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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.

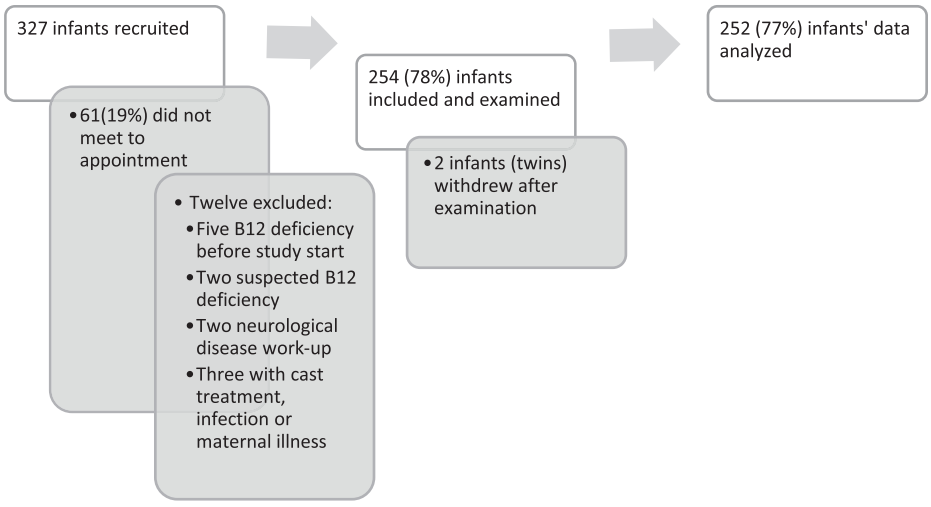

Fig. 1. Online. Flowchart of recruitment, inclusions, and exclusions in the present study.

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

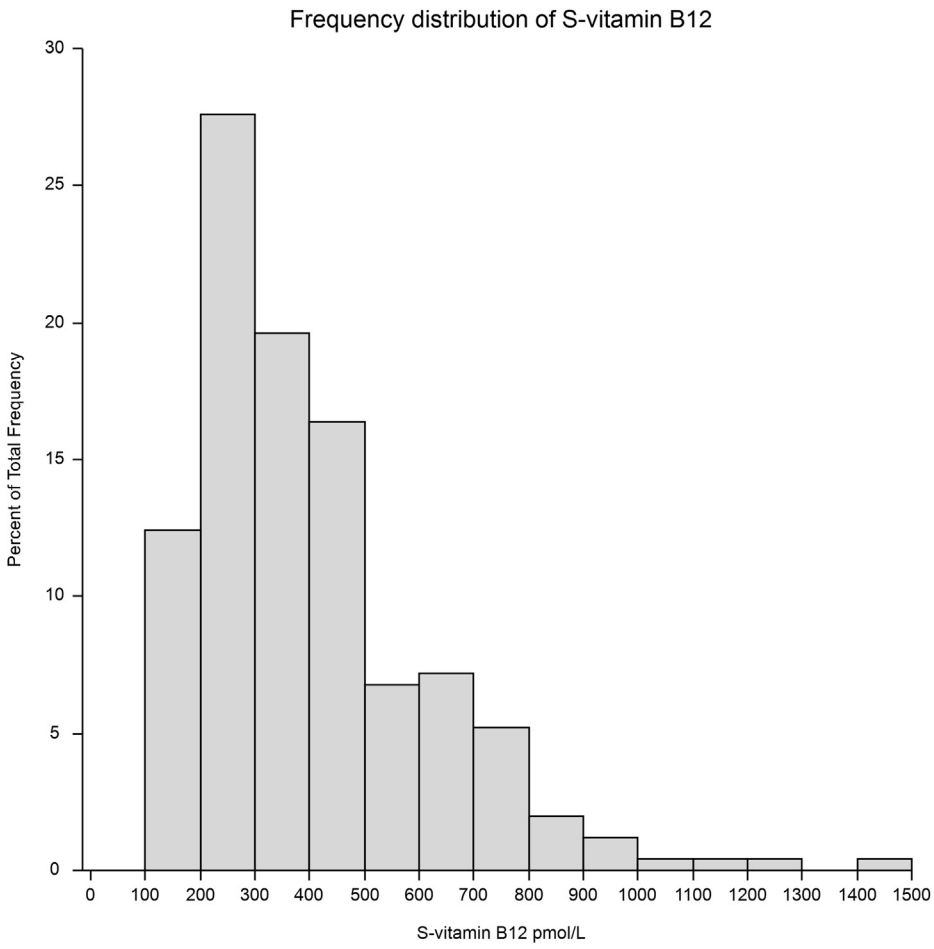

Fig. 2. Online. Frequency distribution of *S*–B12 values for n = 250 infants.

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

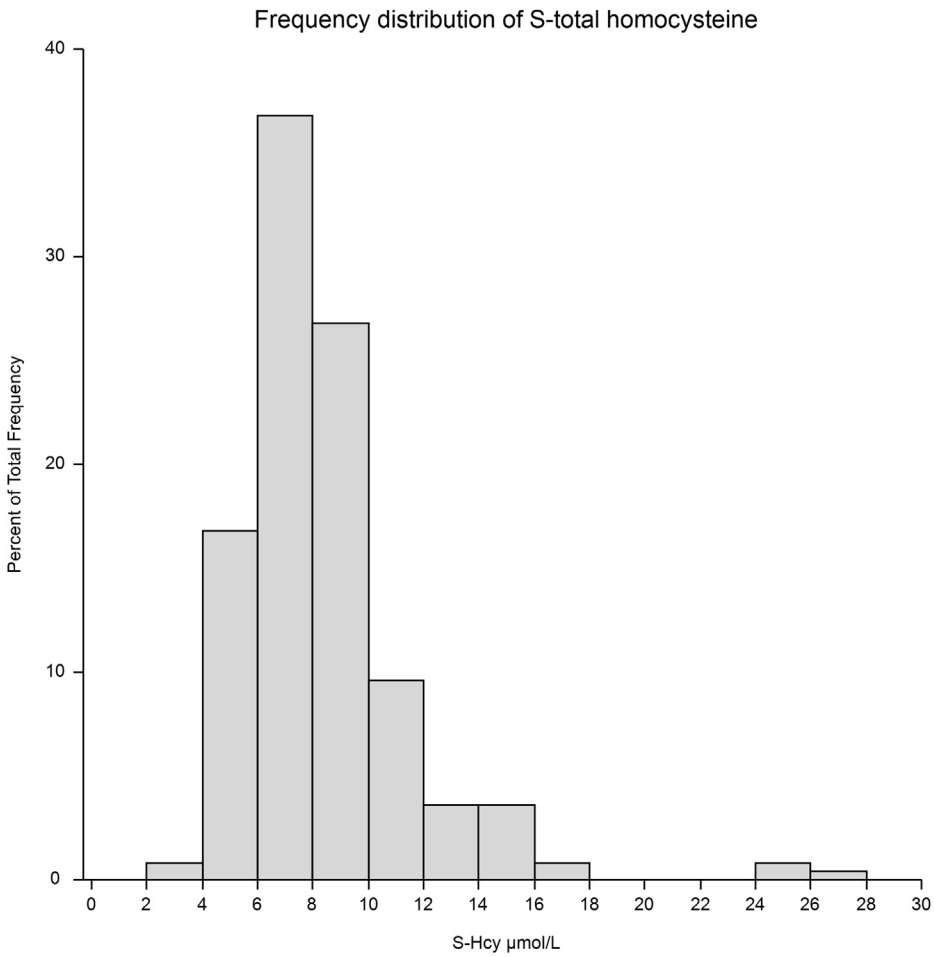

Fig. 3. Online. Frequency distribution of *S*-tHcy for n = 250 infants.

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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