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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

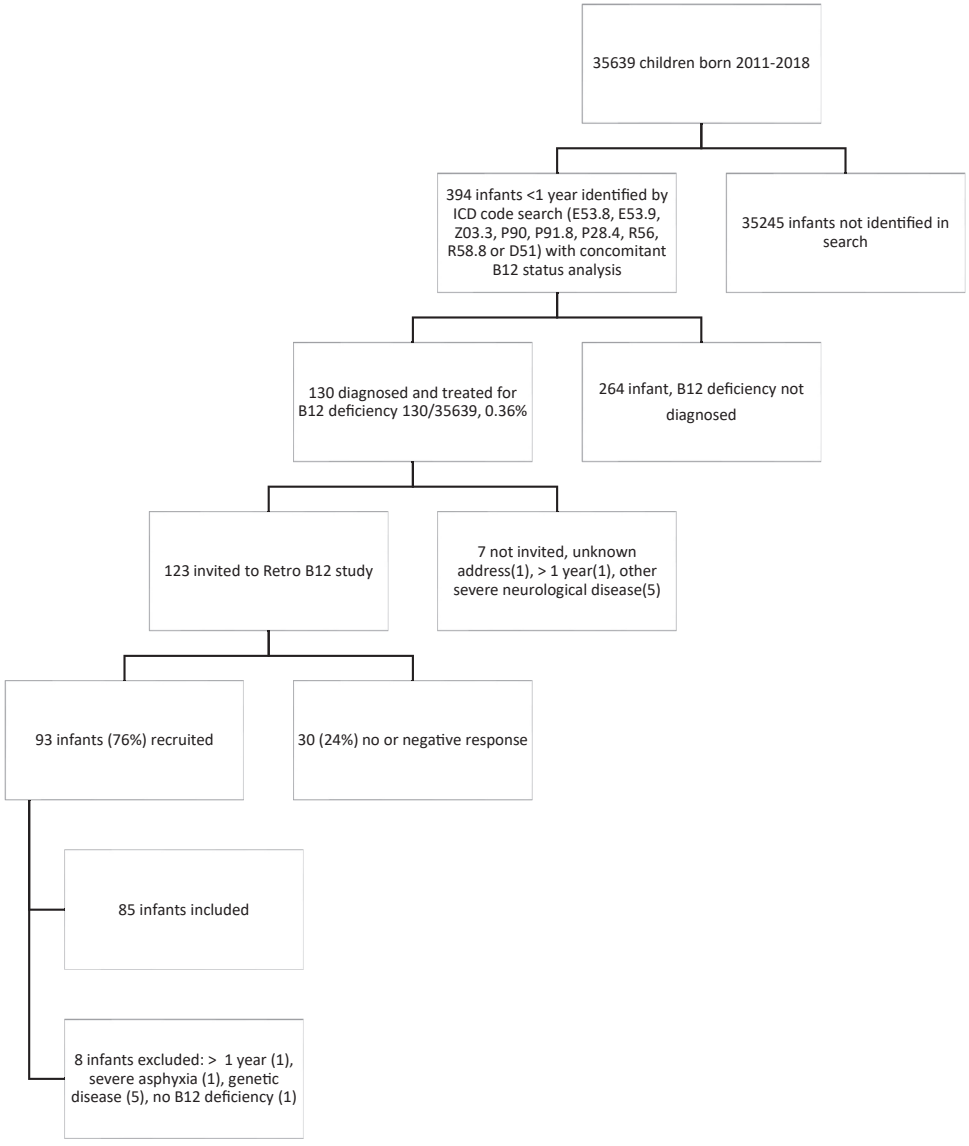

FIGURE 1. Search and selection process for cases.

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.

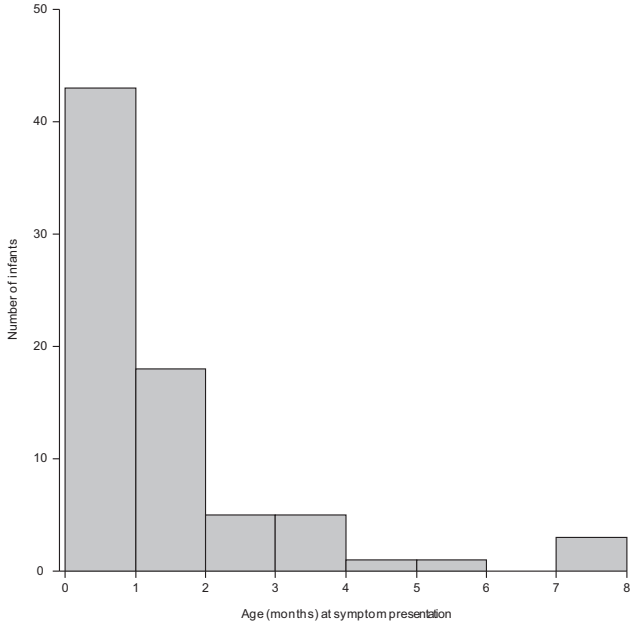

FIGURE 2. Age at symptom presentations (N = 76/85)[1]. [1]Nine infants were excluded because they were tested after NBS recall or because the mother or previous siblings had been diagnosed with B12 deficiency.

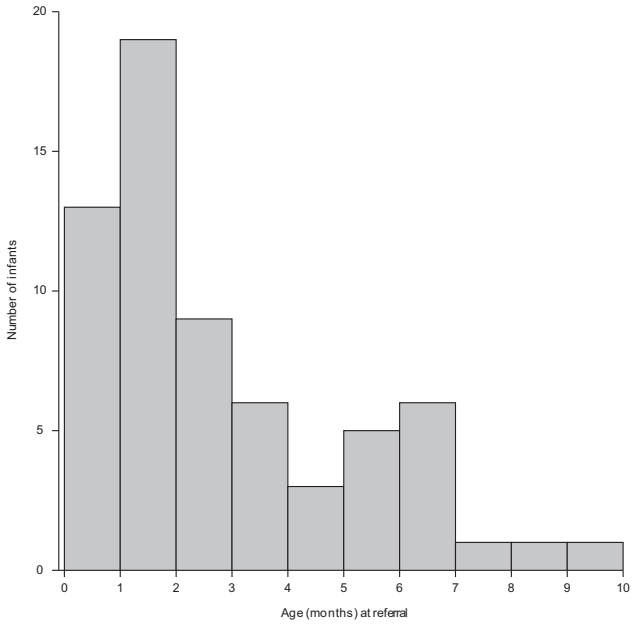

FIGURE 3. Age in months at referral (n = 64/85)[1]. [1]2 infants missing; 9 infants tested after NBS results or family risk, not referred; 10 in-house patients, not referred.

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.

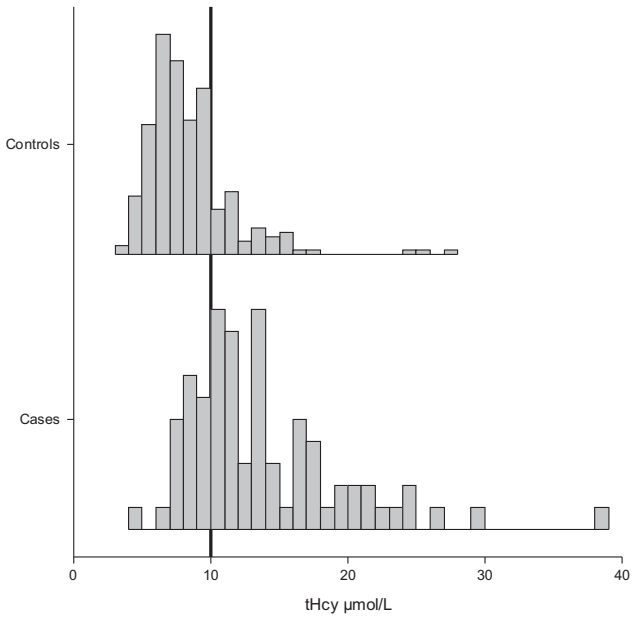

FIGURE 4. Comparison of frequency distribution of tHcy between cases (n = 79) and controls (n = 250). tHcy, total homocysteine.

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.

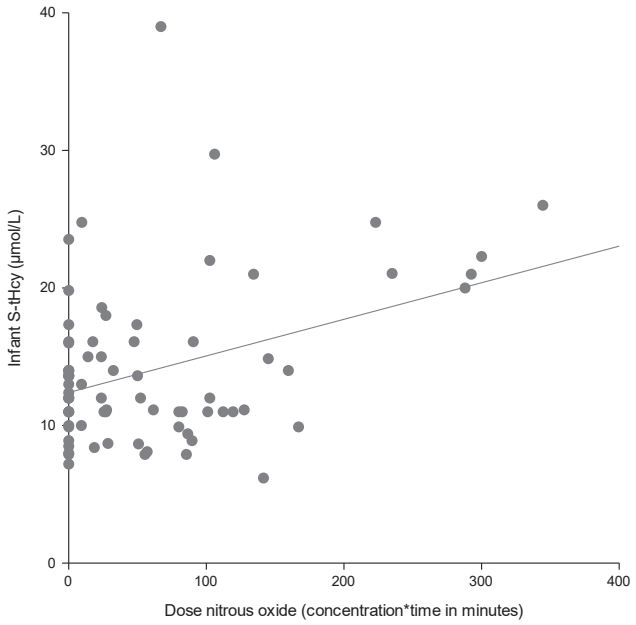

FIGURE 5. Scatterplot of relationship between dose of N_2O to mother during labor and infant tHcy (n = 76) with regression line (r = 0.37). tHcy, total homocysteine.

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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