

# **B vitamin and iron status during infancy**

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Det største mysterium er ikke mer  
enn det: at en ørliten kropp  
er våknet til jorden. Den nyfødte *ser*.  
To luker i himlen går opp.

Selv femtrinns-raketter og kjernefysikk  
blir puslingers puslespill,  
når det nyfødte barn med et eneste blikk  
beviser at Gud er til.

*André Bjerke*

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## **Abbreviations**

AGA: Appropriate for gestational age  
AI: Adequate intake  
AIMS: Alberta Infants Motor Scale  
ASQ: Ages and Stages Questionnaires  
BSID-II: Bayley Scales of Infant Development  
BW: Birth weight  
CHR: Reticulocyte hemoglobin content  
CNS: Central nervous system  
CoA: Coenzyme A  
FMN: Flavin mononucleotide  
FAD: Flavin adenine dinucleotide  
G: Grams  
GA: Gestational age  
GAM: Generalized additive models  
Hb: Hemoglobin  
Hcy: Homocysteine  
%Hypo: Percentage of hypochromic red cells  
ID: Iron deficiency  
IDA: Iron deficiency anemia  
IM: Intramuscular  
ITT: Intention to treat  
LBW: low birth weight  
MCV: Mean corpuscular volume  
MLBW: Marginally low birth weight  
MMA: Methylmalonic acid  
Met: Methionine  
NTD: Neural tube defects  
PDMS: Peabody Developmental Motor Scale–2  
PL: Pyridoxal  
PLP: Pyridoxal 5'-phosphate

PM: Pyridoxamine  
PMP: Pyridoxamine phosphate  
PN: Pyridoxine  
PNP: Pyridoxine phosphate  
RDA: Recommended dietary allowances  
SD: Standard deviation  
SGA: Small for gestational age  
TfR: Transferrin receptor  
sTfR: Soluble transferrin receptor  
TC: Transcobalamin  
tHcy: Total plasma homocysteine  
WHO: World Health Organization  
ZPP: Zinc protoporphyrin



## List of papers

**Paper I:** Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation. Bjørke-Monsen AL, Ingrid Torsvik, Hege Sætran, Trond Markestad, Per Magne Ueland: *Pediatrics* 2008; Jul;122(1):83-91.

**Paper II:** Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study. Torsvik I, Ueland PM, Markestad T, Bjørke-Monsen AL: *Am J Clin Nutr.* 2013 Nov; 98(5):1233-40.

**Paper III: B** Vitamin status and motor development at 6 months are better in formula fed than breastfed infants with a birth weight between 2000-3000 g. Ingrid Kristin Torsvik, Trond Markestad, Per Magne Ueland, Øivind Midttun, Anne-Lise Bjørke Monsen: Submitted

**Paper IV:** Evaluating iron status and the risk of anemia in young infants using erythrocyte parameters. Torsvik IK, Markestad T, Ueland PM, Nilsen RM, Midttun O, Bjørke Monsen AL: *Pediatr Res.* 2013 Feb;73(2):214-20.

## Summary

Adequate nutrition during infancy is essential to ensure normal growth and development. Since 2001, World Health Organization (WHO) has recommended exclusive breast-feeding for the first 6 months of life, which puts great nutritional demands on the mothers. Breast milk is important for the growing infant, but deficiency of both vitamins D and K have been well described in breastfed infants. There has also been concern about low levels of other micronutrients like iron, cobalamin (vitamin B12) and pyridoxal 5'-phosphate (PLP, the active form of vitamin B6), micronutrients that are important for brain development. Both cobalamin and iron deficiency may cause impaired psychomotor development with potential permanent intellectual defects. Gross motor function is an important marker of neurodevelopment in early infancy and known to be related to micronutrient status.

The objectives of this thesis were to 1) investigate whether cobalamin supplementation can normalize the metabolic profile related to cobalamin status commonly observed in breastfed infants, 2) evaluate the effect of cobalamin supplementation in infants with developmental delay and/or feeding difficulties and biochemical signs of a moderate cobalamin deficiency and 3) investigate vitamin B and iron status in infants with a subnormal birth weight (BW) (2000-3000 grams (g)) during the first 6 months of life according to nutrition, and relate this to nutrition and gross motor development.

The study included three populations with a total of 309 infants aged 6 weeks to 8 months, and their 298 mothers. These populations and main results are:

1. Healthy term infants with an appropriate BW for gestational age (AGA), n=107.

Two-thirds of these mainly breastfed infants aged 1½ – 4 months had moderate cobalamin deficiency judged from total plasma homocysteine (tHcy) level > 6.5 µmol. In a randomized intervention study, cobalamin supplementation changed all markers of impaired cobalamin status toward a profile observed in cobalamin-replete older children and adults.

2. Infants less than 8 months, admitted to a pediatric outpatient clinic at Haukeland University Hospital due to feeding difficulties, subtle neurological symptoms and/or delayed psychomotor development, n=105. The majority of these infants, 80%, had

indices of moderate cobalamin deficiency and a randomized intervention study showed that cobalamin supplementation resulted in significant improvements in regurgitations and motor skills compared to infants who were not supplemented.

3. Healthy infants with BW 2000-3000 g, n=97. In these infants with a suboptimal BW, formula feeding was associated with a better B vitamin status and better motor development at 6 months compared to infants who were mainly breastfed. In infants with a BW  $\leq$  2500 g, iron supplementation resulted in an improved iron status at 6 months compared to non-supplemented infants with BW 2501 – 3000 g. A high weight gain, exclusively breastfeeding and male gender were associated with a poorer iron status in the non-supplemented infants.

As B vitamins and iron play an important role in the development of the brain, an optimal nutrition during the first 6 months is important. Breast milk is important for the infant, but prolonged exclusive breastfeeding during a period characterized by rapid growth and development, may not provide sufficient B vitamins and iron for the infant, particularly for those with a suboptimal BW. We suggest that the recommendation of exclusive breastfeeding for 6 months needs to be reconsidered, and more data on micronutrient status in exclusively breastfed infants are warranted.

## **1. Introduction**

Infancy is a period characterized by rapid physical growth, maturation and neurodevelopment, and an adequate nutrition, including both micronutrients and macronutrients, is of greatest importance during this period (1). Substantial evidence indicates that early nutrition has profound implications for long-term health (2). Unlike macronutrients, which comprise protein, carbohydrate and fat, micronutrients are only needed in a very small amount. However, these substances consisting of vitamins and minerals, enable the body to produce functional enzymes, hormones and others substances that are essential for appropriate growth and development.

Vitamins cannot be synthesized by humans and need to be provided in the diet or, in case of vitamin D, from sun exposure. For infants, the micronutrient status depends on gestational age (GA), BW, maternal micronutrient status during pregnancy and also micronutrient status postpartum for those who are exclusively breastfeed (3-5). For AGA infants born at term, human milk is recommended as the exclusive nutrient source for the first six months of life, but with added vitamin D and K (6). However, exclusive breastfeeding for 6 months has been associated with poorer iron status (7-9) and a biochemical profile indicative of impaired cobalamin status (10-13), both important micronutrients for neurodevelopment. Preterm infants, low BW infants and infants born small for gestational age (SGA) have lower stores of micronutrients (14-16), and iron and folic acid are commonly prescribed for infants with a BW < 2500 g (17-19).

### **1.1. Micronutrients - vitamin B and iron status in infants during the first year of life**

The brain is the metabolically most active organ in the body (20) and maybe the first organ to reflect an inadequate diet. The growth rate of the brain is particularly high during the first 6 months of life (21). At birth, the weight of the brain is 25% and at 2 years of age it has accomplished 77% of its final weight (20, 22). It is known that a dietary deficiency at critical stages of development can results in permanent changes in

brain structure, leading to impaired cognitive function. It is therefore of vital importance to secure an optimal micronutrient status during this period (1).

Micronutrients, like cobalamin and iron, play an important role in the brain development, and developmental delay is common in infants with severe cobalamin deficiency (23-26) and with iron deficiency anemia (IDA) (27, 28). Gross motor function is an important marker of neurodevelopment in early infancy (29-31) and is known to be related to micronutrient status (32, 33).

The Food and Nutrition Board of the Institute of Medicine (34) establishes dietary reference intakes for all micronutrients and the recommended dietary allowance (RDA) is the average daily intake level of a nutrient sufficient to meet the requirements of almost all (97.5%) healthy individuals according to age and gender. If an RDA cannot be determined, an adequate intake (AI) recommendation is set. In children, these intake recommendations are based on data regarding average micronutrient intakes of children and also on certain criteria for micronutrient adequacy. However, because of limited data, many of the micronutrient intake recommendations for children are extrapolated from recommendations for adults using a formula that accounts for metabolic body weight and growth.

### **1.1.1. Cobalamin (Vitamin B12)**

#### **Sources and metabolism of cobalamin**

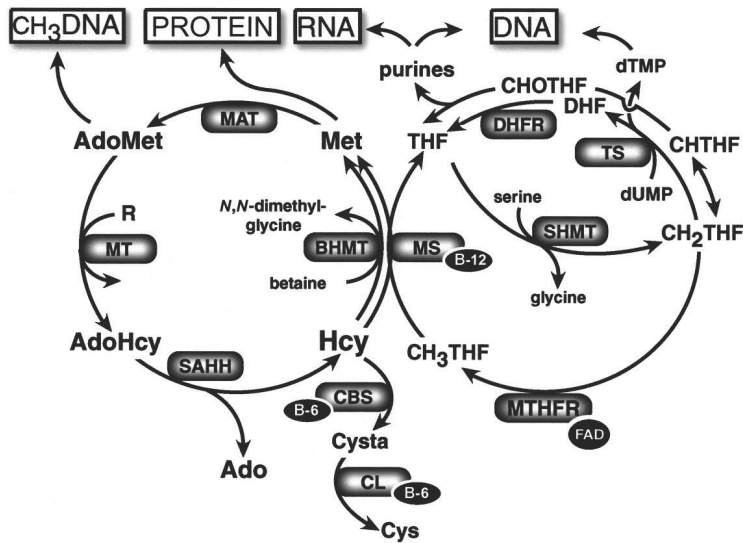
Cobalamin is a water-soluble vitamin synthesized by bacteria and algae. Dietary cobalamin is exclusively derived from animal sources such as meat, fish and dairy products, or from foods that have been fermented. Strict vegetarians are at high risk of nutritional vitamin B12 deficiency, and a low intake of animal food products, which is the situation for many people, especially in low-income countries (35), is associated with a risk for cobalamin deficiency (11).

During digestion, cobalamin is released from dietary proteins in the stomach forming a cobalamin-intrinsic factor complex, and this complex binds to specific receptors located in terminal ileum. The complex dissociates in the enterocyte and cobalamin enters the portal circulation bound to transcobalamin (TC) II, which transports cobalamin to the tissues (10). Most of the circulating cobalamin is bound to

haptocorrins and it is primarily stored in the liver (10). Other storage sites are the heart, spleen, brain, kidneys, bones and muscles (10). Within the cell, cobalamin is a cofactor for two metabolic reactions, i.e. methylation of homocysteine (Hcy) to methionine (Met) (Figure 1) and conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA (11, 36) (Figure 2). Lack of cobalamin will result in increased plasma concentrations of tHcy and methylmalonic acid (MMA), and measurements of these metabolites are useful for diagnosis of cobalamin deficiency (37, 38).

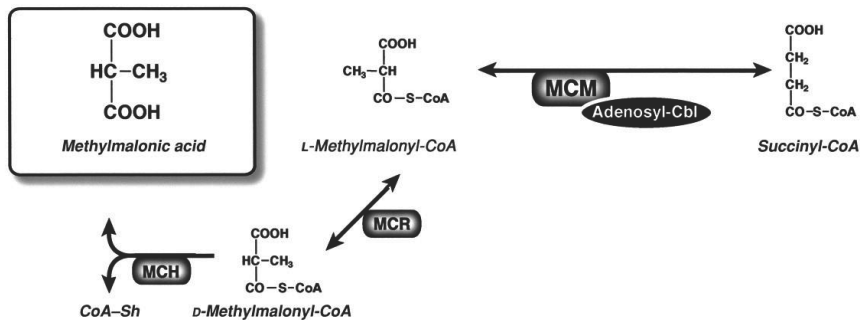
Cobalamin has a significant role in cellular metabolism, i.e. formation of all cells, including erythrocytes, synthesis of DNA, and metabolism of the fatty acids need to produce myelin, the sheath around the axon of the neuron (24).

**FIGURE 1.** Homocysteine formation, remethylation, and transsulfuration and the enzymes and B vitamins involved in these process.



Hcy is formed from *S*-adenosylhomocysteine (AdoHcy). Remethylation to methionine (Met), in most tissues, is catalyzed by the ubiquitous methionine synthase (MS), which requires cobalamin (B-12) as cofactor and 5-methyltetrahydrofolate (CH<sub>3</sub>THF) as substrate. CH<sub>3</sub>THF is formed by the action of the flavin adenine dinucleotide-dependent enzyme methylenetetrahydrofolate reductase (MTHFR), which thus resides at a critical metabolic locus directing the folate pool to Hcy remethylation at the expense of folate used for DNA and RNA biosynthesis. Ado, adenosine; AdoMet, *S*-adenosylmethionine; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine β-synthase; CH<sub>2</sub>THF, 5,10-methylenetetrahydrofolate; CH<sub>3</sub>DNA, methylated DNA; CHOTHF, formyltetrahydrofolate; CHTHF, methenyltetrahydrofolate; CL, cystathionine lyase; Cys, cysteine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; MT, methyltransferase; R, methyl acceptor; SAHH, *S*-adenosylhomocysteine hydrolase; THF, tetrahydrofolate; TS, thymidylate synthase. Reprinted with permission from AJCN (2003)

**FIGURE 2.** The role of the cobalamin-dependent enzyme l-methylmalonyl-CoA mutase in the metabolism of methylmalonic acid.



Adenosyl-Cbl, adenosylcobalamin; MCH, d-methylmalonyl-CoA hydrolase; MCM, l-methylmalonyl-CoA mutase; MCR, d,l-methylmalonyl-CoA racemase. Reprinted with permission from AJCN (2003)

### Measurement of cobalamin concentrations in infants

During infancy there are substantial physiological changes in plasma proteins and renal function, which may affect blood concentrations of both vitamins and metabolic markers. To establish adequate cut-off levels for cobalamin and the metabolic markers tHcy and MMA indicating deficiency, are challenging during infancy, a period characterized by substantial changes in markers of cobalamin status (39). Different studies have reported various levels of cobalamin, tHcy and MMA in infants (Table 1), and elevations of tHcy and MMA have been shown to reflect a functional lack of cobalamin, even when the cobalamin level is within established normal reference ranges (11, 38, 40). Various cut-off levels for cobalamin, tHcy and MMA deficiency have been considered (41-43), and a plasma tHcy level of 6.5  $\mu\text{mol/L}$  has been proposed as a cut-off level for defining moderately impaired cobalamin function in infants (44). This plasma tHcy cut-off represents the 97.5 percentile in infants aged 4 months, who were given a single intramuscular dose of 400  $\mu\text{g}$  hydroxycobalamin at 6 weeks, rendering them cobalamin replete (44).

In infants, cobalamin is the main determinant of plasma tHcy, while the main determinant of plasma tHcy in older children and adults is folate (39, 45, 46). There is only a weak inverse association between cobalamin and MMA in infants, and the MMA concentrations, particularly in breastfed infants, are normally higher over the



entire cobalamin distribution compared to older children (39, 47), hampering the interpretation of high MMA concentrations. One possible explanation may be that degradation of odd-chain fatty acids present in breast milk can lead to elevation of MMA levels.

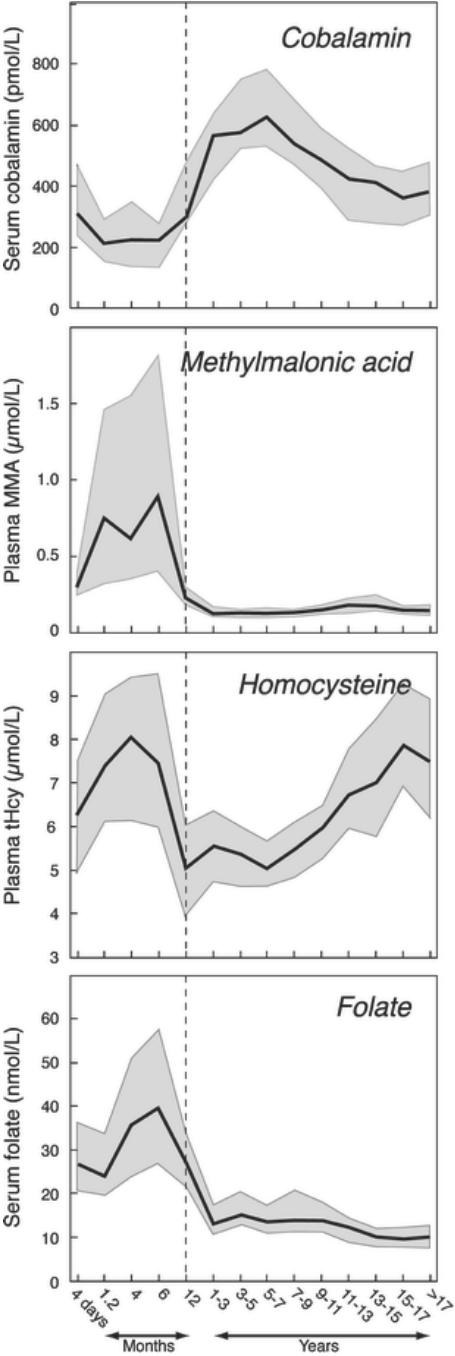
### **Cobalamin status during infancy and early childhood**

During fetal life and infancy an adequate cobalamin status is important for the growth and development of the central nervous system (11, 48-51). Infants of well-nourished women are born with a store of 50 µg cobalamin (25-30 µg in the liver), which has been predicted to be sufficient until the end of the first year (10). However, the fetal storage is correlated to the mother's cobalamin level prior to and during pregnancy (11, 52, 53) and premature and low-birth-weight infants have lower levels compared with full-term infants with normal BW (14, 15).

According to earlier research by our research group on Norwegian children, there is a considerable decrease in serum cobalamin level during the first weeks of an infant's life, and the lowest levels are commonly seen between 6 weeks to 6 months of age (median 217 (interquartile range 147-290) pmol/L) (39). During the same period plasma tHcy and MMA increase remarkably (3, 39, 54, 55). In infants older than 6 months, serum cobalamin increases and reaches a peak between 3-7 years, while plasma tHcy and MMA decrease after 6 months and remain low until the age of 7 years (tHcy < 6 µmol/L, MMA < 0.26 µmol/L) (39) (Table 3). A study of 123 healthy neonates aged 1 to 28 days had a serum cobalamin levels ranging from 86-939 pmol/L with a median of 264 pmol/L(54), and Davis et al observed a serum cobalamin range (5-95 percentile) of 120-800 ng/L in 509 infants aged 3-54 weeks (56).

Studies have reported higher cobalamin levels in infants who are mainly formula-fed than exclusively breastfed (57-59). RDA for cobalamin in infants is 0.4 µg from 0-6 months and 0.5 µg from 7-12 months (Table 2.) (34).

**FIGURE 2:** Changes in serum cobalamin and folate and plasma total homocysteine (tHcy) and methylmalonic acid (MMA) in children from day 4 throughout adolescence. The solid lines indicate the values; shaded areas indicate the 25th and 75th percentiles. Reprinted with permission from Monsen et al. (2003)



**Table 1. Cobalamin and folate levels in infants and children according to age**

Publication	Age	No.	S-Cobalamin pmol/L	S-Folate nmol/L	Comments
Specker(57)	0.8-6.8m	24	496 (207-1202) <sup>1</sup>		Breastfed
		32	264 (93-750) <sup>1</sup>		Formula-fed
Minet(54)	4-20 w	123	264 (86-939) <sup>1</sup>	35.6 (17.4-111) <sup>1</sup>	
	4-20 w	30	263 (62-703) <sup>1</sup>		Breastfed
		30	456 (121 886) <sup>1</sup>		Formula-fed
Bjørke Mønsen(3)	4 d	173	314 (238-468) <sup>2</sup>	27.0 (20.4-36.3) <sup>2</sup>	
	6 w	45	230 (158-287) <sup>2</sup>	22.7 (19.0-31.3) <sup>2</sup>	
Bjørke Mønsen(39)	1.5-6 m	118	217 (147-290) <sup>2</sup>	31.6 (21.3-43.3) <sup>2</sup>	
	1-10 y	172	551 (456-683) <sup>2</sup>	14.9 (12.0-21.1) <sup>2</sup>	
Hay(60)	At birth	361	120-686 <sup>3</sup>	26-95 <sup>3</sup>	
	6 m	221	121-517 <sup>3</sup>	23-122 <sup>3</sup>	Breastfed
		41	195-640 <sup>3</sup>	23-112 <sup>3</sup>	Formula-fed
	12 m	115	165-580 <sup>3</sup>	19-79 <sup>3</sup>	Breastfed
		128	221-717 <sup>3</sup>	14-72 <sup>3</sup>	Formula-fed
Karademir(58)	2 d	204	238 pg/ml (94-706) <sup>4</sup>	16 ng/ml (4.2-20) <sup>4</sup>	Breastfed
			257 pg/ml (151-885) <sup>4</sup>	14 ng/ml (5.2-20) <sup>4</sup>	Formula-fed
	2 m	204	243 pg/ml (141-615) <sup>4</sup>	12.8 ng/ml (4.8-20) <sup>4</sup>	Breastfed
			558 pg/ml (267-1232) <sup>4</sup>	20 ng/ml (7-20) <sup>4</sup>	Formula-fed
	6 m	204	263 pg/ml (110-9589) <sup>4</sup>	13.2 ng/ml (7.5-20) <sup>4</sup>	Breastfed
			600 pg/ml (225-1500) <sup>4</sup>	20 ng/ml (12-20) <sup>4</sup>	Formula-fed

m, months; w, weeks; d, days; y, years

<sup>1</sup>Mean (95% CI)

<sup>2</sup>Median (range)

<sup>3</sup>The 5th and 95th percentiles

<sup>4</sup>Mean (range)

**Table 2. Recommended Dietary Allowance for B vitamins and iron in infants, children and adults<sup>1</sup>**

		Riboflavin (mg/d)	Vitamin B6 (mg/d)	Vitamin B12 (µg/d)	Folate (µg/d)	Iron (mg/d)
Infants	0-6 m <sup>*</sup>	0.3 <sup>2</sup>	0.1 <sup>2</sup>	0.4 <sup>2</sup>	65 <sup>2</sup>	0.27 <sup>2</sup>
	6-12 m	0.4 <sup>2</sup>	0.3 <sup>2</sup>	0.5 <sup>2</sup>	80 <sup>2</sup>	11
Children	1-3 y <sup>**</sup>	0.5	0.5	0.9	150	7
	4-8 y	0.6	0.6	1.2	200	10
Females	9-13 y	0.9	1.0	1.8	300	8
	14-18 y	1.0	1.2	2.4	400	15
	≥19 y	1.1	1.3	2.4	400	18
	>50 y	1.1	1.5	2.4	400	8
Pregnant women		1.4	1.9	2.6	600	27
Lactating women		1.6	2.0	2.8	500	9.5

m, months; y, years

Recommended dietary allowance (RDA) and adequate intake are used as goals for individual intake. RDAs are set to meet the needs of almost all (97 to 98 per cent) individuals in a group.

<sup>1</sup>Based on Institute of Medicine (2013)(34)

<sup>2</sup>Adequate intake

### Symptoms and sign of cobalamin deficiency

Severe cobalamin deficiency in infants has been considered to be rare. The most frequent reports of deficiency occurred in breastfed infants of mothers who had undiagnosed cobalamin deficiency because of low cobalamin diet including vegetarianism (11, 48, 49), malabsorption due to gastric bypass surgery (61), short gut syndrome (62) and unrecognized pernicious anemia (48, 62). However, in recent years, several reports have documented that apparently healthy infants who are born to mothers on a westernized diets and exclusively breastfed for extended periods, have low s-cobalamin concentrations combined with elevated concentrations of the metabolic markers tHcy and MMA (3, 39, 54, 55, 63).

Infants born to mothers with severe cobalamin deficiency may have symptoms at birth, but often the symptoms are apparent by the age of 4-8 months (13) and depend on the severity and duration of cobalamin deficiency (64). It is a continuum of symptoms, from subtle developmental delay in small infants to life-threatening clinical conditions, and as many physicians are not familiar with symptoms of cobalamin deficiency, it is not easily diagnosed and there tend to be a diagnostic delay (65).

### *Neurological symptoms.*

Developmental delay is common in infants with cobalamin deficiency, but in young infants this may be difficult to assess due to the large normal variation and the limited repertoire in this age group. It has been reported that the infants usually have a normal psychomotor development for the first months of life followed by non-specific symptoms such as irritability, apathy and failure to thrive (11). Clinical examination may reveal insufficient head control, delayed turning from supine to prone position and delayed sitting and walking (24, 65, 66). Other symptoms are hypotonia, drowsiness, reduced eye contact and smiling, and abnormal movements such as tremors, twitches, chorea or myoclonus (24, 65, 67, 68). In severe cases of cobalamin deficiency even developmental regression may occur (23, 26, 48, 69).

Delayed myelination has been documented with magnetic resonance studies in cobalamin deficient infants (25, 68, 70). Myelination is an indicator of functional brain maturation and is correlated to psychomotor development (71, 72).

### *Gastrointestinal symptoms.*

Infants with cobalamin deficiency may have feeding difficulties like dysphagia, regurgitation, vomiting and refusal of complementary food (24, 65, 73). In a report from Zengin et al, 41% of the infants with cobalamin deficiency had vomiting and regurgitation (65), and persistent solid food refusal is a known problem (74). Refusal to wean is reported to be present in 53-83% of cobalamin deficient infants, probably induced by hypotonia and difficulty in consuming and swallowing solid foods (24). Obstipation has been reported in cobalamin deficient infants (65, 73).

### *Hematological symptoms.*

Most commonly, infants with mild or moderate cobalamin deficiency only have neurological and/or feeding symptoms without hematological abnormalities, which are late symptoms in infants (69). However, in severe and longstanding cobalamin deficiency, the majority of the infants have megaloblastic anemia with mean

corpuscular volume (MCV) ranging from 104-117 fl and hemoglobin (Hb) levels down to 5 g/dL (24, 65).

#### *Growth.*

Growth retardation affecting weight, length and head circumference is associated with severe cobalamin deficiency (24, 69, 75), but normal weight and even overweight are more common (33).

#### *Long-term prognosis of cobalamin deficiency.*

There are limited data on long-term prognosis after prolonged cobalamin deficiency during infancy, but case reports show that long-term neurological consequences depend on the severity and duration of deficiency (64). Permanent developmental disabilities have been reported, even in infants who were successfully treated with cobalamin (48, 49, 68, 76). Louwman et al reported impaired cognitive performance in adolescents from macrobiotic families, despite the fact that they had eaten animal products since the age of 6 years (76). These findings emphasize the importance of adequate cobalamin level for the developing central nervous system during the first part of life.

### **Diagnosis and treatment of cobalamin deficiency**

The diagnosis of cobalamin deficiency in infants is challenging because the symptoms are often nonspecific and difficult to detect, and the variation in normal development is large in this age group (26, 73). In addition, many physicians are unfamiliar with the symptoms, as demonstrated in a review of 48 published cases of infantile cobalamin deficiency, where the median diagnostic delay was 4 months after the first symptoms appeared (24, 65).

In a pediatric setting cobalamin deficiency is mainly due to low stores at birth and /or low dietary intake, i.e. exclusive breastfeeding for an extended period from a cobalamin deficient mother. Mild cobalamin deficiency can be treated with introduction of food of animal origin, but in severe cobalamin deficiency intramuscular (IM) cobalamin is needed. Various regimens have been proposed (49, 77-

79) and hydroxycobalamin (250-1000 µg) can be given as an IM injection 3-7 times per week for 1-2 weeks and then once weekly for 1 month or until blood counts are normal. Maintenance doses every 2-3 months are necessary if the underlying condition is not corrected. In a randomized study of infants assumed to have moderate cobalamin deficiency at 6 weeks of age (median serum cobalamin 170 pmol/l and median plasma tHcy 7.46 µmol/L) an injection of 400 µg hydroxycobalamin given IM at 6 weeks resulted in normalized cobalamin status at 4 months (median serum cobalamin 420 pmol/L; median plasma tHcy 4.57 µmol/L) (44).

Several reports describe an immediate response within days to cobalamin treatment in symptomatic infants (49, 77-80). Most of the infants experience reversal of apathy, muscle hypotonia and anorexia, and they start to eat and drink normally within days (24). Comatose infants and children regain consciousness within hours after treatment (49).

Normal growth parameters, including a normal head circumference, recover within months (48), and hematological parameters change rapidly with reticulocytosis appearing after a few days and a normal bone marrow within few weeks (33, 80, 81).

The central nervous system pathology improves rapidly after initiation of treatment, and in a report from Kuhne et al, atrophy of the optic nerve was completely resolved after 6 months (82). Other infants with structural abnormalities on cerebral MRI due to longstanding cobalamin deficiency, showed a normal cerebral MRI 10 weeks after cobalamin treatment was started (49, 80).

### **1.1.2. Vitamin B6**

#### **Sources and metabolism of vitamin B6**

Vitamin B6 includes six related pyridine derivatives: pyridoxal (PL) and pyridoxal phosphate (PLP), pyridoxine (PN) and pyridoxine phosphate (PNP), and pyridoxamine (PM) and pyridoxamine phosphate (PMP). The main form of vitamin B6 in animal tissue is PLP, the active form of vitamin B6, which acts as a cofactor for numerous enzymes involved in amino acid metabolism and is the most widely used marker of vitamin B6 status in the last decade (83). The condensation of tHcy with serine to form

cystathionine, is dependent on vitamin B6, and tHcy is a sensitive marker of both functional and intracellular deficiency of vitamin B6 (84)

Vitamin B6, like cobalamin and riboflavin, is a water-soluble vitamin. Food sources of vitamin B6 comprise potatoes, bananas, meat, fish and poultry, and in some countries, fortified cereals. Food of animal origin contains mainly PLP and PMP, while food from plants contains PN and PNP (85, 86). Bioavailability is estimated to be above 75% from food in a mixed Western diet and for vitamin supplements above 90%. The RDA is between 0.2-0.3 mg/d for infants during the first year of life (34) (Table 2).

### **Vitamin B6 status and deficiency during infancy**

The prevalence of vitamin B6 deficiency during infancy and childhood is very uncertain, because vitamin B6 status is rarely measured or studied scientifically in infants and children. Leklem et al suggested a plasma PLP above 30 nmol/L to be considered as an adequate vitamin B6 status (87). In a study on Indonesian children (ages 8-9 years) the mean plasma PLP concentration was 54 nmol/L ( $\pm$  30 SD), and 25% of the children had PLP concentrations  $\leq$  30 nmol/L (88). Several reports suggest that breast milk concentration and infant status reflect maternal intake and status (89, 90), and the Second National Health and Nutrition Examination Survey from USA stated that approximately 90% of young women consumed less vitamin B6 than the RDA, rising concern about deficiency during pregnancy and associated implications for fetal brain development (91).

### **Symptoms and signs of vitamin B6 deficiency**

Vitamin B6 adequacy is known to be critical to the developing central nervous system (92, 93). Overt deficiency is probably rare, but marginal deficiency has been associated with microcytic anemia (94), nonspecific stomatitis, seborrheic dermatitis (95), irritability, confusion and convulsive seizures (96, 97). Low vitamin B6 in human milk has been associated with slower growth in breast-fed infants (89, 98), and a report from Egypt found that low B6 concentrations in breast milk were associated with poorer consolability and response to aversive stimuli in their infants (99).



### **1.1.3. Riboflavin (Vitamin B2)**

#### **Sources and metabolism of riboflavin**

Riboflavin is found in many foods including green vegetables and foods of animal origin like meat, fish, eggs and milk. In food, riboflavin is bound to albumin and riboflavin-specific carrier proteins. In the proximal small intestine, after being released from the carrier-proteins by the gastric acid and proteolytic enzymes, riboflavin is absorbed passively across the intestinal mucosa (100). In the cytoplasm of cells, particularly in the liver, heart and kidney, riboflavin is first phosphorylated to form flavin mononucleotide (FMN). FMN can be further phosphorylated into flavin adenine dinucleotide (FAD) (101). FAD is the common form in humans, and is incorporated in complexes with enzymes or proteins to form flavoproteins, that act as catalysts in a number of mitochondrial oxidative and reductive reactions (100). Riboflavin is involved as coenzymes in multiple cellular metabolic pathways, including the energy producing respiratory pathways. The RDA is between 0.3-0.4 mg/d for infants during the first year of life (34) (Table 2).

#### **Riboflavin status and deficiency during infancy**

The prevalence of riboflavin deficiency is uncertain, but deficiency is often endemic in many populations with low intake of foods of animal origin and green vegetables. A high prevalence of riboflavin deficiency has been reported in various population groups in high-income countries like USA, France and the United Kingdom (102, 103). A report from National Diet and Nutrition Surveys of the United Kingdom has revealed a prevalence of marginal biochemical riboflavin deficiency in 95% of adolescent girls (104, 105), and a report from the same country showed that 66% of low milk consumer adults had marginal riboflavin status which was correctable by supplementation (106). The high prevalence of riboflavin deficiency among young adults is reason for concern, since infants born by deficient mothers will be deficient at birth (107).

The prevalence of riboflavin deficiency in low-income countries has been reported to be 30% in Kenyan school children (108) and in 14% of Ghanaian infants

(109). Many cases are undetected due to the mild nature and nonspecific signs and symptoms of deficiency (110).

### **Symptoms and sign of riboflavin deficiency**

Symptoms associated with riboflavin deficiency are sore throat, stomatitis, glossitis and normocytic-normochromic anemia (111, 112), but deficiency is often accompanied with deficiency of other water-soluble vitamins, which can cause similar symptoms (112, 113). Some possible consequences of riboflavin deficiency in human might be reduced motor skills and attention span (114).

### **1.1.4. Folate**

#### **Sources and metabolism of folate**

Animal products and leafy vegetables contain folate in the polyglutamate form, which undergo hydrolysis in the gut to monoglutamates before intestinal absorption into plasma (115). Folate passes into the hepatocytes and other cells by binding to a folate receptor, and once inside the cell, folate is polyglutamated mainly in the form 5-methyl-tetrahydrofolate (116, 117). The RDA of folate is 65-80 µg/day, and about half of the total body content (5-10 mg) is stored in the liver.

Folate acts as coenzyme for enzymes involved in one-carbon metabolism and the synthesis of pyrimidines and purines, and different amino acids, like serine and methionine (118).

Folate status is often assessed by measurement of serum folate and plasma tHcy, as folate deficiency impairs remethylation of Hcy and causes elevated tHcy. However, in the first years of life, folate levels are usually high and weakly related to tHcy, and the main determinant of tHcy is cobalamin (3, 54, 119, 120), whereas, in older children and adults, serum folate is strongly correlated to and the main determinant of plasma tHcy (39, 45, 46).

#### **Folate status and deficiency during infancy**

Folate deficiency is one of the most common micronutrient deficiencies worldwide, and frequent causes are malnutrition and starvation (121, 122). Inadequate folate status

in women of reproductive age has been linked to adverse pregnancy outcomes (123), particularly neural tube defects (NTD) (124, 125).

Folate deficiency is rare in healthy term newborns (11), and several studies of healthy infants in high-income countries have reported higher serum folate in infants than in adults and older children, with a common pattern showing an increase from birth to 6 months with a subsequent decline until age 1-3 years (39, 60). Breastfed infants are usually protected against folate deficiency because the human milk contains relatively high folate concentrations with high bioavailability (126-128). Furthermore, the supply of folate from breast milk is relatively independent of maternal folate status (129, 130). A study of healthy Norwegian infants showed no differences in serum folate between breastfed and non-breastfed infants (60). Low folate levels are reported to be common in low BW (LBW) and premature infants, due to small fetal stores and the great demand during growth, and folic acid supplementation is commonly recommended for the first 3 months of life in infants with a BW < 2500 g (14, 15).

### **Symptoms and sign of folate deficiency**

Infants with folate deficiency may develop pancytopenia, including megaloblastic anemia, neutropenia and thrombocytopenia. Mental retardation has been demonstrated in infants born with inborn errors of folate metabolism (131-133). The relationship between maternal folate deficiency during pregnancy and cognitive development of their offspring has been questioned (134), and the issue is not settled (135).

Folate has a documented protective role in NTD (124, 125) and as the neural tube closes at day 25 (23-26) after conception, most countries, including Norway, recommend a daily folate intake of 400 µg from one month before conception through pregnancy week 12, to all fertile women planning a pregnancy (136-138). However, data from 13 European countries over a 10-year period, before and after the recommendation was introduced, showed that the current recommendation has not yet had any measurable impact on the rates of NTD (136). The prevalence of NTD has only shown a reduction in countries where flour has been fortified with folic acid (139).

### 1.1.5. Homocysteine (Hcy)

#### Metabolism of Hcy

Hcy is a non-protein amino acid formed from Met after removing the terminal methyl group. Hcy can be remethylated into Met or during Met excess, converted to cystathionine by the condensation of Hcy and serine, called the transsulfuration pathway, reactions which use PLP as a cofactor. The remethylation of Hcy back to Met is catalysed by the enzyme methionine synthase using 5-methyl tetrahydrofolate as a co-substrate and cobalamin as a cofactor (figure 1).

Both folate and cobalamin deficiency hampers remethylation of Hcy, and in this situation Hcy is exported out of the cell and into the extracellular compartment. The plasma level of tHcy is a sensitive marker of both intracellular folate and cobalamin status (140). In serum and plasma Hcy exists in several forms with the protein-bound Hcy as the predominant form, and different Hcy forms are collectively measured as tHcy (141).

#### *tHcy during infancy*

The tHcy concentration in the umbilical artery of the fetus is reported to be lower compared to the maternal tHcy concentration (142), but is highly related to maternal folate and cobalamin status (120, 143). In various trials the mean tHcy concentration in newborns and infants is reported to be 6-9  $\mu\text{mol/L}$ , considerably higher than the mean tHcy concentrations reported in children 1-15 years of age (4-8  $\mu\text{mol/L}$ ) (39, 54, 119, 120) (Table 3). Total Hcy is high during the first 6 months of life, decreases thereafter and then increases from age 7 years until adult levels are reached after puberty (39, 40, 144, 145). Some studies have shown slightly higher tHcy concentrations in boys than in girls (145, 146), and variation according to ethnicity with higher tHcy in black than in white and Hispanic children (46, 147). Poor nutrition with low vitamin intake and vegetarian diets are associated with high tHcy concentrations. Several case reports of infants, who were exclusively breastfed for extended periods, report signs of impaired cobalamin status with elevated tHcy (44, 47).

Both folate and cobalamin deficiency leads to elevated tHcy in plasma in infants, older children and adults. In the first year of life, plasma tHcy shows a strong inverse correlation to serum cobalamin and a weak or absent relationship to folate (3, 54, 119, 120). In comparison, in children above 2 years and adults there is a strong inverse correlation between plasma tHcy and serum folate, and a weaker inverse correlation between plasma tHcy and serum cobalamin (39, 45, 46, 144).

**Table 3. Total Hcy and MMA in infants and children according to age**

Publication	Age	No.	tHcy $\mu\text{mol/L}$	MMA $\mu\text{mol/L}$	Comments
Minet(54)	1-28 d	123	7.8 (3.1) <sup>1</sup>		
	4-20 w	30	10.4 (3.4) <sup>1</sup>		Breastfed
		30	7.0 (1.7) <sup>1</sup>		Formula-fed
Fokkema(55)	40 d	15	9.1 (2.4) <sup>1</sup>		Breastfed
	40 d	53	7.4 (1.6) <sup>1</sup>		Formula-fed
Bjørke-Monsen(3)	4 d	173	6.2 (5.0-7.5) <sup>2</sup>	0.29 (0.24-0.38)	Increase with age
	6 w	45	7.4 (6.5-8.9) <sup>2</sup>	0.81 (0.37-1.68)	
Bjørke-Monsen(39)	4 d	173	6.2 (5.0-7.5) <sup>2</sup>	0.29 (0.36-1.51) <sup>2</sup>	
	1.5-6 m	118	7.5 (6.1-9.2) <sup>2</sup>	0.78 (0.36-1.51) <sup>2</sup>	
	1-10 y	172	5.2 (4.7-6.0) <sup>2</sup>	0.13 (0.11-0.17) <sup>2</sup>	
Hay(60)	At birth	361	3.9-9.6 <sup>3</sup>	0.17-0.50 <sup>3</sup>	
	6 m	213	4.7-12.0 <sup>3</sup>	0.14-2.20 <sup>3</sup>	Breastfed
		41	3.8-8.8 <sup>3</sup>	0.16-0.50 <sup>3</sup>	Formula-fed
	12 m	115	3.6-7.9 <sup>3</sup>	0.12-0.83 <sup>3</sup>	Breastfed
		127	3.1-6.8 <sup>3</sup>	0.12-0.40 <sup>3</sup>	Formula-fed
Karademir(58)	2 d	204	7.1 (2.9-25.5) <sup>4</sup>		Breastfed
			8.18 (3.2-19.6) <sup>4</sup>		Formula-fed
	2 m	204	12.8 (5.1-41.8) <sup>4</sup>		Breastfed
			10.2 (4.7-17.0) <sup>4</sup>		Formula-fed
	6 m	204	8.1 (3.4-40.7) <sup>4</sup>		Breastfed
			6.2 (3.6-17.7) <sup>4</sup>		Formula-fed

Hcy, Homocysteine; MMA, Methylmalonic acid; d, days; w, weeks; m, months;

<sup>1</sup>Mean (95% CI)

<sup>2</sup>Median (range)

<sup>3</sup>The 5th and 95th percentiles

<sup>4</sup>Mean (range)

### **1.1.6. Methylmalonic acid (MMA)**

#### **Metabolism of MMA**

MMA is a structural isomer of succinyl-CoA and is normally derived from propionyl-CoA as a part of the catabolic pathways of isoleucine, valine, threonine, Met, cholesterol and odd-chain fatty acids. For the conversion of D-methylmalonic CoA to succinyl-CoA, two enzymes are involved; methylmalonyl CoA racemase which forms L-methylmalonic CoA, and methylmalonyl CoA mutase which converts the L-methylmalonic CoA to succinyl-CoA. The latter enzyme requires adenosylcobalamin as a co-factor. Deficiency of cobalamin causes accumulation of MMA and its precursors (148), and MMA is therefore a sensitive and specific marker of cobalamin status (140).

#### **MMA during infancy**

Plasma and urine MMA levels vary during infancy, and the highest values are seen during the first 6 months. Bjørke-Monsen et al observed median MMA concentrations above 0.78  $\mu\text{mol/L}$  in infants of 6 weeks to 6 months of age, plasma MMA decreased after 6 months and remained low (MMA < 0.26  $\mu\text{mol/L}$ ) (39). In another study of infants born to healthy non-vegetarian mothers, newborns had a median MMA concentration of 0.29  $\mu\text{mol/L}$ , and after 6 weeks, the plasma MMA had increased to 0.81  $\mu\text{mol/L}$ . In a Dutch study 10-20 months' old children had plasma MMA levels ranging from 0.06-0.51  $\mu\text{mol/L}$  (40) (Table 3).

The reference range for urine MMA in infants has been reported to be higher and wider than in children and adults (57, 63). Several reports have demonstrated higher urinary and plasma MMA levels in breastfed infants compared to formula-fed infants (47, 57). The explanation for this phenomenon is unclear, but one reason could be degradation of odd-chain fatty acids present in breast milk to MMA precursors or increased production of propionate and MMA precursors by intestinal bacteria (39, 47, 149, 150). Alternatively, high plasma MMA may reflect impaired cobalamin status in the breastfed infants, as formula is supplemented with cobalamin and deficiency is therefore rare in formula-fed infants (54, 55, 57, 59).

### **1.1.8. Iron**

#### **Sources and metabolism**

Iron is a vital micronutrient for all cells and is used in more than 400 different enzymes and proteins in the human body. The majority of iron is bound in heme proteins, particularly Hb and myoglobin. The remainder is bound in the storage proteins ferritin and hemosiderin, and only a small amount (3%) is bound in enzyme systems such as catalase and cytochromes (151).

Dietary iron is present in two main forms, heme iron and non-heme iron. Meat, poultry and fish contain heme iron, which has a higher bioavailability than non-heme of vegetables and fruits, as well as iron-fortified food, which usually contains non-heme iron. Uptake and metabolism of iron is strictly regulated in the body and involves a number of specific proteins in a complex interplay between iron absorption, iron recycled from the breakdown of old red blood cells, release of iron from stores and iron loss from the body. The iron absorption is strongly regulated since there is no mechanism for excreting iron (152)

#### **Measurement of iron status in infants**

Iron status is investigated by using a battery of various laboratory analyses like Hb, MCV, percentage of hypochromic red cells (%HYPO), ferritin, zinc protoporphyrin (ZPP) and transferrin receptor (TfR). During the first months of life, interpretation of iron status may, however, be difficult due to substantial physiological changes in the erythrocyte parameters and other common iron markers like ferritin and soluble TfR (sTfR) (153, 154). During the first days after birth the erythropoiesis decreases dramatically as a result of the greater oxygen supply available outside the womb and because of the degradation and the shorter life span of fetal red blood cells (60-70 days). The Hb level and erythrocyte count decrease and reach their lowest values approximately 2 months after birth (155, 156). The size of the erythrocyte, determined by MCV, also decreases after birth. It is smallest at 6 months of age, but increases gradually thereafter until adult's levels are reached (156).

Iron is an essential component of Hb, and erythrocyte parameters are good markers of iron status (157). Ordinary red cell parameters, like Hb and MCV, reflect



mean values of an erythrocyte population, which has an average lifespan of 120 days. Reticulocytes represent the youngest erythrocytes in peripheral blood, and their hemoglobin content can be measured specifically on the basis of RNA residues in these cells. The measurement of reticulocyte hemoglobin content (CHr), however, is a direct assessment of the incorporation of iron into erythrocyte hemoglobin and provides an indirect measure of functional iron available for new red blood cell production in the bone marrow. CHr therefore provides a real time evaluation of the erythropoiesis in the preceding 24-48 hours (158).

Determination of CHr concentration is useful during periods with rapid physiological and hematological changes, as during pregnancy, lactation and infancy (157). CHr has also been shown to be an early indicator of iron-restricted erythropoiesis in patients receiving erythropoietin therapy (159, 160). Mast et al. showed that CHr was found to be a better predictor of iron stores than ferritin, transferrin saturation and MCV when bone marrow iron analysis was used as the criterion standard (161). In several studies, CHr has been suggested as a useful marker of iron deficiency in children (157, 162, 163), and superior to traditional iron parameters like serum ferritin and transferrin saturation (157).

Due to the physiological lower MCV and mean corpuscular hemoglobin levels in infants and toddlers, lower CHr cut-off levels in the range of 25.0-27.5 pg have been suggested as indicators of iron deficiency in this age-group (157, 162, 163)

### **Iron status during infancy**

Apart from maternal iron status, GA and BW strongly affect the infant's iron stores at birth. A study from 1951 where whole-body iron content was assessed in aborted and stillborn fetuses, a linear relation between body iron and BW was found and estimated to be 75mg/kg body weight (164). The concentration of iron in liver, spleen and kidney remains relatively constant during gestation (165), but during the last 8 weeks of gestation, the liver increases in size resulting in increased total amount of liver iron (166).

The time point for clamping the umbilical cord is another factor, which may affect infant iron status. Chaparro et al. found that a 2-minute delay in clamping the

umbilical cord at birth significantly increased infant iron status at 6 months of age (167).

The heme iron released during lysis of fetal red blood cells, the iron stores at birth and the postnatal diet are the main sources of iron during the first months of life. The rapid postnatal weight gain and the related expanding hemoglobin and myoglobin mass, are associated with increased iron requirements (168, 169), and the minimum daily requirements for iron in infants are about 10 mg (34).

In term infants with a normal BW, fetal iron stores are considered sufficient for the first 6 months of life, even when exclusively breastfed (168), whereas daily iron supplementation from 1- 2 months throughout the first year of life is commonly recommended for LBW infants (< 2500 g) due to low fetal iron stores and rapid catch-up growth (16). However, recent data suggest that 6 months of exclusive breastfeeding may be associated with a poorer iron status also in children with higher BW (7-9). The iron content in breast milk is only 1.5 mg/L compared to formula milk with 7-15 mg/L, but the bioavailability is much higher in human milk. Different studies estimate iron absorption to be 12-56% from human milk, depending on assay methods, infant age and iron status (170-173), and 3-11% from formula milk (170, 174, 175).

During the second half year of life the requirements for exogenous iron increase and the introduction of pureed meats and iron-fortified cereals is recommended (18).

#### *Prevalence of iron deficiency in infants.*

There is no consensus concerning which iron parameters should be used to confirm iron deficiency (ID) and IDA, and there is no consensus on the cut-off levels for either ferritin or Hb. In 6-12 months old infants, the usual requirement for identifying ID is serum ferritin < 10-12  $\mu\text{g/L}$  (176, 177) and for diagnosing IDA, Hb < 110 g/L (178, 179), but the cut-off value of Hb < 110 g/L has been questioned (180). In a Norwegian study from 2004 (181), the prevalence of IDA (Hb < 110 g/l and ferritin < 15  $\mu\text{g/l}$ ) at 6 months of age was 3% and increased to 10% at 12 months, similar to that found in Danish and Swedish infants (182, 183).

#### *Gender differences in iron status during infancy.*

Some studies have described substantial differences in iron status between infant girls and boys, and Domellof et al observed lower Hb, MCV, ferritin and higher ZPP and sTfR in boys at 4, 6 and 9 months compared to girls, and a prevalence of IDA of 17% in boys vs. 2% in girls at 9 months(184). The differences remained significant when controlling for BW and postnatal weight gain (184). Other studies have confirmed that boys have lower iron- stores, predisposing them for iron deficiency during infancy (185-187).

### **Symptoms and signs of iron deficiency in infancy**

Symptoms of iron deficiency with or without anemia depend on the degree of deficiency and the rate at which the anemia develops. Infants with iron deficiency without anemia have seldom symptoms. The first sign of anemia is pallor, and other symptoms attributing to the degree of anemia are fatigue, excessive sleepiness, tachycardia and systolic murmurs. Long-term iron deficiency may affect growth (188, 189).

Impaired psychomotor development and cognitive function are well described in infants with IDA (27, 28), but also iron deficiency without anemia in the growing infant may cause impaired psychomotor development with potential permanent intellectual deficits (28, 190, 191). Early recognition of iron deficiency and intervention are therefore essential. In some trials iron supplementation prevented or even corrected impairments in psychomotor development (192-196), but other studies suggest that a complete recovery may not be accomplished in moderate to severe IDA in infants (197, 198).

### **Treatment**

Treatment of iron deficiency is easy and inexpensive and given the concern of impaired neurological development in infants with IDA and ID, supplementation with iron is recommended (18). However, iron is a pro-oxidant and potentially toxic and also an essential nutrient for bacterial proliferation, and humans have no ability to excrete iron. In a study from Africa, involving children with IDA and protein malnutrition, iron supplementation lead to reactivation or progression of infections like

malaria and tuberculosis (199). However, a systematic review of randomized controlled trials with iron supplementation in children, showed no increased risk of infectious illness (200, 201).

## **1.2. Infant nutrition**

### **1.2.1. The rationale behind exclusive breastfeeding for six months**

Human milk is perceived to be a complete and perfect food for the infant during a period of rapid growth and development, and the WHO recommended in 2001 that human milk should be the exclusive nutrient source for term infants for the first six months of life (6, 202). Exclusively breastfeeding defined by WHO (203) is feeding the infant only with breast milk, without any additional food and drink with the exception of drops or syrups consisting of vitamins, mineral supplements or medicines. Predominant breastfeeding means that the predominant source of nourishment is breast milk, but the infant may receive water and water-based drinks and juice, in addition to drops or syrups consisting of vitamins, mineral supplements or medicines. No food-based fluid is allowed under this condition. Full breastfeeding is defined as exclusively breastfeeding and predominant breastfeeding together and complementary feeding means that the infant receives breast milk together with solid or semi-solid food (formula). The advice of six months of exclusive breastfeeding rested largely on Kramer and Kakumas systematic review of infant and maternal health effects of exclusive breast-feeding for six months versus three to four months (202). The review included 23 studies, 11 from low-income countries including two controlled trials from Honduras, and 12 observational studies from high-income countries.

The overall conclusion was that infants exclusively breastfed for six months had a reduced risk of gastrointestinal infection and no observable deficits in growth. However, no protection against obesity or allergic disease was revealed, nor any benefits in cognitive ability or behaviour, compared with infants exclusive breastfeed for three to four months.

The Norwegian health authorities have also recommended exclusive breastfeeding for the first 6 months of life and thereafter gradual introduction of appropriate complementary foods along with continued breastfeeding (18, 204). In Norway, we have a positive attitude to extensive breastfeeding (205) and our long and paid maternal leave supports the possibility to breastfeed.

### **1.2.2. Breast milk**

The composition of human milk is complex. It contains multiple nutrients, immune-related components and various biologically active substances that contribute to efficient nutrient uptake and utilization and gives the infant active and passive protection against infections (206). The concentration of some nutrients is relative constant while others depend on the nutritional status of the mother and duration of lactation (206). The various nutrients can be categorized into two groups according to their secretion into breast milk (5, 10). Some vitamins like thiamine (vitamin B1), riboflavin, vitamin B6, cobalamin, choline, vitamin A and vitamin D, and minerals, like selenium and iodine, easily enter breast milk, and both maternal status and supplementation during lactation affect breast milk concentrations and supply to the infant (5, 10). In contrast, the concentration of folate and iron, calcium, copper and zinc in breast milk are relatively unaffected by maternal intake or status (5, 10).

Different studies have reported variable amounts of B vitamin levels in human milk, depending upon the method used for assay, stage of lactation and maternal plasma level during lactation (58, 59, 63, 207).

The concentrations of cobalamin in human milk vary from 150-700 pmol/L (59, 63, 207, 208) and correlate significantly with maternal plasma levels (63, 209). Several reports show a progressively fall in total cobalamin concentrations during the lactation period (210, 211), while Specker et al did not observe a decrease from 2 to 14 months postpartum (63). During lactation the reduced cobalamin content in the human milk is thought to be compensated by increased milk output to the infant (210), but a study from USA estimated that the total cobalamin intake for the infants (based on a daily milk intake of 150ml/kg body weight) was at its peak at 12 weeks postpartum and then declined progressively to about 50% at 27-35 weeks (59).

For riboflavin, the concentration in human milk from well-nourished women has been reported to be in the range of 0.18-0.80 mg/L (212), while studies from low-income countries have reported concentrations between 0.16-0.22 mg/L (107, 213, 214). Reports from low-income countries showed that maternal riboflavin deficiency rapidly resulted in low milk concentrations (107, 213, 214), and a strong correlation between maternal intake and concentration of riboflavin in breast milk was observed in

a study of well-nourished Russian women (215). Maternal supplementations post-delivery increased the milk concentration of riboflavin (214) and during the lactation period, an increase in both riboflavin and vitamin B6 in breast milk has been reported (98, 207, 215).

The mean concentration of vitamin B6 in human milk from Egyptian peri-urban mothers was found to be 0.11 mg/L, and about 40% of the them had low levels, i.e. < 0.10 mg/L (99). A report from USA demonstrated a rapid increase in milk concentration of vitamin B6 in mothers supplemented with pyridoxine hydrochloride (216) and other reports have shown that most mothers, despite a vitamin B6 intake consistent with the RDA, produce milk that does not meet the RDA for infants (217, 218).

During the lactation period folate in human milk is reported to increase, decrease and remain stable (128, 129). Reported values of folate in human milk range from 81 to 85 µgram/L (85, 130), and breastfed infants are usually protected against folate deficiency during lactation, because human milk contains relatively high folate concentrations (126) which is fairly independent of maternal folate status (126, 129, 130). In a study from rural Mexico the milk concentration of folate was unrelated to maternal status and unaffected by maternal supplementation, but the mothers became more depleted as lactation progressed (219). To preserve the maternal folate stores, folic acid supplementation during lactation is important considering maternal health, later conception and pregnancy (220).

During lactation, a wide range of iron values in breast milk have been reported (0.1-1.6 mg/L) (221-228) with the highest content in colostrum (1.0 mg/L) (228) and early transitional milk (0.97 mg/L) (229). With duration of lactation the iron content decreases steadily from an approximate level of 0.35 mg/L at 1 month to 0.20 mg/L at 6 months (230). An infant consuming 750-800 ml breast milk/day (at 1 month) will receive approximately 0.27 mg iron per day, but although the bioavailability is high in human milk, different studies estimate an iron absorption of only 12-56% from human milk, depending on the assay methods, infant's age and iron status (170-173) Also diurnal variation has been observed for the iron content of human milk, with lower mean value in the early morning compared to late evening (231).

The majority of reports mainly from high-income countries, report no correlation between iron content in breast milk and Hb level and iron status in lactating women (225, 232-234) and Shashiraj et al confirmed this lack of association in a study from India where iron content in breast milk from non-anemic and anemic mother were measured (235).

### **1.2.3. Infant formula**

Infant formula is a manufactured food designed for feeding infants below 12 months of age. The content is roughly based on content of human milk 1 to 3 months postpartum, including whey and casein as a protein source, a blend of vegetable oils as a fat source, lactose as a carbohydrate source and a vitamin-mineral mixture (236). As formula is supplemented with many vitamins (57-59, 207), studies show that formula-fed infants have a better micronutrient status compared to breastfed infants (54, 55, 63). Compared to formula milk, variable, but always lower B vitamin status is reported in breast milk, depending somewhat on the assay used, the stage of lactation and maternal vitamin levels (57, 58, 237).

In 2001 the WHO concluded that infant formula was a safe and suitable breast milk substitute, but the use of formula in low-income countries is linked to poorer health outcomes because of the prevalence of unsanitary preparation conditions, including lack of clean water and lack of sanitizing equipment (238).



**Table 4. Vitamin and iron contents in human milk and in different formula milk**

Vitamin per L prepared milk	Human milk	NAN H.A.1 <sup>1</sup>	NAN 1 <sup>2</sup>	NAN 2 <sup>3</sup>	Collett <sup>4</sup>	Nutramigen <sup>5</sup>
Riboflavin, B2, mg	350 <sup>*</sup> /384 <sup>**</sup>	1.6	1.4	1.4	1.0	0.6
Pyridoxine, B6, mg	93-205 <sup>*</sup> /57 <sup>**</sup>	0.5	0.5	0.5	0.2	0.4
Cobalamin, B12, µg	0.5-1.0 <sup>*</sup> /0.4 <sup>**</sup>	1.4	2.4	0.9	1.0	2.0
Folic acid, µg	24-50 <sup>*</sup> /62 <sup>**</sup>	110	95	150	60	109
Iron, mg	0.1-1.6 <sup>†</sup>	6.9	4.1	10.0	7.0	12.2

<sup>1</sup>NAN H.A.1 is a specially treated formula (partly hydrolysed) that is easy to digest, used from birth to 6 months. Produced by Nestle Barnemat<sup>®</sup>, Norway

<sup>2</sup>NAN1 Formula used from birth to 6 months. Produced by "Nestle Barnemat", Norway

<sup>3</sup>NAN2 Formula used from 6 months of age. Produced by "Nestle Barnemat", Norway

<sup>4</sup>Collett Formula used from birth to 6 months. Produced by Axellus AS, Norway

<sup>5</sup>Nutramigen1 Lipil is a special formula for infants with cow's milk protein allergy and/or lactose intolerance. Produced by Mead Johnson Nutrition, Norway

<sup>\*</sup>Do breastfed infants need supplemental vitamins? Frank R Greer

<sup>\*\*</sup>Fat-soluble and water-soluble vitamin contents of breast milk from Japanese woman. Sakurai et al

<sup>†</sup>Mothers iron status, breast milk iron and lactoferrin –are they related? Shashiraj et al

#### **1.2.4. Prevalence of breastfeeding**

In Norway we have relatively high breastfeeding rates and almost all mothers breastfeed their infants after birth (205). However, despite the current recommendation of 6 months' exclusive breastfeeding, only 7-10% of Norwegian infants are exclusively breastfed at 6 months compared to 84-90% at one month, 65-70% at 3 months and 44-48% at 4 months of age (239, 240). In a recent Norwegian study, the total number of breastfed infants (including exclusive and partial breastfeeding) slowly decreased from 96% at 1 month, to 82% at 6 months and to 46% at 12 months of age (239). Only 1-1.5% of Norwegian infants had never been breastfed (239, 240). Similar high rates of initiating breastfeeding are found in other Nordic countries (182, 241, 242), while countries like UK (243) and USA (244) have a much lower rates (66% and 60%, respectively).

#### **1.2.5. Characteristics of the breastfeeding woman**

Both initiation and duration of breastfeeding, both exclusive and partial, are influenced by a number of factors. The maternal decision to breastfeed is associated with social status, maternal age, marital status, education, parity and smoking (239). A study from Norway revealed significant positive trends for exclusively breastfeeding during the first 6 months with level of maternal education and number of children (239). Smoking has been associated with shorter periods of exclusively (245) and partial breastfeeding compared to non-smoking mothers (239, 240). Some studies have also shown that breastfeeding occurs more frequently among married women and that they also breastfed for a longer period than mothers living in partnership or alone (240, 246). In the literature the association between parity and breastfeeding is inconsistent (246, 247), but one report showed that exclusively breastfeeding increased with rising number of children (239).

Breastfeeding duration is also found to be associated with others factor like social status, maternal work situation (248, 249) and insufficient milk supply (250, 251). Infant health problems like infant disease, low BW, prematurity and being a twin, are all factors associated with shorter duration of breastfeeding (182, 240, 245, 246).

### **1.2.6. Short and long-term effects of exclusive breastfeeding for 6 months**

In a systematic literature review concerning short- and long-term health effects of breastfeeding in a Nordic setting (252), the authors concluded that breastfeeding had a protective effect against infections like acute otitis media, gastrointestinal and respiratory tract infections, and overweight and obesity in childhood. The authors also concluded that there were some evidence that breastfeeding was a protective factor against inflammatory bowel disease, celiac disease, and diabetes (type 1 and 2), and had beneficial effects on IQ and developmental scores of children, as well as a small reductive effect on blood pressure and blood cholesterol levels in adulthood (252). However, several of the studies used in this review, did not differentiate between exclusive and partial breastfeeding.

The scientific rationale behind the WHO recommendation of exclusive breastfeeding for the first 6 months has been questioned (253). Fewtrell et al express concern about higher risk of IDA, a higher incidence of food allergies and a higher risk of coeliac disease in infants exclusively breastfed for six months versus four to five months (253). The European Food Safety Authority's panel concluded that complementary foods may be introduced safely between four to six months and that exclusively breastfeeding for six months not always provided sufficient nutrition for optimal growth and development (254).

A recent study from Norway indicated that introduction of gluten later than 6 months of age was associated with an increased risk of coeliac disease (255). During 1984-1996, Sweden experienced an epidemic of coeliac disease in children less than 2 years old (256). In the same period, the Health authority recommended exclusively breastfeeding for 6 months, and subsequent studies have shown a positive correlation between delayed introduction of gluten and the diagnosis of coeliac disease (257, 258). The prevalence of coeliac disease was reduced when gluten was introduced in an earlier age (257).

An association between breastfeeding and better cognitive ability in children was reported more than half a century ago (259), but a causal relationship has not been establish. Breastfeeding is often associated with socioeconomic advantage in

industrialized communities, and the quality of home environment plays an important role in child development (260). Many of the studies on the relation between breastfeeding and cognitive development in children do not adjust for such confounding factors and do not differentiate between exclusive and partial breastfeeding. The results are also diverging, as some author claim a positive effect of breastfeeding on IQ, whereas others do not (260-265). There is only one study demonstrating a higher IQ related to exclusive breastfeeding, but in this study exclusive breastfeeding was limited to 3 months (262).

Deficiency of vitamins D and K have been well described in breastfed infants (266-269), but also iron and cobalamin deficiency have been reported in infants exclusively breastfed for a longer period (10-13, 270). There are scarce data on other B vitamins in lactating infants, but riboflavin and B6, have been documented to be rather low in pregnant and lactating women in both high-income and low-income countries and this may have serious implications for their breastfed infants as maternal riboflavin and B6 status during lactation is known to affect the breast milk concentration (99, 214, 271)

### **1.3. Infants with BW 2000 – 3000 g**

BW less than 2500 g is defined as LBW, between 2000-2500g defined as marginally LBW (MLBW) and BW less than 1500 g as very low BW. Normal BW is defined as BW between 2500-4500g. In our work we have elected to describe BW between 2000-3000g as subnormal BW.

The global prevalence of LBW is estimated to be 14% and 7% in high-income countries (272). In Norway, approximately 5% of the infants are born LBW (273). The majority of the LBW infants have a BW between 1500 and 2500g, and more than 50% have a MLBW. LBW may be due to prematurity defined as live birth before 37 completed weeks of pregnancy and/or being SGA at birth. There is no established definition of SGA, but in a clinical setting it is usually defined as a BW less than the 10<sup>th</sup> percentile for GA, while in epidemiological studies it is often defined as a BW less than 2 standard deviations (SD) below the mean for GA, i.e. less than the 2.5<sup>th</sup>

percentile. SGA infants include infants with poor intrauterine growth or constitutionally small infants with genetic predisposition for small size.

Infants with BW between 2000 - 3000 g are a heterogeneous group, including infants born moderately preterm or at term with or without SGA status. There are scarce data on this group as these infants are often excluded from clinical studies of both preterm and term infants. However, during recent years these infants have been more commonly studied, and both increased mortality and morbidity, perinatal as well as later in life, have been reported (274, 275).

Evidence from both animal and human studies shows that normal variations in fetal size at birth, maternal body composition and diet, and nutrition and growth during early life, have implications for later health (276). The “fetal origins hypothesis” proposes that fetal under-nutrition in middle to late gestation causes a disturbance in metabolic programming leading to cardiovascular, metabolic and endocrine disease in adult life (277). One explanation behind this observation may be that growth acceleration in low birth weight infants after birth leads to inadequate metabolic imprinting (278-282). The greatest acceleration in growth takes place in the first weeks after birth (283, 284), and factors that promote growth during this crucial window could be especially important in adversely programming later cardiovascular health. It has been suggested that reduced growth and nutritional restriction during this period may reduce the risk for later cardiovascular disease (285, 286).

LBW is a known risk factor for lower stores of several micronutrients, and iron, folic acid, and multi-vitamin supplements are commonly recommended for infants with a BW < 2500 g (14, 16, 17, 19). However, infants with a suboptimal BW (2500-3000 g) may also have insufficient stores of micronutrients, but so far routine supplementations to these infants have not been implemented.

#### **1.4. Neurodevelopment in infant**

Neurodevelopment in infants is often measured in terms achievement of developmental milestones according to age. The domains of development are usually categorized into 4 major areas: fine and gross motor, language, cognitive, and personal and social development (31). Several factors like genetic and cultural differences,

parental sociodemographic factors and child characteristics influence neurodevelopment (287, 288).

Developmental delay is a term used to describe a child who does not reach developmental milestones at the expected age. Approximately 5-10% of the pediatric population has a developmental impairment (289) and factors like LBW; shorter GA and being a twin have been associated with an increased risk of delayed motor development (287, 288).

#### **1.4.1. Gross motor development during infancy**

Gross motor function is an important and good marker of neurodevelopment in early infancy (29-31). Motor development is influenced by several factors, like cultural and sociodemographic factors, and maternal nutrition (32, 287, 288), as well as infant characteristics, such as LBW, short gestation and being a twin (287, 288).

Additionally, infant nutrition, including micronutrient status, plays an important role in the initial development of the brain and infant motor development (1, 32, 33), as demonstrated by a study from Bangladesh where weekly multivitamins, iron and zinc supplementation from 6 to 12 months of age was reported to have a beneficial effect on infant motor development (290).

There are different ways of understanding motor development and motor learning. “The neuromaturational theories” suggest that motor development is intrinsically preprogrammed in the central nervous system (CNS) and that motor skills are based on predestined sequences of maturation of the CNS and not changeable by the environment. Alternatively, “the dynamic systems theory” proposes that motor development depends on interactions between multiple factors like body weight, muscle strength, the infant’s mood and specific environmental conditions together with brain development. A third variant, “the neuronal group selection theory”, combines the two aforementioned theories. According to this theory, the brain is dynamically organized into variable networks forming neuronal groups. These groups act as functional units, and development, experience, behaviour and environment can modify the structure and the function of the neuronal networks (291, 292).

### **1.4.2. Evaluating motor development**

To evaluate motor development, a major developmental function in early infancy (29-31) is challenging (293). Infants develop discontinuously, and the age of achieving developmental gross motor milestones varies substantially between healthy term infants (294). The strategy to identify developmental delay is usually through developmental surveillance, and/or by using developmental screening tests.

Developmental surveillance is a continuous procedure where health professionals gather information on the infant's development and behaviour by observing the infant in different situations and by obtaining information from the parents and other relevant professionals. Additionally, the physician takes a relevant developmental history and uses an age appropriate checklist to record developmental milestones. Research has shown that subjective clinical impressions may not be sufficient to detect developmental disabilities in children (295), and a survey revealed that only 30% of children with language disabilities, mental retardation and other developmental problems were detected when based only on subjective clinical judgment (296).

An adjunct to developmental surveillance is a developmental screening test, which may be defined as "a brief assessment procedure designed to identify infants and children who should receive more intensive diagnosis and further assessment" (297). Screening tests have shown to be superior compared to using developmental surveillance for detecting developmental delays in infants (296), and several professional organization, included the American Academy of Pediatrics, have recommended that all infants and children should be routinely screened for developmental delays (298, 299).

One of the most commonly used test to detect developmental disorders, is the Bayley Scales of Infant Development version II or III (BSID-II/III) (300), which may be considered a gold standard for evaluating psychomotor development in infant and children. The test evaluates the progressive functional development of children from 1 to 42 months of age divided into three scales; motor, mental and behavioural. The motor scale consist of 111 items that assess fine and gross motor development and the child's development is classified as significantly delayed, moderately delayed, normal

or accelerated. At 6 months BSID-II has 20 test items for motor development, with 8 dedicated to fine motor abilities and any items to be observed in the prone positions.

Another test is the Peabody Developmental Motor Scale-2 (PDMS-2) (301), an early childhood motor development program that provides both in-depth assessment and training or remediation of gross and fine motor skills. The assessment is composed of 6 subtests that measure interrelated motor abilities that develop early in life. It is designed to assess the motor skills of children from birth through 5 years of age.

### **Alberta Infants Motor Scale**

Alberta Infants Motor Scale (AIMS) is a neuromotor-screening test arising from the dynamic systems theory of development. The test is a norm-referenced observational instrument for evaluating gross motor development in infants from birth to 18 months (29). The test contains 58 items divided into 4 subscales; prone, supine, sitting and standing. Each item is described in terms of the weight-bearing surface of the body, the posture necessary to achieve the gross motor skill and the antigravity or voluntary movement performed by the infant in the position (302). The clinician observes the infant, and each item is scored as “observed” or “not observed” and the obtained score, 0 to 60 points, is converted to a normative age-dependent percentile rank (5th to 90th percentile).

The AIMS test was developed in the province of Alberta and based on the assessment of 2202 randomly sampled gender-age-stratified term infants. It has a high inter-rater and test-retest reliability and is considered to be among the most reliable tests for assessing gross motor function (303). To estimate the reliability, two therapists observed the same sample of 253 infants, and they obtained an inter-rater value of 0.96-0.99 and an intra-rater value ranging between 0.85 and 0.99(302, 303). Concurrent validity tested against other standardized motor testes, e.g. PDMS and BSID II, ranged from 0.84 to 0.99 (302, 303).

### **Ages and Stages Questionnaires**

Ages and Stages Questionnaires (ASQ) is originally a validated American screening tool recommended by the American Academy of Paediatrics for detection of



developmental delay in infants and small children (299). It consists of 19 age-specific questionnaires intended for use from 4 to 60 months of age. The questionnaire covers 5 developmental domains; communication, gross motor function, fine motor function, personal-social functioning and problem solving abilities, and each domain has 6 questions on developmental milestones. The parents evaluate whether the child has achieved a milestone (yes, 10 points), has partly achieved the milestone (sometimes, 5 points) or has not yet achieved the milestone (no, 0 points). Infants and children scoring at or below the cut-off on one or more areas should be considered for referral for further assessment.

ASQ has been validated in many countries and settings (304-307), and in most of the reports the questionnaire appears accurate in detecting true problems in apparently healthy children, even in children with biological risk factors (308, 309). Gollenberg et al assessed the concurrent validity of the ASQ compared with BSID II, for 24 months' old children. The ASQ domains communication, personal-social functioning and gross motor function were moderately correlated with the BSID II Mental Scale ( $R=0.45-0.52$ ,  $p<0.01$ ), but ASQ problem solving and fine motor function were not significantly correlated to BSID II (310).

## **1.5. The influence of B vitamin and iron status on neurodevelopment in infants**

It has been difficult to establish a causal relationship between B vitamin and iron deficiency and impaired neurodevelopment because several environmental indices such as maternal IQ or education, stimulation at home, occurrences of stressful events or even growth and other nutritional deficiencies may have confound the results in different trials (20, 311).

### **1.5.1. Significance of cobalamin on neurodevelopment**

Cobalamin plays an important role in the development of the brain, and long-term deficiency of cobalamin results in impaired myelination and demyelination in the spinal cord and brain (68, 70, 312-314). The pathophysiological mechanism is not fully understood, but involves reduced supply of methyl groups as a result of

inadequate remethylation of Hcy to Met and inappropriate conversion of methylmalonyl CoA to succinyl CoA. The result is excess propionyl CoA that leads to odd chain fatty acid synthesis and altered and deranged myelin structure (80, 315). Myelination is an indicator of functional brain maturation and is correlated to psychomotor development (71, 72). However, improved myelination cannot be the only neurological effect of cobalamin supplementation, since significant improvements after cobalamin supplementation have been reported within days in severely deficient infants (65, 316).

### **1.5.2. Significance of other B vitamins on neurodevelopment**

Vitamin B6 adequacy is known to be critical for the developing central nervous system, and deficiency may inhibit brain development and cognitive function (92, 317). Studies in animal models suggest that vitamin B6 deficiency during gestation and lactation alters the function of N-methyl-D-aspartate receptors, a subtype of receptors of the glutamatergic neurotransmitter system, which is thought to play an important role in learning and memory (317). A report from the 1950s described irritability and convulsive seizures in infants fed on a formula deficient in vitamin B6 (318), and changed behaviour with depression and mental confusion were observed in adults with low intakes of vitamin B6 (97). A low vitamin B6 status in breastfed infants was associated with slower growth (98), and recently there has been particular interest in the relationship between Vitamin B6 and autism, although no conclusions have been drawn (1, 319).

Folate plays a key role in brain development, and involves nucleotide synthesis, DNA integrity and transcription (320). Adequate maternal folate status is important for normal fetal growth and development (124, 125). One report revealed that infants born to mothers with severe folate deficiency during pregnancy showed abnormal or delayed development (134). Other studies are inconclusive in regard to cognitive development and folate status of mothers during pregnancy (125). Folic acid supplementation during pregnancy has been associated with reduced incidence of immaturity and periconceptional folic acid supplementation reduces the risk for NTD (124, 125).

### **1.5.3 Significance of iron on neurodevelopment**

Iron is an essential nutrient for normal brain development and function and ID in early life can affect both myelination and neurotransmitter function which may explain the association between iron deficiency and delayed neurodevelopment. Impaired psychomotor development and cognitive function are well described in infants with IDA (27), but also ID without anemia may cause impaired psychomotor development with potential permanent intellectual deficits (28, 190, 191). Causality and question of reversibility are not clear. In some trials iron supplementation prevented or even corrected impairments in psychomotor development (192, 194, 195, 321), while no complete recovery was observed after iron supplementation in children with moderate to severe IDA in other studies (321-323).

ID may alter behaviour in infants, as shown in a study of anaemic infants between 11-13 months. The anaemic infants were unhappier, more fearful and less attentive than non-anaemic infants (324, 325). Altered behaviour may isolate the child and contribute to enhanced impairment of psychomotor development.

A recent study by Berglund et al revealed a significant reduction in behavioural problems in 3 ½ year old children supplemented with iron from 6 weeks to 6 months of age (326). Another explanation of the reported behaviour changes related to iron deficiency may be an increased lead absorption. This is found in infants and children with ID and is known to have negative effects on cognitive function (327).

## 2. Aims of the study

An adequate micronutrient status during infancy, a period characterized by rapid growth and development, is important for optimal growth and development (1). Gross motor function, which is a major developmental function in early infancy (29-31), is known to be related to micronutrients status (32, 33).

The objectives for this study were

- To investigate whether cobalamin supplementation influence the biochemical profile of cobalamin status in healthy, term infants.
  - Our hypothesis was that cobalamin supplementation would improve the cobalamin status in term infants toward a profile observed in cobalamin-replete older children and adults.
- To evaluate the biochemical and clinical effects of cobalamin supplementation in infants with biochemical signs of cobalamin deficiency and developmental delay and /or feeding difficulties.
  - Our hypothesis was that cobalamin supplementation would improve psychomotor development and feeding difficulties.
- To investigate B vitamin status in infants with a subnormal BW (2000-3000 g) during the first 6 months of life according to nutrition, and relate nutrition and B vitamin status to gross motor development.
  - Our hypothesis was that infants with a subnormal BW who were mainly formula-fed had a better B vitamin status and motor development at 6 months compared to mainly breastfed infants.
- To investigate iron status in infants with subnormal BW during the first 6 months of life according to iron supplementation and nutrition.
  - Our hypothesis was that infants with a BW 2501-3000 g who were mainly breastfed had a risk of iron deficiency at 6 months.

### 3. Study populations and methods

#### 3.1. Study populations

The study involved 3 cohorts recruited between 2004 and 2010:

- Cohort 1: Healthy term infants (n=107) and their mothers (n=104)
- Cohort 2: Infants less than 8 months of age referred for subtle neurological symptoms and/or delayed psychomotor development or feeding difficulties (n=105)
- Cohort 3: Healthy infants with BW 2000-3000 g (n=97) and their mothers (n=89).

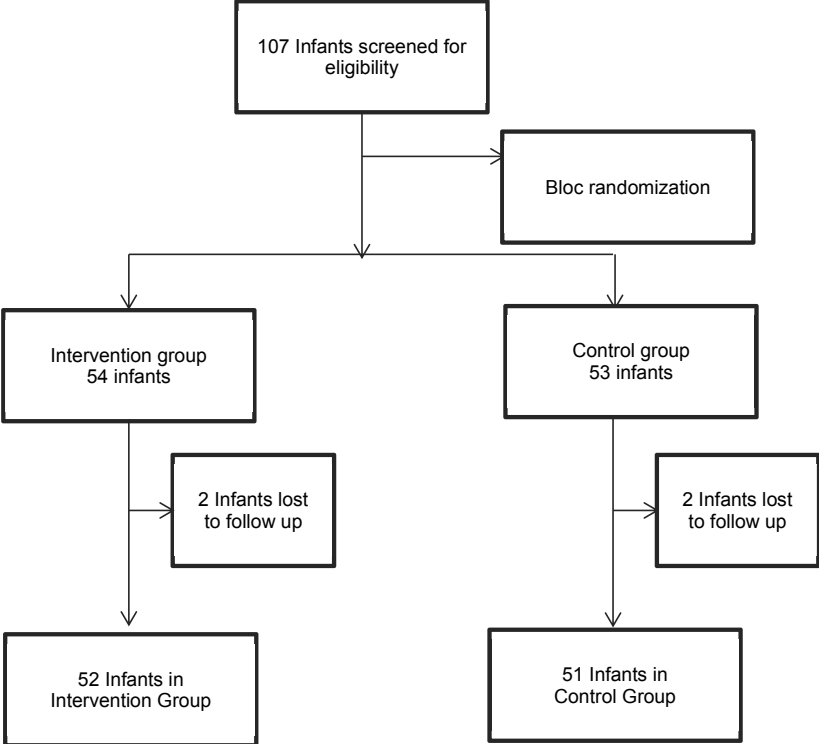
#### 3.2. Design of the studies

##### 3.2.1. A randomized, placebo-controlled trial of cobalamin supplementation

**Cohort 1.** (Figure 3) Healthy term infants (n=107) and their mothers (n=104) were recruited by local public health nurses during well baby visits at 5 different public health care services in the city of Bergen and municipalities around Bergen, Norway, between December 2004 and April 2006.

At 6 weeks the infants were assigned through block randomization to receive either an intramuscular injection of 400 µg hydroxycobalamin (Vitamin B<sub>12</sub> depot; Nycomed Oharma, Zurich, Switzerland) (intervention group, n=54) or no treatment (control group, n=53). They were invited back for a second investigation at 4 months of age. The laboratory personnel responsible for blood sampling and analyses were blinded with respect to group assignment.

**FIGURE 3.** Flow of participants through the randomized, placebo-controlled trial.

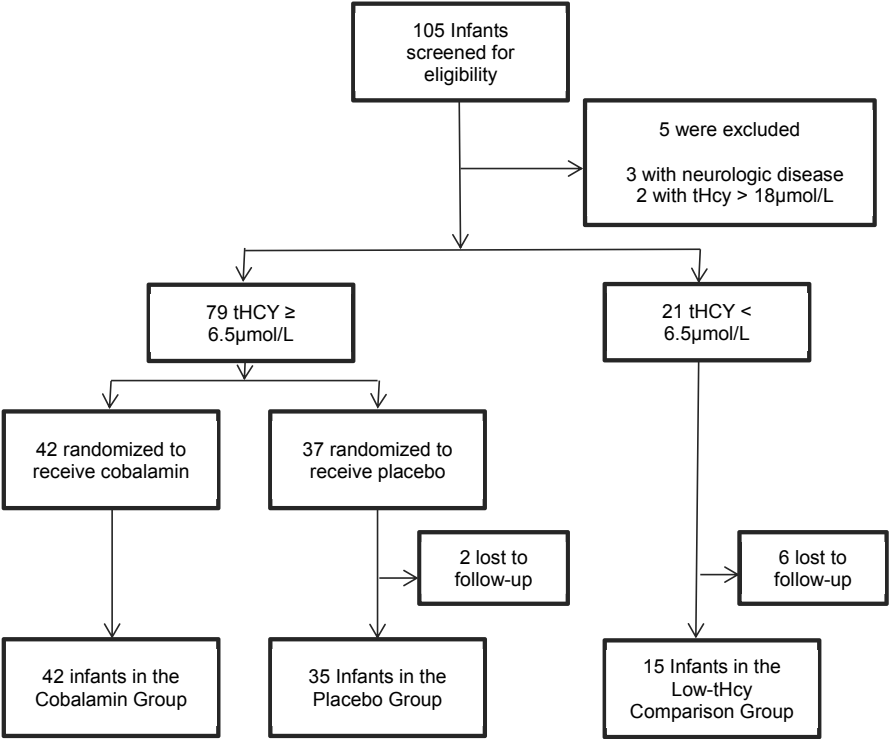


### **3.2.2. A randomized, double blind placebo controlled trial of cobalamin treatment**

**Cohort 2.** (Figure 4) Infants less than 8 months of age with feeding difficulties, minor neurological symptoms and/or developmental delay (n=105) were recruited between January 2008 and May 2010. The recruitment was done among referrals to the Pediatric Outpatient Clinic at Haukeland University Hospital, Bergen, Norway; 120 mothers were contacted and 105 chose to participate. The infants underwent a pediatric examination and had a blood test. A plasma tHcy level of 6.5  $\mu\text{mol/L}$  was chosen as a cut-off for defining cobalamin deficiency, as this level represented the 97.5 percentile in 4 months old infants given a single intramuscular dose of 400- $\mu\text{g}$  hydroxycobalamin at 6 weeks (44). Infants with a tHcy above 18  $\mu\text{mol/L}$  were considered having a more severe cobalamin deficiency and they were excluded from the study and received regular pediatric follow-up (n=2). Infants with neurological disease were also excluded from the study (n=3).

Infants with a moderate cobalamin deficiency (defined as P-tHcy level between 6.5 and 18.0  $\mu\text{mol/L}$ ), were included in the intervention trial (n=79) and randomized to cobalamin or placebo by block randomization to receive either an intramuscular injection of 400  $\mu\text{g}$  hydroxycobalamin (cobalamin group, n=42), or a sham injection (placebo group, n=37). Infants with a tHcy level < 6.5  $\mu\text{mol/L}$  were considered as having adequate cobalamin status and were included as an additional comparison group (low tHcy group, n= 21). One physician did all the injection procedures, and the parents, the pediatrician who performed all the clinical and developmental assessments and the laboratory personnel were blinded with respect to group assignment. The infants were invited back for a second investigation after 1 month.

**FIGURE 4.** Flow of participants through the double blind randomized controlled trial.

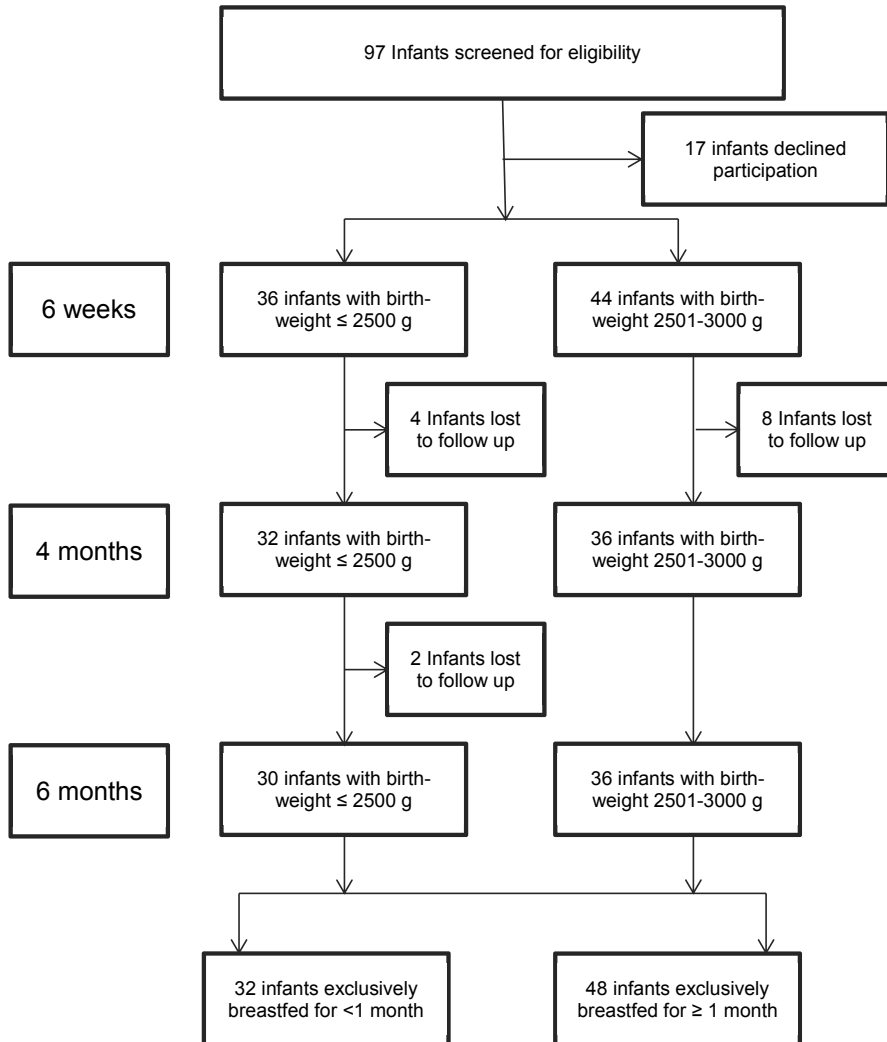




### **3.2.3. An observational study including infants with BW between 2000-3000 g**

**Cohort 3.** (Figure 5) Healthy infants with a BW between 2000-3000 g (n=97) and their mothers (n=89) were consecutively recruited during December 2008 to April 2010 at the Department of Obstetrics and Gynecology, Haukeland University Hospital, Bergen, Norway. Eighty infant-mother dyads came back for the first investigation at 6 weeks and were included in the study. According to routine, infants with a BW  $\leq$  2500 g (n=36) were recommended iron supplementation as ferrous fumarate mixture 9 mg daily from 6 weeks to 6 months, thereafter 18 mg daily to 12 months of age, folic acid 0.1 mg daily from day 3 to 3 months and a multivitamin supplement 0.5 ml daily for the first three weeks after being discharged from hospital. Infants with a BW  $>$  2500 g (n=44) did not receive any vitamin or iron supplementation. They were invited back for investigation at 4 months and 6 months.

**FIGURE 5:** An observational study including infants with a birth weight between 2000-3000 grams.



### **3.3. Data collection**

#### **3.3.1. Clinical information**

At each visit the infants' growth parameters were measured and data on the infant's diet, vitamin and iron supplementation were obtained through a questionnaire filled out by the mother. As the various types of formulas and cereals contained iron and vitamins, infant nutrition at each visit was categorized as exclusive breastfeeding or mixed feeding which included breastfeeding combined with formula, exclusive formula feeding or either of these combined with solid foods. Intake of vitamin and iron supplementation was categorized as never, 2-3 times a week or daily. Gestational age at birth was based on ultrasonography at 17-18 weeks' gestation.

In the intervention study on infants with developmental delay and/or feeding difficulties, an additional questionnaire concerning specific neurological symptoms like twitching or tremors and gastrointestinal symptoms, i.e. occurrence of regurgitations (defined as  $\geq 2$  per day (328)), number of regurgitations and food refusal, was answered by the mother. Severity of symptoms was described as no, minor and major problems. On follow-up, changes in symptoms were reported as no improvement or improvement.

Maternal data on diet, intake of vitamin and iron supplementation before, during and after pregnancy, smoking during and after pregnancy and information about current and former pregnancy history were obtained through an interview with the mother. Intake of vitamin and/or iron supplementation was categorized as daily, 2-3 times a week or never. Parity was classified as para 0 (no previous children) and para  $\geq 1$  (one or more former children).

#### **3.3.2. Clinical assessments**

In the intervention study on infants with developmental delay and/or feeding difficulties, the infants underwent a standard pediatric examination and an AIMS tests on entry and at follow up. If the infant still had symptoms of concern after one month, he/she was referred to an ordinary pediatric follow-up.

Infants in the observational study underwent a standard pediatric examination and a neurodevelopmental evaluation, including AIMS and ASQ at 6 months.

### **3.3.3. Blood sampling and storage**

Blood samples from the infants and the mothers were obtained by antecubital venipuncture at each visit. The prandial status was not registered, but all samples were collected before noon.

Serum was obtained by collecting blood into Vacutainer Tubes with no additive (Becton Dickinson). Blood was allowed to clot at room temperature for 30 min before the serum fraction was transferred to an empty glass vial. The blood samples used for preparation of EDTA-plasma were collected into Vacutainer Tubes (Becton Dickinson), placed in ice water, and plasma was separated within 4 hours. The samples were stored at  $-80^{\circ}\text{C}$  until analysis.

### **3.3.4 Biochemical analysis**

A complete set of vitamins, metabolites and erythrocyte parameters was not available for all infants at all time points, due to various problems, both technical and blood sampling problems and in a very few instances, refusal by the mother.

#### **Serum folate and serum cobalamin**

Serum cobalamin levels were determined by a *Lactobacillus leichmannii* microbiological assay (329) and serum folate by a *Lactobacillus casei* microbiological assay (330)

#### **Plasma tHcy and plasma MMA**

Plasma levels of tHcy and MMA were assayed using a GC-MS method based on methylchloroformate derivatization (331).

#### **Plasma riboflavin and PLP**

Plasma levels of riboflavin and PLP were analyzed using a LC-MS/MS assay (332).

#### **The hematological parameters**

Erythrocyte parameters (Hb, MCV, Red blood cells (RBC), Red blood cell distribution width (RDW), %Hypo and CHr) were analyzed within 4 hours with an automated hematology analyzer (ADVIA 120, Bayer Diagnostics, Tarrytown, NY).

### **3.4. Methodology**

We have included two randomized controlled trials and one observational study. Randomized controlled trials are considered the gold standard for testing the effect of various types of interventions, e.g. effects of vitamin supplementation. For such trials it is important to secure an appropriate randomization and a proper assessment at follow-up in order to avoid systematic errors.

We have included one observational study, known to have its limitations. A major challenge in observational studies is to draw inferences that are acceptably free from influences by overt biases, as well as to assess the influence of potential hidden biases.

#### **3.4.1. Validity**

##### **Internal validity**

In scientific research internal validity refers to what extent a conclusion is correct based on the data from the study, particularly with reference to avoiding or controlling for possible confounding or other bias. A study with high degree of confounding, which is factors that are related to both the exposure and outcome variable, will result in low internal validity.

Systematic errors are factors affecting the results of the study and may lead to incorrect conclusions. Selection bias refers to the problem that the selection of participants in a study may not be representative of the cohort and may interact with the independent variable, i.e. the selection is related to exposure or outcome measures. Information bias is systematic errors in the variables used in the study.

In our randomized trials, in the first study, Cohort 1, a public health nurse recruited the infants to secure a random selection and in the second study, Cohort 2, the infants were consecutive enrolled after being referred for pediatric assessment from primary care. In both intervention studies the infants were assigned by block

randomization where we used envelopes, 10/10, to decide which infant should receive cobalamin supplementation given as an intramuscular injection of 400 µg hydroxycobalamin. In the first intervention study, Cohort 1, we did not use placebo. In the second intervention study, Cohort 2, the placebo group got a sham injection, i.e. the skin was punctured by a needle connected to an empty syringe. These procedures were performed by one physician (ALBM) and the parents were blinded to whether their infant received cobalamin or not (both syringes were wrapped in aluminium foil in order to hide the content, and while the infant was placed on the mother's lap, the mother was asked to turn her head away and not observe the actual injection).

In both studies, the two study-groups were similar with reference to baseline characteristics, suggesting that the randomization was appropriate and the selection bias was insignificant.

Randomized controlled trials often suffer from two major complications, noncompliance and missing outcomes. One solution to this problem is a statistical concept called intention-to-treat (ITT). This analysis includes every subject who is randomized according to randomized treatment assignment whether the treatment and follow-up is adhered to or not. It ignores noncompliance, protocol deviations, withdrawal, and anything that happens after randomization. ITT analysis maintains prognostic balance generated from the original random treatment allocation. In ITT analysis, estimate of treatment effect is generally conservative. In the double-blind randomized controlled trial, the analyses were based on the intention to treat principle. All analyses were conducted according to treatment assignment and all available data were incorporated. We phoned and sent a letter with a new appointment within 1-3 days to the mothers who did not come back for follow-up. No assumptions were made for missing biochemical data, as these are continuous. We investigated the potential effect of missing clinical data by assuming an outcome.

AIMS data was missing for 5 infants (from the placebo group), but when assuming that these 5 infants showed an increment in AIMS percentiles, there was still a significantly better outcome in the cobalamin versus the placebo group. This assumption emphasizes that cobalamin supplementation alone improved motor development in infants with moderate cobalamin deficiency.

The ASQ is a validated parent-completed developmental screening tool with a high sensitivity and specificity to detect developmental delay (304, 305, 310). To minimize information bias related to maternal assessment, we used a simple questionnaire addressing specific gastrointestinal and neurological symptoms and a simple description of change at follow-up, i.e. improvement or no improvement. However, we know that maternal assessment of infant symptoms is to a large extent subjective and may be hard to relay on.

In the observational study, cohort 3, the data were collected prospectively and there were no significant differences in infant or maternal characteristics between the two groups that could explain the differences in clinical outcome. It was a weakness of the study that the examiner (IT) was not blinded to the nutrition of the infants, but all analyses of hematological parameters and vitamins were performed by the laboratory staff and after the clinical examinations were completed, which reduce the possibility of bias.

A confounding variable is an extraneous variable interfering with both the exposure and the outcome and may lead to incorrect conclusions. In our observational study, maternal educational level was a confounding factor, known to be associated with both the decision to breastfeed and neurodevelopment in the infants. However, the formula-fed infants showed a better motor development compared to the breastfed infants, despite the fact that a higher proportion of the mothers in the formula-fed group had lower education. This may suggest that that the effect of nutrition in favor of formula feeding was even greater than observed.

### **External validity**

External validity is the extent to which the results of the trials can be generalized to the whole infant population.

In Cohort 1, public health nurses recruited infants during well baby visits at 5 different public health care services in the city of Bergen and municipalities around Bergen, Norway during December 2004 to April 2006. We believe this secured a random selection, which make it possible to generalize the findings to the Norwegian infant population for the same age group. As the biochemical data on B vitamins are highly

dependent on both maternal and infant nutrition, changes in dietary recommendations will of course affect this aspect.

Cohort 2: This study population included infants admitted to a pediatric outpatient clinic due to feeding difficulties and/or delayed neurodevelopment. The symptoms they presented are quite common among infants, but one would assume that these infants were more than usually of concern. Feeding difficulties like regurgitations are frequently reported in young infants and are usually a self-limiting problem that resolves by age 12 – 18 months. In some infants, however, regurgitations may cause reduced weight gain, respiratory symptoms, irritability and crying, and it is one of the most common causes for physician consultation. Evaluation of both inclusion criteria and confirmation of the findings should be done in a larger study, also including infants from primary care.

Cohort 3: In the observational study, the high follow-up rate implies good external validity. The overall inclusion criterion was a BW less than 3000 gr and SGA, AGA, premature, twin and term infants were mixed together. The results pertain to infants with BW less than 3000 g, and relevance to infants with higher BWs remains speculative.

### **3.4.2. Reliability**

Reliability means that the results of a test or measure are identical or closely similar each time it is conducted.

### **Biochemical analyses**

B vitamins and related metabolites have been analyzed at Bevital AS, which has implemented both internal and external quality control. The within- and between-day imprecision (CVs) for the various parameters are: tHcy 1 % and 2 %, MMA 2 % and 3 %, cystathionine 2 % and 6 %, PLP 3 % and 6-7 %, riboflavin 6 % and 11-13 %, cobalamin 4 % and 5 %, and folate 4 % and 5 % ([www.bevital.no](http://www.bevital.no)).

Hematological parameters have been analysed at Laboratory of Clinical Biochemistry at Haukeland University Hospital. This is an accredited hospital laboratory, which has implemented both internal and external quality control for all



analyses. The within- and between-day imprecision (CVs) for the various parameters are: Hb 1 % and 2 %, MCV 2 % and 3 %, RBC 2 % and 6 %, CHr 6 % and 11-13 %, %Hypo 4 % and 5 %.

### **Psychomotor evaluation**

The AIMS test is considered to be among the most reliable tests for the assessment of gross motor function (29-31) and each infant was evaluated according to deviation from its original AIMS percentile trajectory. Only one pediatrician (IT) performed all the clinical and developmental assessments of the infants and she was blinded to the randomization, i.e. placebo, treatment and additional comparison group, to avoid information bias. She was, however, not blinded to infant nutritional information in the observational study.

#### **3.4.3. Predictive values**

The sensitivity of a test means the ability of the test to identify correctly those who have a specific condition, i.e. delayed motor development, and the specificity of the test is the ability to identify correctly those who do not have this condition. Positive predictive value reflects the portion of infants who test positive who actually have the condition, i.e. delayed motor development, and negative predictive value reflects the proportion of infants who test negative who are actually free from the condition, i.e. normal developmental status. For the AIMS test, the 10<sup>th</sup> percentile was used as a cut-off level for motor delay at 4 months and reported sensitivity was 77%, specificity 82% and negative predictive value was 96%. At 8 months, the 5<sup>th</sup> percentile was recommended as a cut-off level for motor delay, and a sensitivity of 86%, specificity of 93% and a negative predictive value of 98% have been reported. The positive predictive value was 40% at 4 months and 66% at 8 months (30).

ASQ has a high reported sensitivity (100%) and specificity (87%) to detect infants with severe psychomotor delay, but for infants with mild delay the sensitivity was only 39% and the specificity 93% (310). Another study reported a sensitivity of 87% and a specificity of 82% with ASQ for detecting children (aged 12 – 60 months) with delay psychomotor development (333). In a study of 18 months old children at a

community clinic a moderate sensitivity (67%) and poor specificity (39%) were reported (334).

### **3.5. Statistical analysis**

#### **3.5.1. Sample size**

Calculation of necessary sample size for the 2 intervention studies was based on data from previous studies on cobalamin status in infants (3, 39, 44) with the assumption that cobalamin supplementation would result in tHcy levels located in the lower quartile  $< 6.14 \mu\text{mol/L}$  or  $< 6.50 \mu\text{mol/L}$  for infants at 4 months. A calculated sample size of 65; i.e. 33 in each group, would give the study a statistical power of more than 90 per cent to detect a 25 per cent relative reduction in tHcy levels at 5 % significance level. However, based on our experience from earlier studies, a drop-out of about 40-50 % was expected, and a total of approximately 100 infants were considered necessary in order to have the estimated 65 infants at all time points.

#### **3.5.2. Statistical analysis**

Descriptive statistics were presented as mean and SD for measures with approximately symmetrical distributions, or as medians and interquartile range (IQR) for data with asymmetrical distributions. Means were compared with Student's t-test and medians were compared by Mann-Whitney U test. Correlation between pairs of continuous variables was estimated using the Spearman's correlation coefficient, whereas the association between two categorical variables was examined by using the Chi-square test. In general, hematological parameters show a normal distribution, whereas B vitamins and related metabolic markers show asymmetrical distributions.

In Cohort 1 a multivariate linear regression models were used to assess relationships between intervention, infant nutrition, and maternal vitamin status to infant's serum cobalamin, serum folate, plasma tHcy, MMA and cystathionine levels at 6 weeks and 4 months. In Cohort 2 predictors of infant cobalamin status were assessed by using multiple linear regression models including maternal vitamin status, infant's nutrition and gender, and the interventions as independent variables. In Cohort 3 multivariate linear regression models were used to assess the relation between AIMS

scores at 6 months with gender, SGA, weight at 6 months, folic acid and iron supplementation, number of months with exclusive breastfeeding and maternal education, and to assess the relationship between gender, nutrition and percent weight gain from 6 weeks to 6 months and the hematological parameters at 6 months.

A mixed linear effects model with random intercept was used to examine whether hematological parameters of the supplemented and non-supplemented infants changed differently over time (6 weeks, 4 months and 6 months). This was tested by adding a product term of time and supplementation in the regression model including their main effects, using Wald test. Post-hoc analyses were further performed to test for differences in hematological parameters across time points within the two supplemented groups. In the abovementioned analyses, %Hypo was log-transformed to better meet the normality-assumption of regression models.

Graphical illustration of dose-response relationship between CHR at 4 months and Hb level and %Hypo at 6 months was obtained by generalized additive models (GAM). We tested for a non-zero difference in slope of a segmented linear relationship by regressing hemoglobin and %Hypo at 6 months on CHR at 4 months at baseline, using Davies' test. The CHR-Hb and CHR-%Hypo relationships at baseline were also fitted by segmented regression using the breakpoint value from Davies' test as the starting estimate for the breakpoint by segmented regression. Iron supplementation was included in the Davis test and the segmented regression model. ROC analysis was used to establish the best sensitivity and specificity for predicting anemia (Hb < 11.0 g/dl) at 6 months for a given CHR cut-off at 4 months.

Graphical illustration of the dose-response relationship between months of exclusive breastfeeding and concentrations of cobalamin, folate, PLP, riboflavin, tHcy and MMA levels at 6 months and between AIMS score and tHcy and MMA levels at 6 months were obtained by GAM. The models were adjusted for folic acid and iron supplementation (i.e. for BW  $\leq$  2500 g).

Statistical analyses were performed by using SPSS (version 18 for windows, Chicago, IL) and R version 2.8.1 (The R Foundation for Statistical Computing, Wien, Austria) and SAS (Statistical Analysis System) version 9.2 (SAS Institute, Inc., Cary, North Carolina). GAM models were computed using the mgcv-package (version 1.4–

0) and segmented regression by the segmented-package (version 0.2–6), both in R (version 2.8.1). Two-sided p-values  $<0.05$  were considered statistically significant.

### **3.6 Ethical considerations and approval**

The Regional Committee on Medical Research Ethics granted ethical approval of the protocols, and the mothers gave written, informed consent. In the double-blinded randomized intervention trial, the Ethics Committee advised a sham injection in the placebo group.

All infants received sugar water for pain relief before and during injection and blood sampling (335). In the randomized controlled intervention study on infants referred to a pediatric outpatient clinic, a follow-up period of one month was chosen. This may be considered too short for evaluating potential effects of cobalamin supplementation on development and non-specific symptoms. However, the short period was chosen for two reasons; firstly, these infants were admitted for pediatric assessment and treatment, and a short observational period was therefore necessary for clinical reasons, and secondly, a short observation period was desirable to minimize confounding, e.g. from changes in feeding practice, which may affect cobalamin status.

## 4. Summary of results

### Paper I

#### **Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation**

In this study, 107 healthy mother-infant dyads (term infants) were recruited consecutively at 6 weeks of infant age for assessment of cobalamin status and of factors affecting cobalamin status. All enrolled infants were milk fed at 6 weeks and 80% were exclusively breastfed. The infants were randomized to receive 400 µg hydroxycobalamin IM or no treatment. Those who received hydroxycobalamin showed a marked increase in median serum cobalamin (75%) and remarkable reductions in median plasma total tHcy (39%), MMA (66%) and cystathionine (65%) levels at 4 months. Those who did not receive hydroxycobalamin had a moderate increase in median serum cobalamin, while plasma tHcy and MMA levels remained unchanged and plasma cystathionine level decreased (48%).

Predictors of infant serum cobalamin and plasma tHcy levels were maternal cobalamin level at 6 weeks, and cobalamin supplementation, maternal cobalamin level and type of infant nutrition at 4 months.

For the infants given hydroxycobalamin, the 97.5<sup>th</sup> percentile for plasma tHcy concentration at 4 months was 6.50 µmol/L. Rendering infants with a plasma tHcy < 6.50 µmol/L cobalamin replete, we observed that 73/107 (68%) infants at inclusion had tHcy > 6.50 µmol/L, indicating a moderate cobalamin deficiency. In the control group, approximately the same proportion of infants 35/51 (69%) had a tHcy concentration above 6.50 at 4 months. Cobalamin supplementation changed all markers of impaired cobalamin status (low cobalamin, high tHcy, high MMA) toward a profile observed in cobalamin-replete older children and adults. The results show that high levels of tHcy and MMA do not reflect organ immaturity, but reflect low cobalamin levels, as in older age groups.

## **Paper II**

### **Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study.**

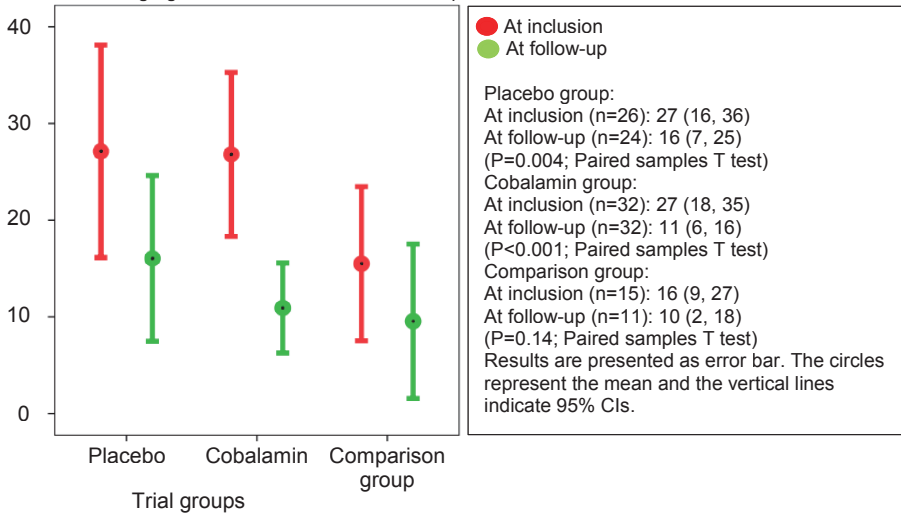
The results from Paper I indicated that breastfed infants commonly have a biochemical profile suggestive of cobalamin deficiency. Frequent signs and symptoms in severely cobalamin-deficient infants are feeding difficulties, such as regurgitations and food refusal, developmental delay and progressive neurological symptoms. These are also common symptoms, albeit less severe, in exclusively breastfed infants, and our hypothesis was that cobalamin deficiency may be a cause of such symptoms in exclusively breastfed infants.

Infants less than 8 months of age referred for pediatric evaluation for feeding difficulties or general developmental delays were recruited. Around 80% of the infants had moderate cobalamin deficiency defined as tHcy between 6.5-18.0  $\mu\text{mol/L}$ , and almost all (79%) of them were exclusively breastfed. The deficient infants were randomized to receive either an injection with 400  $\mu\text{g}$  hydroxycobalamin IM or a sham injection. Group assignment was unknown to the parents and the pediatrician performing the follow-up examinations after one month.

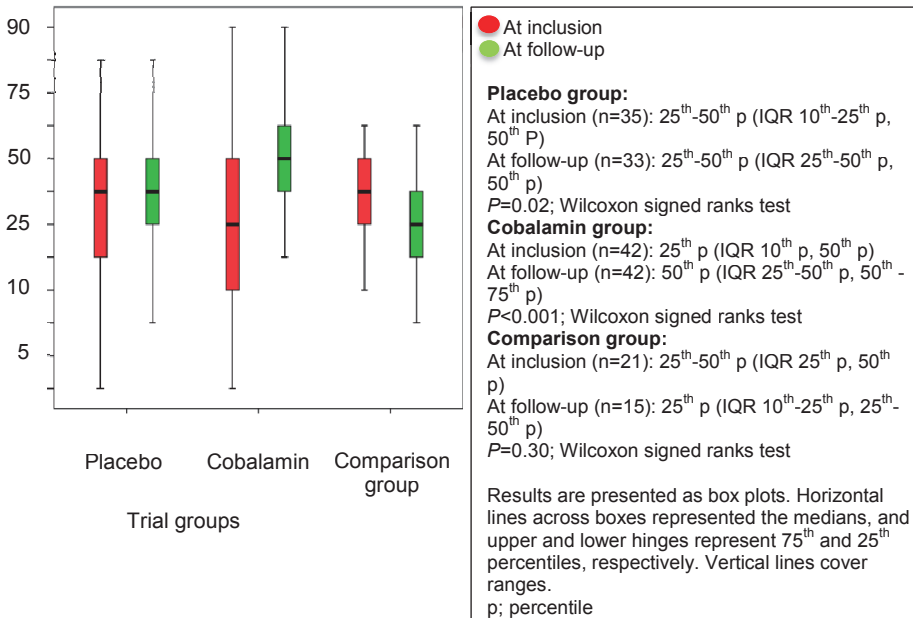
Cobalamin supplementation resulted in a normal cobalamin status and significant improvement in regurgitations (69% vs. 29% in the placebo group,  $p=0.003$ ), and motor function as judged from higher median AIMS score ( $p=0.003$ ).

The results demonstrated that cobalamin supplementation not only improved biochemical status, but also gastrointestinal symptoms and motor development in moderately cobalamin-deficient infants, a result which may imply that such moderate symptoms and delays are not always innocuous.

**FIGURE 7.**  
Number regurgitations at inclusion and follow-up



**FIGURE 8.**  
AIMS percentile at inclusion and follow-up



### **Paper III**

#### **B vitamin status and motor development at 6 months are better in formula fed than breastfed infants with a birth weight between 2000-3000 g**

Infancy is a period of rapid growth and development, and infants with low BW may be at increased risk of micronutrient deficiencies, which may have consequences for growth and development if exclusively breast-fed. Therefore, iron, folic acid and multi-vitamin supplements are commonly recommended for infants with a BW  $\leq$  2500 g. Our hypothesis was that infants with BWs above 2500 g may also have a risk of micronutrient deficiencies if not supplemented.

Infants with BW 2000-3000 g were recruited within days after birth. Infants with BW  $\leq$  2500 were supplemented with iron, folic acid and multivitamins according to guidelines while those with BW of 2501-3000 g only received cod liver oil. B vitamin status (cobalamin, riboflavin, PLP, tHcy and MMA) was determined at 6 weeks and 4 and 6 months. At 6 months, B vitamin status was related to nutrition and gross motor function evaluated by using AIMS and ASQ.

Infants who were mainly formula-fed from one month of age had significantly higher levels of cobalamin, riboflavin and PLP at all end point, and at 4 and 6 months also significantly lower levels of the metabolic markers tHcy and MMA compared to infants who were mainly breastfed ( $p < 0.01$ ). A longer duration of breastfeeding was associated with poorer gross motor development at 6 months. In the breastfed group, 66% of the infants had AIMS scores below the 50<sup>th</sup> percentile, compared to 39% of the infants who were mainly formula-fed ( $p = 0.04$ ). According to ASQ, the breastfed infants had a significantly lower median gross motor score than formula-fed infants ( $p = 0.01$ ), and the median fine motor score showed a similar trend ( $p = 0.06$ ).

The results suggest that prolonged exclusive breastfeeding may not provide sufficient B vitamins during the first months of life for infants with suboptimal BW, and that insufficient provision may have negative consequences for motor development. The current recommendation of exclusive breastfeeding for 6 months needs to be reconsidered, and more data on micronutrient needs in exclusively breastfed infants, particular in those with a suboptimal BW, are warranted.



**TABLE 5 Clinical outcome in infants at 6 months (n=66) according to infant's nutrition**

Parameters	Duration of exclusive breastfeeding (Group)		P value
	0 - 1 month (Formula fed)	>1 month (Breastfed)	
Number	26	40	
Gender, male, n (%)	13 (50)	20 (50)	1 <sup>1</sup>
Birth weight, grams, mean (SD)	2458 (294)	2561 (224)	0.12 <sup>2</sup>
Gestational age, weeks, mean (SD)	36.9 (1.9)	37.3 (1.8)	0.42 <sup>2</sup>
Premature, n (%)	10 (39)	16 (40)	0.90 <sup>1</sup>
Small for gestational age <sup>4</sup> , n (%)	7 (30)	13 (33)	0.63 <sup>1</sup>
Twins, n (%)	10 (39)	4 (10)	0.006 <sup>1</sup>
Exclusive breastfeed, months, median (25 <sup>th</sup> , 75 <sup>th</sup> )	0 (0)	5 (3.4, 5.4)	0.02 <sup>3</sup>
Folate and iron supplementation <sup>5</sup> , n (%)	16 (62)	14 (35)	0.03 <sup>1</sup>
Multivitamin supplementation <sup>6</sup> , n (%)	11 (42)	12 (30)	0.31 <sup>1</sup>
Mother higher education <sup>7</sup> , n (%)	10 (42)	28 (70)	0.03 <sup>1</sup>
<b>Vitamins and metabolites in infants at 6 months according to nutrition</b>			
Serum cobalamin, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	497 (387, 622)	321 (198, 451)	<0.001 <sup>3</sup>
Serum folate, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	53.9 (34.2, 67.0)	50.5 (39.9, 62.5)	0.69 <sup>3</sup>
Plasma PLP, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	184 (123, 278)	122 (93, 162)	<0.001 <sup>3</sup>
Plasma riboflavin, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	33.5 (22.7, 49.5)	14.8 (10.6, 18.5)	<0.001 <sup>3</sup>
Plasma tHcy, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	5.38 (4.38, 6.96)	7.35 (5.78, 9.02)	0.001 <sup>3</sup>
Plasma MMA, µmol/L, median (25 <sup>th</sup> , 75 <sup>th</sup> )	0.19 (0.16, 0.36)	0.59 (0.33, 1.20)	<0.001 <sup>3</sup>
<b>Clinical outcome in infants at 6 months (n=66) according to infant's nutrition</b>			
AIMS <sup>1</sup> , score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	24 (22, 27)	21 (18, 25)	0.03 <sup>3</sup>
AIMS, percentile, median (25 <sup>th</sup> , 75 <sup>th</sup> )	50-75 (25-50, 75)	25-50 (25, 50)	0.01 <sup>3</sup>
ASQ, communication, score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	48 (40, 50)	45 (35, 50)	0.35 <sup>3</sup>
ASQ, gross motor, score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	40 (35, 49)	35 (25, 40)	0.01 <sup>3</sup>
ASQ, fine motor, score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	50 (36, 60)	35 (30, 50)	0.06 <sup>3</sup>
ASQ, problem solving, score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	50 (50, 60)	50 (40, 58)	0.22 <sup>3</sup>
ASQ, personal-social, score, median (25 <sup>th</sup> , 75 <sup>th</sup> )	45 (35, 50)	45 (35, 53)	0.66 <sup>3</sup>

PLP, Pyridoxal 5'-phosphate; tHcy, total homocysteine; MMA, Methylmalonic acid; AIMS, Alberta Infant Motor Scale, AIMS was missing for 5 infants; ASQ, Ages and stages questionnaires, ASQ was missing for 5 infants

<sup>1</sup>Chi-square test

<sup>2</sup>Student's t-test

<sup>3</sup>Mann-Whitney U test

<sup>4</sup>< 10percentile

<sup>5</sup>Folic acid supplementation 0.1 mg daily from day 3 to 3 months

<sup>6</sup>Multivitamin supplementation the first 3 weeks of life

<sup>7</sup>Minimum 3 years of college or university education (one missing in each group)

## **Paper IV**

### **Evaluating iron status and the risk of anemia in young infants using erythrocyte parameters**

Iron is a vital micronutrient for all cells and iron deficiency in infants, even without anemia, may cause impaired psychomotor development and potentially permanent intellectual deficits. Early recognition of iron deficiency is therefore essential. It has been suggested that the hemoglobin content in reticulocytes (CHr), which reflects iron available for bone marrow hemoglobin production during the last 24-48 hours, is superior to common iron parameters for detecting iron deficiency. Daily iron supplementation is recommended from 6 weeks to 12 months in infants with a BW  $\leq$ 2500 g. Our hypothesis was that exclusively breastfed infants with BW 2501- 3000 g also are at risk of iron deficiency, and that CHr is a better predictor of IDA than more traditional parameters.

In this study we used erythrocyte parameters to evaluate iron status and the risk of developing anemia (defined as Hb<11.0 g/dl) in infants with LBW ( $\leq$  2500 g) who were supplemented with iron from 6 weeks and in non-supplemented infants with a BW of 2501-3000 g.

At 6 weeks, infants with BW  $\leq$  2500 g had poorer iron status than non-supplemented infants with a higher birth weight, but the supplemented infants had better iron status at 6 months. From 4 to 6 months the number of infants with anemia decreased from 4 (13%) to 1 (3%) in the supplemented group and increased from 2 (6%) to 9 (26%) in the non-supplemented group. The non-supplemented infants who were exclusively breastfed at 4 months had a significantly lower mean Hb and CHr, and a higher median %Hypo at 6 months, compared to infants who were not exclusively breastfed at 4 months. No differences due to nutrition were observed within the supplemented group. Rapid weight gain, exclusive breastfeeding and male sex were associated with a poorer iron status in the non-supplemented group at 6 months.

At four months CHr was a more accurate predictor of anemia at 6 months than Hb. The best sensitivity (91%) and specificity (79%) was obtained by defining iron

deficiency as CHr < 26.9 pg. At 4 months, 48% of the infants from the non-supplemented group and 27% from the supplemented group had a CHr < 26.9 pg, and infants with a CHr < 26.9 pg at 4 months had significantly poorer erythrocyte status at 4 months (Hb 11.7 vs. 12.1 (p=0.04), MCV 77.9 vs. 82.1 (p < 0.001), %Hypo 1.9 vs. 0.3 (p < 0.001), CHr 25.4 vs. 28.4 (p < 0.001)) and at 6 months (Hb 11.3 vs. 12.3 (p=0.002), MCV 74.3 vs. 79.9 (p < 0.001), %Hypo 2.3 vs. 0.4 (p < 0.001), CHr 25.8 vs. 28.1 (p=0.001)).

Infants with a moderately low birth weight, especially if exclusively breastfed, are at risk of iron deficiency during the first 6 months of life. The need for iron supplementation in infant populations with certain risk factors should be further evaluated in randomized intervention studies.

## **5. Discussion**

We have observed that breastfeeding compared to formula feeding is associated with poorer B vitamin status in infants during the first 6 months of life. In infants with a subnormal BW (2000-3000 g), exclusive breastfeeding for more than 1 month was associated with poorer B vitamin and gross motor development at 6 months compared to infants who were mainly formula-fed. Infants with a BW 2501-3000 g also had a poorer iron status compared to iron supplemented infants with lower BW (2000-2500 g). A biochemical profile indicative of moderate cobalamin deficiency is commonly observed among Norwegian term, mainly breastfed, young infants, so are also gastrointestinal complaints and minor delays in psychomotor development. We have demonstrated that cobalamin supplementation improves biochemical status, and also gastrointestinal symptoms and motor development in moderately cobalamin-deficient infants, a result, which may imply that such moderate symptoms and delays are not always innocuous.

### **5.1 Study design and limitations**

The thesis includes 2 randomized intervention trials and 1 observational study.

#### **5.1.1 Two randomized intervention studies**

In Cohort 1, healthy infants were randomized to receive cobalamin or no treatment. Different public health nurses at 5 different public health care services recruited the infants during well baby visits to secure a random selection, and the two study-groups were similar according to baseline characteristics, which strengthen the study.

However, it may be considered a weakness that the control group did not receive placebo medication and that the investigators and the mothers were not blinded to who received the cobalamin injection. These decisions were done due to ethical constraints. However, the aim of the study was to examine the biochemical response to cobalamin supplementation, in a group of infants where a biochemical profile indicative of moderate cobalamin deficiency is common. The study did not involve any clinical

assessments of the infants' health and development, the cobalamin status was unknown to the researchers and the families until after the end of the study, and technicians who did the biochemical analyses, were blinded to group assignments. It is therefore unlikely that the design limited the internal or external validity of the study.

The strengths of the study of Cohort 2, the double-blind randomized placebo-controlled trial, were the consecutive enrolment of infants referred for paediatric assessment from primary care and the similar clinical characteristics of the infants in both cobalamin and placebo groups, confirming a successful randomization. The mother, the pediatrician who performed all the motor assessments (IT) and the laboratory staff were all blinded to group assignments.

Various cut-off levels for cobalamin, tHcy and MMA to define deficiency have been considered and are much debated in adults (41, 42, 43). To find correct cut-off levels to define a moderate cobalamin deficiency in infants is even more challenging, because this period is characterized by substantial changes in markers of cobalamin status making evaluation difficult (39). Elevation of tHcy and MMA have been shown to reflect a functional lack of cobalamin, even when plasma cobalamin level is at the lower limit of ordinary used reference levels (11, 38, 40). We decided to use plasma tHcy to define cobalamin deficiency as tHcy in infants is strongly and inversely correlated to serum cobalamin (3, 54). The chosen cut-off level for defining cobalamin deficiency, i.e. plasma tHcy of 6.5  $\mu\text{mol/L}$ , represents the 97.5 percentile in 4 months old infants given a single intramuscular dose of 400  $\mu\text{g}$  hydroxycobalamin at 6 weeks, rendering them cobalamin replete optimized (44).

The clinical methods used in this study, including the clinical assessments, the AIMS and ASQ tests, and the parents' assessments of their children's development on the specific questionnaire, involve subjective elements, but because of the blinding procedures uncertainties were expected to be evenly distributed between the two groups.

Motor development is a major developmental function in early infancy (29-31), and a good marker of brain development. However, motor evaluation in young infants is challenging. Infants develop discontinuously and the age of achieving gross motor milestones, varies substantially between healthy term infants (294). There are a limit

number of diagnostic tests which can be used in this age-group (31), and whether other screening tools are better for assessing motor development in early infancy than AIMS is a matter of debate. The chosen test, AIMS, is considered to be among the most reliable tests for assessing gross motor function (29, 30), and each infant was evaluated according to deviation from its original percentile trajectory. The AIMS test not only focuses on the achievement of milestones, but also on spontaneous movements leading to the different milestones.

Common findings in infants with severe cobalamin-deficiency are feeding difficulties, developmental delay and progressive neurological symptoms (24, 65, 73). A clinical picture resembling this, albeit less severe and probably commonly considered to be within normal variation, is frequently observed in young infants. Usually the symptoms resolve without any treatment and there is no clear agreement among parents and doctors concerning the degree of infant symptomatology and motor delay that deserve evaluation and intervention (336). Our population was infants admitted to a paediatric outpatient clinic and one would consider them to more than usually troubled. However, the decision to refer for a paediatric evaluation is also influenced by the experience of the parents and the doctor. In order to be able to generalize our findings, this study should be repeated in a general infant population.

A follow-up period of only one month may be considered too short for evaluating potential effects of cobalamin supplementation on motor development and non-specific symptoms and signs. However, the short period was chosen for two reasons; first, these infants were admitted for paediatric assessment and treatment, and a short observational period was therefore necessary for clinical and ethical reasons. Second, a short observation period was desirable to minimize confounding, e.g. from changes in feeding practice, which may affect cobalamin status.

### **5.1.2 An observational study**

The last two papers in this thesis are based on an observational study, which is known to have its limitations. A major challenge in observational studies is to draw inferences that are acceptably free from influences by overt biases, as well as to assess the influence of potential hidden biases. However, data were collected prospectively and

there were no significant differences in infant or maternal characteristics between the two groups based on nutritional practice that could explain the differences in clinical outcome. Furthermore, the high follow-up rate implies good external validity. Our conclusion that prolonged breastfeeding in infants with subnormal BW carries a higher risk of iron and B vitamin deficiency than formula feeding is well founded. However, the effect on motor function carries uncertainties since the study was not blinded to the mothers who completed the ASQ questionnaires or the paediatrician who performed the AIMS assessments. However, it seems unlikely that the parents should be biased in favor of formula feeding and gives them higher ASQ scores than the others. The principally same observation by the parents and the paediatrician strengthens the conclusion that the breastfed infants had a developmental delay, and that it is likely that the delay was caused by lack of micronutrients such as B vitamins and iron.

The inclusion criterion was BW less than 3000 g, irrespective of the cause of low BW, i.e. whether it was related to preterm birth or intrauterine growth restriction. This means that it was a mixture of various degrees of SGA and AGA infants. We cannot, therefore, make firm statements as to the significance of gestational age vs. limited intrauterine growth, and we cannot extrapolate our findings to new-borns larger than 3000 g.

## **5.2 Interpretations and comparison of results**

### **5.2.1 Development of B vitamin status and the metabolic markers tHcy and MMA from 6 weeks to 6 months**

During infancy and childhood, vitamin B status and the metabolic markers tHcy and MMA, undergo marked changes (39) and during the first months of life of term infants, serum cobalamin levels decrease (39, 337) and serum folate, plasma tHcy and MMA levels tend to increase (39, 126, 127).

#### **Term infants with normal birth weight**



In term infants we observed relatively low levels of cobalamin (170 -172pmol/l), and high levels of folate (21.7 -23.7 nmol/L), tHcy (7.46 – 7.66 pmol/L) and MMA (0.50 - 0.58 pmol/L) at 6 weeks of age compared to older infants and children 1-10 years of age(39). Seventy-three of 107 infants (68%) had tHcy levels of >6.50pmol/L at age 6 weeks. The same observation has been reported in other studies (3, 54, 55) and in infants nursed by mothers with a low cobalamin status due to insufficient diet or malabsorption (11).

In the cobalamin supplemented infants, median serum cobalamin levels increased markedly (from 172 to 421 pmol/L) and serum folate increased (from 21.7 to 35.2 nmol/L) together with a considerable reduction in median plasma tHcy (from 7.46 to 4.57 pmol/L) and plasma MMA (from 0.58 to 0.20 pmol/L) during the follow-up period to 4 months. In the control group who did not receive supplemental cobalamin, median serum cobalamin increased moderately (from 170 to 240 pmol/L), serum folate (from 23.7 to 47.9 nmol/L) increased more than in the supplemented group, while median plasma tHcy (from 7.66 to 7.40 pmol/L) and MMA (from 0.50 to 0.51 pmol/L) levels remained stable. In the non-supplemented infants at age 4 months, 35/51 (69%) had tHcy levels of >6.50pmol/L. Our findings are in accordance with reports of lower tHcy and MMA in infants given cobalamin supplementation or formula, which usually contains higher cobalamin concentration than human milk (54, 55, 57, 59).

### **Infants with subnormal BW (2000 – 3000 g)**

Infants who were mainly formula-fed for the first months of life, had at all time points (6 weeks, 4 and 6 months) significantly higher level of cobalamin, PLP and riboflavin and at 4 and 6 months also significantly lower levels of the metabolic markers tHcy and MMA, compared to infants who were mainly breastfed. In the formula-fed group, the proportion infants with tHcy levels above 6.50 pmol/L, indicating cobalamin deficiency, decreased from 69% at 6 weeks to 33% at 4 months and 31% at 6 months. For infants who were mainly breastfed, the proportion of infants with tHcy levels above 6.50 pmol/L was approximately 70% at all end points. Formula is supplemented with vitamins, including B vitamins (57, 58) and higher B vitamin levels are reported in formula than in breast milk (54, 55). Consistent with our findings, several reports

have demonstrated higher cobalamin levels in infants who were mainly formula-fed compared to infants on exclusive breastfeeding (57-59), and we have strengthened that notion by showing that extended breastfeeding may lead to further deficiencies in cobalamin and other B vitamin.

### **5.2.2 Determinants of B vitamin status in infants**

We observed that cobalamin level in maternal blood was the strongest predictor of infants' cobalamin level at 6 weeks, while duration of exclusive breastfeeding was inversely correlated to cobalamin and other B vitamin status at 4 and 6 months of age in infants. This is in accordance with previous observations showing that cobalamin status in infants is related to infant nutrition (60) and maternal cobalamin status (3). There are scarce reports on PLP and riboflavin in infants, but studies from both high-income and low-income countries have reported a rather high deficiency incidence of both vitamins among pregnant and lactating women (99, 107, 271), something which may have serious implication for their breastfed infants.

### **5.2.3 Effects of cobalamin intervention**

#### **B vitamin status**

For the infants in the intervention studies who were given cobalamin supplementation, we observed a significant increase in serum cobalamin level, and a considerable reduction in plasma tHcy, MMA and cystathionine levels during follow-up. In the control groups, plasma tHcy and MMA levels remained unchanged, while the cobalamin concentration showed a slight increase. Cobalamin supplementation normalized the cobalamin biomarkers tHcy and MMA in young infants toward a profile observed in cobalamin-replete older children and adults.

Our results are in accordance with reports of lower tHcy and MMA levels in infants given cobalamin supplement or formula milk, which usually contains higher cobalamin concentrations than human milk (54, 55, 57, 59). The great magnitudes of the observed reductions in tHcy and MMA levels in response to one dose of cobalamin exceed the effects observed in most published intervention studies in adults (338).

Reports of low cobalamin concentrations combined with elevated concentrations of the metabolic markers of tHcy and MMA in healthy breastfed infants born to mothers on a westernized diet have been reported (3, 11, 39, 44). The question is whether this is an innocuous phenomenon caused by immature function of the liver or kidney or reflects any underlying clinical condition. However, our findings that the biochemical profile responded similarly to what is expected for older individuals, suggest that even the youngest infants have the same ability to regulate cobalamin metabolism and that the observed initial profile was due to relative cobalamin deficiency.

### **Gastrointestinal symptoms and motor development**

We observed a substantial improvement in regurgitations and motor function after cobalamin supplementation in infants with biochemical signs of cobalamin deficiency, and we suggests that mild to moderate cobalamin deficiency may be the cause of such symptoms during early infancy.

Our results are in accordance with published case studies demonstrating improvement of hypotonia, physical activity and ability to roll over within the first week of cobalamin treatment in severely deficient infants (65, 78, 339). Several case reports have described gastrointestinal and neurological symptoms and delayed psychomotor development in severely cobalamin-deficient children (23, 26, 73, 78, 339-343). The high frequency of regurgitations in the infants in our study may reflect developmental immaturity of the lower esophageal sphincter, esophageal dysmotility and delayed gastric emptying suggesting neurological dysregulation (24).

Although our infants were by no means severely affected, and most of them would probably have been considered to have only mild deviations from the normal developmental pattern, signs and symptoms were similar to what has been described in established cobalamin deficiency during infancy. Our findings may therefore have revealed the top of an iceberg and our results showing improvement on motor development and regurgitations imply that such moderate symptoms and delays are not always innocuous. Based on the high prevalence of a biochemical profile indicative of cobalamin deficiency we find among young mainly breastfed infants, one

may suspect that many infants may have a delayed motor development and have feeding difficulties due to a moderate cobalamin deficiency. Vitamin B deficiencies spontaneously improve when the diet includes food with a high B vitamin content, as when the infant is formula-fed and during weaning with animal food. According to the WHO this should be postponed until 6 months of age. The question is whether a moderate cobalamin deficiency during the first months of life has any implications for final neurodevelopment. This issue was not addressed in this thesis. However, there may be reasons for concern.

Cobalamin plays an important role in the initial development of the brain, and long-term deficiency of vitamin B12 has been associated with impaired myelination and demyelination of the spinal cord and brain (68, 70, 312-314). The pathophysiological mechanisms for this observation are not fully understood (24), but involve reduced supply of methyl groups, as a result of inadequate remethylation of homocysteine to methionine and inappropriate conversion of methylmalonyl CoA to succinyl CoA. The results area changes in myelin structure and delayed myelination in the central nervous system, which may lead to neurological symptoms and deregulation and delayed psychomotor development in the infants (80, 315). Indeed, it has been reported that children and adolescents being fed a macrobiotic diet in infancy and childhood, had poorer intellectual functioning compared to controls raised on an omnivore diet, suggesting that cobalamin deficiency may lead to later impaired cognitive function (48, 76).

#### **5.2.4 Nutrition and neurodevelopment in subnormal birth weight infants**

In our observational study of infants with subnormal BW (2000-3000 g), those who were mainly formula-fed had a better gross motor development at 6 months compared to infants who were exclusively breastfed for more than one month. We observed that duration of exclusive breastfeeding in months was inversely correlated to gross motor development at 6 months. Based on the results from our cobalamin intervention study in infants with delayed motor development, one may consider that the better B vitamin status in the formula-fed infants during follow-up may explain the better motor outcome. Infants' nutrition, including micronutrients, plays an important role in the

initial development of the brain and infant motor development (290, 344), and gross motor function is an important marker of neurodevelopment in early infancy (29-31).

Motor development in infants is influenced by several factors, like cultural differences and parental sociodemographic factors (287, 288), as well as infant characteristics (287, 288). We observed a better motor development in the infants mainly formula-fed compared to the mainly breastfed infants, despite the fact that this group included more twins and mothers with lower educational level, both factors known to be associated with a negative psychomotor development (287, 288).

LBW is associated with lower stores of many micronutrients (19, 20) and may have at higher risk for micronutrient deficiency compared to infants with higher BW. In spite of this, motor development was not related to whether the infants had a BW below 2500 g or between 2500 and 3000 g, although we know that LBW and shorter gestation are factors known to influence the motor development in a negative way (287, 288).

Several reports conclude that breastfeeding improves cognitive development in later childhood (345, 346), but such an association is still uncertain (347, 348). Most of the studies on IQ and neurodevelopment in relation to breastfeeding do not differentiate between exclusive and partial breastfeeding, and the results are also diverging, as some authors claim a positive effect of breastfeeding on IQ, whereas others do not (260, 262-265, 349, 350). It is also important to note that women of higher social class and education more often breastfeed and also breastfeed for longer periods than women of lower social class and education. Family background is the most important predictive factor for later IQ and school functioning (260, 349, 350) as well as for breastfeeding. Although this factor is commonly adjusted for in studies of breastfeeding and cognitive development, a possibility for remaining confounding persists.

### **5.2.5 Iron status according to iron supplementation**

Several reports have shown a higher risk of ID in infants with a MLBW especially if exclusively breastfed (7-9), and iron supplementation is commonly recommended to

infants with a BW  $\leq$ 2500 g (16, 351). In our observational study, infants with a BW > 2500g showed signs of an iron-restricted erythropoiesis from 6 weeks to 6 months, as their Hb level did not increase, %Hypo remained high and CHr decreased, despite introduction of iron-enriched formula and cereals before 6 months for most of them. Iron-supplemented infants with a BW  $\leq$ 2500 g acquired a better iron and erythrocyte status during the first 6 months of life, despite a poorer starting point.

We observed that for non-supplemented infants the most influential factor for iron status was exclusive breastfeeding, and infants who were exclusively breastfed at 4 months had significantly lower Hb and CHr levels and higher %Hypo at 6 months compared to infants supplemented with iron. Male sex and a high weight gain during the first months of life were also associated with lower iron status, but only among the non-supplemented infants. This is in accordance with several published studies showing that infants with a MLBW on exclusive breastfeeding have a higher risk of iron deficiency (7, 352, 353), and the explanation is that breast milk contains only small amounts of iron (8, 354). Fetal iron stores are considered to be exhausted at 4-6 months of age, and even before that for those with LBW because of lower micronutrient stores at delivery (19, 20). A lower iron status related to male sex and a high weight gain during the first months of life are also confirmed in other studies (169, 184, 270). A study from Domelløf et al observed that male infants had significantly lower Hb, MCV and ferritin and higher ZPP and TfR than female infants. The differences remained significant after controlling for confounders like growth variables and dietary factors (184). One possible explanation might be hormone-mediated differences in metabolism among male and female infants (184).

### **5.2.6 Evaluating iron status and the risk of anemia using erythrocytes parameters**

Since iron is an essential component of Hb, erythrocyte parameters are good markers of iron status (157), especially CHr, which reflects iron available for bone marrow Hb production during the last 24-48 hours (157, 162, 163). A study from Mast et al showed that CHr was a better predictor of iron stores than ferritin, transferrin saturation or mean MCV, commonly used parameters for evaluating ID, when bone marrow analysis was used as the gold standard (161). In our study we obtained the best

sensitivity (91%) and specificity (79%) for predicting anemia at 6 months by using a CHr cut-off level of 26.9 pg at 4 months. A significantly larger proportion of the non-supplemented infants had a CHr < 26.9 at 4 months, compared to the supplemented infants, and infants with a CHr < 26.9 pg at 4 months had significantly poorer erythrocyte status, both at 4 and 6 months. Different CHr cut-off levels for defining ID with and without anemia have been suggested in children, but there is no clear agreement and different authors suggest different CHr cut-off levels within the range 25.0-27.5 (157, 162, 163). As ID in infants, even without anemia, may cause impaired psychomotor development with potential intellectual deficits (28, 190, 191), early recognition of ID and intervention are essential. CHr has shown to be a sensitive marker of iron-restricted erythropoiesis as well as an early indicator of response to iron supplementation (159, 355).

### **5.3 Conclusion and Perspectives**

In conclusion, we have shown that young infants respond biochemically to cobalamin like older children and adults. Based on this finding we have shown that moderate cobalamin deficiency defined as tHcy level above 6.5  $\mu\text{mol/L}$  is very common in infants who are mainly breastfed, and that feeding difficulties and developmental delays in infants with mild to moderate cobalamin deficiency can be ameliorated with cobalamin supplementation. In infants with suboptimal BW, formula feeding was associated with better B vitamin status and motor development in early infancy when compared to infants who were breastfed, and we suggest that deficiencies in B vitamins might influence the observed differences in motor development. Erythrocyte parameters including CHr, prove to be useful for evaluation of iron status and risk of subsequent anemia in young infants. Despite a poorer iron status at 6 weeks for the infants with a BW  $\leq 2500$  g, iron supplementation in this BW group lead to a better iron status at 6 months than in infants with BW 2501 – 3000 g. Rapid weight gain, exclusively breastfeeding and male sex were associated with poorer iron status in the non-supplemented infants with BW 2501 – 3000 g, but not with iron status in the

supplemented group with BW equal or less than 2500 g. The findings suggest that these somewhat larger infants may also need iron supplementation.

This thesis suggests that prolonged breastfeeding may carry a risk of deficiencies of several micronutrients that are important for somatic growth and organ development, including development of the central and peripheral nervous system and hematological parameters. Early infancy is considered the most important period for brain development, with myelination, maturation and growth of the brain, and securing an optimal micronutrient status, particularly for cobalamin, is therefore vital.

Today, there is no clear consensus on what constitutes the best diet, including micronutrients supplementation, for both the infant and the lactating mother. The Norwegian Health authorities recommend human milk, only supplemented with vitamin D, as the exclusive nutrient source for term infants during the first 6 months of life. There is international consensus that breast milk is the preferred nutrient for infants, but there seems to be an increasing debate as to how long exclusive breastfeeding is beneficial for infants, and when to supplement with vitamins and other micronutrients and with solid food. This thesis suggests that prolonged breastfeeding is associated with micronutrient deficiency, which may have a negative effect on early neurodevelopment. An effort should be made to establish the optimal duration of breastfeeding and the need for micronutrient supplementation based on characteristics of the newborn infant and the mother.



## **6. Appendix**

Questionnaire developed for the study

# Undersøkelse av barn med i B12 prosjektet

Dato: \_\_\_\_\_

Mors navn og fødselsdato: \_\_\_\_\_

Barnets navn og fødselsdato: \_\_\_\_\_

Adresse/telefon: \_\_\_\_\_

## Barn

Født i uke: \_\_\_\_\_ Problemer under svangerskapet:  nei  ja \_\_\_\_\_

Ved fødsel: Vekt: \_\_\_\_\_ Lengde: \_\_\_\_\_ Hodeomkrets: \_\_\_\_\_

Nå, dato: \_\_\_\_\_ Vekt: \_\_\_\_\_ Lengde: \_\_\_\_\_ Hodeomkrets: \_\_\_\_\_

Får barnet :            **Daglig**            **2-3/uke**            **Sjelden**

Vitamin tilskudd                                 **Biovit**     **Sanasol**     **Folinsyre**    \_\_\_\_\_

Tran                                                Fullammet i antall md.: \_\_\_\_\_ md.

Jern                                                Kun melk i antall md.: \_\_\_\_\_ md.

Morsmelk:                                        Alder ved introduksjon av grøt: \_\_\_\_\_ md.

Kumelk                                            Alder ved introduksjon av middag: \_\_\_\_\_ md.

Tillegg                                            Type tillegg: \_\_\_\_\_

Ant. måltider/d i dag: Grøt \_\_\_\_\_ Middag \_\_\_\_\_ Skive \_\_\_\_\_ Frukt \_\_\_\_\_

Medikamenter:     Ja     Nei            Hvilke \_\_\_\_\_ Ant. md. \_\_\_\_\_

## Mor

Ant. barn: \_\_\_\_\_ F.dato forrige barn: \_\_\_\_\_ Vekt før sv.sk: \_\_\_\_\_ Vekt i dag: \_\_\_\_\_ Høyde: \_\_\_\_\_

**BRUKER DU:**    **Daglig**            **2-3/uke**            **Sjelden**            HVA HETER PREPARATET

Sv.sk  I dag

Vitamin tilskudd                                           \_\_\_\_\_

Tran                                                           \_\_\_\_\_

Jern                                                           \_\_\_\_\_

Tobakk                                                       Ant./dag: \_\_\_\_\_

Medikamenter:     Ja     Nei            Hvilke \_\_\_\_\_

Variert kosthold:     Ja     Nei

Spiser ikke:             Melprod.     Fisk     Kjøtt     Melkepro.     Grønnsaker

## Spørsmål om barnet

Kryss av på skalaene nedenfor omtrent hvordan du synes barnet passer inn:

Gulper barnet (kaster opp): Ant. gulp pr. døgn?

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Ant. gulp pr. døgn? \_\_\_\_\_

Har barnet kolikk/magesmerter:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Har barnet spisevegring:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelde

Svært ofte

Har barnet problemer med å svelge:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelde

Svært ofte

Skriker barnet:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Er barnet aktivt:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Gir barnet god kontakt:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Er barnet rolig:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Virker barnet fornøyd/ i fin form:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Sover barnet:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært mye

Svært lite

Antall timers søvn på dagtid? \_\_\_\_\_

Har barnet hatt anfall:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Antall timers søvn om natten? \_\_\_\_\_

Har barnet hatt apnoeanfall:

<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
1	2	3	4	5	6	7	

Svært sjelden

Svært ofte

Barnets vekt ved (korrigert) alder: 2 mndr. \_\_\_\_\_ 4 mndr. \_\_\_\_\_ 6 mndr. \_\_\_\_\_ 12 mndr.

Barnets ho ved (korrigert) alder: 2 mndr. \_\_\_\_\_ 4 mndr. \_\_\_\_\_ 6 mndr. \_\_\_\_\_ 12 mndr.

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