Ulf Wike Ljungblad

Infant Vitamin B12 Deficiency

Vitamin B12, derived from animal food products, is vital for neurological function and infant development. The aim was to explore infant vitamin B12 deficiency in a Norwegian population, including its frequency and clinical relevance, risk factors, presenting symptoms, and whether newborn screening could have detected it.

We performed a retrospective study with 85 infant cases diagnosed with B12 deficiency and one prospective, observational study with 252 healthy infants who were also controls for the cases, in addition to 850 newborn screening dried blood spot controls.

Ten percent of presumed healthy infants had tHcy>8 μmol/l and tremor or excessive sleep, suggesting infant B12 deficiency. B12 deficiency often presented with severe symptoms like spells of apneas and seizures in infant cases. None of their mothers were vegans or vegetarians. The combination of nitrous oxide and exclusive breastfeeding was associated with an earlier presentation of infant B12 deficiency. We suggest nitrous oxide during labor as a novel risk factor for infant B12 deficiency. Newborn screening failed to identify ≥90% of infants diagnosed with symptomatic B12 deficiency after the newborn period.

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Summary of the Thesis

Background: Vitamin B12 is vital for the nervous system, and deficiency in infancy may cause neurological disease and impaired development. Maternal B12 deficiency due to a diet with a low B12 content or pernicious anemia are recognized risk factors for inducing B12 deficiency in breastfed infants. Symptom debut age in the Czech Republic and India has been reported to be 5-6 months, in contrast to 1.7 months in Sweden. The most common presenting symptoms reported from the Czech Republic and India were anemia, failure to thrive, and delayed development, compared to seizures, apneas, and apparent life-threatening events reported from Sweden. The prevalence of biochemical B12 deficiency in newborns is 5-10% in Norway, but the clinical relevance, associated risk factors, and presenting symptoms are less explored. Nitrous oxide is extensively used for analgesia during labor, irreversibly inhibiting methionine synthase, leading to a lack of S-adenosyl-methionine and accumulation of homocysteine. Total homocysteine (tHcy) is the preferred marker of infant B12 status.

Aims: To explore and describe infant vitamin B12 deficiency in a Norwegian population: its frequency and clinical relevance, risk factors, presenting symptoms, and if we could have detected it on newborn screening.

Methods: We performed one retrospective study with 85 infants diagnosed with B12 deficiency (cases) in the first year of life between 2012-2018 and one prospective observational study with 252 healthy infants who also were controls for the infants in the retrospective study. In addition, we included 850 newborn screening (NBS) dried blood spot (DBS) controls. We clinically examined the 252 infants aged 3-7 months with standardized neurological tests before analyzing B12 deficiency markers. We searched medical records, and parents completed questionnaires. We retrieved NBS results and analyzed tHcy and methylmalonic acid (MMA) on stored NBS DBS filtercards from the third day of life. We performed linear and logistic regressions to examine associations and compared groups with the chi-squared test, Mann-Whitney U test, and t-test.

Results: The incidence of infants <1 year treated for B12 deficiency in the catchment area during the study period was 0.36%. Ten percent of infants had tHcy>8 µmol/l associated with tremor and excessive sleep, defined as clinically relevant increased tHcy. Infants who scored below normal on fine motor skills in the Ages and Stages Questionnaire assessment had higher tHcy levels than those who obtained normal scores. Eighty percent of the infants diagnosed with B12 deficiency showed symptoms of B12 deficiency within the first two months of life. 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%). Head lag, tremor, irritability, and abnormal eye contact were other symptoms seen significantly more often in the cases

compared to the controls. Among the 85 cases, the dose of nitrous oxide correlated with infant plasma/serum levels of tHcy and MMA. In the 35 exclusively breastfed infants whose mothers received nitrous oxide analgesia during labor, the symptom presentation age was 1.2 months, whereas in the 39 infants not exclusively breastfed or whose mothers had not received nitrous oxide, the symptom presentation age was 2.0 months. Exclusive breastfeeding and self-reported maternal B12 deficiency were risk factors for infant B12 deficiency, while B12 supplementation during pregnancy was protective. There were no vegans or vegetarians among the case mothers. NBS failed to identify ≥90% of infants diagnosed with symptomatic B12 deficiency after the newborn period. tHcy and MMA were higher on NBS DBS in infants later clinically diagnosed with B12 deficiency than in controls. Multiple regression analysis showed that the dose of nitrous oxide during labor was the strongest predictor for tHcy level in NBS DBS for all infants, with a larger effect in infants later clinically diagnosed with vitamin B12 deficiency than controls. tHcy was higher for the unmatched controls born in hospitals providing nitrous oxide than hospitals without this opportunity.

Conclusions: We have demonstrated associations between increased levels of tHcy and symptoms suggestive of infant B12 deficiency in presumed healthy infants with a suggested prevalence of 10%, a substantial proportion in our highly selected cohort. The nitrous oxide dose during labor was a predictor for both tHcy and MMA at diagnosis for cases with symptomatic B12 deficiency. However, since the nitrous oxide dose was a predictor for tHcy only at the NBS for both cases and controls, we suggest it to be a risk factor for infant B12 deficiency. The combination of exclusive breastfeeding and nitrous oxide during labor was associated with an earlier presentation of infant B12 deficiency. However, a prospective study, including mothers' B12 status, is needed to confirm causality, and a randomized controlled treatment study could confirm the suggested prevalence of mildly symptomatic B12 deficiency among healthy infants. NBS showed low sensitivity for symptomatic B12 deficiency in our cohort of infants presenting beyond the neonatal period, and the shortcomings of NBS in detecting all infants prone to developing B12 deficiency should be acknowledged. However, B12 screening and treatment are not typically included in pregnancy care, making NBS essential for early detection and treatment of B12 deficiency in breastfed newborns. Unnecessary hospital referrals could be mitigated 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 gastrointestinal disease or known B12 deficiency, regardless of the mother`s diet preferences.

Sammendrag (Summery in Norwegian)

Bakgrunn: Vitamin B12 er viktig for nervesystemet, og mangel i spedbarnsalderen kan føre til nevrologisk sykdom og forsinket utvikling. Kjente risikofaktorer for B12-mangel hos spedbarn som ammes er B12-mangel hos mor på grunn av vegansk kosthold og pernisiøs anemi. I Tsjekkia og India er gjennomsnittlig alder for debut av symptomer rapportert å være 5-6 måneder, mens den i Sverige er 1,7 måneder. Vanlige debutsymptomer i Tsjekkia og India er anemi, dårlig vekst og forsinket utvikling, mens i Sverige er det rapportert om krampeanfall, pustestopp og livløshetsanfall. Forekomsten av B12-mangel i blodprøver hos nyfødte er 5-10% i Norge, men vi vet ikke helt hva det betyr klinisk, hvilke risikofaktorer som er involvert, eller hva symptomene er. Lystgass brukes mye som smertestillende under fødsel. Lystgass hemmer enzymet metioninsyntase irreversibelt, noe som fører til mangel på S-adenosyl-metionin og opphopning av homocystein. Totalt homocystein (tHcy) er den foretrukne markøren for B12-status hos spedbarn.

Mål: Å utforske og beskrive vitamin B12-mangel hos spedbarn i en norsk populasjon, inkludert hyppighet og klinisk relevans, risikofaktorer, debutsymptomer og om B12 mangelen kunne ha blitt oppdaget på nyfødtscreening.

Metoder: Vi gjennomførte en retrospektiv studie med 85 spedbarn som ble diagnostisert med B12-mangel løpet av det første leveåret mellom 2012-2018, samt en prospektiv studie med 252 friske spedbarn som også fungerte som kontroller for spedbarna i den retrospektive studien. I tillegg inkluderte vi 850 blodprøvekontroller fra nyfødtscreeningen. Vi gjennomførte kliniske undersøkelser av de 252 spedbarna i alderen 3-7 måneder med standardiserte nevrologiske tester før vi analyserte blodprøver for B12-mangel. Vi gjennomgikk journaler, og foreldrene fylte ut spørreskjemaer. Vi hentet inn resultater fra nyfødtscreeningen og gjorde nye analyser av tHcy og metylmalonsyre (MMA) på nyfødtscreeningprøver fra en biobank, spart fra prøven tatt på tredje levedøgn. Vi gjorde linjere og logistiske regresjoner og jamførte grupper med Chi-kvadrattest, Mann-Whitney U test og t-tester.

Resultater: Forekomsten av spedbarn under 1 år som ble behandlet for B12-mangel i studieområdet i løpet av studieperioden var 0,36%. Ti prosent av spedbarna hadde tHcy over 8 µmol/l assosiert med tremor og økt søvnbehov og som vi definerte som klinisk relevant økt tHcy. Spedbarn som skåret under normal poengsum på finmotorisk utvikling i testen Ages and Stages Questionnaire hadde høyere tHcy enn de som skårte normalt. Åtti prosent av spedbarna som ble diagnostisert med B12-mangel viste symptomer på mangel i løpet av de to første levemånedene. Den vanligste årsaken til henvisning var pustestopp (11/76, 14%), fjernhetsanfall (8/76, 11%) eller krampeanfall (13/76, 17%), som vi samlet benevnte som anfall (28/76, 37%). Symptomer som dårlig hodekontroll, tremor, irritabilitet og avvikende øyekontakt

ble også observert hyppigere hos spedbarna med B12 mangel sammenlignet med kontrollene. Blant de 85 barna med B12 mangel korrelerte mengden lystgass under fødsel med nivåene av homocystein og metylmalonsyre i blodet. Hos de 35 spedbarna som utelukkende blitt ammet og der mødrene hadde fått lystgass under fødselen, var symptomdebuten i gjennomsnitt 1,2 måneder, mens det blant de 39 spedbarna som ikke blitt utelukkende ammet eller der mødrene ikke hadde fått lystgass, var symptomdebuten i gjennomsnitt 2,0 måneder. Utelukkende amming og selvrapportert B12-mangel hos mor var risikofaktorer for B12-mangel hos barnet, mens B12-tilskudd under graviditeten var beskyttende. Ingen av mødrene til barn med B12 mangel var veganereeller vegetarianere. Nyfødtscreening for B12 mangel misset å identifisere ≥90% av spedbarna som senere ble diagnostisert med symptomatisk B12-mangel etter nyfødtperioden. Homocystein og metylmalonsyre var høyere på nyfødtscreeningprøver hos spedbarn som senere ble klinisk diagnostisert med B12-mangel sammenlignet med kontrollene. Mengden lystgass under fødsel var den sterkeste prediktoren for homocystein-nivået på nyfødtscreeningprøven for alle spedbarn, med større effekt hos spedbarn som senere ble klinisk diagnostisert med vitamin B12-mangel enn hos kontrollene. Homocystein var høyere på nyfødtscreeningprøver fra barn født på sykehus som tilbød lystgass enn på sykehus som ikke gjorde det.

Konklusjoner: Vi har påvist sammenhenger mellom økte nivåer av homocystein og symptomer som indikerer B12-mangel hos tilsynelatende friske spedbarn, med en prevalens på hele 10% i vår selekterte kohort. Mengden lystgass under fødsel var en prediktor for både homocystein og metylmalonsyre ved diagnose av symptomatisk B12 mangel. Dog predikerte mengden lystgass ved fødselen kun homocystein på nyfødtscreeningen, og vi foreslår derfor at lystgass er en risikofaktor for B12-mangel hos spedbarn. Kombinasjonen av fullamming og lystgass under fødsel var knyttet til tidligere debut av B12-mangel hos spedbarn. Imidlertid er det behov for en studie der mødrenes B12-status også tas med, for å bekrefte årsakssammenhenger, og en randomisert kontrollert behandlingsstudie som bekrefter den foreslåtte hyppige forekomsten av mild symptomatisk B12 mangel blant friske spedbarn. Nyfødtscreening viste lav følsomhet for symptomatisk B12-mangel i vår kohort av spedbarn som debuterte med B12 mangel etter nyfødtperioden, og disse begrensningene ved nyfødtscreening for B12 mangel bør det tas høyde for. Likevel kan nyfødtscreening fortsatt være viktig for å oppdage og derved tidlig kunne behandle nyfødte som fullammes og har B12-mangel, spesielt når B12-screening og behandling ikke er en del av svangerskapsomsorgen. Unødvendige sykehusinnleggelser kan muligens unnvikes ved å øke kunnskapen om B12 mangel hos spedbarn, slik at behandlere kan undersøke B12-status på spedbarn som fullammes og med nevrologiske symptomer, spesielt hvis moren har mage-tarm-sykdom eller kjent B12-mangel.

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Tønsberg, November 6, 2023

Ulf Wike Ljungblad

Scientific Environment

The Ph.D. candidate has conducted his research at Vestfold Hospital Trust and Oslo University Hospital. At both Vestfold Hospital Trust and Oslo University Hospital, he has been a part of the Pediatric Research Group, the latter also endorsed by the Institute of Clinical Medicine, Medical Faculty, University of Oslo, where he has conducted his Ph.D. studies.

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List of Papers

Paper I

Ljungblad UW, Paulsen H, Mørkrid L, Pettersen R, Hager HB, Lindberg M, Astrup H, Eklund EA, Bjørke-Monsen AL, Rootwelt T, Tangeraas T

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

Eur. J. Paediatr. Neurol. 2021, 35, 137–146. Doi: 10.1016/j.ejpn.2021.10.008

Paper II

Ljungblad UW, Astrup H, Mørkrid L, Hager HB, Lindberg M, Eklund EA, Bjørke-Monsen AL, Rootwelt T, Tangeraas T

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

Pediatr Neurol. 2022. 131, 4-12. Doi: 10.1016/j.pediatrneurol.2022.03.003

Paper III

Ljungblad UW, Lindberg M, Eklund EA, Sæves I, Bjørke-Monsen AL, Tangeraas T

Nitrous Oxide in Labour Predicted Newborn Screening Total Homocysteine and Is a Potential Risk Factor for Infant Vitamin B12 Deficiency

Acta Paediatr. 2022, 111,2315–2321. Doi: 10.1111/apa.16530

Paper IV

Ljungblad UW, Lindberg M, Eklund EA, Sæves, I, Sagredo, C, Bjørke-Monsen AL, Tangeraas T

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

Int. J. Neonatal Screen. 2022, 8, 66. Doi: 10.3390/IJNS8040066/S1

Paper I	*"The prevalence and clinical relevance of hyperhomocysteinemia suggesting vitamin B12 deficiency in presumed healthy infants"*. Participants: 252 presumed healthy infants 3- 7 months old.
Aims	To investigate prevalence/relevance, symptoms/findings, and risk factors for suboptimal B12 status.
Methods	A prospective observational study with standardized neurological and developmental tests to evaluate clinics before biochemical tests for B12 status. T-test, Mann Whitney U, Chi-squared, Fisher's exact, linear, and logistic regressions.
Results	Total homocysteine above 8 µmol/L was associated with tremor, excessive sleep, and subnormal fine motor scores, and 10% had symptoms and findings suggesting B12 deficiency.
Conclusions	We demonstrated clinically relevant total homocysteine above 8 µmol/L, suggestive of B12 deficiency, in 10% of presumed healthy infants.
Paper II	*"Breastfed Infants with Spells, Tremor, or Irritability: Rule Out Vitamin B12 Deficiency".* Participants: 85 infants <1 year treated for B12 deficiency, controls same cohort as Paper I.
Aims	To describe presenting symptoms and biochemical profiles and to identify risk factors in infants diagnosed with B12 deficiency.
Methods	A case-control study with data collected from files, newborn screening results, and homocysteine and methylmalonic acid analyses on stored filtercards. T-test, Mann Whitney U, related samples Wilcoxon signed-rank test, Chi-square, Fisher's exact, Pearson's correlation, linear and logistic regressions.
Results	80% presented within the first two months of life, with spells (37%) of apneas, motor seizures, or absences. Tremor (29%) and irritability (18%) were the most common findings at the first examination. None of the mothers were vegetarians. The dose of nitrous oxide given during labor was associated with the infant's total homocysteine level at diagnosis for cases but not for controls.
Conclusions	Spells, tremor, and irritability were common findings in early infant vitamin B12 deficiency. Nitrous oxide given during labor is proposed as a contributing risk factor to developing early infant vitamin B12 deficiency.
Paper III	*"Nitrous Oxide in Labour Predicted Newborn Screening Total Homocysteine and Is a Potential Risk Factor for Infant Vitamin B12 Deficiency".* Participants: The same cohorts from Paper I and II and 400 filtercard controls.
Aims	To assess predictors and frequency distribution of total homocysteine and methylmalonic acid analyzed in newborn screening (NBS) dried blood spots (DBS) from infants with clinically diagnosed B12 deficiency.
Methods	A case-control study with data collected from files, newborn screening results, and homocysteine and methylmalonic acid analyses on stored NBS DBS. Mann Whitney U, Chi-square, Fisher's exact, linear regressions.
Results	Both total homocysteine and methylmalonic acid were higher on stored NBS DBS in infants later clinically diagnosed with vitamin B12 deficiency than controls. The dose of nitrous oxide during labor was the strongest predictor for total homocysteine level for all infants, with a larger effect in infants later clinically diagnosed with vitamin B12 deficiency than controls.
Conclusions	Nitrous oxide dose during labor predicted 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 developing vitamin B12 deficiency.
Paper IV	*"Retrospective Evaluation of the Predictive Value of Newborn Screening for Vitamin B12 Deficiency in Symptomatic Infants Below 1 Year of Age*". Participants: 70 infants diagnosed with symptomatic B12 deficiency (from Paper II) compared to 646 matched and 434 unmatched DBS controls to evaluate the Austrian and Heidelberg B12 NBS algorithms.
Aims	To evaluate the predictive value using NBS algorithms in detecting infants that later were clinically diagnosed with symptomatic B12 deficiency. To investigate whether being born in a hospital using nitrous oxide as pain relief in labor may have impacted total homocysteine at NBS.
Methods	A case-control study with retrospectively retrieved NBS data and analyses of total homocysteine, methylmalonic acid, and methyl citrate on stored NBS 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 the median age of 10.9 weeks was ≤10%. Total homocysteine was higher in DBS for the unmatched controls born in hospitals providing nitrous oxide than in other hospitals.
Conclusions	NBS algorithms could not identify most infants diagnosed with symptomatic B12 deficiency after the neonatal period. Being born in hospitals providing nitrous oxide may impact total homocysteine at NBS.

Abbreviations

AIMS	Alberta Infant Motor Scales
ALTE	Apparent Life-Threatening Event
ASQ-2	Ages and Stages Questionnaire Second Version
B12	Vitamin B12
DBS	Dried Blood Spot
GMA	General Movement Assessment
HINE	Hammersmith Infant Neurological Examination
MMA	Methylmalonic Acid
MS	Methionine Synthase
MUT	Methylmalonyl-CoA Mutase
NBS	Newborn Screening
SGA	Small for Gestational Age
SIDS	Sudden Infant Death Syndrome
tHcy	Total Homocysteine
TIMP	Test of Infant Motor Performance

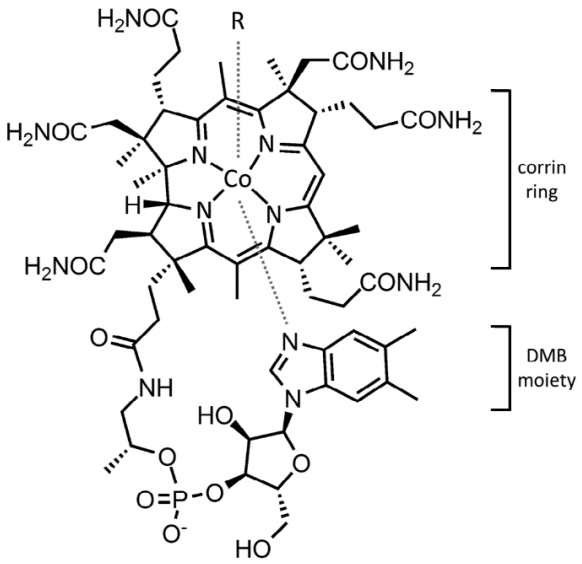
1 Introduction

1.1 Vitamin B12

Vitamin B12 (cobalamin) was first described structurally after crystallography studies by Dorothy Hodgkin in 1956. Still, in 1843, Thomas Addison described pernicious anemia, in which uptake of vitamin B12 is reduced due to autoimmune inflammation, leading to B12 deficiency over time, and in 1926, Minot, Murphy, and Whipple reported that patients with pernicious anemia were cured by eating raw liver (Green & Miller, 2022; Smith et al., 2018). Infant vitamin B12 deficiency was first reported in India in 1956 (Jadhav et al.,1962).

Figure 1

Chemical structure of vitamin B12. Dashed lines = nonessential bonds, R = various upper axial ligands (see text), and DMB = dimethyl benzimidazole. Reprinted with Creative Commons Attribution License Froese et al. Journal of Inherited Metabolic Disease 2019, 42(4), 673–685. Doi:10.1002/jimd.12009

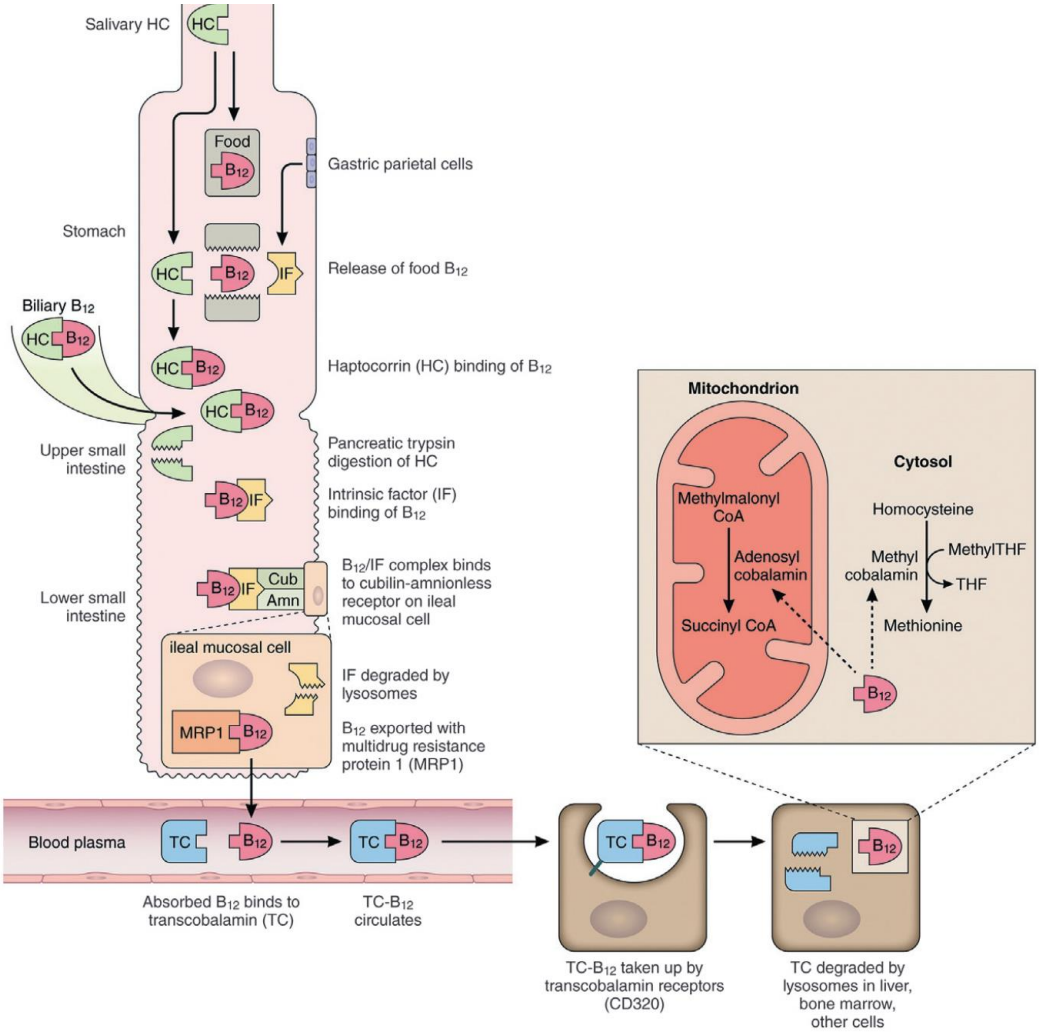


B12 is a chemically complex molecule (Figure 1) that humans do not synthesize. Two human enzymes, the mitochondrial methylmalonyl-CoA mutase (MUT) and the cytosolic methionine synthase (MS), require B12 in the cofactor forms adenosylcobalamin and methylcobalamin, respectively. A complete B12 synthesis only occurs in some Eubacteria and Archaea. B12 has a central cobalt atom that can exist in three different oxidation states. Cob(I)alamin, the most reduced form, can form four bonds; cob(II)alamin can form five bonds; and the most oxidized form, cob(III)alamin, can form six bonds. In the required structure of the B12 molecule, a minimum of four nitrogen atoms from the corrin ring must bind to the cobalt atom. A dimethyl benzimidazole (DMB) moiety is a lower axial ligand to the corrin ring that can bind back to the cobalt atom (Figure 1), a state called "base-on," opposed to "base-off" when it is not bound. The cobalt atom may bind an upper ligand called the R-group. Different cobalamin cofactor roles require different R-groups. In humans, the R-group may comprise an adenosyl group (adenosylcobalamin), a methyl group (methylcobalamin), in addition to hydroxo- (hydroxocobalamin), gluthathionyl- (glutathionylcobalamin) or a cyano-group (cyanocobalamin) (Froese et al., 2019).

Proteolysis in the stomach releases B12, which is then bound by the protein haptocorrin, again degraded in the duodenum, where intrinsic factor (IF) binds B12. Parietal cells in the stomach produce IF. After binding to B12, IF protects vitamin B12 until the IF-B12 complex binds to the heterodimer amnionless and cubulin (cubam) receptor in the distal ileum (Froese et al., 2019).

Figure 2

Overview of B12 absorption, transport, and coenzyme functions. Reprinted with permission. This article was published in Blood, 129(19), Green, R, Vitamin B12 deficiency from the perspective of a practicing hematologist, 2603-2611, copyright Elsevier (2017).



B12 is bound to transcobalamin (holotranscobalamin, holoTC) when exported from ileal cells into the portal circulation. Many cells in the body express the receptor CD320 that binds

holoTC. The half-life of holoTC is 90-120 min; thus, the holoTC level describes how much B12 is recently absorbed. Haptocorrin also transports B12 in the blood and has a half-life of 10 days, which is considerably longer than holoTC. In contrast to holoTC, only the liver takes haptocorrin up by unspecific asialoglycoprotein receptors on the liver cells. Total B12 includes both haptocorrin and holoTC. HoloTC comprises 20-30% of the total B12 (Green & Miller, 2022). B12 is further transported and processed by different intracellular mechanisms beyond the scope of this thesis, ultimately forming the cofactor forms of adenosyl- and methylcobalamin (Froese et al., 2019)

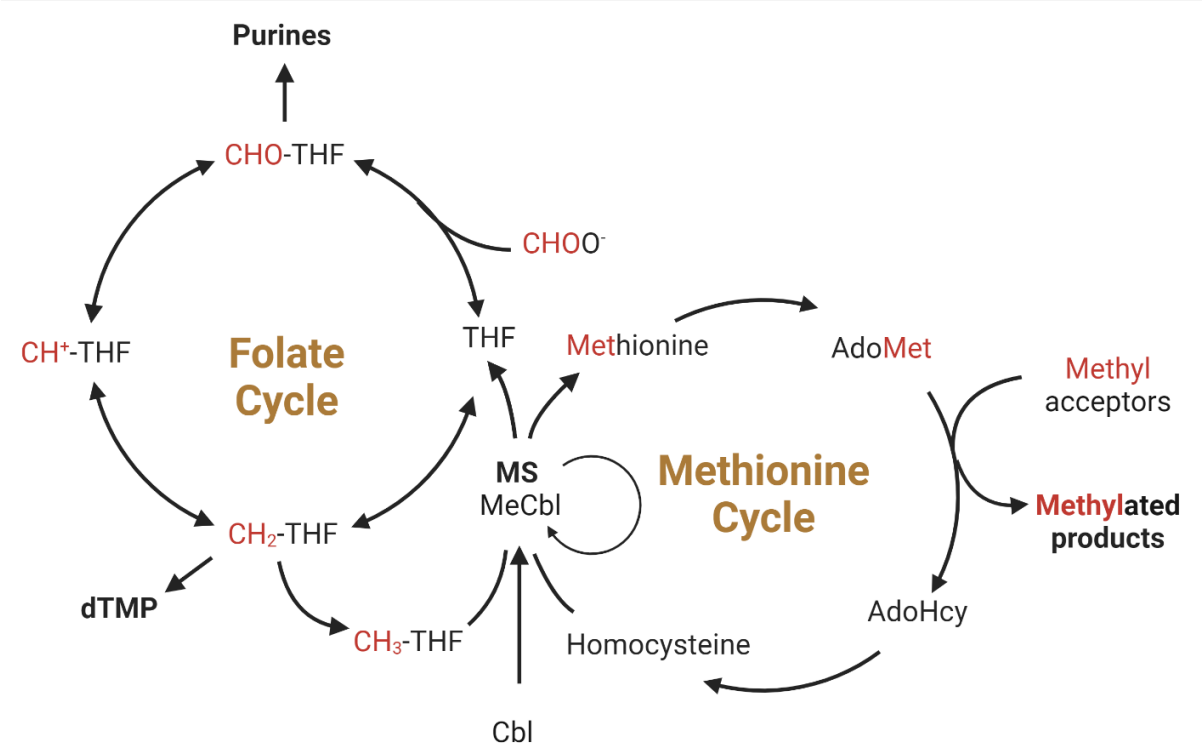
After delivery of B12 to MS, B12 is reduced to cob(I)alamin, the most reactive form, and primed to bind a methyl group provided from 5-methyltetrahydrofolate. This methyl group is transferred to Hcy using methylcobalamin as the methyl donor, yielding methionine. With inhibited MS activity, the level of Hcy increases, and methionine decreases. During this MS-catalyzed remethylation reaction, the cob(I)alamin is oxidized every 1:200-1000 turnovers to inactive cob(II)alamin, which needs to be reduced back to cob(I)alamin, catalyzed by MS reductase (MSR). Further, the remethylation of homocysteine is intimately intertwined with the folate cycle and regenerates tetrahydrofolate from 5-methyltetrahydrofolate (Figure 3) (Froese et al., 2019; Green & Miller, 2022).

Methionine can form *S*-adenosylmethionine (AdoMet) catalyzed by methionine adenosyltransferase. AdoMet is the principal methyl donor in humans and is a substrate for many methyltransferases, making up approximately 1% of human genes (Froese et al., 2019). These enzymes are required in methylation reactions involving DNA, RNA, histones, proteins, neurotransmitters, and guanidinoacetate (Froese et al., 2019; Hannibal & Jacobsen, 2022). In the liver and kidney, AdoMet is required to synthesize creatine from guanidinoacetate and phosphatidylcholine from phosphatidyl ethanolamine, which may account for 80% of all methylation reactions in the human body. After delivering the methyl group from AdoMet, adenosylhomocysteine (AdoHcy) is formed. AdoHcy is cleaved by AdoHcy hydrolase (AHCY), rendering adenosine and Hcy. Hcy can again be approached by MS, completing the methionine cycle (Figure 3), or enter the transsulfuration pathway by combining it with serine to form cystathionine catalyzed by cystathionine beta-synthase (Froese et al., 2019).

The mitochondrial methylmalonyl-CoA mutase (MUT) uses adenosylcobalamin as a cofactor, and the dysfunction of MUT or lack of B12 leads to a build-up of precursors such as methylmalonic acid (MMA) and causes downstream impaired anapleurosis of the citric cycle and mitochondrial energy production (Sobczyńska-Malefora et al., 2021).

Figure 3

An overview of the remethylation pathway showing the methionine- and folate cycles. Red represents the single carbon groups originating from formate and ending in methylated products. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; Cbl, cobalamin (B12); CH+-THF, 5,10-methenyltetrahydrofolate, CH2-THF, methylenetetrahydrofolate; CH3-THF, methyltetrahydrofolate; CHO-THF, 10-formyltetrahydrofolate; CHOO-, formate, dTMP, deoxythymidine monophosphate; MeCbl, methylcobalamin, MS, methionine synthase. Adapted from Froese et al. Journal of Inherited Metabolic Disease 2019, 42(4), 673–685. Doi: 10.1002/jimd.12009. Created with BioRender.com.



1.2 Homocysteine

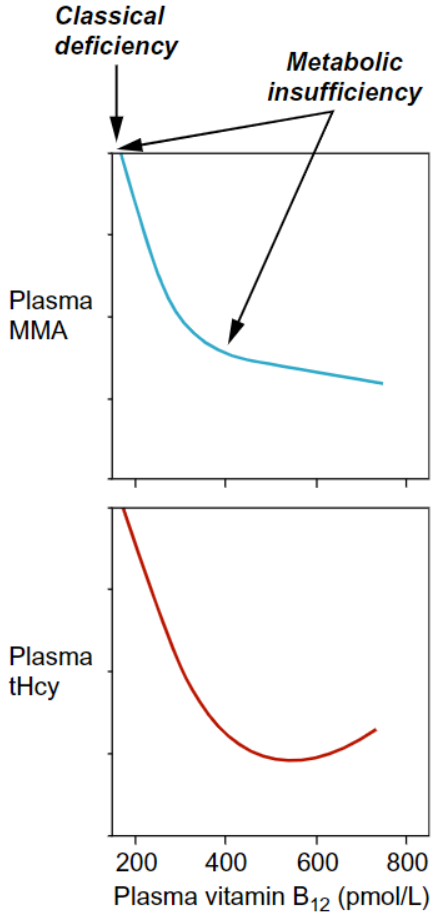
Mildly or moderately elevated tHcy levels are seen in B12 deficiency, folate, pyridoxine deficiency, and renal insufficiency (Green & Miller, 2022). tHcy rises before B12 decreases to subnormal levels (Figure 4)(Smith et al., 2018). Rare metabolic diseases (inborn errors of remethylation) usually cause more pronounced increased levels of tHcy. In some countries or regions, newborn screening programs have included remethylation disorders and B12 deficiency. Low methionine is the primary marker, followed by homocysteine as a second-tier analyte (Gramer et al., 2020; Rozmarič et al., 2020). B12-optimized plasma-tHcy is <6.5 µmol/L at four months of age (Bjorke-Monsen et al., 2008).

1.3 Methylmalonic Acid

The B12-dependent enzyme MUT isomerizes methylmalonyl CoA to succinyl-CoA. Elevated levels of MMA may indicate tissue B12 deficiency. MMA rises before B12 decreases to subnormal levels (Figure 4)(Smith et al., 2018). A common single nucleotide polymorphism in the gene for 3-hydroxyisobutyryl-CoA-hydrolase (HIBCH) results in an increase of MMA not related to vitamin B12 deficiency (Green & Miller, 2022). The MMA levels in breastfed infants are relatively high compared to the B12 level in older children and adults. The cause is unknown but may be due to intestinal absorption of propionate or the metabolism of odd-chain fatty acids from breast milk (Green et al., 2017). Renal insufficiency may also result in an increased level of MMA (Green & Miller, 2022). In some countries, NBS laboratories analyze MMA as a second-tier metabolite for methylmalonic aciduria, combined remethylation disorders, and vitamin B12 deficiency (Gramer et al., 2020).

Figure 4

Defining B12 inadequacy using markers of metabolic insufficiency. Reprinted with permission from Smith, A. D., Warren, M. J., & Refsum, H. (2018). Vitamin B12. In Advances in Food and Nutrition Research (Vol. 83, pp. 215–279). National Library of Medicine (US). Copyright Elsevier. Doi: 10.1016/bs.afnr.2017.11.005; and Smith, A. D., & Refsum, H. (2012). Do we need to reconsider the desirable blood level of vitamin B12? Journal of Internal Medicine, 271(2), 179–182. Copyright Wiley. Doi: 10.1111/j.1365-2796.2011.02485.x



1.4 Vitamin B12 Deficiency

B12 deficiency has many causes. An adequate amount of B12 is only found in food of animal origin, mainly meat, fish, egg, and dairy products. Consequently, vegetarianism and low intake of animal products due to social or economic reasons are essential causes of B12 deficiency (Zengin et al., 2009). Impaired vitamin B12 absorption is the most crucial cause of deficiency due to disease processes (Honzik et al., 2010). In pernicious anemia, an autoimmune process damages the parietal cells in the ventricle. Therefore, they cannot produce the intrinsic factor needed to absorb B12 in the ileum. Gastric reduction surgery to treat obesity or ileal resection will have the same effect and lead to B12 deficiency if not supplemented. The use of gastric acid-lowering drugs like proton pump inhibitors and H2-antagonists, as well as metformin, may impair the absorption of B12. Rare congenital metabolic disorders of B12 metabolism may impede the delivery of B12 to methionine synthase and methylmalonyl CoA-mutase (Green & Miller, 2022). The most common cause of infant B12 deficiency is maternal B12 deficiency, and most initial data on clinical outcome came from case studies of exclusively breastfed infants of B12 deficient mothers on vegan, vegetarian, or lacto-ovo vegetarian diets (Black, 2008; Smith et al., 2018). Even short-term maternal dietary restrictions during pregnancy and lactation can result in poor infant B12 status because the adequacy of maternal intake and absorption of the vitamin has a more substantial influence on infant status than maternal stores (Dror & Allen, 2008; Michael Whitehead, 2006). The infant B12 status declines during the first months of life, with a nadir at 4-6 months in exclusively breastfed infants. Breast milk B12 levels correlate well with maternal B12 levels varying between 150 and 700 pmol/L (Green et al., 2017). There is a significant fall in breast milk B12 levels between 6-12 and 19-25 weeks postpartum (Ford et al., 1996). Infant formulas contain 800-1200 pmol B12 per liter, and formula-fed infants have better B12 status than breastfed infants (Green et al., 2017). Maternal B12 deficiency and breastfeeding are the critical causes of infant B12 deficiency (Honzik et al., 2010).

1.5 Symptoms and Findings in B12 Deficient Infants

Research groups from around the world have reported a wide range of symptoms and findings to be associated with infant B12 status: Hypotonia, insufficient head control, hypokinesia, lethargy, ataxia, twitching and tremor, involuntary movements, irritability, seizures, apparent life-threatening events (ALTE), apneas, nystagmus, swallowing dysfunction, regurgitations, food refusal, failure-to-thrive, abnormal pigmentation, pancytopenia, diarrhea, constipation, brain atrophy and white matter disease (Akcaboy et al., 2015; Bjørke-Monsen & Ueland, 2011; Black, 2008; Goraya et al., 2015; Green et al., 2017; Honzik et al., 2010; Irevall et al., 2016; Kaul et al., 1963; Smith et al., 2018). The most common symptoms of infant B12 deficiency in studies from the Czech Republic (Honzik et al., 2010) and India (Azad et al., 2020) were failure to thrive, anemia, and delayed development, with a 4–6-month debut. The reported presenting symptoms from a Swedish study were seizures, apneas, and ALTE starting at an average age of 1.7 months (Irevall et al., 2016). Long-term consequences of severe infant B12 deficiency may lead to stunting, impairment of social and visuospatial perception, attention, short-term memory, and academic performance, as well as lower mental development scores in childhood (Black, 2008; Green et al., 2017; Smith et al., 2018).

1.6 Developmental and Neurocognitive Outcome after Infant B12 Deficiency

Severe, long-lasting infant B12 deficiency may result in neurological sequelae even after treatment. Australian pediatricians reported six cases of infant B12 deficiency, all exclusively breastfed with mothers who were either vegetarian or diagnosed with pernicious anemia. Their age of symptom onset was four months in two infants and 8-10 months in the other four infants. The age of diagnosis was between 8 and 15 months. They all had a prompt response to B12 with diminishing neurological symptoms. Psychometric testing showed that two children had borderline intellectual disability at 4 to 5 years, two had normal test results at 9 to 11, and two were lost to follow-up (Graham et al., 1992). Czech pediatricians reported 40 cases of breastfed infants with B12 deficiency, all responding quickly to B12 supplementation. However, seven children had delayed speech development, learning, or behavioral problems, and two

infants developed infantile spasms and developmental delay (Honzik et al., 2010). Our group has also reported on two infants aged eight and nine months referred for developmental delay starting at four months, diagnosed with severe B12 deficiency and supplemented where the outcome was a developmental delay in one child and attention deficit disorder for the other (Tangeraas et al., 2023).

Infants with mild to moderate B12 deficiency may have suboptimal motor development compared to infants with better B12 status, and they may also benefit from supplementation, at least concerning short-term gross motor development. In Norway, six-month-old infants with a birthweight between 2000-3000 grams, exclusively breastfed for over one month, and with plasma tHcy >6.5 µmol/L had poorer short-term gross motor development than formula-fed infants. A single injection of 0.4 mg hydroxocobalamin in breastfed infants improved short-term gross motor function (Torsvik et al., 2015). In a group of 79 infants with a mean age of four months, with feeding difficulties and plasma tHcy >6.5 µmol/L, an injection of 0.4 mg hydroxocobalamin resulted in a better short-term gross development compared to infants that received a sham injection after randomization (Torsvik et al., 2013).

1.7 Associations between B12 Status and Developmental and Neurocognitive Outcome

Researchers have studied associations between B12 status and developmental and neurocognitive outcomes in India and Nepal with ambiguous results. In an observational study in India, researchers found a correlation between maternal B12 and folate during pregnancy and mental and social infant development at two years (Bhate et al., 2012). Furthermore, there is a linear, positive association between B12 status and cognitive performance, also through the assumed normal range of B12 status markers, in infants 12-18 months old in India (Strand et al., 2013). In a randomized controlled trial, infants in North India had better development (Kvestad et al., 2015) evaluated with ASQ when supplemented with low-dose peroral B12 and folate for six months. The effect was most significant in infants with tHcy >10 µmol/L, who were stunted or younger than 24 months at the end of the study. In a prospective, observational study in Nepal, infant B12 status was positively associated with cognitive performance at five years of

age (Kvestad et al., 2017). On the contrary, in a randomized controlled clinical trial with 183 women in each group, 50 µg vitamin B12 per day during pregnancy did not affect the neurocognitive outcome in 178 infants at nine months assessed with Bayley Scales of Infant Development (Srinivasan et al., 2017), nor neurophysiological outcome using event-related potentials at six years (Srinivasan et al., 2020). In a prospective, randomized intervention study in Nepal where 6-11 months old infants with a risk of vitamin B12 deficiency were given two µg vitamin B12 per day in a multivitamin preparation or non-vitamin B12 containing multivitamin, no differences in neurodevelopment could be measured after one year of B12 supplementation (Strand et al., 2020) or two years after end of the one-year supplementation (Ulak et al., 2022).

1.8 Nitrous Oxide

Anesthesiologists have been using nitrous oxide for over 150 years. Supraspinal activation of opioidergic and noradrenergic neurons induces the analgesic effect. Opioid release in the brainstem removes the inhibitory tone in descending noradrenergic inhibitory pathways. These inhibitory pathways give analgesia via adrenoreceptors in the spinal cord's dorsal horns (Sanders et al., 2008). During labor, nitrous oxide is a widely used option for managing pain (Collins, 2017; Likis et al., 2014). The first clinical report of toxicity of nitrous oxide was published in 1956 when 50% nitrous oxide in oxygen was used to control the spasms in patients with tetanus and pancytopenia, and megaloblastic hematopoiesis was observed (Lassen, 1956). In 1968, it was suggested that nitrous oxide might oxidize cobalamin required by MS (Amess et al., 1978), confirmed the same year (Deacon et al., 1978). Chanarin described nitrous oxide to irreversibly oxidize methyl-cobalamin from its 1+ to its 3+ state (Chanarin, 1980). The irreversibility may not be due to the oxidation itself but from forming a rogue hydroxyl radical at the active site and, thereby, irreversibly inactivating MS (Banerjee & Matthews, 1990; Frasca et al., 1986). High levels of folate enhance the catalytic activity of MS and favor the inactivation process, whereas high methionine levels protect the enzyme (Banerjee & Matthews, 1990; Christensen et al., 1992; Christensen & Ueland, 1993; Guttormsen et al., 1994). Recovery of enzyme activity after inhibition of nitrous oxide depends on the re-synthesis of MS, requiring 3-4 days in humans (Nunn & Nunn, 1987). In a study of 40 patients exposed to nitrous oxide anesthesia for

70-720 minutes compared to 12 control patients, a postoperative increase of folate with 220% and tHcy with 310% was found. The increase correlated with exposure time. In 20% of the patients, tHcy had not resumed preoperative values in one week. The authors concluded that tHcy could be used to monitor nitrous oxide cobalamin inactivation (Ermens et al., 1991). For a review of MS and its interaction with nitrous oxide, see (Banerjee & Matthews, 1990).

1.8.1 Placental Transmission of Nitrous Oxide

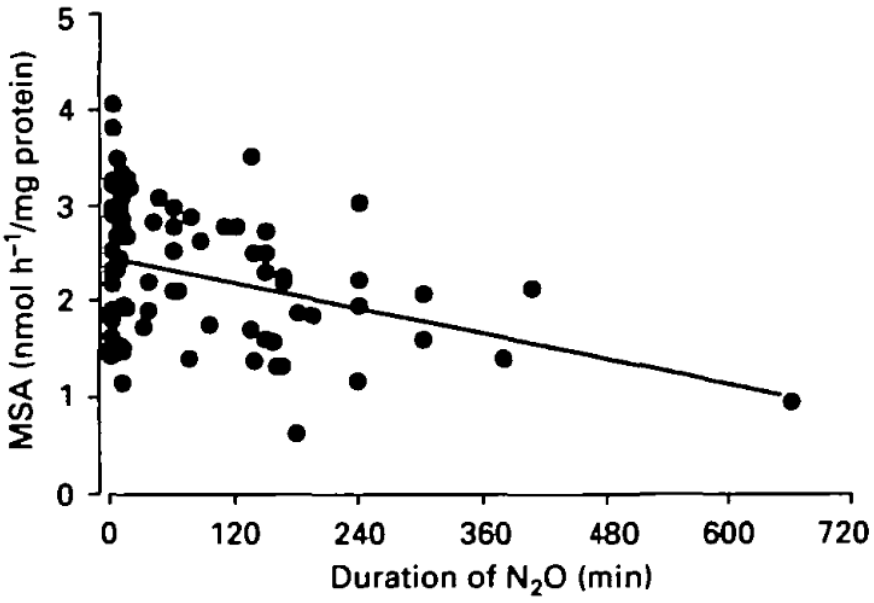
Umbilical vein nitrous oxide levels ranged from 55-91% (mean 79%) of maternal values after 2-19 minutes of inhalational anesthesia with 50-70% nitrous oxide during cesarean section. Umbilical artery nitrous oxide levels ranged from 34-90% of the umbilical vein levels. The ratios increased with exposure time. Umbilical cord blood samples were drawn from a cord doubly clamped before the infant's first breath. Thus, nitrous oxide transmits readily over the placenta to the fetus. Further, a substantial part of the nitrous oxide is taken up and is retained in the fetus until the infant starts breathing (Marx et al., 1970).

1.8.2 Placental Methionine Synthase Inactivation

Landon et al. found a linear reduction in placental MS activity with nitrous oxide exposure time during labor (Figure 5)(Landon et al., 1992). Landon et al. also studied the influence of maternal B12 status on the inactivation of placental MS by nitrous oxide during labor. The duration of nitrous oxide exposure, maternal B12 status, and MS activity correlated, and inactivation was more rapid in women with reduced B12 levels (Landon et al., 1992).

Figure 5

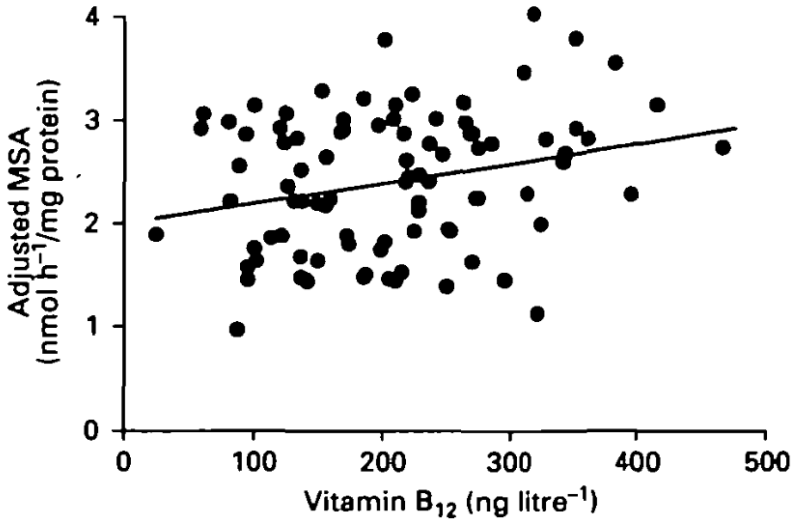
Relation between placental methionine synthase activity and duration of exposure to nitrous oxide (r=-0.347, p=0.001). Reprinted with permission. This article was published in the British Journal of Anaesthesia, 69(1), Landon et al., Influence of vitamin B12 status on the inactivation of methionine synthase by nitrous oxide, 81–86. Doi: 10.1093/bja/69.1.81. Copyright Elsevier (1992).



There was also a direct linear relationship between the placental MS activity and maternal B12 levels after adjusting for nitrous oxide duration (Figure 6) (Landon et al., 1992).

Figure 6

Relation between the placental methionine synthase activity and maternal level of B12 after adjustment for nitrous oxide exposure. Reprinted with permission. This article was published in the British Journal of Anaesthesia, 69(1), Landon et al., Influence of vitamin B12 status on the inactivation of methionine synthase by nitrous oxide, 81–86. Doi: 10.1093/bja/69.1.81. Copyright Elsevier (1992).



Landon et al. stipulated that an average exposure to nitrous oxide of two hours would be sufficient to reduce the hepatic MS to 25% of normal, given that the fetal liver approaches the adult liver in its MS turnover (Landon et al., 1992).

1.9 Newborn Screening

In the Newborn Screening Program (NBS) in Norway, propionyl carnitine (C3) and C3/acetyl carnitine are the primary biochemical screening markers for methylmalonic aciduria and propionic aciduria, two of the current 26 diseases screened for (Tangeraas et al., 2020). A quarter of the newborns reported as newborn screening positive due to elevated C3 in Norway were instead associated with B12 deficiency (Tangeraas et al., 2023). A higher incidence of B12 deficiency in newborn screening programs has recently been demonstrated when

remethylation disorders were introduced as primary targets and after implementing algorithms designed explicitly for B12 deficiency (Gramer et al., 2020; Pajares et al., 2021; Rozmarič et al., 2020). Newborn screening for B12 deficiency mainly reveals maternal B12 deficiency, a primary risk factor for infant B12 deficiency. However, B12 deficiency developing after the first month of life with prolonged exclusive breast milk feeding might not be detected (Gramer et al., 2020). In a retrospective study of 40 breastfed infants with clinical B12 deficiency diagnosed after the first few months of life, all but one had methylmalonic aciduria, 80% had tHcy >12 µmol/L, but only 2/25 had elevated propionyl carnitine (Honzik et al., 2010).

2 Aims of the Studies

The overall aim was to explore and describe infant vitamin B12 deficiency in a subpopulation representative of Norway. The overall proposed clinical benefit was to increase knowledge about infant B12 deficiency to detect it in time and avoid futile investigation and treatment.

Aims of Paper I: a) To investigate the prevalence of serum-tHcy>8 µmol/L and its clinical relevance in presumed healthy infants. b) To evaluate risk factors and associations of increased tHcy with infant symptoms and neurodevelopment.

- Hypothesis: Premature and low birth weight infants have suboptimal B12 status compared to infants born at term with appropriate weight for gestational age. Infants with suboptimal and clinically deficient vitamin B12 status have symptoms and findings detectable with neurological tests, and there are associations between infant vitamin B12 status and neurodevelopmental outcomes.

Aims of Paper II: To describe presenting symptoms and biochemical profiles and to identify risk factors in infants diagnosed with B12 deficiency.

- Hypothesis: Nitrous oxide in labor is a risk factor for infant vitamin B12 deficiency.

Aims of Paper III: To assess predictors and frequency distribution of tHcy and MMA analyzed in NBS DBS infants with clinically diagnosed B12 deficiency.

- Hypothesis: Nitrous oxide in labor is a risk factor for infant vitamin B12 deficiency.

Aims of Paper IV: To evaluate the predictive value using NBS algorithms to detect infants clinically diagnosed with symptomatic B12 deficiency in infancy.

- Hypothesis: Newborn screening can detect infants with symptomatic B12 deficiency presenting after the neonatal period.

The specific proposed clinical benefit was investigating the sensitivity and specificity of newborn screening for B12 deficiency.

3 Study Populations and Methods

3.1 Subjects

The study involved two clinical cohorts of infants:

- The Combo study cohort with 252 infants
- The Retro study cohort with 85 infants

In addition, we included 850 NBS DBS controls:

- NBS DBS matched to the clinical cohorts (n=400)
- Un-matched NBS DBS (n=450)

3.2 Design of the Studies

Two studies comprised the base for this thesis: one retrospective case-control study and one prospective observational study. The location was the south-east of Norway, a high-income country. Almost all pregnant women are followed up during pregnancy by a nationally standardized program, most babies are born in hospitals (*Medical Birth Registry of Norway - NIPH*, n.d.), and nearly all participate in the national NBS program (Tangeraas et al., 2020).

3.2.1 The Combo Study

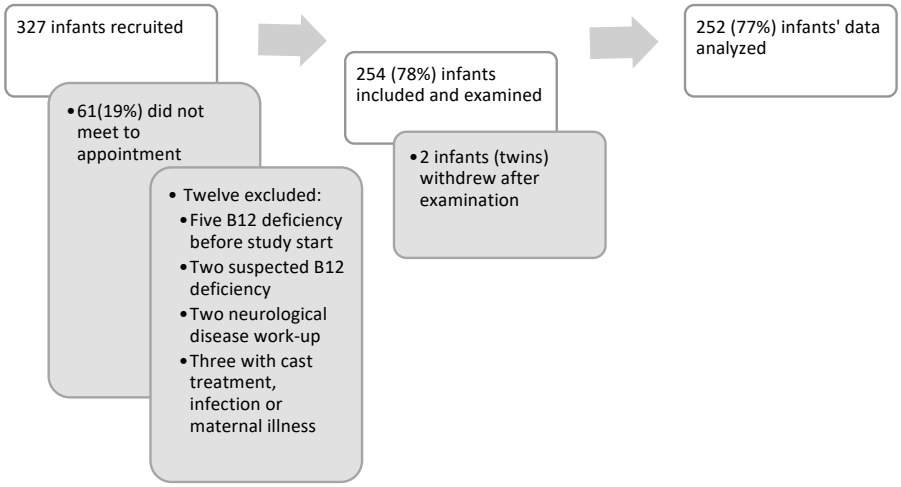
We examined a prospective cohort of 252 presumed healthy infants called the Combo Study. We chose this designation since we both planned to include this group in a prospective, explorative study and use these infants as healthy controls to the Retro Study in a retrospective case-control study. A physician routinely examines all healthy newborns in the well-baby unit on the second day of life. The physiotherapists routinely arrange information meetings in groups to advise parents. The infants invited from the neonatal unit were healthy at discharge with low risk for neurological sequelae. Physicians and physiotherapists consecutively invited 327 infants without identified neurological disease and their mothers from the well-baby clinic and neonatal unit, Vestfold Hospital Trust, between May 2018 and March 2019. Parents

received both oral and written information about the study. The parents had to sign a consent form to participate with their infant. We invited them to meet at the hospital outpatient clinic for a neurological examination, neurodevelopment testing, and infant blood sampling to participate in our research.

We excluded seven of the 327 invited infants since they had a work-up for B12 deficiency done after the invitation but before the first appointment, and 5 (1.5% of the total) of these infants were diagnosed with B12 deficiency. We also excluded five infants due to work-up for neurological disease, cast treatment, infection, or maternal illness. Another 61 infants did not meet at the appointment for unknown reasons. After repeatedly trying to reschedule, we interpreted their lack of response as withdrawing their consent and excluded them from the study. One set of twins withdrew after the appointment. Finally, we included 252 infants and stratified them into three groups: 170 infants born with normal weight at term, 39 infants born at term but small for gestational age with a weight below 10th percentile for gestational age, and 43 infants born moderately preterm at gestational age 32-36+6 weeks. We classified infants born both preterm and small for gestational age as preterm (7/43, 15%).

Figure 6

Recruitment, inclusion, and exclusions of the Combo Study from Paper 1, reprinted under the CC BY 4.0 license.



Data from the Retro study was unavailable when we planned and performed the Combo Study. There was one single study visit, and we scheduled it consecutively to cover the age span between 3 and 7 months when clinical B12 deficiency is most often diagnosed (Honzik et al., 2010). We only had information of age corrected for term date when we examined the infants. We instructed the parents not to tell us anything about their infants before the tests were completed and recorded. We videotaped the infants younger than four months corrected age for two to five minutes of active wakefulness. Later, the physiotherapist Henriette Paulsen evaluated the movements anonymously and calculated a motor optimality score after the Prechtl General Movement Assessment (GMA) (Einspieler & Prechtl, 2005). The same physiotherapist also performed the Test of Infant Motor Performance (TIMP) (K. Campbell, 2012) on infants younger than four months corrected age and an Alberta Infant Motor Scales (AIMS) (Piper & Darrah, 1994) on all infants. Both examiners performed the standardized infant neurological examination (HINE) (Haataja et al., 1999).

Blood sampling failed in two infants, leaving 250 infants with available blood test results.

After the study visits, we collected the three questionnaires completed by the parents, described in 3.4/3.4.2.3. Three of the Ages and Stages Questionnaire (see 3.4.2.3) responses were missing, one from the small for gestational age group and two from the preterm group.

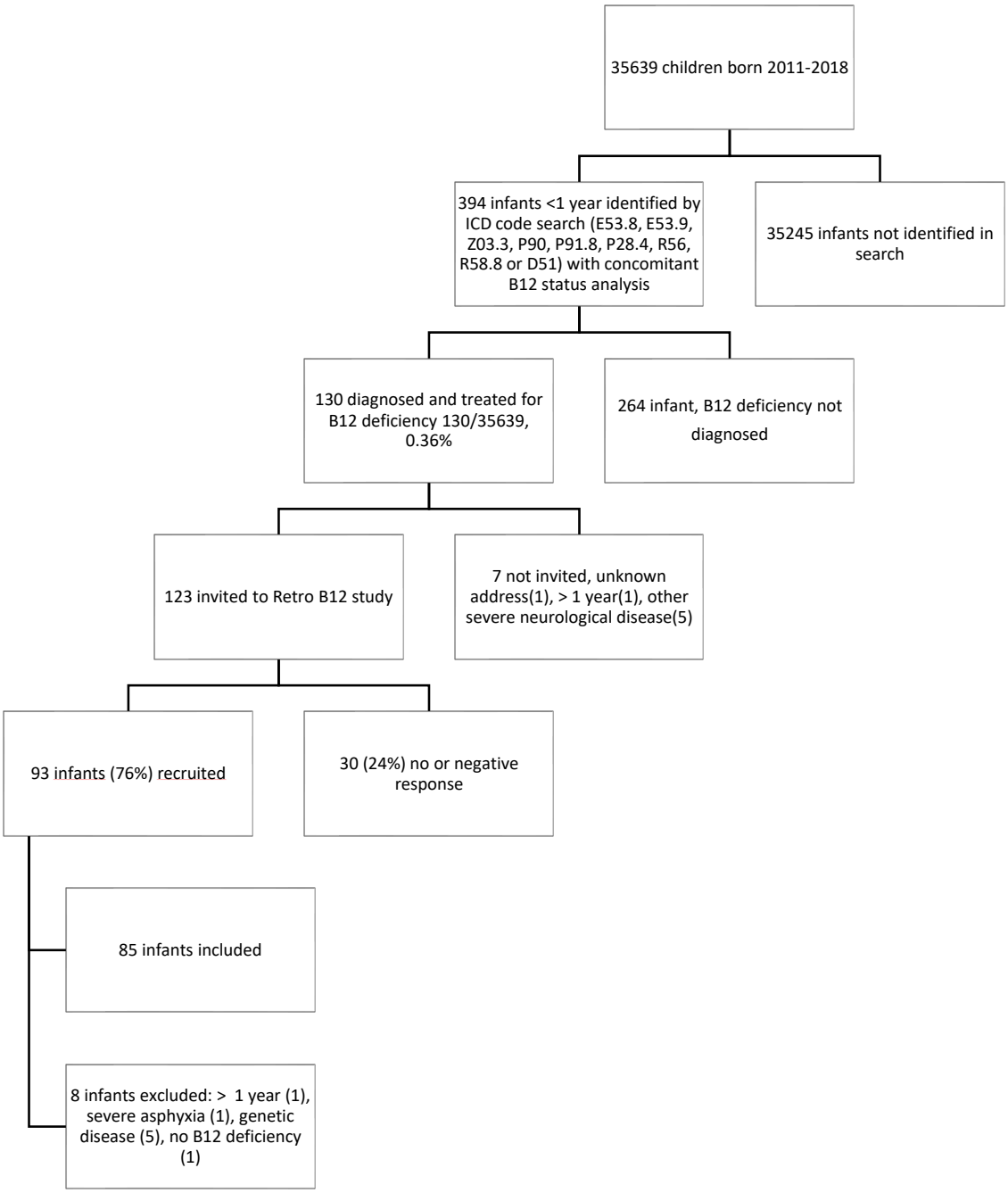
3.2.2 The Retro Study

We included a retrospective cohort of 85 infants diagnosed and treated for vitamin B12 deficiency during their first year of life, and we called it the Retro Study. We searched the medical file database at Sørlandet Hospital Trust and Vestfold Hospital Trust. We restricted our search to infants diagnosed in 2012-2018 since the NBS was extended in 2012 (Tangeraas et al., 2020). Since vitamin B12 deficiency can cause many different symptoms, we performed a broad search, including the 10th International Classification of Diseases codes E53.8, E53.9, Z03.3, P90, P91.8, P28.4, R56, R58.8 or D51, and retrieved cases where also a concomitant B12 status analysis was included. This broad search identified 394 infants under the age of 1 year. According to the file, 130 were diagnosed with B12 deficiency and treated with B12

supplementation. We invited 123 infants. We did not invite seven infants due to unknown addresses, age above one year, and five with other concomitant, severe neurological diseases not attributed to B12 deficiency. Of the invited, 30 did not answer our invitation. After the invitation, we excluded another eight infants: one found to be over one year of age, one with severe asphyxia, five with genetic disorders, and one with no B12 deficiency diagnosis. This left us with 85 infants to include in the Retro Study (Figure 7).

Figure 7

Search and selection process of the Retro Study from Paper 2, reprinted under the CC BY 4.0 license.

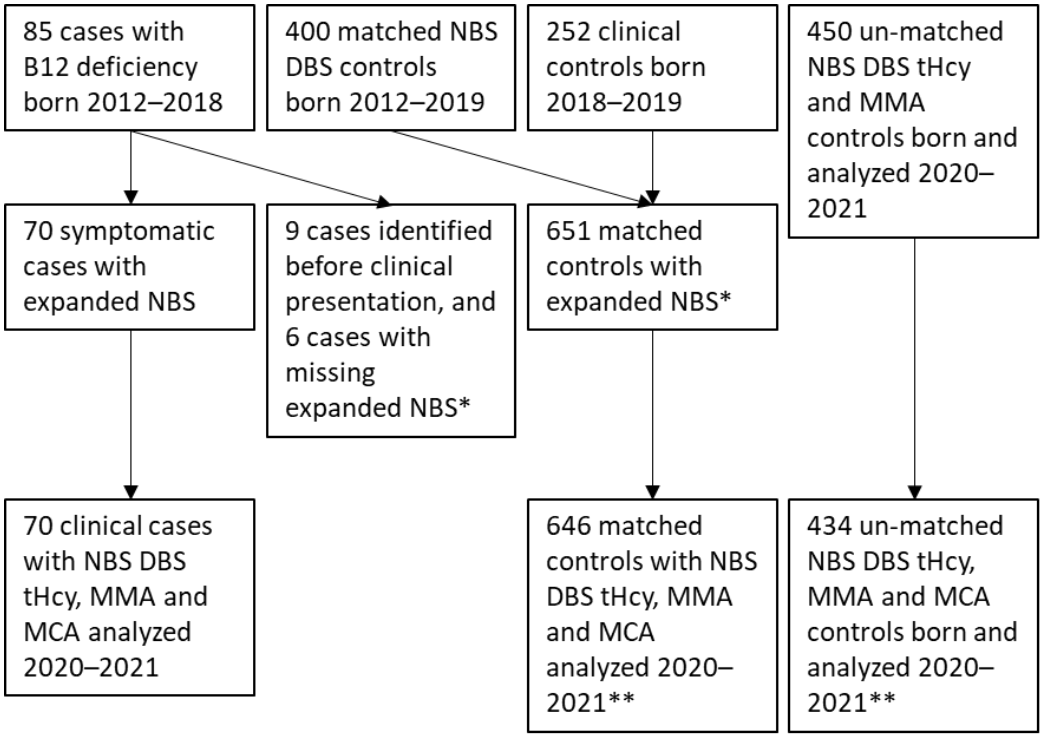


3.2.3 Newborn Screening Studies

To evaluate newborn screening results and re-analyses of B12 deficiency markers, we retrieved bio-banked NBS DBS filtercards from the infants in the Combo and Retro studies. We also included anonymous NBS DBS controls with gender, birth weight, hospital of birth gestational age, and NBS results data. We included a matched control group with 400 NBS DBS filtercards; 94 NBS DBS controls for the Retro group cases and 306 for the Combo group cases, and we matched for sex, date of birth, age at test, hospital, birth weight, and gestational age. We also included a non-matched control group of 450 infants to calculate the percentiles for our analyses (Figure 8).

Figure 8

*Inclusion and exclusion process for the Newborn Screening Study in Paper 4, reprinted under the CC BY 4.0 license. * = 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.*



3.3 Data Collection

The primary data sources we used in the studies were:

- Patient files from mother and infant
- A symptom questionnaire
- A nutritional questionnaire
- Biochemical blood tests and stored results
- Biochemical tests and stored results from the NBS
- Neurological examinations and psychomotor tests

Ultrasonography was performed in standard maternity care and dated the pregnancies. We registered gestational age at birth and calculated the age corrected for the due term date for all infants. We retrieved perinatal information, like maternal health and disease, pregnancy, delivery, and use of nitrous oxide, from the obstetric patient file database. We retrieved the time of start and stop of intermittent use of nitrous oxide and the concentration of the nitrous oxide/oxygen blend. We multiplied the time of intermittent administration in minutes with concentration to get a measure of the dose of nitrous oxide. We collected clinical information from the patient file database for the cases in the Retro Study Group. We manually completed proformas for neurological tests and registered them with questionnaires and clinical information in EpiData version 4.4 from EpiData Association, Odense, Denmark. We then exported files from Epidata and imported them into SPSS for statistical analyses. To avoid manual handling, we harvested blood test results directly to a data file. We stored data on a research server provided by Vestfold Hospital Trust according to board approval. The Ph.D. candidate continuously logged the data collection process, data washing, variables, and dataset handling in our Study Book.

3.4 Methodology

We constructed two questionnaires (Appendix) to cover the variables planned for the studies. One of the questionnaires covered maternal and infant nutrition, supplements, and known risk

factors for infant B12 deficiency, and the other questionnaire contained known symptoms of infant B12 deficiency inspired by previous literature (Akcaboy et al., 2015; Bjørke-Monsen & Ueland, 2011; Black, 2008; Goraya et al., 2015; Green et al., 2017; Honzik et al., 2010; Irevall et al., 2016; Kaul et al., 1963; Smith et al., 2018). The parents answered twelve specific symptoms with one of three choices: Do not agree, partly agree, and fully agree. We dichotomized the answers, classifying partly agree and fully agree as agree. In the retrospective study group, we included the same symptoms as in the prospective study. However, the questionnaire in the retrospective study group comprised three parts instead of one to describe symptoms before and after B12 substitution as well as which symptoms changed after the substitution with B12. Apart from this, the questionnaire was similar in the prospective and retrospective study groups.

3.4.1 Blood Analyses

For the Combo study, we collected venous blood samples non-fasting in 4 mL serum tubes with serum separator and clot activator (Vacuette®, Greiner Bio-One, Austria) from 250 infants. We analyzed them at the Department of Medical Biochemistry at Vestfold Hospital Trust. Venipuncture failed in two infants. We informed the families about their infants' blood test results, and, where appropriate, we gave nutritional advice, including the need for supplementation with iron or vitamins.

We performed analyses of serum B12, holoTC, and folate on Cobas e801 from Roche Diagnostics GmbH, Mannheim, Germany. The measuring range of serum folate was 4.5 to 45 nmol/L. We reported results above 45.4 nmol/L as ">45 nmol/L".

We analyzed hematology samples using XN-9000 analyzers from Sysmex Co., Kobe, Japan.

We determined MMA and tHcy simultaneously by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method in serum. To obtain serum, we left the blood samples at room temperature for a minimum of 30 minutes to allow for coagulation and centrifuged them within two hours.

In the Retro Study, we devoted considerable effort to aligning results from the different laboratories used by the two study sites. Infants in Vestfold had their B12, holotranscobalamin, and folate 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) after that. This shift introduced a bias for holotranscobalamin results corrected in our studies using a documented regression algorithm (Y[Roche]=9.887+0.865X [Abbott], n=56, r=0.985). B12 and folate were measured in serum using an immunoassay on Cobas 6000 e601 from Roche Diagnostics during the whole period at Sørlandet Hospital. Vestfold and Sørlandet analyzed hematology samples using Sysmex instruments (Sysmex XE 5000, Sysmex Corporation, Japan) until February 2017 and XN-analyzers afterward. Telemark Hospital Trust analyzed serum MMA and plasma tHcy from samples collected in Vestfold 2011-2015 using gas chromatography-mass spectrometry and high-performance liquid chromatography, respectively. In 2016, Vestfold started determining MMA and Hcy by serum liquid chromatography/tandem mass spectrometry. Sørlandet analyzed tHcy in plasma using an enzymatic assay and sent MMA for analysis at Oslo University Hospital by liquid chromatography/tandem mass spectrometry. Hcy is released from erythrocytes during the serum preparation, causing approximately 1 μmol/L higher values in serum than in plasma. 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). We reported all tHcy values in serum according to this regression algorithm, and we converted the cutoff limit of 6.5 μmol/L in plasma for vitamin-optimized tHcy (Bjorke-Monsen et al., 2008) to 8.0 μmol/L in serum.

3.4.2 Testing Infant Neurodevelopment

Different tests evaluate the infant's neurodevelopment during the first year of life. Some examine the neurological status, while others evaluate motor or global development. We examined the infants with well-known and standardized neurological and psychomotor tests. The HINE encompasses many variables used to describe neurological symptoms and findings. For the HINE, we constructed and published a reference interval for infants aged 3-7 months,

not included in this thesis. We also showed excellent inter-rater reliability (Ljungblad et al., 2022). We assessed the infant's neurodevelopment by the AIMS, the GMA, the TIMP, and the parent-reported ASQ-2.

3.4.2.1 The Hammersmith Infant Neurological Examination

The HINE is a standardized neurological examination. It may be used from 3 months until the infant starts walking. It is divided into three sections. We used only section 1, the neurological examination, in our studies. Section 1 consists of 26 items assessing cranial nerve function, posture, movements, tone, and reflexes. The items are scored 0-3 points in 0.5-point increments with a total maximum score of 78 (Haataja et al., 1999; Ljungbladet al., 2022). The validity of detecting neurological deficits in high-risk (Romeo et al., 2016) and low-risk (Ljungblad et al., 2022) infants has been proven high.

3.4.2.2 The Alberta Infant Motor Scales

The AIMS is an observational scale created to evaluate the motor development of infants from birth until independent walking (Piper & Darrah, 1994). It contains 58 items assessing the control of antigravity postures evaluated in four subscales: prone, supine, sitting, and standing. The score describes a dichotomized choice, 'observed' (1 point) or 'not observed' (0 points). The infant's age percentile in the normative material is calculated from the total score (Spittle et al., 2008). In infants aged three to eight months, a cut-off at the 10^{th} percentile provides the highest validity for identifying delayed motor function (Darrah et al., 1998).

3.4.2.3 The Ages and Stages Questionnaire

The ASQ assesses global development (Squires, J; Potter, L; Bricker, 1999), and the second edition has been translated into Norwegian (Janson & Squires, 2004). The questionnaire encompasses the five developmental domains: communication, gross and fine motor function, personal-social functioning, and problem-solving. The possible score for each domain is 0 – 60. According to the manual, we defined motor function as typical if the infant scored above the cut-off in the gross and fine motor domains (Squires et al., 1999). The cut-off scores are age-

dependent: 40.1 and 27.5 points for gross and fine motor at four months, 25.0 points for gross and fine motor at six months, 36.0 and 27.5 points for gross and fine motor at 24 months, and 25.0 points at 27 months (Janson & Smith, 2003). The reliability of the Norwegian ASQ-2 is satisfactory, but its validity is limited (Richter & Janson, 2007).

3.4.2.4 The General Movement Assessment

The GMA is based on a gestalt perception of video-recorded, age-specific normal or atypical general movements (Einspieler & Prechtl, 2005; Novak et al., 2017; Spittle et al., 2008) to assess neurological function and to perform scoring using the Motor Optimality Score - Revised (Einspieler et al., 2019). In healthy infants at three to five months, the motor repertoire consists of fidgety movements and other movements, such as leg lift, foot-to-foot contact, kicking, and swiping. Summing the scores of five subcategories yields the motor optimality score, ranging from a minimum of five to a maximum of 28 points. A score between 25 and 28 is considered optimal, and scores ≤ 25 indicate suboptimal or reduced motor performance (Einspieler et al., 2019). The GMA has demonstrated a high validity in detecting severe neurological deficits in high-risk infants (Novak et al., 2017).

3.4.2.5 The Test of Infant Motor Performance

The TIMP is validated for infants up to 17 weeks of corrected age. The assessment has 42 items: posture, handling, movement, reactions, and postural control. Scores range from 0-1 for observed items and 1-6 for elicited items, with a maximum total score of 170. (Campbell, 2012). According to the normative references, a cut-off value of -0.5 standard deviations below the mean is highly sensitive for detecting developmental problems in high-risk infants (Campbell & Hedeker, 2001; Heineman & Hadders-Algra, 2008). It is considered one of the best tools for discriminating between age-appropriate and delayed motor function in preterm and term-born infants (Heineman & Hadders-Algra, 2008; Spittle et al., 2008).

3.4.3 Data Analysis and Statistics

We knew the prevalence of hospital-diagnosed symptomatic infant B12 deficiency during the first year of life of 0.31% in the North of Sweden (Irevall et al., 2016). With 35639 infants born between 2011 and 2018 in our catchment area, we could expect 0.31% of 35639 = 110 infants diagnosed with B12 deficiency, according to the Swedish study.

We performed a power calculation to ensure we included sufficient infants in the case-control study. The tHcy level in infants was used as the primary marker of B12 deficiency and as the dependent variable in the planned regressions. A Norwegian study (Bjørke-Monsen et al., 2008) found that the mean plasma level of Hcy was 7.99 µmol/L at four months of age with an SD of 2.31 µmol. We assumed a difference in Hcy of ≥ 1.4 µmol/L would give a clinically significant difference between cases and controls. With a power of 80% and an SD of 2.31 µmol/L, we calculated that we would need 43 infants in our sample. To cover for a non-response rate of 50%, we would need 86 infants. Accordingly, we concluded that we would have enough power to avoid a type 1 error when we finally included 85 infants as clinical cases and the 252 infants from the Combo as controls. We used a two-sided t-test and 5% significance in the power calculations.

Combo B12 was an observational, explorative, prospective study. To calculate the prevalence of infant B12 deficiency, we learned the biochemical prevalence of B12 deficiency of 5-10% from a Norwegian study on NBS (Refsum, 2004). We then assumed we would need to invite at least 200 infants to calculate the prevalence of B12 deficiency in our material. Since each clinical visit would take 1 hour, we had to consider the feasibility and not include more infants than necessary. Since we expected a 50% dropout rate between the invitation and the clinical visit, we planned to invite 400 infants. We started to examine the infants while the invitation was still ongoing. We experienced that we had overestimated the dropout rate and stopped further invitations at 327 infants. We finally included 252 infants, requiring new approval from the ethics board.

The Ph.D. candidate has performed all statistical analyses, partly but independently re-analyzed by Morten Lindberg. Professor emeritus Lars Mørkrid, a professor in both statistics and biochemistry, has advised us on statistical methods. We used the statistical packages from IBM SPSS Statistics version 28 (IBM Inc., New York, NY, USA) and NCSS 2021 Statistical Software (NCSS, LLC., Utah, USA).

We presented symmetric continuous variables as mean and standard deviation or, if skewed, as median and interquartile range. We gave categorical variables as proportions and percentages (Altman, 1991).

We compared categorical variables between groups using the Chi-squared test of proportions or Fisher's exact test for small samples. We quantified differences between independent groups regarding normally distributed variables with the two-sample t-test or Mann-Whitney U test in case of uncorrectable skewness in the data (Altman, 1991).

We decided a priori to include the corrected age in all regression models. The presented models were statistically significant with $p < 0.001$ and did not violate assumptions.

All statistical tests were two-sided; a p-value <0.05 was considered statistically significant.

3.4.3.1 Data Analysis and Statistics in Paper 1

We naturally logarithmically transformed not normally distributed variables to ensure normality before analyses. We then converted to original units for interpretation, presented as geometric means. 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. We applied linear and logistic regression to evaluate possible significant covariates for non-optimal tHcy and test results in infants. We used candidate variables from Tables 1 and 2 in Paper 1 to identify significant exposure variables in regressions for risk factors. We included variables correlating with the independent variable with Spearman's rho over 0.1 in a crude model. Then, we removed nonsignificant variables for a more saturated model. We reintroduced the excluded variables one at a time and retained them if they became significant. In the final model, we only kept biologically relevant variables significant at a 0.05 level.

3.4.3.2 Data Analysis and Statistics in Paper 2

We analyzed differences in tHcy and MMA before and after treatment with related samples Wilcoxon signed-rank test. The strength of the association between continuous variables was measured using Pearson's correlation coefficient (Altman, 1991). We defined biologically relevant differences when covariates in regressions caused a change of the dependent variable>0.25 standard deviation (SD) when the covariate changed 2 SD. We applied linear and logistic regressions to evaluate possible covariates for tHcy and 'B12 deficiency', respectively. We used candidate variables from Tables 1 and 2 in Paper 2 to identify significant exposure variables in regressions. We did not include dichotomous variables with fewer than 5 in a category. We entered variables with a Spearman correlation rho >0.1 in a crude linear regression model and removed nonsignificant variables for a more saturated model. We reintroduced the excluded variables one at a time and retained them if they became significant. We only kept biologically relevant variables significant at a 0.05 level in the final models. To obtain normally distributed residuals, we applied log-transformed tHcy for use as the dependent variable in linear regression analyses.

3.4.3.3 Data Analysis and Statistics in Paper 3

We performed linear regressions to identify predictors for DBS tHcy and MMA. We used a forward method with a criterion probability of F to enter ≤0.05 to calculate significant variables (Field, 2017). The variables entered in regressions of tHcy and MMA to identify risk factors were maternal Norwegian origin, smoking during the last two 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 labor, prematurity, growth restriction, gender, and vaginal delivery. Significant variables were re-analyzed by the enter method. There was no need to transform tHcy or MMA to meet the assumptions for the regressions. We showed that the storage time of DBS filtercards was associated with an increase of 0.35 µmol/L per year for tHcy but not for

MMA. We consequently chose to include the storage time of DBS filtercards in all regressions for tHcy to correct this systematic error.

3.4.3.4 Data Analysis and Statistics in Paper 4

We used receiver operating characteristic (ROC) curves to test the NBS analytes' performance as classifiers. Being a 'symptomatic case of infant B12 deficiency' was the outcome variable. We only presented data for cases and controls with the combined results from expanded NBS and second-tier analyses available. We used the published flowcharts from the Austrian NBS program (Rozmarič et al., 2020) and the Heidelberg NBS program (Gramer et al., 2020) to retrospectively categorize our study cohort´s NBS results into NBS negative or NBS positive for symptomatic infant B12 deficiency. We entered absolute NBS values from our program (Tangeraas et al., 2020) corresponding to their suggested percentile cutoffs. We calculated the cutoff values for the tHcy equivalent of the percentiles used by Rozmaric et al. from the unmatched controls (Rozmarič et al., 2020). We could not compute the 99.9 percentile for MMA used by Gramer et al. due to an insufficient number of controls, and we, therefore, chose to use their absolute cutoff value (Gramer et al., 2020). We compared matched controls to cases since DBS tHcy increased by 0.35 μmol per year with storage time, as shown in Paper 3. DBS MMA was not affected by storage (Paper 3).

3.6 Ethical Approval

We conducted our studies following the Declaration of Helsinki (World Medical Association, 2013), and the Regional Committee for Medical Research Ethics Northern Norway (179/2018) approved them on 12 February 2018.

4 Summary of Results

4.1 Summary of Results Paper 1

Our main findings were associations between increased tHcy and tremor, excessive sleep, lower fine motor scores on the ASQ-2, and that 10% of presumably healthy infants had clinically relevant tHcy >8 µmol/L.

We found that 13/251 (5.2%) infants with tremor at examination had a significantly higher geometric mean tHcy of 11.0 µmol/L compared to 8.0 µmol/L in infants with no tremor.

The 21/247 (8.5%) infants reported to sleep excessively had a significantly higher tHcy with a geometric mean of 10.8 µmol/L compared to 7.9 µmol/L in infants not reported to sleep excessively.

Tremor was present, and excessive sleep was reported significantly more often in infants with tHcy >8 µmol/L, and we thus defined both as clinically relevant symptoms. Consequently, we defined clinically relevant increased homocysteine as tHcy >8 µmol/L in the presence of tremor or when parents reported excessive sleep. We found 25 (10%) infants with clinically relevant increased tHcy. Infants with clinically relevant increased tHcy had lower B12 than the other infants.

Of 250 infants, 107 (43%) exhibited hypotonia, when defined as being hypotonic in vertical suspension or exhibiting head lag, scoring 0 or 1 on the HINE item pull to sit. Sixteen of 25 (64%) infants with MMA over 1.49 µmol/L (90th percentile) were hypotonic. Infants with or without hypotonia showed no significant differences in B12 or tHcy levels.

The 23/249 (9.2%) infants scoring below normal on fine motor skills on ASQ-2 had a significantly higher tHcy with a geometric mean of 9.4 µmol/L compared to 8.0 µmol/L in infants obtaining normal scores.

Months of infant formula use was the strongest negative predictor for tHcy >8 µmol/L. High-dose B12 substitution during pregnancy in all cases but one protected against symptomatic

tHcy >8 µmol/L /L in the infant. Exclusive breastfeeding increased the odds three times for symptomatic tHcy >8 µmol/L when correcting for prematurity, SGA, and age (OR 2.93).

We found that 89/114 (78%) infants with tHcy >8 µmol/L were asymptomatic. The type of feeding was associated with biochemical test results but not with clinically relevant tHcy >8 µmol/L. We found no associations between biochemical markers of B12 status and total scores in HINE, AIMS, TIMP, or GMA scores. There were no differences in clinically relevant tHcy >8 µmol/L between infants born preterm, small for gestational age, and with normal weight at term, nor did infants born preterm or small for gestational age have lower B12 status than infants with normal weight born at term.

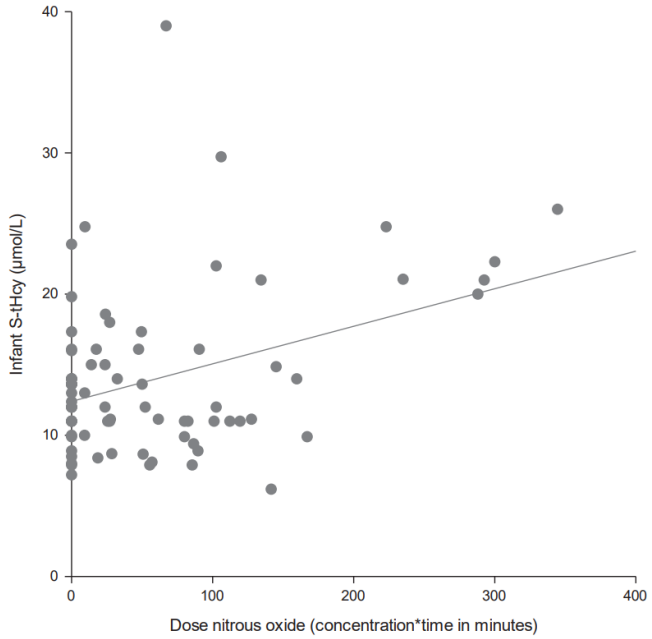
4.2 Summary of Results Paper 2

Our main findings were that 80% of the infants showed symptoms of B12 deficiency within the first two months of life, and the most common presenting symptoms were spells (37%) of apneas, absences, or motor seizures. Further, the dose of nitrous oxide given to the mothers in labor was associated with their infants' levels of tHcy and MMA.

Among the cases, the dose of nitrous oxide correlated significantly with infant levels of tHcy (Figure 9) and MMA but not with B12 at the time of diagnosis. We found no associations between the dose of nitrous oxide and tHcy, MMA, or B12 at 3-7 months in the control group.

Figure 9

Relation between the dose of nitrous oxide and infant tHcy, r=0.37, from Paper 2, reprinted under the CC BY license.



The prevalence of infants <1 year treated for B12 deficiency in the catchment area during the study period was 0.36%. The reasons for referral were apneas (11/76, 14%), absences (8/76, 11%), or motor seizures (13/76, 17%), collectively termed as spells (28/76, 37%). Head lag, tremor, irritability, and abnormal eye contact were other symptoms seen significantly more often in the cases compared to the controls. The primary care referrers never suggested B12 deficiency as a differential diagnosis. Eighty percent showed symptoms of B12 deficiency within the first two months of life, and the age of referral peaked at 1-2 months and six months.

In the 35 exclusively breastfed infants whose mothers received nitrous oxide analgesia during labor, symptom presentation and referral age were 1.2 and 2.2 months, whereas in the 39 infants not exclusively breastfed or whose mothers had not received nitrous oxide, symptom presentation and referral age were 2.0 and 3.6 months, respectively. The difference in referral

age was significant with p=0.016, while the difference in symptom presentation did not reach significance with p=0.051.

Exclusive breastfeeding and self-reported maternal B12 deficiency were risk factors for infant B12 deficiency, while B12 supplementation during pregnancy was protective. There were no vegans/vegetarians among the case mothers. There were no differences between the cases and the controls in the number of infants born preterm or small for gestational age.

Median tHcy and MMA before treatment were 12.4 µmol/L and 1.54 µmol/L and after treatment, 5.8 µmol/L and 0.17 µmol/L, representing 53% and 89% reductions in tHcy and MMA, respectively. Parents reported improvement in symptoms after the B12 supplementation of their infants.

4.3 Summary of Results Paper 3

Our main findings were that tHcy and MMA were higher on NBS DBS in infants with symptomatic B12 deficiency presenting after the newborn period than in controls. Multiple regression analysis showed that the dose of nitrous oxide during labor was the strongest predictor for tHcy in NBS DBS for all infants, with a larger effect in infants that were later clinically diagnosed with vitamin B12 deficiency than controls, but also the only predictor for the clinical controls. Storage time of DBS and nausea in pregnancy were associated with DBS tHcy for the cases.

We found an association between symptomatic infant B12 deficiency and DBS MMA. We also found associations between celiac disease and nausea in pregnancy and MMA for all symptomatic infants, but storage time of DBS did not affect DBS MMA.

4.4 Summary of Results Paper 4

Our main findings were that NBS markers failed to identify ≥90% of infants diagnosed with symptomatic B12 deficiency after the newborn period. Restricting B12 deficiency to symptomatic infants with B12 <160 pmol/L or holoTC <35 pmol/l did not substantially increase

NBS algorithms' sensitivity. We also indirectly showed that nitrous oxide could interfere with interpreting second-tier NBS tHcy.

C3/C2 had the strongest correlation with tHcy at diagnosis of symptomatic B12 deficiency and had the best diagnostic accuracy of the first-tier tests. C3/C2 correlated with NBS second-tier tHcy. Of the second-tier tests, NBS tHcy had the strongest correlation with tHcy at diagnosis of symptomatic B12 deficiency. NBS tHcy had the best diagnostic accuracy of the second-tier tests with an area under the receiver operating curve of 0.665, followed by MMA with an area under the curve of 0.639. Methionine or methionine/phenylalanine did not correlate with diagnostic markers.

In total, 55% of unmatched controls were born in hospitals providing nitrous oxide. tHcy was significantly higher for the unmatched controls born in hospitals providing nitrous oxide than hospitals not, while MMA did not differ.

5 Discussion

5.1 Discussion of the Results

5.1.1 Symptoms and Findings Associated with B12 Deficiency

Paper 1 found associations between increased tHcy and tremor, excessive sleep, and lower fine motor scores on the ASQ-2, and 10% of presumably healthy infants had clinically relevant tHcy >8 μmol/L. Tremor and excessive sleep could reflect a lack of neurological maturation or immaturity, and the presence of a tHcy >8 μmol/L may indicate a suboptimal B12 status and a potential deficiency of methyl donors, transiently delaying neurological maturation. For instance, when clinically examining a three-month B12 deficient baby with a slight tremor, the neurological finding corresponds to a few weeks-old baby, where the tremor would have been seen as normal jitteriness. Our results support this assumption of delayed maturation as increased tHcy was associated with subnormal scores on the fine-motor subscale on ASQ-2, adding to the findings by Torsvik et al. that infants with suboptimal tHcy supplemented with B12 had better development scores than placebo (Torsvik et al., 2015). We, therefore, propose that some symptoms of these infants may be signs of a transiently delayed neurological maturation rather than overt disease.

Paper 2 found that tremor, hypotonia, and reduced eye contact were common presenting symptoms of infant B12 deficiency, aligning with our findings of associations between biomarkers of infant B12 deficiency and tremor, hypotonia, and excessive sleep in Paper 1. These symptoms could again reflect immaturity and suboptimal development rather than a disease, where B12 deficiency causes a delay in neurological maturation.

Irevall et al. published a retrospective study based on a search for infants below one year of age with B12 deficiency in a patient file registry from Sweden (Irevall et al., 2016). They describe the clinical presentation of 35 infants. They defined B12 deficiency as total B12 <160 pmol/L or tHcy >10 μmol/L or increased MMA in addition to an ICD-10 diagnosis of B12 deficiency and stated as B12 deficiency in the medical records. We defined B12 deficiency differently, considering

only the treating physician's conclusion irrespective of B12 status. However, their rate of 74% of infants with tHcy >10 μmol/L was comparable to the rate of 77% in the infants diagnosed with B12 deficiency in Paper 2. In their study, symptoms presented at a mean age of 1.7 months, comparable to our findings in Paper 2, where 80% showed symptoms within the first two months of life. In the Swedish study, 45% had seizures or spasms, 23% had apneas, and 14% had ALTE. These figures were comparable to our findings in Paper 2, where 39% had motor seizures, apneas, or absences at examination. They calculated an infant B12 deficiency prevalence of 0.31% compared to 0.36% in Paper 2.

We further compare Paper 2 and the Swedish study by Irevall et al. to the retrospective study by Honzik et al. that describes the clinical presentation and metabolic consequences of 40 infants with B12 deficiency in the Czech Republic (Honzik et al., 2010). They included 40 breastfed infants recognized in the participating clinics between 2002-2006, representing about 1% of infants in their selective screening, with low or borderline total B12 and increased tHcy > 12 μmol/L or urinary MMA, reversible on enteral B12 supplementation. Of the 40 infants, 63% had anemia, and 80% had tHcy > 12 μmol/L. Only 2/25 infants had elevated propionyl carnitine (C3). They dichotomized their infants to compare mild to severe B12 deficiency. Of the 17 infants in the severe deficiency group, 41% had brain atrophy on sonography and 24% cortical atrophy on MRI, of which half of the infants had delayed myelinization. Their mean (SD) age at first clinical symptoms was 5.4 (2.8) months, diagnosis at 8.3 (4) months, total B12 51 (13) pmol/L, tHcy 99 (33) μmol/L, 94% anemia, 88% failure to thrive, 71% apathy, 88% psychomotor delay, 94% hypotonia, 12% epilepsy, 18% movement disorder, and maternal total B12 was 90 (38) pmol/L. The cause of maternal B12 deficiency was chronic gastritis, verified with gastroscopy in 53%, and 47% had inadequate absorption of B12. Their findings are both in line with a pooled analysis of case studies of infants with B12 deficiency to vegetarian mothers or mothers with untreated pernicious anemia (Dror & Allen, 2008) and a retrospective study of 27 infants from Turkey with megaloblastic anemia (Zengin et al., 2009).

The infants in our studies present much earlier and have biochemically much milder B12 deficiency, and no anemia compared to the studies on Czech, Turkish, and Indian infants. We

speculate that this is why we did not find associations between B12 status and total scores on neurodevelopmental tests, compared to the Czech, Turkish, and Indian infants, where the deficiency has been standing for longer, gradually causing both anemia and developmental delay. Interestingly, the infants in the Nordic studies not only present at an earlier age but also more often with acute symptoms compared to the Czech, Turkish, and Indian infants. We suspect this is due to different risk factors and, thus, causes of deficiency.

5.1.2 Risk Factors Associated with Infant B12 Status

Veganism and poverty are major risk factors for B12 deficiency worldwide (Azad et al., 2020; Zengin et al., 2009). In contrast, the mothers in our studies were well-educated and not poor; neither did veganism, gastritis, and malabsorption explain our cases (Papers 1 and 2), like in the study by (Honzik et al., 2010). Our studies confirmed exclusive breastfeeding as a risk factor for infant B12 deficiency (Papers 1 and 2). Previously unknown risk factors or a combination thereof may have contributed. As shown in Papers 2, 3, and 4, nitrous oxide given to the mother as birth analgesia may be a new risk factor.

In paper 2, we showed an association between the dose of nitrous oxide to the mother in labor 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 labor, the less B12 remains in her infant months later. This has not been reported before. However, Paper 2 found no associations between the dose of nitrous oxide and B12 status in the control group at several months of age. This discrepancy could be explained by insufficient maternal B12 status and a higher breastfeeding rate among the cases, leaving them more susceptible to B12 depletion by nitrous oxide. Accordingly, when we in Paper 3 analyzed 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 the dose of nitrous oxide, indicating decreased MS activity, evident for both cases and controls. This is in line with the findings by Landon et al. discussed in the introduction of the present thesis and depicted in Figure 3 (Landon et al., 1992).

Interestingly, both the tHcy and the MMA–levels were higher on the third day of life when retrospectively analyzed in later, clinically presenting B12-deficient infants compared to controls. Since nitrous oxide affects MS only, not MUT, this finding may indicate a lower B12 status on the third day of life in the cases, rendering them more prone or vulnerable to later symptomatic B12 deficiency than the controls. Furthermore, insufficient maternal B12 status, a well-known risk factor for infant B12 deficiency, could explain the higher infant MMA level at NBS.

According to our results, exclusively breastfed infants whose mothers received nitrous oxide were referred with symptoms of B12 deficiency at a younger age than infants not exclusively breastfed or whose mothers had not received nitrous oxide. We propose that nitrous oxide given as an analgesic during labor may contribute to an early infant presentation of B12 deficiency in exclusively breastfed infants.

We did not find prematurity or growth restriction associated with clinically relevant increased tHcy (Paper 1) or symptomatic B12 deficiency (Paper 2). We interpret the higher rate of formula feeding among preterm and growth-restricted infants as protective.

5.1.3 Newborn Screening for B12 Deficiency

In our study, NBS markers failed to identify ≥90% of infants diagnosed with symptomatic B12 deficiency after the newborn period, also after restricting B12 deficiency to clinical cases with B12 < 160 pmol/L or holoTC < 35 pmol/l. It is in line with the findings by Honzik et al., where C3 analyzed at the time of diagnosis failed to identify symptomatic B12 deficiency in 23/25 cases (Honzik et al., 2010). The NBS studies report a higher positive predictive value of 45–81%, only using biochemical tests on asymptomatic infants to confirm the B12 deficiency (Gramer et al., 2020; Rozmarič et al., 2020). The NBS detects maternally derived infant B12 deficiency, but after birth, additional risk factors modulate the infant's B12 status, where feeding practice seems the most important (Papers 1 and 2); formula feeding may restore a maternally derived low B12 status, and a borderline B12 status at NBS may deteriorate and result in clinical B12 deficiency after long-term exclusive breastfeeding (Reischl-Hajiabadi et al., 2022). In Paper 4,

four of the five infants identified retrospectively with a positive NBS were exclusively breastfed. The fifth infant had only recently been introduced to porridge after exclusive breastfeeding, underpinning the moderation of risk from NBS by feeding practice and strengthening the reservation made by Gramer et al. that B12 deficiency presenting after the neonatal period is poorly detectable at NBS (Gramer et al., 2020). We have shown that formula feeding was protective of infant B12 deficiency (Papers 1 and 2), possibly interrupting the predictability of NBS for infant B12 deficiency (Reischl-Hajiabadi et al., 2022).

Several published NBS algorithms include a repeated DBS to confirm elevated tHcy before recalling the infant for B12 deficiency, pointing to a suboptimal specificity (Gramer et al., 2020; Pajares et al., 2021; Rozmarič et al., 2020). We propose that nitrous oxide given as birth analgesia is one of the confounding factors in that it may transiently increase tHcy and may also explain why a subset of mothers is not diagnosed with B12 deficiency following the detection of her infant at NBS. The tHcy returns to the outset when re-synthesis restitutes the MS enzyme activity. This process requires B12, rendering mothers and fetuses with low B12 stores prone to B12 deficiency (Papers 1 and 2). Thus, our study adds to the discussion of the relevance and feasibility of including B12 deficiency as a primary target in NBS (Mütze et al., 2021). Therefore, we would like to regard infant B12 deficiency from a layered risk model, where the infant needs to have co-occurring risk factors to develop B12 deficiency, where one obligatory risk factor seems to be breastfeeding (Honzik et al., 2010).

NBS programs have reported high sensitivities and specificities for asymptomatic infant B12 deficiency at about one month of age (Gramer et al., 2020; Rozmarič et al., 2020). However, symptomatic infant B12 deficiency manifests later (Paper 2) (Azad et al., 2020; Honzik et al., 2010; Irevall et al., 2016), probably because most infants have sufficient B12 stores to remain asymptomatic initially (Honzik et al., 2010). Further, there is a large discrepancy between the screening and clinical prevalence. The birth prevalence of B12 deficiency reported from NBS programs is 0.01–0.09% (Gramer et al., 2020; Rozmarič et al., 2020). Paper 2 found that 0.36% of infants under one year were diagnosed with symptomatic B12 deficiency, and a Swedish retrospective study estimated an incidence of 0.31% (Irevall et al., 2016). Paper 1 showed that 10% of presumably healthy infants had mild symptoms in combination with biochemical

findings suggesting B12 deficiency. There seems to be a tenfold increase in infant B12 deficiency incidence depending on the diagnostic viewpoint: NBS, selective testing, or clinical screening. Theoretically, our finding of a relatively low, ≤10% sensitivity for NBS to identify symptomatic B12 deficiency was, therefore, to be expected.

Spells were the presenting symptoms in three of the five NBS-positive infants (Paper 4). Spells may be potentially life-threatening and could have been prevented with NBS for B12 deficiency. Larger studies may clarify if NBS for B12 deficiency could reliably prevent the more severe cases of symptomatic B12 deficiency in exclusively breastfed infants because, up till now, the literature suggests that the severity alone is not the only determinant regarding detection by NBS (Tangeraas et al., 2020).

5.2 Methodological Considerations

5.2.1 Study Design

The Combo Study had both a prospective and a cross-sectional, observational design. We had two purposes with this group: to be a control group with healthy infants in the Retro Study and an independent study to examine the prevalence of suboptimal B12 status in a cohort of presumed healthy infants. We chose a prospective design to include healthy newborn babies examined by a pediatrician with normal results on the second day for those recruited from the well-baby unit and at discharge from the neonatal intensive care unit. We examined associations prospectively between risk factors from birth and cross-sectionally between findings at the examination and blood test results from the same day. The study was observational since we made no interventions during the study.

The Retro Study had a retrospective case-control design with the infants from the Combo Study as controls. We chose the retrospective design because clinical infant B12 deficiency is rare, with a reported prevalence of 0.31% in a Swedish material (Irevall et al., 2016), in contrast to biochemical B12 deficiency, which was reported in 5% of infants in a large Norwegian study

with 4992 newborns (Refsum, 2004). A prospective study of infants referred for clinical B12 deficiency would thus be time-consuming and expensive.

Further, we chose to include NBS DBS filtercard controls to account for the difference in the time the filtercards had been stored since we had information on the possibility of storing-time dependent changes in the analytes (Alodaib et al., 2012), but also to build a normative material for the analytes. We decided we did not need more clinical information than what was already included in the standardized NBS laboratory system. By not including the identity of the infants, the regional ethics committee agreed to collect data without additional consent from the carers, saving us much time and effort.

The tHcy was a main outcome measure in all studies and is the preferred single marker for infant B12 status (Green et al., 2017). Elevated tHcy levels are seen in vitamin B12 deficiency, folate and rarely pyridoxine deficiency, and renal insufficiency (Green & Miller, 2022). None of our infants had folate deficiency (Paper 1) or increased creatinine as a sign of renal insufficiency (unpublished data). Pyridoxine is not associated with tHcy in this age group (Minet et al., 2000).

It was demanding to plan the studies since we did not know how many cases with B12 deficiency we would include. According to the power calculation, we would need 86 infants to cover a non-response rate of 50%. Assuming the same prevalence as in the North of Sweden of 0.31% (Irevall et al., 2016), there should be enough infants if we included infants from two hospitals. We also planned to include infants from the large Combo Study as controls to improve the power in the retrospective study since there would be at least two controls per case.

Further, we did not know what symptoms and findings, if any, could be associated with insufficient B12 status in supposedly healthy infants in the Combo Study. Therefore, we examined the infants thoroughly and with standardized examination tools. In that way, we also felt confident that we did not overlook concurrent, significant diseases. The use of several examination tools generated much data and increased the workload. There is a risk of finding random associations when examining many variables for associations (Altman, 1991). Since our studies were explorative and thus hypothesis-generating, we chose not to statistically correct

for this. Consequently, our results should be viewed as explorative and tested prospectively to control for bias and establish cause and effect.

The strengths of the Combo study were the prospective design and the rigorous adherence to standardized examinations. We obtained a high participation rate in the Combo and the Retro studies, with 77% and 76%, respectively. A thorough work-up in both studies minimized other overlapping diagnoses. Also, we draw the blood for analysis only after examining the infants in the Combo Study, minimizing the assessment to be biased by us knowing the results beforehand.

A significant limitation in our studies was the lack of a systematic blood sample analysis of maternal B12 status. Since B12 status is not included in standard pregnancy blood tests in Norway, we could not reliably obtain B12 status data for mothers of either cases or controls. For the cases where we retrieved a maternal B12 status, it was not always obtained simultaneously with the infants. We decided not to include a maternal B12 status for the controls when planning the study because B12 status reference ranges postpartum differ from before and during pregnancy (Varsi et al., 2017). However, in hindsight, the mother's B12 status is a probable confounder that could have moderated our findings, particularly the associations between nitrous oxide and infant B12 status, where we know from Landon and coworkers (Landon et al., 1992) that a better maternal B12 status may protect the infant from the adverse effects of nitrous oxide.

In the Combo Study, there was a risk of selection bias in that mothers interested in nutrition and vitamin adequacy were more attracted to the study. We could expect their infants to be even healthier compared to infants of mothers without this interest. Moreover, we excluded seven infants due to a workup of B12 deficiency between invitation and the study appointment, after which we supplemented five of the seven with B12. Consequently, we could underestimate the prevalence of moderately increased tHcy. Further, we regard our sample of mothers as highly selected, healthy mothers, of whom 97% reported no diet restrictions, 65% reported use of B12 supplements during pregnancy, and 69% had a university education, which may be limiting the external validity regarding the prevalence and risk factors. On the contrary,

we found no significant difference in the rate of non-Norwegian origins of the mothers between cases and controls (Paper 2). The percentage of mothers of non-Norwegian origin was representative of the Norwegian population (Paper 1) (*Fakta Om Innvandring - Statistisk Sentralbyrå*, n.d.). However, participating immigrants might have been more educated compared to all immigrants. Reischl-Hajiabadi et al. found a higher percentage of immigrant mothers to infants with B12 deficiency in their NBS study (Reischl-Hajiabadi et al., 2022). Our study must be repeated in other populations to validate our findings.

The Retro Study entailed risks for both selection and recall bias. In the Retro Study, the inclusion criterium was that an infant had been treated for B12 deficiency, but no predefined criteria existed for the B12 deficiency diagnosis. Thus, the Retro Study cohort may have included infants with symptoms unrelated to B12 deficiency and with concomitant mild, biochemical B12 deficiency. The treating physician decided upon the B12 deficiency diagnosis. However, 92% had tHcy≥ 8 mmol/L, corresponding to 6.5 mmol/L when measured in plasma, a decision level for diagnosing B12 deficiency in infants (Bjorke-Monsen et al., 2008). Some of the Retro Study cohort infants were diagnosed with B12 deficiency for up to six years before the parents completed questionnaires on symptoms, posing an increased risk for recall bias since the accuracy of the parents' memories may have been influenced by subsequent experiences and events.

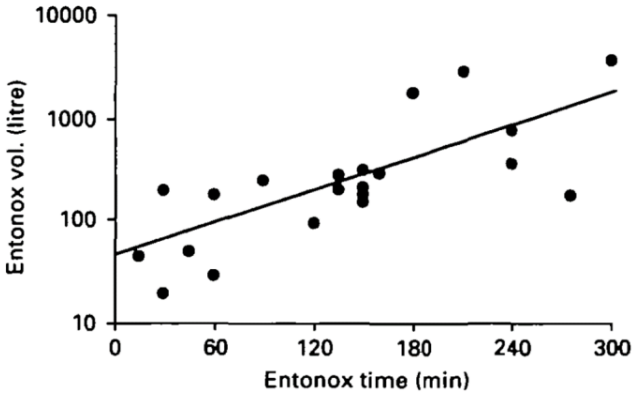
The infants in the Combo group were, on average, six weeks older compared to the Retro group. Therefore, we corrected for age in regressions. The younger age may partly explain the higher rate of exclusive breastfeeding among the cases, but exclusive breastfeeding remained a strong predictor for B12 deficiency after correction for age.

The medical records described a resolution of symptoms after B12 substitution for most 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. Systematic reporting on alleviating symptoms after treatment would increase the likelihood of a correct diagnosis and, thus, the quality of the results.

Our measure of nitrous oxide dose was imprecise since it was limited to calculating the highest possible dose from the start and stop of intermittent use. To have a precise measure for future prospective studies, we should better register the volume of administered nitrous oxide, which was unavailable to us in our retrospective data collection. The imprecise measure may have introduced a bias. However, since the associations we have found between the dose of nitrous oxide and tHcy are strong and align with the findings of other researchers, we assume the associations would be even stronger with a more precise measure of nitrous oxide dose. Landon et al. found a good correlation between the duration of nitrous oxide use and measured volume (Figure 10). They concluded that the volume made no significant contribution to the relation between MS activity and duration of exposure to nitrous oxide (Landon et al., 1992). Their study invariably used a concentration of 50% nitrous oxide. In comparison, the concentration varied between 30-50% in our studies, so we calculated a dose of nitrous oxide by multiplying the concentration by the time of intermittent exposure.

Figure 10

Relation between duration of time with intermittent Entanox® use and volume of Entanox in liters. Entanox® = 50% nitrous oxide and 50% oxygen for intermittent use as inhalational analgesia. Reprinted with permission. This article was published in the British Journal of Anaesthesia, 69(1), Landon et al., Influence of vitamin B12 status on the inactivation of methionine synthase by nitrous oxide, 81–86. Doi: 10.1093/bja/69.1.81. Copyright Elsevier (1992).



Our study in Paper 4 was original in combining clinical cases with symptomatic B12 deficiency with their respective NBS results and re-analysis of stored DBS filtercard. In the unmatched 450 DBS controls, we only had access to whether nitrous oxide was available at the birth hospital; however, data on the individual mothers receiving nitrous oxide was not available for this cohort. This information would probably have strengthened the association between mothers' nitrous oxide intake and tHcy in DBS, as we showed in Paper 3. Additionally, as shown in Paper 3, tHcy increases with DBS storage time, introducing a bias to our cohort. This may, in Paper 4, theoretically, have overestimated some of the few oldest cases picked up by the Austrian algorithm second-tier tHcy test. Still, it would not change the conclusion of the study.

When we retrospectively collected blood test results analyzed over several years and at different laboratories, we had a challenge transforming the results to make them comparable, refer to section 3.4.1. This could have introduced a systematic bias through analytical validity.

Of the questionnaires we used for the mothers, only the ASQ-2 questionnaire was standardized. We constructed our questionnaires to collect data on nutrition and symptoms. There are existing standardized nutritional questionnaires, but we deemed them too exhaustive to justify, fearing increased drop-out, since they should be answered by busy infant parents also taking their time to visit the hospital for a clinical examination. Nevertheless, the scientific value could have been improved by using standardized nutritional questionnaires, and we must consider using them for follow-up studies. The follow-up studies can then better control for bias, while this thesis has met the need to explore. We could not find standardized questionnaires on the symptoms we were researching.

5.2.2 Statistical Considerations

We used conservative statistical methods to keep it simple and transparent for our papers' research team and reviewers.

We used the t-test or the Mann-Whitney U test to quantify differences between independent groups. The assumptions of random samples, normal distribution of samples or residuals, equal or homogeneity of variance, and independence of samples must be met to use the t-test. We

used Levine's test to assess if the samples had unequal variances. Then, other variants of t-tests were used, allowing for unequal variances. We used both kinds of t-tests in our papers. One can use the Shapiro-Wilks test for normality if the sample sizes are small (<50). If the sample sizes are larger, the Shapiro-Wilks test gets too sensitive, and using the QQ-plot to inspect for normality visually is preferred. If the sample sizes are large, the t-test is also robust to violating the normality assumption (Altman, 1991; Field, 2017).

In case of skewness in the data, we either logarithmically transformed or used the Mann-Whitney U test to test for differences between groups. The Mann-Whitney U test assumes that the variables are either ordinal or continuous, the samples are independent, and the shapes of the samples are similar (Altman, 1991).

We used the Chi-squared test for samples greater than five and Fisher´s exact test for smaller samples to compare ratios of categorical values in Papers 1 and 2 to assess associations.

We consulted Professor Emeritus Lars Mørkrid (Statistical Editor of the Journal of Inherited Medicine) when constructing the explorative linear regressions for risk factors. We ensured the regressions fulfilled the required assumptions (Field, 2017). In some (Paper 1 and 2), but not all (Paper 3), of the regressions on tHcy, we had heteroscedasticity that we could amend by logarithmically transforming tHcy. Heteroscedasticity is when the variance of the residuals systematically increases or decreases over the range of the dependent variable. Heteroscedasticity could introduce bias since the confidence interval could differ with different values of the dependent variable, and the assumption is that the variance of the residuals should be equal along the interval of the dependent variable, called homoscedasticity (Field, 2017).

5.2.3 Considerations of the Study Environment

The Ph.D. candidate has been working in an academically interdisciplinary stimulating team across both professions and medical specialties: The Ph.D. candidate’s main supervisor, MD Ph.D. Trine Tangeraas is a pediatric specialist interested in metabolic diseases and NBS. The co-supervisor MD Ph.D. Erik A. Eklund is a specialist in neuropediatrics with metabolic in-depth

knowledge. MD, Ph.D. Professor Dr. Anne-Lise Bjørke-Monsen, co-supervisor, is an internationally acknowledged infant vitamin B12 deficiency researcher. The co-workers at our local hospital have supported the Ph.D. candidate in the day-to-day work of the project. Henriette Paulsen is a pediatric physiotherapist specializing in infant neurological examination. MD PhD. Morten Lindberg is a clinical chemistry specialist, and both have been important in the theoretical and practical research process. The NBS laboratory team, headed by Ph.D. Ingjerd Sæves has been an essential partner in NBS method interpretation, analyses, and development. These people have provided a safe yet effective environment to acquire academic knowledge to complete this thesis according to the plan (Figure 11).

Figure 11

Timeline and effectuation of the Ph.D. studies.

	2017	2018	2019	2020	2021	2022	2023	2024
Preparation and applications								
PhD programme 6 years 50%		sept.						sept.
Data collection								
Data analyses								
PhD courses								
Writing and publishing papers								
Writing thesis								
Dissertation								

The Ph.D. candidate has contributed to all parts of the studies: Planning and preparation, funding applications, data collection inclusive of physical and neurological examinations of all the 252 infants in the Combo Study, data analyses, drafting, and writing all four included papers.

5.3 Ethical Considerations

When doing research on humans, ethical standards must be followed (*WMA International Code of Medical Ethics – WMA – The World Medical Association*, n.d.). To be able to follow them, you first need to know about them, as emphasized in the Declaration of Helsinki, Article 12 (World Medical Association, 2013). When researching children, it is imperative to be aware of ethical rules and principles and to follow them carefully – to do good.

Our project involved research on newborn babies. We had some demanding ethical issues when we recruited presumably healthy babies from our well-baby Postnatal Ward to our control group. They were examined with extensive neurological and biochemical testing.

When Hippocrates opened a school of medicine on Kos some hundred years BC, he used empirical methods in addition to practicing a moral code of conduct. This moral code has become known as the Oath of Hippocrates. This code of conduct puts the needs of the patient first. In an editorial, Srinivasan discusses whether practitioners still hold on to the patients' beneficence, a key component of patient-centeredness (Srinivasan, 2012). Our research occasionally raised concerns about disease when examining healthy infants. In my everyday work as a pediatrician, sick infants are referred to me, and my job is to describe their symptoms and signs to elucidate any disease. We often found irregularities when we examined the presumed healthy infants in our research. Those were often well within the normal range, not necessarily pointing to disease. But how and what do you communicate to the parents? If an expert on infant neurology like me is examining their infant, they expect an expert opinion. But would the whole truth, pointing out the irregularities we found, be in the patient's interest if we concluded that our findings were within the wide range of normality? Our project was well prepared before we started. We followed the Helsinki recommendations (World Medical Association, 2013), according to which we first found that our project would generate knowledge. Then, we wrote a meticulous protocol and made plans for how to handle accidental findings. However, we still struggled with the feeling of ambiguity when we found suboptimal B12 status, signaling the insufficiency we sought. When the infant had no apparent symptoms or findings, it was probably to be regarded as normal and should not be treated according to our protocol. Nevertheless, it could potentially restrict psychomotor development. We did not know, which was a knowledge gap our project hopefully could fill. I informed the parents about the biochemical findings and advised them to contact us should their infant develop symptoms, which I also explained.

On the other hand, in case of borderline clinical findings on an infant in the project, possibly but probably not signaling disease, it was important not to ignore our findings, not to worry the

parents, but instead act to the best for the infant. This is much easier when an infant is referred to me as a clinician.

Maximizing goodness ethically justifies an act according to consequentialism (Holm et al., 2015). When doing research with the intention of doing good, we shall not harm. The original Oath of Hippocrates says that physicians shall not harm their patients. More recently, the World Medical Association International Code of Medical Ethics binds members to act in the patient's best interest when providing medical care (*WMA International Code of Medical Ethics – WMA – The World Medical Association*, n.d.). The Declaration of Helsinki refers to this as a general principle in Article 3, continuing in Article 5 with that research also is to be done on humans to ensure medical progress, but further specifies in Article 9 that the researcher must protect the health and integrity of the research subject (World Medical Association, 2013). In the application for our project to our Regional Ethical Committee, we had to explain why we had to do our research on infants instead of on older children or adults and how we could protect them, carefully weighing benefits for the whole infant population against the possible negative impact on the baby participating in our study. This is considered in Article 20 of the Declaration of Helsinki (World Medical Association, 2013).

The participants in all medical research on humans must give informed consent, which assumes that the information must be understandable, that the researcher ensures that the information is understood, and communicates the possibility of withdrawing without negative consequences. The subject must not feel obliged to participate. This and many other issues are clarified in the Declaration of Helsinki (World Medical Association, 2013), an essential document in all phases of research.

Less than ten years ago, the WHO concluded that there was little guidance for clinical research in children, and a group working with standards for research with children was formed (*Survey of Current Guidance for Child Health Clinical Trials*, n.d.). They have published guidelines on different subjects. Since getting informed consent from a young child is impossible, it is still essential to get assent (Caldwell et al., 2012) and respect dissent. When it comes to infants, this is not possible either. Then, it is even more important to follow general ethical principles closely.

Researching infants comes with a vast ethical responsibility. I have intended to do good. According to virtue ethics, my research is ethically defensible (Holm et al., 2015).

5.4 Conclusion, Clinical Perspectives, and Future Studies

We have demonstrated associations between increased levels of tHcy and symptoms suggestive of infant B12 deficiency in presumed healthy infants with a suggested prevalence of 10%, a substantial proportion in our highly selected cohort. The nitrous oxide dose during labor was a predictor for both tHcy and MMA at diagnosis for infants with symptomatic B12 deficiency but not for controls. However, the dose of nitrous oxide predicted only tHcy at NBS on the third day of life, but then both for cases and controls, and we suggest nitrous oxide to be a risk factor for infant B12 deficiency. The combination of exclusive breastfeeding and nitrous oxide during labor was associated with an earlier presentation of infant B12 deficiency. However, a prospective study, including mothers' B12 status, is needed to confirm causality, and a randomized controlled treatment study could confirm the suggested prevalence of mildly symptomatic B12 deficiency among healthy infants. NBS showed low sensitivity for symptomatic B12 deficiency in our cohort of infants presenting beyond the neonatal period, and the shortcomings of NBS in detecting all infants prone to developing B12 deficiency should be acknowledged. However, NBS may still be essential in detecting and treating breastfed newborns with B12 deficiency, mainly if B12 screening and treatment are not part of pregnancy care. Unnecessary hospital referrals could be mitigated 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 gastrointestinal disease or known B12 deficiency, regardless of the mother`s diet preferences.

It is easy to treat B12 deficiency, but it is challenging to decide whom to treat. In our studies, we had infants with suggestive symptoms but only mild biochemical B12 deficiency and infants with apparent biochemical B12 deficiency without symptoms. Treating infants with symptoms suggestive of B12 deficiency with tHcy above known vitamin-optimized values is reasonable. Our clinical practice is to give 1 mg of hydroxocobalamin intramuscularly once to all infants with moderate to severe symptoms, like spells, and infants below the age of 3-4 months with any

symptoms of B12 deficiency. We use peroral cyanocobalamin to treat infants over 3-4 months with less severe symptoms and mature enough to introduce complementary food. We do not have a liquid hydroxocobalamin formula of B12, so we use one 9 µg cyanocobalamin tablet daily that we crush and mix with food. We intend to persuade the pharmacies to import a liquid hydroxocobalamin formula to supplement infants with B12 deficiency with less severe symptoms (Mütze et al., 2021). We have chosen not to treat infants with non-vitamin-optimized B12 status without symptoms. However, we inform the parents and the primary healthcare providers about the symptoms. For now, we strongly encourage all infants with demonstrated B12 deficiency or risk for B12 deficiency to introduce complementary feeding containing meat, fish, or liver pate twice daily as soon as the infant shows interest in solid food and has started with voluntary grasping. Early introduction of complementary feeding will concomitantly meet the need to increase iron intake and reduce the risk for food allergies without affecting the breastfeeding rate at six months (Fewtrell et al., 2017; Ljungblad et al., 2022; Skjerven et al., 2022).

To fill in knowledge gaps and test the causality of associations found in our present studies, we suggest a prospective study from the first trimester until six months after birth. B12 status would be obtained in the second trimester of pregnancy in all participants to confirm previously published reference values (Varsi et al., 2018). Then, participants would be randomized to either a low- or high-dose oral B12 substitution arm, or to a controlgroup without B12 supplementation. A high-dose B12 substitution ensures a passive gastrointestinal uptake if the active uptake is impaired in case of gastritis or pernicious anemia (Green et al., 2017). With an application program downloaded to a mobile device (app) allowing communication with the parents and gathering data, stricter control with adherence to the substitution and acquisition of more precise data would be possible. During delivery, if the woman chooses to use nitrous oxide for pain relief, the dose of nitrous oxide could be measured exactly by a flowmeter to increase the precision. Infant nutrition status could also be checked weekly by communicating with the parents via an app to control for recall bias. Maternal and infant B12 status should be analyzed at the time of the NBS and at three months. An infant neurodevelopment assessment should be performed at three months with the GMA, AIMS, and HINE and at six months with

the HINE, AIMS, and ASQ. If a large enough cohort were included, the suggested B12 deficient infant group should be divided into two groups to allow for a randomized controlled trial to test if the symptoms are reversible with B12 substitution to ensure a correct diagnosis. Bakken et al. are conducting a similar study, where a large cohort of pregnant women are being recruited from the second trimester to study the association between maternal and infant B12 status. After delivery, the infants' B12 statuses are analyzed. Infants with a total B12 above 148 pmol/L are randomized to either a screening group or a control group to study the impact of an injection of hydroxocobalamin on infants with a plasma tHcy above 6.5 µmol/L on development measured with the Bayley Scales of Infant and Toddler Development at 12 months of age (Bakken et al., 2023). Our proposed study could elucidate if the subtle symptoms we suggest are related to B12 deficiency should be treated. In contrast, the study by Bakken et al. will answer whether subclinical B12 deficiency should be supplemented, as indicated by Torsvik et al. ten years ago (Torsvik et al., 2015).

Finally, sudden infant death syndrome (SIDS) and apparent life-threatening event (ALTE) rates peak between one and four months of age (Moon et al., 2011; Semmekrot et al., 2010). Is vitamin B12 deficiency a risk factor for SIDS and ALTE, given the similar presenting age and events with apneas and seizures (Paper 2)? In Norway, DBS filtercards from children with SIDS and children diseased for other reasons have been biobanked but stored at room temperature. MMA is reported to be stable on filtercards (McCann et al., 1996), unlike Hcy (Bowron et al., 2005). By using MMA as a marker for B12 deficiency and comparing cases and controls, it could be possible to investigate an association between B12 deficiency and SIDS.

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7 Papers I-IV

I

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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.

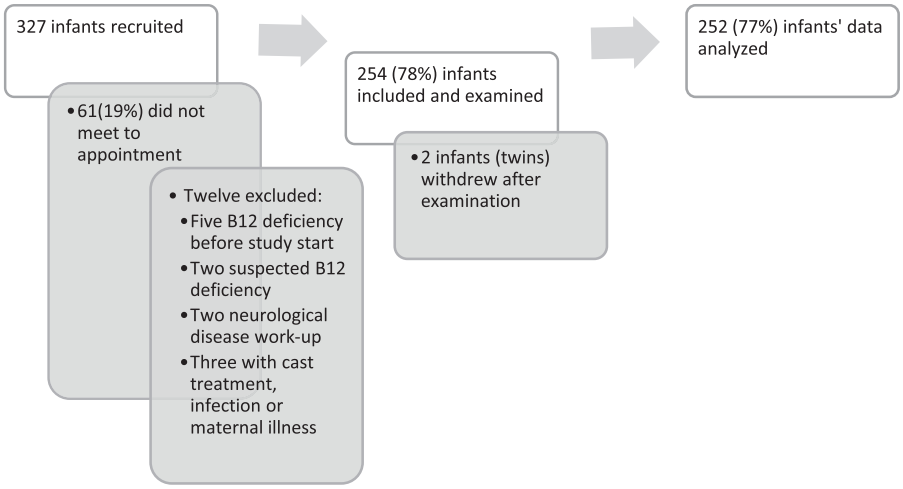

Fig. 1. Online. Flowchart of recruitment, inclusions, and exclusions in the present study.

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

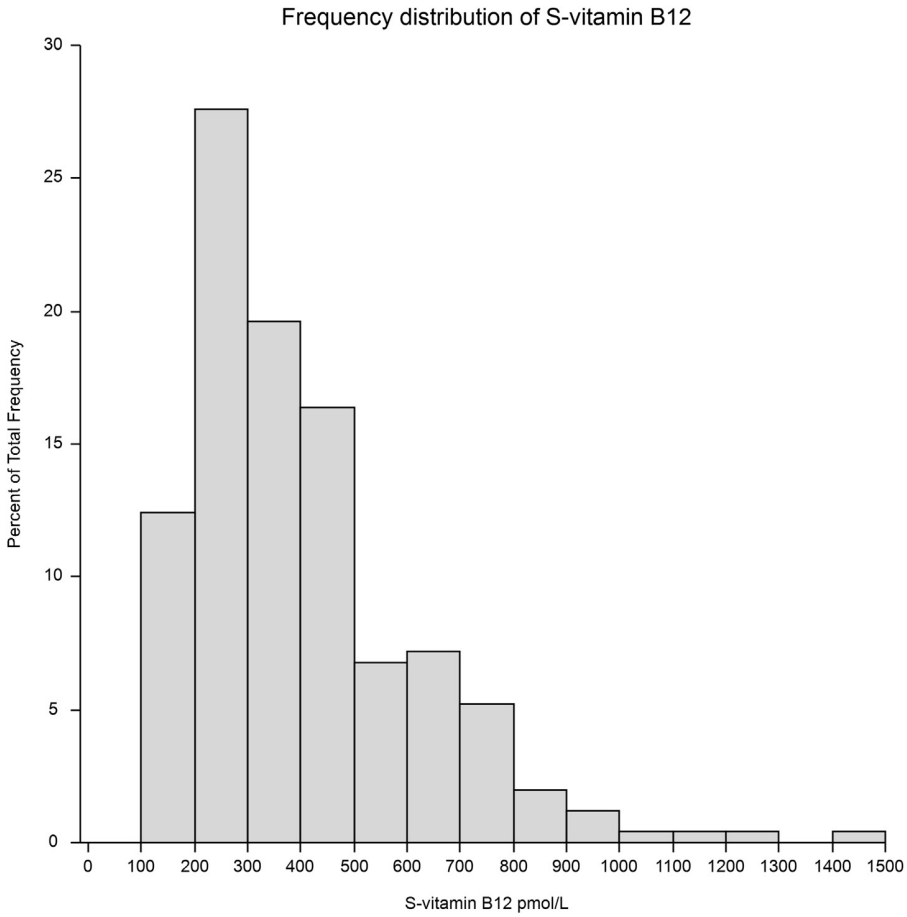

Fig. 2. Online. Frequency distribution of *S*–B12 values for n = 250 infants.

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

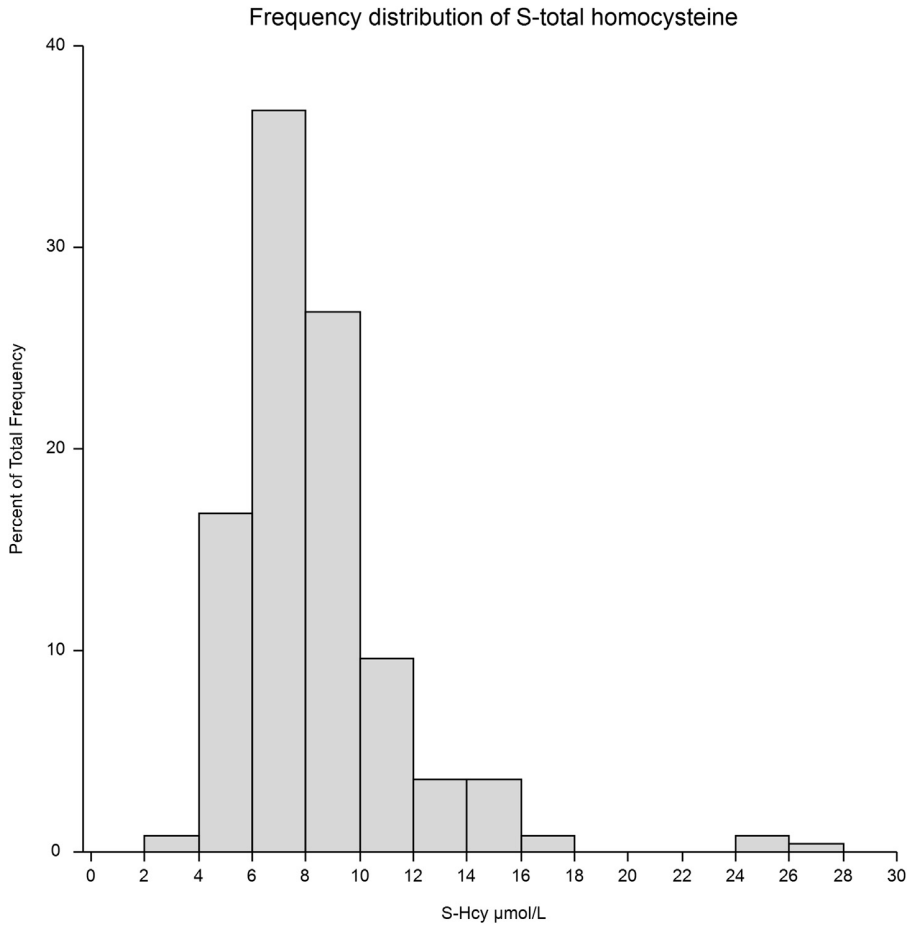

Fig. 3. Online. Frequency distribution of *S*-tHcy for n = 250 infants.

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

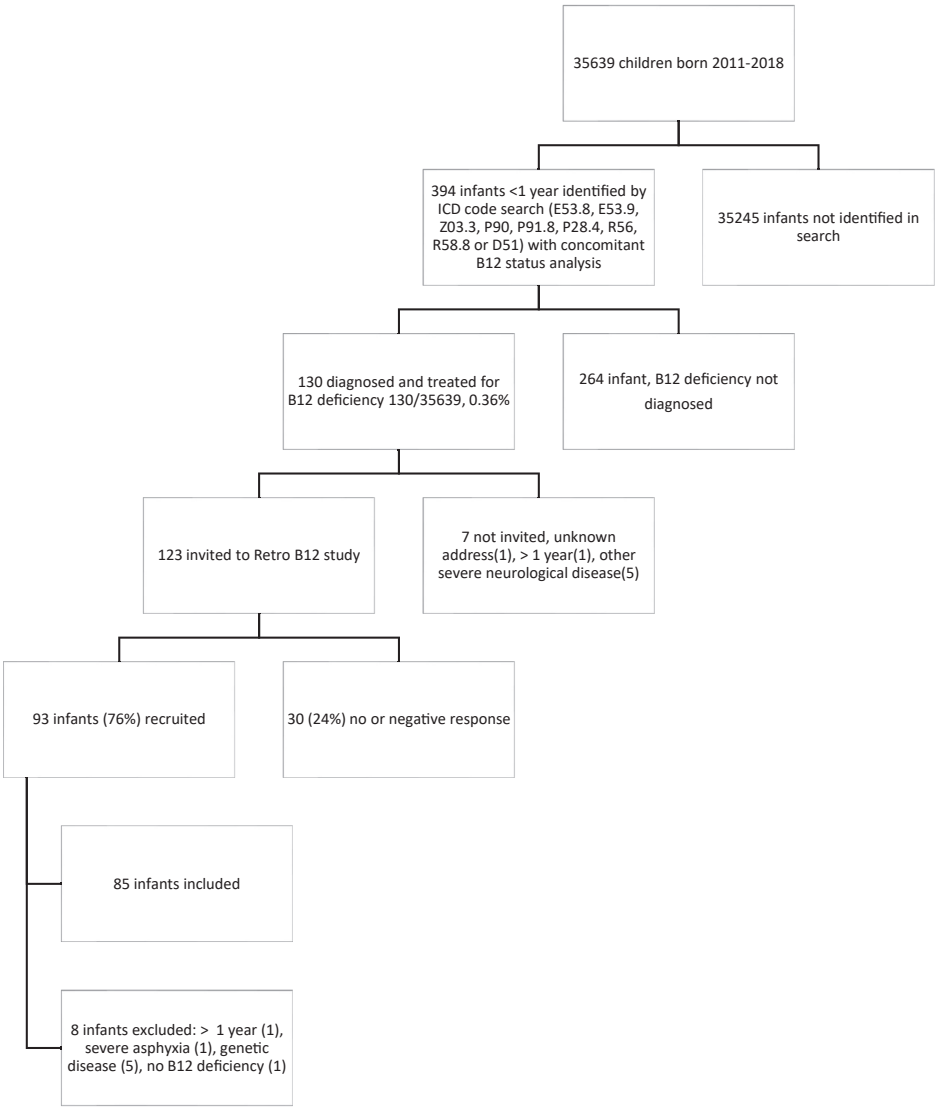

FIGURE 1. Search and selection process for cases.

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.

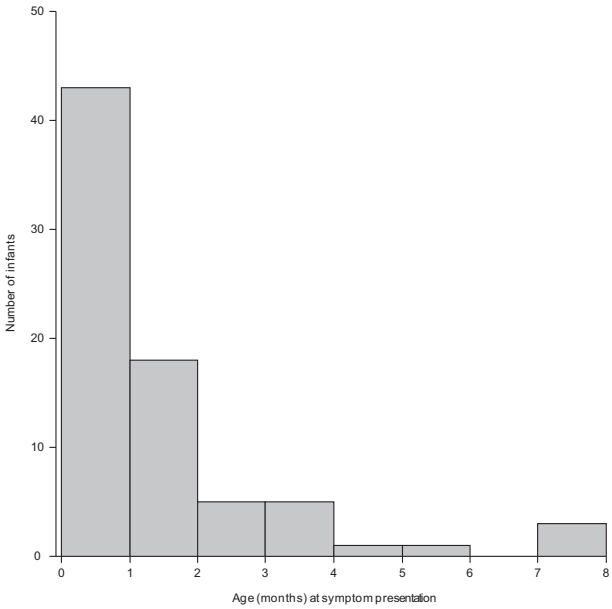

FIGURE 2. Age at symptom presentations (N = 76/85)[1]. [1]Nine infants were excluded because they were tested after NBS recall or because the mother or previous siblings had been diagnosed with B12 deficiency.

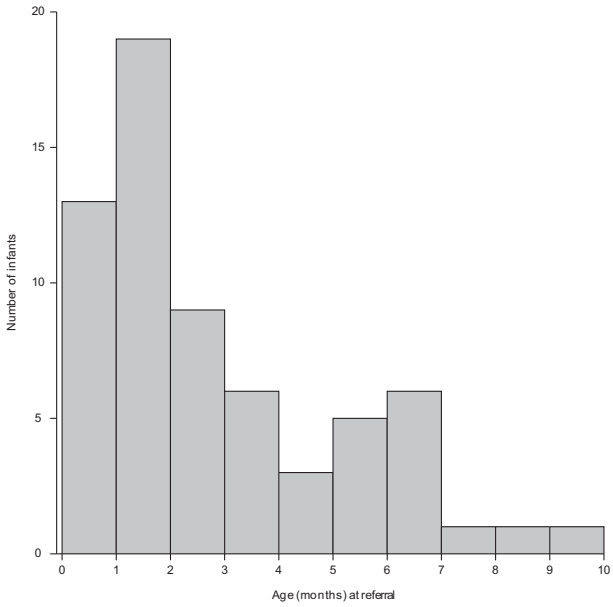

FIGURE 3. Age in months at referral (n = 64/85)[1]. [1]2 infants missing; 9 infants tested after NBS results or family risk, not referred; 10 in-house patients, not referred.

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.

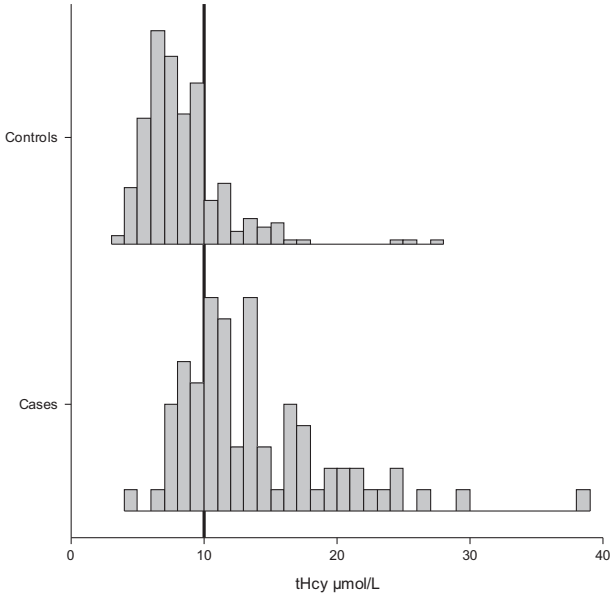

FIGURE 4. Comparison of frequency distribution of tHcy between cases (n = 79) and controls (n = 250). tHcy, total homocysteine.

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.

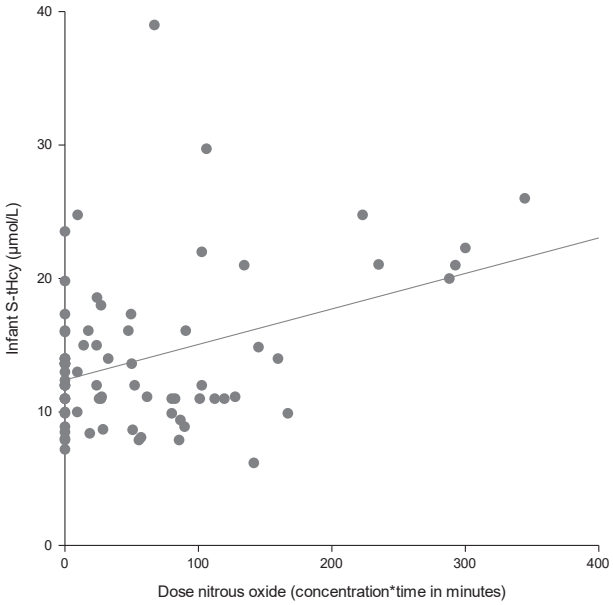

FIGURE 5. Scatterplot of relationship between dose of N_2O to mother during labor and infant tHcy (n = 76) with regression line (r = 0.37). tHcy, total homocysteine.

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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III

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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.

Trine Tangeraas Member of the European Reference Network for Rare Hereditary Metabolic Disorders (MetabERN).

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).

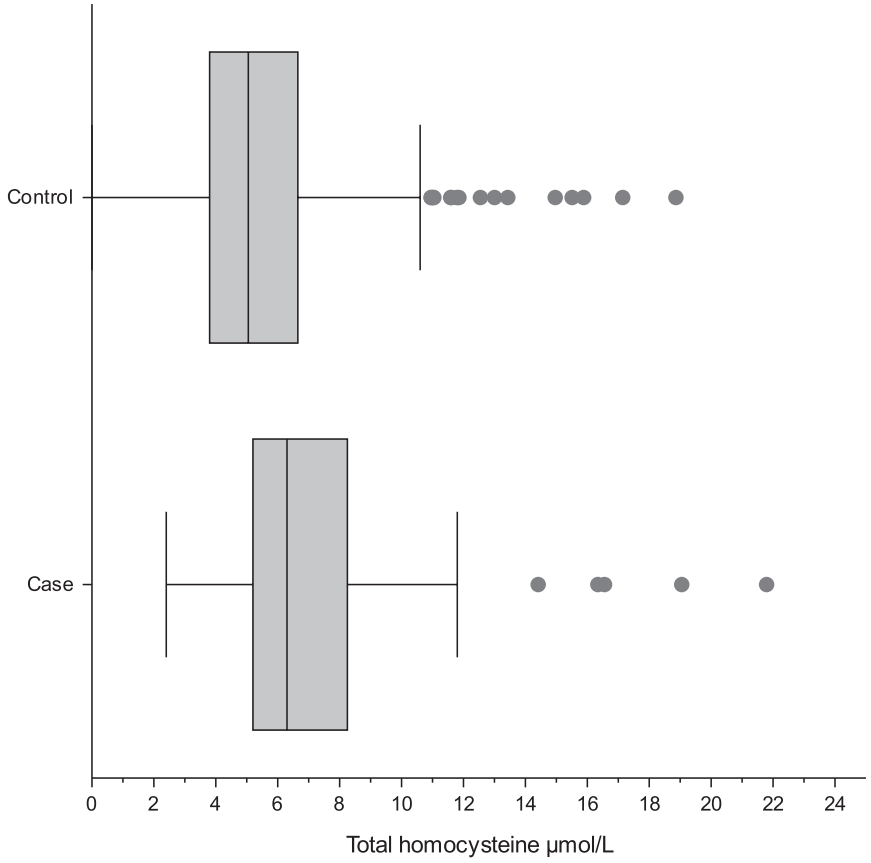

FIGURE 1 Comparison of frequency distribution for total homocysteine (tHcy) between the 79 clinical cases and the 652 controls.

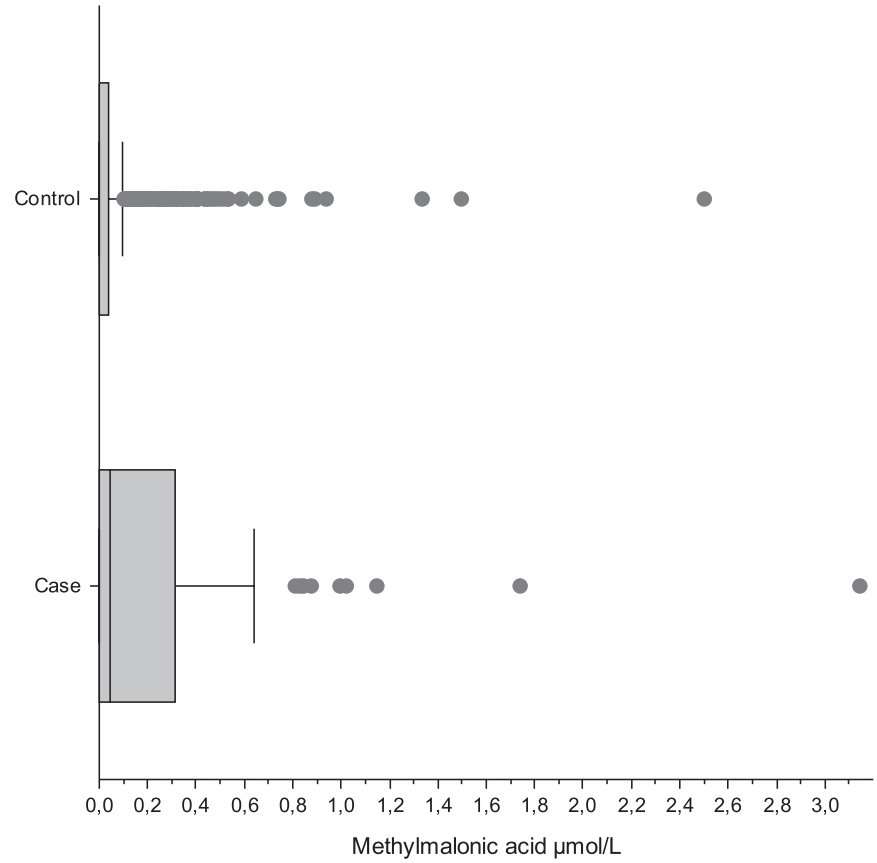

FIGURE 2 Comparison of frequency distribution for methylmalonic acid (MMA) between the 79 clinical cases and the 652 controls.

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

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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.

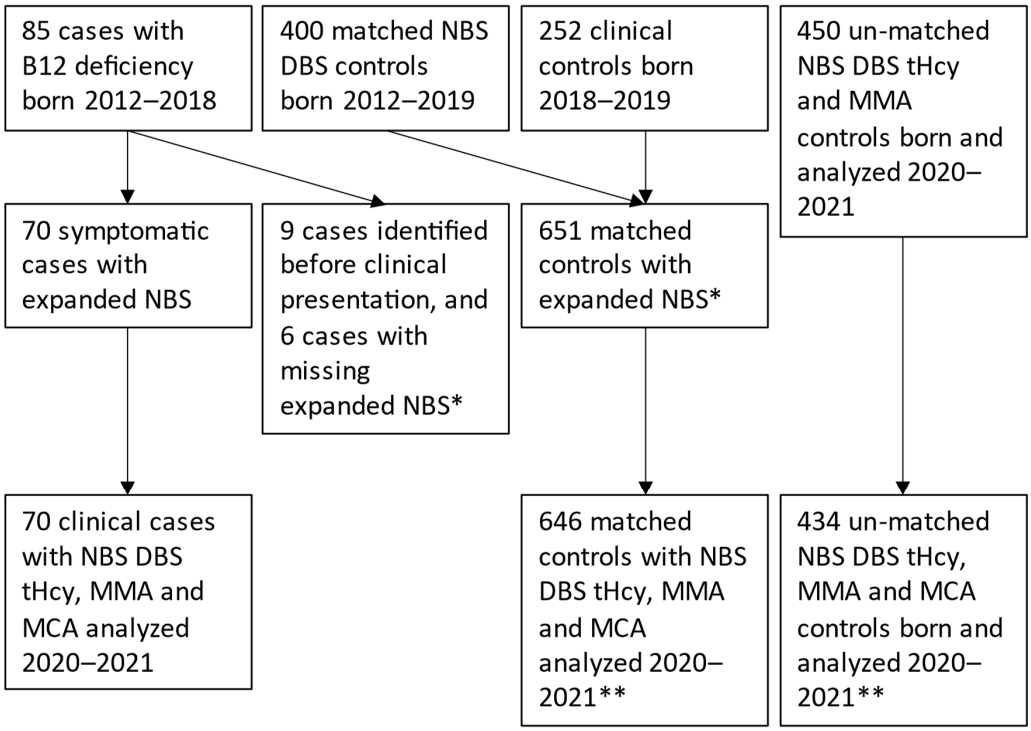

Figure 1. Inclusion of clinical cases and controls, * = missing infants born before 2012, missing expanded NBS, ** = missing infants with unsuccessful 2nd tier analyses, NBS = newborn screening, DBS = dried blood spot, tHcy = total homocysteine, MMA = methylmalonic acid, MCA = methyl citric acid.

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.

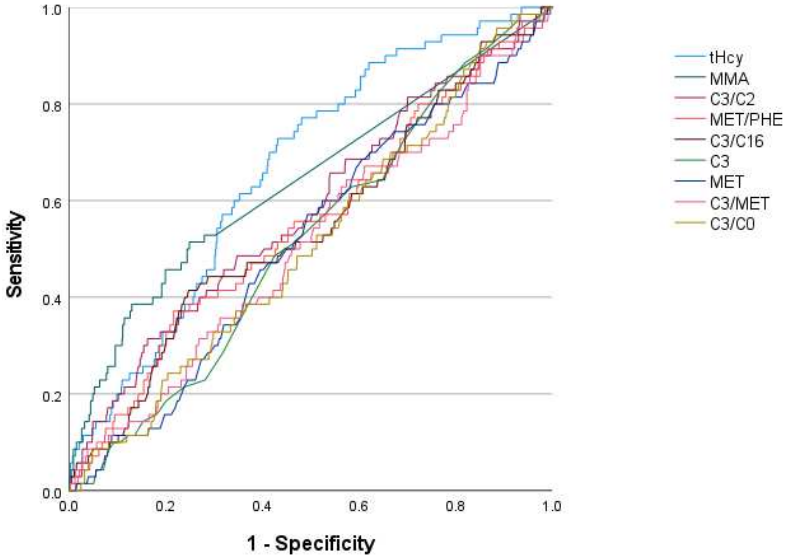

Figure 2. ROC curves of newborn screening parameters' diagnostic accuracy of being a case with B12 deficiency, cases and matched controls, n = 716. MET = methionine, PHE = phenylalanine, C0 = carnitine, C2 = acetylcarnitine, C3 = propionylcarnitine, C16 = palmitoylcarnitine, tHcy = total homocysteine, 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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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by Regional Committee for Medical Research Ethics Northern Norway (179/2018) on 12 February 2018.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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8 Appendix with Questionnaires

Spørreskjema til mor

Takk for at du og ditt/dine barn ønsker å delta i vårt prosjekt «Vitamin B12 mangel hos spedbarn».
For å bedre kunne forstå sammenhengene ber vi deg svare på følgende spørsmål:

Barnets navn: _____

Barnets fødselsnummer: _____

Mors navn: _____

Mors fødselsdato og nummer (11 siffer): _____

Yrke: _____

Land mor er født i: _____

Mors høyeste utdanning: _____

Alene forelder? Ja ☐ Nei ☐

Gift/sambo? Ja ☐ Nei ☐

I arbeid (noe) siste 2 år før fødsel av barnet som er med i prosjektet? Ja☐ Nei ☐

Familiens (min, hvis jeg er alene) totale årsinntekt? _____

Hvor mange barn har du født tidligere? _____

Hvilket/hvilke år var ev. tidligere barn født? _____

Har du røyket siste 2 år før du fikk barnet ditt? Ja ☐ Nei ☐

Røyket du under svangerskapet? Ja ☐ Nei ☐

Når ev. sluttet du å røyke? _____

Kryss av en eller flere: Spiser du kjøtt? ☐ Fisk? ☐ Melkeprodukter? ☐ Egg ☐ Utdyp på baksiden!

Har du sykdom i mage eller tarm? Ja ☐ Nei ☐ Utdyp gjerne på baksiden!

Har du kjent vitamin B12 mangel? Ja☐ Nei☐

Har du fått vitamin B12 injeksjon/sprøyte hos legen? Ja ☐ Nei ☐

Hvis ja, hvor ofte? _____

Når var siste? Før/under svangerskap? _____

Har du tatt vitamintilskudd før svangerskapet? Ja □ Nei □

Om ja, hvilken type og hvor ofte? _____

Har du tatt vitamintilskudd under svangerskapet? Ja □ Nei □

Om ja, hvilken type og hvor ofte? _____

Har du tatt vitamintilskudd under amming? Ja □ Nei □

Om ja, hvilken type og hvor ofte? _____

Har du tatt metformin (Competact, Eucreas, Glucophage, Janumet, Jentadueto, Synjardy, Xigduao) under svangerskapet? Ja □ Nei □

Hadde du svangerskapsdiabetes? Ja □ Nei □

Hadde du svangerskapskvalme (hyperemesis)? Ja □ Nei □

Hvis ja, gikk du ned i vekt? Ja □ Nei □

Ble du slanke-operert før svangerskap? Ja □ Nei □ Om ja (ring inn riktig) Gastric by-pass Sleeve

Vekt før svangerskapet: _____

Vekt før fødsel: _____

Lengde: _____

Hvor mange måneder har du gitt morsmelk til barnet ditt? _____

Hvor mange måneder gav du barnet ditt utelukkende morsmelk? _____

Har barnet fått annet enn morsmelk før denne time? Ja □ Nei □

Hvilke typer morsmelks erstatninger eller annen mat hadde barnet ditt fått før denne timen?

_____

Hvor mange måneder har barnet ditt fått erstatning? _____

Har du gitt barnet ditt noen vitaminer eller kosttilskudd før denne timen? Ja □ Nei □

Om ja, spesifiser:

_____

Annet du ønsker å tillegge som du tenker har betydning? Skriv på baksiden!
Tusen takk for din hjelp!

Spørreskjema til mor

Takk for at du og ditt/dine barn ønsker å delta i vårt prosjekt «Vitamin B12 mangel hos spedbarn».
For å bedre kunne forstå sammenhengene ber vi deg svare på følgende spørsmål:

Barnets navn: _____

Barnets fødselsnummer: _____

Mors navn: _____

Mors fødselsdato og nummer (11 siffer): _____

Yrke: _____

Land mor er født i: _____

Mors høyeste utdanning: _____

Alene forelder? Ja ☐ Nei ☐

Gift/sambo? Ja ☐ Nei ☐

I arbeid (noe) siste 2 år før fødsel av barn henvist for mulig B12 mangel? Ja ☐ Nei ☐

Familiens (min, hvis jeg er alene) totale årsinntekt? _____

Hvor mange barn har du født tidligere? _____

Hvilket/hvilke år var ev. tidligere barn født? _____

Har du røyket siste 2 år før du fikk barnet ditt? Ja ☐ Nei ☐

Røyket du under svangerskapet? Ja ☐ Nei ☐

Når ev. sluttet du å røyke? _____

Kryss av en eller flere: Spiser du kjøtt? ☐ Fisk? ☐ Melkeprodukter? ☐ Egg ☐ Utdyp på baksiden!

Har du sykdom i mage eller tarm? Ja ☐ Nei ☐ Utdyp gjerne på baksiden!

Har du kjent vitamin B12 mangel? Ja☐ Nei☐

Har du fått vitamin B12 injeksjon/sprøyte hos legen? Ja ☐ Nei ☐

Hvis ja, hvor ofte? _____

Når var siste? Før/under svangerskap? _____

Har du tatt vitamintilskudd før svangerskapet? Ja ☐ Nei ☐

Om ja, hvilken type og hvor ofte? _____

Har du tatt vitamintilskudd under svangerskapet? Ja ☐ Nei ☐

Om ja, hvilken type og hvor ofte? _____

Har du tatt vitamintilskudd under amming? Ja ☐ Nei ☐

Om ja, hvilken type og hvor ofte? _____

Har du tatt metformin (Competact, Eucreas, Glucophage, Janumet, Jentadueto, Synjardy, Xigduao) under svangerskapet? Ja ☐ Nei ☐

Hadde du svangerskapsdiabetes? Ja ☐ Nei ☐

Hadde du svangerskapskvalme (hyperemesis)? Ja ☐ Nei ☐

Hvis ja, gikk du ned i vekt? Ja ☐ Nei ☐

Ble du slanke-operert før svangerskap? Ja ☐ Nei ☐ Om ja (ring inn riktig) Gastric by-pass Sleeve

Vekt før svangerskapet: _____

Vekt før fødsel: _____

Lengde: _____

Hvor mange måneder gav du morsmelk til barnet ditt? _____

Hvor mange måneder gav du barnet ditt utelukkende morsmelk? _____

Hadde barnet fått annet enn morsmelk før henvisning for B12 mangel? Ja ☐ Nei ☐

Hvilke typer morsmelks erstatninger eller annen mat hadde barnet ditt fått før henvisning for B12 mangel?_____

Hvor mange måneder fikk barnet ditt erstatning? _____

Fikk barnet ditt noen vitaminer eller kosttilskudd før diagnosen B12 mangel? Ja ☐ Nei ☐

Om ja, spesifiser:

_____

Annet du ønsker å tillegge som du tenker har betydning? Skriv på baksiden!
Tusen takk for din hjelp! Var vennlig og send dette sammen med samtykkeerklæringen tilbake til oss i vedlagt frankert konvolutt.

Symptomregistrering

Namn og fødselsnummer på barn:

Namn og fødselsnummer på mor:

Dagens dato:

Marker beste svaret (stryk under eller ring rundt)!

Mitt barn har ofte vært irritert, urolig, grått mye og/eller ikke virket fornøyd	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har gitt lite øyekontakt	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har sovet mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har vært vanskelig å få til å spise	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har gulpet mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt pustestopp	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt mye rykkninger	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt fjernhetsanfall	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt lav kroppsspenning*	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt langsom utvikling	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har hatt dårlig vektoppgang	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn har slimete avføring	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke

*lav kroppsspenning, slapp muskulatur, vanskelig å holde hodet

Mitt barn har vart innlagt for pustestopp	Ja	Nei
Mitt barn har vart innlagt for kramper	Ja	Nei
Mitt barn har vart innlagt for fjernhetsanfall	Ja	Nei

Mitt barn har vart innlagt for andre årsaker, beskriv:

Har barnet fått vitamin B12? JA NEI

Har barnet fått annen behandling? Beskriv:

Symptomregistrering *før* barnet fikk vitamin B12

Namn og fødselsnummer på barn:

Namn og fødselsnummer på mor:

Dagens dato:

Marker beste svaret (stryk under eller ring rundt)!

Mitt barn var ofte irritert, urolig, gråtт mye og/eller virket ikke fornøyd	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn gav lite øyekontakt	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn sov mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn var vanskelig å få til å spise	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn gulpet mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde pustestopp	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde mye rykkninger	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde fjernhetsanfall	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde lav kroppsspenning*	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde langsom utvikling	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde dårlig vektoppgang	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde slimete avføring	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke

*lav kroppsspenning, slapp muskulatur, vanskelig å holde hodet

Mitt barn var innlagt for pustestopp	Ja	Nei
Mitt barn var innlagt for kramper	Ja	Nei
Mitt barn var innlagt for fjernhetsanfall	Ja	Nei

Mitt barn var innlagt for andre årsaker, beskriv:

Fikk barnet intramuskulær injeksjon vitamin B12? JA NEI

Fikk barnet annen behandling? Beskriv:

Symptomregistrering *etter* vitamin B12

Navn og fødselsnummer på barn:

Namn og fødselsnummer på mor:

Dagens dato:

Marker beste svaret (stryk under eller ring rundt)!

Mitt barn var ofte irritert, urolig, gråter mye og/eller virker ikke fornøyd	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn gav lite øyekontakt	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn sov mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn var vanskelig å få til å spise	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn gulpet mer enn vanlig	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde pustestopp	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde mye rykninger	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde fjernhetsanfall	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde lav kroppsspenning*	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde langsom utvikling	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde dårlig vektoppgang	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke
Mitt barn hadde slimete avføring	Stemmer i stor grad	Stemmer i noen grad	Stemmer ikke

*lav kroppsspenning, slapp muskulatur, vanskelig å holde hodet

Endret seg barnet etter tilskudd med vitamin B12?

Mer våken	Ingen endring	Mindre våken
Mer fornøyd	Ingen endring	Mindre fornøyd
Bedre øyekontakt	Ingen endring	Dårligere øyekontakt
Oftere smil	Ingen endring	Sjeldnere smil
Mindre gulping	Ingen endring	Mer gulping
Mer motorisk aktiv	Ingen endring	Mindre motorisk aktiv
Spiser bedre	Ingen endring	Spiser dårligere
Mindre rykkninger	Ingen endring	Mer rykkninger
Mindre fjernhetsanfall	Ingen endring	Mer fjernhetsanfall
Bedre kroppspenning	Ingen endring	Dårligere kroppspenning
Raskere utvikling	Ingen endring	Treigere utvikling
Mindre slimete avføring	Ingen endring	Mer slimete avføring
Mindre pustestopp	Ingen endring	Mer pustestopp

Hvis barnet fått annen behandling siden første timen, beskriv:

Errata

PhD candidate Ulf Wike Ljungblad

“Infant B12 Deficiency”

The reference «Bjørke-Monsen, A.-L., Torsvik, I., Saetran, H., Markestad, T., & Ueland, P. M. (2008). Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation. Pediatrics, 122(1), 83–91. https://doi.org/10.1542/peds.2007-2716” was listed twice on page 67. This was corrected to list it only once.