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The prevalence and clinical relevance of hyperhomocysteinemia suggesting vitamin B12 deficiency in presumed healthy infants

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ABSTRACT

Background: Previous studies have demonstrated a high prevalence of biochemical vitamin B12 deficiency in infants in Norway. Increased total homocysteine (tHcy) is the most important marker of B12 deficiency in infants. There is a need to evaluate its clinical relevance.
Aims: To investigate the prevalence of hyperhomocysteinemia (*S*-tHcy > 8 μmol/L) suggestive of suboptimal B12 status and the prevalence of clinically relevant hyperhomocysteinemia in presumed healthy infants in Norway. Further, to evaluate risk factors, presence of symptoms and psychomotor development in these children.
Methods: In a prospective study we clinically examined 252 infants aged 3–7 months using standardized neurological and psychomotor tests prior to analyzing biochemical B12 deficiency markers in 250 infants.
Results: Twenty-five of 250 (10%) infants had hyperhomocysteinemia combined with clinically relevant symptoms suggestive of B12 deficiency. Hyperhomocysteinemia was associated with tremor, excessive sleep, and sub-normal scores in the fine motor section of the Ages and Stages Questionnaire. One-hundred and fourteen of 250 (46%) infants had hyperhomocysteinemia. Multiple regression analysis showed months of infant formula use as the strongest negative predictor for hyperhomocysteinemia.
Conclusion: We have demonstrated associations between symptoms suggestive of infant B12 deficiency and increased levels of tHcy in presumed healthy infants The combination of hyperhomocysteinemia and associated relevant symptoms suggestive of B12 deficiency was a common finding, albeit most infants with hyperhomocysteinemia did not show symptoms.

Abbreviations: AGA, appropriate weight for gestational age; B12, vitamin B12; CA, corrected age for term date; GA, gestational age; HHcy, hyperhomocysteinemia; holoTC, holotranscobalamin; MMA, methylmalonic acid; P, plasma; S, serum; SGA, small for gestational age; tHcy, total homocysteine.

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1. Introduction

During the first year of life the brain is growing, myelinating and maturing rapidly; processes in which vitamin B12 is vital [1,2]. In B12 deficient infants, symptoms such as hypotonia, excessive reflux, tremor, seizures, apneas, and delayed psychomotor development have been reported. It is also common with irritability, failure to thrive, apathy and food refusal [1,3–7]. The symptoms and signs of B12 deficiency in infants can be subtle and diffuse and overlap with other common diseases. Infant B12 deficiency may impair cognitive development later in childhood [2,8,9].

In Canada, it was found that 5% of women aged 20–45 years were B12 deficient, and 20% had marginal stores [10]. Pregnant women are not routinely screened for B12 deficiency in Norway [11], and the prevalence in Norway is not known. Maternal B12 status in pregnancy is strongly correlated with infant B12 status [12]. The infant's hepatic B12 reserves and mother's breast milk B12 content are reduced when her B12 status is poor, and thus her infant is at risk of developing clinically relevant B12 deficiency within 4–10 months of age if predominantly breastfed [2,5,6,13]. In Norway, recommendations from WHO are followed, and exclusive breastfeeding until 6 months of age is recommended [14]. Thirty-nine percent of 4 month-old infants were exclusively breastfed according to a recent national survey [15]. In a Norwegian cohort of 107 healthy, breastfed infants, two-thirds were moderately biochemically B12 deficient at 6 weeks and 4 months of age [16]. B12 supplementation significantly improved motor development in these infants [17].

To our knowledge, retrospective case studies and cohort studies have so far only explored the biochemical B12 deficiency prevalence without concurrent evaluation of the clinical and developmental status of the child [16,18,19], or only hospitalized infants have been recruited [20]. The measurement of total B12 in isolation has limited diagnostic value as a discriminator of B12 deficiency, and the diagnosis of B12 deficiency requires the use of additional biomarkers such as methylmalonic acid (MMA), total homocysteine (tHcy) and holotranscobalamin (holoTC). During the first two years of life, tHcy reflects B12 status rather than folate status while folate is the main determinator of tHcy later in life, and tHcy is therefore the best marker of infant B12 status [7]. Vitamin-optimized plasma-tHcy is < 6.5 μmol/L at 4 months of age [16].

The primary aim of our exploratory, prospective study was to investigate the prevalence of hyperhomocysteinemia (HHcy) and its clinical relevance in presumed healthy infants in Norway. Secondary aims were to evaluate risk factors for HHcy and its association with infant symptoms and psychomotor development.

2. Materials and methods

2.1. Study population

327 infants without identified perinatal neurological disease, and their mothers, were consecutively invited from the Postnatal and Neonatal Units at Vestfold Hospital, Norway, between May 2018 and March 2019 (Fig. 1) to come to the hospital out-patient clinic for a neurological examination and blood sampling to participate in our study. Seven of 327 infants were excluded due to work-up for suspected B12 deficiency after invitation. Five of the seven infants were diagnosed with B12 deficiency (5/327, 1.5%) with *S*-tHcy >8 μmol/L and suggestive symptoms. Sixty-one infants did not attend the clinical appointment and were therefore not included. One set of twins underwent testing but were then withdrawn from the study by the family. Two-hundred and fifty-two infants were included after informed written consent and were stratified into three groups: n = 170 born at gestational age (GA) ≥37 weeks and appropriate weight for gestational age (AGA), n = 39 born at GA≥37 weeks and small for gestational age (SGA, weight below the 10th percentile in relation to GA [21]) and n = 43 born at GA 32–36 + 6 weeks. Infants that were both preterm and SGA were classified as 'preterm' (7/43, 16%). Blood sampling failed in two infants leaving 250 infants with available blood test results. One set of triplets and 13 pairs of twins, of which 3 were monochorionic and 10 dichorionic, were among the participants.

2.2. Questionnaires

The parents completed three questionnaires prior to clinical examination in the hospital; 1) presumed risk factors for vitamin B12 deficiency and mother and infant nutrition, 2) symptom scoring of their infant and 3) Ages and Stages Questionnaire (ASQ) [22], either the four or six months version according to age. The first and second questionnaires were developed specifically for this study and have not been validated. ASQ is a standardized screening tool for global development. The symptom scoring reported by the parents consisted of questions concerning twelve specific symptoms that could be answered with one of three choices: Do not agree, partly agree, and fully agree. The answers were dichotomized (yes (partly agree/fully agree) or no). The selection of covariates, *i.e.* suggested risk factors and symptoms of B12 deficiency, was based on previous reports [3–7]. We measured exclusive breastfeeding in total months and as a dichotomous variable. We measured formula feeding in total months of either formula complementing breastfeeding or as exclusive formula feeding hereafter named 'formula/mixed feeding'. Folate supplement could be used either as a sole folate supplement or folate contained in a multivitamin, both in a dose of 400 μg/daily, the dose recommended in Norway for the first trimester, hereafter named 'folate supplement'. B12 supplement could be used as low dose (2–2.5 μg) contained in a multivitamin for daily use or prescribed as high dose cyanocobalamin 1 mg or parenteral hydroxocobalamin 1 mg.

2.3. Neurological examination and psychomotor testing

The visit for the study infant examination was chosen consecutively to cover the age span between 3 and 7 months when clinical B12 deficiency most often is diagnosed [2,4–6,13]. We examined infants once, with information of age corrected for term date (CA) only, and without prior knowledge of B12 status or clinical and perinatal history. The parents were asked not to inform the examiners before the tests were completed and recorded. All infants were examined by the same pediatrician (UWL) and in 248/254 cases (98%) by the same pediatric physiotherapist (HP). UWL and/or HP performed a standardized infant neurological examination using the Hammersmith Infant Neurological Examination (HINE) [23–25]. The HINE is divided in three sections. Section one consists of 26 items assessing cranial nerve function, posture, movements, tone, and reflexes, and the items are scored zero to three points in 0.5-point steps. Section two is a short, non-scorable development assessment, in this study substituted with the scorable, more comprehensive and standardized Alberta Infant Motor Scale (AIMS) [25,26], and section three is assessment of state at examination. In 149 of 252 (59%) infants, HINE was repeated independently by HP to evaluate reliability. HP also tested the infants with AIMS, Test of Infant Motor Performance (TIMP) [25,27,28] and General Movement Assessment including assessment of motor repertoire producing a motor optimality score (GMA/MOS) [25,29]. The latter two tests are only feasible before infants start with intentional movements at four months of age.

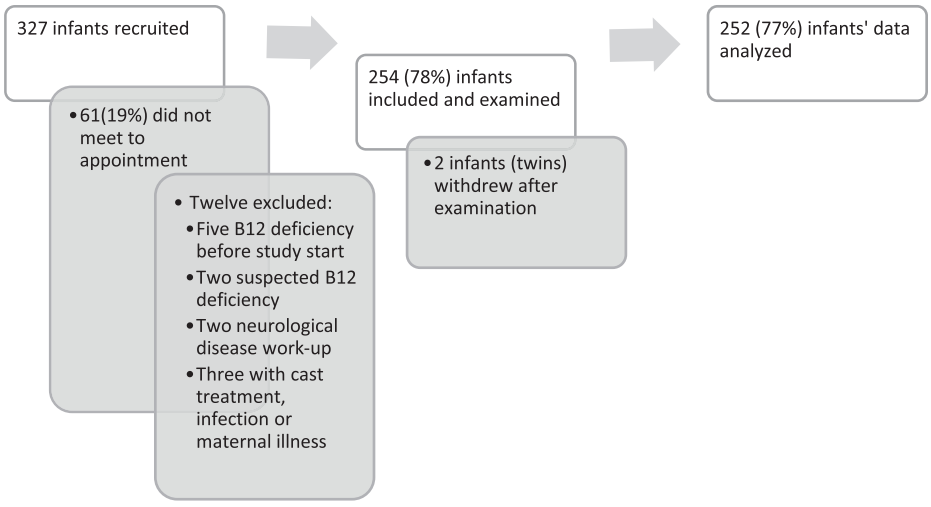

Fig. 1. Online. Flowchart of recruitment, inclusions, and exclusions in the present study.

2.4. Biochemical analyses

Venous blood samples were collected non-fasting in 4 mL serum tubes with serum separator and clot activator (Vacuette®, Greiner Bio-One, Austria) from 250 infants and analyzed at the Department of Medical Biochemistry at Vestfold Hospital Trust. Venipuncture failed in two infants. Analysis of serum B12, holoTC and folate were performed on Cobas e801 from Roche Diagnostics GmbH, Mannheim, Germany. The measuring range of serum folate was 4.5 nmol/to 45 nmol/L. Results above 45.4 nmol/L are reported as "> 45 nmol/L". Hematology samples were analyzed using XN-9000 analyzers from Sysmex Co., Kobe, Japan, while MMA and tHcy were simultaneously determined by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method in serum. To obtain serum, the blood samples were left at room temperature for a minimum of 30 min to allow for coagulation and centrifuged within 2 h. During this time, tHcy is released from erythrocytes, causing slightly higher values in serum than in plasma (~+1 μmol/L). Duplicate measurement in serum (*S*-) and plasma (*P*-) from 75 blood donors with tHcy in plasma below 10.0 μmol/L yielded equation *P*-tHcy = 0.006153 + 0.8074 * *S*-tHcy (r = 0.925). The cut off limit of 6.5 μmol/L in plasma was converted to 8.0 μmol/L in serum according to the regression algorithm. The families were informed about their infants' blood test results and, where appropriate, given nutritional advice including the need for supplementation with iron or vitamins.

2.5. Definitions

We defined HHcy as *S*-tHcy>8 μmol/L. We defined clinically relevant HHcy as whenever any of the following symptoms were occurring significantly more often in infants with HHcy than in infants with *S*-tHcy<8 μmol/L: feeding difficulties, regurgitations, failure-to-thrive, irritability, spells of absence, apneas and seizures, abnormal movements, tremor, reduced spontaneous motor activity, excessive sleep, abnormal eye contact, hypotonia, developmental delay and cytopenia, reported as associated with B12 deficiency in infants in the literature [1,3–7]. Tongue fasciculations as an associated sign of HHcy was not included for systematic observation when the study was planned.

2.6. Statistics

Data were registered in EpiData version 4.4 from EpiData Association, Odense, Denmark. Symmetric continuous variables were presented as mean and standard deviation or if skewed, as median and interquartile range. Categorical variables were given as proportions and percentages. Not normally distributed variables were natural logarithmically transformed to ensure normality before analyses, and when converted back to original units for the sake of interpretation, presented as geometric means. Differences between independent groups regarding normally distributed variables were quantified with the two-sample *t*-test, or Mann Whitney *U* test in case of uncorrectable skewness in the data. Categorical variables were compared between groups using the Chi squared ($\chi 2$) test for homogeneity (test of proportions) or Fisher's exact test for small samples. All statistical tests were two-sided, and a p-value <0.05 was considered statistically significant. We defined biologically significant differences when Cohen's d was >0.25, or covariates in regressions that caused a change >0.25 SD of the dependent variable when the covariate changed 2 SD. To evaluate possible significant covariates for HHcy and test-results in infants, linear or logistic regression was applied. Presented models were statistically significant with $p < 0.001$ and did not violate assumptions. To identify significant exposure variables in regressions for risk factors, we used candidate variables in Tables 1 and 2. Variables correlating with the independent variable with Spearman's rho over 0.1 were included in a crude model. Then non-significant variables were removed for a more saturated model. The variables were again re-introduced one at a time and retained if they became significant. This was repeated, and in the final model we only kept biologically relevant variables significant at a 0.05 level. We decided a priori to include CA in all regression models. All analyses were performed in IBM SPSS Statistics version 27 (SPSS Inc, Chicago, IL, USA). NCSS 2021 Statistical Software (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss) was used for figure 2 and 3.

The study was approved by the Regional Ethics Committee (179/2018) and conducted according to the Helsinki declaration. Written informed consent was collected for all participants.

Table 1
Descriptive characteristics of mothers and infants.

		AGA Term (N=170)	SGA Term (N=39)	Preterm (N=43)	Total (N=252)
		N (%)	N (%)	N (%)	N (%)
Origin of mother[a]	Norway	134 (79)	28 (72)	31 (72)	193 (77)
	Other Nordic	4 (2.4)	1 (2.6)	1 (2.3)	6 (2.4)
	Europe	18 (11)	6 (15)	3 (7)	27 (11)
	Non-Europe	14 (8)	4 (10)	8 (19)	26 (10)
Education	Elementary	6 (3.6)	0	0	6 (2.4)
	High school	45 (27)	18 (47)	8 (21)	71 (29)
	University	118 (70)	20 (53)	31 (79)	169 (69)
Parity	0	100 (59)	24 (62)	14 (33)	138 (55)
	1	50 (29)	14 (36)	20 (47)	84 (33)
	2 or more	20 (12)	1 (3)	9 (21)	30 (12)
Known maternal B12 deficiency		11 (7)	9 (23)	4 (10)	24 (10)
Metformin use in pregnancy		6 (4)	2 (5)	1 (2)	9 (4)
Smoking last 2 years		14 (8)	6 (15)	10 (25)	30 (12)
Diabetes in pregnancy		9 (5)	2 (5)	5 (12)	16 (6)
Preeclampsia		5 (3)	4 (10)	5 (12)	14 (6)
Hyperemesis		10 (6)	4 (12)	2 (5)	16 (7)
B12-containing[b] supplements during pregnancy		106 (62)	28 (72)	29 (71)	163 (65)
High-dose 1000 μg oral B12 supplement during pregnancy		7 (4.1)	2 (5.1)	4 (9.8)	13 (5.2)
Parenteral 1000 μg B12 during pregnancy		3 (1.8)	3 (7.7)	2 (4.9)	8 (3.2)
Folate[c] during pregnancy		150 (88)	30 (77)	39 (95)	219 (88)
B12-containing[b] supplements during breastfeeding		52 (31)	19 (49)	16 (39)	87 (35)
High-dose 1000 μg oral B12 supplement during breastfeeding		3 (1.8)	5 (13)	1 (2.5)	9 (3.6)
Parenteral 1000 μg B12 during breastfeeding		1 (0.6)	3 (7.7)	2 (5.0)	6 (2.4)
N_2O analgesia		129 (76)	22 (56)	19 (46)	170 (68)
Multiple birth	Duplex infants	7 (4)	3 (8)	16 (37)	26 (10)
	Triplex infants	0	0	3 (7)	3 (1)
Delivery	Vaginal	146 (86)	29 (74)	21 (49)	196 (78)
	Cesarean section	24 (14)	10 (26)	22 (51)	56 (22)
Cord clamping	Immediately	17 (11)	5 (14)	16 (42)	38 (16)
	1–3 min	38 (24)	11 (30)	12 (32)	61 (26)
	over 3 min	106 (66)	21 (57)	10 (26)	137 (58)
Sex	Female	84 (49)	23 (59)	17 (40)	124 (49)
Type of feeding	Exclusively breastmilk	63 (37)	11 (29)	8 (20)	82 (33)
tHcy>8 μmol/L[d]		83 (49)	16 (41)	15 (36)	114 (46)
Clinical hyperhomocysteinema[e]		16 (9.5)	3 (7.7)	6 (14)	25 (10)

[a] the mother was inquired for country of birth.
[b] B12 content 2–2.5 μg and/or high dose.
[c] Folate 400 μg/day is recommended in Norway during the first trimester.
[d] venipuncture failed in one AGA and one preterm.
[e] co-occurrence of *S*-tHcy >8 μmol/l (HHcy) and tremor or excessive sleep.
AGA = appropriate for gestational age, SGA = small for gestational age. Preterm = gestational age 32–36 weeks.

3. Results

3.1. Characteristics of population

The characteristics of the study population are summarized in Tables 1 and 2. Biochemical test results are presented in Tables 3 and 4.

In our cohort of selected, presumed healthy infants, 114 of 250 (46%) infants had tHcy >8 μmol/L at a mean CA of 19 (5.1) weeks.

Nine of 250 (3.6%) infants had B12 < 148 pmol/L, 30 (12%) B12 < 200 pmol/L and 99 (40%) B12 < 300 (Fig. 2). Almost 1/5 (47/250, 19%) had tHcy >10 μmol/L (Fig. 3) whereas 60/250 (24%) had either tHcy>10 μmol/L or B12 < 200 pmol/L and 17/250 (6.7%) of the infants had tHcy>10 μmol/L combined with B12 < 200 pmol/L.

The results of s-folate were highly skewed (range 18 to->45 nmol/L, median >45 nmol/L). Hence, all infants in the present study were folate replete according to the reference intervals of our laboratory.

3.2. Symptoms, signs, and associations with HHcy and increased methylmalonic acid

Infants with tremor at examination (13/251, 5.2%) had a significantly higher geometric mean tHcy = 11.0 μmol/L compared to the others with mean tHcy = 8.0 μmol/L (p = 0.001, Cohen's d = 0.33). Ten of 113 (8.8%) infants with tHcy >8 μmol/L had tremor compared to 3/136 (2.2%) infants with tHcy ≤8 μmol/L (p = 0.023). Ten of thirteen (77%) infants with tremor had tHcy>8.0 μmol/L. Five infants in the study had tHcy>16 μmol/L, and three of them had tremor. In a logistic regression analysis, with tremor as the dependent variable and tHcy and CA as independent variables, an increase in tHcy of 1 μmol/L was associated with 18% increased odds for tremor (OR 1.18, 95% CI 1.06–1.33, p = 0.004). There were no significant differences in B12 and MMA in infants with or without tremor. Four of the infants with tremor also had fasciculations in the tongue, three of whom had tHcy>8 μmol/L.

Six of the AGA term infants (6/168 (3.6%)) had tremor at

Table 2
Descriptive characteristics of mothers and infants presented as mean (SD) or median [interquartile interval].

	AGA Term (N=170)		SGA Term (N=39)		Preterm (N=43)		Total (N=252)	
	N		N		N		N	
Birthweight (grams)	170	3652 (433)	39	2648 (304)	43	2458 (462)	252	3293 (668)
Birthweight z-score[a]	170	−0.06 (0.93)	39	−2.31 (0.74)	43	−0.06 (0.77)	252	−0.41 (1.20)
Exclusively breastmilk, total months	169	3.2 [1.5,4.0]	38	2.75 [0.5,4.0]	40	2 [0.3,4.0]	247	3.1 [1.0,4.0]
Formula/mixed feeding, total months	167	0 [0,2.5]	36	1 [0,3.5]	39	3 [0,4.5]	242	0,5 [0,3.0]
Infant age in weeks	170	20.5 (5.4)	39	19.7 (5.0)	43	23.0 (3.7)	252	20.8 (5.2)
Infant age in weeks corrected for term date	170	20.1 (5.2)	39	18.5 (4.8)	43	17.7 (3.4)	252	19.5 (5.0)
Weight (kg)	170	7.44 (1.09)	39	6.22 (0.94)	43	6.95 (1.06)	252	7.17 (1.15)
Weight z-score[b]	170	0.30 (0.90)	39	−1.06 (0.79)	43	−0.77 (0.96)	252	−0.09 (1.06)
Mother's age at birth	170	30 (4.6)	39	30 (5.0)	43	31 (4.7)	252	30 (4.7)
Mother's BMI before pregnancy	169	23.0 [21.4,27.5]	39	22.8 [21.5,27.5]	43	23.0 [20.3,25.4]	251	22.9 [21.4,27.3]
Yearly household income (Euros)	125	99,000 (37,000)	28	93,000 (32,000)	31	93,000 (23,000)	184	97,000 (34,000)

[a] Norwegian growth charts for term infants, Fenton growth charts for infants with GA<37 weeks [21,37].
[b] Norwegian growth charts [38].

Table 3
Infant B12-related laboratory test results at mean corrected age of 19 (5.1) weeks presented as mean (SD) or median (interquartile interval).

	AGA Term N=169[a]	SGA Term N=39	Preterm N=42[a]	All N=250
***S*-vitamin B12** pmol/L	323 [236–455]	396 [258–624]	414 [277–606]	341 [250–496]
Holotranscobolamine pmol/L	61 [41–108]	62 [47–109]	88 [46–123]	62 [43–112]
tHcy μmol/L	8.0 [6.4–10]	7.7 [6.5–9.2]	7.5 [6.4–9.2]	7.8 [6.4–10]
MMA μmol/L	0.34 [0.22–0,88]	0.24 [0.18–0.44]	0.34 [0.23–0.54]	0.33 [0.21–0.76]
Folate nmol/L	45 [34–>45]	>45 [39–>45]	>45 [45–>45]	>45 [36–>45]
Hb g/100 mL	11.6 (0.9)	11.4 (0.9)	11.7 (0.8)	11.6 (0.9)
MCV fL	79 (4.2)	80 (4.5)	77 (2.5)	79 (4.1)

[a] A single infant missing in AGA term and preterm groups, respectively.

Table 4
Comparison between infants with and without clinically relevant HHcy [1]. Blood test results presented as mean (SD), median [interquartile interval], dichotomous variables as n (%).

	Clinically relevant HHcy[a]		
	Yes (n = 25)	**No** (n = 225)	p
***S*-vitamin B12** pmol/L	300 [207–402]	349 [255–503]	0.041
Holo-transcobalamin pmol/L	45 [31–68]	64 [44–113]	0.022
tHcy μmol/L	10 [9.1–15]	7.7 [6.4–9.4]	
MMA μmol/L	0.49 [0.23–0.92]	0.32 [0.21–0.72]	0.151
Folate nmol/L	>45 [37–>45]	>45 [36–>45]	0.597
Hb g/100 mL	11.6 (1.23)	11.6 (0.83)	0.749
MCV fL	79 (5)	79 (4)	0.485
Primiparous	10 (40)	126 (56)	0.128
Smoking last 2 years before pregnancy	5 (20)	24 (11)	0.182
Known maternal B12 deficiency	0	24 (11)	0.146
B12-containing[b] **supplements during pregnancy**	16 (64)	145 (65)	0.769
High-dose 1000 μg oral B12 supplement during pregnancy	0	12 (5.8)	0.373
Parenteral 1000 μg B12 during pregnancy	0	8 (3.6)	1.00
Folate[c] **during pregnancy**	22 (88)	195 (87)	0.936
B12-containing[b] **supplements during breastfeeding**	9 (36)	78 (35)	0.999
High dose 1000 μg oral B12 supplement during breastfeeding	1 (4.0)	8 (3.6)	1.00
Parenteral 1000 μg B12 during breastfeeding	0	6 (2.7)	1.00
Mother's age at birth	31.3 (4.0)	29.9 (4.8)	0.100
Mother's BMI before pregnancy	22.1 [19.8–25.2]	23.0 [21.5–27.8]	0.100
Multiple birth	7 (28)	22 (10)	0.007
Preterm	6 (24)	36 (16)	0.310
SGA	4 (16)	42 (19)	0.744
Age (weeks) uncorrected	20.0 (4.6)	20.8 (5.2)	0.549
Age (weeks) corrected	17.9 (4.2)	19.6 (5.0)	0.101
Formula/mixed feeding, total months	0 [0–1.5]	0.5 [0–3.4]	0.121

[a] Co-occurrence of *S*-tHcy >8 μmol/l (HHcy) and tremor or excessive sleep.
[b] B12 content 2–2.5 μg and/or high dose 1000 μg.
[c] Folate 400 μg/day is recommended in Norway during the first trimester.

examination, and they had a significantly higher geometric mean tHcy = 11.2 μmol/L compared to the others who had a mean tHcy = 8.1 (p = 0.013, Cohen's d = 0.31).

Infants reported to sleep excessively (21/247, 8.5%) had a significantly higher tHcy with geometric mean 10.8 μmol/L compared to 7.9 μmol/L (p = 0.004, Cohen's d = 0.32) in infants not

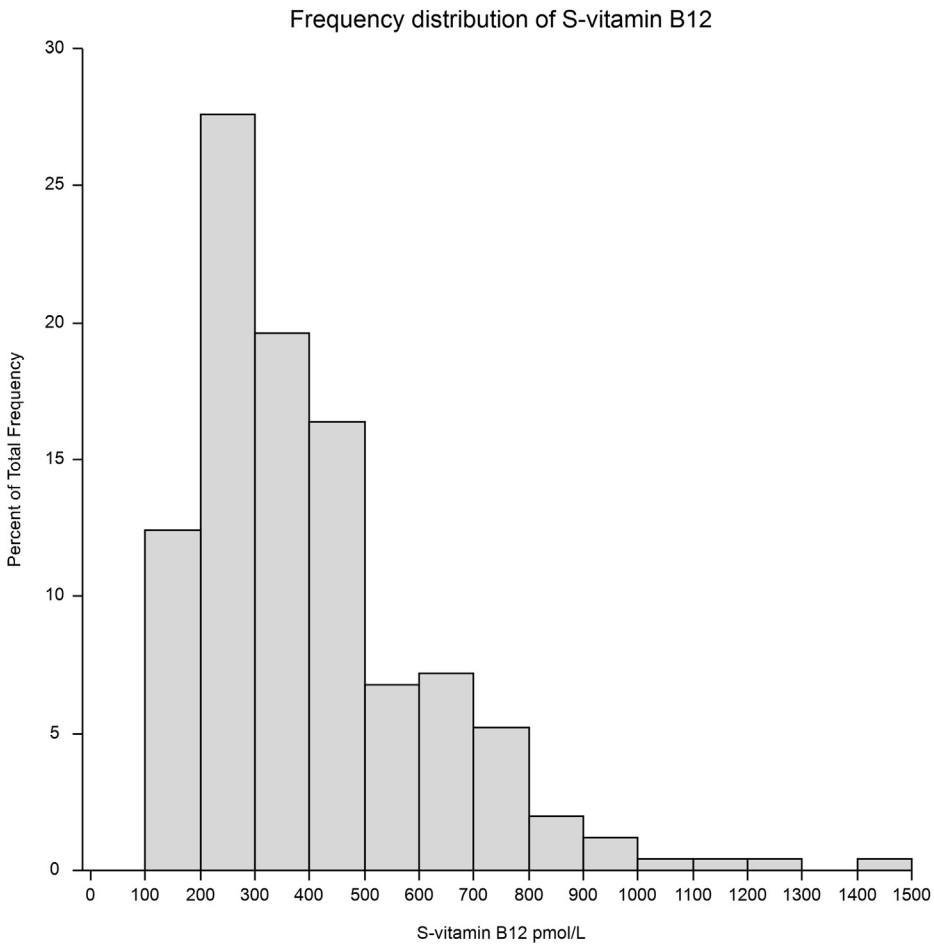

Fig. 2. Online. Frequency distribution of *S*–B12 values for n = 250 infants.

reported to sleep excessively. Sixteen of 112 (14%) infants with tHcy >8 μmol/L were reported to sleep excessively compared to 5/133 (3.8%) infants with tHcy ≤8 μmol/L (p = 0.003). Sixteen of 21 (76%) infants reported to sleep excessively had tHcy>8.0 μmol/L. Fourteen infants in the study had tHcy>14 μmol/L, and seven of them were reported to sleep excessively. In a logistic regression analysis with reported excessive sleep as the dependent variable, and with tHcy and CA as independent variables, an increase in tHcy of 1 μmol/L was associated with 21% increased odds for reported excessive sleep (OR 1.21, 95% CI 1.08–1.35, p = 0.001).

Thirteen of the AGA term infants (13/168 (7.7%)) were reported to sleep excessively, and they had a significantly higher geometric mean tHcy = 10.9 μmol/L compared to the others with mean tHcy = 8.0 μmol/L (p < 0.001, Cohen's d = 0.30).

Hypotonia was found in 107 of 250 infants (43%) when defined as being hypotonic in vertical suspension or by head lag when pulled to sit with score 0 or 1 on the corresponding HINE item. Sixteen of 25 (64%) infants with MMA over 90th percentile (1.49 μmol/L) were hypotonic. In a logistic regression analysis with hypotonia as dependent variable, and CA and MMA over 90th percentile as independent variables, MMA over 90th percentile was associated with 2.5 times higher odds for being hypotonic (OR 2.5, 95% CI 1.04–6.0, p = 0.041). Geometric mean tHcy in infants with MMA over 90th percentile was 11 μmol/L and significantly higher (p < 0.001, Cohen's d = 0.32) than in infants with MMA under 90th percentile (tHcy 7.9 μmol/L) and in 22/25 cases tHcy was >8 μmol/L. There were, however, no significant differences in B12 or tHcy in infants with or without hypotonia.

3.3. Clinical relevance of HHcy

Tremor was present and excessive sleep was reported significantly more often in infants with tHcy>8 μmol/L and both were thus defined as clinically relevant symptoms. Consequently, we defined clinically relevant HHcy as tHcy> 8 μmol/L in the presence of tremor or when excessive sleep was reported. Twenty-five of 250 (10%) infants were categorized with clinically relevant HHcy. Clinically relevant symptoms were absent in 89/114 (78%) of infants with HHcy.

Infants classified with clinically relevant HHcy did not differ in CA compared to infants with tHcy< 8 μmol/L, mean 18 (4.2) weeks and 20 (5.0) weeks, respectively (p = 0.074). There was no difference in occurrence of clinically relevant HHcy between infants born preterm, SGA or term AGA (p = 0.565) (Table 1). Comparisons of test results and characteristics of infants with and without clinically relevant HHcy are presented in Table 4.

3.4. Risk factors and predictors of infant B12 and total homocysteine

A multiple linear regression analysis was run with transformed infant vitamin B12 (LnB12) as dependent variable and with

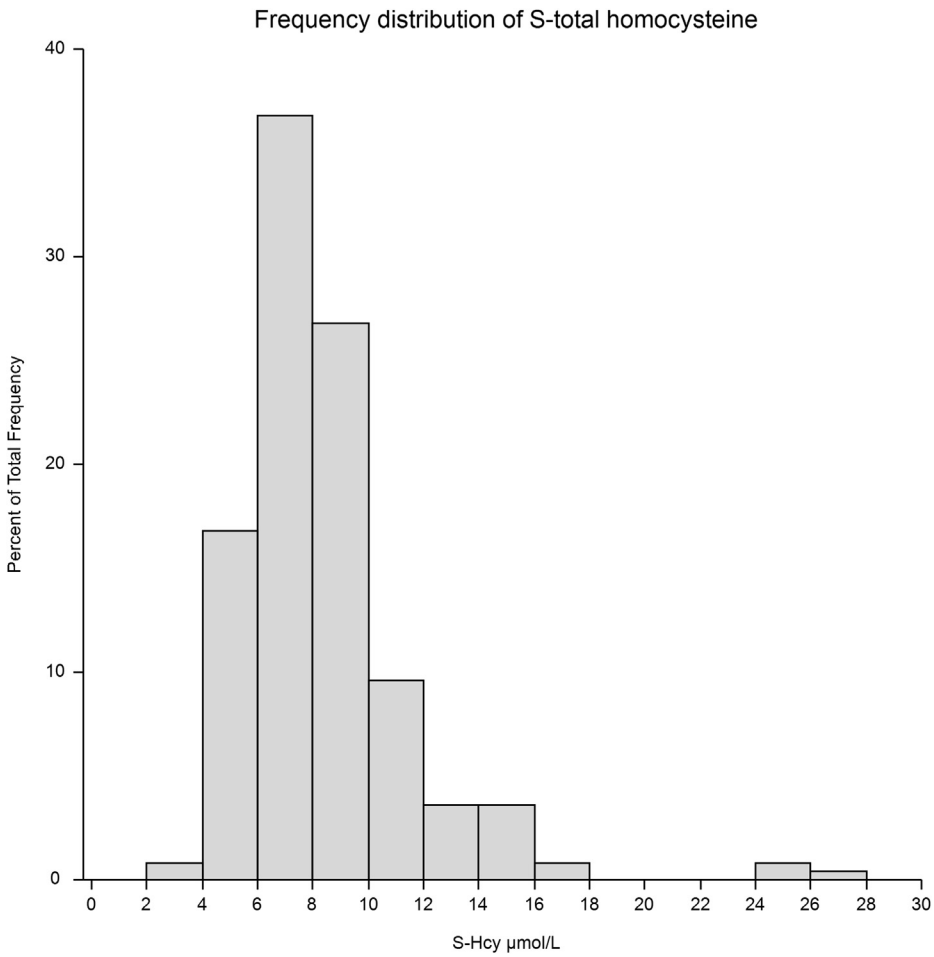

Fig. 3. Online. Frequency distribution of *S*-tHcy for n = 250 infants.

independent variables from Table 1 and 2. Multiple birth, age of infant and smoking were associated with lower B12, while months of formula/mixed feeding, and para 1 or more were associated with higher B12 (Table 5).

A multiple linear regression analysis was run with transformed infant tHcy (LntHcy) as dependent variable and with independent variables from Table 1 and 2. Multiple birth was associated with higher tHcy while months of formula/mixed feeding and use of B12-containing supplement during pregnancy were associated with lower tHcy (Table 5).

Most mothers (245/252) did not indicate any diet precautions. Only one mother was vegan. She gave her infant imported, oral vitamin B12 mixture, and her infant had normal B12 status. Six other mothers excluded meat from their diet, but included fish, egg, and milk. Infants born to meat-excluding mothers had a median B12 of 255 [184–467] pmol/L and tHcy 8.5 [6.5–12] µmol/L. Infants to mothers eating meat had a median B12 of 338 [250–495] pmol/L and tHcy 7.8 [6.4–10] µmol/L. The differences were not statistically significant, Mann-Whitney *U* test p = 0.33 and 0.37, respectively.

Thirteen mothers used high dose oral B12 supplement, and eight mothers received parenteral B12 during pregnancy. None of their infants had clinically relevant HHcy. Nine mothers used high dose oral B12 supplement, one of their infants had clinically significant HHcy. Six mothers received parenteral B12 during breastfeeding, and none of their infants had clinically relevant HHcy. None of those differences between groups with or without clinically significant HHcy were statistically significant (Table 4). Low-dose B12 supplement (2–2.5 µg daily) was used by 154/250 (62%) mothers during pregnancy and by 79/249 (32%) during breastfeeding.

3.5. Type of feeding and associations with markers of infant B12 status

Univariate associations between type of feeding and markers of B12 status are shown in Table 6. In supplementary linear regression analyses with dependent variable LntHcy, and independent variables CA and either total months with exclusive breastfeeding or total months with formula/mixed feeding, both were significantly ($p < 0.001$) associated with LntHcy, with standardized beta = 0.47 (beta = 0.087, 95% CI 0.066–0.107) for total months exclusive breastfeeding and −0.47 (beta = −0.076, 95% CI -0.094 to −0.057) for total months with formula/mixed feeding. Both models and standardized betas were significant ($p < 0.001$).

In a logistic regression analysis with tHcy>8 µmol/L as the dependent variable, and with age, SGA, prematurity, and exclusive feeding with breastmilk as independent variables, exclusive feeding with breastmilk was the only significant predictor (OR 2.93, 95% CI 1.59–5.4], p = 0.001), while neither SGA, prematurity, nor age reached statistical significance. In supplementary linear regression analyses with dependent variable LnB12, and independent variables CA and either total months exclusive breastfeeding or total months with formula/mixed feeding, both were significantly ($p < 0.001$) associated with LnB12 with standardized beta = −0.62 (beta = −0.169, 95% CI -0.196 to −0.141) for total months exclusive breastfeeding and 0.64 (beta = 0.151, 95% CI

Table 5
Linear model coefficients of predictors for transformed infant vitamin B12 and tHcy.

	Ln Infant B12 pmol/L (n = 237)		**Ln Infant tHcy μmol/L** (n = 239)	
	B-coefficient (95% CI)	Std. β[a]	B-coefficient (95% CI)	Std. β[a]
Multiple birth	−0.21 (−0.378;-0.042)	−0.135	0.180 (0.061; 0.298)	0.170
Total months formula/mixed feeding	0.168 (0.143; 0.193)	0.711	−0.084 (−0.102;-0.065)	−0.520
Age (days)	−0.002 (−0.004;-0.001)	−0.168	−0.001 (−0.002; 0)	−0.083
Parity; para 1 or more	0.146 (0.047; 0.244)	0.147		
Smoking last 2 years before pregnancy	−0.192 (−0.352;-0.032)	−0.124		
B12-containing supplement[b] during pregnancy			−0.117 (−0.192;-0.042)	−0.167

Std. β[a] = standardized beta, [b]all forms and doses of B12. Variables entered in the crude model, removed from the final model and not shown in the table, were mother born in another country than Norway, university education, known maternal B12 deficiency, mother's BMI, mother's age, family income, preeclampsia, metformin use, diabetes in pregnancy, hyperemesis, use of B12 containing supplement during breastfeeding, folate during pregnancy, dose of nitrous oxide, vaginal delivery, sex, preterm and SGA status.

0.126–0.176) for total months of formula/mixed feeding. Both models and estimates were significant ($p < 0.001$). Eleven of 25 (44%) of infants classified with clinically relevant HHcy were fed exclusively with breastmilk compared to 71/221 (32%, $p = 0.233$) of the remaining infants.

3.6. Socio-economic factors and infant B12 status

We compared socio-economic factors of mothers to infants with and without clinically relevant HHcy. There were no significant differences between groups in income ($p = 0.27$), education ($p = 0.77$) or nationality ($p = 0.53$). Sixty-nine per cent of women in our cohort had university education.

3.7. Clinical tests and association with B12 status

Infants scoring below normal (<5-percentile) on fine motor skills on ASQ (23/249, 9.2%) had a significantly higher tHcy with geometric mean 9.4 μmol/L compared to 8.0 μmol/L in infants obtaining normal scores ($p = 0.027$, Cohen's $d = 0.33$). We found no other direct associations between biochemical markers of B12 status and total scores in HINE, AIMS, TIMP or GMA scores, corrected for age. We could not show any associations between time with exclusive or formula/mixed feeding and HINE, AIMS, TIMP, ASQ or GMA scores, corrected for age.

4. Discussion

In our study of 250 presumed healthy infants aged 3–7 months, we showed significant associations between increased infant tHcy levels and tremor or excessive sleep, well recognized symptoms of B12 deficiency [1–7]. Further, we demonstrated an association between increased tHcy levels and subnormal scores on the fine-motor subscale on ASQ. Twenty-five of 250 (10%) infants had clinically relevant HHcy defined as a co-occurrence of s-tHcy>8 μmol/L and tremor or excessive sleep. Since all infants in this study were folate replete, and the tHcy level is considered a reliable marker of B12 status in the first two years of life [7], we assume the tHcy level in these infants indicates their B12 status. Bjørke-Monsen et al. showed that B12 optimized infants had a tHcy<8 μmol/L at 4 months of age [16]. Consequently, we propose that the clinically relevant HHcy represents clinically relevant B12 deficiency and that our study adds a clinical aspect to other prevalence studies defining B12 deficiency from biochemical test results only [16,18,19,30]. On the other hand, 89 of 114 (89%) infants with HHcy did not present any of the associated symptoms, why it is challenging to decide whom and when to replenish.

The finding of exclusive breastfeeding in 37% of AGA infants in our cohort was in line with a recent national dietary survey reporting 39% of infants being exclusively breastfed at 4 months of age [15]. In our cohort with mean age of 19 weeks, 46% of our infants had HHcy and 33% were breastfed compared to 69% and 75% respectively in a Norwegian study of 4 months old infants [16]. The discrepancy in HHcy could simply be explained by a higher proportion of exclusively breastfed infants in the latter study. In accordance with previous studies [2,7,13], the regression analyses for predictors of tHcy and B12 levels showed that infant nutrition was the single most important determinant of B12 status. Formula feeding and use of B12-containing supplements in pregnancy were associated with a higher infant B12 status whereas smoking and multiple birth were associated with lower infant B12 status. Multiple birth was also associated with a higher rate of clinically relevant HHcy. Hay et al. showed significant differences in B12 status between infants that never received breastmilk, and infants fully or partly breastfed, and interpreted their data as if breastmilk by itself resulted in deranged B12 status [18]. By contrast, our data

Table 6
Univariate associations of markers of B12 status and clinically relevant[a] hyperhomocysteinemia (HHcy) with type of feeding.

	***S*-vitamin B12** (n = 250)	***S*-tHcy** (n = 250)	***S*-MMA** (n = 250)	**Clinically relevant[a] HHcy** (n = 250)
	r_s	r_s	r_s	r_s
Breastfeeding total months (n = 249)	−0.425[b]	0.238[b]	0.277[b]	−0.002[c]
Exclusive breast-feeding total months (n = 247)	−0.600[b]	0.432[b]	0.281[b]	0.096[c]
Exclusive breastfeeding (n = 248)	−0.382[b]	0.344[b]	0.106[c]	0.076[c]
Formula/mixed feeding total months (n = 242)	0.627[b]	−0.510[b]	−0.236[b]	−0.100[c]

r_s = Spearman's rho.
[a] Co-occurrence of *S*-tHcy >8 μmol/l (HHcy) and tremor or excessive sleep.
[b] $p < 0.001$.
[c] $p > 0.05$, non-significant.

suggested an almost equal effect of total months of breastfeeding compared to formula/mixed with breastfeeding on both tHcy- and B12 levels in a dose-responsive way. Feeding practices were not directly associated with psychomotor test results, as opposed to findings in other studies [31,32] showing breastfeeding to be associated with better outcome in psychomotor tests. As our study was not designed for this outcome our inconsistent findings should be interpreted with caution. It is interesting to note that in our study, feeding practice was not significantly associated with the rate of clinically relevant HHcy. This may be due to lack of power for analysis of subgroups, but we also speculate on a counteractive effect of exclusive breastfeeding, where the effect is dependent on whether the mothers were B12 sufficient during pregnancy or not, and that formula feeding could compensate for maternal B12 insufficiency.

In a prevalence study of newborns with biochemical B12 deficiency in Norway, Refsum et al. analyzed 4992 serum samples from the Norwegian newborn screening program and estimated a 5% prevalence of B12 deficiency at birth using the combination of *S*-tHcy>10 pmol/L and *S*−B12 < 200 pmol/L as cut-off values [30]. These are rather strict biochemical criteria for newborn B12 deficiency and may underestimate the true prevalence. Infants attain their highest B12 levels at birth, followed by a decrease in B12 during the first weeks of life, while tHcy and MMA levels increase [3,7]. Nevertheless, applying the same definition to our cohort for comparison would render 17/250 (6.8%) of our infants B12 deficient. This is still a substantial proportion considering our highly selected subpopulation of educated, healthy mothers of whom 97% reported no diet restrictions, 163/252 (65%) reported use of B12 supplement during pregnancy, and five infants had been excluded due to diagnosed B12 deficiency prior to study visit. The absence of 'the mothers' diet' and 'poverty' as explanatory variables for B12 deficiency is in line with the findings of other infant B12 studies in western countries [7,33,34], underlining the importance of mixed explanatory factors in high-income populations.

We speculate that tremor and excessive sleep are symptoms which could reflect a younger developmental stage, and that the presence of HHcy reflects a suboptimal B12 status and a potential deficiency of methyl donors which delays neurological maturation. Our results support this assumption as increased tHcy was associated with subnormal scores on the fine-motor subscale on ASQ, adding to the findings by Torsvik et al., that infants with suboptimal tHcy supplemented with B12 had better development scores than placebo [35]. Thus, we suggest an association between suboptimal neurological maturation and higher tHcy. If this is the case, the symptoms of these infants are a sign of suboptimal development rather than overt disease.

The prospective study design, with clinical examination and testing of infants prior to analyzing B12 status and rigorous adherence to standardized neurological and psychomotor testing, were strengths in the present study. A limitation to our study was the lack of mother's B12 status, a very important determinator of infant B12 status [12]. Given the observational design of the study, only associations and no cause-and effect relationship between infant symptoms and tHcy could be established. Our results must be viewed in the light of a particularly healthy cohort of mothers and infants and its rather small sample size, under-powered to do further analyses on the subgroup with clinically relevant HHcy. Deficiency of pyridoxine and betaine could theoretically result in raised tHcy and be possible confounders not analyzed in the present study but a previous study of 123 infants with median age 12 weeks did not support any association between pyridoxine and tHcy [36].

In conclusion, we have demonstrated associations between symptoms suggestive of infant B12 deficiency and increased levels of tHcy in presumed healthy infants. To determine causality and the impact of suboptimal B12 status on psychomotor development, a randomized intervention study is warranted.

Declarations of interest

None.

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Declaration of competing Interest

None of the authors of the submitted manuscript have any conflicts of interest to declare.

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Research Paper

Breastfed Infants With Spells, Tremor, or Irritability: Rule Out Vitamin B12 Deficiency

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ABSTRACT

Background: In Norway, 5-10% of neonates and infants have biomarkers suggesting vitamin B12 deficiency from newborn screening tests and unselected clinical screening, respectively.
Aims: The aims were to identify risk factors and describe presenting symptoms and biochemical profiles in infants diagnosed with vitamin B12 deficiency.
Methods: In this case-control study, we searched hospital medical records for infants younger than one year born in 2011-2018, diagnosed with vitamin B12 deficiency. We compared 85 cases with a control group of 252 infants aged 3-7 months. Parents completed questionnaires.
Results: Of the 85 cases with vitamin B12 deficiency, 80% presented with spells (37%) of apneas, motor seizures, or absences within the first two months of life. Tremor (29%) and irritability (18%) were the most common findings at the first examination. Serum total homocysteine $\geq$10 μmol/L was found in 77% of cases compared to 28% of controls ($P < 0.001$). None of the mothers were vegetarians, but 25% reported a previous history of vitamin B12 deficiency and 7% had celiac disease. The dose of nitrous oxide given during labor was significantly associated with infant serum total homocysteine level at diagnosis ($r = 0.37$, 95% confidence interval = 0.16-0.55, $P < 0.001$) for cases, but not for controls.
Conclusion: Spells, tremor, and irritability are common findings in early infant vitamin B12 deficiency. Nitrous oxide given during labor is proposed as a contributing risk factor to the development of early infant vitamin B12 deficiency.

Introduction

Exclusively breastfed infants are at risk of developing vitamin B12 (B12) deficiency when born to asymptomatic B12 depleted mothers. The symptoms mainly emerge in the first 4-10 months of the infants' lives.[1,2] In 2004, Refsum *et al* analyzed 4992 newborn screening serum samples in Norway and found that five percent of the newborns had biomarkers suggesting B12 deficiency when

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applying the criteria serum total homocysteine (tHcy) > 10 μmol/L, B12 < 200 pmol/L or tHcy >10 μmol/L, and serum methylmalonic acid (MMA) > 0.40 μmol/L.[3] We recently demonstrated a 10% prevalence of clinically relevant hyperhomocysteinemia suggestive of B12 deficiency in infants in Norway.[4] tHcy is the preferred functional biochemical marker of infant B12 status, and vitamin-optimized plasma-tHcy is <6.5 μmol/L at 4 months of age.[5,6]

Many studies of B12 deficiency in infancy originate from parts of the world where women are either vegetarians or otherwise deprived of animal sources of B12, and infant B12 deficiency is common and often severe.[6,7] In high-income countries, other risk factors for infant B12 deficiency may be more important.[8] Nitrous oxide (N_2O) is widely used for analgesia during labor.[9,10] N_2O oxidizes the methionine synthase–bound cob(I)alamin to cob(II)alamin, which irreversibly inhibits this enzyme, leading to the accumulation of tHcy and lack of adenosyl-methionine.[11,12] tHcy increases significantly when N_2O is given to children with a strong dose-response correlation.[13,14] The effects of N_2O, during labor, have only been studied to document short-term safety for obstetric use.[9,10] Whether N_2O is a risk factor for early infant B12 deficiency is unknown.

The aims of this retrospective case-control study were to identify risk factors and describe presenting symptoms and biochemical profiles in infants diagnosed with B12 deficiency.

Patients and Methods

Study population

We searched the medical record databases of two hospitals in the South East of Norway for infants born in 2011-2018 that were treated for B12 deficiency before one year of age (Fig 1), hereafter defined as "cases." A control group of 252 healthy infants aged 3-7 months was recruited in 2018-2019 from the Postnatal and Neonatal Unit at Vestfold Hospital Trust, Norway. Data from this control group have been published elsewhere.[4] For cases, obstetric data, presenting symptoms and findings, and results from the hospital investigation and treatment were collected from hospital records. Some infants had more than one symptom. For controls, obstetric data from hospital records were retrieved, and the infants were neurologically examined before the blood test results were

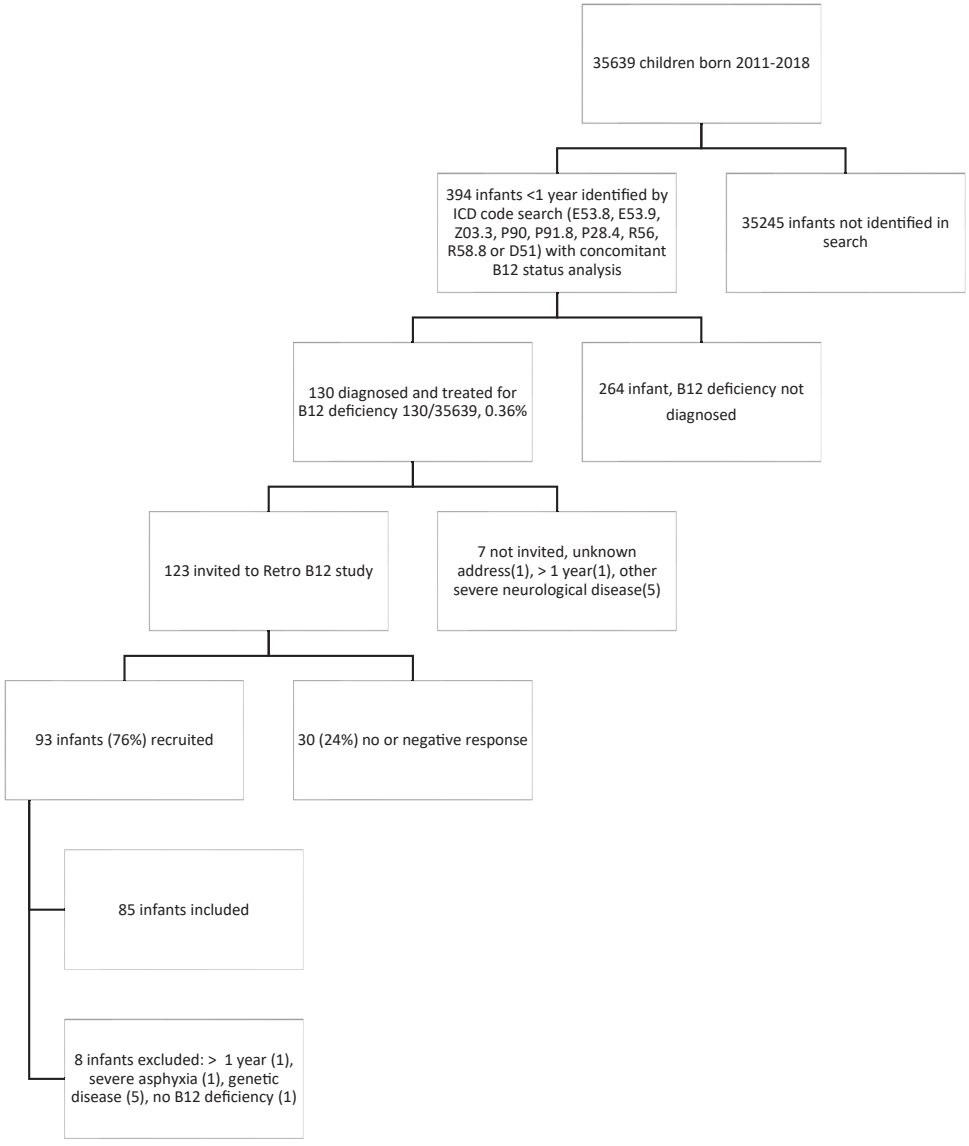

FIGURE 1. Search and selection process for cases.

available. The parents of both cases and controls completed the same nonstandardized questionnaires on diet, vitamin supplementation, and symptoms. The parents of the cases completed two questionnaires, examining symptoms before and after B12 treatment. Data on the use of N_2O during labor came from the mothers' obstetric files, including the time of usage and the concentration of N_2O given. We calculated the total dose of N_2O as the product of administration time in minutes and the concentration of N_2O. The study was approved by the Regional Ethics Committee (179/2018) and conducted according to the Helsinki declaration. Written informed consent was collected for all participants.

Biochemical analyses

B12, holotranscobalamin, and folate in Vestfold residents were measured in serum using a chemiluminescence method on Architect i2000SR (Abbott Diagnostics, IL) until October 2017 and on Roche Cobas 8000, e801 (Roche Diagnostics GmbH, Germany) thereafter. The shift introduced a bias for holotranscobalamin results corrected using a documented regression algorithm (Y [Roche] = 9.887 + 0.865X [Abbott], n = 56, r = 0.985). At Sørlandet Hospital, B12 and folate were measured in serum using an immunoassay on Cobas 6000 e601 from Roche Diagnostics during the whole period. Hematology samples were analyzed using Sysmex instruments in both Vestfold and Sørlandet (Sysmex XE 5000, Sysmex Corporation, Japan) until February 2017 and XN-analyzers thereafter. During 2011-2015, serum MMA and plasma tHcy from samples collected in Vestfold were analyzed at Telemark Hospital Trust using gas chromatography–mass spectrometry and high-performance liquid chromatography, respectively. From 2016, MMA and Hcy have been determined in Vestfold by liquid chromatography/tandem mass spectrometry in serum. In patients from Sørlandet, tHcy was analyzed in plasma using an enzymatic assay and MMA was determined at Oslo University Hospital by liquid chromatography/tandem mass spectrometry. During the preparation of serum, Hcy is released from erythrocytes, causing slightly higher values in serum than in plasma (~+1 μmol/L). Duplicate measurement in serum and plasma from 75 blood donors with tHcy in plasma below 10.0 μmol/L yielded the equation plasma-tHcy = 0.006153 + 0.8074 * serum-Hcy (r = 0.925). All tHcy values are reported in serum according to this regression algorithm.

Statistics

We registered data in EpiData, version 4.4 (EpiData Association, Denmark). Continuous variables are presented as mean and standard deviation or, if skewed, as median and interquartile range. Categorical variables are given as proportions and percentages. Differences between independent groups regarding normally distributed variables were quantified with the two-tailed t-test, or the Mann Whitney U test in case of skewness in the data. Differences in tHcy and MMA before and after treatment were analyzed with related-samples Wilcoxon signed-rank test. Categorical variables were compared between groups using the Chi-squared test for homogeneity or Fisher's exact test for small samples. The strength of association between continuous variables was measured using Pearson's correlation coefficient. All statistical tests were two-sided, and a *P*-value <0.05 was considered statistically significant. We defined biologically relevant differences when covariates in regressions caused a change >0.25 standard deviation (SD) of the dependent variable when the covariate changed 2 SD. To evaluate possible covariates for tHcy and B12 deficiency, linear and logistic regressions were applied, respectively. All regression models were significant with $P < 0.001$. To identify significant exposure variables in regressions, we used candidate variables in Tables 1 and 2, excluding dichotomous variables with fewer than 5 in a category. In linear regressions, variables with a Spearman correlation rho >0.1 were entered in a crude model and nonsignificant variables were removed for a more saturated model. The variables excluded were reintroduced one at a time and retained if they became significant. In the final models, only biologically relevant variables significant at a 0.05 level were retained. To obtain normally distributed residuals, log-transformed Hcy was applied for use as a dependent variable in linear regression analyses. Assumptions for regressions were then met. We decided a priori to include infant age in all regression models. Analyses were performed in IBM SPSS Statistics, version 27 (SPSS Inc, IL) or in NCSS 2021 Statistical Software (NCSS, LLC., Utah, ncss.com/software/ncss).

Results

Characteristics of infants

In the catchment area, a total of 35 639 births were registered during the study period, and 130 of these infants were treated for B12 deficiency (0.36%). Eighty-five B12-deficient infants participated in our study (Fig 1, Tables 1 and 2). Referrals from primary health care comprised 50 of 85 (59%) and emergency referrals 16 of 85 (19%), whereas 19 of 85 (22%) were diagnosed in infants already in-house, including 9 of 85 (11%) infants tested because their mothers or siblings had been diagnosed with B12 deficiency (n = 6) or identified by newborn screening (NBS) with increased propionyl carnitine (C3)[18] (n = 3) (Table 3). Infants identified by NBS or family risk were tested at a median age of 7 days and were excluded from analyses on age and symptom presentation. In primary care referrals, B12 deficiency was never suggested as a differential diagnosis. The most common reason for referral was apneas (11/76, 14%), absences (8/76, 11%) or motor seizures (13/76, 17%), collectively termed as spells (28/76, 37%).

Eighty percent (61/76) showed symptoms of B12 deficiency within the first two months of life, and the 'age of referral' peaked at 1-2 months and 6 months (Figs 2 and 3). In exclusively breastfed infants whose mothers received N_2O analgesia during labor (n = 35), mean (SD) symptom presentation and referral age were 1.17 (1.40) and 2.21 (1.72) months, whereas in infants not exclusively breastfed or whose mothers had not received N_2O (n = 39), symptom presentation and referral age were 2.03 (2.23) and 3.57 (2.52) months, ($P = 0.051$, Cohen's d = 0.45 and $P = 0.016$, Cohen's d = 0.63, respectively).

Symptoms and findings at the first examination are presented in Table 4. Unusual findings included a solitary skin ulcer on nates (n = 1), vertical nystagmus (n = 1), and neutropenia of unknown cause (n = 3); these all resolved after a B12 injection except for one case of neutropenia. B12 status, including the biomarkers MMA and tHcy at diagnosis, is shown in Table 5. Sixty-one of 79 (77%) cases compared to 70 of 250 (28%) controls had tHcy ≥10 μmol/L ($P < 0.001$) (Fig 4). Urine organic acid test was performed in 22 of 85 cases (26%), and 18 of 22 (82%) showed elevated secretion of MMA (qualitative analysis). All 85 cases received intramuscular B12 injections, the majority a single dose of 1 mg of hydroxocobalamin. Median (interquartile range) tHcy and MMA pretreatment were 12.4 μmol/L (10.0-16.1) and 1.54 μmol/L (0.56-2.83) and post-treatment 5.8 μmol/L (4.7-6.3) and 0.17 μmol/L (0.12-0.22), respectively. This represents reductions of 53% and 89% in tHcy and MMA, respectively (both $P < 0.001$). Forty-three of 85 cases (51%) underwent brain imaging (magnetic resonance imaging: n = 12, cerebral ultrasound: n = 35), revealing enlarged ventricles in one infant and delayed myelination in one infant. Twenty-eight of 85 (33%) were assessed with electroencephalography. Epileptic

TABLE 1.
Descriptive Characteristics of Mothers and Infant Cases and Controls

Descriptive	Cases (n = 85)	Controls (n = 252)	*P*
	n (%)	n (%)	
Origin of mother[*]			
Norway	72 (85)	193 (77)	0.114[†]
Other Nordic	1 (1.2)	6 (2.4)	0.684[‡]
Europe	9 (11)	27 (11)	0.974[†]
Non-Europe	3 (3.5)	26 (10)	0.071[‡]
Education			
Elementary	5 (6)	6 (2.4)	0.156[‡]
High school	26 (31)	71 (29)	0.717[†]
University	53 (63)	169 (69)	0.345[†]
Parity			
0	36 (42)	138 (55)	**0.048**[†]
1	33 (39)	84 (33)	0.358[†]
2 or more	16 (19)	30 (12)	0.108[†]
Married/cohabitant	76 (89)	249 (99)	**<0.001**[†]
Smoking last 2 years	11 (13)	30 (12)	0.800[†]
Employment last 2 years	63 (78)	220 (91)	**0.003**[†]
Celiac disease	6 (7.1)	8 (3.2)	0.121[†]
Known maternal B12 deficiency	21 (25)	24 (9.7)	**<0.001**[†]
Metformin use in pregnancy	2 (2.8)	9 (3.6)	1.00[‡]
Diabetes in pregnancy	5 (6)	16 (6.3)	0.896[†]
Preeclampsia	4 (4.8)	14 (5.6)	1.00[‡]
Hyperemesis (self-reported)	32 (38)	67 (27)	0.055[†]
B12-containing supplement during pregnancy	37 (45)	163 (65)	**0.001**[†]
Folate during pregnancy	68 (82)	219 (88)	0.194[†]
N_2O analgesia	54 (64)	170 (68)	0.531[†]
Multiple birth			
Twins	2 (2.3)	26 (10)	**0.021**[‡]
Triplets	0	3 (1)	0.575[‡]
Delivery			
Vaginal	69 (81)	196 (78)	0.509[†]
Cesarean section	16 (19)	56 (22)	0.509[†]
Cord clamping			
Immediately	16 (35)	38 (16)	**0.003**[†]
1-3 min	30 (65)	61 (26)	**<0.001**[†]
Over 3 min	0	137 (58)	**<0.001**[‡]
Preterm GA 32-36 weeks	11 (13)	43 (17)	0.370[†]
Small for gestational age <10p	14 (17)	46 (18)	0.710[†]
Sex			
Female	36 (42)	124 (49)	0.274[†]
Type of feeding			
Exclusively breastmilk	59 (71)	82 (33)	**<0.001**[†]

Bold indicates significant *P*-values <0.05.
[*] The mother was asked country of birth.
[†] Chi-square.
[‡] Fisher's exact, GA = gestational age.

TABLE 2.
Descriptive Characteristics of Mothers, Cases, and Control Infants

Descriptive	n	Cases (n = 85)	n	Controls (n = 252)	*P*
Birthweight (grams)	85	3375 (671)	252	3293 (668)	0.721[*]
Birthweight z-score[†]	85	−0.26 (1.12)	252	−0.41 (1.20)	0.333[*]
Exclusively breastmilk, months	81	4.0 [3.0,5.0]	247	3.1 [1.0,4.0]	**<0.001**[‡]
Infant age in weeks[§]	76	14 (10)	252	21 (5)	**<0.001**[*,‖]
Infant age in weeks[§] corrected for term date	76	13 (11)	252	19 (5)	**<0.001**[*,‖]
Weight (kg)	73	5.74 (1.75)	252	7.17 (1.15)	**<0.001**[*,‖]
Weight z-score[¶]	71	−0.50 (1.18)	252	−0.09 (1.06)	**0.006**[*]
Mother's age at birth	85	30.7 (4.4)	252	30.0 (4.7)	0.206[*]
Mother's BMI before pregnancy	77	23.7 [20.8,26.9]	251	22.9 [21.4,27.3]	0.995[‡]
Yearly household income (Euros)	70	90,000 (35,000)	184	97,000 (34,000)	0.120[*]

Abbreviations:
BMI = Body mass index
SD = Standard deviation
Data presented as mean (SD) or median [interquartile interval].
Bold indicates significant *P*-values <0.05.
[*] 2-tailed t-test.
[†] Norwegian growth charts for term infants,[15] Fenton growth charts for infants with gestational age<37 weeks.[16]
[‡] Mann-Whitney U-test.
[§] Excluding 9 infants where NBS/risk was indication for test.
[‖] Unequal variances assumed.
[¶] Norwegian growth charts.[17]

TABLE 3.
Main Indication for Case Referral

Indication for Referral	Emergency Referral	Primary Health Care	In-House	Total
Spells*	9 (56%)	14 (28%)	5 (26%)	28 (33%)
Tremor	3 (19%)	10 (20%)	1 (5.3%)	14 (17%)
Irritability	1 (6.3%)	11 (22%)	0 (0%)	12 (13%)
Hypotonia†	1 (6.3%)	6 (12%)	1 (5.3%)	8 (9.4%)
NBS or risk	0 (0%)	0 (0%)	9 (47%)	9 (11%)
Other‡	2 (13%)	9 (18%)	3 (16%)	14 (17%)
Total	16 (100%)	50 (100%)	19 (100%)	85 (100%)

* Spells of apneas, absences, or motor seizures.
† hypotonia or slow motoric development commented in letter of referral.
‡ reflux (n = 7), failure-to-thrive (n = 4), neutropenia (n = 3), excessive sleeping (n = 3), diarrhea (n = 3), refusal to eat (n = 2), other neurological symptoms (n = 4), and other non-neurological symptoms (n = 5), sometimes coexisting.

activity was reported in one patient. Cerebrospinal fluid analyses were normal (n = 6).

B12 status was determined within a week from referral in 47 of 66 (71%) cases. Ten of 66 (15%) had their first B12 test between one and five months from referral. In 40 of 85 (47%), injection of B12 was given within a fortnight after the B12 test, but in 18 of 85 (21%), there was a delay between 33 and 271 days. Parents reported improvement in symptoms after B12 supplementation of their infants (Table 6). Resolution of symptoms was described in the medical records for most of the infants; however, consistent information on the treatment effects was not available in several hospital medical records, as the follow-ups were transferred to primary health care.

Characteristics of mothers

Six mothers (7.1%) were diagnosed with celiac disease compared to 8 of 252 (3.2%, $P = 0.126$) in the control group. Of these, 4 of the mothers of cases and 3 of the mothers of controls had fed their infants with breastmilk exclusively. There were no vegans/vegetarians among the case mothers. The case mothers' serum B12 (n = 73) was median 246 (185-328), and 50 of 73 (68%) had B12 of <300 pmol/L.

Associations between B12 deficiency and risk factors

A multiple logistic regression analysis was run with cases versus controls as the dependent variable and infant age, B12-containing supplement use during pregnancy, exclusive breastmilk feeding, and self-reported maternal B12 deficiency as independent variables. An increase in infant age and use of B12-containing supplement during pregnancy were associated with lower odds for B12 deficiency. Exclusive breastmilk feeding and self-reported maternal B12 deficiency were associated with higher odds for B12 deficiency (Table 7). A multiple linear regression analysis was run with log-transformed infant tHcy as the dependent variable and infant age, maternal B12-containing supplement use during pregnancy, and exclusive breastmilk feeding as independent variables. Increasing infant age and maternal B12 supplement use were associated with lower tHcy and exclusive breastmilk feeding with higher tHcy (Table 8).

Among the cases, the dose of N_2O correlated significantly with infant level of tHcy (r = 0.372, 95% confidence interval [CI] = 0.159-0.549, $P < 0.001$) (Fig 5) and MMA (r = 0.290, 95% CI = 0.070-0.482, $P = 0.011$), but not with B12 (r = −0.127, 95% CI = −0.338 to 0.097, $P = 0.266$). There were no associations between the dose of N_2O and tHcy, MMA, or B12 in the control group.

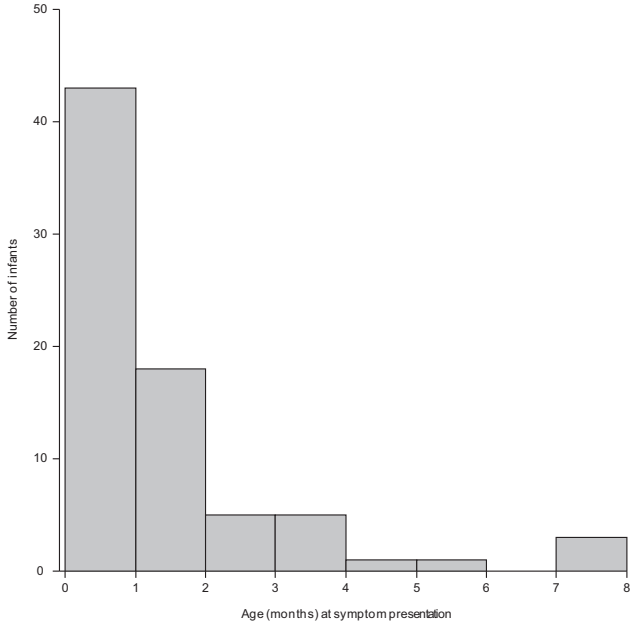

FIGURE 2. Age at symptom presentations (N = 76/85)[1]. [1]Nine infants were excluded because they were tested after NBS recall or because the mother or previous siblings had been diagnosed with B12 deficiency.

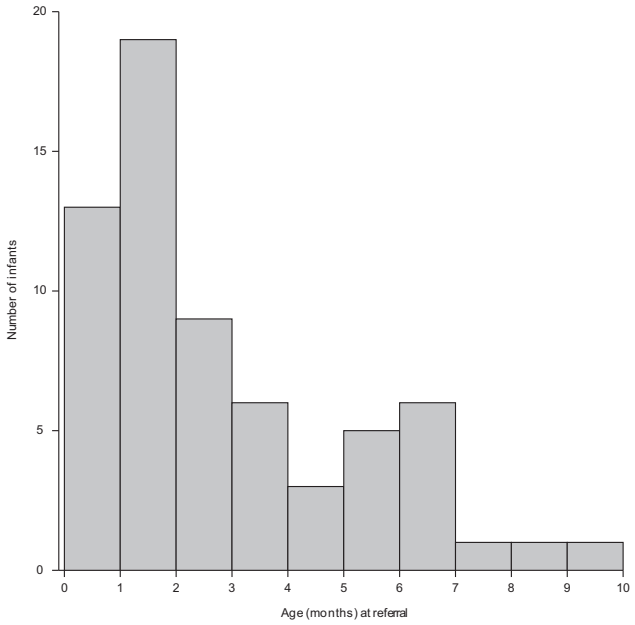

FIGURE 3. Age in months at referral (n = 64/85)[1]. [1]2 infants missing; 9 infants tested after NBS results or family risk, not referred; 10 in-house patients, not referred.

TABLE 4.
Symptoms and Findings at Examination in Cases and Controls

Symptom or Finding	Cases n = 85	Controls n = 252	*P*
Spells (motor seizures, apneas, or absences)	30/76[*] (39%)	0/250 (0%)	<0.001[†]
Tremor	21/72[‡] (29%)	13/250 (5.2%)	<0.001[§]
Irritability	12/68[‡] (18%)	19/252 (7.5%)	0.012[§]
Head lag at pull-to-sit	26/53[‡] (49%)	38/250 (15%)[‖]	<0.001[§]
Abnormal eye contact	9/67[‡] (13%)	0/250 (0 %)	<0.001[†]

[*] N = 9 infants evaluated after newborn screening test results or due to family history of B12 deficiency are excluded.
[†] Fisher's exact.
[‡] Number of patients evaluated for this symptom, as described in the medical records. All infants examined were indirectly evaluated for spells when spells were not commented in the medical records.
[§] Chi-Square.
[‖] Head lag at pull-to-sit as commented in medical records for cases, for control infants head lag was defined as score 0 or 1 on pull-to-sit item on Hammersmith Infant Neurological Examination.

In cases only, a multiple linear regression analysis was run with log-transformed infant tHcy as the dependent variable and dose of N_2O during labor, body mass index (BMI) of mother before pregnancy, prematurity, B12-containing supplement during pregnancy, maternal age, and infant age as independent variables. An increasing dose of N_2O, mothers' BMI, and prematurity were associated with higher infant tHcy, while B12 supplement use during pregnancy and increasing maternal age were associated with lower tHcy (Table 9).

Discussion

In our cohort of B12-deficient infants, the most common presenting symptoms were tremor and spells of apneas, motor seizures, or absences. For the vast majority, onset occurred within the first two months of life. In none of the referrals was B12 deficiency suspected as a cause, and none of the mothers were vegetarians. Further, this is a pioneering study in that it shows an association between the dose of N_2O given during labor and biomarkers indicating B12 deficiency in the exposed symptomatic infant.

The incidence of infant B12 deficiency and symptoms presenting within the first two months of life were in accordance with the findings in a Swedish study (18), but in contrast to the mean (SD) symptom debut ages of 5.4 (2.8) months and 5.9 months (3.3) reported from the Czech Republic and India, respectively.[1,2] Our study further highlights the findings in the Swedish study[19] with an acute spell-like presentation, including apneas, absences, and motor seizures, also elsewhere reported.[20,21] Spells were only exceptional findings in reports of B12-deficient infants from the Czech Republic and India,[1,2] probably since they were older and thus neurologically more mature. The older presenting age in these studies possibly reflects an etiologically more homogenous group with maternal B12 deficiency as the main determinant for age at clinical presentation since it takes a certain time to deplete infant B12 stores. In the present study, we noticed two possible peaks in age of referral, 6 weeks and 6 months; the latter peak overlapped with the aforementioned studies.[1,2] This age overlaps with the timing of routine well-child visits which may have prompted the referrals. For the younger cases in our study, other factors beyond maternal B12 deficiency probably play a role.

It has been shown by Landon et al. that N_2O given to mothers in labor inactivates methionine synthase in the placenta in a dose-responsive way[22] by oxidizing the cob(I)alamin bound to the enzyme.[12] We showed that exclusively breastfed infants whose mothers received N_2O were referred to hospital at a younger age than infants that were not exclusively breastfed or whose mothers had not received N_2O. We propose that N_2O given as an analgesic during labor may contribute to an early infant presentation of B12 deficiency in exclusively breastfed infants. The inactivation of B12 bound to methionine synthase is irreversible, thus requiring *de novo* synthesis of methionine synthase, depleting the limited infant B12 stores in the meantime. This can cause an early debut of symptoms of the kind one would expect in a younger and less mature infant, namely spells. The B12 reserves transferred from mother to child are meant to last through the breastfeeding period since breastmilk does not contain enough B12 to replenish depleted

TABLE 5.
Comparison of Biomarkers for B12 Status Between Cases and Controls

Biomarker	Cases	Controls	*P*
	All n = 85	All n = 252	
Total homocysteine μmol/L	12.4 [10.0-16.1]	7.8 [6.4-10.0]	<0.001[*]
Methylmalonic acid μmol/L	1.54 [0.56-2.83]	0.33 [0.21-0.76]	<0.001[*]
Vitamin B12 pmol/L	197 [144-249]	341 [250-496]	<0.001[*]
Holotranscobalamin pmol/L	38 [29-47]	62 [43-112]	<0.001[*]
Folate nmol/L	33 [26-40]	>45 [36->45]	<0.001[*]
Neutrophils giga/L	1.89 [1.40-2.95]	1.73 [1.29-2.40]	0.118[*]
Hemoglobin g/100 mL	12.7 (2.70)	11.6 (0.87)	<0.001[†]
Mean corpuscular volume fL	85 (7)	79 (4)	<0.001[†]

Data presented as median (interquartile range) and mean (SD).
[*] Mann-Whitney U test.
[†] 2-tailed t-test, unequal variances assumed.

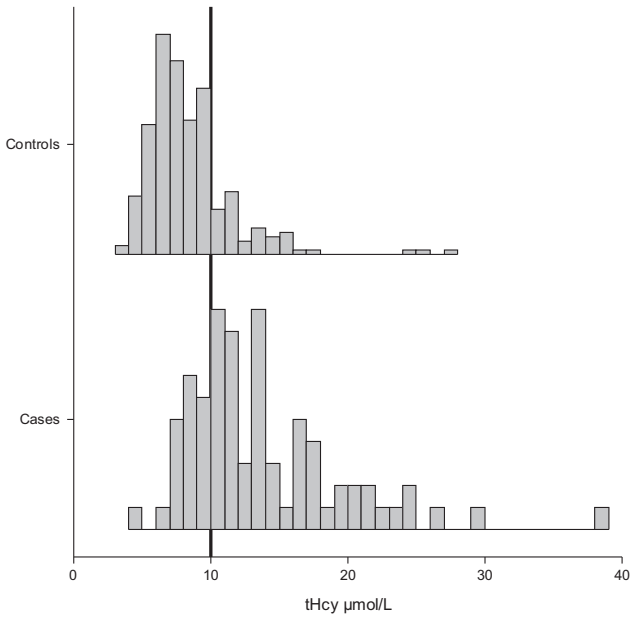

FIGURE 4. Comparison of frequency distribution of tHcy between cases (n = 79) and controls (n = 250). tHcy, total homocysteine.

TABLE 6.
Comparison of Parent-Reported Symptoms Before and After B12 Injection and in Controls

Symptom	Cases Before*	Cases After†	Controls‡	P ($Case_{before}$ vs $Case_{after}$)
Irritability	44%§	27%	30%	0.024
Reduced eye contact	25%‖	7.3%¶	1.6%	0.002
Sleepiness	32%‖	16%	8.5%	0.013
Food refusal	32%‖	16%	14%	0.013
Regurgitations	39%	27%	29%	0.098
Apneas	27%‖	15%#	3.6%	0.04
Tremor	42%‖	11%	7.6%	<0.001
Absence spells	27%‖	6.0%¶	1.6%	<0.001
Hypotonia	38%‖	13%¶	4.9%	<0.001
Slow development	34%‖	20%¶	6.8%	0.034
Slow weight increase	29%‖	19%¶	11%	0.146
Mucous stools	37%§	18%	23%	0.007

* n = 82-85.
† n = 81-83.
‡ n = 247-249, $Case_{before}$ vs control.
§ $P < 0.05$.
‖ $P < 0.001$, $Case_{after}$ vs control.
¶ $P < 0.05$.
# $P < 0.001$, all tests are Chi-squared.

reserves.[23] In this study, the dose of N_2O given to the mothers in labor correlated significantly with the case infants' levels of tHcy and MMA, indicating that the more N_2O the mother inhales, the less the B12 remains in her infant several months after birth. There were no associations between the dose of N_2O and B12 status in the control group. We suggest that this discrepancy may be explained by the combination of insufficient maternal B12 status and a higher rate of breastfeeding among the cases, leaving them more susceptible to B12 depletion by N_2O.

The majority (71%) of cases were exclusively breastfed at diagnosis, recognized as one of the major predictors of infant B12 deficiency in other studies.[6] In B12-replete women, B12 is readily transmitted to her breast milk.[24] However, we found that breastfeeding is one of the risk factors of infant B12 deficiency, along with maternal B12 deficiency and a pregnancy devoid of B12 supplements as independent predictors. Breastfeeding was significantly more frequent among cases compared to 33% in the control group, which in turn was in level with a recent national dietary survey with 39% exclusively breastfed term-born infants at 4 months of age.[25] Exclusive breastfeeding and self-reported maternal B12 deficiency were associated with increased odds for infant B12 deficiency, and exclusive breastfeeding was associated with a higher tHcy, as previously suggested.[6] It has been discussed whether recommending B12-containing supplement during pregnancy reduces the risk for infant B12 deficiency,[7] which our data support. Cases also had lower folate levels, yet not below the threshold for folate deficiency, and their growth rates were below expected and 0.4 SD lower than controls.

Celiac disease, a known cause of B12 deficiency,[6] was seven times more prevalent among mothers to B12 deficient cases than in the general population,[26] but the difference was not significant compared to mothers of controls where the prevalence was also three times higher than expected. Sixty-eight percent of the case infants' mothers were B12 insufficient,[7] though to a lesser extent than in other reports.[1] Varsi et al. recommended a maternal B12 > 394 pmol/L by microbiological assay, corresponding to >275 pmol/L by immunoassay,[27] at week 18 of pregnancy to decrease the risk of infant B12 deficiency in the first six months.[24] The mothers' B12 status was not routinely investigated when infants were diagnosed, and the obtained maternal B12 status was often not corresponding in time with that of her infant's. Therefore, maternal-infant B12 status associations in our study must be carefully interpreted.

Our study supports the findings in other studies that infant B12 deficiency is an important diagnosis also in affluent societies, however with far less classical risk factors such as veganism and poverty.[8,19] In fact, none of our case mothers were vegetarians or vegans, though they were more often multiparous, unemployed, and single than controls. N_2O may be the less recognized risk factor in high-income countries,[10] explaining the shortage of classical risk factors.[2,6]

We also reported two rare manifestations of infant B12 deficiency: skin ulcer and nystagmus. Brain stem and cerebellar symptoms with vertical nystagmus from B12 deficiency have been reported in adults.[28] Further, we found that tremor, hypotonia, and reduced eye contact were common presenting symptoms in addition to spells. This is also supported by our earlier findings of associations between biomarkers of infant B12 deficiency and tremor, hypotonia, and excessive sleep.[4] These are symptoms that could reflect immaturity and suboptimal development rather than disease, where B12 deficiency causes delay in neurological maturation.[7,29] Both sudden infant death syndrome (SIDS) and apparent life-threatening event (ALTE) rates peak between 1 and 4 months of age.[30,31] Given the coinciding presenting age, and events with

TABLE 7.
Risk Factors for B12 Deficiency in a Logistic Regression Model

Covariate	Beta	Wald	Odds Ratio (95% CI)	P
Infant age (days)	−0.018	26.4	0.98 (0.98-0.99)	<0.001
B12 supplement*	−0.972	10.1	0.38 (0.21-0.69)	0.002
Exclusive breastmilk	0.948	8.28	2.58 (1.35-4.92)	0.004
Maternal B12 deficiency†	0.801	3.85	2.23 (1.00-4.97)	0.050

Dependent = case (n = 80), controls (n = 247).
* B12 containing supplement during pregnancy.
† self-reported.

TABLE 8.
Linear Model Coefficients of Predictors for Transformed Infant tHcy, Cases and Controls (n = 320)

Covariate	Beta (95% CI)	Std beta	P
Exclusive breastmilk	0.224 (0.135; 0.312)	0.278	<0.001
B12 supplement*	−0.166 (−0.246; −0.087)	−0.205	<0.001
Infant age (days)	−0.001 (−0.002; −0.001)	−0.199	<0.001

Abbreviation:
CI = Confidence interval
* B12-containing supplement during pregnancy.

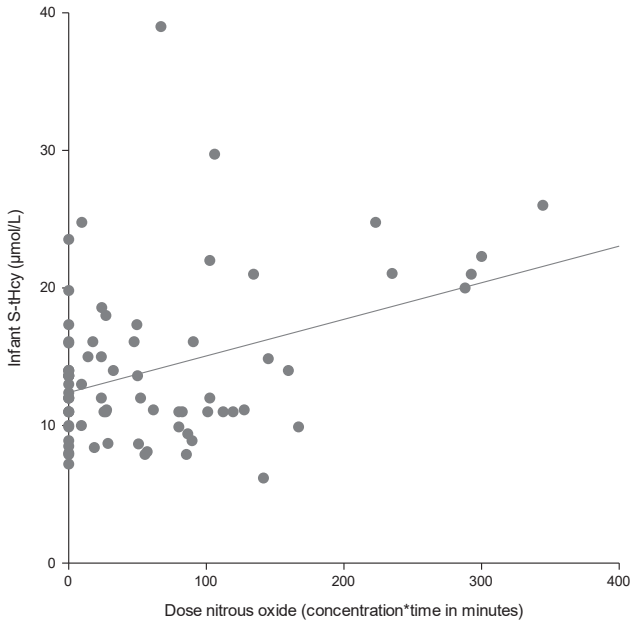

FIGURE 5. Scatterplot of relationship between dose of N_2O to mother during labor and infant tHcy (n = 76) with regression line (r = 0.37). tHcy, total homocysteine.

apneas and seizures, we speculate that vitamin B12 deficiency could be an unrecognized vulnerability factor for SIDS and ALTE. Associations between SIDS, ALTE, and infant B12 deficiency should be addressed in future studies.

Strengths and limitations

High participation (76%) was a strength in our study. Further, the infants underwent thorough workup, minimizing other diagnoses overlapping with symptoms of B12 deficiency. Since this is a retrospective, explorative study, it has important limitations from both selection and recall biases. Recall bias may influence the replies in questionnaires completed years after delivery. The infants in the control group were six weeks (corrected age) older in average than the cases and did not fully cover the cases age-wise. This may partly explain the higher rate of exclusive breastfeeding among cases that nevertheless remained a strong predictor for B12 deficiency also after correction for age. It probably also explains the higher average weight and lower Hb and MCV in controls. Delayed cord clamping has been recommended only recently, and controls were born in later years than cases, probably explaining the higher rate of delayed cord clamping in controls than in cases. Even though the treating physician decided upon B12 deficiency diagnosis without predefined criteria, 92% had tHcy≥ 8 µmol/L, corresponding to 6.5 µmol/L when measured in plasma, a well-acknowledged decision level for diagnosing B12 deficiency in infants.[6] We could not reliably analyze clinical outcome response after B12 supplementation due to lack of or imprecise information in the medical records.

TABLE 9.
Linear Model Coefficients of Predictors for Transformed Infant tHcy in Cases Only (n = 67)

Covariate	Beta (95% CI)	Std beta	*P*
Dose N_2O (min.*conc.)	0.002 (0.001; 0.003)	0.350	0.002
Mother's age	−0.026 (−0.045; −0.007)	−0.295	0.008
B12 supplement*	−0.186 (−0.339; −0.032)	−0.261	0.019
Prematurity	0.264 (0.030; 0.499)	0.240	0.028
Mother's BMI†	0.016 (0.002; 0.029)	0.239	0.029
Infant age (days)	<0.001 (−0.001; 0.002)	0.094	0.392

Abbreviations:
BMI = Body mass index
CI = Confidence interval
* B12-containing supplement during pregnancy.
† Prior to pregnancy.

Conclusion

Maternal use of N_2O in labor should be considered a novel risk factor to be included in a prospective study. Unnecessary referrals and hospital admissions could be reduced with increased education in pediatric health care to include B12 status in breastfed infants with subtle and overt neurological symptoms and signs, especially if the mother has celiac disease or known B12 deficiency. To reduce risk of infant B12 deficiency, we advise screening for maternal B12 status in early pregnancy.

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ACTA PÆDIATRICA NURTURING THE CHILD WILEY

ORIGINAL ARTICLE

Nitrous oxide in labour predicted newborn screening total homocysteine and is a potential risk factor for infant vitamin B12 deficiency

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Abstract

Aim: Risk factors for vitamin B12 deficiency in infants are not fully understood. The aim of the study was to assess predictors of total homocysteine and methylmalonic acid analysed in newborn screening dried blood spots.

Methods: In a Norwegian case control study, we analysed total homocysteine and methylmalonic acid in newborn screening dried blood spots of 86 infants clinically diagnosed with vitamin B12 deficiency during 2012–2018. Results were compared to 252 healthy infants and 400 dried blood spot controls. Medical records were reviewed, and mothers completed questionnaires.

Results: Both total homocysteine and methylmalonic acid were significantly higher on newborn screening dried blood spots in infants later clinically diagnosed with vitamin B12 deficiency than controls. Multiple regression analysis showed that the dose of nitrous oxide during labour was the strongest predictor for total homocysteine level in newborn screening dried blood spots for all infants, with larger effect in infants later clinically diagnosed with vitamin B12 deficiency than controls.

Conclusion: Nitrous oxide dose during labour was a predictor for total homocysteine and may impact the interpretation of total homocysteine analysis in newborn screening. Nitrous oxide is suggested as a contributing risk factor for infants prone to develop vitamin B12 deficiency.

KEYWORDS
homocysteine, newborn screening, nitrous oxide, risk factor, second-tier, vitamin b12 deficiency

Abbreviations: B12, vitamin B12; CI, confidence interval; DBS, dried blood spot; IQR, interquartile interval range; MMA, methylmalonic acid; NBS, newborn screening; SD, standard deviation; tHcy, total homocysteine.

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1 | INTRODUCTION

Analyses of total homocysteine (tHcy) and methylmalonic acid (MMA) are used in newborn screening (NBS) programs as second-tier tests for cystathionine-β-synthase deficiency, remethylation diseases, and methylmalonic and propionic acidaemia.[1] More recently, they have also been recommended for detection of vitamin B12 (B12) deficiency in newborn infants,[2–4] tHcy being regarded as the best marker of infant B12 deficiency.[5] Maternal–foetal transfer of B12 results in a higher concentration of B12 in the newborn infant than in the mother, consistent with active transport of the vitamin.[6] In B12-replete mothers, B12 stores are gradually accrued in the foetal liver during gestation, achieving 25–30 μg at term, compared to 2–5 μg in newborn infants of B12-deficient mothers.[6] A B12-deficient mother is also the strongest predictor for B12 deficiency in a breastfed infant.[3,5] However, maternal B12 deficiency is not always evident in B12-deficient newborn infants detected by NBS.[3] Vegetarianism is only an exceptional cause of maternal B12 deficiency in high-income countries[2,3,7] and thus other risk factors for infant B12 deficiency need to be considered. Nitrous oxide is extensively used for analgesia during labour.[8,9] It oxidises the methionine synthase bound cob(I)alamin to cob(II)alamin, thereby irreversibly inhibiting this enzyme, which leads to accumulation of Hcy and lack of S-adenosyl-methionine.[10–12] Nitrous oxide does not affect methyl malonyl-CoA-mutase activity.[12] tHcy increases significantly after nitrous oxide has been given to children during anaesthesia for surgery, with dose–response kinetics.[13] Furthermore, already 30 years ago, nitrous oxide given as pain relief during labour was shown to inhibit methionine synthase in the placenta in a dose-responsive manner.[14] Nitrous oxide is distributed to and accumulates in the foetus when provided to the mother prenatally.[15] Only short-term safety for obstetric use has been documented,[8,9] but the longer-term effect of the inhibition of methionine synthase has not been evaluated. In a previous publication, we found that nitrous oxide correlates with both infant tHcy and MMA levels several months after birth in infants with clinically diagnosed B12 deficiency, suggesting nitrous oxide as a possible risk factor for early infant B12 deficiency.[16]

The aims of this retrospective case–control study were to explore predictors for tHcy and MMA levels, analysed in dried blood spots (DBS) obtained from NBS, and to analyse the frequency distribution of tHcy and MMA levels for infants later diagnosed with B12 deficiency, compared to controls.

Key Notes

- Total homocysteine and methylmalonic acid were significantly increased at newborn screening in infants later clinically diagnosed with vitamin B12 deficiency compared to healthy controls.
- The dose of nitrous oxide used in labour was the strongest predictor for the total homocysteine level in newborn screening.
- Nitrous oxide is suggested as a contributing risk factor for infants prone to develop vitamin B12 deficiency.

2 | MATERIALS AND METHODS

2.1 | Study population

We performed a retrospective case–control study. We included infants below 1 year of age, born between 2012 and 2018, who were treated for clinical B12 deficiency, designated as cases. They were identified after search in medical record databases of two hospitals in South-East Norway. As controls, we used a cohort of healthy, age-matched infants (Figure S1), referred to as clinical controls, since they were recruited for postnatal clinical follow-up in 2018–2019 from the Postnatal and Neonatal Unit at Vestfold Hospital Trust, Norway. Details on inclusion, background characteristics, and clinical and biochemical findings have previously been published.[16–19] We also included DBS controls, matched for date of birth, age in days, sex, hospital, birth weight, and gestational age of the cases and clinical controls. Data on pregnancy, delivery, and clinical follow-up were not available for the DBS controls. The study was approved by the Regional Committee for Medical Research Ethics Northern Norway (179/2018) and was conducted according to the Declaration of Helsinki. Written informed consent was collected for all participants.

2.2 | Background data

We collected obstetric data from hospital records. Mothers completed non-standardised questionnaires on vitamin-supplementation and self-reported health. We retrieved information on the use of nitrous oxide during labour from the mothers' obstetric files and included time for start and stop of intermittent administration of nitrous oxide and its concentration in percentage in the nitrous oxide/oxygen blend. We calculated the total dose of nitrous oxide as the concentration of nitrous oxide multiplied by the time for intermittent administration in minutes. The selection of covariates, that are suggested risk factors for infant B12 deficiency, was based on previous reports.[5,16,17,20] We also calculated the storage time of DBS card from birth until tHcy and MMA analyses were performed since storage possibly could influence the levels.

2.3 | Newborn screening analyses

Blood samples were collected on filter cards 48–72 h after birth and sent by prioritised mail to the Norwegian National NBS laboratory at Oslo University Hospital.[21] After the standard NBS analyses were performed, filter cards were first collected in a fridge at 2–4°C for up to some weeks before being stored in a biobank at −20°C

until they were retrieved for second-tier tHcy and MMA analysis in 2020–2021. A combined second-tier method for tHcy, MMA, and 2-methylcitric acid was established in DBS by LC–MS/MS, partially adapted from Fu et al.[22] tHcy was introduced in 2020 as second-tier analysis for cystathionine β-synthase deficiency and MMA for methylmalonic aciduria and propionic aciduria (Appendix S1). Only NBS filter cards obtained after the expansion of the NBS program in Norway, on 1 March 2012, were available for second-tier analysis.

2.4 | Statistical analysis

Data were registered in EpiData version 4.4 (EpiData Association, Odense, Denmark). Continuous variables were presented as mean and standard deviation or if skewed, as median and interquartile range (IQR). Categorical variables were given as proportions and percentages and compared between groups using the chi-square test of proportions or Fisher's exact test for small samples. Differences between independent groups were quantified with the Mann–Whitney *U* test because of skewness in the data. All statistical tests were two-sided, and a *p* value <0.05 was considered statistically significant. All regression models were significant with $p < 0.001$. Linear regression analyses were performed to identify predictors for DBS tHcy and MMA. A forward method with criterion probability of F to enter ≤0.05 was used to calculate significant variables. Variables entered in regressions of tHcy and MMA to identify risk factors were maternal Norwegian origin, smoking during the last 2 years before pregnancy, meat-consumer, known self-reported B12 deficiency, B12 supplements during pregnancy, diabetes in pregnancy, metformin use, self-reported nausea in pregnancy, age, body mass index at pregnancy start, primiparity, hospital-diagnosed celiac disease, folate supplement, nitrous oxide dose during labour, prematurity, growth restriction, gender, and vaginal delivery. Significant variables were re-analysed by the enter method. Analyses were performed in IBM SPSS Statistics version 28 (IBM Corp, New York, USA), and graphs were created in NCSS 2021 Statistical Software (NCSS LLC, Utah, USA).

3 | RESULTS

3.1 | Characteristics of population

We included 85 clinically diagnosed B12-deficient infant cases, 252 clinical controls (Table S1-S2), and 400 DBS controls. DBS tHcy and MMA were analysed in 79/85 (93%) cases. Six filter cards for children born prior to 1 March 2012 had been destroyed according to Norwegian NBS regulations. tHcy and MMA were analysed in all clinical and DBS controls (Figure S1).

Storage time of DBS (age of DBS) before second-tier analyses [IQR, total range] for cases was median 3.5 years [2.8–5.4, 7] and for all the 652 control median 2.0 years [1.8–2.4, 7]. Mean (SD) birth weight for cases was 3375 g (671) and for all controls 3293 g (668). The median [IQR, total range] case gestational week was 39 [38–41, 15] and for all controls 39 [38–41, 13]. The median [IQR, total range] case age in hours at collection of blood for NBS DBS was 58 h [51–66, 110] and for all controls 58 h [51–67, 113].

3.2 | DBS tHcy and MMA

For the 79 clinical cases, the median [IQR] tHcy was 6.29 μmol/L [5.18–8.23] (Figure S2) and MMA 0.043 μmol/L [0.00–0.31] (Figure S3). For the 652 controls, the median [IQR] tHcy was 5.04 μmol/L [3.82–6.66] (Figure S4) and MMA 0.00 μmol/L [0.00–0.039] (Figure S5). Both DBS tHcy and MMA were significantly higher in cases than in controls (Mann–Whitney *U* test, $p < 0.001$) (Figures 1 and 2).

3.3 | Associations between predictors and DBS tHcy

A multiple linear regression was run with DBS tHcy in μmol/L as the dependent variable, storage time of DBS in years, and DBS case/control as the independent variables, in total $n = 730$. Both the storage time of DBS (beta = 0.350, 95% CI 0.239–0.460, $p < 0.001$, standardised beta = 0.229) and DBS case versus control (beta = 1.178, 95% CI 0.576–1.780, $p < 0.001$, standardised beta = 0.142) predicted DBS tHcy significantly. Multiple linear regression analyses were run separately to identify predictors for DBS tHcy for clinical cases and clinical controls (Table S2 and Table 1). The dose of nitrous oxide given to the mother during labour was the strongest predictor for tHcy for the clinical cases (standardised beta 0.413, $p < 0.001$) and the only significant predictor for the clinical controls (standardised beta 0.240, $p < 0.001$). For the clinical cases, nausea in pregnancy was associated with DBS tHcy (standardised beta 0.301, $p = 0.003$) (Table 1).

3.4 | Associations between predictors and DBS MMA

A multiple linear regression was run with DBS MMA in μmol/L as the dependent variable, storage time DBS in years, and DBS case/control as the independent variables, $n = 731$. DBS case/control predicted DBS MMA significantly (beta = 0.173, 95% CI 0.117–0.228, $p < 0.001$, standardised beta = 0.229), while the storage time of DBS (beta = 0.006, 95% CI -0.004 to 0.016, $p = 0.23$, standardised beta = 0.045) did not. Multiple linear regression analyses were run to identify predictors for DBS MMA for all clinical infants (Table 2). Later clinical infant B12 deficiency was associated with increased DBS MMA (standardised beta 0.284, $p < 0.001$). Celiac disease and nausea in pregnancy predicted MMA for all clinical infants (standardised betas 0.157 and 0.122, $p = 0.003$ and 0.019, respectively) (Table 2).

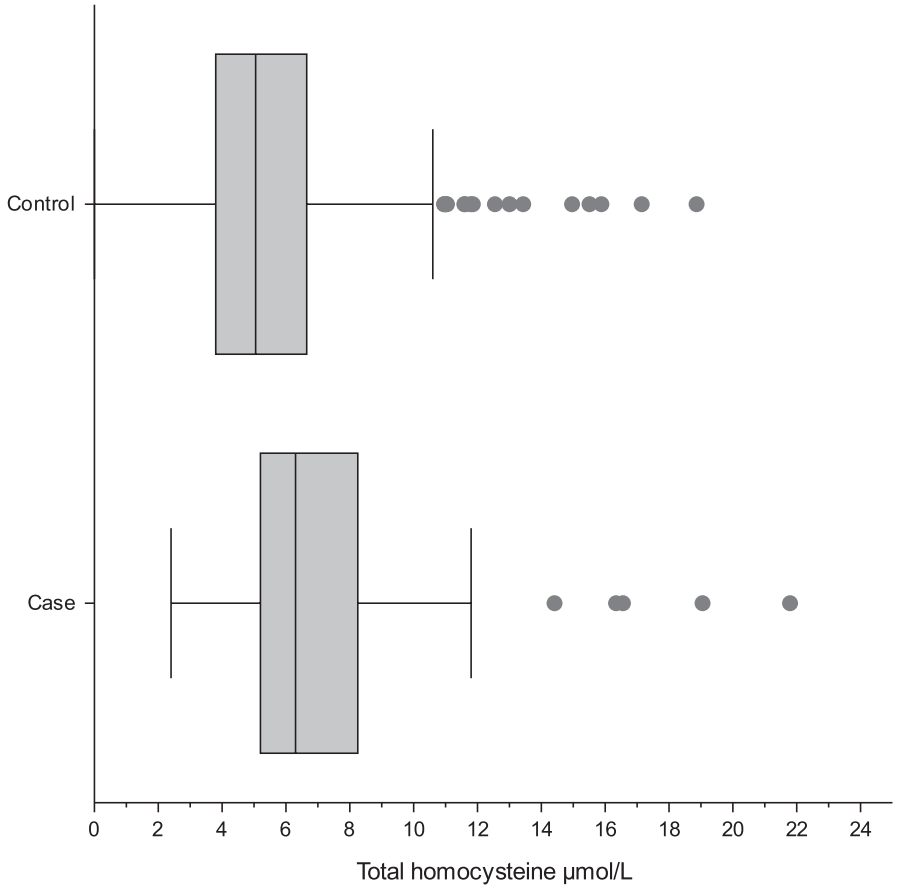

FIGURE 1 Comparison of frequency distribution for total homocysteine (tHcy) between the 79 clinical cases and the 652 controls.

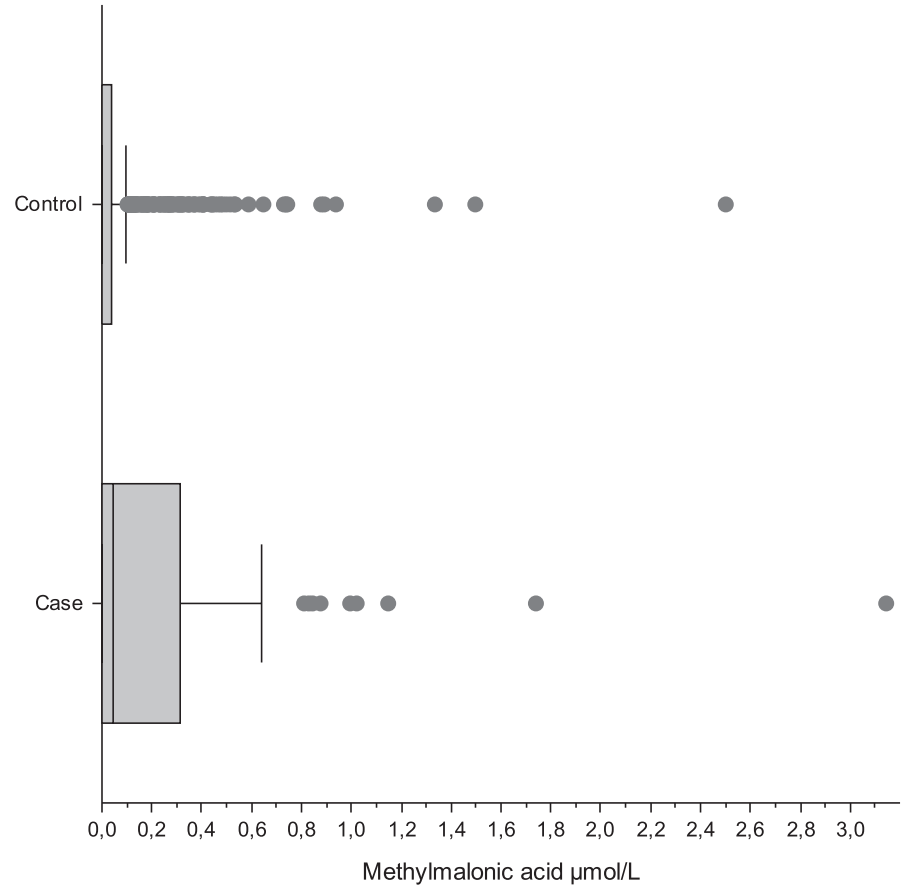

FIGURE 2 Comparison of frequency distribution for methylmalonic acid (MMA) between the 79 clinical cases and the 652 controls.

TABLE 1 Linear model coefficients of predictors for dried blood spot (DBS) total homocysteine for clinical cases and controls separately

	Clinical cases ($n = 76$)			Clinical controls ($n = 243$)		
	Beta (95% CI)	**Std beta**	***p***	**Beta (95% CI)**	**Std beta**	***p***
Dose N_2O[a]	0.017 (0.009–0.025)	0.413	<0.001	0.006 (0.003–0.008)	0.240	<0.001
Nausea in pregnancy	2.19 (0.751–3.62)	0.301	0.003			
Storage of DBS (years)	−0.538 (−0.898 to −0.178)	−0.297	0.004			

[a]Dose of nitrous oxide (N_2O) is the product of concentration of N_2O and the administration time in minutes.

TABLE 2 Linear model coefficients of predictors for dried blood spot methylmalonic acid, all 326 clinical infants

	Beta (95% CI)	**Std beta**	***p***
Infant B12 deficiency	0.180 (0.115–0.246)	0.283	<0.001
Celiac disease	0.211 (0.074–0.348)	0.157	0.003
Nausea in pregnancy	0.073 (0.022–1.52)	0.122	0.019

4 | DISCUSSION

This case–control study investigated predictors for tHcy and MMA, analysed in DBS obtained from newborn screening for healthy infants and infants with known B12 deficiency, clinically diagnosed during the first year of life. We showed that the strongest predictor for tHcy was the dose of nitrous oxide given to the mother during labour followed by self-reported nausea in pregnancy. Celiac disease and nausea in pregnancy predicted MMA.

We have previously published an association between dose of nitrous oxide to the mother in labour and both serum tHcy and MMA retrieved several months later in life in clinically diagnosed B12-deficient infants, hypothesizing that the more nitrous oxide delivered to the mother in labour, the less B12 remains in her infant months later.[16] Accordingly, when we in the present study analysed the DBS collected on the third day of life from the same, clinically presenting B12-deficient infants and their controls, only tHcy but not MMA, was associated with dose of nitrous oxide, indicating decreased methionine synthase activity. This was evident for both cases and controls. In contrast, both the tHcy and the MMA–levels were higher on the third day of life in later, clinically presenting B12-deficient cases compared to controls. Since nitrous oxide has been shown to affect methionine synthase only, not methylmalonyl-CoA-mutase,[12,23] this finding indicates a lower B12 status in the cases rendering them more prone to later B12 deficiency. Furthermore, the higher infant MMA level at NBS could be explained by insufficient maternal B12 status, a well-known risk factor for infant B12 deficiency.[5]

Nitrous oxide chemically inactivates B12 through irreversible oxidation of its coenzyme form, methyl cobalamin, at the active site of the B12-dependent methionine synthase reaction.[10–12] The nitrous oxide-induced homocysteine response depends on the cobalamin status of the individual exposed to the gas and will be higher with lower cobalamin status.[5,14] The irreversible inactivation requires re-synthesis of methionine synthase and B12 stores are consumed. Hence, nitrous oxide given during labour will decrease B12 stores in both the mother and the newborn infant, the effect being relatively larger if the mother is B12-deficient or have a suboptimal B12 status during pregnancy. The exclusively breastfed infant is at risk to develop symptomatic B12 deficiency since breastmilk B12 content is accordingly reduced.[5] Consequently, our results propose nitrous oxide to be an unrecognised contributor for B12 deficiency in vulnerable infants with lower B12 status. This is both in line with results recently reported by us,[16] and with findings by Landon et al. over 30 years ago, in that nitrous oxide inactivated placental methionine synthase in a dose-responsive manner and more so if maternal B12 was low.[14] Low maternal B12 status, nitrous oxide in labour, and breastfeeding may reinforce the risk of infant B12 deficiency. If the mother's B12 status is sufficient though, or if the infant is formula fed, the risk for B12 deficiency is low. Nitrous oxide during labour should also be considered when interpreting increased tHcy in NBS. Transient elevation of tHcy in the newborn infant may be one of the factors explaining why a subset of mothers are not diagnosed with B12 deficiency following detection of her infant at NBS.[3,7,24,25]

We also found associations between MMA and plausible risk factors for maternal B12 deficiency such as self-reported nausea in pregnancy and celiac disease, both potentially impacting on the pregnant women's B12 stores.[5] We assume, like others before us, that this may be explained by decreased intake or uptake of B12 from the food in the pregnant woman with nausea or celiac disease.[5] This is also in accordance with a previous study of infants with confirmed B12 deficiency suggested by NBS, in which nausea and food aversion were reported in 28% and gastrointestinal disorder in 8% of 19 mothers as a cause for maternal B12 deficiency.[24] We did not find associations between self-reported maternal B12 deficiency and DBS tHcy or MMA in the infants, presumably because this variable was inaccurate since we did not collect temporal information on when the mothers were B12-deficient. In the light of our findings, we encourage to screen and treat mothers for B12 deficiency early in pregnancy to reduce risk of infant B12 deficiency.

Maternal B12 status, recognised as the most important determinant of neonatal B12 status,[20] was not available and was a limitation to our study. B12 status is not included as part of standard

pregnancy blood tests in Norway and could only have been accessed through a planned prospective study. Since this was a retrospective case–control study, our associations were mainly found in linear regression models and causality was not proven. We showed that storage time of DBS was associated with an increase of 0.35 μmol/L per year for tHcy, but not for MMA. Therefore, we could not infer the differences we found in tHcy between cases and controls for tHcy directly without correction for storage time of DBS since the time elapsed was longer for cases than for controls. Our finding showing increased concentration with time for tHcy for DBS stored in a cold environment has not been reported before. A decrease in tHcy has been observed for DBS stored in dry, sealed plastic bags.[26,27] The reason suggested for the latter situation is that whole blood with erythrocytes contains less homocysteine than plasma. It has previously been shown that tHcy increases in plasma if whole blood is stored uncentrifuged after sampling, explained by the release of homocysteine from the erythrocytes even at storage at 4°C, but we do not know if this applies for whole blood sampled on filter paper.[28] However, preanalytical factors, such as collection devices, humidity, and temperature may all potentially influence long-term stability of the analytes.[29] We consequently chose to include storage time of DBS in all regressions for tHcy to correct for this systematic error. Our study was not designed to analyse the relation between storage time of DBS and tHcy and this association should therefore be interpreted with caution. We measured time between the use of nitrous oxide started and stopped. The use was intermittent, and since we did not measure the volume used, the measure is inexact.

5 | CONCLUSION

In conclusion, nitrous oxide dose during labour was a predictor for tHcy at NBS and is suggested as a risk factor for infant B12 deficiency. We recommend to routinely analyse B12 status of mothers prior to use of nitrous oxide in labour and that mothers should be informed of the potential risks to their infants.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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A Retrospective Evaluation of the Predictive Value of Newborn Screening for Vitamin B12 Deficiency in Symptomatic Infants Below 1 Year of Age

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Abstract: Background: The sensitivity of newborn screening (NBS) in detecting infants that later develop symptomatic vitamin B12 deficiency is unknown. We evaluated the predictive value using NBS algorithms in detecting infants that later were clinically diagnosed with symptomatic B12 deficiency. Furthermore, we investigated whether being born in a hospital using nitrous oxide (N_2O) as pain relief in labor may have had an impact on total homocysteine at NBS. Methods: We retrospectively retrieved NBS data and analyzed total homocysteine, methylmalonic acid and methyl citrate on stored NBS dried blood spots (DBS) of 70 infants diagnosed with symptomatic B12 deficiency and compared them to 646 matched and 434 unmatched DBS controls to evaluate the Austrian and Heidelberg B12 NBS algorithms. Results: The sensitivity of NBS in detecting infants later diagnosed with symptomatic B12 deficiency at median age 10.9 weeks was ≤10%. Total homocysteine was higher in DBS for the unmatched controls who were born in hospitals providing N_2O compared to in hospitals not providing N_2O, with median total homocysteine 4.0 µmol/L compared to 3.5 µmol/L (n = 434, 95% CI 0.04–0.87, p = 0.03). Conclusion: NBS algorithms were unable to identify most infants diagnosed with symptomatic B12 deficiency after the neonatal period. Being born in hospitals providing N_2O may impact total homocysteine at NBS.

Keywords: vitamin B12 deficiency; homocysteine; infant; newborn screening; nitrous oxide; second-tier; vitamin B12

1. Introduction

Vitamin B12 (B12) is important for neurodevelopment and even moderate deficiency during the first months of life may cause disease with tremor, apneas, seizures, and developmental delay [1,2]. Prompt B12 substitution effectively resolves the deficiency [3], but severe long-standing B12 deficiency may result in long-term neurological disabilities even if treated [4]. A higher incidence of B12 deficiency in newborn screening (NBS) programs have recently been demonstrated after the implementation of algorithms specifically designed for this purpose and when remethylation disorders have been introduced as primary targets of the NBS programs [5,6]. Total homocysteine (tHcy) is recognized as the best marker of B12 deficiency in this age group [2]. The B12 deficiency NBS algorithms

published from Austria [6] and Heidelberg [5] utilized first and second-tier markers deriving from both B12-dependent pathways. Propionylcarnitine (C3) with different ratios and methylmalonic acid (MMA)/methylcitrate (MCA) were primary and secondary markers from the conversion of methylmalonyl-CoA to succinyl-CoA-pathway, whereas methionine with its ratio to phenylalanine and tHCy were first-tier and second-tier tests emanating from the remethylation of homocysteine to methionine [5–9]. These studies reported a positive predictive value of 67–81% [6] and 45% [5] using B12, holotranscobalamin (holoTC), tHcy and MMA to confirm the biochemical diagnosis of B12 deficiency. Since NBS for B12 deficiency mainly reveals maternal B12 deficiency, recognized as a main risk factor for infant B12 deficiency, it has the potential not only to detect the still asymptomatic newborn, but also the mother, allowing both to be treated and thus preventing symptoms and deficiency in the next pregnancy [5]. In Canada, 5% of women in fertile age has been found to have vitamin B12 deficiency [10]. Nitrous oxide (N_2O), commonly used as pain relief in labor, accumulates in the fetus and is known to irreversibly inhibit methionine synthase by oxidizing the cobalt atom in a dose–response manner [11–15].

The aims of this study were to evaluate the Austrian and Heidelberg NBS algorithms applied retrospectively for infants clinically diagnosed with symptomatic vitamin B12 deficiency, and to assess if the availability of N_2O, and thus its possible use as pain relief during labor at hospital of birth, could affect the NBS interpretation.

2. Materials and Methods

2.1. Study Population

We performed a case–control study with a group of symptomatic B12 deficiency cases and two groups of controls (Figure 1). We included infants below one year of age, born between 2011 and 2018, that were diagnosed and treated for symptomatic B12 deficiency. The treating physician decided upon B12 deficiency diagnosis from clinical symptoms and findings, and B12 status without any predefined criteria. These infants were designated as clinical cases and were identified after search for the International Classification of Disease 10 codes E53.8, E53.9, Z03.3, P90, P91.8, P28.4, R56, R58.8 or D51, with a concomitant B12 status analysis in medical record databases of two hospitals in the South-East of Norway [1]. We recruited a cohort of healthy, age-matched infants, referred to as clinical controls, scheduled for postnatal clinical follow-up in 2018–2019 from the Postnatal and Neonatal Unit at Vestfold Hospital Trust, Norway [16]. Details on inclusion, background characteristics, clinical and biochemical findings have been published elsewhere [1,15–18]. We also included NBS dried blood spot (DBS) controls, matched for date of birth, age in days, sex, hospital, birth weight and gestational age of the clinical cases and clinical controls, designated as matched NBS DBS controls. Additionally, another 450 unmatched NBS DBS controls were collected in 2020–2021 (Figure 1). The included hospitals were stratified according to the availability of nitrous oxide as pain relief during delivery.

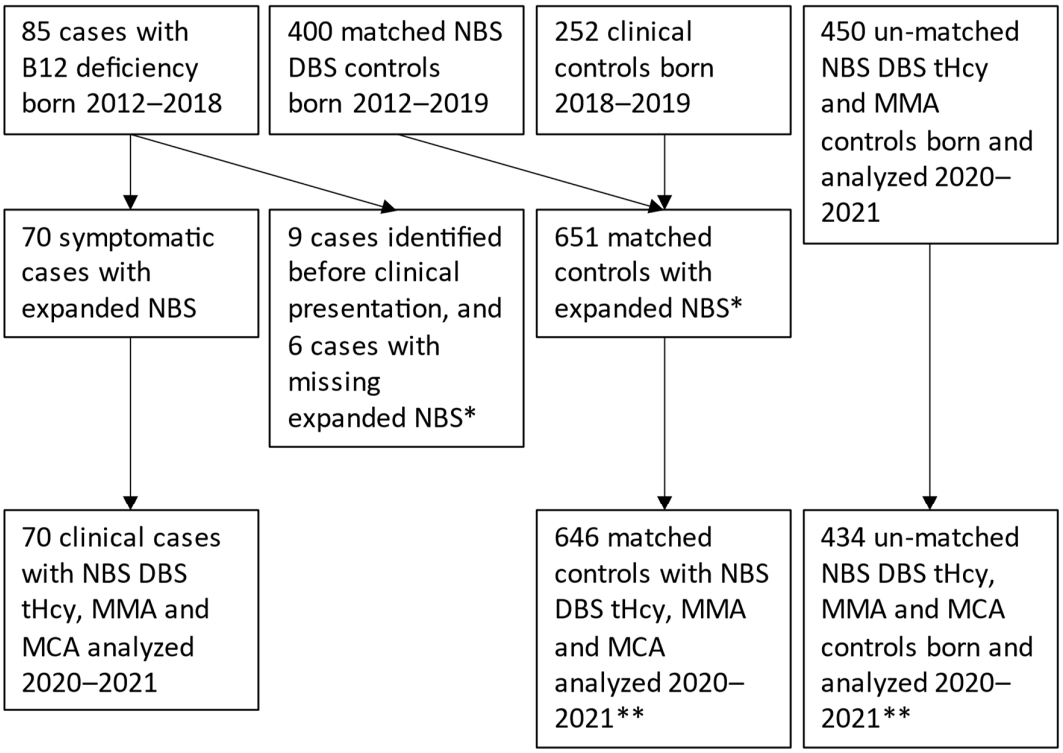

Figure 1. Inclusion of clinical cases and controls, * = missing infants born before 2012, missing expanded NBS, ** = missing infants with unsuccessful 2nd tier analyses, NBS = newborn screening, DBS = dried blood spot, tHcy = total homocysteine, MMA = methylmalonic acid, MCA = methyl citric acid.

2.2. Newborn Screening Analyses

Blood samples were collected on filter cards 48–72 h after birth and sent by prioritized mail to the Norwegian National NBS laboratory at Oslo University Hospital [16]. Only infants born after the expansion of the NBS program in Norway, on 1 March 2012, were included, as DBS before this date were destructed in accordance with Norwegian law. First-tier analyses of acylcarnitines were performed using the NeoBase 2 Non-Derivatized MSMS Kit (PerkinElmer, Turku, Finland) on an Acquity UPLC coupled to a Xevo TQS-micro mass spectrometer (Waters, Milford, MA, USA), after being punched (3.2 mm disc) with a Panthera-Puncher 9 (PerkinElmer, Turku, Finland). After the standard NBS analyses were performed, the DBS were first kept at +2–4 °C, for 1–3 months, followed by storage in a biobank at −20 °C until the second-tier analyses of tHcy, MMA and MCA were undertaken twice during 2020–2021. The second-tier analyses were performed at the time of the standard NBS analyses for the unmatched controls. A combined method for second-tier analysis of tHcy, MMA and MCA in DBS was set up using an LC-MS/MS method described elsewhere [15], and systematically introduced as second-tier analysis for cystathionine β-synthase deficiency and methylmalonic- and propionic aciduria in 2020. Readings without a tHcy peak were considered unreliable and therefore excluded. We used the previously published flowcharts from the Austrian NBS program [6] and the Heidelberg NBS program [5] to retrospectively categorize our study cohort's NBS results into NBS positive or NBS negative B12 deficiency. We entered absolute NBS values from our own program corresponding to their suggested percentile-cutoffs [19]. We calculated the cutoff values for tHcy equivalent of the percentiles used by Rozmaric et al. [6] from the unmatched controls. We could not calculate the 99.9 percentile for MMA used by Gramer et al. [5] due to insufficient number of controls, and we therefore chose to use their absolute cutoff value. We compared matched controls to cases since DBS tHcy increased

0.35 µmol/L per year with storage time as shown previously. DBS MMA was not affected by storage [15].

2.3. Statistics

Continuous variables are presented as mean and standard deviation or if skewed, as median and interquartile range (IQR). Categorical variables are given as proportions and percentages and are compared between groups using the Fisher's Exact test. Differences between independent groups are quantified with *t*-tests. We use receiver operating characteristic (ROC) curves with being a 'symptomatic case' as outcome variable to test the NBS analytes' performance as classifiers. All statistical tests are two-sided, and a p-value < 0.05 is considered statistically significant. We present data for cases and controls where the combined results from expanded NBS and from second-tier analyses (Figure 1) are available. Data analyses were performed in IBM SPSS Statistics version 28 (IBM Inc., New York, NY, USA).

3. Results

During the study period 35,639 children were born in the catchment area. By the search string presented in the methods we identified 394 infants < 1 year. Of these, 130 were diagnosed and treated for B12 deficiency (130/35,639, 0.36%) and in 264 infants, B12 deficiency was not diagnosed. We invited 123 of the infants diagnosed with B12 deficiency [1], of which 93 infants were recruited and 30 did not reply or declined the invitation. We excluded 8 infants due to age over 1 year (n = 1), severe asphyxia (n = 1), genetic disease (n = 5) or no B12 deficiency (n = 1, erroneously included) [1]. Of the remaining 85 infants (Figure 1), nine were diagnosed presymptomatically with B12 deficiency, and six infants were excluded due to missing tHcy analyses, five because DBS had been destroyed and one case was born before the expanded NBS was introduced (Figure 1). Thus, 70 infants with symptomatic B12 deficiency were included for analyses in the present study.

At work-up, median [IQR] age was 10.9 [4.7–18] weeks, and the symptomatic B12 deficient cases had median [IQR] S-B12 197 [148–249] pmol/L, S-tHcy 12 [10–15] µmol/L, and S-MMA 1.50 [0.51–2.60] µmol/L. Twenty-eight (40%) had either S-B12 < 148 pmol/L or S-holoTC < 35 pmol/L, 34/67 (51%) had S-B12 < 200 pmol/L and 62/70 (89%) had either S-B12 < 200 pmol/L or S-tHcy > 10 µmol/L. Sixty of 66 (91%) had tHcy $\geq$ 8 µmol/L The mothers (n = 60) had median S-B12 254 pmol/L [187–342]. In a subgroup of 30 infants with S-B12 < 160 pmol/L (n = 20) or holoTC < 35 pmol/L (n = 10), median S-B12 was 144 [129–188] pmol/L, S-holoTC 31 [25–39] pmol/L, S-tHcy 12 [11–16] µmol/L, S-MMA 1.34 [0.43–2.42] µmol/L and the median of 25 maternal S-B12 was 229 [183–287] pmol/L.

We applied the same percentiles for tHcy as the Austrian published NBS algorithm [6]. The tHcy 89.2 percentile and the 96.7 percentile in the unmatched control group (n = 434) corresponded to 6.3 µmol/L and 8.6 µmol/L, respectively. The unmatched NBS controls were collected from 34 different hospitals with maternity wards, and N_2O was available as birth analgesia at 25 (74%) of these hospitals. In total, 239/434 (55%) of unmatched controls were born in hospitals providing N_2O. tHcy was higher for the unmatched controls who were born in hospitals providing N_2O compared to in hospitals not providing N_2O, with tHcy = 4.0 µmol/L compared to 3.5 µmol/L (n = 434, p = 0.03), while mean MMA was 0.26 µmol/L compared to 0.21 µmol/L, respectively (p = 0.131). The clinical cases and the matched controls were all born at two hospitals which provided N_2O [15]. Descriptive characteristics are presented in Tables 1 and 2. Clinical presentation and findings in cases and controls are presented in Table 3. None of the cases or controls were diagnosed with an inherited disorder of cobalamin metabolism.

Table 1. Descriptive characteristics of cases and controls, mean (SD) and n (%).

	Positive NBS (n = 5)	**Clinical Cases with B12 < 160 or holoTC < 35 pmol/L (n = 30)**	**Clinical Cases (n = 70)**	**Clinical Controls (n = 252)**	**Matched Controls (n = 646)**	**Unmatched Controls (n = 434)**
Gestational age (weeks)	38 (4)	39 (2)	39 (2)	39 (2)	39 (2)	39 (2)
Birthweight (grams)	3152 (840)	3327 (554)	3401 (627)	3296 (666)	3427 (588)	3493 (540)
NBS DBS age (hours)	59 (15)	59 (10)	59 (14)	62 (16)	62 (15)	57 (18)
DBS storage time (years)	3.7 (1.5)	4.0 (1.8)	4.0 (1.8)	1.9 (0.2)	2.6 (1.6)	0
Female	2 (40)	12 (40)	29 (41)	125 (50)	301 (47%)	216 (50%)

NBS = newborn screening, DBS = dried blood spot.

Table 2. Descriptive characteristics of clinical cases and controls, mean (SD) and n (%).

	Positive NBS (n = 5)	**Clinical Cases with B12 < 160 or holoTC < 35 pmol/L (n = 30)**	**Clinical Cases (n = 70)**	**Clinical Controls (n = 252)**	**Difference Compared to Clinical Controls (Fisher's Exact Test or *t*-Test, *p*)**	
					Positive NBS	**Clinical Cases with B12 < 160 or holoTC < 35 pmol/L**
Married/cohabitant	5 (100)	27 (90)	63 (90)	249 (99)	1.0	**0.01**
Higher education	4 (80)	20 (67)	45 (64)	169 (69)	1.0	0.84
Origin outside the Nordic countries	1 (20)	7 (23)	11 (16)	53 (21)	1.0	0.81
Employment last 2 years	4 (80)	20 (77)	50 (76)	220 (91)	0.40	**0.045**
Smoking last 2 years	0	5 (17)	9 (13)	30 (12)	1.0	0.56
Meat-eater	5 (100)	29 (97)	69 (99)	241 (97)	0.13	0.60
Known maternal B12 deficiency	1 (20)	7 (24)	17 (25)	24 (9.7)	0.41	**0.03**
Celiac disease	0	1 (3.3)	5 (7.1)	8 (3.2)	1.0	1.0
Primipara	4 (80)	15 (50)	30 (43	138 (55)	0.38	0.70
Diabetes in pregnancy	0	1 (3.3)	3 (4.3)	16 (6.3)	1.0	1.0
Metformin use	0	1 (3.6)	2 (3.4)	9 (3.6)	1.0	1.0
Hyperemesis (self-reported)	3 (60)	14 (47)	23 (33)	67 (27)	0.13	**0.03**
Folate during pregnancy	5 (100)	25 (83)	56 (81)	219 (88)	1.0	0.56
B12 containing supplement during pregnancy	3 (60)	11 (37)	28 (41)	163 (65)	1.0	**0.005**
Preeclampsia	0	2 (6.7)	4 (5.8)	14 (5.6)	1.0	0.68
N_2O analgesia	4 (80)	20 (67)	43 (62)	170 (68)	1.0	1.0
Cesarian section	0	5 (17)	13 (19)	56 (22)	0.59	0.64
Female	2 (40)	12 (40)	29 (41)	124 (49)	1.0	0.44

Table 2. *Cont.*

	Positive NBS (n = 5)	Clinical Cases with B12 < 160 or holoTC < 35 pmol/L (n = 30)	Clinical Cases (n = 70)	Clinical Controls (n = 252)	Difference Compared to Clinical Controls (Fisher's Exact Test or *t*-Test, *p*)	
					Positive NBS	Clinical Cases with B12 < 160 or holoTC < 35 pmol/L
Multiple birth	0	2 (6.7)	2 (2.9)	29 (12)	1.0	0.55
Preterm GA 32–36 weeks	1 (20)	4 (13)	6 (8.6)	43 (17)	1.0	0.80
Small for GA < 10p	1 (20)	3 (10)	10 (14)	46 (18)	1.0	0.32
Exclusively breastmilk	4 * (80)	23 (79)	49 (72)	82 (33)	**0.047**	**<0.001**
Yearly household income (NOK)	742,800 (375,312)	860,960 (392,077)	894,293 (329,007)	971,884 (341,984)	0.14	0.14
Mother's BMI before pregnancy	22.8 (3.7)	25.1 (6.5)	24.8 (5.5)	24.7 (5.0)	0.39	0.74
Mother's age at birth	26 (3.9)	31 (4.1)	31 (4.3)	30 (4.7)	0.06	0.43
Dose N_2O ** (min × conc)	85 (83)	71 (105)	63 (90)	62 (81)	0.54	0.58
Gestational age in weeks	38.3 (3.7)	39.1 (2.4)	39.3 (2.5)	39.1 (2.2)	0.46	0.99
Birthweight z-score	−0.32 (1.31)	−0.40 (1.06)	−0.28 (1.12)	−0.41 (1.20)	0.86	0.96
Infant age in weeks	14.3 (8.0)	16.7 (11.8)	13.5 (10.7)	20.8 (5.2)	0.007	0.001
Weight z-score	−0.34 (1.25)	−0.51 (1.23)	−0.46 (1.15)	−0.09 (1.06)	0.6	0.06

* The single case not exclusively breastfed had recently introduced porridge in addition to breastmilk. ** Dose of nitrous oxide (N_2O) is the product of concentration of N_2O and the intermittent administration time in minutes. NBS = newborn screening, NOK = Norwegian krone, BMI = Body Mass Index. Significant *p*-values (<0.05) are written in bold.

Table 3. Clinical symptoms and findings of cases and controls, n (%).

	Positive NBS (n = 5)	Clinical Cases with B12 < 160 or holoTC < 35 pmol/L (n = 30)	Clinical Cases (n = 70)	Clinical Controls (n = 252)	Difference Compared to Clinical Controls (Fisher's Exact Test, *p*)	
					Positive NBS	Clinical Cases with B12 < 160 or holoTC < 35 pmol/L
Spells (motor seizures, apneas, or absences)	3/5 (60)	10/23 (43)	29/60 (48)	0/250 (0)	**<0.001**	**<0.001**
Tremor	1/4 (25)	8/22 (36)	20/58 (34)	13/250 (5.2)	0.20	**<0.001**
Irritability	1/4 (25)	4/21 (19)	10/56 (18)	19/252 (7.5)	0.28	0.09
Head lag at pull-to-sit	2/4 (50)	9/18 (50)	23/44 (52)	38/250 (15)	0.12	**0.001**
Abnormal eye contact	2/5 (40)	4/22 (18)	7/54 (13)	0/250 (0)	**<0.001**	**<0.001**

NBS = newborn screening. Significant *p*-values (<0.05) are written in bold.

First-tier pathways identified clinical cases in 19% using the Heidelberg algorithm [5] and 5.7% when incorporating the Austrian algorithm [6]. In a subgroup analysis restricting B12 deficiency to clinical cases with B12 < 160 pmol/L or holoTC < 35 pmol/L the ratio increased to 30% and 10%, respectively, using the above algorithms. For the matched controls, 14% were identified according to the Heidelberg algorithm and 4.5% using the Austrian algorithm, whereas for unmatched controls the corresponding proportions were 20% and 8.8%, respectively. When adding the second-tier analytes, the Heidelberg algorithm identified three clinical cases (4.3%), all three also identified in the subgroup (10%), compared to 0.6% and 0.7% of the matched and unmatched controls, respectively. When tHcy > 6.3 µmol/L was applied as second-tier cutoff-limit, the Austrian algorithm identified two clinical cases (2.9%), both to be found in the subgroup (6.7%), whereas 1.1% and 0.2% of matched and unmatched controls would have been subjected to repeat DBS, respectively. When tHcy > 8.6 µmol/L was attempted as second-tier cutoff, the Austrian algorithm did not identify any of the clinical cases but 0.2% of both matched and unmatched controls (Figure S1, Tables 4 and S1).

Table 4. Results from applying B12 deficiency algorithms according to the Austrian and Heidelberg NBS for cases and controls (n, %).

	Clinical Cases with B12 < 160 pmol/L or holoTC < 35 pmol/L (n = 30)	Clinical Cases (n = 70)	Matched Controls (n = 646)	Un-Matched Controls (n = 434)
Heidelberg 1st tier positive	9 * (30%)	13 * (19%)	93 (14%)	85 (20%)
Heidelberg 1st and 2nd tier positive	3 ** (10%)	3 ** (4.3%)	4 (0.6%)	3 (0.7%)
Austrian 1st tier positive	3 * (10%)	4 * (5.7%)	29 (4.5%)	38 (8.8%)
Austrian 1st tier positive and tHcy > 6.3	2 *** (6.7%)	2 *** (2.9%)	7 (1.1%)	1 (0.2%)
Austrian 1st tier positive and tHcy > 8.6	0	0	1 (0.2%)	1 (0.2%)

* = one in C3 pathway, the rest in MET pathway ** = only MET pathway, *** = one in C3 and MET pathways, respectively.

C3/C2 had the strongest correlation with plasma or serum tHcy at diagnosis of clinical B12 deficiency with r = 0.225 ($p < 0.001$, Table 5) and had the best diagnostic accuracy among the first-tier tests. C3/C2 correlated with NBS second-tier tHcy (r = 0.293, $p < 0.001$). Of the second-tier tests, tHcy had the strongest correlation (r = 0.492, $p < 0.001$) with serum or plasma tHcy at diagnosis of symptomatic B12 deficiency. NBS tHcy had the best diagnostic accuracy among the second-tier tests with AUC = 0.665, followed by MMA with AUC = 0.639. Methionine and methionine/phenylalanine did not correlate with diagnostic markers (Figure 2, Tables 5 and 6).

Table 5. Univariate correlations between tHcy, MMA, B12 at mean (SD) 19 (7.4) weeks of age and NBS parameters at mean (SD) age 62 (15) hours of age (Pearson, n = 316–317).

Newborn Screening Parameter	tHcy µmol/L	MMA µmol/L	S-Vitamin B12 pmol/L
tHcy	0.492 **	0.275 **	−0.208 **
MMA	0.235 **	0.187 **	−0.100
MET	−0.076	0.066	0.022
MET/PHE	−0.029	0.049	−0.044
C3	0.134 *	0.085	−0.117 *
C3/C2	0.225 **	0.165 **	−0.174 **
C3/C16	0.120 *	0.126 **	−0.069
C3/MET	0.178 **	0.065	−0.143 *
C3/C0	0.094	0.001	−0.106

* = correlation significant <0.05, ** <0.001, MET = methionine, PHE = phenylalanine, C0 = carnitine, C2 = acetylcarnitine, C3 = propionylcarnitine, C16 = palmitoylcarnitine, tHcy = total homocysteine, MMA = methylmalonic acid.

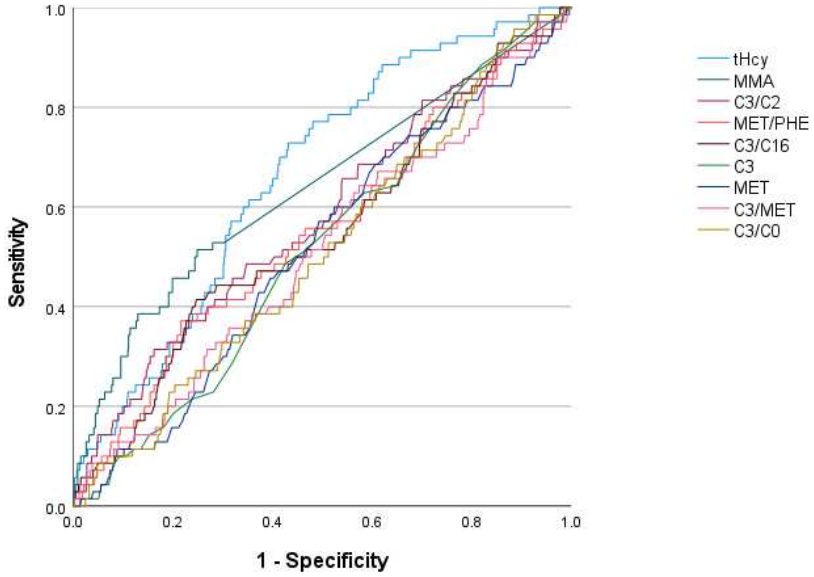

Figure 2. ROC curves of newborn screening parameters' diagnostic accuracy of being a case with B12 deficiency, cases and matched controls, n = 716. MET = methionine, PHE = phenylalanine, C0 = carnitine, C2 = acetylcarnitine, C3 = propionylcarnitine, C16 = palmitoylcarnitine, tHcy = total homocysteine, MMA = methylmalonic acid.

Table 6. ROC areas under the curve, diagnostic accuracy of newborn screening parameters for being a case with B12 deficiency; clinical cases (n = 70), clinical cases with B12 < 160 or holotranscobalamin < 35 pmol/L (n = 30) and matched controls (n = 646).

Newborn Screening Variable	Area under the Curve	
	Clinical Cases	Clinical Cases with B12 < 160 pmol/L or holoTC < 35 pmol/L
tHcy	0.665	0.708
MMA	0.639	0.636
C3/C2	0.579	0.600
MET/PHE	0.550	0.579
C3/C16	0.547	0.525
C3	0.517	0.560
MET	0.515	0.542
C3/MET	0.512	0.544
C3/C0	0.510	0.515

MET = methionine, PHE = phenylalanine, C0 = carnitine, C2 = acetylcarnitine, C3 = propionylcarnitine, C16 = palmitoylcarnitine, tHcy = total homocysteine, MMA = methylmalonic acid.

4. Discussion

Our study showed that NBS markers failed to identify ≥90% infants diagnosed with symptomatic B12 deficiency after the newborn period. Restricting B12 deficiency to clinical cases with B12 < 160 pmol/L or holoTC < 35 pmol/l did not increase the sensitivity of NBS algorithms substantially. We also indirectly showed that N_2O could interfere with the interpretation of second-tier NBS tHcy. It is generally agreed that tHcy is the best functional test for B12 deficiency in this age group, but the specificity is suboptimal as several of the published NBS algorithms contain a second DBS to show the persistence of elevated tHcy before the infant is recalled for confirmatory testing [5,6,20]. We propose N_2O given as birth analgesia is one of the confounding factors that transiently increases tHcy. tHcy returns to the outset when the methionine synthase enzyme activity has been restituted by re-synthesis. This process requires B12, rendering mothers and fetuses with low B12 stores prone to B12 deficiency [1,15]. Our results confirmed the reservation made by Gramer et al. [5] that B12 deficiency presenting after the neonatal period is poorly

detectable at NBS. Thus, our study adds to the discussion of the relevance and feasibility of including B12 deficiency as a primary target in NBS [21].

When authors of published NBS programs have reported high sensitivities and specificities for infant B12 deficiency [5,6], a biochemical definition of B12 deficiency on blood tests drawn at recall at median 4.5 weeks of age have been applied [6] and all cases have been reported to be symptom free [5,6]. Symptomatic infant B12 deficiency has been shown to manifest later than the first month of life [1,22–24] probably because most infants have sufficient B12 stores to remain asymptomatic the first month(s) of life. Further, there is a large discrepancy between the prevalence reported from NBS programs compared to the clinical settings: The birth prevalence of B12 deficiency reported from NBS programs are in the magnitude 0.01–0.09% [6,25]. In the southeastern part of Norway, a retrospective study found that 0.36% of infants under 1 year were diagnosed with B12 deficiency [1], while a Swedish retrospective study estimated an incidence of 0.31% [23]. Moreover, 10% of presumably healthy infants had mild symptoms and biochemical findings suggestive of B12 deficiency in a prospective study [16]. About two thirds of mainly breastfed infants below the age of six months have a biochemical profile indicative of vitamin B12 deficiency, which responds to B12 supplementation [26]. Intervention studies have shown that B12 supplementation to moderately B12 deficiency infants may improve both motor function and regurgitations, which suggests that an adequate B12 status is important for a rapidly developing nervous system [3]. There seems to be a ten times increase in infant B12 deficiency incidence depending on the diagnostic viewpoint: NBS, selective testing, or clinical screening. Theoretically then, our finding of a rather low, $\leq$10% sensitivity for NBS to identify symptomatic B12 deficiency was expected. Other risk factors beyond maternal B12 deficiency may come into play for infants with B12 deficiency during the first year of life. In the present study, we found associations between symptomatic B12 deficiency in infants with B12 < 160 or holoTC < 35 pmol/L and single parenthood, lack of employment, lack of B12 supplementation, known maternal B12 deficiency, self-reported hyperemesis, and exclusive breastfeeding. Of the five infants identified retrospectively with a positive NBS, four were exclusively breastfed, and the fifth infant had only recently been introduced to porridge after exclusive breastfeeding. NBS detects prenatal, maternal B12 deficiency. Breastfeeding is a postnatal risk factor. Infants to B12 deficient mothers are first born with diminished B12 stores and then fed with milk that contains less B12 [27]. Thus, the risk identified with NBS is propagated through exclusive breastfeeding. We have previously shown that formula feeding was protective of infant B12 deficiency [1,15,16], so if the infant is formula fed, this chain of risks is broken, and the predictability of NBS for infant B12 deficiency is lost. This is unique for B12 deficiency NBS. In no other disease screened for is the source feeding the only factor decisive for symptom presentation. Another factor may be maternal use of N_2O during labor, a common form of pain relief. We found that N_2O was provided as an analgesia option at 74% of the hospitals from where the un-matched controls were collected, in a distribution representative for Norway. We have previously shown that N_2O was used by 64–68% of women in labor [1]. In the present study, we showed that tHcy was higher in newborns at hospitals where N_2O was optionable as birth analgesia compared to where N_2O was unavailable. Previously, we found the maternal dose of N_2O to be a significant predictor for tHcy (but not MMA) at NBS. However, at diagnosis of symptomatic infant B12 deficiency, maternal dose of N_2O was associated with both tHcy and MMA. We therefore suggested that N_2O is a risk factor for later presenting symptomatic infant B12 deficiency [1,15,16].

Presenting symptoms in three of the five NBS positive infants were spells of apneas, absences, or seizures, and two of five showed abnormal eye contact. These are potentially life-threatening symptoms that could have been prevented with NBS for B12 deficiency. Half of the cases with B12 < 160 or holoTC < 35 pmol/L had head lag at pull-to-sit and a third had tremor, which were significant findings compared to clinical controls. The yield of NBS was doubled in this subgroup with a stricter B12 definition, although the sensitivity remained $\leq$10%. We speculate in a difference in sensitivity of having symptoms from low

B12 between different genotypes of the B12 dependent enzymes, and then there is a risk that the more sensitive and vulnerable infants will be missed in NBS.

Our study was original in the design of combining clinical cases with symptomatic B12 deficiency with their respective NBS results and re-analysis of DBS. In the unmatched 450 DBS controls, we only had access to whether N_2O was available at the hospital of birth or not; however, data on the individual mothers receiving N_2O or not was not retrieved for this cohort. This information would probably have strengthened the association between mothers N_2O intake and tHcy in DBS as we have shown in a recent study [15]. Maternal B12 parameters were neither available during pregnancy nor at birth, representing a limitation to our study. Additionally, as we previously showed, tHcy increases with storage time of DBS [15], and this introduced a bias to our cohort. This may, theoretically, have overestimated some of the few oldest cases picked up by the Austrian algorithm second-tier tHcy test [6], but it would not change the conclusion of our study.

5. Conclusions

To summarize, NBS showed a low sensitivity for symptomatic B12 deficiency in our cohort of infants presenting beyond the neonatal period. However, NBS may still play an important role in detecting and treating breastfed newborns with B12 deficiency but the shortcomings of NBS in detecting all infants prone to develop B12 deficiency should be acknowledged and sustain awareness of B12 deficiency as a cause of subtle and overt neurological symptoms in infancy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijns8040066/s1, Table S1: Proportions of single metabolites and ratios above cutoff limits, and results from applying algorithms according to the Austrian and Norwegian NBS for cases and controls. Figure S1: Results from applying the Austrian and Heidelberg algorithms for B12 deficiency on 70 clinical cases and on a subgroup of 30 clinical cases with B12 < 160 or holoTC < 35 pmol/L.

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