



High incidence of low vitamin B12 levels in Estonian newborns

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ABSTRACT

Vitamin B12 deficiency seems to be more common worldwide than previously thought. However, only a few reports based on data from newborn screening (NBS) programs have drawn attention to that subject. In Estonia, over the past three years, we have diagnosed 14 newborns with congenital acquired vitamin B12 deficiency. Therefore, the incidence of that condition is 33.8/100,000 live births, which is considerably more than previously believed. None of the newborns had any clinical symptoms associated with vitamin B12 deficiency before the treatment, and all biochemical markers normalized after treatment, which strongly supports the presence of treatable congenital deficiency of vitamin B12. During the screening period, we began using actively ratios of some metabolites like propionylcarnitine (C3) to acetylcarnitine (C2) and C3 to palmitoylcarnitine (C16) to improve the identification of newborns with acquired vitamin B12 deficiency.

In the light of the results obtained, we will continue to screen the congenital acquired vitamin B12 deficiency among our NBS program. Every child with aberrant C3, C3/C2 and C3/C16 will be thoroughly examined to exclude acquired vitamin B12 deficiency, which can easily be corrected in most cases.

1. Introduction

Vitamin B12 deficiency is a worldwide problem, especially in developing countries where deficiency is common in all age groups [1]. In developed countries, vitamin B12 deficiency is most frequent at advanced age; however, the prevalence in younger age groups may be higher than previously thought [1,2]. The worldwide spread of NBS programs using tandem mass spectrometry (MS) has also greatly contributed highlight to the high incidence of vitamin B12 deficiency even in industrialized countries, and has also pointed out that non-inherited conditions are mainly secondary to maternal deficiency [3–8], associated with strict vegan diet, poverty and malnutrition, occult pernicious anemia, previous gastric bypass surgery, and short gut syndrome [9].

Throughout childhood, an adequate vitamin B12 status is important for normal growth and development, as demonstrated by the clinical picture in children with inborn errors of vitamin B12 absorption, transport, and metabolism [10–12]. The onset and severity of symptoms differ according to age. In infants, neurological impairment with hypotonia, seizures, developmental delay, and brain atrophy are

typical, and the damage is thought to occur during the first 6 months, which is a critical period for maturation of oligodendrocytes, and brain myelination [2,13].

Early detection and intervention is critical to prevent irreversible neurologic damage caused by prolonged vitamin B12 deficiency [14]. Numerous recently published case reports have shown that NBS with tandem MS has great potential to identify vitamin B12 deficiency, an important and treatable condition in asymptomatic stage [3,5,7,8,15].

Here we report the three-year summary of expanded newborn screening in Estonia and draw attention to high incidence of acquired vitamin B12 deficiency in our region.

2. Method

Screening samples from 2014 to 2016 consisted of capillary blood drawn from heel prick, and were collected on Whatman 903® filter paper. Samples were dried and sent by mail or courier from local hospitals to the screening laboratory (Tartu University Hospital) for analysis. The NBS coverage was close to 100%. The recommended age for obtaining a screening sample was 3–5 days after birth. All the

Abbreviations: NBS, newborn screening; MMA, methylmalonic acid; tHcy, total homocysteine; MS, mass spectrometry; DBS, dried blood spots; C3, propionylcarnitine; C2, acetylcarnitine; C16, palmitoylcarnitine; Met, methionine

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samples collected from patients with vitamin B12 deficiency were taken on the 3rd or 4th day of life.

Amino acids and acylcarnitine analysis of dried blood spots (DBS) were performed on a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, Milford, MA, USA), and MMA from urine and serum, collected during the first paediatric consultation, was measured with the Agilent 7890B GC with 5977A MSD running on MassHunter software (Agilent Technologies, Santa Clara, CA, USA). All the used reference intervals were age-specific and based on the previous experience of our laboratory, except for the ratios of C3/C2 and C3/C16, which were taken from literature [7]. Folate and vitamin B12 were measured on Cobas 601 Immunoassay Analyzer (Roche Diagnostics GmbH, Mannheim) and tHcy measurement was performed by enzymatic assay on Cobas Integra 400 Plus Analyzer (Roche Diagnostics GmbH, Mannheim). The reference intervals were taken from CALIPER database (<http://www.sickkids.ca/caliperproject/index.html> - 22.03.2017).

3. Results

In Estonia, a population-based extended newborn screening by using tandem MS was introduced on January 1st, 2014. Over the past three years, we have screened 41,453 children and diagnosed 14 newborns with congenital acquired vitamin B12 deficiency (Table 1). The incidence of this condition is 33.8/100,000 live births in Estonia.

All newborns with a C3 value higher than 4.31 $\mu\text{mol/L}$ on DBS analysis, with or without altered C3/C2 (abnormal value > 0.18 [7]) and/or C3/C16 (abnormal value > 1.80 [7]) ratios, underwent paediatric evaluation and further laboratory testing, including the measurement of vitamin B12, folate and tHcy in serum, and MMA content in urine. At that period we could not implement the MMA measurement as a second-tier test from the same DBS. Instead, this was done from fresh urine samples collected during the first paediatric consultation. MMA from serum were measured posteriorly, therefore, there were not enough serum left from all newborns who underwent the paediatric evaluation. We were able to analyse only 8 newborn's serums and all of them contained elevated amount of methylmalonic acid: 2.41–17.02 $\mu\text{mol/L}$ (abnormal value > 0.3). These results are reported in Table 1.

None of the newborns had any clinical symptoms associated with vitamin B12 deficiency before the treatment. All biochemical markers normalized after treatment, which strongly suggests the presence of impaired vitamin B12 status. After a thorough prospective clinical follow-up and laboratory testing, we can confirm that all well-known genetic disorders that could cause neonatal onset of elevated MMA were also excluded in all of the treated children.

As the most common cause for vitamin B12 deficiency among newborns is maternal vitamin B12 deficiency, the blood tests for mothers were also performed. Most of the mothers showed values of vitamin B12 within the reference interval (141–489 pmol/L) with tendency towards lower normal limit and they described themselves as healthy and nourished, without serious chronic medical conditions, except for one mother, who had been diagnosed with systemic lupus erythematosus. Her autoimmune disease was well-controlled during the pregnancy. In our study, only one mother had low vitamin B12 without any clinical complaints and she was mainly vegetarian for religious reasons. All mothers, except one, used prenatal folic acid supplements (no other vitamins added), mostly 400 micrograms per day (Table 1), which is quite widespread practice in Estonia.

4. Discussion

Present study describes fourteen cases of secondary vitamin B12 deficiency identified during newborn screening, and characterized by altered C3 concentration on DBS, with or without altered ratios of C3/C2 and/or C3/C16. The incidence of congenital acquired vitamin B12 deficiency was found to be very high in Estonia – 33.8/100,000 live

births (1:2959). Although the study period was quite short, we believe that the three-year experience is long enough to reflect the current situation of this condition in Estonia, since Italy's newborn screening program showed that the incidence of congenital acquired vitamin B12 deficiency remained similar over the years [16].

Our incidence of acquired vitamin B12 deficiency is higher than reported in most of the literature [3,7,8,16]. Sarafoglou et al. described a population-based study of 363,649 infants born in Minnesota. During the six-year study period, 11 newborns with vitamin B12 deficiency were found, thus the incidence was 3.02/100,000 live births (1:33,113). All of them had secondary changes due to maternal vitamin B12 deficiency [8]. Higher incidence was detected in the Italian population, where a six-year extended metabolic newborn screening pilot project showed an incidence of acquired vitamin B12 deficiency of 20/100,000 (1:5000) and evidenced that this condition is also mainly a consequence of maternal vitamin B12 deficiency [7]. However, it is important to point out, that not all screening results are fully comparable to one another, because most NBS programs are unique in each country/region and therefore they are using different strategies and cut-off values.

The most exceptional hypothesis was proposed by Refsum et al., who concluded that 10% of screened newborns had vitamin B12 levels below 150 pmol/L , which is the lower reference limit for adults [6]. Therefore, it would be beneficial to detect most of those children in the symptom-free phase. For this purpose, some newborn screening programs have lowered their cut-off level of C3 and started to use more actively the ratios of some metabolites. For example, Minnesota Department of Health, Mayo Clinic, and University of Minnesota markedly lowered the cut-off level of C3 from 9.2 $\mu\text{mol/L}$ to 5.25 $\mu\text{mol/L}$ and the vitamin B12 detection rate increased more than three times [8]. A similar decision was also made by Scolamiero et al. [7]. Although, Campbell et al. pointed out that the C3 is a marker used to detect organic acidurias and remethylation defects and its level alone is not sensitive or specific enough to detect all newborns with B12 deficiency, more-over C3 levels may not be sufficiently high during the first few days of life when the DBS is collected [15]. Therefore, there is some evidence that the C3 to C2 ratio and/or C3 to C16 ratio together with C3 are better than C3 level alone for detecting or identifying the babies with nutritional B12 deficiency [7,8,17]. Some authors have also suggested to measure tHcy from first DBS and if it is elevated, then evaluate the second-tier markers like methionine (Met), Met to phenylalanine ratio, C3 and C3/C2, which is considered to be valuable method to detect remethylation disorders and vitamin B12 deficiency [18]. The MMA, measured from DBS as a secondary test, might also add value to increase sensitivity and specificity in detection of vitamin B12 deficiency [8]. During the screening period in Estonia, we could not implement the second-tier testing of MMA from the DBS; however it will be introduced in our laboratory in the near future. We measured the MMA level in freshly collected urine (most of the urine samples were collected at least a week after the first DBS) and only three of the fourteen children with acquired vitamin B12 deficiency had elevated MMA in urine. The MMA concentration in serum were measured posteriorly, therefore, there were not enough serum left from all newborns who underwent the first paediatric evaluation but still it is notable, that all eight newborns, with whom we could measure serum MMA, had it elevated. (Table 1).

For a long time, the causes of maternal B12 deficiency were considered to be associated with strict vegan diet, poverty and malnutrition, occult pernicious anemia, previous gastric bypass surgery, and short gut syndrome [9]. Nowadays, the maternal B12 deficiency can be subclinical, the mothers may not be anaemic and their vitamin B12 levels are normal or low-normal. Maternal vitamin B12 concentrations during pregnancy are thought to be closely associated with fetal [19,20] and early infant [21,22] vitamin B12 status. Some authors even suggest that maternal dietary intake during pregnancy is a stronger determinant of infant vitamin B12 status than are maternal vitamin B12 stores [23].

Table 1
Biochemical and clinical parameters of the 14 newborns and their mothers, who underwent paediatric evaluation and further laboratory testing.

No	Sex	C3 (µmol/L) (abnormal value < 4.31; 99th percentile)	C3/2 (abnormal value < 0.18) ^a	C3/C16 (abnormal value < 1.8) ^a	Methionine (µmol/L) (reference intervals: 4.23–44.30; 1st to 99th percentile)	Vitamin B12 (pmol/L) (reference intervals: 216–891)	Folate (nmol/L) (reference intervals: 7.0–46.5)	Hcy (µM) (abnormal value: > 10)	MMA (Mmol/mol Cr) in urine (abnormal value: > 10)	MMA (µmol/L) in serum (abnormal value: > 0.3)	Exclusively breast-fed	Mother's vitamin B12 (pmol/L) (reference intervals: 141–489)	Mother's prenatal folic acid supplementation ≥ 400 µg/day
1.	M	4.8	0.19	1.23	8.52	162	27.7	15.7	8.75	^b	yes	251	yes
2.	M	5.27	0.3	2.17	9.01	176	38.1	6	not detected	^b	yes	316	yes
3.	M	5.88	0.23	1.8	11.89	184	32	10.3	1.5	^b	yes	Normal (autoimmune disease)	yes
4.	F	5.91	0.23	2.46	14.93	110	36.9	12.2	7.36	^b	yes	Normal	yes
5.	F	5.96	0.08	0.47	9.09	143	31.2	22.9	62.08	6.71	yes	174	yes
6.	M	7.07	0.18	2.67	6.44	103	38.5	14.1	132	4.92	yes	Normal	yes
7.	M	7.27	0.3	2.43	6.27	60	59.9	23.6	24.07	4.0	yes	Low (vegetarian)	n.a.
8.	M	7.35	0.17	2.19	13	201	^b	^b	4.97	^b	yes	168	yes
9.	M	7.94	0.14	1.42	20.21	184	67.2	8	2.51	17.02	yes	404	yes
10.	M	8.08	0.45	2.21	21.27	135	43	14	not detected	^b	yes	Normal	yes
11.	M	8.45	0.21	2.52	8.4	187	^b	^b	7.7	3.19	yes	Normal	yes
12.	M	8.94	0.18	2.46	10.6	188	79	10.3	4.6	14.74	yes	246	yes
13.	M	9.91	0.17	1.52	10.7	208	35.6	11.3	9.01	2.41	yes	347	yes
14.	M	12.16	0.16	1.58	18.99	168	30.4	6.3	1.31	14.9	yes	230	yes

Biochemical and clinical parameters of the 14 newborns and their mothers, who underwent paediatric evaluation and further laboratory testing.

^a Scolamiero et al.

^b Not measured.

Interestingly, most of our study group mothers had normal or low-normal levels of serum vitamin B12, which is similar to reports providing evidence of subclinical vitamin B12 deficiency in presence of normal serum B12 levels [24]. This phenomenon is explained by the fact that tissue levels become depleted before serum levels [25]. Some people with borderline or low-normal serum vitamin B12 levels may have symptoms that resolve with B12 treatment, suggesting that levels previously considered borderline or normal may represent deficiency in some patients [26]. Therefore, it is important to highlight that all 14 children were exclusively breast-fed.

In Estonia, majority of pregnant women use folic acid supplementation exclusively (no other vitamins added), as recommended by clinicians. The standard dose is 400 micrograms of folic acid per day and most of them are taking their supplements conscientiously. That is reflected in our study group as well – most of the mothers had taken pure folic acid during their pregnancy. Only in one case we could not verify mother's supplementation status (Table 1, patient no. 7). Selhub et al. suggested that both pathways of vitamin B12 metabolism are adversely affected by high serum folate, despite the fact that folate is directly involved only in methionine synthase activity [27]. A similar finding was described by Monsen et al., concluding that high serum folate during infancy is attributable to low B12 status and resulting methyl folate trap phenomenon [28]. More extensive studies are needed for specifying the precise connection between folic acid supplementation during pregnancy and newborns' low vitamin B12 level to make any fundamental conclusions.

Also, we should not forget that vitamin B12, folate, tHcy and MMA undergo marked changes during childhood. In the first weeks of life, there is a considerable decrease in serum vitamin B12 level, accompanied by a marked increase in plasma tHcy and MMA [29,30]. The lowest vitamin B12 levels and the highest tHcy and MMA levels in childhood are seen in infants 6 weeks to 6 months of age [28]. Therefore, the use of correct age-appropriate reference values is indispensable to prevent hyper-diagnostics. Although, we believe that significantly more harm can be done by diagnostic delay, since vitamin B12 deficiency is associated with biomarkers of genomic instability. Global DNA hypomethylation and decreased purine and pyrimidine synthesis impair genomic stability, causing chromosomal breaks and aberrations [31]. A study in Turkey noted that DNA damage was increased in vitamin B12 deficient children and their mothers [32]. Equally important is the fact that vitamin B12 deficiency is associated with retardation of neural myelination in some studies [13,33,34], and long-term consequences of neurological deterioration may persist even after vitamin B12 deficiency has been treated [35–37]. In addition, it is difficult to make the diagnosis on the clinical ground and there tends to be substantial diagnostic delay [38].

5. Conclusion

We have found very high incidence of congenital acquired vitamin B12 deficiency in Estonia (33.8/100,000 live births), which is considerably more than previously believed. According to Wilson and Jungner's principles of screening, the intention with newborn screening is to identify babies with serious and treatable conditions before symptoms arise [39], therefore we are continuing to screen the congenital acquired vitamin B12 deficiency among our NBS program. Every child with aberrant C3, C3/C2 and C3/C16 will be thoroughly examined with the second-tier test for MMA and/or tHcy to exclude acquired vitamin B12 deficiency, which can easily be corrected in most cases.

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