Iron, folate and cobalamin status
in infants and toddlers

A longitudinal study of Norwegian children 0-2 years

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Contents

Summary

Background

Iron and cobalamin are necessary for the development of the nervous system in infancy, and together with folate they are key components in the formation of red blood cells. Deficiency of either iron or cobalamin may have serious adverse long-term consequences related to cognitive, mental and motor skills, possibly irreversible, and prevention is thus essential. To prevent nutritional deficiencies in infancy and childhood, it is vital to understand how nutrient status in the infant is affected by maternal lifestyle in pregnancy and during lactation, as well as infant feeding mode.

Aim

This thesis aims to describe iron, cobalamin and folate status in a group of healthy children in a high-income country without iron or folic acid fortification and to identify determinants of the status of these nutrients from birth to 2 years of age.

Methods

A cohort of children born to mothers of Nordic decent at Aker University hospital during the spring 1997 was followed longitudinally from birth until 2 years of age. The number of children included was 364 at birth, gradually decreasing to 231 children at 24 months. A sample of the cord blood was collected at birth, and venous plasma samples were drawn at 6, 12 and 24 months. In addition, plasma samples from a proportion of the mothers (n=149, 41%) during pregnancy (17-19 weeks) were obtained. Iron status was assessed by a combination of two or more of the following indexes; haemoglobin (Hb), mean cell volume (MCV), serum ferritin (SF) and serum transferrin receptor (sTfR). Folate status was determined by serum folate and total homocysteine (tHcy), and cobalamin status was determined by the indexes serum cobalamin, holotranscobalamin (holoTC), holohaptocorrin (holoHC), total homocysteine and methylmalonic acid (MMA). At birth, a questionnaire was completed with information about the mothers and their supplement use during pregnancy. At 6, 12 and 24 months, semi-quantitative food frequency questionnaires (SFFQ) were completed for the children, and at 12 and 24 months, a dietary survey using the 7 days weighed food record method was carried out. In general, we used parametric univariate and multivariate regression models. However, in some cases, nonparametric methods were

applied, due to the skewed distribution of data. To control for confounding factors, multivariate models were used.

Results

The prevalence of iron deficiency anaemia (IDA) differed with the cutoffs used; 1-3% at 6 months, 2-10% at 12 months and 2-12% at 24 months (paper I). Iron stores at birth, as indicated by serum ferritin, predicted iron status in the first 2 years of life (paper II). Maternal smoking was inversely associated with cord serum ferritin. Maternal iron status, measured early in pregnancy, was not associated with iron status in the newborn infant; but use of iron supplements during pregnancy had long-lasting effect on the iron reserves throughout infancy (paper II).

For folate and cobalamin, we found that all serum indexes changed markedly from birth to age 24 months (paper III) and that maternal folate and cobalamin status is important for the B-vitamin status of breastfed children for at least the first 6 months of life (paper IV).

Multiparity, high birth weight and female sex were associated with lower cobalamin status at birth, but not at 6 months of age (paper IV). At all ages, cobalamin status was lower in breastfed than in non-breastfed infants (paper III). However, low cobalamin levels were not associated with impaired haematological status. Complementary feeding in breastfed infants did not alter cobalamin status. The difference between breastfed and non-breastfed infants remained even after adjusting for total cobalamin intake from the diet at 12 months of age. The effect of breastfeeding was only apparent with holoTC (reflecting short-term changes in cobalamin status), while holoHC (reflecting long-term changes) did not differ. At 24 months, all children had cobalamin intake above Nordic Nutrition Recommendations and only 1-3% had low serum concentrations. Cobalamin status was significantly associated with intake of dairy products, cobalamin supplements and liver pâté (paper V). Folate status at birth was associated with maternal folate status and folic acid intake. At 6 months, folate status was positively correlated with exclusive breastfeeding. At 24 months, 35% of the children had a folate intake below the recommended intake; however, only ~6% of the subjects had low serum folate. In multivariate models, serum folate was significantly associated with the consumption of fruits and berries and grain products.

At birth, girls had better iron and folate status than boys, but lower holoTC, indicating lower cobalamin status. At 6 months, the difference remained for iron status, and MCV was lower in boys than in girls also at 12 and 24 months. Boys may be at special risk of low iron status in infancy.

Conclusions

These data show that mild IDA (defined as Hb <110 g/L in combination with serum ferritin <12 µg/L) exists, but is uncommon among otherwise healthy children, with the highest prevalence at 24 months. Folate status was good, in particular in breastfed infants. Low cobalamin status is a normal finding in breastfed infants.

This thesis adds valuable information about iron, folate and cobalamin status in infancy and has contributed to a better understanding of what is normal nutrient status in infancy and early childhood, as well as predicting factors.

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The work presented in this thesis was carried out when I was a PhD-student in the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, 1996-2000, and since then, part time besides other work. The project was designed as a longitudinal study, originally to investigate iron status in infancy and early childhood, however, folate and cobalamin status was later included.

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List of Papers

PAPER I

Hay G, Sandstad B, Whitelaw A, Borch-Iohnsen B. Iron status in a group of Norwegian children aged 6-24 months. Acta Pædiatr 2004;93:592-8 (1).

PAPER II

Hay G, Refsum H, Whitelaw A, Melbye EL, Haug E, Borch-Iohnsen B. Predictors of serum ferritin and serum soluble transferrin receptor in newborns and their associations with iron status during the first 2 y of life. Am J Clin Nutr 2007;86:64-73 (2).

PAPER III

Hay G, Johnston C, Whitelaw A, Trygg K, Refsum H. Folate and cobalamin status in relation to breastfeeding and weaning in healthy infants. Am J Clin Nutr 2008; 88:105-14 (3).

PAPER IV

Hay G, Clausen T, Whitelaw A, Trygg K, Johnston C, Henriksen T, Refsum H. Maternal folate and cobalamin status predicts vitamin status in newborns and 6-month-old infants. J Nutr 2010;140:557-564 (4).

PAPER V

Hay G, Trygg K, Whitelaw A, Johnston C, Refsum H. Folate and cobalamin status in relation to diet in healthy 2-year-old children. Am J Clin Nutr 2011;93:727-35 (5).

Frequently used abbreviations

AI   Adequate Intake

DRI   Dietary Reference Intakes

EAR   Estimated Average Requirement

FFQ   Food frequency questionnaire

Hb   Haemoglobin

HoloHC   Holohaptocorrin

HoloTC   Holotranscobalamin

IDA   Iron deficiency anaemia

MCH   Mean corpuscular haemoglobin

MCV   Mean corpuscular volume

MMA   Methylmalonic acid

MoBa   The Norwegian Mother and Child Cohort Study

NNR   Nordic Nutrition Recommendations

RDA   Recommended Dietary Allowance

RNI   Recommended Nutrient Intake

SFFQ   Semi-quantitative food frequency questionnaire

sTfR   Serum transferrin receptor

tHcy   Total homocysteine

THF   Tetrahydrofolate

WHO   World Health Organization

UL   Tolerable upper intake level

1 Introduction

Optimal growth and development in early childhood depend on sufficient supply of essential nutrients to the pregnant woman as well as her child. Advice on dietary intake should be based on an understanding of the nutrient intake and serum profile associated with good health and development at various ages, as well as the impact of maternal and infant diet and life style factors on nutritional status. Nutrient status is generally believed to be good in children in high-income countries, but deficiencies or impaired nutrient function still exists. Iron deficiency is the most common nutritional deficiency in infancy (6, 7), and deficiencies of iron, cobalamin and folate are the dominant causes of anaemia world-wide. Anaemia is, however, a late sign of suboptimal intake. Furthermore, while sufficient folate status has proved to be essential in pregnancy to prevent neural tube defects (NTD), iron and cobalamin status are of special concern in infancy as deficiency of either of these micronutrients is associated with long-term adverse neurological development (8-10).

Although iron intake and status in children have been quite thoroughly studied, data on folate and cobalamin status and the associations with dietary intake are sparse. Nutrient status should optimally be assessed by biomarkers that can indicate early deficiency before clinical symptoms become apparent.

Lack of reference and reliable cutoff values for biomarkers is a challenge in infancy and early childhood. The main problem is the lack of functional and clinical outcome measures related to nutrient status. Assessment of micronutrient status is further complicated because serum indicators are strongly affected by confounding factors as infection (for iron) and breastfeeding status (for cobalamin).

Anaemia

Anaemia is caused either by decreased production of red cells or by increased loss of red cells because of haemorrhage or haemolysis. Iron deficiency is the most common cause of anaemia in infancy and early childhood (7).

Anaemia is defined as haemoglobin (Hb) below an established cutoff value. For children ≤5 years of age, the World Health Organization (WHO) has set the cutoff at 110 g/L (11), derived by extrapolating data from young children to cover infancy. However, these cutoffs

are challenged (see 1.1.6.). Although iron deficiency is the most common cause of anaemia in children (7), a low Hb is not specific for iron deficiency anaemia (IDA) but is frequently related to deficiency of other nutrients, particularly folate and cobalamin. Furthermore, anaemia may arise following infection, inflammation or vaccination (12-14). Most nutritional anaemias, including those of iron, folate and cobalamin deficiency, are associated with a restriction of cell proliferation or differentiation resulting in a decreased release of young red blood cells from the bone marrow (7).

Anaemia is usually described as microcytic or macrocytic, depending on the size of the red blood cells. Microcytosis can be caused by mutations in the formation of blood cells (hereditary microcytosis) or by impaired synthesis of Hb, as in iron deficiency anaemia, characterized by small and pale blood cells (hypocrome microcytic anaemia) (6, 7). Microcytic anaemia may, however, also be caused by chronic inflammatory disease or lead poisoning, and very rarely copper deficiency.

Macrocytosis is a common sign of folate/cobalamin deficiency, and is believed to result from a decreased number of cell divisions during the development of erythroid precursors, following a diminished rate of DNA synthesis (7).

The size of the red blood cells is measured by the mean corpuscular volume (MCV), which is valuable in separating iron deficiency (low MCV) from folate or cobalamin deficiency (high MCV) (7).

Concomitant presence of iron deficiency and cobalamin deficiency may lead to a finding of red blood cells with a normal size (normal MCV), making it more difficult to reach the correct diagnosis (15). It is thus essential to determine both cobalamin and iron status in all cases of otherwise unexplained severe anaemia in infants (15). WHO reports that MCV is lowered more by iron deficiency than it is raised by folate or cobalamin deficiency. Thus, high MCV may be a more sensitive measure of deficiency of these vitamins in populations where iron deficiency is less prevalent (16).

1.1 Iron

1.1.1 History

The historical background to iron nutrition has been described by Hoffbrand & Herbert (17) and Yip & Dallman (18). The "strengthening properties" of iron were recognized by the early Greeks and the Romans, and iron has been used to cure anaemia for more than 200 years. Early in the 18th century, iron was found to be a major constituent of blood. In 1832, the widespread therapeutic use of iron tablets started with a report on the efficacy of treating young women in whom "colouring matter was lacking in the blood". In the 20th century, it became apparent that iron deficiency is the most common cause of anaemia in every country of the world, affecting about half the population in some of the poorest countries (17). Castle and co-workers proved that inorganic iron could be used for Hb synthesis in 1932. McCance and Widdowson estimated iron absorption and loss in adults in 1936, and they described balance studies for estimating iron needs carried out as early as 1897. In 1892, Bunge described the special vulnerability of infants to iron deficiency. He found that milk was a poor source of iron, and that excessive milk feeding could lead to iron deficiency after neonatal iron reserves were depleted. Mackay showed in her investigations in 1925-1927 that anaemia was present in most artificially fed and many breastfed infants in London, and that a substantial increase in Hb and prevention of anaemia could be achieved by the addition of iron citrate to powdered milk. Her studies changed the understanding of normal Hb concentrations in infants, and excluded other suggested causes of anaemia, such as lack of ultraviolet light, lack of vitamins, or milk fat toxaemia. Nevertheless, not until the 1960s it became common to add iron to infants' diets in the US and other countries (17, 18).

1.1.2 Sources in the diet

Breast milk has low iron content (0.2-0.4 mg/L), but a high proportion of the iron is absorbed (16-49%) (19, 20). It has even been suggested that the absorption may be as high as 100% (45-100%) (21-23). The iron content of breast milk is not affected by maternal nutritional status (24). Recent Nordic Nutrition Recommendations (NNR 2012) conclude that breast milk is sufficient for iron status until 6 mo of age, and that after the age of 6 mo, the total diet and the choice and amount of breast milk, cow's milk or formula are of main importance (25, 26).

Infant formula frequently contains iron sulphate and has been shown to be effective in preventing iron deficiency in infants (12, 27, 28). Iron-enriched infant cereal is a good source of iron, depending on the form of the iron that is added (iron fumarate is easily absorbed, iron orthophosphate or pyrophosphate less so) (29). Other sources of iron in the infant diet include meat, bread, liver pâté, cereals and green vegetables (25, 30). The composition of the diet is of equal importance as the total iron content (31, 32). The bioavailability of iron depends on the proportion of haem iron from meat and fish (readily absorbed) compared with non-haem iron primarily from plant-based foods (not so readily absorbed), and the content of enhancing and inhibiting factors in the diet (21, 25). The absorption of non-haem iron is increased by enhancing factors in animal muscle tissue and by vitamin C, or inhibited by phytates, tannins, calcium, polyphenols, and soybean proteins, or possibly other soy components (6, 33). In addition, the absorption of iron also depends on the iron status of the individual (18, 33). In adults, the absorption of dietary iron is regulated in response to the size of the iron stores or inflammatory signals, involving a peptide called hepcidin (6). This regulation may not be fully developed in infants (25).

A nation-wide study of more than 1200 Norwegian children, aged 12 months, in 1999 showed that among non-breastfed children, the most important dietary sources of iron were commercial infant cereal (43%), bread (10%), formula as drink (10%), meat and meat products, including liver (9%), and commercial baby dinners with meat/liver (6%), and potatoes/vegetables (3%) (34). However, the study did not relate iron intake to iron status, so it is not possible to determine the relative importance of the various food groups or food items.

Low iron status in children is usually assumed to be a result of low iron intake. However, a relationship between iron intake and iron status (Hb or serum ferritin) is not always found (35, 36), possibly because it is difficult to assess the influence of enhancing and inhibiting factors.

1.1.3   Recommendations

Iron requirements are relatively high in childhood, particularly during periods of rapid growth (6 to 24 months) and adolescence (21). In the Dietary Reference Intakes (DRI) (21), the criteria for determining iron requirements are based on the average iron intake in infants consuming exclusively human milk (0 through 6 months) and factorial modelling (37), taking into account the requirements for basal losses as well as the amount needed for maintenance of growth and fetal development (7 through 12 months and 1 through 70 years) (38). Also in the Nordic Nutrition Recommendations (NNR 2012) (25), the iron supplied by breast milk is considered to be sufficient for term exclusively breastfed infants during the first 6 months of age, due to infant iron stores and the high bioavailability of iron in breast milk. Hence, no recommendation is given for the first 6 months. It is stated that there is no evidence that preventive iron supplementation before 6 months of life would give any beneficial effects on cognition or psychomotor development in healthy, breastfed infants of normal birth weight in low-risk populations such as in the Nordic countries. On the contrary, excessive iron intake during the first 6 months should be avoided because infants may not down-regulate their absorption as efficiently as children and adults (25).

Low birth weight infants should be supplemented (6 weeks-6 months). From 7 months to 5 years of age the recommendation is 8 mg per day, assuming that the basic iron requirements are similar in this period (25). This recommendation is justified by the finding that infants with an iron intake of 9 mg per day (mainly from iron-fortified phytate-rich cereal) have adequate iron status towards the end of infancy, the observation that iron status improves from 1 to 2 years of age, and that a higher recommendation would require an unrealistic high iron density in the diet (25). In **Table 1** recommendations on iron intake in children are listed.

Table 1. Recommendations on intake of iron, children 0-3 years

	Iron, mg per day		
Age	**0-6 months**	**7-12 months**	**1-3 years**
Institute of Medicine (21)			
Adequate Intake (AI)	Iron supplied in breast milk: 0.27 (0.78 L/day x 0.35 mg iron/L)	-	-
Estimated Average Requirement (EAR)	-	6.9	3.0
RNI (=RDA)	-	11	7
UL	40	40	40
FAO/WHO (39)			
Recommended Nutrient Intake (RNI)[1]	As supplied in breast milk	6.2-18.6[2]	3.9-11.6 (15-5% iron bioavailability)
Nordic Council of Ministers			
NNR 2012 (25, 40) (unchanged from 2004)	As supplied in breast milk	8	8
Norwegian Directorate of Health			
Norwegian infant recommendations (41)	5	8	8

[1]RNI = RDA, Recommended Dietary Intake. Based on the 95th percentile of the absorbed iron requirements

[2]15-5% iron bioavailability

1.1.4 Function

Iron is involved in oxygen transport, and transitional tissue storage and cellular use of oxygen, as an essential part of Hb and myoglobin (6). Oxygen is bound to an iron-containing porphyrin ring, either as part of the prosthetic group of Hb within red blood cells or as part of myoglobin as the facilitator of oxygen diffusion in tissues (21). Iron is also a part of various enzymes and participates in electron transfer by interconversion of iron oxidation states (ranging from –2 to +6) (21). In cytochrome P450 in the liver and intestine, iron contributes to the degradation of endogenous compounds and environmental toxins. A recent overview of the various enzymes that iron is a part of is given by McDermid & Lönnerdal (6).

In the developing brain of infants, iron appears to be required for the production of myelin isolating the nerve fibers (42). Iron deficiency impairs myelination of nerve fibers in the central nervous system, which is a plausible explanation for the long lasting effects of iron deficiency (43). The effects on transmission in the auditory and visual systems persist into childhood (44). Studies of IDA and behaviour in the developing human and in animal models suggest persistent functional changes (43, 44). In monkeys, it has been demonstrated different behavioural consequences depending on the timing of iron deficiency relative to brain developmental stages (45).

1.1.5 Iron indexes

Combined with haematologic measurements (e.g., Hb and MCV), serum ferritin and sTfR are believed to provide a good picture of iron status (46). However, a recent or concurrent infection or an inflammatory condition can cause anaemia and also result in confusing results of other laboratory tests, e.g., decreased transferrin saturation and increased serum ferritin (47). Recent studies suggest that serum ferritin is the most useful biomarker in the screening of iron depletion in healthy children in high-income countries, whereas sTfR has limited value in the detection of iron depletion (48). An overview of the most commonly used iron biomarkers is given in **Table 2**.

Table 2. Biomarkers of iron status

Index	Description/function
Haemoglobin (Hb)	Haem protein that accounts for more than 60% of body iron. Transports oxygen via the blood stream from the lungs to the tissues. Often determined using an electronic counter, which also gives haematocrit and red cell indices (MCV, MCH) (7).
Haematocrit (Hct)	Originally calculated by comparing the volume (height in a column) of packed red cells with the volume of red cells and plasma (height of entire column). Less reliable than Hb in diagnosing anaemia when measured by electronic counter (7).
MCV, MCH 3rd *stage of ID*	Red cell indices. A low MCV and/or MCH reflects microcytosis and supports diagnosis if iron deficiency– together with low Hb or haematocrit (7, 47).
Serum ferritin (SF) 1st *stage of ID*	Iron is stored as ferritin in the liver, spleen, and bone marrow. Small quantities of glycosylated ferritin, proportional to the size of body iron stores, also circulate in the blood (termed SF). Low SF reflects depletion of storage iron/early iron deficiency in healthy individuals (7, 49). In children, 1 µg/L of SF reflects an iron store of about 0.14 mg iron/kg bodyweight, and a SF below 12 µg/L reflects depleted iron stores (49). However, the thresholds are debated (see Discussion).
Serum transferrin receptor (sTfR)	Reflects cellular iron needs. An increased sTfR reflects increased cellular iron needs. sTfR is not affected by infection (46). May be difficult to interpret in infants and young children (50).
Free erythrocyte protoporphyrin (EP) 3rd *stage of ID*	EP accumulates in red blood cells when insufficient iron is available for formation of haem from protoporphyrin. Thus, elevated EP indicates low iron status (18). EP is also elevated in lead poisoning, infection and inflammatory disease. May be difficult to interpret in infants and young children (7, 25, 50, 51).
Transferrin	Iron-binding protein that carries iron in plasma (7).
Serum iron 2nd *stage of ID*	Additional test: Decreases in iron deficiency (7). Diurnal variation; reduced in infection/inflammation (51).
Total iron binding capacity (TIBC) 2nd *stage of ID*	Total iron-binding capacity of transferrin. Increases in iron deficiency (7). Low reproducibility due to wide biological variation. Requires newly separated plasma. Rarely used today (51).
Transferrin saturation 2nd *stage of ID*	Ratio between serum iron and TIBC. Decreases in iron deficiency consistent with low ratio of iron to total iron-binding capacity. It is also decreased during infection. In general, 12-16% indicates iron deficiency in children (7, 47).
Hepcidin	New and promising biomarker for iron status. Regulates dietary iron absorption and hence systemic iron homeostasis (6).
Reticulocyte haemoglobin content (CHr)	Provides a real time evaluation of the erythropoiesis in the preceding 24-48 hours. This makes CHr useful during periods with rapid physiological changes, and CHr has been suggested as the best predictor of iron deficiency in infants, and superior to serum ferritin and transferrin saturation (52) referring to (53, 54).

Abbreviations: Hb, haemoglobin; Hct, haematocrit; MCV, mean cell volume; MCH, mean corpuscular haemoglobin; SF, serum ferritin; ID, iron deficiency; sTfR, serum transferrin receptor; TIBC, total iron binding capacity; EP, free erythrocyte protoporfyrin; CHr, reticulocyte haemoglobin content.

1.1.6 Deficiency

The development of iron deficiency (and overload) as depicted by Herbert in 1987 (55) is shown in Figure 1. The figure suggests three stages of iron deficiency corresponding to stage 1-3 in **Table 2**. In the first stage, called iron depletion, iron stores are empty, but circulating iron and erythrocyte iron are intact. In the second stage, called iron deficient erythropoiesis, there is a reduction in circulating iron. In the third stage, IDA, erythrocyte iron is reduced, resulting in anaemia. The sequence of events and corresponding reduction in the various indexes are complicated to identify, due to factors such as individual variation, infection, and pregnancy. The picture may also be different in children. An operational definition of IDA (sometimes referred to as the "gold standard") is a therapeutic trial of iron supplementation resulting in increased Hb (such as ≥ 1.0 g per dl), indicating that the Hb production was restricted by lack of iron (7).

	IRON OVERLOAD	NORMAL	IRON DEPLETION	IRON DEFICIENT ERYTHROPOIESIS	IRON DEFICIENCY ANEMIA
Iron Stores	EXCESS				
Circulating Iron					
Erythron Iron					
RE Marrow Fe	4+	2-3+	0-1+	0	0
Transferrin IBC (µg/100mL)	<300	330±30	360	390	410
Plasma Ferritin (µg/L)	>300	100±60	20	10	<10
Iron absorption (%)	>15	5-10	10-15	10-20	10-20
Plasma Iron (µg/100mL)	>175	115±50	115	<60	<40
Transferrin saturation (%)	>60	35±15	30	<15	<15
Sideroblasts (%)	40-60	40-60	40-60	<10	<10
RBC Protoporphyrin	30	30	30	100	200
Erythrocytes	Normal	Normal	Normal	Normal	Microcytic/ Hypochromic

Figure 1. Sequence of events in developing iron deficiency and overload. For explanations of all the variables, see original article. To convert µg iron to SI (nmol), multiply by 17.9. Reproduced from Herbert 1987 (55) with permission from *The American Journal of Clinical Nutrition*.

Pregnancy

Maternal anaemia in pregnancy is associated with unfavourable pregnancy outcome, including premature delivery, low birth weight, and increased perinatal infant mortality (56). Iron deficiency is a likely cause of maternal anaemia, leading to restricted expansion of erythrocyte cell mass. Also iron depletion with low maternal serum ferritin concentration (<20 μg/L) is associated with unfavourable pregnancy outcome (57). Decreased Hb in pregnancy is a normal consequence of the expansion of plasma volume. A high Hb usually reflects decreased plasma volume associated with maternal hypertension and eclampsia. Hence, there is a U-shaped relationship between Hb concentration and unfavourable pregnancy outcome, with increased risk at Hb concentration below 90 g/L or above 130 g/L (21). Iron supplementation in pregnancy effectively prevents maternal anaemia and iron deficiency and reduces the risk of low birth weight (58).

Iron deficiency anaemia (IDA) in infants and children

IDA in the first 2 years of life may delay or impair the mental and physical development of children, causing long-lasting poor cognitive and behavioural performance, including inattentiveness and psychomotor disturbances (21, 25, 59, 60). An association between early iron deficiency and later developmental scores has been observed also in the Nordic setting (8, 61). IDA may also lead to decreased infection resistance and impaired temperature regulation (6). In older children, low iron status is associated with worse performance on standardized math tests (62), and lower mental and motor functioning (9). Some, but not all, studies suggest that delayed development can be reversed by iron treatment (9, 63-65).

The prevalence of iron deficiency in infants is highest between about 4 months and 3 years of age (66). IDA is usually diagnosed by a Hb concentration below an age-appropriate cutoff combined with other indexes indicative of iron deficiency, such as low serum ferritin, transferrin saturation or MCV or an elevation in erythrocyte protoporphyrin or TfR (51). Indicators of iron deficiency are difficult to interpret in infants because of the effect of coincident changes in physiology and metabolism during growth and development and because of frequent infections (50). A combination of several tests increases specificity (18).

The cutoff values for some iron indexes in infants and children, are listed in Table 3.

Table 3. Cutoff values for iron status indexes in infants and children (NNR 2012) (25).

Age/age group	Hb, g/L	Serum ferritin, µg/L	sTfR, mg/L	MCV, fL[1]
4 months	<105 (67)	<20 (67)	>11 (67)	<73 (67)
6 months	<105 (67)	<9 (67)	>11 (67)	<71 (67)
9 months	<100 (67) <105 (68)	<5 (67)	>11 (67)	<71 (67)
Children and adolescents	<105 (69) <110 (60)	<12 (60, 70, 71)	>8.5 (21, 72)	<74 (73)

[1] 1 fL is 10^{-15} L.

1.1.7 Adverse effects of excessive intake

Symptoms of excessive iron intake, usually due to genetic causes such as haemochromatosis, are associated with oxidative damage (50, 51). Other possible adverse effects of special relevance in infancy include increased risk of infection (bacteria need iron for growth) and interactions with other minerals (50, 51). Some studies, (74-76) but not all (77), suggest that iron supplementation to replete infants may cause negative effect on growth in children. Domellöf *et al* (40) summarise that there is no conclusive evidence that iron supplements impair growth in iron-replete children. Lower score for visual-motor–integration has also been suggested; however, any such effect cannot be concluded (40).

1.1.8 Status in children

Few studies of iron status have been conducted in Norway. Before the present study, smaller or unpublished studies of ethnic Norwegian children showed IDA in 2-4% of children at 12 months and 0-9% at 24 months. Depleted iron stores (serum ferritin <10/12 µg/L) were found in 0-13% at 12 months, and 9-16% at 24 months (78, 79) (personal communication, Berit Borch-Iohnsen, see also **Table 12**). The prevalence of IDA at 12 months was similar in studies of Swedish and Icelandic infants (3%) (69, 80); however in these studies a higher proportion of infants had depleted iron stores (serum ferritin <12 µg/L; 26 and 41%, respectively). In Swedish children at 24 months of age (81), depleted iron stores were found in 10%, comparable with the Norwegian studies.

1.2   Folate

1.2.1   History

In the late 1920s and early 1930s, Lucy Wills, a consultant haematologist at the Royal Free Hospital and School of Medicine in London, conducted studies in India to find out what caused the severe macrocytic anaemia found among poor women with dietary deficiencies, particularly those in the textile industry. She discovered a "new haemopoietic factor" found in yeast and liver, which cured tropical macrocytic anemia in humans and experimental anaemia in monkeys and chicks (82). The factor was initially named "Wills factor".

Two other research groups, led by Robert Stokstad and Joseph John Pfiffner also isolated factors with the biological properties of Wills' factor, from bacterial culture and from yeast and liver, respectively. Pteroylglutamic acid was isolated (crystallized) between 1943 and 1945 and named folic acid, because extracts from leafy greens proved to be a rich source (82).

In the 1960s, Hibbard and coworkers linked abnormal folate metabolism to pregnancy complications. In the 1970s Smitthels and coworkers related vitamin deficiency, including folate deficiency, to neural tube defects, and in 1980 they published a paper confirming that periconceptional intake of folic acid protected against recurrence of neural tube defects (83). In 1991 it was conclusively shown that 4 mg per day of folic acid prevents 75% of neural tube defects (84).

In Norway, Ek & Magnus in 1979 showed that breastfed infants born to mothers that were not supplemented with folic acid had normal growth and red cell status. The authors suggested that folate status of normal, breastfed infants was optimal. Furthermore they suggested that the folate status found in breastfed infants should be used as the standard to reach for folic acid addition to infant formula (85).

1.2.2   Sources in the diet

Folate is found in a wide variety of foods, however in relatively low amounts. Certain foods contains ample folate: leafy green vegetables such as spinach; other fresh, green vegetables; legumes such as dried or fresh beans; sunflower seeds; citrus fruits; egg yolk and animal liver (39, 86). Good sources include oranges and orange juice, strawberries and cereals/whole meal. Naturally occurring folate is chemically labile and as much as 50-75% of initial folate activity may be lost over periods of days or weeks during harvesting, storage, processing, and preparation (39). The synthetic form of the vitamin, folic acid, is more bioavailable than natural folates (87) and is resistant to chemical oxidation and thus stable for months or years. This form is used in fortified foods and dietary supplements. However, folic acid is not metabolically active; it has to be reduced to tetrahydrofolate (THF) or to other THF derivatives, to participate in cellular metabolism (86).

In breast milk, folate is categorized as a "Group II nutrient", meaning that the folate content in breast milk is maintained even if the mother is deficient and irrespective of maternal supplementation. However the folate status of the mother may deteriorate (88).

Fortification and supplementation

In the United States, folic acid in the amount of 140 µg per 100 g is added to cereal or grain products in an attempt to reduce the incidence of neural tube defects. The program has been estimated to provide 100–200 µg of folic acid per day to women of childbearing age (89). In Norway, there is no general folic acid fortification, but women planning to get pregnant are advised to take a folic acid supplement of 400 µg per day prior to and during the first months of pregnancy (90). Fortification with folic acid in the US has doubled serum folate concentrations and decreased the prevalence of elevated total homocysteine concentrations by half, from 20% to 10%. Rates of neural tube defects decreased by about 20% and there are indications of a similar decrease in the death rates due to stroke (83). However, in an elderly US population (>65 y), high folate intake has been associated with a faster rate of cognitive decline (91). It has been hypothesized that this effect may be due to unmetabolized folic acid in the circulation caused by limitation in the capacity to metabolize folic acid to the active form THF (83).

Trials of cardiovascular disease prevention by prophylactic oral folic acid have been carried out (92). In a group of Norwegian patients with a history of heart disease, folic acid supplementation did not have any effect on the risk of coronary heart disease, however, cancer incidence increased in the supplemented group (93). Thus, based on data from the Norwegian trials, there is concern that folic acid fortification of food items also may increase cancer risk, and mandatory fortification has not been introduced. There is an on-going controversy whether this risk is outweighed by the reduction of neural tube defects incidence as a result of the folic acid fortification (94-97). A recent meta-analysis including data on 50 000 individuals showed that supplementation with folic acid, with doses that are higher than those provided by fortification of food items, was associated with a relative risk of cancer of 1.06 (95% CI 0.99-1.13) the first 5 years of treatment (98), a finding that did not completely reach significance.

1.2.3 Recommendations

In the NNR (99), no recommendation is given on folate intake for infants <6 months of age. For the age group 6-11 months, 50 µg per day is recommended, and for the age group 11-23 months, 60 µg per day is recommended. The recommendation is based on a small study showing that a diet that supplied 3.5-5.0 µg of folate per kilogram of body weight maintained growth, haemopoiesis and clinical well-being in 20 infants aged 2-11 months during a period of 6-9 months (26, 100). The values have been adjusted to older children. In the DRI (101), the AI is based on the observed mean folate intake of infants consuming exclusively human milk. The human milk folate concentration used to estimate AIs for infants is 85 µg/L, based on four reports (101). Based on that and the average milk intake according to age, the AI for infants 0-6 months is 65 µg per day, equaling about 9.4 µg per kilogram of body weight. The EAR and RDA are based on extrapolation from adult values because of lack of data in children. This probably explains the marked difference in recommendations between the NNR and DRI. In NNR 2012 (99), the recommended intake for fertile women was 400 µg per day. However, as opposed to the Norwegian recommendation (102), the NNR 2012 does not specifically recommend folic acid supplement. The goal according to NNR 2012 was that provision of 400 µg per day from the diet would facilitate adequate folate supply also to women experiencing unplanned pregnancies.

For pregnant women, the NNR 2012 recommendation is 500 µg per day, based on studies indicating that 400-500 µg per day are sufficient to meet the increased requirement from fast growing tissues in pregnancy (99). Lactating women are also recommended 500 µg per day, an amount that for most women only can be achieved by supplements, as average intakes in the Nordic countries are about half of the recommended amount (103).

Recommendations on folate intake in children are given in **Table 4**.

Table 4. Recommendations on intake of folate, children 0-3 years

Recommendation	Folate, µg per day[1]		
	0-6 months	**7-12 months** (6-11 mo) (103)	**1-3 years** (12-23 mo) (103)
Institute of Medicine (101)			
Adequate Intake (AI)	65 9.4 µg/kg bw[2]	80 8.8 µg/kg bw	120
Estimated Average Requirement (EAR)	-	-	120
Recommended Dietary Allowance (RDA)	-	-	150
Upper Limit (UL)	Not possible to establish for supplemental folate[3]	Not possible to establish for supplemental folate	300 (fortified foods/ supplements)
FAO/WHO (39)			
Estimated Average Requirement EAR	65[4]	65	120
Recommended Nutrient Intake (=RDA)	80[4]	80	150
Nordic Council of Ministers			
NNR 2012 (99, 40) (unchanged from 2004)	As supplied in breast milk	50	60 80 (2-5 y)
Norwegian Directorate of Health (41)			
Norwegian infant recommendations	35	50	75

[1] If not otherwise stated
[2] Per kilogram of body weight
[3] To prevent high levels of intake, the only source of intake for infants should be from food
[4] Based on a human milk intake of 0.75 L per day.

1.2.4 Function

Folate is a generic term that includes both naturally occurring folates (food folate or pteroylpolyglutamates) and folic acid (pteroylmonoglutamic acid, PGA) used in dietary supplements and fortified foods. Folic acid is the most stable form of folate (37). Pteroylmonoglutamic acid is the parent compound of a large group of components with the same vitamin activity, differing from each other by degree of reduction (oxidized, dihydro, or tetrahydro forms), presence of different single carbon units (for example methyl, methylene, methenyl formyl), and by variation in number of glutamate residues (17).

Folate plays a central role cell multiplication and tissue growth. Folate is required in many critical cellular pathways as a one-carbon source, including DNA, RNA (ribonucleic acid) synthesis and protein and DNA methylation. Folate is needed in the biosynthesis of purines and pyrimidines, in the metabolism of some amino acids, and in the catabolism of histidine. Folate can be a limiting factor in all these reactions (104). The role of folate in biology is closely interrelated to that of cobalamin through their link in the methionine synthase reaction converting 5-methyl-THF into THF, which is then converted into to the active THF forms (37). Methionine synthase simultaneously catalyses the conversion of homocysteine to methionine. The enzyme requires 5-methyl-THF as substrate and methylcobalamin as a cofactor (39).

1.2.5 Folate indexes

A recent systematic review concludes that both serum and erythrocyte folate values reflect folate intake in the paediatric age group; however, serum folate reacts more rapidly to folate intake than erythrocyte folate (86). An overview of folate indexes is given in **Table 5**.
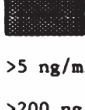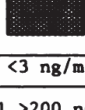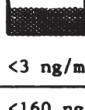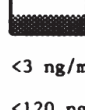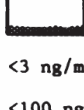
Table 5. Folate indexes

Index	Description/function
Serum/plasma total homocysteine (tHcy)	- Elevated total homocysteine is a strong predictor of inadequate folate status. Other factors, including deficiencies of the vitamins B2, B6 and cobalamin, lifestyle factors, drugs and renal impairment, also increase total homocysteine values (29, 105). Elevated total homocysteine is thus not specific for low folate status. Normal total homocysteine suggests adequate folate supply. - A cutoff value of ~15-16 μmol/L for total homocysteine is commonly used (101, 105), but ranges from 10-20 μmol/L, depending on age, sex and population.
Erythrocyte (RBC) folate	- Reflects tissue stores and is an indicator of long-term dietary intake (29). - Concentrations <340 nmol/L[1] (150 mg/L) suggests low folate status (16, 106). The DRI suggests a cutoff of 305 nmol/L (140 ng/ml) (101). - Red cell folate >340 nmol/L (150 mg/L) is adequate to prevent anaemia but is associated with increased risk of neural tube defects (39, 107).
Serum folate	- A sensitive indicator and the most widely used method of assessing folate status (101). Useful diagnostic test if used and interpreted correctly together with other folate status indexes (108). - The most practical and least expensive measurements at the population level (16). - A good indicator of recent dietary folate intake (101). Affected by recent or transient changes in intake. Serum folate is strongly correlated to intake in population studies (109). - A low concentration is a sign of negative folate balance (folate depletion) at the time the sample was drawn and about two weeks before. Serum folate drops prior to tissue depletion and does not reflect tissue stores or whether there is sufficient folate for biochemical function (55, 110). - Plasma folate is subject to greater fluctuation than is red cell folate; but a drop in serum folate is paralleled by a reduction in red blood cell folate. (110). - Concentrations <10 nmol/L suggests low folate status (16). In the DRI (101), a cutoff for serum folate of 7 nmol/L (3 ng/mL) is suggested, citing (111).
Hb, MCV	- Anaemia due to folate and cobalamin deficiency is relatively uncommon worldwide (16). Haematologic indicators such as raised MCV and hypersegmentation of neutrophils together with anaemia are indicators of reduced folate status. These signs appear at a late stage of deficiency (55, 105) and is thus of limited value in diagnosing negative folate balance, folate depletion or mild folate deficiency (16).

[1] To convert conventional units (ng/mL) to SI units (nmol/L), multiply by 2.265. http://www.cdc.gov/nchs/nhanes/nhanes2007-2008/FOLATE_E.htm

1.2.6 Deficiency

In both folate and cobalamin deficiency, the lack of adequate 5,10-methylene-THF will result in megaloblastic changes in the bone marrow and other replicating cells (101). The risk of folate deficiency is increased in populations with low dietary intake, especially when combined with malabsorption. Pregnant women have increased requirements of folate and are thus at risk of deficiency. Also during lactation requirements are increased. **Figure 2** depicts the sequence of events in the development of folate deficiency. In negative folate balance, serum folate decreases first, followed by a gradual and simultaneous decrease in liver folate and erythrocyte folate during the subsequent stages 1) folate depletion, 2) folate deficient erythropoiesis and 3) folate deficiency anaemia, paralleling the development of iron deficiency (in Figure 1). In accordance with Figure 2, serum folate >5 ng/ml (11.3 nmol/L) is generally believed to reflect normal folate status, whereas serum folate <3 ng/ml (6.8 nmol/L) is indicative of negative folate balance or even folate deficiency anaemia (55).

	Normal	Negative Folate Balance	Folate Depletion	Folate Deficient Erythropoiesis	Folate Deficiency Anemia
Liver folate Plasma folate Erythron folate					
Serum folate	>5 ng/ml	<3 ng/ml	<3 ng/ml	<3 ng/ml	<3 ng/ml
RBC folate	>200 ng/ml	>200 ng/ml	<160 ng/ml	<120 ng/ml	<100 ng/ml
Diagnostic dU suppression	Normal	Normal	Normal	Abnormal*	Abnormal*
Lobe average§	< 3.5	< 3.5	< 3.5	> 3.5	> 3.5
Liver folate	>3 µg/g	>3 µg/g	<1.6 µg/g	<1.2 µg/g	<1 µg/g
Erythrocytes	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	> 12 g/dL	> 12 g/dL	> 12 g/dL	> 12 g/dL	< 12 g/dL
Plasma clearance of intravenous folate	Normal	Normal	Normal	Increased	Increased

Figure 2. Sequence of events in developing folate deficiency. For explanations of all the variables, see original article. To convert folate ng/ml to SI (nmol), or µg/g to SI (nmol/g), multiply by 2.266. Reproduced from Herbert 1987 (55) with permission from *The American Journal of Clinical Nutrition.*

In Indian infants and young children, poor folate status was an independent predictor of persistent diarrhea (112) and low mental development index scores when children with poor cobalamin status were excluded (113). Lower cognitive scores have been reported also in Western school children with low folate status (16). The data on cognitive development and behavioral problems in childhood should be further investigated (114).

In 2008, a WHO Technical Consultation summarized the evidence on folate and vitamin B12 deficiencies (16):

Strong evidence of:
- A causal relationship between folate deficiency and megaloblastic anaemia.
- An inverse association between blood folate concentrations and risk of low birth weight.
- A causal association between low maternal folate intake or lower folate status and increased risk of neural tube defects.
- A protective effect of folic acid supplementation or consumption of fortified foods, in the periconceptional period, against neural tube defects.

Moderate evidence of:
- An association between low folate status and cognitive impairment and some measures of dementia. Evidence for a beneficial effect of folic acid supplementation on cognitive performance or dementia is inconsistent.
- An association between low serum or red blood cell folate concentrations and prevalence or a longer duration of depression.

Weaker evidence of:
- An association between serum/plasma folate concentrations and cognitive function in children than in adults. However, some studies have reported lower scores in school children with low folate status.

1.2.7 Prevention of neural tube defects

Poor folate status increases the risk of neural tube defects 10-fold. Neural tube defects include spina bifida, anencephaly, and encephalocoele. These conditions are congenital malformations caused by improper closure of the spinal cord and cranium, a process that is completed within 28 days after conception (115). A recent Cochrane-review (115) concluded that folic acid, alone or in combination with other vitamins and minerals, prevents neural tube defects with no evidence of short-term side effects. The risk reduction of periconceptional folic acid supplementation has been estimated to be almost three-quarters (116). Supplementation has also been suggested to help prevent other foetal malformations (117). There is, however, not a clear effect on other birth defects (114, 115). The systematic review underlying NNR 2012 (114) concludes that evidence is insufficient to support or contradict current recommendations for dietary folate or supplemental folic acid during pregnancy and lactation or to recommend a particular periconceptional folic acid dose (114).

In Norway, 70% of women use folic acid when they know they are pregnant, however, only 27% take such supplements prior to pregnancy. Thus, folic acid is frequently taken too late (118). As a result, about 15 children are born with NTD every year. The number is reduced since the 1990s, however, there has been an increase in the number of abortions after week 12 following ultrasound diagnoses of neural tube defects, and the total number of pregnancies with neural tube defects is assumed to be constant at about 60 per year (118, 119). The Norwegian Mother and Child Cohort Study (MoBa) is a population-based pregnancy cohort study that between 1999 and 2008 recruited 90,723 women with 106,981 pregnancies and 108,487 children (120). One study in MoBa showed no association between maternal folate intake or status and pregnancy length or infant growth (120, 121). However, both in MoBa (122) and another study (123) prenatal folic acid supplementation was associated with an increased risk of certain infant respiratory diseases in early childhood, possibly due to epigenetic mechanisms (120). The NNR 2012 review (114) also refers to studies that do not find such an association, as well as data from NHANES (National Health And Nutrition Examination Survey) which totally contradicts the findings in MoBa, and the authors conclude that further research is needed.

1.2.8   Adverse effects of excessive intakes

A potential risk of consuming excess supplemental folate is the onset or progression of neurological complications in people with cobalamin deficiency. Excess folate can obscure or mask and thus potentially delay the diagnosis of cobalamin deficiency, which can result in an increased risk of progressive, unrecognized neurological damage (37). High concentrations of folate, and possibly exposure to unmetabolized folic acid, has been suggested to influence brain development adversely, perhaps through epigenetic mechanisms (97). A high proportion of US children have intakes of folic acid exceeding the tolerable upper intake (1-3 y: 300 µg per day) due to the general folic acid fortification and widespread use of folate containing supplements (97, 124). It has been pointed out that the potential risk of high folate intake in children deserves further review (124). The potential risk of high folic acid intake is, however, of little relevance in the Nordic countries without general folic acid fortification.

1.2.9   Status in children

It is well known that serum folate is higher in infants than in adults and older children (105, 125-130). Most previous studies on folate and cobalamin status in infants have been confined to newborns. In studies with data from later infancy, the effect of breastfeeding has often not been reported or only a limited number of folate and cobalamin indices have been reported. In only one study, based on newborn children, (105) reference limits for these indices during infancy has been reported. In Indian children (6-30 months) poor folate status was independently associated with persistent diarrhea (112). However, supplementation with folic acid and cobalamin did not reduce the incidence of diarrhea or lower respiratory infections. On the contrary, administration of folic acid significantly increased the risk of persistent diarrhea (131). Breastfeeding was identified as the most important protective factor in the prevention of persistent diarrhea (132).

1.3   Cobalamin

1.3.1   History

The history of cobalamin has been reviewed by Chanarin (133). George Hoyt Whipple (1878-1976) studied the rate of Hb regeneration in anaemic dogs, by giving them various foods and comparing their effect on the Hb concentration. Liver was the most effective, primarily because of the high iron content. However, liver also contains high amounts of cobalamin and folate. Whipple received the Nobel Prize in medicine and physiology for his work in 1934, together with Minot and Murphy. George Richard Minot (1885-1950) and William Murphy (1892-1987) provided a diet high in liver to patients with PA, who lack the intrinsic factor (IF) in the stomach needed to absorb cobalamin. However, about 1% of cobalamin can be absorbed by passive diffusion, and because of the high content in liver, a response was found in patients with pernicious anaemia given liver concentrates orally and subsequently by injections (133). In 1948, a pure antianaemic factor was isolated almost simultaneously by Folkers and co-workers in the US and Lester-Smith and co-workers in England. They isolated a red crystalline substance termed vitamin B_{12} and subsequently renamed cobalamin (133-135). In 1958 the co-enzyme deoxyadenosyl cobalamin was described, which is the major form of cobalamin in tissues. The compound was found to be light sensitive and rapidly changed into hydroxo cobalamin. Deoxyadenosyl cobalamin takes part in the metabolism of MMA.

The other functional form of cobalamin, methyl cobalamin, participates in the conversion of homocysteine to methionine (133). In patients with pernicious anaemia, gastric acid is lacking. The American William Castle found (about 1930) a response in patients with pernicious anaemia after he transferred partly digested beef and gastric juice from a healthy man to the stomach of pernicious anaemia patients. He concluded that a reaction was taking place between an unknown intrinsic factor (IF) in the gastric juice and an unknown extrinsic factor in beef muscle (133). In 1964 it was demonstrated that intrinsic factor was produced in the gastric parietal cell. These cells produce both HCL in gastric acid and the intrinsic factor, which is a glycoprotein (133). In 1962 nutritional cobalamin deficiency was for the first time reported in children. A syndrome was described in Indian children, with neurological symptoms and alterations in skin pigmentation, occurring in the first year of life (136).

1.3.2 Sources in the diet

Cobalamin is synthesized only in microorganisms, including bacteria and algae, and enters the human food chain through incorporation into food of animal origin. Animal-source foods including meat, eggs, fish, and milk, are thus the only natural source of cobalamin (16, 136). Cobalamin is stored in large concentrations in the liver. The cobalamin required by humans is not derived from micro flora in any appreciable amounts, although vegetable fermentation preparations have been reported as being possible sources of cobalamin (39).

The cobalamin content in breast milk has previously been shown to be 0.4-0.8 µg/L (39), and the DRI is 0.43 µg/L (310 pmol/L) (88, 101)[1]. Cobalamin in breast milk is firmly bound to haptocorrin**.** When a new and better method for separating cobalamin from haptocorrin was developed in 2009 (137), it became clear that previous methods had over- or underestimated the cobalamin content in breast milk. The content was estimated to be 1.2 µg/L (865 pmol/L)[2] in a group of Californian mothers with high intake of cobalamin (including supplements), whereas it was found to be less than 100 pmol/L in breast milk from women in a low income Guatemalan community (88). In a recent Danish study median concentrations of cobalamin in hindmilk were 760 (1.05), 290 (0.4), and 440 (0.6) pmol/L (µg/L) at 2 weeks, 4 months, and 9 months, respectively, with slightly lower values in foremilk (138).

Cobalamin in breast milk has been classified as a Group I nutrient, defined as nutrients in breast milk that are affected by maternal status/depletion. The content of cobalamin in breast milk reflects the cobalamin status of the mother (88). If the infant is already cobalamin depleted, supplementing the mother with cobalamin during breastfeeding only moderately increases the content in breast milk and does not provide enough cobalamin to improve the cobalamin status of the infant (88). Other sources of cobalamin in the infant diet (first year of life) include multivitamin supplements, formula, fortified cereals (porridge), meat and fish in infant dinners. As the child gradually starts eating family foods from the age of 1 year, adult diet cobalamin sources, including cow's milk, become more important.

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[1] A conversion factor of 721 (310/0.43) can be calculated.
[2] 1.2 µg/L multiplied with 721.

1.3.3 Recommendations

The criteria for determining cobalamin requirements in infants and children in the DRI are as follows: 0 through 6 months, human milk content; 7 through 12 months, extrapolation from younger infants; 1 through 18 years, extrapolation from adults (37, 101). The AI for infants 0-6 months as well as the WHO recommendations (39) are based on the observation/assumption that cobalamin deficiency does not occur in infants fed milk from mothers with adequate cobalamin status and that human milk contains enough cobalamin for optimum health in infants (39, 101). Based on an average content of cobalamin in the breast milk from well-nourished women of 0.4-0.8 µg/L and an average milk production of 0.75 L per day, cobalamin intake by infants would be between 0.3 and 0.6 µg per day, giving an AI/ RNI of between 0.4 and 0.7 µg per day. It was considered appropriate to use the lower RNI figure of 0.4 µg per day for infants aged 0–6 months and the higher RNI figure of 0.7 µg per day for infants aged 7–12 months (39). With the new method for estimating cobalamin in breast milk (137), future recommendations are likely to be somewhat higher. Recommendations on intake of cobalamin are presented in **Table 6**.

Table 6. Recommendations on intake of cobalamin, children 0-3 years

	Cobalamin, µg per day		
Age	0-6 months	7-12 months (6-11 mo) (103)	1-3 years (12-23 mo) (103)
Institute of Medicine (101)			
AI	0.4 ≈ 0.05 µg/kg	0.5 ≈ 0.05 µg/kg	-
EAR	-	-	0.7
RDA	-	-	0.9
UL	Not sufficient data	Not sufficient data	Not sufficient data
FAO/WHO (39)			
EAR	0.3	0.6	0.7
RNI (=RDA)	0.4	0.7	0.9
Nordic Council of Ministers			
NNR 2012 (26) (unchanged from 2004)	As supplied in breast milk	0.5	0.6 0.8 (2-5 y)
Norwegian Directorate of Health			
Norwegian infant recommendations (41)	0.3	0.6	1.0

1.3.4 Function

An adequate supply of cobalamin is essential for normal blood formation and neurological function (39, 101). Cobalamin is necessary for the development and initial myelination of the central nervous system as well as for the maintenance of its normal function (139). In mammalian cells, there are only two cobalamin-dependent enzymes: methionine synthase and l-methylmalonyl–coenzyme A mutase (39, 140, 141). These enzymes are involved in two reactions, as depicted and described by Stabler (139) in **Figure 3**: **A)** methionine synthase is responsible for a critical methyl transfer reaction that converts homocysteine to methionine. Methionine synthase requires methylcobalamin as a cofactor for the methyl transfer from methyl-THF to homocysteine to form methionine and THF. Insufficient supply of cobalamin will interfere with the methylation transfer, causing an increase in homocysteine (139, 142), **B)** a reaction that converts L-methylmalonyl-coenzyme A (CoA) to succinyl-CoA. The enzyme responsible for this reaction is called L-methylmalonyl-CoA mutase. L-methymalonyl-CoA mutase requires adenosylcobalamin to convert L-methymalonyl-CoA to succinyl-CoA in an isomerization reaction. In the absence of cobalamin the increased methylmalonyl-CoA is hydrolyzed to MMA that can be detected in body fluids (139, 142).
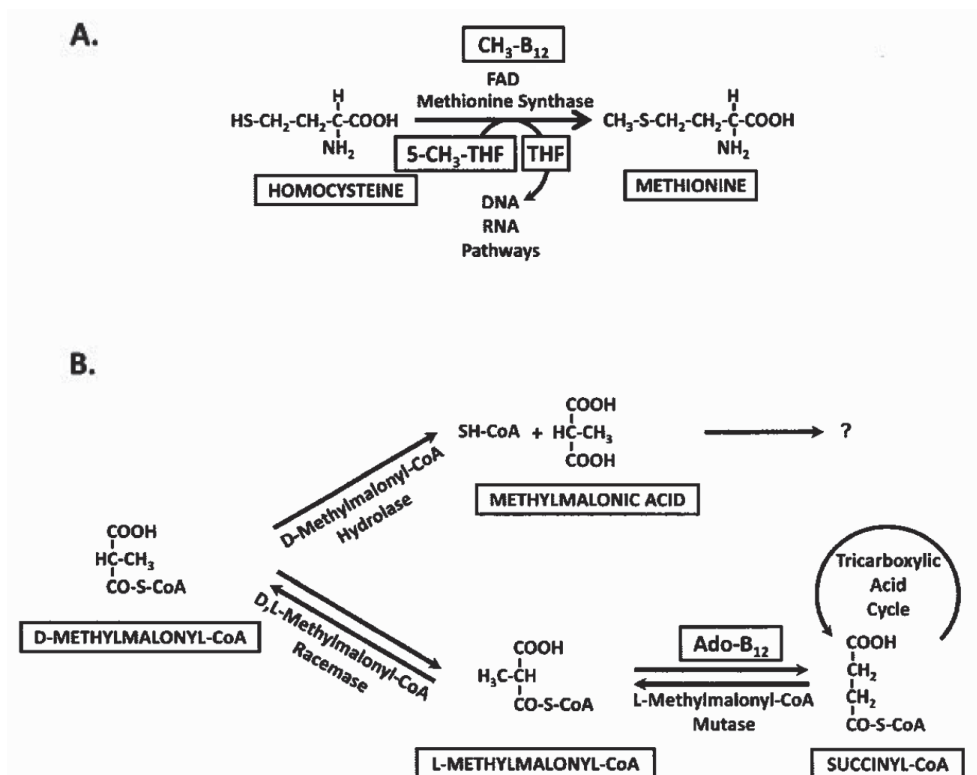
Figure 3. Function of cobalamin. Vitamin B12 (cobalamin) is required for two reactions in animals. (A) Methylation of homocysteine to methionine utilizing methyl-B12 (CH3-B12) as a cofactor and demethylating 5-methyltetrahydrofolate (5-CH3-THF), which supplies tetrahydrofolate, a cofactor for the synthesis of purines and pyrimidines required in DNA and RNA synthesis. The block in methionine synthase causes a buildup of homocycteine in both vitamin B12 and folate deficiency. (B) The adenosyl-B12 (Ado-B12) dependent conversion of L-methylmalonyl-CoA to succinyl-CoA. In the absence of vitamin B12 the increased methylmalonol-CoA is hydrolyzed to methylmalonic acid which can be detected in body fluids. Reproduced with permission from (139), supplemental material, Copyright Massachusetts Medical Society.

1.3.5   Cobalamin indexes

There are no gold standards for the assessment of cobalamin status. Haematological variables, such as Hb and MCV, are affected at a late stage of deficiency (105). Conventionally, low cobalamin status is detected by a combination of various indices, including low serum cobalamin concentration, combined with elevated concentrations of the functional markers total homocysteine and MMA (105). Total cobalamin in serum is bound to two main transport proteins, haptocorrin and transcobalamin (143, 144). Haptocorrin also carries the inactive forms of the vitamin, the so-called analogs (144). The metabolites total homocysteine and MMA mirror any lack of cobalamin within the cells (144). Sex and age influence serum indexes, both in adults (145), in adolescents (146) and children. Some of the most commonly used cobalamin status indexes are presented in **Table 7**.

Table 7. Cobalamin status indexes in serum/plasma

Index	Description/function
Cobalamin	Total cobalamin, which includes cobalamin bound to transcobalamin and haptocorrin (144). The usual cutoff for serum cobalamin is 150 pmol/L indicating deficiency, and 150–221 pmol/L[1] (200–300 pg/mL) indicating depletion (147).
HoloTC	Cobalamin bound to transcobalamin, HoloTC, is the biologically active cobalamin in serum and is available to all cells. HoloTC is a sensitive indicator of change in cobalamin intake and absorption (143). HoloTC is increasingly used to assess cobalamin status and is suggested to be a better marker than total cobalamin to identify deficiency (individuals, populations) (143, 144). The usual cutoff for holoTC is 30 pmol/L (147).
HoloHC	HoloHC is cobalamin bound to haptocorrin. It constitutes the major part of circulating cobalamin, but has no known function (144, 148, 149).
MMA	MMA accumulates in cobalamin deficiency. MMA is a sensitive marker of cobalamin deficiency, and elevated MMA is often used as a gold standard for classification of a patient's status as vitamin deficient (144). However, results can be affected by poor renal function (39). Marginally elevated MMA has low specificity (144).
Total homocysteine	Accumulates in cobalamin deficiency. Total homocysteine has low specificity for cobalamin deficiency because it also increases in folate deficiency and possibly thiamine and vitamin B6 deficiency (144).

[1] 150–221 pmol/L[1] = 200–300 pg/mL. 1 pmol/L = 1.355 pg/mL, 1 pg/mL = 0.738 pmol/L
http://www.endmemo.com/medical/unitconvert/Vitamin_B12.php

1.3.6 Deficiency

Cobalamin deficiency may be caused by lack in the diet, as is observed in vegetarians, or lack of intrinsic factor and thus deficient absorption in the stomach as is seen in patients with pernicious anaemia. Thus, low serum cobalamin is always present in untreated pernicious anaemia, however there may be other reasons than pernicious anaemia for low serum cobalamin concentration (133).

Figure 4 depicts the sequence of events in the development of cobalamin deficiency. In negative cobalamin balance, holoTC decreases first, followed by a gradual and simultaneous decrease in liver cobalamin and erythrocyte and white blood cell cobalamin during the stages 1) cobalamin depletion, 2) cobalamin deficient erythropoiesis and 3) cobalamin deficiency anaemia, paralleling the sequence seen in the development of iron and folate deficiency.

Stage:	Normal	Negative B12 Balance	B12 Depletion	B12-Deficient Erythropoiesis	B12-Deficiency Anemia
Liver B12 / HoloTC II / RBC+WBC B12					
HoloTC II	>30 pg/ml	<20 pg/ml	<20pg/ml	<12 pg/ml	<12 pg/ml
TC II % sat.	>5%	<5%	< 2%	< 1%	< 1%
Holohap	>150 pg/ml	>150pg/ml	<150 pg/ml	<100 pg/ml	<100 pg/ml
dU suppression	Normal	Normal	Normal	Abnormal	Abnormal
Hypersegmentation	No	No	No	Yes	Yes
TBBC*% sat.	>15%	>15%	>15%	<15%	<10%
Hap % sat.	>20%	>20%	>20%	<20%	<10%
RBC Folate	>160 ng/ml	>160	>160	<140	<140
Erythrocytes	Normal	Normal	Normal	Normal	Macroovalocytic
MCV	Normal	Normal	Normal	Normal	Elevated
Hemoglobin	Normal	Normal	Normal	Normal	Low
TC II	Normal	Normal	Normal	Elevated	Elevated
Methylmalonate	No	No	No	?	Yes
Myelin damage	No	No	No	?	?

Figur 4. Sequential stages in the development of vitamin B12 deficency as suggested by Herbert 1987. To convert B12, holotranscobalmin or holohaptocorrin from pg/mL to SI (pmol/L), multiply by 0.74. For further explanations of all the variables, see original article. Reproduced from (55) with permission from *The American Journal of Clinical Nutrition.*

Cobalamin deficiency is prevalent when intake of food of animal origin is low. This may be due to high cost, lack of availability, or cultural or religious beliefs (16). Low or deficient cobalamin status is reported in around 40% of the population in Latin America and reach 60-80% in some populations in Africa and Asia, affect all ages, and are associated with low intake of food from animal sources (88). Deficiency is more prevalent in strict vegetarians, but lacto-ovo vegetarians also have substantially lower intakes and status than omnivores (16, 88). In high-income countries, deficiency is usually found in elderly people and persons consuming a strict vegetarian diet (150). Breastfed infants born to deficient mothers are in numerous case reports described with clinical symptoms of cobalamin deficiency, usually presented around 4-7 months of age. Symptoms include growth stunting (length, weight, and head circumference), cerebral atrophy and various muscular, behavioural, and other developmental problems, some of which are not reversed by treatment in up to 50% of the cases (88, 136). These infants are typically exclusively breastfed. Symptoms and disabilities associated with cobalamin deficiency in infants and children are presented in **Table 8**.

There are two main reasons for maternal deficiency; 1) insufficient dietary intake, due to low intake of food with animal origin and 2) malabsorption, due to pernicious anaemia caused by lack of intrinsic factor, achlorhydria, ileal damage, or gastric bypass surgery (136). Dror and Allen (136) have described the underlying mechanisms of the neurological symptoms seen in cobalamin deficient infants. Cobalamin deficiency leads to impaired folate function (151). In cobalamin deficiency, folate may accumulate in the serum as a result of slowing of the cobalamin-dependent methyltransferase. This can be explained by the "folate-trap-hypothesis". A cobalamin–containing enzyme removes the methyl group from methylfolate, thereby regenerating THF for the formation of 5,10-metylene-THF, which is required for thymidylate synthesis. Because methylfolate is the predominant form of the vitamin in human serum and liver and because methylfolate only returns to the body's folate pool via the cobalamin-dependent step, cobalamin deficiency results in folate being trapped as methylfolate and thus becoming metabolically useless (152).

Table 8. Abnormalities in infants and children related to cobalamin deficiency[1]

Symptoms or disability associated with cobalamin deficiency
Failure of brain development and overall growth and development
Developmental delay or regression, which may be permanent
Does not smile
Feeding difficulties
Hypotonia, lethargy, coma
Hyperirritability, convulsions, tremors
Myoclonus
Microcephaly
Choreoathetoid movements

[1] Modified from (139, 147)

1.3.7 Adverse effects of excessive intakes

No adverse effects have been associated with excess cobalamin intake from food or supplements in healthy individuals. In the DRI, the evidence was not sufficient for deriving a Tolerable Upper Intake Level. When orally ingested there is a limitation in absorption from the gastrointestinal tract (101). However, even when administered intramuscularly at 300–3000 times the recommended dietary allowance, no toxic effect has been identified (147). Infants may, however, be especially vulnerable.

1.3.8 Status in children

Prior to the present study, some studies had reported cobalamin status in newborns (105, 153, 154) and during infancy (126, 127, 155, 156). However, the effect of breastfeeding was either not reported (125) or the studies included a limited number of cobalamin indexes (126, 127, 156). One study reported reference limits in newborns (105), but none of the studies reported reference limits in infancy beyond the newborn period. Most of these studies showed that infants have lower serum cobalamin and higher MMA than do older children and adults and the prevailing opinion was that this metabolic profile reflects immature organ systems, rather than impaired cobalamin status (157). However, some interpreted the results as cobalamin deficiency and suggested that cobalamin deficiency, particularly in breastfed infants, is

prevalent (157-159). Low cobalamin status has been found in populations of infants and young children consuming a diet low in food with animal origin. Dutch infants fed a macrobiotic diet had low serum cobalamin status compared with controls. They were also smaller, and a higher proportion had iron indexes indicative of iron deficiency (160, 161). MMA was increased 8-fold and total homocysteine 2-fold (162, 163). In a population of Indian children 12-18 months, with a high prevalence of folate and cobalamin deficiency, cobalamin status was significantly and positively associated with cognitive performance (113).

1.4   Breastfeeding and complementary feeding

WHO has since 2001 recommended exclusive breastfeeding for six months (164). This recommendation has been adopted by 20 out of 24 European countries and the US (personal communication, Anne Bærug, National Resource Center for Breastfeeding, Rikshospitalet). However, there has been a debate in developed countries about the micronutrient adequacy of this recommendation, as well as the existence and magnitude of health benefits. These questions have been addressed in a systematic Cochrane-review by Kramer and Kakuma from 2002 (165), updated in 2012 (166), comparing exclusive breastfeeding for 6 months with exclusive breastfeeding for 3-4 months followed by mixed breastfeeding. This systematic review concludes as follows: *"Infants who are exclusively breastfed for six months experience less morbidity from gastrointestinal infection than those who are partially breastfed as of three or four months, and no deficits have been demonstrated in growth among infants from either developing or developed countries who are exclusively breastfed for six months or longer. Moreover, the mothers of such infants have more prolonged lactational amenorrhea. Although infants should still be managed individually so that insufficient growth or other adverse outcomes are not ignored and appropriate interventions are provided, the available evidence demonstrates no apparent risks in recommending, as a general policy, exclusive breastfeeding for the first six months of life in both developing- and developed-country settings.*"

A WHO statement was published in 2011, saying: "*WHO recommends mothers worldwide to exclusively breastfeed infants for the child's first six months to achieve optimal growth, development and health. Thereafter, they should be given nutritious complementary foods and continue breastfeeding up to the age of two years or beyond*" (167).

The WHO position is in agreement with the position taken by American Academy of Paediatrics (AAP) (168, 169) and the ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition) Committee on Nutrition (170), all concluding that exclusive breastfeeding for about 6 months is a desirable goal. ESPGHAN, however, adds that complementary feeding (i.e. solid foods and liquids other than breast milk or infant formula and follow-on formula) should not be introduced before 17 weeks and not later than 26 weeks (170).

It is well known that exclusive breastfeeding for 6 months protects against gastrointestinal infections in developing countries (166, 171). Also in the developed world setting with good healthcare standards, exclusive breastfeeding for 6 months has been shown to lessen the frequency and severity of infectious episodes (166). Partial breastfeeding did not provide the same effect (172).

Iron: In developing-country settings where newborn iron stores may be suboptimal, the evidence suggests that exclusive breastfeeding without iron supplementation through six months may compromise haematologic status (165, 166). In a randomized trial on exclusive breastfeeding for 4 versus 6 months, serum ferritin was lower in a group exclusively breastfed for 6 months compared with a group exclusively breastfed for 3-4 months and given complementary food together with breast milk until 6 months (173). No indications or evidence of differences of biological or clinical importance was found. On the other hand, exclusive breastfeeding for 9 months has been negatively associated with iron status (27).

Folate: Folate status was not included in the Cochrane-review (166). Folate status is regarded to be good in exclusively breastfed infants in both high-income and more poorly nourished mother-child populations (147).

Cobalamin: Cobalamin status was neither addressed in the Cochrane review (166). The recommendation of 6 months exclusive breastfeeding is challenged with reference to cobalamin status in infancy (138, 174) (see discussion).

2 Aims of the study

The overall objective of the study was to examine the status of iron, cobalamin and folate in healthy infants and young children, and factors predicting their status.

The specific aims were:

1. To examine and assess nutritional status with regard to the micronutrients iron, folate and cobalamin in a group of healthy Norwegian children 0-2 years of age.
   a. To assess iron status from birth to two years of age (Paper I) (1).
   b. To assess folate and cobalamin status in children 6-24 months (Paper III and V) (3, 4).

2. To determine how maternal factors affects nutrient status at birth and later in infancy.
   a. To identify predictors of iron status at birth, and relate iron status at birth to iron status in the first two years of life (Paper II) (2).
   b. To determine predictors of serum folate and cobalamin status at birth and 6 months (Paper IV) (4).

3. To explore the relationship between dietary intake of folate and cobalamin and relevant serum indexes.
   a. To examine the influence of diet, including breastfeeding, on serum folate and cobalamin status in children 0-2 years of age (Paper III and V) (3, 5).

3 Study populations and methods

The thesis relates to nutritional sufficiency in the vulnerable first two years of life in a prosperous, Western population (mothers of Nordic decent). It is in part a description of iron, folate and cobalamin status, and in part a description of the relationship between dietary intake and serum indexes of these micronutrients. With the inclusion of maternal vitamin status, and the finding of the sources in the diet, it was possible to assess status in the children as probably normal as opposed to low.

3.1 Subjects

The study was designed as a prospective, longitudinal study from pregnancy to two years of age, to determine predictors of nutrient status in infancy and early childhood. We started out by assessing iron status, but since the collected data also allowed for assessment of folate and cobalamin status, these nutrients were included in the thesis.

The study population was recruited between April and June 1997 from Aker University Hospital in Oslo, which at the time of the study had the largest maternity ward in Oslo and provided care for 14 of the 25 local authorities within the city of Oslo. The participants had been invited by mail during pregnancy or they were recruited by admission to the hospital for delivery. A total of 364 mothers fulfilled the following inclusion criteria: Norwegian or other Nordic origin, singleton birth, gestational period of 37-42 weeks and child's birth weight above the 2.5 percentile (2600 and 2700 g for girls and boys, respectively). Exclusion criteria were twin birth and congenital malformations. See Papers I-V for further details.

A flow chart showing the number of children participating, not participating and lost to follow up at the various stages is given in **Figure 5**. The number of blood/serum measurements is given in **Table 10**. An overview of the data collected (including the number of blood samples and dietary data) at the various ages is presented in **Figure 1** in Paper II.

Pregnancy weeks 17-19
Serum samples: n=149

Pregnancy/birth
Included: n=364 (100%)
Available blood samples: n=364

Lost to follow up: n=11

6 months
Participants: n= 295
Blood samples: n=287, Questionnaires: n=294

Did not participate: n=58

Lost to follow up: n=2

9 months
Participants: n=309
FFQ: n=309

Did not participate: n=42

Lost to follow up: n=12

12 months
Participants: n=289
Blood samples: n=249, 7-d records: n=219, FFQ: n=280

Did not participate: n=50

Lost to follow up: n=9

18 months
Participants: n=290
FFQ: n=290

Did not participate: n=40

Lost to follow up: n=40

24 months
Participants: n=290
Blood samples: n=231, 7-d records: n=197, FFQ: n=276

Figure 5. The figure shows the number of participants at birth, 6-, 9-, 12-, 18- and 24 months, and also the numbers at each stage that did not participate and the numbers lost to follow up.

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3.2   Data collection

In brief, data were collected at birth (cord blood + interview) and at 6, 12 and 24 months (venous blood samples + questionnaires). In addition, 7 days weighed food records were completed at 12 and 24 months and simple questionnaires were completed at 9 and 18 months. For a subsample, plasma measurements from pregnancy, in weeks 17-19, were obtained (collected in another project). Shortly after delivery (1-2 days), data on the mothers were collected from the pregnancy record and the partogram. An interview with a questionnaire was completed at the hospital 1-2 days after birth. An overview of the methods used to collect background and dietary data, is given in **Table 9**.

Table 9. Collected data: Characteristics and dietary data

	Data collection time	N	Source of data on characteristics and dietary intake	Paper				
				I	II	III	IV	V
Mother	Pregnancy	364	Pregnancy record Gestational age, smoking, body weight, height, serum ferritin and Hb in pregnancy.		x	x	x	
	Delivery	364	Partogram/medical journal, and questionnaire Age, education, marital status, number of previous births, and number of years since last birth, use of vitamin and mineral supplements during pregnancy		x		x	
Child	Birth	364	Newborn medical record Birth weight, length, head circumference and sex		x	x		
	6 months	292 275	SFFQ Questionnaire about sickness/fever in previous week and month	x		x	x	
	9 months	312	Simple SFFQ			x		
	12 months	280 246	SFFQ Questionnaire about sickness/fever in previous week and month	x		x		
	12 months	219	7-d weighed record			x		
	18 months	290	Simple SFFQ			x		
	24 months	146	Weight and height data from health care centers	x		x		x

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3.3   Blood analyses

The number of blood samples was: Cord, 363; 6 months, 287; 12 months, 249; 24 months, 231; pregnancy, 149. Because of limited sample volume, the number of most measurements is lower than the total number of blood samples. The number of measurements and methods of analyses are given in **Table 10**.

Iron status was assessed by measurements of haematology (Hb and MCV), serum ferritin and sTfR. All analyses of iron indexes were performed at Aker University Hospital; the Central Laboratory (haematology and serum ferritin) and the Hormone Laboratory (sTfR), by standard methods.

Folate status was assessed by measurements of serum folate and total homocysteine, whereas cobalamin status was assessed by measurements of serum cobalamin, holoTC, holoHC, MMA and total homocysteine. The analyses of folate and cobalamin indexes were performed at the University of Bergen and the University of Oxford. The method used for analysing folate has been described by Molloy *et al* (175), the method for cobalamin by Kelleher *et al* (176, 177), the method for TC by Refsum *et al* (145), and the method for MMA and total homocysteine by Husek *et al* (178).

Haematology was measured shortly after the samples were drawn. All serum samples were stored at -70 °C until they were analysed. The analyses of serum ferritin and sTfR were performed after few weeks/months, whereas folate and cobalamin indexes were performed in samples that had been frozen for about 8 years. All analyses were performed at quality-controlled laboratories in samples that previously had not been thawed (infant serum samples). Maternal samples had been thawed once, which may have resulted in lower concentrations of folate. These lower concentrations may weaken the associations but are unlikely to create false associations (179).

Table 10. The number of blood/serum measurements and methods of analyses

Paper	Aim of the study	Variables; number of measurements	Serum analyses
I	To describe iron status in healthy Norwegian children	Hb, MCV, serum ferritin 6 months: 281-284 12 months: 249 24 months: 221-231	*Hb and MCV:* Sysmex haematology instrument (model 8000/9000; Sysmex Corp, Kobe, Japan). CV <1%. Serum ferritin: 180 Ferritin assay; Chiron Diagnostics ACS, Medfield, MA. CV <5%.
II	To identify predictors of iron status in healthy newborns, and to relate iron indexes at birth to iron status until 2 years	Serum ferritin, sTfR Birth: 350-363 6 months: 264-281 12 months: 242-249 24 months: 226-229	Serum ferritin: 180 Ferritin assay; Chiron Diagnostics ACS, Medfield, MA. CV <5%. *sTfR:* IDeA sTfR IEMA assay; Orion Diagnostica, Turku, Finland. CV of 5-6%.
III	To examine the influence of breastfeeding on serum folate and cobalamin status in healthy infants	Folate, cobalamin, holoTC, holoHC, MMA, total homocysteine Birth: 325-361 6 months: 186-262 12 months: 223-243 24 months: 219-224	*Cobalamin and folate:* Microbiological assays with a colistin sulfate-resistant strain of Lactobacillus leichmanii (cobalamin) (176, 177) or L. casei (folate) (175). *HoloTC:* Magnetic beads (microspheres) with immobilized monoclonal antibody specific for human transcobalamin to isolate transcobalamin, followed by a microbiological assay for cobalamin. CV: 5-8%. (145). *HoloHC:* Subtraction of holoTC from cobalamin (145). *MMA and total homocysteine:* Modified gas chromatography–mass spectrometry method based on ethylchloroformate derivatization. CV <5%. (178).
IV	To determine predictors of serum folate and cobalamin status in infancy	Same as for Paper III Pregnancy: 146-149 Birth: 325-361 6 months: 158-221	Same as for Paper III
V	To examine the influence of diet on folate and cobalamin status in an unfortified population	Same as for Paper III 24 months: 178	Same as for Paper III

3.4   Statistics

The statistical methods used have been described in each Paper. An overview of the statistical presentations and methods used in the various papers is given in **Table 11**. Descriptions of basic characteristics, serum indexes and dietary intake are given as means (standard deviations), geometric means (95% confidence intervals) and/or medians (quartile 1 – quartile 4), as well as proportions of the total sample. Because of the skewed distribution of serum indexes, these results were presented as geometric means (and 95% confidence intervals). The geometric mean was calculated by back-transformation of the mean of log-transformed data. Dietary intake data were presented as medians and interquartile ranges. Reference interval for vitamin status is given as 5th and 95th percentiles.

Univariate analyses

Comparisons between two independent groups were performed using Student's t-test and Mann-Whitney U test. For comparisons of proportions Fisher's exact test and Chi-square test were used. To correlate two variables, we used Pearson's correlation test and Spearman rank order correlation test. To compare serum indexes in different age groups, Paired-samples t-test was applied.

Multivariate models

We used linear regression analyses and analyses of variance (ANOVA) to estimate the relative influence of various factors on iron, folate and cobalamin status with control for potential confounders. Regression models and analysis of variance for a continuous variable assume that the dependent variable is normally distributed. Because this is not the case for serum indexes, we used log-transformed data in these analyses. To allow for adjustment for relevant factors, the intake of energy, cobalamin and folate at 24 months was also presented as geometric mean (and 95% confidence intervals).

Table 11. Statistical methods used[1]

Statistical methods/ data analyses	Description of test	Paper I	II	III	IV	V
Mean (SD)	Parametric average and variation from the average. The mean (arithmetic mean) is the sum of all the observations divided by the number of observations. The standard deviation is a measure of the average distance from the mean. Used for description of continuous variables, e.g., serum indexes, basic maternal and child characteristics	x	x		x	
Geometric mean (95% confidence interval)	Parametric average and variation from the average using log-transformed values with back-transformation. Used for continuous variables with an asymmetric (skewed) distribution, e.g., for serum indexes with skewed distribution, intake of energy and nutrients.		x	x	x	
Median (Q1-Q3):	Non-parametric average and variation from the average (25th and 75th percentiles). The median is the value half-way when the data are ranked in order. Quartiles together with the median divide the data into four equally populated subgroups. Used for non-normally distributed variables, e.g., intake of nutrients and food items.		x	x		x
Reference interval; 90% central range	The range within which the central 90% of values lie (i.e., excluding 5% at each end of the distribution). Used to present the variation of serum folate and cobalamin indexes (continuous variables) in healthy infants and toddlers using 5th and 95th percentiles.			x		
Two sample *t* test (Student's t-test)	Parametric test for comparing two independent groups of data (continuous variables). Used for comparison of serum status indexes in two independent groups, e.g., according to sex or breastfeeding status.	x	x	x	x	x
Paired *t* test	Parametric test for the comparison of a continuous variable with two groups of paired observations; e.g., measurement of serum indexes at two different ages.			x	x	
Analyses of variance, one way (ANOVA)	Parametric test for comparing three or more independent groups of observations (continuous data). Used when there is a single way of classifying individuals, e.g., cord iron indexes according to maternal iron intake (three groups) and to depict the dose-concentration relation between various food items and serum indexes, with adjustments.		x	x	x	x
Analyses of variance, two way (ANOVA)	Extension of the one-way ANOVA that examines the influence of two different categorical independent variables on one continuous dependent variable. Used when there are two factors classifying the observations. Used also to test for interaction, e.g., between parity and birth weight.				x	
ANOVA with adjustment for potential confounders	A test to determine whether a fixed factor has an effect on the outcome variable after controlling for potential confounding variables (also called ANCOVA), i.e., removing the variance caused by covariates (confounders), e.g., cord indexes according to smoking, adjusting for gestational age and birth weight.		x			
Repeated measures ANOVA	The equivalent of the one-way ANOVA test, but for related, not independent groups, e.g., paired comparison of serum indexes at three or more different ages.		x	x		

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Method	Description	I	II	III	IV	V
Mann–Whitney U test	Non-parametric test for comparing two independent groups of data (continuous variables), e.g., comparison of food intake in boys vs. girls.		x			x
Kruskal-Wallis test	Similar to Mann–Whitney U test, but for three groups or more, e.g., iron intake from supplements according to categories of maternal iron stores or cord iron indexes according to categories of maternal supplement use.		x			
Fisher's exact test and Chi-square test	Test used to assess the relationship between two categorical variables. Chi-square test requires that each cell has a frequency of five or more, while the Fisher's exact test can be used regardless of expected frequency. Used to compare the proportion with low serum indexes, e.g., in girls vs. boys.	x				
Pearson's correlation test	Parametric method of analysis of the association between two continuous variables, e.g., maternal serum indexes vs. cord serum indexes.	x			x	
Spearman rank order correlation test	Non-parametric method of analysis of the possible association between two continuous variables, using the ranks of the observations, e.g., serum indexes at 6 mo vs. days since change of feeding mode.		x		x	x
Multiple linear regression analyses	A statistical process for modelling the relationship between two or more explanatory (independent) variables and a response (dependent) variable by fitting a linear equation to observed data. The model describes how a dependent variable changes according to variation in one independent variable, while other independent variables are held at fixed values. E.g., serum cobalamin (dependent variable) as a function of cobalamin intake and number of breastfeedings (independent variables).		x	x	x	x
Stepwise linear regression analysis	As linear regression, but with sequential analyses to identify independent variables to be included or excluded in the following steps. Used to identify factors that were independently associated with cord and vitamin status at 6 months.		x			x
Linear model for repeated measurements (linear mixed models)[2]	Statistical model used for the assessment of possible interaction between age and sex of the child versus serum iron indexes.		x			
Extension of logistic regression to repeated measurements[2]	A method used to assess potential interactions between categories of cord serum ferritin and the age or the sex of the child versus the proportion of those with low serum ferritin at 6 months.		x			
Gaussian generalized additive regression models	Statistical procedure used for the generation of a graphic representation of the nonlinear relation between cord SF and sTfR.		x			x
Bonferroni correction	Adjustment for multiple pairwise comparisons following ANOVA.		x		x	
Fisher's r-to-z transformation	Analysis of the significance of the difference between two correlation coefficients, e.g., measuring the correlation between iron indexes assessed at different ages.		x			
Variance inflation factor	Assessment of the possibility of multicollinearity between dependent variables, e.g., intakes of relevant food items or food groups, cobalamin and supplement use (categories).					x

[1] See Papers I–V for further details. The table does not include all statistical procedures applied.
[2] Analyses performed by statistician Abderrahim Oulhaj, University of Oxford.

4 Synopsis of results

PAPER I

Iron status in a group of Norwegian children aged 6-24 months (1) .

Background
Adequate iron status is of vital importance for health and development in early childhood.

Objectives
We aimed to describe iron status in a group of healthy, Norwegian infants and toddlers, 6-24 months of age.

Design
In a longitudinal study, we measured Hb, MCV and serum ferritin at 6, 12 and 24 months, in order to assess iron status at these ages.

Results
The proportion of children with low iron status depended on the criteria used. By defining IDA as Hb <110 g/L in combination with serum ferritin <15 µg/L, the proportion of children with IDA was 3, 10 and 12% at 6, 12 and 24 months of age, respectively. If the number of children with depleted iron stores, defined as serum ferritin <10 µg/L but not low Hb, were added, the proportions of children with "low iron status" were 4% at 6 months, 12% at 12 months and 17% at 24 months. With more restrictive criteria for defining IDA, as Hb <110 g/L or <105 g/L combined with serum ferritin <12 µg/L, the prevalence decreased to 1–2% at 6 months and 2–5% at 12 and 24 months of age. If children with a history of fever in the previous month were excluded, the proportion of children with depleted iron stores (serum ferritin <10 µg/L) increased from 2 to 3% at 6 months, from 5 to 7% at 12 months and from 9 to 13% at 24 months.

Conclusions
Low iron status was relatively common in this group and mild iron deficiency does exist among otherwise healthy Norwegian children. Prevention and early treatment of iron deficiency should be a priority for the child health services.

PAPER II

Predictors of serum ferritin and serum soluble transferrin receptor in newborns and their associations with iron status during the first 2 y of life (2).

Background
Adequate iron status at birth may prevent iron deficiency in early childhood.

Objectives
We aimed to identify factors in pregnancy that may influence iron status at birth in healthy newborns, and to relate iron status at birth to iron status at 6, 12 and 24 months of age.

Design
In a longitudinal study, various factors in pregnancy were related to infant iron status at birth, assessed by serum ferritin and sTfR, and these measurements at birth (in cord serum) were compared with the corresponding measurements at 6, 12 and 24 months. Cord serum ferritin reflects neonatal iron stores, while cord sTfR is believed to reflect erythropoietic activity in the newborn. Good iron status would thus be associated with a high serum ferritin and a low sTfR.

Results
Cord serum ferritin was significantly (negatively) correlated with cord sTfR. Serum ferritin at birth was positively associated with maternal use of iron supplements in pregnancy, but negatively associated with maternal smoking. The concentration of cord sTfR increased with maternal first trimester BMI, gestational age and male sex of the baby. Cord serum ferritin was positively correlated with serum ferritin at 6, 12, and 24 months. At 6 months, 16 out of 17 infants with low iron stores (serum ferritin <15 µg/L) were boys.

Conclusions
We concluded that cessation of smoking and adequate iron prophylaxis during pregnancy may improve iron status in infancy. Iron status at birth exerts a long-term influence on iron status in early childhood. Cord serum ferritin is a predictor of iron status in the first 2 years of life. Boys are at particular risk of low iron status in early infancy.

PAPER III

Folate and cobalamin status in relation to breastfeeding and weaning in healthy infants (3).

Background

Folate and cobalamin status changes markedly during infancy and reference limits for these vitamins in the age group 0-2 years are lacking.

Objective

We aimed to describe folate and cobalamin status in a group of children 0-24 months of age, and how breastfeeding, weaning and supplemental feeding affect status of these vitamins.

Design

In a longitudinal study, we measured a broad range of folate and cobalamin indexes at birth and at the ages of 6, 12, and 24 months. Breastfeeding status and nutrient intake were assessed by use of questionnaires and 7-d weighed food records (at 12 months).

Results

All indexes of folate and cobalamin status changed significantly from birth to age 24 months. Serum folate was high until age 6 months and then declined. Serum folate did not differ significantly between breastfed and non-breastfed infants at 6, 12 or 24 months, however, exclusive breastfeeding was significantly and positively associated with serum folate at 6 months. Cobalamin status in breastfed children declined from birth to age 6 months, with a gradual increase thereafter. A different pattern was found in non-breastfed children, with a continuous increase from birth onwards, and with values at all ages above the breastfed children. In breastfed children, complementary feeding did not increase cobalamin status at 6 months and only modestly increased cobalamin status at 12 months.

Conclusions

Whereas folate status was highest in exclusively breastfed infants, breastfeeding was associated with low cobalamin status. This is a normal finding in breastfed children, possibly caused by a metabolic effect of breastfeeding itself on cobalamin homeostasis, and may prove to be appropriate and even beneficial. Reference limits according to age and breastfeeding status should be considered in early childhood.

PAPER IV

Maternal folate and cobalamin status predicts vitamin status in newborns and 6-month-old infants (4).

Background

Maternal factors in pregnancy may influence folate and cobalamin status at birth and in infancy. Sparse data exist on whether this influence lasts beyond the newborn period and how newborn folate and cobalamin status is related to status later in infancy and childhood.

Objective

We aimed to determine predictors of folate and cobalamin status in infancy by relating maternal factors in pregnancy to folate and cobalamin status at birth and at 6 months.

Design

In a longitudinal study, maternal vitamin status, parity, lifestyle variables and anthropometry were related to indexes of folate and cobalamin status in cord serum and venous serum samples of the baby at 6 months. Also cord serum indexes of folate and cobalamin status as well as newborn sex and anthropometry, were related to serum indexes at 6 months.

Results

The strongest predictors of folate status at birth and 6 months were maternal folate and cord folate. Cobalamin status at birth was best predicted by maternal holoTC, whereas at 6 months maternal and cord holoHC were the strongest predictors of cobalamin status. The association between cobalamin status and parity was negative at birth but positive at 6 months. Birth weight, female sex of the baby, and smoking were associated with low cobalamin or high total homocysteine at birth, but showed no or opposite associations at 6 months.

Conclusions

We found that maternal folate and cobalamin status exerts long-term positive effect on infant vitamin status. Multiparity, high birth weight and female sex were associated with lower cobalamin status at birth, but not at 6 months. Maternal smoking appeared to have little independent effect on B-vitamin status. Maternal holoTC was the superior predictor of newborn cobalamin status, while holoHC could be a valuable marker for predicting cobalamin status later in infancy.

PAPER V

Folate and cobalamin status in relation to diet in healthy 2-y-old children (5).

Background

Limited data exist on sources of folate and cobalamin in the toddler diet.

Design

We examined the influence of diet on folate and cobalamin status in healthy children, aged 24 months, in an unfortified population, by comparing results from the 7-d weighed food record-method to indexes of folate and cobalamin status obtained from venous blood samples.

Results

We found that although 35% of the children had folate intake below the Norwegian recommendations (80 µg per day), only 5.8% had low serum folate (<10 nmol/L). All children reached the recommended cobalamin intake of 0.8 µg per day. Intakes of both folate and cobalamin were positively associated with serum indexes. However, concentrations of the functional markers total homocysteine and MMA did not change according to intake, suggesting that functional vitamin status was sufficient. Serum folate was positively associated with fruits and berries and grain products; but not with dairy products, which was the food group that contributed most to folate intake. Cobalamin status was associated with dairy products, cobalamin supplements and liver pâté. Girls had higher holoTC and lower MMA than boys, indicating better cobalamin status in girls.

Conclusions

The best sources of folate were fruit and berries and grain products, whereas the best cobalamin sources were dairy products, liver pâté, and supplements. In the assessment of vitamin sources, vitamin availability must be considered.

5 Discussion

5.1   Methodological considerations

5.1.1   Study population

The results presented in this thesis apply to a group of children followed from birth to 24 months of age. The study was confined to healthy, term infants of mothers with Norwegian or other Nordic heritage. Infants of mothers with immigrant background, who are at increased risk of nutritional deficiencies, were not included. Most Norwegians adhere to a non-vegetarian diet with relatively high intake of meat, fish and milk (180). Thus, these healthy infants were probably at low risk of nutrient deficiencies. The high percentage of families participating at all stages (78, 68 and 63% of those invited at 6, 12, and 24 months, respectively) strengthens the credibility of the results.

5.1.2   Study design

The longitudinal design allowed us to gather a lot of data on the same mother-child pair from pregnancy, through birth until 24 months of age. By this approach, we could explore how maternal and newborn iron, folate and cobalamin status, affect status of these nutrients in the first two years of life. A weakness with the longitudinal design is that the participants may have been influenced during the study, with an increased awareness about diet and nutrient status. Indeed, the groups at each age stage cannot be viewed as independent and random samples. Even if dietary advice was not given, the individual child's iron status at each stage was reported to the parents, together with advice on use of iron medication when needed. Furthermore, children with low iron status (defined as Hb <110 g/L in combination with serum ferritin <15 µg/L at 6 (n=2), 12 (n=5) and 24 (n=5) months were advised to take iron supplements to improve iron status. These children were not excluded at the next follow-up. Thus, at 12 and 24 months, some few cases of IDA may have been prevented. Furthermore, it is possible that participation in the project led to increased awareness of the importance of adequate iron supply in the diet. On the other hand, normal iron status at one test may have led to a feeling of false safety from iron deficiency. The long duration of the study, with frequent follow-ups and blood sampling from the children at three times after birth may have

limited participation rate. However, out of 471 mothers that were invited, we included a total of 364 mother-child pairs (77%). It is unlikely that reward in the form of free diapers and other infant products has had any influence on the infant's diet in the families who participated.

5.1.3 Dietary assessment

The 7-days weighed food record used to collect dietary data at 12 and 24 months (Papers III and V) is the method that provides the best estimate for energy intake in children 0.5-4 years of age compared with the "gold standard", the doubly labelled water method (181). This method allows for ranking of individuals and thus correlations with serum nutrient concentrations (182).

Underreporting leads to the underestimation of nutrient intake. However, only seven children at 2 years of age had an energy intake <3MJ (183), which suggest overall good data on most children. Nelson *et al* (184) calculated the number of daily food records necessary to correctly classify 80% of toddlers into the extreme thirds of the distribution. They found that a period of six days was enough for folate, 12 days were necessary for cobalamin, whereas only four days were enough for calcium. Taking into consideration the finding that dairy products, and not fish and meat, was the main source of cobalamin in the diet, data-recording over seven days was probably sufficient for calculation of average cobalamin intake, but may underestimate the potential importance of fish and meat as dietary sources. The FFQs used at 12 months and 24 months were produced for use in a national survey of infant and toddler diet. A validation of the FFQ at 24 months against the 7-days weighed food record showed only moderate correlation (183). On a group level, FFQ is a useful tool because it makes it possible to collect data from a large number of infants/children. However, the FFQ may be too crude to reveal associations between dietary intake and serum indexes, and actual associations may be lost due to ranking errors.

5.1.4 Iron, folate and cobalamin status

A strength of the study is the use of multiple iron, folate and cobalamin serum markers. Importantly, these are only indicators of nutrient status and not linked to clinical outcome measures such as mental and motor score or development. Serum markers are difficult to interpret in children due to lack of age-specific reference values, and because their concentration is affected by common conditions in childhood such as infections, and also by breastfeeding. Furthermore, usual (normal) levels are not always optimal levels, where the latter are the levels associated with optimal health or minimal disease risk. The only clinical outcomes assessed in this study were the haematologic markers Hb and MCV. Ideally, we should also have assessed other clinical outcomes such as growth, infection risk and mental and motor development in the children from birth to 24 months, and preferably later in childhood. However, this was outside the scope of this study, which originally aimed to measure iron status. Furthermore, previous smaller studies indicated that few children would be identified with measurable anaemia or deficiency severe enough to cause adverse consequences of iron deficiency (21).

Iron

The combination of haematologic measurements (i.e., Hb and MCV), together with serum ferritin and sTfR, provides a good picture of iron status (46). Anaemia can be caused by deficiency of iron or other nutrients or arise following infection, inflammation or vaccination (12-14). A low serum ferritin is considered specific for iron deficiency. However, a normal or high serum ferritin may be caused by other circumstances, in particular inflammatory states, including infections (13, 185). Thus a high serum ferritin cannot preclude iron deficiency. Despite these limitations, according to NNR (25), serum ferritin is considered the best single indicator of iron status and is also the most widely used. However, studies on the correlation between storage iron and serum ferritin concentrations in infants and young children are lacking (186). Hence, we do not know whether serum ferritin of 10 and 15 µg/L, i.e., cutoffs frequently used in adults, is indicative of low iron stores in children (21). It has also been suggested that a cutoff for serum ferritin of 12 µg/L does not reflect depleted iron stores (50, 187).

The relatively high frequency of infections in infancy complicates the use of serum ferritin as a diagnostic tool for iron deficiency in this age group (50). Serum ferritin is an acute phase protein that increases in the presence of infection or inflammatory disease (188). Recent infections may increase serum ferritin, whereas values of Hb and MCV are decreased (13, 185). Furthermore, C-reactive protein (CRP) has been described to have a shorter half time than serum ferritin, making it an unreliable criterion regarding iron status assessment (189). Since we lack good diagnostic tools to separate infection anaemia from other anaemias, all children were included in Paper I. Omitting children with fever in the previous month before blood sampling, increased the number with low serum ferritin (<10 µg/L) somewhat, but it did not change the overall picture.

A finding of a low MCV provides strong supportive evidence for iron deficiency (47). In the age group 1-2 y, the lower MCV limit based on the 95% reference range is 73 fL. (51). This cutoff was used in Paper I. Although about 10% of the children had low MCV; only about 3% had the combination of anaemia, low serum ferritin (e.g., <15 µg/L) and low MCV.

In recent years (after iron status was assessed in our material), new markers of iron status have been developed. For instance, the hormone-like peptide hepcidin is a promising diagnostic tool for assessing iron status (190). Hepcidin is very low in patients with IDA or with low serum ferritin. Hepcidin interacts with the cellular iron exporter ferroportin and is thus a key regulator of systemic iron homeostasis. However, assays for hepcidin are still not available for clinical routine use. Furthermore, there is a need for the standardization of hepcidin assays and to determine clinically useful reference ranges (190, 191). In children, hepcidin correlates with serum ferritin and C-reactive protein, and reference ranges for children 0.5-3 years has recently been provided for commercial methods (192).

Folate and cobalamin

Folate and cobalamin status are evaluated by assessment of serum indexes (folate, cobalamin, holoTC, holoHC) as well as functional markers (total homocysteine and MMA). Low serum folate reflects recent negative folate balance (folate depletion), but not tissue stores or whether there is enough folate for biochemical function. Our results suggest that holoHC may be a more useful indicator of cobalamin status than previously recognized.

The serum concentration of total homocysteine is a functional indicator of folate and cobalamin status in adults. However, total homocysteine is an unspecific marker that can be raised in several circumstances (16, 17). Serum MMA is considered to be specific for cobalamin status in adults (16), but is often elevated in infants independent of cobalamin concentrations, possibly due to renal immaturity. We found a relationship between cobalamin and total homocysteine, but not between folate and total homocysteine. Other researchers have reported that folate/folic acid was not associated with total homocysteine in newborns or at 0-6 months, but in older children and adults (125, 193). Some studies have shown that plasma total homocysteine is more strongly correlated with serum cobalamin than with serum folate in infancy (126, 194). Thus, total homocysteine is not a good marker for infant B-vitamin status.

This is illustrated by the strong inverse relationship between maternal plasma folate in pregnancy and infant plasma total homocysteine at 6 months in an Australian study, even after adjustments (195). In a population with general folic acid fortification and with more than 70% of the mothers using folic acid supplements in pregnancy, it is very unlikely that high total homocysteine was caused by folate deficiency in pregnancy. Rather it is suggested that this is caused by intrauterine metabolic programming in response to the maternal nutritional environment, which persists after birth, as has been demonstrated in animal models (195). It has been suggested that one-carbon-metabolism is different in newborns and in adults (193). A similar relationship may exist for MMA. The serum concentration of MMA is elevated when cobalamin status is low, but also under certain other circumstances, such as renal insufficiency. The elevation of MMA, in itself, is thus not diagnostic (39), and renal immaturity may explain the high MMA concentrations frequently found in infants.

5.2   Discussion of results

5.2.1   Iron

Iron status

In Paper I iron status 6-24 months is described. We conclude that mild IDA exists among otherwise healthy Norwegian children. However, the appropriate definition of IDA has not yet been established, and the prevalence of low iron status in this group of infants and toddlers depends on the cutoffs and definitions used. At the time the present study was carried out and the iron results were published (1997-2004), a definition of IDA, Hb <110 g/L + serum ferritin <15 μg/L, proposed by Dallman (51), was frequently used. However, more strict criteria to define IDA, Hb <105 g/L + serum ferritin <12 μg/L were suggested by an ESPGHAN committee as a more appropriate definition of iron deficiency anaemia after studies of Danish infants (68) and Swedish infants (67). According to the ESPGHAN committee, the use of the WHO-cutoff for Hb (110 g/L) for defining anaemia in this age group and serum ferritin 12 μg/L as a cutoff for depleted iron stores, overestimate the prevalence of anaemia and of iron deficiency (50). This view is supported by others (196). The ESPGHAN committee in 2002 suggested that more appropriate cutoff values for anaemia in infants and young children should be established (50). In 2002, Domellöf (67) suggested new cutoff values for Hb of 105 g/L at 6 months and 100 g/L at 9 months, based on iron-replete, breastfed, unsupplemented infants. In 2014 ESPGHAN published a position paper with Hb <105 and serum ferritin <10-12 as suggested cutoffs for children 6-24 months (186). Due to the uncertainty regarding appropriate cutoff values, prevalence data on iron status in infants and young children should be interpreted with caution.

Using the more restrictive definition, as suggested (Hb <105 g/L in combination with serum ferritin <12 μg/L) (50), the prevalence of IDA in this group of children was 1% at 6 months and 2% at 12 months and 24 months of age, which is quite low.

The proportion of infants with depleted iron stores (serum ferritin <12 μg/L) at 12 months in our study (10%) corresponds to findings in a previous smaller Norwegian study from the 1980s (13%) (197, 198) and in a recent study from Sweden (10%) (199). However, higher prevalence has previously been found in Sweden (26%) (80), Iceland (41%) (69) and in the

Euro Growth study (serum ferritin <10 µg/L: 16%) (187) (**Table 12**). In Iceland, iron status has improved after a change in infant feeding recommendations resulting in replacement of unmodified cow's milk by iron enriched infant formula. Following this, the prevalence of depleted iron stores was reduced from 41% to 6% (200). In NNR 2012 (25) it is stated that the prevalence of iron deficiency in the Nordic countries is low and has decreased since the 1990s.

Table 12 shows, that depending on the criteria used, IDA in the Nordic countries has been found in 0-3% in children 0-6 months, 0-10% in children at 12 months, and 0-12% at 24 months. The corresponding proportions of children with depleted iron stores (serum ferritin <12 µg/L) are 0-4%, 6-41% and 10-13%.

The results from the present study, with IDA of 1-3% at 6 months, 2-10% at 12 months and 2-12% at 24 months, with the ranges representing IDA definitions as suggested by the ESPGHAN committee and Dallman 1993 (50, 51, 68), seem to be in accordance with findings from the other Nordic countries. Using the ESPGHAN criteria, the prevalence of iron deficiency anaemia in the present study was in good agreement with prevalence in European infants and toddlers, as recently reported by the ESPGHAN committee; <2% before 6 months, 2%–3% at 6–9 months, and 3%–9% at 1–3 years of age (186).

Table 12. Proportion of children with iron indexes below cutoff values in Nordic surveys

Country	Age (mo)	N	IDA Definition Hb, g/L+SF, µg/L	%	Anaemia Definition Hb, g/L	%	Iron depletion Definition SF, µg/L	%
Norway (1) (Paper I)	6	278-284	110 + 15	3	<110	26	<10	2
			110 + 12	2	<105	9	<12	4
			105 + 12	1	<100	5	<15	6
Denmark (201)	6	47-57	105 + 13	0	<110	32	<13	0
					<105	5		
Sweden (202)	4-9	70	110 + [1]	3				
	9				<110	20		
Norway (1) (Paper I)	12	249	110 + 15	10	<110	39	<10	5
			110 + 12	5	<105	16	<12	10
			105 +12	2	<100	3	<15	21
Norway (197, 198)	12	159-190	[2]	4	<110	7	<10	5
					<100	1	<12	13
Norway (78)	12	33	110 + 15	0	<110	9	<10	0
							<15	15
Norway (203)	12	93	110 + 15	2	<110	11	<10	13
							<15	34
Sweden (80)	12	56-76	110 + 12	3	<110	13	<12	26
					<105	8		
					<100	1		
Iceland (69)	12	111-114	105 + iron deficiency[3]	3	<105	9	<12	41
Iceland (200)	12	110-141	105 + iron deficiency[3]	0	<105	0	<12	6
Norway (1) (Paper I)	24	229-231	110 + 15	12	<110	31	<10	9
			110 + 12	5	<105	10	<12	13
			105 + 12	2	<100	3	<15	29
Norway (204)	24	44	110 + 15	9	<110	16	<10	16
Norway (79)	24	37	110 + 15	0	<110	5	<10	9
							<15	32
Sweden (81)[4]	30	3674	110 + iron deficiency[2]	7	ND		<12	10

[1] SF=serum ferritin; SF <12 µg/l + MCV <70 fl
[2] Multiple criteria
[3] SF <12 µg/l + one abnormal iron test, or SF 12-19 µg/l + 2 abnormal iron tests
[4] 32% immigrants

The prevalence of IDA in toddlers has been estimated to be 10% in Western societies, to around 50% in less developed societies (205), or about 25% among pre-school children worldwide (206). In NHANES III, serum ferritin <15 µg/L and <10 µg/L was found in 26% and 13% of non-breastfed (US) children 1-3 years (21).

In Paper II we found that low values of cord serum ferritin were associated with high values of cord sTfR. A sharp increase in cord sTfR was seen when cord serum ferritin declined below 100 µg/L. Nearly 20% of the infants in our study had cord serum ferritin values below this threshold. Combined with the observation that cord serum ferritin was a strong predictor of serum ferritin at 6, 12 and 24 months, and also significantly associated with sTfR at 6 months, this finding may suggest that 20% of newborn infants are at risk of developing low iron status in infancy or early childhood. However, the actual numbers of children with low iron status at 6, 12 or 24 months, proved to be lower, even according to the cutoffs set by Dallman (51); 4, 12 and 17% respectively. A tracking effect of serum ferritin, i.e., a strong correlation of serum ferritin at one time point with later measurements, has been seen in Danish infants 2-9 months (68) and in Irish children up to 36 months (207).

Predictors of iron status 0-2 y

The results in Paper II suggest that iron status at birth is a predictor of iron status during the first two years of age, and that adequate iron prophylaxis and avoidance of smoking during pregnancy may improve iron status at birth by increasing infant iron stores.

Maternal iron status

In Paper II we did not find an association between maternal serum ferritin as measured in early pregnancy and serum ferritin at birth. This is not surprising, given that the measurement was done early in pregnancy and that the women were advised to use iron supplementation according to their serum ferritin values (208, 209). A potential influence of maternal iron stores in early pregnancy on infant iron stores (both measured by serum ferritin) would thus likely have been evened out throughout the course of the pregnancy. Based on several trials, Allen (24) concludes, that maternal iron status strongly affects the iron stores of the infant at birth. Under circumstances with poor nutritional status and high prevalence of iron deficiency an association between maternal and infant iron status is found (210, 211). However, also in a Finnish study (212) and a British study (213) a significant association between maternal and

cord serum ferritin was found. The difference between these studies and our study may be the differential advice on supplement use according to early pregnancy serum ferritin.

Iron supplementation in pregnancy

Our results indicate that irrespective of maternal iron status, the use of selective iron supplements in pregnancy, even at low doses, may increase serum ferritin at birth and thus prevent iron deficiency in infancy and early childhood. Infants born to women taking iron supplements had significantly higher cord serum ferritin than did infants born to women not taking such supplements, despite the lower maternal serum ferritin in the former group. Furthermore, there was a clear association between cord serum ferritin and serum ferritin at 6 months, 12 months and 24 months, strongest at 6 months, but still significant at 24 months. As part of the standard pregnancy care routine at the time, all pregnant women received advice on iron supplements based on selective iron prophylaxis, after measurement of serum ferritin in early pregnancy (214). We found that iron intake from supplements in pregnancy reflected early pregnancy serum ferritin, indicating that the mothers did in fact follow the advice. However, there was no difference in cord serum ferritin between infants born to mothers with a high or low intake of iron from supplements. Other researchers did not find an effect of supplements in pregnancy on infant iron status. Zhou *et al* (215) showed in a randomized controlled trial that routine iron supplementation in pregnancy had no effect on iron status of children at 6 months and 4 years of age. Systematic reviews by Pena-Rosas *et al* (58, 216) conclude―that there is a lack of convincing evidence that routine iron supplementation during pregnancy improves infant iron status, even though this was suggested by a single study performed in a Nigerian population with a very high prevalence of IDA. Due to adverse side effects and particularly high pregnancy Hb, they summarise that there is a need to update recommendations on doses and regimens for routine iron supplementation (58).

In accordance with this, Domellöf *et al* (40) conclude that there is no conclusive evidence that iron supplementation of pregnant women in a Scandinavian setting would improve infant iron status. Nor has iron supplementation in pregnancy proved to have any effect on cognitive function in the offspring at 4 years or behavioural scores at 6-8 years in the offspring (40). The lack of conclusive evidence does not mean that there is no effect of maternal iron supplementation of infant iron status. Few studies exist, probably due to the challenges regarding collection of serum samples in infants and young children.

Domellöf *et al* (40) conclude that supplementation with 20-40 mg Fe per day (corresponding to a total iron intake of 34-49 mg per day) from week 18-20 of gestation would be effective in preventing iron deficiency and IDA during pregnancy. This conclusion is based on studies by Sandstad *et al* (209), Makrides *et al* (217) and Milman *et al* (218, 219). Furthermore, Domellöf *et al* (40) conclude, based on the studies by Sandstad *et al* (209) and Milman *et al* (219), that iron supplementation on an individual basis according to serum ferritin in early pregnancy, may be of some benefit. Women with serum ferritin below 20-30 μg/L would benefit from an iron dose of 40-60 mg Fe per day, while iron replete women (serum ferritin >60-70 μg/L) would not need to take iron supplements.

In the current recommendations for pregnancy care by health care personnel (220), it is advised against routine recommendation of iron supplementation to healthy women. It is claimed that a) iron deficiency is a small problem in Norway, b) that serum ferritin probably does not reflect iron status in pregnant women, and c) that provision of iron to healthy women has no effect on the mother or the foetus. In contrast, Borch-Iohnsen *et al* (221) argue against these recommendations and document that a) about 15% of Norwegian fertile women have depleted iron stores, b) that serum ferritin measured early in pregnancy is a good indicator of iron stores in healthy pregnant women, and c) that iron supplements are absolutely necessary to cover pregnancy iron needs in these women. The authors argue that the previous guidelines for selective iron supplementation in pregnancy should be reinforced. This is in accordance with the recent Nordic Nutrition Recommendations (25).

The present Norwegian National Guideline for pregnancy care is up for revision. The recommendation on iron supplementation in pregnancy should be based on the NNR 2012 (25) and the underlying systematic review (40). The current recommendations for pregnancy care without routine measurement of serum ferritin and advising against routine iron supplementation, may result in overlooking women with a sub-optimal iron status, and the birth of babies with suboptimal iron stores, resulting in increased risk of iron deficiency in childhood.

Sex

We found that boys were at higher risk of iron deficiency than girls from birth on. In Paper II we show that boys at birth had higher cord sTfR concentrations than did girls, which may reflect greater cellular iron needs in boys than in girls. Higher sTfR in boys than in girls in infancy has been found in some (222, 223), but not all (224) studies. We speculate that the difference in sTfR at birth could be due to hormonal factors.

Cord serum ferritin concentrations in boys did not differ from those in girls. However, low cord serum ferritin (<40 µg/L) was more prevalent in boys than girls, suggesting that boys already at birth are at risk of low iron status, as a consequence of the long lasting effect of iron stores at birth on iron status throughout infancy (6-24 months). The risk of low serum ferritin at 6 months was most apparent in boys, and in particular, boys born with cord serum ferritin concentrations below the median.

In Paper I we showed that there was a sex difference in iron status at 6 months, reflected not only by higher concentrations of serum ferritin in girls, but also higher Hb and MCV. The MCV difference remained at both 12 and 24 months (Paper I). Pooled data from six studies in four countries showed that male sex was the main predictor of both iron deficiency and IDA, together with low birth weight (225). Better iron status in girls was found among Swedish 6-9-month-old infants (223) and Icelandic 12-months-olds (69). After increasing the iron content in the Icelandic infant diet, no significant gender difference was found in MCV and Hb, but girls still had significantly higher serum ferritin (200). Domellöf *et al* (223) conclude that some of the differences may be genetically determined while others seem to reflect an increased incidence of true deficiency in boys.

Smoking in pregnancy

Smoking in early pregnancy was associated with significantly lower cord serum ferritin compared with non-smoking, in line with findings in other studies (226, 227). The smokers did have a lower intake of iron supplements during pregnancy. Smokers have previously been shown to have a less nutritious diet compared with non-smokers throughout pregnancy (228). However, even after controlling for iron supplement use, as well as intake of cod liver oil (as an indicator of healthy lifestyle (229)), the effect of smoking on cord serum ferritin remained in the present study, indicating an independent effect of smoking on serum ferritin at birth.

Other possible explanations include hypoxia leading to increased erythrocyte production, impaired uterine blood flow or interference with transplacental availability of iron (se Paper II).

Infant diet

This thesis does not include iron status related to iron intake. However, this was investigated in two master theses that were part of the project, at 12 and 24 months (230, 231). Both at 12 and 24 months total iron intake was significantly and positively correlated with Hb, but not with serum ferritin (except in girls at 24 months). This may reflect suboptimal Hb caused by the high iron needs and low iron stores at this age, as described by Engelmann (232) and hence priority given to Hb before iron stores. Children may not have the same ability as adults to mobilize iron from stores (233). At 12 months, infant food products including formula, contributed 63% of iron intake in breastfed children and 68% in non-breastfed children. Bread and meat were also significant sources of iron. At 24 months, the food groups that contributed most to the iron intake were bread, meat, infant cereals and iron enriched Norwegian brown whey cheese.

The iron status results from the present study were used in the revision of the Norwegian National Recommendations on infant nutrition (41). The finding of low iron status in a considerable proportion of the children in the present study, contributed to a change in the recommendations towards postponing the introduction of cow's milk from 6 months until 12 months of age, and the emphasis on the inclusion of iron rich food items from 6-12 months. These changes were in accordance with international recommendations. Hopefully, this change has contributed to an even lower prevalence of iron deficiency in Norwegian infants and toddlers.

5.2.2 Folate
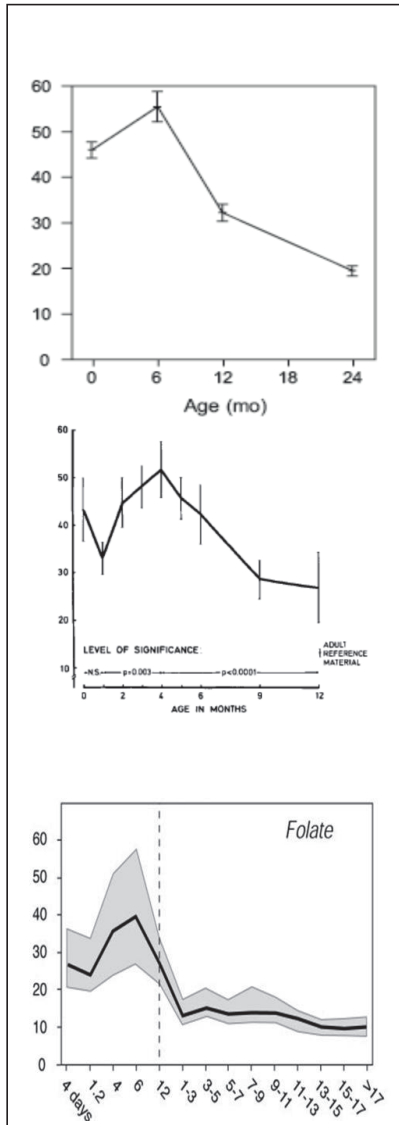
5.2.3 Folate status

We have reported folate status at birth, 6, 12 and 24 months as well as maternal folate status in pregnancy (Paper III-V).

The high values at birth compared with the relatively low maternal values in pregnancy, may reflect an active transport process in utero (101).

Serum folate was high also at 6 months and then declined, in accordance with previous findings (85, 125) (see **Figure 6**).

Few infants had low serum folate; however at 24 months, when most children were eating family food, 6% had serum folate <10 nmol/L.

Serum concentrations of total homocysteine was relatively unaffected by serum folate in early infancy, but showed the typical inverse association at 24 months.

Figure 6. Serum folate in healthy infants and children. *Top*: Hay 2008 (paper IV) (3); *middle*: Ek & Magnus 1979 (85) (plasma folate), reproduced with permission from *Acta Paediatrica*; *bottom:* Monsen 2003 (158), reproduced with permission from *The American Journal of Clinical Nutrition.*

Influence of maternal factors

Our data suggest that there is a consistency of maternal folate status during pregnancy and lactation. Cord serum folate was predicted by maternal folate status and use of folate supplements in pregnancy. Cord serum folate, in turn, predicted folate status at 6 months (Paper IV).

Folate supplements

The finding that supplementation of folate and cobalamin in pregnancy influences the cord serum concentrations of these vitamins has been reported by others (195). Half of the mothers in our study (51%) reported use of folic acid supplements (at least once) during pregnancy, a somewhat lower proportion than what was found in the later MoBa (61-72%) (234, 235). Although supplements contributed with 65% (median 400 μg per day) of total intake (607 μg per day) in MoBa, 57% of the women still had a total intake of folate below recommended intake (235). As reported in the NNR 2012 review (114), the periconceptional use of folic acid supplements in 2000–2003 was low, at 10.2% (234). Thus only few women follow the recommendation to take 400 μg of supplemental folic acid before or around the conception. NNR 2012 recommends 500 μg per day throughout pregnancy and the lactation period. Very few women in the Nordic countries achieve this level of intake without using supplements (114). A diet rich in fruits, berries, whole meal grain products and vegetables will have a positive effect on both maternal and infant intake of vitamins and minerals, including folate, iron, and vitamin C. Outside the scope of this thesis, the reported possible adverse effects of folate supplementation in early pregnancy on infant health outcomes should be further explored and weighed against the possible benefit of taking supplements (96).

Parity

Although folate status overall was good in infants, we observed that increasing parity was associated with lower infant serum folate at 6 months. One possible explanation is that maternal folate stores during pregnancy and lactation are reduced with increasing number of pregnancies and infants breastfed. However, we did not find an association between maternal folate status in pregnancy and infant folate status at 6 months. Furthermore, the folate content of breast milk is relatively unaffected by maternal status (88, 236). An alternative explanation is that the number of siblings in a family affects infant feeding mode and diet, including breastfeeding, exclusive breastfeeding and/or the provision of folate-rich complimentary

foods. However, a Norwegian survey showed that the odds of exclusive breastfeeding at 4 months and breastfeeding at 6 months increased with increasing number of children (237). Thus, we do not have any explanation for this association.

Folate status related to diet

In infants, aged 6 months, folate status was positively associated with exclusive breastfeeding. The folate content in breast milk, relatively unaffected by maternal intake or status, including supplementation (US women), protects the infant from deficiency if maternal intake is low (88, 236). These results support the view that exclusive breastfeeding for 6 months will maintain sufficient folate status (88). Ek & Magnus (238) found that the average folate content in human milk of non-supplemented Norwegian mothers fed to 3-month-old babies was 55 µg/L, somewhat lower than the average milk folate concentrations reported by the Institute of Medicine (85 µg/L) (101). This may be related to methodological differences, but may also be due to differences in general folate fortification of foods. Furthermore, they found that a formula containing 78 µg folic acid/L resulted in serum folate concentration in non-breastfed children close to breastfed children (238). The authors concluded that formula-fed infants need 70 to 80 µg per day during the first months of life to match the serum folate concentrations of breastfed infants, and that formulas should have a folate content ~40% higher than mothers milk (238). ESPGHAN states that the goal for the formula composition is that the effect on physiological or biochemical components (e.g., plasma markers) should be as similar as possible to exclusively breastfed infants (239). Therefore, it can be questioned if the content of folic acid should be increased in the formulas on the Norwegian market, in order to reach the serum levels of exclusively breastfed infants. The European Commission directive on infant formula and follow on milk give a broad limit of 10-50 µg folate per 100 kcal of formula (240). However, the folic content of cereals and supplements should also be considered. At the time of our study, infant cereals were fortified with folate, but almost none of the infant multivitamin supplements contained folic acid. In our cohort, only the Floradix formula contained folic acid (50 µg per 5 ml), and it was used by only two infants at 12 months. As could be expected, the use of multivitamin supplements at 6- or 12 months did not influence folate status (Paper III, supplemental tables). Now folic acid is added to most vitamin supplements on the marked, as well as to infant cereals and other baby products.

At 12 months, the median folate intake from foods and formulas (61 and 75 µg per day in breastfed and non-breastfed infants, respectively), was above the NNR recommendation of 60

µg per day for infants 6-11 months, and well above the recommendation of 5 µg/kg body weight per day (103). With a median body weight for 12-month-olds (241), this translates into about 50 µg per day.

Serum folate at 12 months was about half of the cord serum concentration; still one third higher than the concentrations found at 24 months. In fact, the 5th percentile of serum folate at 12 months[3] was above the WHO cutoff of 10 nmol/L (242). Baby products, including formula and baby cereals, yielded about one fourth of the folate intake at 12 months, whereas fruits and berries only contributed to 12-14%. It is possible that the substantially higher serum folate concentration at 12 months compared with 24 months was due to the much higher percentage of folate from fortified infant products, with highly available folic acid, at 12 months. Other possible explanations are breastfeeding (positively associated with infant folate status) or expansion of body volume.

At 24 months, the median folate intake (87 µg per day) was in accordance with the recommendation of 60 µg per day at 6-23 months and 80 µg per day at 2-5 years (99). Thirty five per cent of the children had intakes below 80 µg per day, however, this recommendation encompasses children up to the age of 5 years. The fact that 98% of the children had folate intake below the RDA/WHO recommendation of 150 µg folate per day (101), while only 6% had low serum folate concentrations (<10 nmol/L), suggest that this recommendation may be too high. Median intake in our group of children in a population without general fortification was – as expected – considerably lower than the mean intake of dietary folate equivalents found in US children 1-3 years of 385 µg per day from diet and 498 µg per day in total (124). Despite the relatively low intake in our population, the serum folate appeared to be sufficient to maintain low total homocysteine concentrations.

Only a few children still got some breast milk at 24 months of age. At 24 months, fruit and berries contributed most significantly to serum folate, even though both dairy products and grain products to a larger degree contributed to folate intake. Thus, availability of folate appears to be more important than the total intake. The availability of folate in fruits and berries appears to be high, while it seems lower from dairy products.

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[3] 19 nmol/L in breastfed and 14 nmol/L in non-breastfed infants, see Paper III, Table 3.

Contrary to our findings, most previous studies have reported higher serum folate in non-breastfed (=formula fed) infants compared with breastfed infants (127, 130, 156). This discrepancy may be caused by the relatively low folate content in Norwegian formulas which at the time was 63 µg of folic acid per 1000 ml ready to drink formula. In contrast, Indian breastfed children had higher folate concentrations compared with non-breastfed infants (243). This may reflect cobalamin deficiency in the breastfed children in this largely vegetarian population, as indicated by a positive association between folate and total homocysteine (243). Increased serum folate has been reported in cobalamin-deficient subjects (244) and is probably related to the "folate trap" mechanism (245, 246). It has been suggested that such an association could complicate the assessment of folate status in cobalamin-deficient subjects (247).

5.2.4   Cobalamin

Cobalamin status

We have shown that cobalamin status differs markedly according to breastfeeding status. Cobalamin status declined after birth in breastfed, but increased in non-breastfed infants (Paper III). Cobalamin status in the newborns and infants in our study was in line with results from other Western studies (105, 125, 126, 153, 154), confirming that concentrations change markedly during the first year of life. Our finding that breastfed infants have a different serum profile for cobalamin indexes (lower serum cobalamin and higher MMA and total homocysteine) compared with non-breastfed infants and older children, is in accordance with findings both prior to (125) and after our study (195). Interpretation of these findings is challenging because reference values for serum cobalamin indexes in infancy and children are scarce or lacking, as is functional outcomes measures, i.e., mental and motor development measurements related to cobalamin status. Traditionally, cobalamin deficiency is considered to be rare in Western countries. A screening program in USA detected and reported 3 cases of cobalamin deficiency in newborns per 100 000 live births, that is 0.003% (248). On the other side, concern has been expressed that cobalamin deficiency is common in otherwise healthy infants of non-vegetarian mothers (158, 159).

In our group of healthy breastfed infants, the 5th percentile for serum cobalamin concentrations was 121 pmol/L at 6 months, 165 pmol/L at 12 months and 183 pmol/L at 24 months. These numbers are lower than the corresponding figures for the non-breastfed infants of 195, 221 and 237 pmol/L, respectively. However, only 5% of the breastfed infants in our population had serum cobalamin values characterized as mild cobalamin deficiency in a Czech study (167 ng/L = 123 pmol/L) (249). Notably, the age-interval in the Czech study was larger, ranging up to toddlers who usually have higher concentrations, with varying feeding mode. Thus the actual proportion of infants with mild cobalamin deficiency in our population of 6-month-old breastfed infants was probably lower than 5%.

In our study, the geometric mean serum cobalamin was above 300 pmol/L in the breastfed group at 12 months of age, compared to ~400 pmol/L in the non-breastfed group (Figure 2, Paper III). Dutch infants 10-20 months, fed a macrobiotic diet, with a red cell profile indicative of cobalamin deficiency, but without significant clinical symptoms of neurological degeneration, had half of these values (mean serum cobalamin 149 pmol/L) (160).

Newborn boys had a modestly better cobalamin status compared with girls (significant for holoTC). This difference has been observed before (105) and may possibly be explained by sex differences in the cobalamin-binding proteins in the amniotic fluid (250). However, at 6 months, the opposite pattern was observed, with higher holoTC in girls. Also at 24 months, girls had higher holoTC and lower MMA than did boys. The reason for this finding is not clear, and the functional and clinical consequences have not been assessed.

Maternal status

Maternal serum cobalamin in pregnancy in our study (geometric mean 282 pmol/L and 95% confidence interval 269, 295) was quite similar to concentrations observed in a group of Danish pregnant women (149). Comparable cobalamin concentrations were also found in another group of pregnant women with high cobalamin intake (251). In a Scottish study, the lower reference limits (2 SD from the mean) for serum cobalamin were 141 ng/L (104 pmol/L) in the 1st trimester and 92 ng/L (68 pmol/L) in the 3rd trimester, respectively (252). So even if 15 out of 149 (10%) of the pregnant women in our study had cobalamin concentrations <200 pmol/L (unpublished data), none had levels below the lower limit of the Scottish study (252). The cobalamin status in our group of pregnant women thus seem to be

very good, as expected in a population with a generally high intake of dairy products, meat and fish, and a high proportion using cobalamin supplements (53% at any time of pregnancy and 38% ≥5 days per week during the 3rd trimester).

Concentrations of cobalamin (as well as serum ferritin and folate) decrease during pregnancy (149, 251, 253, 254), in spite of normal cobalamin stores, because of haemodilution and preferential uptake of absorbed cobalamin in the third trimester by the placenta and transfer to the fetus (255). The decline in cobalamin is related to alterations in haptocorrin-bound cobalamin, with no change in the biological active holoTC (149).

Maternal – infant status

In Paper IV, we have shown that maternal cobalamin status in pregnancy as indicated by holoTC predicted infant cobalamin status at birth, and that both maternal and cord cobalamin status (as indicated by holoHC) predicted cobalamin status at 6 months. The association between maternal cobalamin status in or before pregnancy and infant cobalamin status at birth has been shown in several other studies as well (88, 256, 257). Cobalamin deficiency in infants is traditionally viewed as being caused by maternal deficiency (248, 249, 258), which may result from insufficient cobalamin absorption due to chronic gastritis (249), pernicious anaemia or vegetarianism (88, 147). Furthermore, dietary insufficiency with low intake of animal-derived proteins is common in many developing countries (147).

A long-term effect of maternal cobalamin status on status in the offspring has also been found in a study of 12-month-old Guatemalan children (259) as well as in a Danish study (at birth, 4 months and 9 months) (138). Because maternal cobalamin stores in women eating a mixed diet are at ~3 mg (3000 μg) and the cobalamin requirement of the fetus is ~50 μg, it may be assumed that the event of a single pregnancy has minimal impact on maternal stores. However, it appears that newly absorbed cobalamin is more available to the fetus than is maternal cobalamin stores (101). Cobalamin deficiency has been reported in infants 4-6 months that were breastfed by mothers who had been strict vegetarians for only 3 years (101, 260). Thus, adequate cobalamin intake in pregnancy is important (101).

In the Danish study (138), the authors suggested that breastfed infants were cobalamin deficient, in spite of sufficient status in the mothers. This challenges the perception of normality. In most of the world the intake of food of animal origin (containing cobalamin) is much lower than it is in Scandinavia. Is there any reason why healthy, term infants born to and breastfed by women living in prosperous countries, as the Scandinavian, eating a varied diet with meat, fish and milk, should be cobalamin depleted?

Nature is, however, not perfect. For example, breast milk does not supply sufficient amounts of vitamin D to sustain the needs of breastfed infants, without exposure to sunlight (261-263). Notably, vitamin D differs from cobalamin in several ways. Vitamin D does not increase in breast milk with increasing intakes in the mother, unless she consumes very large quantities. Greibe *et al*, on the other hand, found a strong positive association between maternal cobalamin status and breast milk cobalamin content (138). Furthermore, for vitamin D there is an alternative source, namely sunlight, and the basic requirement for vitamin D can be satisfied by exposing the skin to the sun (103).

The critical question is whether breastfed infants need additional supplement with cobalamin. Based on the findings in the Danish study, Greibe (138) argues that the higher cobalamin status in infants fed complimentary foods at 4 months compared with exclusively breastfed infants (contrary to the findings in our study at 6 months), is indicative of deficiency. In our study, maternal use of cobalamin supplements improved cobalamin status in infants at birth but not at 6 months, suggesting an overall good cobalamin status in both mother and child (Paper IV).

Functional outcomes

There were no significant differences in the haematological variables haemoglobin or MCV between breastfed and non-breastfed infants, neither at 6 nor 12 months, supporting the view that the low cobalamin status found in breastfed infants is not associated with negative functional outcome. In another study (52, 174), no differences in the haematological indexes haemoglobin, MCV or reticulocyte haemoglobin content were found between a group of children injected with cobalamin and a control group, in spite of a reported improvement in motor development and regurgitations in the cobalamin group compared with the control group. Notably, in contrast to our cohort, the children in the latter study were recruited from a clinic after admittance for feeding difficulties, neurologic symptoms, or developmental delay (174). In a recent randomized controlled trial in India, in children aged 12-18 months, with markedly lower cobalamin status than in our group, treatment with cobalamin improved growth and stunting, but only in those with elevated total homocysteine (>10 µmol/L) (personal communication Tor Strand). Thus, the interpretation of the results by Torsvik *et al* is unlikely to be relevant for healthy children with a physiological intake of the vitamin. Notably, the improvement appeared to happen very quickly after injection. The authors' explanation for the reported improvements would be increased cobalamin available for myelination. However, myelination is a slow process, and an alternative explanation is that the treatment enhanced substrate supply for oxidative phosphorylation and energy production (264).

In the Indian study, supplementation of children with cobalamin or folate or both for 6 months, improved motor and problem solving skills compared with placebo. The supplementation with each of the vitamins alone only had an effect on gross motor functioning, while an improvement in problem solving skills was only seen when the vitamins were supplied together. The overall effect and the effects on subscales were strongest in stunted children, and in children with evidence of cobalamin deficiency, i.e., in children at risk (personal communication, Tor Strand).

There is a general agreement that the principal way to determine requirements of infants should be to examine the levels in milk from mothers on adequate diets (39), and similarly, that the serum profile in infants breastfed by well nourished women should be the standard for the content of nutrients in formula (239). Consequently, in lack of functional outcomes to

measure cobalamin status, the serum cobalamin profile in exclusively breastfed, healthy infants, born and breastfed by well-nourished mothers, should be considered the gold standard.

A systematic review of the literature comparing infants exclusively breastfed until 6 months with infants introduced to supplemental feeding from 3-4 months, did not reveal any differences in functional outcomes, including motor and cognitive development, apart from lower morbidity from gastrointestinal infections in infants exclusively breastfed until 6 months (166). Thus, there is no reason to assume that the observed lower cobalamin status found in healthy breastfed infants has any negative effect on functional outcome measures and there is no contradiction between cobalamin status and exclusive breastfeeding until 6 months.

Possible mechanisms

We speculate that the characteristic serum profile found in breastfed infants is caused by some properties of breast milk and not lack of cobalamin in the breast milk. These properties may exert a metabolic or hormonal effect on cobalamin status. It is possible that breastfeeding increases the utilization of holoTC. In support of this view, is the finding of a strong and long-term effect of pregnancy cobalamin status on infant status, with no or little association between breast milk cobalamin and infant cobalamin status (138, 259). Similarly, intake of cobalamin supplements in pregnancy increased cobalamin in cord serum, but not in the infants at 6 months of age (Paper III, Table 2). This is in accordance with the findings in the Australian study (195). Mixed feeding at 6 months and cobalamin from other sources is, an alternative explanation in line with the interpretation in the Danish study (138).

The main difference between breastfed and non-breastfed infants is a difference in holoTC, the active form of the vitamin (Paper III). It seem plausible that breastfed infants have a special ability to utilize cobalamin more efficiently and that priority is given to the most important of the two reactions that involve cobalamin in the metabolism, i.e., the methionine synthase reaction. The less critical reaction converting L-methylmalonyl-coenzyme A (CoA) to succinyl-CoA, may be given less priority, resulting in increasing amounts of methylmalonyl-CoA being hydrolysed to MMA, thus explaining the increased concentrations of MMA found in breastfed infants.

Cobalamin status related to diet

The lower cobalamin status in our group of breastfed children remained, both at 6 months after introduction of complementary foods, at 12 months after adjustment for total cobalamin intake from the diet, and even at 24 months when the contribution of breast milk to total energy intake was small/modest. Greibe *et al* (138) reported higher cobalamin status (significant for holoTC, borderline significant for total cobalamin, not significant for MMA) in infants fed a mixed diet at 4 months compared with infants exclusively breastfed at this age. In contrast to our larger study, they did not compare partly breastfed with non-breastfed infants. The breastfeeding mothers were assessed as having good cobalamin status; however compared with 2 weeks and 9 months after birth, the breast milk content was lowest at 4 months, as was the cobalamin status of the babies at this age. Unfortunately, in the Danish study, the results from our study were misinterpreted, as if infants consuming a mixed diet had better cobalamin status than did those exclusively breastfed. This misinterpretation is used to argue in favor of the view that the low cobalamin status represents deficiency and not a normal physiological state (138). However, the authors conclude that it remains to be determined whether these findings indicates that cobalamin intake through breast milk is insufficient to sustain a child's needs at this age (138). With the limitation that it was a small group that was exclusively breastfed until about 6 months, our findings do not support the view that complementary food should be introduced before 6 months of age in order to increase cobalamin status.

At 24 months of age, we found that cobalamin status was associated with intakes of dairy products, liver pâté and supplements. The good availability of cobalamin from dairy products has also been demonstrated in adults (265, 266). Our results suggest that liver pâté is an excellent source of cobalamin, as a consequence of the large quantities of cobalamin stored in liver (39) (83 µg per 100 g) (267). Although the cobalamin content in liver pâté is lower (12 µg per 100 g) (267), the cobalamin content is still markedly higher than in almost all other food items.

5.2.5   Breastfeeding and complementary feeding

We found that breastfed infants had lower cobalamin status than did non-breastfed infants at 6-24 months. The same was true for iron status at 12 months; breastfed children had significantly lower serum ferritin and higher sTfR than non-breastfed children (serum ferritin: 25 vs 31 µg/L, p=0.03; sTfR: 4.3 vs 3.9 mg/L; p=0.02), even if no difference was found in Hb (unpublished data). Cobalamin status remained lower in breastfed infants even after controlling for cobalamin in the diet at 12 months. We hypothesize that metabolic effects of breast milk or breastfeeding may cause a change in cobalamin homeostasis. Whatever reason, low cobalamin status seems to be a normal finding in healthy breastfed children.

In the past, a higher content of energy and protein in formula compared with breast milk resulted in non-breastfed children being heavier when compared with breastfed children. Growth charts developed in the 1960's, based on infant populations with a large proportion of formula fed infants, resulted in normal weight, breastfed children frequently being assessed as underweight. New WHO growth charts based on exclusively breastfed children (241) has corrected this. Similarly, the content of iron in formula has been reduced, with the aim of attaining a serum profile in non-breastfed children more similar to that found in breastfed children (268).

In a recent systematic NNR 2012 review, Domellöf *et al* (40) concludes that iron supplementation of breastfed infants results in a transient increase in iron status reflected by Hb or serum ferritin. "*However, since no reduction in IDA was observed, the data do not support that term, normal birth weight, healthy, breast-fed infants need additional dietary iron before 6 months of life in populations with a low general prevalence of IDA such as the Nordic countries.*" (40).

The same reasoning can probably be applied for cobalamin, i.e., that supplementation with cobalamin to breastfed infants will increase serum cobalamin and probably also lower total homocysteine serum and MMA. There is, however, no available data to suggest that cobalamin provided to healthy, breastfed infants will improve haematological status, prevent anaemia, promote growth or enhance cognitive development.

In a Medical position paper, Global standard for the composition of infant formula recommendations, by an ESPGHAN Coordinated International Expert Group (IEG) (239), it is stated *"...Therefore, the adequacy of infant formula composition should be determined by a comparison of its effects on physiological (e.g. growth patterns), biochemical (e.g. plasma markers) and functional (e.g. immune responses) outcomes in infants fed formulae with those found in populations of healthy, exclusively breast-fed infants."*... *"The IEG concludes that infant formulae should only contain components in such amounts that serve a nutritional purpose or provide another benefit. The inclusion of unnecessary components, or unnecessary amounts of components, may put a burden on metabolic and other physiologic functions of the infant. Those components taken in the diet, which are not utilized or stored by the body, have to be excreted, often as solutes in the urine. Since water available to form urine is limited and the infant's ability to concentrate urine is not fully developed during the first months of life, the need to excrete any additional solutes will reduce the margin of safety, especially under conditions of stress, such as fever, diarrhoea or during weight loss."*

According to this, exclusively breastfed infants should be the standard, and the lower serum cobalamin indexes found in breastfed infants compared with formula fed infants, should result in a reflection that the amount of cobalamin added to formula and possibly infant cereals, is too high and should be reduced, rather than to supplement breastfed infants in order to attain a similar cobalamin status. Future research will have to show whether the low iron and cobalamin status in infancy reflects increased cellular utilization or has a physiological purpose, i.e., to counteract bacteria and infections and thus protect the vulnerable infant.

5.3   Conclusions and future implications

In this longitudinal study, we have examined serum markers of iron, folate and cobalamin status in 364 healthy children followed from birth to 24 months of age. Maternal and child factors associated with these serum biomarkers have been described, and the importance of newborn status for values later in infancy and early childhood have been examined.

Iron

According to established definitions, IDA was relatively uncommon in this cohort of infants and toddlers. Using ESPHAN criteria (50, 186), prevalence of IDA was 1-5% and depleted iron stores were found in about 10% (Paper I). Cord serum ferritin was a strong predictor of iron status during the first 24 months of life (Paper II). As selective iron supplementation in pregnancy was positively associated with iron stores in the newborn, these data support the suggestion that iron should be provided during pregnancy, and especially in mothers with low serum ferritin in the beginning of pregnancy (40), – to prevent iron deficiency in infancy and early childhood. As shown by others, we confirmed that boys were at higher risk of iron deficiency than girls, at birth and later in infancy (Paper I-II). Further studies should be performed to evaluate potential long-term risk associated with low iron status in infants.

Folate

In this cohort, maternal serum folate predicted infant serum folate at birth, which, in turn, was positively associated with serum folate at 6 months (Paper IV). Folate status at 6 months was positively associated with exclusive breastfeeding (Paper III), consistent with the notion that folate status is protected in breastfed infants (88, 236). At 24 months, folate status was associated with intakes of fruit and berries and grain products, i.e., similar to that observed in older children and adults.

Throughout infancy, plasma folate levels were high relative to the results observed later in childhood and in adults. This probably explains the lack of concern related to this nutrient in well-nourished infants. The possibility that infants need higher folate levels than later in life should not be ignored. Thus, future studies should look at long-term effects of low folate status in infants, even if folate levels are within the defined normal range for older children and adults.

Cobalamin

As reported by others, low serum cobalamin status was a common finding in the breastfed infants, compared with formula-fed infants and adults. However, breastfeeding status did not affect haematological variables. It is well documented that breastfeeding has beneficial effects on functional outcomes, including risk of infections and cognitive development (269, 270) and that exclusive breastfeeding for 6 months has several advantages over exclusive breastfeeding for 3-4 months followed by mixed breastfeeding (166). Given that the infants in this study were healthy and well-nourished and born to mothers with excellent cobalamin status, the low serum cobalamin levels cannot be equated with deficiency. Rather, our results indicate that low cobalamin status is a characteristic finding in healthy, breastfed infants. Thus, the finding that breastfed infants have different biomarker levels than non-breastfed infants, older children and adults, is an observation, but cannot be used to determine prevalence of deficiency or to suggest changes of breastfeeding recommendations.

In 2-year-old children, cobalamin levels were substantially higher than in infants, and cow's milk was the most important source of cobalamin. The relatively high content of cow's milk in the diet of most Norwegian children probably ensures an excellent cobalamin status after infancy.

This thesis emphasises the need to establish age-specific reference values for both breastfed and non-breastfed infants for relevant nutrient biomarkers. In the assessment of cobalamin status in infants, reference intervals from healthy, well-nourished infants and children should be applied, in the same way as is done in the assessment of iron status. The controversial issue of how to interpret low cobalamin levels in breastfed infants should be further examined in carefully designed trials in healthy breastfed children, with one group of infants receiving only breast milk and the other group receiving cobalamin supplements in addition. Relevant outcome variables should include serum biomarkers, and more importantly, clinical outcomes, such as cognitive development, motor development, growth, haematology and infection risk.

Policies on breastfeeding recommendations should be based on longitudinal studies in infancy and childhood that include relevant functional outcomes and preferably with additional data from proper clinical trials.

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