasma (performed in Qatar)	**194.0**	**7**	-	-	-	-

Out-of-range results with regard to cut-offs in our proposed new strategy are marked in bold. In several cases of maternal vitamin B_{12} deficiency (patients N6, N8, N9), homocysteine had already decreased in the second DBS. In patient N6 this is explained by the fact that the second sample was taken under vitamin B_{12} supplementation. In the other children this could be explained by feeding of infant formula, which contains vitamin B_{12}. However, unfortunately we do not have information if these children were breast or bottle fed. *: twin to patient N7; †: in patient N11 final subspecification of the isolated remethylation disorder was still pending at time of publication, therefore diagnosis is stated as "solated remethylation disorder" (includes cbl-D-Hcy, E, G, MTHFR). Confirmational diagnostics initiated in Qatar showed normal concentration of methylmalonic acid in DBS and a vitamin B_{12} level of 111 pmol/L. "-": none; CBS: cystathionine ß-synthase; Hcy: homocysteine; Met: methionine; Phe: phenylalanine; C3: propionylcarnitine; C2: acetylcarnitine; C0: free carnitine; DBS: dried blood spots; cbl: cobalamin; MTHFR: methylenetetrahydrofolate reductase.

measurement could be obtained due to technical reasons (Hcy invalid, "0 µmol/L"). For selection of cut-offs (Tables 1 and 2), we used data on population percentiles from our own center and published experiences and recommendations from other centers.[1,8] The selection of the optimal marker and cut-off for the second-tier strategy for detection of CBS deficiency has been established in a previous study.[6] This study reevaluated screening results from our center in samples from Qatar between 2006 and 2013, including 30 confirmed cases of classical homocystinuria under consideration of the reliability of Met/Phe results from repeated measurements. The cut-off for Met/Phe established to achieve 100% sensitivity for classical homocystinuria was the 89th percentile of Met/Phe (0.56), and this cut-off was therefore also used in the study reported here. Met (high) was not used as a second-tier marker in our present study. However, for information of the reader also results for Met are stated in the Tables 1 and 2, and for orientation the 99.5 percentile is included as "cut-off" for Met (high).

Depending on the grade of pathology in the first DBS analysis, recommendations given are either to send a repeat sample for analysis of Hcy, acylcarnitines and amino acids profile in DBS or to send also plasma and urine samples for additional analyses. Samples for these analyses were again transported to our laboratory by airplane.

We evaluated our new approach for Qatar in parallel to the previous strategy for newborn screening for Qatar in all samples analysed at our screening laboratory from January 2015 on. Recall rates and detection of true positive cases from this time period were compared applying the old approach (first-tier total Hcy measurement in all samples, Hcy screening targeted at CBS deficiency only) and the new strategy for Qatar (first-tier total Hcy measurement in all samples, second-tier approach targeting CBS deficiency, remethylation disorders and vitamin B_{12} deficiency) (Fig. 1). As first-tier analysis of Hcy would not be feasible in all samples from Germany analyzed in our laboratory (about 120 000/year), an adapted strategy was developed for Germany using Met, Met/Phe ratio, C3 and C3/C2 with the suggested cut-offs from model 1 as first-tier, and Hcy as second-tier if at least one of the first-tier parameters was out of range (Fig. 2).

Retrospective evaluation of the new approach in confirmed cases of remethylation disorders and vitamin B_{12} deficiency

The applied cut-offs of our new approach were evaluated in a total of 12 confirmed cases of remethylation disorders or maternal vitamin B_{12} deficiency from Qatar and Germany, in which newborn screening results were available from our center from the past years. C3, C3/C2, Met, Met/Phe are measured in all children from Germany screened by tandem-MS screening at our center, but according to regulations results have to be blinded in medical validation as respective disorders are not targeted by the current newborn screening panel in Germany. The results for these metabolites from tandem-MS screening are however available in the newborn screening data base and were retrieved for this study. If the original newborn screening DBS was still available, Hcy was determined retrospectively also in samples of patients from Germany after written informed consent of the parents had been obtained.

Results

New approach for Qatar including newborn screening for remethylation disorders

In the evaluation period of 12 months (January 2015 to December 2015; total number of NBS samples from Qatar in this period 26 202) the new approach including

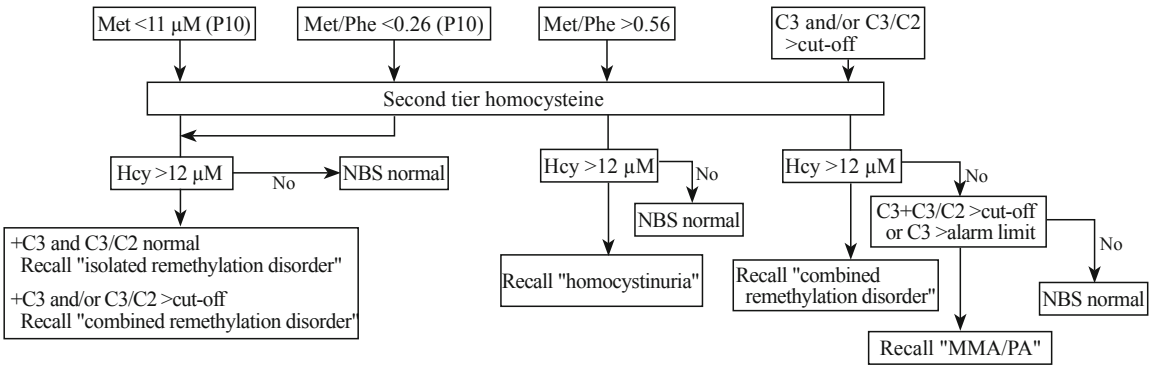

Fig. 2. Proposed new algorithm for the first blood spot in newborn screening for Germany. Met: methionine; Phe: phenylalanine; C3: propionylcarnitine; C2: acetylcarnitine; Hcy: homocysteine; NBS: newborn screening; MMA: methylmalonic aciduria; PA: propionic aciduria.

Table 2. Newborn screening results of 12 patients with confirmed cobalamin defects/remethylation disorders or vitamin B_{12} deficiency from Germany and Qatar

Patient number	Diagnosis	First DBS						Remarks	Patient would have been detected by new strategy	
		Cut-offs	Hcy (μmol/L) 12 (P99.2)	Met (μmol/L) Low 11 (P10) High 35 (P99.5)	Met/Phe Low 0.26 (P10) High 0.56 (P89)	C3 (μmol/L) 5.5 (between P99 and P99.5)	C3/C2 0.22 (P99.5)	C3/C0 0.28 (P99.5)		Qatar	Germany
R1	cblC*		ND	32	0.56	**6.66**	**0.26**	0.15			Yes
R2	cblC†		ND	15	**0.20**	**17.27**	**0.69**	**0.60**	First available sample at our lab taken at 2.5 wk of age		Yes
R3	cblC†		**83.9**	**4**	**0.08**	**7.75**	**0.60**	**0.65**		Yes	
R4	cblC†		**76.0**	**10**	**0.18**	**5.79**	**0.34**	**0.19**		Yes	
R5	cblC†		**150.2**	**7**	**0.20**	**6.48**	**0.65**	**0.43**		Yes	
R6	cblC/D*		ND	**7**	**0.15**	5.50	**0.39**	**0.37**			Yes
R7	cblC*		**57.1**	**6**	**0.15**	**6.51**	**0.36**	**0.50**	Retrospective Hcy determination 7 mon after sampling		Yes
R8	cblE*		ND	**4**	**0.09**	1.22	0.04	0.07			Yes
R9	Maternal vitamin B_{12} def.†		4.8	21	0.33	**13.21**	**0.28**	**0.36**		Yes	
R10	Maternal vitamin B_{12} def.*		**43.3**	**6**	**0.17**	**5.87**	0.17	0.18	Retrospective Hcy determination 9.5 mon after sampling		Yes
R11	MTHFR*		ND	**6**	**0.10**	2.94	0.12	0.11			Yes
R12	MTHFR*		ND	**8**	**0.17**	0.56	0.19	0.04	First available sample at our lab taken at 4 wk of age		Yes

All patients from Qatar were detected by newborn screening. In the patients from Germany, tandem-MS results shown here (except Hcy) were measured in the course of routine newborn screening, but not evaluated according to the German screening panel valid from 2005 onwards. If DBS card was still available at the time of diagnosis by selective screening homocysteine measurement was retrospectively performed from the dried blood spot after parent consent (patients R7 and R10; time span from sampling is stated under remarks). One German patient (patient R2) was detected by newborn screening in the pilot period of tandem-MS NBS before 2005, while there was no regulation on target disorders of tandem-MS NBS in Germany. Out-of-range results with regard to cut-offs in our proposed new strategy are marked in bold. *: patient detected by selective screening; †: patient detected by newborn screening. ND: not done; cbl: cobalamin; DBS: dried blood spot; def.: deficiency; MTHFR: methylenetetrahydrofolate reductase deficiency; tandem-MS: tandem-mass spectrometry; Hcy: homocysteine; NBS: newborn screening; C3: propionylcarnitine; C2: acetylcarnitine; C0: free carnitine.

the second-tier evaluation of Met, Met/Phe, C3, and C3/C2 (Fig. 1) would have led to a reduction of the recall rate in Hcy screening from 1.09% (previous strategy targeting CBS deficiency only, number of recalls 285) to 0.68% (number of recalls 178) while now in addition also systematically targeting remethylation disorders and vitamin B_{12} deficiency. From January 2015 on five patients with CBS deficiency, five patients with maternal vitamin B_{12} deficiency, and one patient with an isolated remethylation disorder have been detected. All patients have been identified by the new approach. Details on screening results for these patients are presented in Table 1. In some cases, no second DBS was sent to our laboratory, but results from plasma measurements performed in Qatar were communicated. In these cases, we have stated the result of the plasma analysis performed in Qatar and have added a comment with this regard (Table 1).

Retrospective evaluation of the new approach in confirmed cases of remethylation disorders and vitamin B_{12} deficiency

Details on newborn screening results of 12 additional patients with remethlyation disorders or vitamin B_{12} deficiency from Germany and Qatar born before 2015 are presented in Table 2. All patients from Qatar were detected by newborn screening and would also be detected by the proposed new strategy shown in Fig. 1. The patients from Germany were diagnosed by selective screening following clinical symptoms, with exception of one patient (patient R2) who was detected by newborn screening in the pilot period of tandem-MS newborn screening in Germany before 2005, while there was no regulation on target disorders of tandem-MS NBS in Germany. In the retrospective evaluation of the newborn screening samples of the patients from Germany, it was evaluated whether they would have been detected by the cut-offs suggested in our new approach for Met, Met/Phe, C3, and C3/C2 if applied as first-tier parameters (Fig. 2). Due to the larger sample size in our newborn screening for Germany (about 120 000 samples/year), using total Hcy as the primary marker in all samples would not be feasible. We therefore suggest using the cut-offs for Met, Met/Phe, C3, and C3/C2 as stated in Fig. 1 as first-tier markers and add total Hcy as secondary marker in samples with one or more out of range first-tier results (Fig. 2). In cases where the original DBS card from NBS was still available (patients R7 and R10), Hcy was determined retrospectively. Despite the fact that Hcy concentration decreases over time of storage,[9] Hcy was still markedly elevated in both cases. Our retrospective evaluation of newborn screening samples of 12 patients with remethlyation disorders or vitamin B_{12} deficiency showed that all of these patients

would have been detected by the cut-offs used in the proposed strategy. Two cases of German patients who were diagnosed by selective screening are presented in some more detail to underscore the significance of newborn screening for early detection of these disorders.

Case 1: severe maternal vitamin B_{12} deficiency

Patient R10 (Table 2) was born at 41 weeks of gestation as the first child of non-related German parents. Anthropometric parameters at birth were normal (weight: 3880 g, length: 54 cm, head circumference: 37 cm). Throughout the pregnancy and for 6 months after birth, the mother took vitamin preparations recommended for pregnancy and lactation containing vitamin B_{12} and folic acid. The newborn period and first months of life were reported as uneventful. The child was fully breast-fed for 4 months. From month 5 of life, in addition to breast milk supplementary food was introduced, but was soon rejected completely and therefore stopped again. From the age of 6 months the parents noticed a regression of motor and language development. The child had previously shown stable support on forearms in prone position and had articulated doubling of syllables. Both abilities were lost, as well as social smiling. The child showed frequent moaning. At the age of nine months the child was admitted for further diagnostic work-up of developmental delay and suspicion of seizures. At that time, the child was unable to sit, showed muscular hypotonia and unstable head-control. Cranial magnetic resonance imaging (MRI) showed brain atrophy and a deficit of myelination. Electroencephalogramm was abnormal with multifocal spikes. Metabolic work-up revealed markedly elevated Hcy in plasma of 155 µmol/L (N<15), markedly elevated methylmalonic acid (quantification using stable isotopes) of 1115 mmol/mol creatinine (N<10) and methylcitric acid (155 mmol/mol creatinine) in urine. Met was very low in plasma (6 µmol/L, N: 15-35) and undetectable in cerebrospinal fluid (0 µmol/L, N: 2.7-5.7). Acylcarnitine profile showed an elevated ratio C3/C2 (0.50, N<0.22). Vitamin B_{12} level in serum was markedly decreased with 42 pmol/L (N: 160-670). The diagnostic work-up revealed vitamin B_{12} deficiency in the mother due to a previously undiagnosed atrophic gastritis. Vitamin B_{12} was immediately administered to the child by daily intramuscular injections over two days (1 mg daily) and by oral supplementation thereafter. Shortly after start of vitamin B_{12} supplementation the child showed improved alertness, activity and muscle tone. All laboratory parameters normalized under vitamin B_{12} supplementation. Retrospective evaluation of the unblinded screening results (C3, C3/C2, Met, Met/Phe) and retrospective analysis of Hcy in the first DBS of newborn screening (Table 2) showed that this patient would have been detected by our proposed new screening strategy for Germany.

Case 2: Cobalamin (cbl) C deficiency

Patient R7 (Table 2) was born at 38 weeks of gestation after an uneventful pregnancy as the second child of parents from Turkish origin. One older brother (aged 4 years) was reported to be healthy. Birth weight was low (2600 g, 3rd percentile), the other anthropometric parameters at birth were normal (length: 51 cm, head circumference: 34 cm). In the first months the child showed feeding difficulties and eczema. Due to suspected intolerance of cow's milk protein, amino acid hydrolyzate formula was introduced. The child was referred to our outpatient clinic aged 7 months due to developmental delay, failure to thrive, and secondary microcephaly. Parents reported delayed development with first eye contact aged 5 months, social smiling 4-5 months, following of objects with the eyes at age 6 months. Aged 7 months the child started to turn to one side, but could not turn from supine to prone position. The child was reported to utter only one monotonic and short sound. On clinical examination only scarce spontaneous movements were observed, muscular tone was low, and head control was insufficient. Eye contact was only of short duration. Pedaudiologic and ophthalmologic work-up was normal. Laboratory work-up showed a pathological profile of acylcarnitines with elevated concentrations of C3 (6.3 µmol/L, N<5.5), ratios C3/C2 (0.49, N<0.22) and C3/C0 (0.63, N<0.28). Met was very low in plasma (4 µmol/L, N: 15-35) and undetectable in cerebrospinal fluid (0 µmol/L, N: 2.7-5.7). Hcy in plasma was markedly elevated with 84 µmol/L (N<15). Methylmalonic acid (quantification using stable isotopes) in urine was markedly elevated with 4700 mmol/mol creatinine (N<10). Vitamin B_{12} level in serum was high with 1209 pmol/L (N: 160-670). Cranial MRI showed a marked deficit of white matter and a pronounced delay in myelination. Based on the biochemical findings, the diagnosis of a combined cobalamin disorders was established and treatment was started with hydroxocobalamin intramuscular injection, betain and folic acid orally. This led to normal plasma Met and a marked decrease of plasma Hcy and urine methylmalonic acid. Shortly after the start of therapy the child showed improved alertness, feeding, and motor activity. Molecular genetic diagnostics confirmed cobalamin C deficiency in this patient.

Retrospective evaluation of unblinded screening results (C3, C3/C2, Met, Met/Phe) and retrospective analysis of Hcy in the first DBS of newborn screening (Table 2) showed that this patient would have been detected by our proposed new newborn screening strategy for Germany.

Discussion

The evaluation of the proposed new screening strategy for Qatar showed that it leads to a decrease in the recall rate in Hcy newborn screening of almost 40%, while allowing for additional systematic and reliable inclusion of remethylation disorders and vitamin B_{12} deficiency into the screening panel. Retrospective evaluation of newborn screening samples of patients from Qatar and Germany with established diagnoses of remethlyation disorders or vitamin B_{12} deficiency showed that all of these patients would have been detected by the cut-offs used in the proposed new strategies. Lowering the cut-offs for Met and Met/Phe from 10th percentile to 1st percentile (Met: 8 μmol/L, Met/Phe: 0.18) would still have clearly detected 2 of the 3 patients with isolated remethylation disorders (patients R8 and R11) (Table 2), while patient R12 (Table 2) would have been on the edge of non-detection. Therefore we propose to keep the suggested first-tier cut-offs for Met and Met/Phe in the prospective study for Germany.

There is evidence for a clear benefit of early identification and treatment in patients with severe MTHFR deficiency and most of the cobalamin disorders, for example late-onset cblC defect.[1,10] However, the majority of patients with cblC defect identified by newborn screening in New York state showed ongoing eye and neurocognitive disease despite early treatment.[11] This is in accordance with a report on a large cohort of patients with cblC defect by Fischer et al,[12] who found an improvement in non-neurological signs and mortality due to treatment, while long-term neurological and ophthalmological outcome were not significantly influenced.

Diekman et al[13] showed that early betaine treatment prevents mortality and allows normal psychomotor development in patients with severe MTHFR deficiency, underscoring the importance of early identification by newborn screening. For the cblE and cblG defects, evidence for the benefit of early treatment is weaker.[14]

The child with severe maternal vitamin B_{12} deficiency diagnosed by selective screening aged 9 months described in our study (patient R10) (Table 2), developed severe neurological sequelae and abnormalities on brain MRI at the time of diagnosis typical for vitamin B_{12} deficiency. There are several reports about the persistence of neurological deficits despite vitamin B_{12} treatment in children diagnosed symptomatically, sometimes even after complete resolution of all MRI abnormalities.[4] This underscores the potentially enormous benefit of early identification by newborn screening for these patients and mothers.

An adapted second-tier strategy for screening in Germany is presented, which would have detected all 12 cases with remethylation disorders or vitamin B_{12} deficiency analysed retrospectively in our study. Although based on different population percentiles, the absolute cut-offs are similar to those suggested in a study by Tortorelli et al[15] in a North American screening population. The cut-offs proposed by us are in accordance with a recent review by Huemer et al.[1] When consulting reports from the Region-4 Stork Collaborative Project, all cases with MTHFR, cblE and G described there would have been detected by our cut-offs for Met and Met/Phe.[8] However, it has to be considered that the data in the Region-4 Stork Collaborative Project are cumulated from different laboratories world-wide, using different methods.

Malvagia et al[16] recently published C17 acylcarnitine as a new biomarker with high specificity in newborn screening for disorders of the propionate metabolism, including combined remethylation disorders. Weisfeld-Adams et al[17] suggested a screening approach for cblC defect based on low methionine as secondary metabolite in cases with elevated C3. However, in our collective two of five patients with cblC defect showed methionine levels well above the 10th percentile (patients R1 and R2) (Table 2). Concerning the cut-off for C3 used for many years in our laboratory, this would miss about 10% of cases with cblC/D and vitamin B_{12} deficiency as published by the Region-4 Stork Collaborative Project.[8] With regard to reasonable recall rates, and given the limited comparability of data from the Region-4 Stork Collaborative Project with our data, we would currently not change the established cut-off for C3 and its respective ratios.

The proposed strategy for Germany allows an extension of the screening panel without necessity to introduce new methodology, as all first-tier parameters are already now obtained in the tandem-MS analysis. This is a major advantage from an economical point of view. The second-tier analysis of Hcy is also done by tandem-MS and only in those samples with abnormalities in the first-tier markers. This means that additional disorders can be included in the screening panel without need for new methodology. It would be desirable that several screening centers use the proposed algorithms to probe their efficiency in screening of methylation disorders. As the first step, the strategy proposed by us will be prospectively evaluated over the next years in our screening center, which performs newborn screening for about 120 000 children from Germany per year, in a pilot project named "Newborn Screening 2020". In this pilot project, an extension of the German newborn screening panel by 21 additional disorders including remethylation disorders and vitamin B_{12} deficiency will be evaluated, using several second-tier strategies. In this project, in addition to Hcy also methylmalonic and 3-OH propionic acid will be analysed as second-tier parameters from DBS to increase specificity of C3 screening. This will presumably also increase

specificity and probably also sensitivity in screening for combined remethylation disorders and vitamin B_{12} deficiency. This project will be essential to judge the false-positive rate and positive predictive value resulting from prospective application of the proposed second-tier strategy in Germany and will be applicable to other countries with a mainly Caucasian population and therefore low frequencies of classical homocystinuria.

Newborn screening for newborns from Qatar using first-tier Hcy in DBS can be extended from CBS deficiency to systematically and reliably include remethylation disorders and vitamin B_{12} deficiency while at the same time lowering the recall rate by second-tier evaluation. An adapted second-tier strategy appears to be feasible for newborn screening in Germany, which will be prospectively evaluated over the next years in a pilot study, and will hopefully be implemented in Germany as well as in many other countries.

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Ethical approval: All procedures followed were 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 parents of patients for report on the clinical presentation where described in detail and for retrospective analysis from newborn screening dried blood spots where applicable (samples from Germany).

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References

1 Huemer M, Kozich V, Rinaldo P, Baumgartner MR, Merinero B, Pasquini E, et al. Newborn screening for homocystinurias and methylation disorders: systematic review and proposed guidelines. J Inherit Metab Dis 2015;38:1007-1019.
2 Wong D, Tortorelli S, Bishop L, Sellars EA, Schimmenti LA, Gallant N, et al. Outcomes of four patients with homocysteine remethylation disorders detected by newborn screening. Genet Med 2016;18:162-167.
3 El Bashir H, Dekair L, Mahmoud Y, Ben-Omran T. Neurodevelopmental and cognitive outcomes of classical homocystinuria: experience from Qatar. JIMD Rep 2015;21:89-95.
4 Whitehead VM. Acquired and inherited disorders of cobalamin and folate in children. Br J Haematol 2006;134:125-136.
5 Gan-Schreier H, Kebbewar M, Fang-Hoffmann J, Wilrich J, Abdoh G, Ben-Omran T, et al. Newborn population screening for classic homocystinuria by determination of total homocysteine from Guthrie cards. J Pediatr 2010;156:427-432.
6 Okun JG, Gan-Schreier H, Ben-Omran T, Schmidt KV, Fang-Hoffmann J, Gramer G, et al. Newborn screening for vitamin B6 non-responsive classical homocystinuria-systematical evaluation of a two-tier strategy. JIMD Rep 2016 June 21. [Epub ahead of print]
7 Schulze A, Lindner M, Kohlmüller D, Olgemöller K, Mayatepek E, Hoffmann GF. Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics 2003;111:1399-1406.
8 McHugh D, Cameron CA, Abdenur JE, Abdulrahman M, Adair O, Al Nuaimi SA, et al. Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: a worldwide collaborative project. Genet Med 2011;13:230-254.
9 Bowron A, Barton A, Scott J, Stansbie D. Blood spot homocysteine: a feasibility and stability study. Clin Chem 2005;51:257-258.
10 Huemer M, Scholl-Burgi S, Hadaya K, Kern I, Beer R, Seppi K, et al. Three new cases of late-onset cblC defect and review of the literature illustrating when to consider inborn errors of metabolism beyond infancy. Orphanet J Rare Dis 2014;9:161.
11 Weisfeld-Adams JD, Bender HA, Miley-Akerstedt A, Frempong T, Schrager NL, Patel K, et al. Neurologic and neurodevelopmental phenotypes in young children with early-treated combined methylmalonic acidemia and homocystinuria, cobalamin C type. Mol Genet Metab 2013;110:241-247.
12 Fischer S, Huemer M, Baumgartner M, Deodato F, Ballhausen D, Boneh A, et al. Clinical presentation and outcome in a series of 88 patients with the cblC defect. J Inherit Metab Dis 2014;37:831-840.
13 Diekman EF, de Koning TJ, Verhoeven-Duif NM, Rovers MM, van Hasselt PM. Survival and psychomotor development with early betaine treatment in patients with severe methylenetetrahydrofolate reductase deficiency. JAMA Neurol 2014;71:188-194.
14 Huemer M, Burer C, Jesina P, Kozich V, Landolt MA, Suormala T, et al. Clinical onset and course, response to treatment and outcome in 24 patients with the cblE or cblG remethylation defect complemented by genetic and in vitro enzyme study data. J Inherit Metab Dis 2015;38:957-967.
15 Tortorelli S, Turgeon CT, Lim JS, Baumgart S, Day-Salvatore DL, Abdenur J, et al. Two-tier approach to the newborn screening of methylenetetrahydrofolate reductase deficiency and other remethylation disorders with tandem mass spectrometry. J Pediatr 2010;157:271-275.
16 Malvagia S, Haynes CA, Grisotto L, Ombrone D, Funghini S, Moretti E, et al. Heptadecanoylcarnitine (C17) a novel candidate biomarker for newborn screening of propionic and methylmalonic acidemias. Clin Chim Acta 2015;450:342-348.
17 Weisfeld-Adams JD, Morrissey MA, Kirmse BM, Salveson BR, Wasserstein MP, McGuire PJ, et al. Newborn screening and early biochemical follow-up in combined methylmalonic aciduria and homocystinuria, cblC type, and utility of methionine as a secondary screening analyte. Mol Genet Metab 2010;99:116-123.

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