SCIENTIFIC OPINION

Scientific Opinion on Dietary Reference Values for cobalamin (vitamin B12)[1]

EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA)[2,3]

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ABSTRACT

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) derived Dietary Reference Values (DRVs) for cobalamin (vitamin B12). The Panel considers that the approach based on a combination of biomarkers of cobalamin status, i.e. serum cobalamin, holotranscobalamin (holoTC), methylmalonic acid (MMA) and plasma total homocysteine (tHcy), is the most suitable approach to derive DRVs for cobalamin. The Panel notes the uncertainties with respect to cut-off values for cobalamin insufficiency of these indicators and that an Average Requirement (AR) cannot be determined from the limited data available. There is consistent evidence in adults that a cobalamin intake of 4 µg/day and greater is associated with serum concentrations of holoTC and cobalamin within the reference ranges derived from healthy subjects, together with MMA and tHcy concentrations below the cut-off values for adults, which indicates an adequate cobalamin status. Therefore, the Panel sets an Adequate Intake (AI) for cobalamin at 4 µg/day for adults based on data on different biomarkers of cobalamin status and in consideration of observed mean intakes, which range between 4.2 and 8.6 µg/day in adults in several EU countries. AIs for infants and children are calculated by extrapolation from the AI for adults using allometric scaling and applying a growth factor. Estimated AIs range from 1.5 µg/day in infants aged 7–11 months to 4 µg/day in children aged 15–17 years. For pregnancy and lactation, additional cobalamin intakes related to the accumulation of cobalamin in fetal tissues and transfer of cobalamin into breast milk are considered and AIs of 4.5 and 5 µg/day, respectively, are proposed.

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KEY WORDS

cobalamin, vitamin B12, Adequate Intake, Dietary Reference Value

SUMMARY

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a Scientific Opinion on Dietary Reference Values for the European population, including cobalamin (vitamin B12).

Cobalamin is a metal complex with a central cobalt atom bonded to six ligands. The upper or β-axial ligand varies (R-group: cyano-, hydroxo-, aquo-, methyl- or adenosyl-group), giving rise to the correspondingly named chemical forms of the vitamin. In humans, two reactions are known to require cobalamin as coenzyme. One is the rearrangement of methylmalonyl-coenzyme A (CoA) to succinyl-CoA in propionate metabolism by methylmalonyl-CoA mutase in mitochondria. The other is the cytosolic transmethylation of homocysteine by 5-methyl-tetrahydrofolate to methionine by methionine synthase. The most frequent clinical expression of cobalamin deficiency is megaloblastic anaemia. Independent of megaloblastic anaemia, neurological dysfunction is another clinical feature of cobalamin deficiency. Cobalamin insufficiency is characterised by biochemical abnormalities, such as elevated total homocysteine (tHcy) and/or methylmalonic acid (MMA) concentrations in blood resulting from impaired cobalamin metabolic activity, with no specific clinical symptoms.

Cobalamin absorption consists of several steps, including its release from proteins, its binding by gastric intrinsic factor and the absorption of intrinsic factor-cobalamin complexes through receptor-mediated endocytosis in the terminal ileum. Fractional absorption of cobalamin is rather variable, depending on the dietary source, the amount of cobalamin ingested, the ability to release cobalamin from food and the proper functioning of the intrinsic factor system. The Panel considers an absorption of 40 % as a conservative estimate.

In plasma, cobalamin is bound to the cobalamin-binding proteins transcobalamin (TC) and haptocorrin. HoloTC is the physiologically active form of cobalamin that delivers the vitamin to cells. Intracellular cobalamin concentration is maintained by modulating the expression of holoTC receptor, with an efflux system that shunts the excess cobalamin out of the cells. In contrast, cobalamin accumulates in the liver and kidney. Various studies have indicated losses of 0.1–0.2 % of the cobalamin pool per day, regardless of the size of the store. The highest losses of cobalamin occur through the faeces, which include cobalamin secreted in the bile. If the circulating cobalamin exceeds the cobalamin binding capacity of the blood, the excess is excreted in the urine.

Main biomarkers of cobalamin status include blood concentrations of cobalamin, holoTC and the metabolites MMA and tHcy. The sensitivity and specificity of these biomarkers can be affected by factors unrelated to cobalamin status. The limitations of all biomarkers make a combination of biomarkers necessary to assess cobalamin status.

From experimental data in individuals with pernicious anaemia in remission, a daily intake of 1.5–2 µg cobalamin represents a minimum requirement for maintenance of a normal haematological status, associated with low body stores of 1–2 mg. Based on a factorial approach and estimating daily obligatory losses of cobalamin, estimated cobalamin requirement ranges between 4 and 20 µg/day, which reflects the large uncertainties associated with this approach. The Panel considers the approach based on a combination of cobalamin biomarkers of status as the most suitable approach to derive DRVs for cobalamin for adults. The Panel notes the uncertainties with respect to cut-off values for cobalamin insufficiency of these indicators and that an Average Requirement (AR) cannot be determined from the limited data available. There is consistent evidence from observational and intervention studies that a cobalamin intake of 4 µg/day and greater is associated with serum concentrations of holoTC and cobalamin within the reference ranges derived from healthy subjects, together with MMA and tHcy concentrations below the cut-off values for adults, which indicates an adequate cobalamin status. Therefore, the Panel sets an AI for cobalamin at 4 µg/day for adults based on data on different biomarkers of cobalamin status and in consideration of observed intakes, which range between 4.2 and 8.6 µg/day in adults in several EU countries.

The Panel considers that there are insufficient data to derive an AR for infants and children. Therefore, AIs are calculated by extrapolation from the AI for adults. Allometric scaling was used on the assumption that cobalamin requirement is related to metabolically active body mass, and growth factors were applied. After rounding, estimated AIs range from 1.5 µg/day in infants aged 7–11 months to 4 µg/day in children aged 15–17 years.

For pregnant women, a cobalamin intake of 0.5 µg/day in addition to the AI for non-pregnant women is proposed in consideration of a fetal accumulation of 0.2 µg cobalamin/day and of an absorption of 40 %. This addition results in an AI of 4.5 µg/day for pregnant women.

For lactating women, an increase in the AI is based on the cobalamin intake required to compensate for the amount of cobalamin secreted in breast milk. Considering a cobalamin concentration of 0.5 µg/L and a mean milk transfer of 0.8 L/day during the first six months of lactation in exclusively breastfeeding women, an average secretion with breast milk of 0.4 µg cobalamin/day is estimated. Taking into account 40 % absorption, a mean cobalamin intake of 1.0 µg/day is required to balance the secretion in milk, and results in an AI of 5 µg/day for lactating women.

Based on data from 13 dietary surveys in nine European Union countries, average cobalamin intake ranges across countries were 0.8–2.1 µg/day in infants < 1 year, 2.2–4.0 µg/day in children aged 1 to < 3 years, 2.6–5.7 µg/day in children aged 3 to < 10 years, 3.3–6.6 µg/day in children aged 10 to < 18 years and 4.2–8.6 µg/day in adults.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The scientific advice on nutrient intakes is important as the basis of Community action in the field of nutrition, for example such advice has in the past been used as the basis of nutrition labelling. The Scientific Committee for Food (SCF) report on nutrient and energy intakes for the European Community dates from 1993. There is a need to review and, if necessary, to update these earlier recommendations to ensure that the Community action in the area of nutrition is underpinned by the latest scientific advice.

In 1993, the SCF adopted an Opinion on the nutrient and energy intakes for the European Community.[4] The report provided Reference Intakes for energy, certain macronutrients and micronutrients, but it did not include certain substances of physiological importance, for example dietary fibre.

Since then new scientific data have become available for some of the nutrients, and scientific advisory bodies in many European Union Member States and in the United States have reported on recommended dietary intakes. For a number of nutrients these newly established (national) recommendations differ from the reference intakes in the SCF (1993) report. Although there is considerable consensus between these newly derived (national) recommendations, differing opinions remain on some of the recommendations. Therefore, there is a need to review the existing EU Reference Intakes in the light of new scientific evidence, and taking into account the more recently reported national recommendations. There is also a need to include dietary components that were not covered in the SCF opinion of 1993, such as dietary fibre, and to consider whether it might be appropriate to establish reference intakes for other (essential) substances with a physiological effect.

In this context EFSA is requested to consider the existing Population Reference Intakes for energy, micro- and macronutrients and certain other dietary components, to review and complete the SCF recommendations, in the light of new evidence, and in addition advise on a Population Reference Intake for dietary fibre.

For communication of nutrition and healthy eating messages to the public it is generally more appropriate to express recommendations for the intake of individual nutrients or substances in food-based terms. In this context EFSA is asked to provide assistance on the translation of nutrient based recommendations for a healthy diet into food based recommendations intended for the population as a whole.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1)(a) and Article 31 of Regulation (EC) No 178/2002,[5] the Commission requests EFSA to review the existing advice of the Scientific Committee for Food on population reference intakes for energy, nutrients and other substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

In the first instance EFSA is asked to provide advice on energy, macronutrients and dietary fibre. Specifically advice is requested on the following dietary components:

- Carbohydrates, including sugars;

- Fats, including saturated fatty acids, polyunsaturated fatty acids and monounsaturated fatty acids, *trans* fatty acids;

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[4] Scientific Committee for Food, Nutrient and energy intakes for the European Community, Reports of the Scientific Committee for Food 31st series, Office for Official Publication of the European Communities, Luxembourg, 1993.

[5] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

- Protein;
- Dietary fibre.

Following on from the first part of the task, EFSA is asked to advise on population reference intakes of micronutrients in the diet and, if considered appropriate, other essential substances with a nutritional or physiological effect in the context of a balanced diet which, when part of an overall healthy lifestyle, contribute to good health through optimal nutrition.

Finally, EFSA is asked to provide guidance on the translation of nutrient based dietary advice into guidance, intended for the European population as a whole, on the contribution of different foods or categories of foods to an overall diet that would help to maintain good health through optimal nutrition (food-based dietary guidelines).

ASSESSMENT

1. Introduction

Vitamin B12 is the generic descriptor for corrinoid compounds exhibiting qualitatively the biological activity of cobalamin. The term cobalamin will be used throughout this opinion.

In 1993, the Scientific Committee for Food (SCF) adopted an opinion on the nutrient and energy intakes for the European Community and derived a Lowest Threshold Intake (LTI), an Average Requirement (AR) and a Population Reference Intake (PRI) for cobalamin for adults (SCF, 1993). The SCF also set PRIs for infants aged 6–11 months and for children. The SCF proposed additional intakes for pregnant and lactating women to be added to the PRI for non-pregnant non-lactating women.

2. Definition/category

2.1. Chemistry

Cobalamin is a metal complex of ~ 1 300–1 500 Da, constituted by a corrin ring and a central cobalt(III) ion bonded to six ligands, four of which are reduced pyrroles forming the corrin ring. The α-axial ligand, extending below the corrin ring, is a 5,6-dimethylbenzimidazole linked through a phosphoribosyl moiety to the corrin ring. The upper or β-axial ligand varies and may be a methyl-, adenosyl-, hydroxo-, aquo- or cyano-group, giving rise to the correspondingly named chemical forms of the vitamin. The cobalt ion cycles from Co(III) to Co(II) and to Co(I) during its catalytic activity (Froese and Gravel, 2010).

Methylcobalamin (MeCbl) and 5′-deoxyadenosylcobalamin (AdoCbl) are the forms that function as coenzymes for metabolic reactions (Sections 2.3.1 and 2.3.5). Hydroxocobalamin (OHCbl) or aquocobalamin are intermediates formed during the synthesis of the coenzyme forms (Green, 2012). Cyanocobalamin (CNCbl) is a stable synthetic form and is used for addition to food and food supplements and drugs.

Other forms, including sulfito-, nitrite- and glutathionyl-derivatives of cobalamin, have also been described (Green, 2012).

2.2. Function of cobalamin

2.2.1. Biochemical functions

In humans, two reactions are known to require cobalamin as coenzyme. One is the rearrangement of methylmalonyl-coenzyme A (CoA) to succinyl-CoA in propionate metabolism by methylmalonyl-CoA mutase in mitochondria (Figure 1). The other is the cytosolic transmethylation of homocysteine by 5-methyl-tetrahydrofolate (5-methyl-THF) to methionine by methionine synthase (Figure 2) (Ludwig and Matthews, 1997; Matthews et al., 1998). Cobalamin and folate interact in the latter reaction.
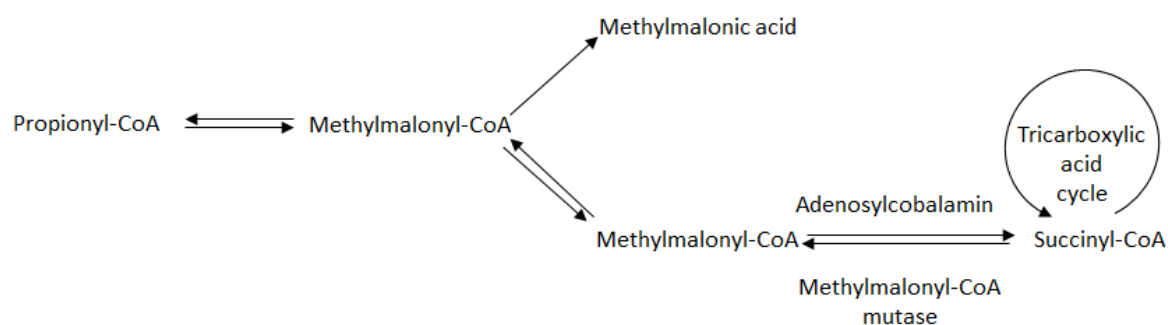
Figure 1: Cobalamin-dependent conversion of methylmalonyl-CoA to succinyl-CoA
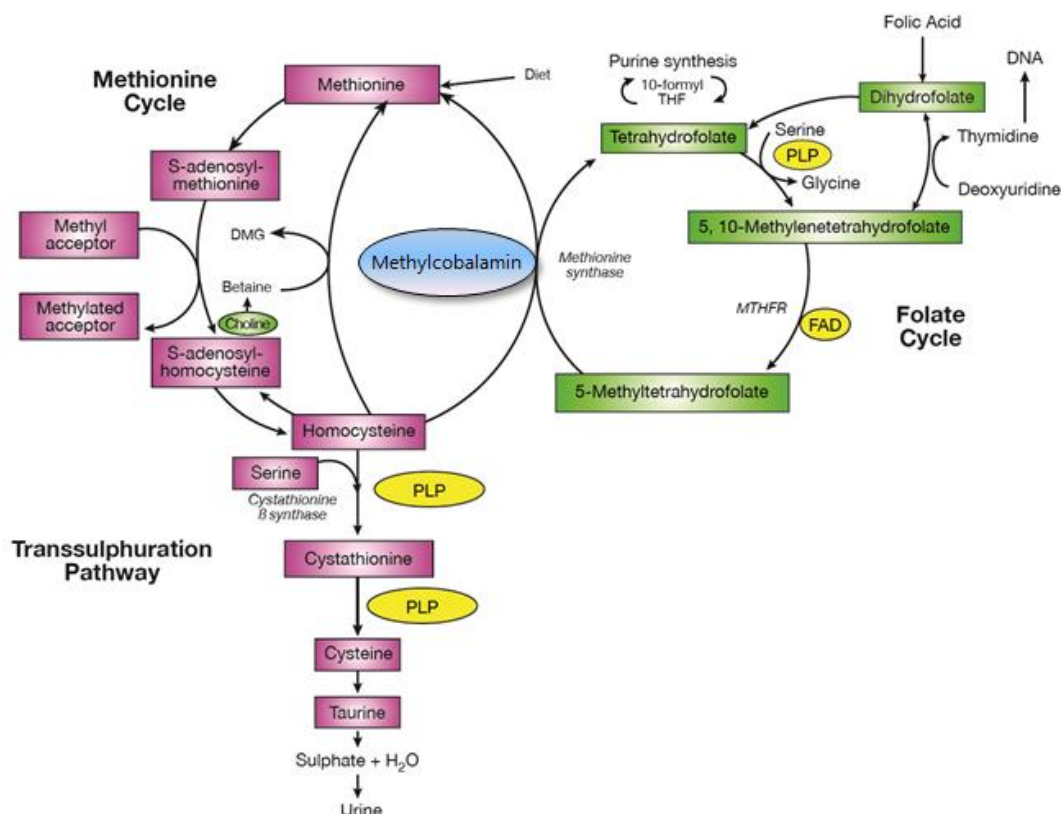
DMG, dimethylglycine; FAD, flavin adenine dinucleotide; PLP, pyridoxal 5´-phosphate; TMP, thymidine monophosphate; UMP, uridine monophosphate (figure kindly provided by J.J. Strain).

Figure 2: Transmethylation of homocysteine to methionine by methionine synthase

Without adequate supplies of both vitamins, the synthesis of methionine and its derivative *S*-adenosyl-methionine (SAM) is disrupted, with profound effects on normal cellular function. Methionine is an indispensable amino acid, whose availability for its various metabolic functions depends critically on recycling through the remethylation pathway. SAM is the universal methyl donor in over 100 transmethylation reactions involving amino acid, nucleotide, neurotransmitter and phospholipid metabolism, as well as detoxification reactions. THF, the fully reduced form of folate, is another product of the methionine synthase reaction (Scott, 1999).

2.2.2. Health consequences of deficiency and excess

2.2.2.1. Deficiency

The most frequent clinical expression of cobalamin deficiency is megaloblastic anaemia, which affects red blood cells and all other blood cells (Chanarin, 1969; Carmel, 2009). The later stages feature symptoms resulting from impaired oxygen delivery, such as fatigue or shortness of breath. Because of the interrelated functions of cobalamin and folate, anaemia and its mechanisms are identical in deficiencies of both vitamins, but the onset occurs later in cobalamin deficiency (Herbert, 1962; Chanarin, 1969; Carmel, 2009).

Independent of megaloblastic anaemia, neurological dysfunction (including myelopathy, neuropathy and neuropsychiatric abnormalities, and less often optic nerve atrophy) is another clinical feature of cobalamin deficiency due to progressive demyelinating lesions of the white matter in the spinal cord and brain, termed subacute combined degeneration or funicular myelosis (Lorenzl et al., 2003). The clinical features of the spinal cord affliction are symmetric dysaesthesia, disturbance of the position

sense and spastic paraparesis or tetraparesis (Russel et al., 1900; Hemmer et al., 1998). The corresponding neuropathological findings are a diffuse, multifocal pattern of axonal loss and demyelination, most severe in the cervical and thoracic spinal cord. Posterior columns are affected predominantly, followed by the antelateral and anterior tracts (Pant et al., 1968; Hemmer et al., 1998). Clinical cerebral features are associated with mental symptoms, such as irritability, memory disturbances, depression and, in severe deficiency or advanced stages, a dementia-like illness, frank psychosis with hallucinations and paranoia may occur (Grober et al., 2013; Hunt et al., 2014). In infants, cobalamin deficiency results in a number of neuromuscular and developmental symptoms and may be associated with cerebral atrophy (Dror and Allen, 2008). Although patients respond to cobalamin treatment, residual neurological abnormalities persist in most (Healton et al., 1991).

Causes of deficiency are inherited (Imerslund–Gräsbeck syndrome) or acquired defects such as pernicious anaemia resulting in malabsorption, or the impairment of transport of the vitamin within the body. Insufficient dietary intake is rare in adults living in developed countries, but is more often reported in vegans (Pawlak et al., 2013) or those living in less developed countries (Stabler and Allen, 2004).

Cobalamin insufficiency is characterised by biochemical abnormalities, such as elevated total homocysteine (tHcy) and/or methylmalonic acid (MMA) concentrations in blood and urine, resulting from impaired metabolic cobalamin activity with no specific clinical symptoms (Section 2.4) (Allen et al., 1993; Ubbink, 1997). Increases in tHcy and/or MMA are observed in conditions of mild malabsorption of food-bound cobalamin that may inevitably not progress to the advanced deficiency stages, resulting in anaemia and/or funicular myelosis, as is the case in severe intrinsic factor-related malabsorption (pernicious anaemia). The pathogenic potential related to such abnormalities is unclear (Carmel, 2011).

The neonatal period is thought to be a period of special vulnerability to cobalamin insufficiency and deficiency (Molloy et al., 2008). Maternal and infant cobalamin status, as measured by serum cobalamin, holotranscobalamin (holoTC), MMA and tHcy concentrations, were strongly associated at birth and six months of age (Doscherholmen et al., 1978; Doets et al., 2013b). A summary of case studies on infants from mothers with undetected pernicious anaemia or adhering to strict veganism indicates that clinical symptoms of deficiency appear in infants at around four to seven months of age (Dror and Allen, 2008). After treatment with cobalamin injection (typically 1 mg intramuscularly for four days), reversal of neuromuscular manifestations was observed in most cases, while psychomotor and cognitive developmental delays were reported not to be reversed in about 40–50 % of cases. In all cases, infants were exclusively breast-fed, indicating that low cobalamin concentration of breast milk due to veganism or pernicious anaemia in some of the mothers was a contributing factor.

Frequent causes of a decline in cobalamin status and/or cobalamin deficiency in older adults are malabsorption of cobalamin bound to food as a consequence of atrophic gastritis (Carmel, 1997) and pernicious anaemia (Matthews, 1995; Andres et al., 2004; Bizzaro and Antico, 2014). Besides *Helicobacter pylori* infection (Andres et al., 2005), long-term ingestion of H2-blockers or proton pump inhibitors (Howden, 2000; Andres et al., 2003) and biguanides (Bauman et al., 2000) are common causes of atrophic gastritis in older adults. The frequency of cobalamin-related biochemical abnormalities is 5–15 % in older adults (Lindenbaum et al., 1994; Carmel et al., 1999; Clarke et al., 2007). The prevalence of pernicious anaemia has been reported to usually occur after the age of 30 years, to increase with age (Bizzaro and Antico, 2014) and to account for 15–20 % of cases of cobalamin deficiency (Andres et al., 2004).

2.2.2.2. Excess

As reported by SCF (2000), no adverse effects have been associated with excess cobalamin intake from food or supplements in healthy individuals. Long-term oral or parenteral administration of daily cobalamin doses between 1 and 5 mg given to patients with compromised cobalamin absorption did not reveal adverse effects. There is no evidence relating cobalamin to teratogenicity or adverse effects on fertility or post-natal development. Cobalamin has not been found to be carcinogenic or genotoxic

in vitro or *in vivo*. Thus, no adverse effects were identified that could be used as a basis for deriving a Tolerable Upper Intake Level (UL) (SCF, 2000).

2.3. Physiology and metabolism

2.3.1. Intestinal absorption

Cobalamin absorption consists of several steps, defects of which can result in reduced or absent uptake of dietary cobalamin.

Cobalamin in food is generally complexed with proteins. The release of cobalamin from food takes place largely in the stomach under the influence of hydrochloric acid and pepsin. During this process, the salivary protein haptocorrin bind with food cobalamin. In the duodenum, cobalamin is released from its complex with haptocorrin through the combined effects of pancreatic bicarbonate and proteolytic enzymes. Free cobalamin is then bound by gastric intrinsic factor (IF). In the terminal ileum, IF–cobalamin complexes are absorbed through receptor-mediated endocytosis. Cobalamin is then released and IF degraded in lysosomes. Cobalamin is finally metabolised to its methyl- and deoxyadenosyl-derivatives (Section 2.1). The vitamin enters plasma primarily in the form of MeCbl (Green, 2012).

The intestinal absorption mediated by IF is saturated at about 1.5–2 µg cobalamin/meal under physiological conditions (Chanarin, 1969). Agents that stimulate acid secretion stimulate IF secretion and an excess of IF is generally available with normal gastric secretion (Chanarin, 1969). Besides the amount of IF, the number of intestinal receptors for the cobalamin–IF complex is one of the factors that limit the absorption of cobalamin ingested in physiological quantities (Grasbeck and Salonen, 1976). Under favourable conditions, healthy persons may absorb more than 10 µg cobalamin/day (Grasbeck, 1984). When increasing doses of cobalamin are given orally, the fraction absorbed decreases rapidly and the amount absorbed approaches a plateau (Glass et al., 1954; Adams et al., 1971). When the IF system capacity is exceeded, cobalamin absorption becomes dependent on passive, non-specific mechanisms which are much less efficient (1–2 % of the dose) (Berlin et al., 1968; Chanarin, 1969).

In a recent systematic review on cobalamin absorption and losses, the results of eight studies using intrinsic cobalamin radiolabelling (^{57}Co, ^{58}Co or ^{60}Co) of various foods were considered, including a total of 115 tests in healthy subjects and subjects with a disease not affecting cobalamin absorption (Doets et al., 2013a). Subjects were between 17 and 55 years of age or described as "young", and four subjects were described as "old". By the faecal excretion method, estimates of fractional absorption obtained ranged from 10 % (coefficient of variation (CV) = 84 %) at a dose of ~ 3 µg from rabbit liver to 65 % (CV = 13 %) at a dose of ~ 0.5 µg from chicken meat (Reizenstein and Nyberg, 1959; Doscherholmen and Swaim, 1973; Doscherholmen et al., 1975, 1976, 1978; Doscherholmen et al., 1981; Kittang et al., 1985). By whole body counting, values from 4.5 % (CV = 38 %) at a dose of ~ 38 µg from mutton liver to 83 % (CV = 11 %) at a dose of ~ 3 µg from mutton meat were reported (Heyssel et al., 1966). Two studies observed cobalamin absorption of more than 50 % with cobalamin doses ranging between 0.42 and 5.11 µg (Heyssel et al., 1966; Doscherholmen et al., 1978), while it was lower in the other studies. Overall, the absolute amount of cobalamin absorbed (A_i) increases with increasing doses of cobalamin (D_i), while fractional absorption decreases. The relationship was estimated as $\ln(A_i) = 0.7694 \times \ln(D_i) - 0.9614$ ($r^2 = 0.78$). Considering habitual cobalamin intakes of 3.5–9.3 µg/day among adults across Europe (Vinas et al., 2011), and assuming that, each day, three meals contribute equal amounts of cobalamin (i.e. 1.2–3.1 µg per meal), the authors estimated that the fractional cobalamin absorption from diet ranges between 29 and 37 %.

The Panel notes that the regression equation by Doets et al. (2013a) is based on cobalamin absorption measures from varying foods and doses in small numbers of subjects, which limit the robustness of the relationship derived from these data. At a given dose level, fractional absorption has been observed to be rather variable and may depend on the dietary source and other factors. Absorption data were

available from a small number of food categories and did not include, for example, milk and dairy products, which are sources of cobalamin (Section 3.1).

On the basis of the same experimental data, other expert bodies have generally assumed that around 50 % of cobalamin in a typical meal is actively absorbed if the IF system is intact (Netherlands Food and Nutrition Council, 1992; IOM, 1998; WHO/FAO, 2004; D-A-CH, 2015) (Section 4).

The Panel notes that the fractional absorption of cobalamin is highly variable, depending on the dietary source, the amount ingested, the ability to release cobalamin from food and the proper functioning of the IF system. No studies have assessed the fractional absorption of cobalamin from specific diets or as a function of cobalamin status. Despite its limitations, the analysis by Doets et al. (2013a) suggests that the generally assumed fractional cobalamin absorption of 50 % from a typical meal may overestimate cobalamin absorption at habitual cobalamin intake levels in Europe. Given the uncertainties and shortcomings inherent in available estimates of cobalamin absorption, the Panel considers a cobalamin absorption of 40 % a more conservative estimate.

2.3.2. Transport in blood

In plasma, cobalamin is bound to the cobalamin-binding proteins transcobalamin (TC) and haptocorrin. TC has a half-life of about 18 hours and is sensitive to changes in cobalamin intake (Hom and Olesen, 1969). Most cells can synthesise TC, including the vascular endothelium, which is, in particular, assumed to maintain the concentration of this protein in the circulation (Quadros et al., 1989; Quadros and Sequeira, 2013). TC combines with cobalamin at the ileal cell to form holoTC and rapidly delivers cobalamin to tissues (Nexø and Gimsing, 1975). Newly ingested cobalamin, as holoTC, can first be detected in the blood three hours after intake with a maximum plasma concentration occurring at 8–12 hours (Hom and Olesen, 1969; Nexø and Gimsing, 1975). Once in the circulation, holoTC has a short half-life of 60–90 minutes (Quadros and Sequeira, 2013). HoloTC is the critical fraction of serum cobalamin, as only TC-bound cobalamin can be taken up by body cells, notably by rapidly proliferating cells, including bone marrow precursors (Seetharam and Li, 2000). Usually, holoTC accounts for 10–30 % of total plasma cobalamin and values for TC saturation have been reported between 3.8 and 17.9 % (5th – 95th percentiles) (Refsum et al., 2006).

The major residual fraction of plasma cobalamin (~ 70–90 %) is attached to haptocorrins (formerly named transcobalamin I and III) (Nexø et al., 2002; Refsum et al., 2006), which are largely saturated with cobalamin, including metabolically inert cobalamin analogues (Hardlei and Nexø, 2009), and have a half-life of 9–10 days (Hom and Olesen, 1969). A high expression of haptocorrin mRNA has been shown in bone marrow, salivary gland and stomach, while there are inconsistencies with regard to the occurrence of haptocorrin mRNA in a number of other tissues (Mørkbak et al., 2007c). The function of haptocorrins is largely unknown; apart from their involvement in cobalamin storage, a role in the clearance of cobalamin analogues has been suggested (Ermens et al., 2003).

2.3.3. Distribution to tissues

HoloTC rapidly delivers cobalamin to all tissues and is internalised by endocytosis through the specific, calcium-dependent transcobalamin receptor (TCblR), which is ubiquitous in the body (Hall and Finkler, 1963; Finkler and Hall, 1967; Quadros et al., 2009; Jiang et al., 2010; Quadros and Sequeira, 2013). Megalin, another calcium-dependent multiligand receptor for holoTC, has been identified in kidneys, intestine, yolk sac and other tissues (Moestrup and Verroust, 2001).

Intracellular cobalamin concentration is maintained by modulating the expression of the receptor, with highest expression in actively proliferating cells and an efflux system that shunts the excess cobalamin out of the cells (Quadros and Sequeira, 2013). In contrast, cobalamin accumulates in the liver and kidney. Cobalamin accumulation in the kidney may be attributed to binding of holoTC to megalin receptors (Moestrup et al., 1996; Moestrup and Verroust, 2001). Uptake of cobalamin bound to haptocorrin by the asialoglycoprotein receptor has been suggested to be the mechanism for cobalamin

accumulation in hepatocytes (Ashwell and Morell, 1974; Alpers, 1999). Haptocorrins are involved in cellular cobalamin uptake solely in hepatocytes (Mørkbak et al., 2006).

In humans, the transfer of cobalamin to fetal circulation appears to involve holoTC binding sites on the trophoblast cell surface which modulate holoTC uptake. No such binding appears mandatory for the uptake of free cobalamin. The human placenta is capable not only of concentrating cobalamin but also of binding cobalamin to placental haptocorrins and TC for release back into the maternal or fetal circulation, thus regulating the transplacental movement of cobalamin (Miller et al., 1993; Perez-D'Gregorio and Miller, 1998).

2.3.4. Storage

The average cobalamin content of the body was estimated to be 2–3 mg in healthy adults (range: 1–6 mg), using whole-body counting after oral or parenteral administration of radioactive cobalamin (Reizenstein et al., 1966; Adams, 1970); lower mean body stores were found in patients with pernicious anaemia (Adams et al., 1972; Bessent et al., 1980). Earlier mean estimates obtained post mortem from patients with various diseases varied between 2 and 5 mg (Grasbeck et al., 1958; Adams, 1962; Heinrich, 1964). Studies included small numbers of subjects (n = 4–22), and large inter-individual variability was observed.

Around 50 % of total body cobalamin is found in the liver, with a mean cobalamin concentration of about 1.0 µg/g of liver tissue in healthy adults (Kato et al., 1959; Stahlberg et al., 1967). Haptocorrin-bound cobalamin accounts for most of the stored cobalamin in liver (Alpers, 1999). Significant amounts of cobalamin are also found in the kidneys (Moestrup et al., 1996; Birn, 2006; Swartzlander et al., 2012). In contrast, cobalamin does not accumulate in most tissues, but is rather recycled by an active transport mechanism (Quadros and Jacobsen, 1995; Beedholm-Ebsen et al., 2010) (Section 2.3.3).

As very little cobalamin is distributed as free cobalamin in the tissues, it is assumed to be mostly bound to methionine synthase and methylmalonyl-CoA mutase (Quadros, 2010) (Figures 1 and 2) and to proteins involved in the synthesis of these enzymes and intracellular cobalamin trafficking (Gherasim et al., 2013). The mechanism of cobalamin release from its storage sites under cobalamin-deficient conditions is not known. Typically, a decrease in serum cobalamin concentration precedes the fall in liver stores (Booth and Spray, 1960).

The Panel notes that the average cobalamin content of the body in healthy adults is 2-3 mg and that around half of total body cobalamin is found in the liver.

2.3.5. Metabolism

The initial step in the synthesis of cobalamin coenzymes is the removal of the upper axial ligand attached to the central cobalt ion, irrespective of the form of cobalamin transported into the cell, as shown by efficient and rapid interconversion of labelled CNCbl, AdoCbl and MeCbl (Section 2.1) (Quadros et al., 1979), by cobalamin reductases (Watanabe and Nakano, 1997). In the cytoplasm, cobalamin exists primarily as MeCbl, which serves as a cofactor for methionine synthase (Figure 2); in mitochondria it is present as AdoCbl, the cofactor of methylmalonyl-CoA mutase (Figure 1). AdoCbl is the predominant form of cobalamin in all tissues, with lower amounts of OHCbl. MeCbl is the major form of cobalamin in the plasma and is disproportionately reduced in cobalamin deficiency. Higher MeCbl concentrations have been observed in fetal tissues in association with higher methionine synthase activity (Linnel, 1975).

Cobalamin is continuously secreted in the bile. Three studies reported on the rate of cobalamin secretion in bile, indicating amounts of 1.1 and 1.5 % of body stores per day (Grasbeck et al., 1958; Reizenstein, 1959a; el Kholty et al., 1991). It has been assumed that 50–80 % is normally reabsorbed, presumably bound to IF (Grasbeck et al., 1958; Reizenstein et al., 1966; el Kholty et al., 1991; Castle, 1998), whereas the remainder is lost in the faeces, along with most corrinoid analogues. In the absence

of IF, all cobalamin from the bile is excreted in the stool and deficiency develops more rapidly than in case of insufficient dietary intake.

On account of the small losses of cobalamin relative to the body store of the vitamin due to the enterohepatic circulation in healthy subjects on mixed diets, development of cobalamin deficiency can take years, even in case of complete absence of intake or absorption of cobalamin (WHO/FAO, 2004; Green, 2012).

2.3.6. Elimination

2.3.6.1. Faeces and urine

Various studies have indicated daily losses of 0.1–0.2 % of the cobalamin pool, regardless of the size of the pool (Bozian et al., 1963; Heinrich, 1964; Heyssel et al., 1966; Reizenstein et al., 1966; Adams and Boddy, 1968; Boddy and Adams, 1968, 1972; Amin et al., 1980). In a systematic review, publications reporting daily cobalamin losses were collected (Doets et al., 2013a), including a pooled analysis of five studies measuring cobalamin losses with whole-body counting (Bozian et al., 1963; Heyssel et al., 1966; Reizenstein et al., 1966; Adams and Boddy, 1968; Boddy and Adams, 1968). Data were available from 52 subjects, comprising healthy subjects and patients with or without pernicious anaemia or "low cobalamin status". A mean daily loss of 0.13 ± 0.03 % (CV = 23 %) of total body stores was estimated (95 % CI = 0.10–0.15). The heterogeneity between studies was high ($I^2 = 91.5$ %, p < 0.001).

The highest losses of cobalamin occur through the faeces. Sources of faecal cobalamin include unabsorbed cobalamin from food or bile, desquamated cells, gastric and intestinal secretions and cobalamin synthesised by bacteria in the colon. Microorganisms of the gastrointestinal tract convert a large portion of ingested cobalamin to cobalamin analogues which account for more than 98 % of the total of cobalamin plus cobalamin analogues measured in the faeces (Allen and Stabler, 2008).

The 24-hour urinary excretion of cobalamin is not correlated with recent dietary intake (Fukuwatari et al., 2009; Tsuji et al., 2010, 2011). Proximal tubule receptor-mediated reabsorption of filtered holoTC, mediated by megalin, efficiently prevents urinary losses of the vitamin and is a saturable process (Birn, 2006). If the circulating cobalamin exceeds the cobalamin binding capacity of the blood, the excess is excreted in the urine (Birn, 2006). Urinary cobalamin was shown to increase 1.3fold with a high oral dose of 1.5 mg of cobalamin (Fukuwatari et al., 2009) and threefold with a dose of 3 mg (Raccuglia et al., 1969).

Losses in faeces (Reizenstein, 1959b) and urine (Mollin and Ross, 1952; Heinrich, 1964; Adams, 1970) decrease when cobalamin stores decrease.

The Panel notes that the main route of cobalamin excretion is via the faeces, and that daily losses of 0.1–0.2 % of the cobalamin pool have been observed.

2.3.6.2. Breast milk

The cobalamin concentration of breast milk reflects maternal cobalamin concentration in blood and it falls progressively during the lactation period (Bjorke Monsen and Ueland, 2011).

Based on IOM (1998), an average content of cobalamin in human milk of 0.31–0.42 µg/L has been noted (EFSA NDA Panel, 2013a).

SCF (2003) referred to a mean (range) concentration of cobalamin in breast milk of 0.38 (0.12–0.48) nmol/L (0.51 (0.16–0.64) µg/L) calculated from 10 studies in unsupplemented mothers from Western countries (Bates and Prentice, 1994). The SCF also noted that there is no, or only a small, increase in the concentration of cobalamin in breast milk following supplementation of cobalamin-replete women.

Cobalamin in breast milk is tightly bound to haptocorrin and has to be released from this binding to be measured accurately (Allen, 2012). Breast milk also contains substantial amounts of unsaturated haptocorrin (apo-haptocorrin) (> 100-fold higher than in serum), which has been found to interfere with measurement of cobalamin by IF binding assays, due to their competitive affinity for cobalamin (Lildballe et al., 2009). Depending on the design of the assay, under- or overreporting of the amount of the vitamin in breast milk has been observed, due to the trapping of the sample cobalamin or of the labelled cobalamin used for measurement, respectively, by apo-haptocorrin. A method using a cobinamide-sepharose column to remove unsaturated haptocorrin has been shown to overcome this issue (Lildballe et al., 2009). Three studies (Lildballe et al., 2009; Deegan et al., 2012; Greibe et al., 2013) report cobalamin concentrations in breast milk using this method. Mean (range) concentration in breast milk of a group of 24 healthy Californian women, most of whom had consumed supplements containing 6 µg cobalamin/day during pregnancy, was 0.57 (0.16–3.70) nmol/L (0.8 (0.2–5.0 µg/L) (measured between one and three months post partum) (Lildballe et al., 2009). In a sample of 183 women of low socio-economic status in peri-urban Guatemala City, breast milk cobalamin concentrations were below the limit of detection (LOD) of 0.05 nmol/L (0.07 µg/L) in 65 % of the participants whose serum cobalamin was < 220 pmol/L. Median (range) breast milk concentration was 0.06 (below LOD–1.30) nmol/L (0.08 (below LOD–1.75) µg/L) in mothers with an "adequate cobalamin status" as defined by a serum cobalamin concentration > 220 pmol/L (n = 55) (Deegan et al., 2012). In the latter sample, breast milk cobalamin concentration was reported to be positively associated (p < 0.05) with maternal cobalamin intake measured by semi-quantitative food frequency questionnaire (FFQ) (r = 0.26) and maternal serum cobalamin concentration (r = 0.30). In a recent longitudinal study, cobalamin concentration of breast milk from 25 Danish women was measured at two weeks, four months and nine months of lactation (Greibe et al., 2013). Most women were taking daily multivitamin supplements containing 1.0–4.5 µg cobalamin. Median (range) concentrations of cobalamin in hindmilk[6] were 0.76 (0.21–1.88) nmol/L (1.0 (0.3–2.5) µg/L), 0.29 (0.14–0.69) nmol/L (0.4 (0.2–0.9) µg/L) and 0.44 (0.16–1.94) nmol/L (0.6 (0.2–2.6) µg/L) at two weeks, four months and nine months, respectively. Slightly lower concentrations were found in foremilk.[7]

The Panel notes that a mean breast milk cobalamin concentration of 0.38 nmol/L (0.51 µg/L) has been found in 10 studies in unsupplemented mothers from Western countries, using analytical methods with uncertain accuracy. Recent data based on a more accurate method report a mean concentration of 0.57 nmol/L (0.8 µg/L) in a group of Californian women and median concentrations of 0.29–0.76 nmol/L (0.4–1.0 µg/L), depending on the lactation stage, in a group of Danish women. The Panel notes the limited number of studies that used this analytical method, their small sample sizes and the inclusion of women receiving cobalamin supplements.

2.3.7. Interaction with other nutrients

There are no known interactions of cobalamin with other nutrients as regards absorption or excretion.

There is a close metabolic interaction with folate which has led to the "methyl-trap hypothesis" (Shane and Stokstad, 1985; EFSA NDA Panel, 2014b). Cobalamin and folate are linked via the enzyme methionine synthase, which converts 5-methyl-THF to the metabolically active form THF, which is needed in one-carbon metabolism (Figure 2). Methionine synthase activity is reduced in cobalamin depletion; this inhibits the formation of THF, with folate becoming "trapped" as 5-methyl-THF. In this situation, cells lack the precursor of 5,10-methylenetetrahydrofolate, and the production of purines and pyrimidines required for DNA synthesis is impaired. This affects the rapidly dividing cells of the bone marrow, leading to the production of megaloblastic blood cells and subsequent megaloblastic anaemia (Section 2.2.2.1). In addition, 5-methyl-THF is a poor substrate for folylpolyglutamate synthase, the enzyme responsible for cellular retention of folate. In order for folate to be retained by the cell, 5-methyl-THF must first be converted to tetrahydrofolate via methionine synthase (Molloy, 2010; Hughes et al., 2013). As a result, cobalamin deficiency usually leads to a rise of 5-methyl-THF and thus serum folate concentration, whereas the tissues and red blood cells are depleted of 5-methyl-THF.

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[6] Hindmilk: milk secreted during the last part of a feed during breastfeeding.
[7] Foremilk: milk secreted in the initial part of a feed during breastfeeding.

Folate deficiency has been associated with lower plasma cobalamin concentration (Klee, 2000; Gibson, 2005). Deficiencies of both vitamins induce an increase in plasma tHcy concentration (Selhub et al., 2008).

2.4. Biomarkers

Main biomarkers of cobalamin status include haematological changes and blood concentrations of cobalamin, holoTC and the metabolites MMA and tHcy (Section 2.2.2.1). Cobalamin, holoTC and MMA can be measured in serum or plasma with equivalent results. In this section and in concluding paragraphs of other sections, the term "serum" refers to either serum or plasma concentrations of these compounds.

2.4.1. Haematological changes

Macrocytosis or macrocytic anaemia with megaloblastic changes and neutrophil hypersegmentation are present in 70–80 % of cases of cobalamin deficiency. Megaloblastic changes are a late event in the development of cobalamin deficiency (Herbert, 1994).

Macrocytosis is easily detected and quantified by measuring the mean corpuscular volume (MCV) of erythrocytes. Nuclear hypersegmentation of neutrophils is the earliest recognisable abnormality of megaloblastic anaemia. Megaloblastosis produces ineffective haematopoiesis. Biochemical markers of massive, premature cell death, notably serum bilirubin and lactate dehydrogenase elevation, become prominent as anaemia advances.

Macrocytosis or macrocytic anaemia with megaloblastic changes are not specific markers of cobalamin deficiency, as their causes are many, including alcohol abuse, folate deficiency, liver disease and use of medication (Carmel, 2008, 2009).

The Panel notes that macrocytosis or macrocytic anaemia with megaloblastic changes are sensitive but not specific markers of cobalamin deficiency.

2.4.2. Serum cobalamin concentration

Serum cobalamin is the most widely used biomarker of cobalamin status comprising the total amount of cobalamin, i.e. both the metabolic active cobalamin bound to TC and the fraction bound to haptocorrin. Haptocorrin is almost fully saturated with cobalamin and carries the major part of circulating cobalamin as well as the inactive cobalamin analogues (Section 2.3.2).

Sex may affect serum concentration of cobalamin, although data are not consistent. In a study that controlled for factors that could affect serum cobalamin concentration (e.g. age, folate status, use of oral contraceptives, pregnancy), higher cobalamin concentrations were found in women than in men (Fernandes-Costa et al., 1985), whereas similar concentrations for men and women have also been reported (Wahlin et al., 2002). An increased prevalence of "low" serum cobalamin concentrations has been observed in older adults (Lindenbaum et al., 1994; Clarke et al., 2007), attributable to atrophic gastritis associated with a decrease in gastric acidity and to pernicious anaemia and subsequent cobalamin malabsorption (Section 2.2.2.1). A decline in serum cobalamin concentrations throughout childhood and adolescence has been observed in several studies (De Laet et al., 1999; Pfeiffer et al., 2005; Papandreou et al., 2006). An age-related decrease in cobalamin intake may be an additional factor (Johnson et al., 2003). A steady fall in serum cobalamin concentration has been observed during pregnancy, starting from the first trimester, resulting from the transfer of the vitamin to the fetal circulation and to plasma volume expansion (Allen, 1994; Koebnick et al., 2002; Milman et al., 2006; Mørkbak et al., 2007b; Murphy et al., 2007; Greibe et al., 2011; Hure et al., 2012). Other factors which influence serum cobalamin concentration include genetic polymorphisms (e.g. haptocorrin gene), as well as liver diseases, renal failure, some blood disorders (e.g. chronic myelogenous leukaemia) and cancers, which may lead to elevated serum cobalamin concentrations (Carmel, 2011; Green, 2011; Andres et al., 2013).

The type of cobalamin assay used may affect serum cobalamin measurements. Earlier radioisotopic dilution assays also measured some of the non-functional analogues of cobalamin and thus may have overestimated serum cobalamin concentration (Kolhouse et al., 1978). Specific assays based on purified IF improved accuracy (Kubasik et al., 1980). Immunoenzymatic luminescence methods have since been developed and have replaced isotopic assays (O'Sullivan et al., 1992; Carmel, 2011). Immunoenzymatic assays appear to lack sensitivity, especially to identify low cobalamin concentrations in patients suffering from pernicious anaemia (Carmel et al., 2000; Carmel, 2011).

Dietary intakes of omnivores are not strongly related to serum cobalamin concentrations (correlation coefficient around 0.10) (Gregory et al., 1990; Vogiatzoglou et al., 2009b). Such low correlations have been linked to the large size of liver cobalamin stores in relation to the usual daily intake of the vitamin, so that cobalamin intake levels slowly influence circulating concentrations of cobalamin (Bates et al., 1997). Lower serum cobalamin concentrations have been observed in vegetarians or vegans than non-vegetarian individuals (Millet et al., 1989; Miller et al., 1991; Krajcovicova-Kudlackova et al., 2000). In intervention studies, cobalamin supplementation significantly increased serum cobalamin concentration in subjects with relatively low serum cobalamin at baseline (Blacher et al., 2007; Duggan et al., 2014). In a sample of Canadian individuals (n = ~ 5 600, aged 6–79 years), daily consumption of cobalamin supplements was associated with higher serum cobalamin concentrations, with no additional increase in serum cobalamin at doses above 10 µg/day in children and adolescents and at doses above 25 µg/day in older adults aged 60–79 years (MacFarlane et al., 2014). Several observational studies have described the dose–response relationship between cobalamin intake and serum cobalamin concentration and showed an increase in serum cobalamin with increasing cobalamin intake which levelled off between approximately 350 and 400 pmol/L at cobalamin intakes between 7 and 10 µg/day (Tucker et al., 2000; Kwan et al., 2002; Bor et al., 2006; Vogiatzoglou et al., 2009b; Bor et al., 2010) (Section 5.1.1.3). Dullemeijer et al. (2013) attempted to characterise the dose–response relationship between cobalamin intake and serum concentration based on data from 37 randomised controlled trials (RCTs) and 19 observational studies (n = 15 968 subjects). There was statistical evidence for substantial heterogeneity between studies included in the meta-analysis, which could not be explained by the differences in study designs (observational studies vs. RCTs), mean age of subjects or the doses or forms of cobalamin consumed, so that the uncertainty around the resulting dose–response relationship is high.

Reference intervals have been proposed for serum cobalamin, based on the distribution of concentrations (5th–95th percentiles) in large populations. The reference range derived from 500 healthy, fasting (7.5 %) and non-fasting (92.5 %), male and female subjects in Norway (18–69 years) was 168–493 pmol/L (Refsum et al., 2006). From a random sample of 961 non-fasting male and female Swedish adults (35–80 years), collected over the course of the day, reference ranges were derived for five age groups; lower and higher bounds ranged between 134 and 178 pmol/L and between 457 and 533 pmol/L, respectively (Wahlin et al., 2002). The reference range derived from a nationally representative sample of ~ 7 300 participants aged ≥ 4 years in the US National Health and Nutrition Examination Survey (NHANES) during 1999–2000 was 179–738 pmol/L (Pfeiffer et al., 2005). For young children, Hay et al. (2008) proposed reference intervals based on data from a cohort of healthy Norwegian children, as follows: 197–677 pmol/L at 12 months (n = 243) and 236–944 pmol/L at 24 months (n = 223). The authors noted lower cobalamin concentrations in breast-fed children than non-breast-fed children and suggested that reference limits according to breastfeeding status should be considered. Reference values for serum cobalamin for older children were derived from a sample of 1 051 (552 females, 499 males) participants aged 12.5–17.5 years in the Healthy Lifestyle in Europe by Nutrition and Adolescence (HELENA) study (Gonzalez-Gross et al., 2012). The 5th–95th percentiles for girls and boys were 173–672 and 169–567 pmol/L, respectively. Based on data from the National Diet and Nutritional Survey (NDNS) in the UK, 5th–95th percentiles of serum cobalamin concentration were 242–749 pmol/L for children aged 4–10 years (n = 317), 172–641 pmol/L for children aged 10–14 years (n = 263) and 139–452 pmol/L for boys (n = 113) and 108–502 pmol/L for girls (n = 132) aged 15–18 years (Kerr et al., 2009).

Different cut-off values for cobalamin concentration in serum have been proposed to define "cobalamin deficiency", ranging from 123 (Valente et al., 2011) to 258 pmol/L (Lindenbaum et al., 1994). Authors have used various criteria to define cut-off values, including the lower bound of the range of concentrations observed in a selected reference population (Valente et al., 2011), the concentration associated with minimal plasma MMA and/or tHcy concentrations (Selhub et al., 2008; Vogiatzoglou et al., 2009a; Bailey et al., 2013) or with MMA concentrations above a predefined cut-off (Clarke et al., 2007; Heil et al., 2012), or metabolic profiling based on the combination of four biomarkers (Fedosov, 2010). A cut-off of 148 pmol/L (200 µg/L) has commonly been used and has shown good sensitivity (95–97 %; specificity < 80 %) for the diagnosis of deficiency (i.e. in patients with megaloblastic anaemia and/or neurological abnormalities), while its sensitivity to diagnose cobalamin insufficiency (i.e. elevated serum MMA and/or plasma tHcy) is moderate (38–39 %) (Carmel, 2011). By using regression analyses to estimate the cobalamin concentration at which the biomarkers of function MMA and tHcy achieved a minimum level, Selhub et al. (2008) derived cut-off values for serum cobalamin concentration of 150 pmol/L based on serum MMA or 300 pmol/L based on plasma tHcy using data from NHANES III, while Vogiatzoglou et al. (2009a) defined a cut-off of 400 pmol/L from breakpoint cobalamin concentrations of 334 pmol/L for plasma MMA and 393 pmol/L for plasma tHcy using data from the Hordaland Homocysteine study in Norway. By modelling the relationship between plasma MMA and serum cobalamin based on data from NHANES 1999–2004 through different statistical models, Bailey et al. (2013) identified two change points and characterised three population subgroups: subjects with serum cobalamin concentrations < 126 pmol/L were identified at high risk of "severe deficiency" (combined abnormalities of MMA and tHcy very frequent; highest MMA and tHcy concentrations) and subjects with cobalamin concentrations > 287 pmol/L as likely to have adequate cobalamin status (lowest MMA and tHcy concentrations), respectively, while the cobalamin status of subjects with serum cobalamin concentrations between 126 and 287 pmol/L was considered difficult to interpret (i.e. neither normal nor clearly deficient).

In an attempt to consider serum cobalamin together with other biomarkers of cobalamin status, i.e. holoTC (Section 2.4.3), MMA (Section 2.4.4) and tHcy (Section 2.4.5), Fedosov (2010) defined a "wellness parameter" (w) based on the combination of the four biomarkers using data from three population groups defined as "healthy volunteers before and after cobalamin supplementation" (n = 74), "individuals suspected of being cobalamin deficient" (n = 647) and "healthy vegans" (n = 144). By modelling the frequency distribution of the concentrations of the four biomarkers, frequency peaks were identified, assumed to be metabolic fingerprints of different (sub)clinical groups of subjects, i.e. "deficient", "transitional", "normal", "excellent". The "wellness parameter" was based on the logarithmic presentation of the geometric mean of the four normalised biomarkers ($w = \log_{10}(\text{holoTC}_n \times \text{cobalamin}_n) - \log_{10}(\text{MMA}_n \times \text{tHcy}_n)$, with e.g. $\text{MMA}_n = \text{MMA}/\text{MMA}_{normal}$) and was calculated for the four subgroups as follows: "deficient" $w = -1.49$, "transitional" $w = -0.516$, "normal" $w = 0.0$ and "excellent" $w = 0.445$. Using this parameter as a criterion to identify assumed deficient ($w \leq -0.516$) or healthy ($w > -0.516$) subjects, cut-offs for serum cobalamin, as well as holoTC, MMA and tHcy, were identified by analysing the frequency of the groups assumed to be deficient or healthy plotted versus the concentration of the biomarkers. A cut-off of 207 pmol/L for serum cobalamin was derived through this method.

The Panel notes that serum cobalamin comprises both the metabolic active cobalamin bound to TC and the fraction bound to haptocorrin. Various criteria have been used in order to derive values for serum cobalamin above which to define adequate cobalamin status. The Panel notes that lower limits of reference intervals for serum cobalamin concentration range between 134 and 178 pmol/L for adults, and between 108 and 242 for older children. Limited data are available on infants and children. For young children, data from one Norwegian cohort indicate lower boundaries at 197 and 236 pmol/L for children aged 12 and 24 months, respectively. The value reported at 24 months of age might indicate that lower boundaries may be somewhat higher in early childhood; however, at 12 months of age, lower boundaries were in a similar range as for adults. There is no consensus on a cut-off value for serum cobalamin above which to define adequate cobalamin status. Divergent criteria have been used for the derivation of cut-off values. Some authors aimed at deriving cut-off values for the

diagnosis of cobalamin deficiency. To that end, the commonly used cut-off of 148 pmol/L has shown good sensitivity. Others have attempted to define cut-offs values below which to diagnose cobalamin insufficiency, as defined by "suboptimal" concentrations of biomarkers of cobalamin function. A conservative approach consists in identifying the serum cobalamin concentration associated with minimal blood MMA and/or tHcy concentrations. Results of this approach have shown considerable variation in serum cobalamin concentrations (from 150 to 400 pmol/L), depending on the reference biomarker considered, the study population, as well as the modelling approach taken.

2.4.3. Serum holotranscobalamin concentration

Serum holoTC has a rapid turnover with a half-life of 60–90 minutes (Section 2.3.2) and is the physiologically active form of cobalamin that delivers the vitamin to cells (Sections 2.3.2 and 2.3.3). It is considered an earlier biomarker for changes in cobalamin status than serum cobalamin concentration (Herzlich and Herbert, 1988; Herbert et al., 1990; Nexø et al., 2002; Green, 2011).

Serum holoTC measurements seem to be only marginally affected by diurnal variation related to cobalamin intake from a normal diet, as no correlations have been observed between time since the last meal and holoTC concentrations (Hvas and Nexø, 2005; Refsum et al., 2006).

In cohorts of healthy pregnant women, concentrations of holoTC have been observed to remain unchanged over the course of pregnancy, despite an increase in total TC and a decrease in serum cobalamin concentration (Mørkbak et al., 2007b; Greibe et al., 2011). HoloTC rises in renal failure (Carmel et al., 2001), but modest renal impairment does not affect holoTC concentration (Loikas et al., 2007; Lewerin et al., 2013). Other suggested confounders include oral contraceptive use, folate disorders, alcoholism and some haematological disorders (Carmel, 2011).

Different reference intervals have been derived for holoTC. A review of eight studies including healthy adult European individuals (n = 65–303) indicate lower limits of reported 95 % reference intervals between 11 and 41 pmol/L and higher bounds between 113 and 204 pmol/L (Mørkbak et al., 2005). Subsequent studies (n = 100–292 subjects) report lower and higher bounds in the same range (19–43 pmol/L and 125–134 pmol/L, respectively) (Brady et al., 2008; Lee et al., 2009; Valente et al., 2011). Based on data from 500 healthy subjects aged 18–69 years, Refsum et al. (2006) suggested sex differences in values, with a lower limit (5th percentile) for younger women (aged 19–45 years) of 34 pmol/L than the rest of the population (41–48 pmol/L, depending on age and sex groups). For young children, Hay et al. (2008) proposed reference intervals (5th–95th percentiles) based on data from a cohort of healthy Norwegian children, as follows: 26–126 pmol/L at 12 months (n = 244) and 38–174 pmol/L at 24 months (n = 224). As for serum cobalamin (Section 2.4.2), the authors noted lower holoTC concentrations in breast-fed than in non-breast-fed children and suggested that references limits according to breastfeeding status should be considered. Reference values for serum holoTC for older children were derived from 551 female and 467 male participants aged 12.5–17.5 years in the HELENA study (Gonzalez-Gross et al., 2012). The 5th–95th percentiles for girls and boys were 29.6–109 pmol/L and 32–105.1 pmol/L, respectively.

Some authors have proposed cut-off values based on the performance of holoTC to identify "high" MMA concentrations in selected populations based on receiver operating characteristics (ROC) curves. Considering the level at which sensitivity for "cobalamin deficiency", defined by MMA > 750 nmol/L, equalled specificity (i.e. both = 77 %), Clarke et al. (2007) derived a cut-off value for holoTC of 45 pmol/L from data on 1 651 older men and women (74–84 years) with normal renal function (serum creatinine < 97 μmol/L in women and < 124 μmol/L in men) in the UK. Using the point at which sensitivity and specificity were "optimised" for the diagnosis of "cobalamin deficiency", defined as MMA > 450 nmol/L, Heil et al. (2012) determined a cut-off for holoTC of 32 pmol/L based on data from 360 subjects (≥ 18 years) with normal renal function (glomerular filtration rate ≥ 60 mL/min per 1.73 m^2) in a multicentre study in the Netherlands. At this level, sensitivity was highest (83 %), with a specificity of 60 %. The combination of cobalamin and holoTC did not improve diagnostic accuracy at this cut-off level. At holoTC concentrations ≤ 21 pmol/L, the

specificity for "cobalamin deficiency" was high (≥ 88 %; sensitivity of 64 %). The authors suggested that for holoTC concentrations between 21 and 32 pmol/L, MMA should be measured to confirm the diagnosis of "cobalamin deficiency".

By the simultaneous modelling of the four biomarkers of cobalamin status (Section 2.4.2), Fedosov (2010) derived a cut-off for holoTC of 36 pmol/L.

With respect to the performance of holoTC to diagnose "cobalamin deficiency" as compared with serum cobalamin, most ROC-based comparisons have shown that holoTC modestly outperforms total cobalamin, with areas under the curves of 0.66–0.90 and 0.62–0.85, respectively (Carmel, 2011; Heil et al., 2012). Various criteria to define "cobalamin deficiency" were applied in these studies. One study concluded that neither test is suitable for the screening of insufficient cobalamin status (as indicated by elevated MMA) because false-positive results outnumbered true-positive ones at the proposed cut-off values (Clarke et al., 2007).

The Panel notes that holoTC is the physiologically active form of cobalamin that delivers the vitamin to cells. As for serum cobalamin, various criteria have been used in order to derive values for holoTC above which to define adequate cobalamin status. The Panel notes that lower limits of reference intervals for serum holoTC range between 11 and 48 pmol/L in adults, depending on the reference population used. For infants and children, limited data are available, and lower limits of reference intervals between 26 and 38 pmol/L are reported, which are in the same range as those derived for adults. Cut-off values have been proposed below which to diagnose cobalamin insufficiency, as defined by "suboptimal" concentrations of biomarkers of cobalamin function. Based on different populations and criteria, cut-off values from 21 to 45 pmol/L have been proposed.

2.4.4. Serum methylmalonic acid concentration

MMA is derived from the hydrolysis of methylmalonyl-CoA and accumulates in serum when methylmalonyl-CoA mutase activity is impaired resulting from an insufficient supply of cobalamin (Stabler et al., 1986; Savage et al., 1994; Bjorke Monsen and Ueland, 2003). The specificity of MMA concentration as a biomarker of cobalamin status has been difficult to determine reliably because of the influence of various other factors (Carmel, 2011). A Norwegian study including 6 946 middle-aged (47–49 years) and older adults (71–74 years) identified creatinine, serum cobalamin, age and sex as the major determinants of serum MMA; however, these variables explained only 16 % of the variation in plasma MMA concentration (Vogiatzoglou et al., 2009a). MMA concentration is affected by impaired renal function. Because of the higher incidence of renal impairment in older adults, this biomarker must be interpreted with caution in this population (Loikas et al., 2007). Small increases in plasma MMA concentration have been observed over the course of pregnancy in cohorts of healthy pregnant women (Milman et al., 2006; Mørkbak et al., 2007b; Murphy et al., 2007; Greibe et al., 2011).

The most commonly applied cut-off value of serum MMA to define cobalamin status is ~ 270 nmol/L (Carmel, 2011). Many laboratories defined cut-offs by three or two standard deviations (SD) from the mean (~ 370 nmol/L or 270 nmol/L, respectively). Other values have been proposed based on the higher bound of serum MMA concentrations observed in selected populations. Pfeiffer et al. (2005) derived a cut-off value (95th percentile) for MMA of 210 nmol/L from a reference population (n = 7 306, aged ≥ 3 years) selected from NHANES 1999–2000 which was "cobalamin-replete" (i.e. with serum cobalamin above the 50th percentile) and did not exhibit elevated creatinine concentration (i.e. creatinine < 133 μmol/L for males and < 115 μmol/L for females). In a reference population of "cobalamin-replete" individuals (i.e. with serum cobalamin ≥ 400 pmol/L) in Norway, Vogiatzoglou et al. (2009a) reported a higher bound (97.5th percentile) for plasma MMA of 280 nmol/L for middle-aged (47–49 years, n = 1 306) and of 360 nmol/L for older adults (71–74 years, n = 1 058). Erdogan et al. (2010) found a higher bound of 450 nmol/L from a "representative range" based on a database of 4 944 serum samples collected in the USA, from which 10 % higher values were removed as they were assumed to belong to unhealthy individuals. When data were classified by age decades, the

higher bounds of the representative ranges for samples of subjects aged 0–10 years (n = 28; 510 nmol/L) and subjects aged 71 years and older (n = 2 149; 480 nmol/L) were higher than the value of the total population. For young children, Hay et al. (2008) proposed reference intervals (5th–95th percentiles) based on data from a cohort of healthy Norwegian children, as follows: 120–530 nmol/L at 12 months (n = 242) and 100–300 nmol/L at 24 months (n = 222). The 10th and the 90th percentiles of plasma MMA concentration in a cohort of 186 healthy Dutch children aged 0–19 years were 110 and 300 nmol/L, respectively (Hogeveen et al., 2008). By the simultaneous modelling of the four biomarkers of cobalamin status (see Section 2.4.2), Fedosov (2010) proposed a cut-off value of 380 pmol/L for MMA. A cut-off value of 750 nmol/L is used for the diagnosis of cobalamin deficiency (Hvas and Nexø, 2006; Clarke et al., 2007; Carmel, 2011; Devalia et al., 2014).

The Panel notes that MMA is a biomarker of cobalamin function with regard to its role in the functioning of methylmalonyl-CoA mutase. Serum MMA concentration increases following an insufficient supply of cobalamin. Its specificity requires further investigation. A large range of cut-off values from 210 to 450 nmol/L has been proposed above which to characterise insufficient cobalamin status on the basis of MMA concentration, derived from selected populations of "cobalamin-replete" or apparently healthy adults. Limited data are available on infants and children. For children aged ≥ 1–19 years, upper limits of reference intervals of MMA concentration distribution ≥ 300 nmol/L have been observed in apparently healthy children. A cut-off value of 750 nmol/L is used above which cobalamin deficiency is diagnosed.

2.4.5. Plasma total homocysteine concentration

Homocysteine may be recycled into methionine through a reaction which is catalysed by methionine synthase (5-methyl-THF homocysteine methyltransferase), which requires 5-methyl-THF as a cosubstrate and cobalamin as a cofactor (Selhub, 1999; Bjorke Monsen and Ueland, 2003) (Figure 2 and Section 2.2.1). Elevated plasma tHcy concentration is observed in patients with cobalamin deficiency (Allen et al., 1990; Carmel et al., 2003).

Plasma tHcy is not a specific marker of cobalamin status since it is affected also by other dietary factors, such as selected B vitamins, choline and betaine, as well as renal insufficiency and some lifestyle factors (e.g. alcohol consumption) (Refsum et al., 2004; da Costa et al., 2005).

Plasma tHcy concentrations are higher in men than in women and increase with age (Selhub, 1999; Refsum et al., 2004; Pfeiffer et al., 2005). Increased plasma tHcy concentrations throughout childhood and adolescence have been observed in several studies (De Laet et al., 1999; Pfeiffer et al., 2005; Papandreou et al., 2006). As for MMA (Section 2.4.4), because of the higher incidence of renal impairment in the aged population, tHcy concentration must be interpreted with caution in older adults (Loikas et al., 2007). In cohorts of healthy pregnant women, increases in tHcy concentration have been observed over the course of pregnancy (Chery et al., 2002; Milman et al., 2007; Mørkbak et al., 2007b; Greibe et al., 2011).

There is no consensus on a desirable cut-off value for tHcy, and acceptable upper reference limits for plasma tHcy from 9 to 16 µmol/L have been proposed (Kauwell et al., 2000; Ubbink, 2001; Refsum et al., 2004; Devalia et al., 2014). Based on 95th–97.5th percentiles of tHcy concentration in presumably healthy populations from large studies, Refsum et al. (2004) proposed the following upper reference limits for populations not supplemented with folate: 10 µmol/L for children < 15 years, 15 µmol/L for older children (≥ 15 years) and adults up to 65 years, 20 µmol/L for older adults (≥ 66 years), and 10 µmol/L for pregnancy. The authors noted that the statistically defined reference interval may be different from the desirable tHcy concentration. There is no consensus on such a desirable tHcy concentration as firm recommendations can only be reached if clear thresholds for disease risk reduction after vitamin intervention have been demonstrated. Hay et al. (2008) proposed reference intervals (5th–95th percentiles) based on data from a cohort of healthy Norwegian children, as follows: 3.3–7.4 µmol/L at 12 months (n = 243) and 3.5–7.7 µmol/L at 24 months (n = 224). Based on data from NDNS in the UK, the 5th and the 95th percentiles of plasma tHcy concentration were 3.0–

8.6 µmol/L for children aged 4–10 years (n = 320), 3.7–10.7 µmol/L for children aged 10–14 years (n = 268) and 4.7–15.3 for girls (n = 132) and 4.6–12.8 µmol/L for boys (n = 117) aged 15–18 years (Kerr et al., 2009). Reference values for plasma tHcy for older children were derived from 552 female and 498 male participants aged 12.5–17.5 years in the HELENA study (Gonzalez-Gross et al., 2012). The 5th and the 95th percentiles for girls and boys were 3.8 and 11.6 and 4.2 and 14.1 µmol/L, respectively. Most laboratories currently consider a tHcy concentration above 15 µmol/L as indicative of hyperhomocysteinaemia (Devalia et al., 2014).

The Panel notes that homocysteine is a biomarker of cobalamin function with respect to its role in the functioning of methionine synthase. Plasma tHcy concentration increases following an insufficient supply of cobalamin. However, this biomarker is of limited specificity. There is no consensus on a desirable cut-off value for tHcy, although a concentration above 15 µmol/L in adults is frequently used as an indicator of hyperhomocysteinaemia.

2.4.6. Conclusions on biomarkers

The Panel notes that macrocytosis or macrocytic anaemia with megaloblastic changes are sensitive but not specific markers of cobalamin deficiency.

HoloTC carries the functional fraction of cobalamin that can be taken up by tissues; despite its short half-life, holoTC is more strongly associated with biomarkers of cobalamin function, MMA and tHcy than serum cobalamin. The Panel considers that serum holoTC is the most specific and therefore the first ranked biomarker to characterise adequate cobalamin status. In addition, the Panel considers that cut-off values of reference ranges for these biomarkers have not yet been clearly defined.

Taking into account that serum cobalamin concentration comprises both the functional and the metabolically inert fractions of cobalamin in serum, that it decreases more slowly than holoTC in negative cobalamin balance and that it shows weaker relationships with the biomarkers of cobalamin function, MMA and tHcy, the Panel considers serum cobalamin a less specific biomarker than serum holoTC for assessing adequate cobalamin status.

Although serum MMA is a specific biomarker of cobalamin function of methylmalonyl-CoA mutase, it does not reflect all cobalamin functions and its specificity is compromised by impaired kidney function and yet unknown factors. The Panel considers that serum MMA can add valuable information in conjunction with serum holoTC and/or cobalamin for assessment of cobalamin status.

As for MMA, plasma tHcy reflects only a part of cobalamin functions, namely the functioning of methionine synthase. It is not a specific biomarker, as it is influenced by some B vitamins, choline, betaine and other factors. The Panel considers that plasma tHcy may be useful to support conclusions based on the other biomarkers of cobalamin status.

Overall, the sensitivity and specificity of these biomarkers can be affected by factors unrelated to cobalamin status (Hvas and Nexø, 2005; Carmel and Sarrai, 2006; Green, 2008; Vogiatzoglou et al., 2009a; Carmel, 2011; Nexø and Hoffmann-Lucke, 2011). Impaired renal function is associated with elevated MMA and tHcy and, to a lesser extent, holoTC and cobalamin concentrations in serum. Individual genetic variation, disease conditions, and pregnancy can also affect all of these biomarkers.

The Panel considers that the limitations of individual biomarkers necessitate a combination of biomarkers to assess cobalamin status and derive the requirement for cobalamin.

2.5. Effects of genotypes

Several genetic abnormalities affect cobalamin status and function. Inborn errors of cobalamin metabolism include hereditary IF deficiency, Imerslund–Gräsbeck syndrome and TC deficiency. Eight disorders of intracellular cobalamin metabolism have also been described that impair the production or utilisation of MeCbl, AdoCbl or both (Froese and Gravel, 2010; Quadros, 2010). Genetic

polymorphisms in either TC or its receptor protein might offer some explanation for the variability in patient responses to treatment (McCaddon, 2013).

The Panel notes that data on the effect of genotypes on cobalamin status and requirement are insufficient to be used for deriving Dietary Reference Values (DRVs) for cobalamin according to genotype variants.

3. Dietary sources and intake data

3.1. Dietary sources

Cobalamin is not a normal constituent of commonly eaten plant foods unless they contain yeast or have been exposed to microbial fermentation that have produced the vitamin (e.g. beer) or have been fortified with cobalamin (e.g. fortified ready-to-eat breakfast cereals). The principal sources of the vitamin are animal products, including meat, fish, dairy products, eggs and liver.

Currently, cyano- and hydroxocobalamin may be added to both foods[8] and food supplements, while 5′-deoxyadenosyl- and methylcobalamin can be added to food supplements only.[9] The cobalamin content of infant and follow-on formulae[10] and processed cereal-based foods and baby foods for infants and young children[11] is regulated.

3.2. Dietary intake

The European Food Safety Authority (EFSA) estimated dietary intake of cobalamin from food consumption data from the EFSA Comprehensive European Food Consumption Database (EFSA, 2011b), individual data from these nationally representative (except for the Finnish surveys in children) surveys undertaken between 2000 and 2012 were available to EFSA, and classified according to the food classification and description system FoodEx2 (EFSA, 2011a). Data from 13 dietary surveys in nine European Union (EU) countries were used. The countries included were Finland, France, Germany, Ireland, Italy, Latvia, the Netherlands, Sweden and the UK. The data covered all age groups from infants to adults (Appendix A).

Nutrient composition data for cobalamin were derived from the EFSA Nutrient Composition Database (Roe et al., 2013). Food composition information from Finland, France, Germany, Italy, Sweden, the Netherlands and the UK were used to calculate cobalamin intake in these countries, assuming that the best intake estimate would be obtained when both the consumption data and the composition data are from the same country. For cobalamin intake estimates of Ireland and Latvia, food composition data from the UK and Germany, respectively, were used, because no specific composition data from these countries were available. In case of missing values in a food composition database, data providers had been allowed to borrow values from another country's database. The amount of borrowed cobalamin values in the seven composition databases used varied between 14 and 97 %. Estimates were based on food consumption only (i.e. without taking dietary supplements into account). Nutrient intake calculations were performed only on subjects with at least two reporting days.

Data on infants (1–11 months) were available from Finland, Germany, the UK and Italy. The contribution of human milk was taken into account if the amounts of human milk consumed (Italian INRAN-SCAI survey and the UK DNSIYC survey) or the number of breast milk consumption events (German VELS study) were reported. In case of the Italian INRAN-SCAI survey, human milk

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[8] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods, OJ L 404, 30.12.2006, p. 26.

[9] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements, OJ L 183, 12.7.2002, p. 51.

[10] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, OJ L 401, 30.12.2006, p. 1.

[11] Commission Directive 2006/125/EC of 5 December 2006 on processed cereal-based foods and baby foods for infants and young children, OJ L 339, 06.12.2006, p. 16.

consumption had been estimated based on the number of eating occasions using standard portions per eating occasion. In the Finnish DIPP study only the information "breast fed infants" was available, but without any indication about the number of breast milk consumption events during one day or the amount of breast milk consumed per event. For the German VELS study, the total amount of breast milk was calculated based on the observations by Paul et al. (1988) on breast milk consumption during one eating occasion at different ages, i.e. the amount of breast milk consumed on one eating occasion was set to 135 g/eating occasion for infants aged 6–7 months and to 100 g/eating occasion for infants aged 8–12 months. The Panel notes the limitations in the methods used for assessing breast milk consumption in infants (Appendices B and C) and related uncertainties in the intake estimates for infants.

Average cobalamin intakes across countries ranged from 0.8–2.1 µg/day in infants < 1 year, 2.2–4.0 µg/day in children aged 1 to < 3 years, 2.6–5.7 µg/day in children aged 3 to < 10 years, 3.3–6.6 µg/day in children aged 10 to < 18 years and 4.2–8.6 µg/day in adults. Average daily intakes were in most cases slightly higher in males (Appendix B) than in females (Appendix C), mainly due to larger quantities of food consumed per day.

The two main food groups contributing to cobalamin intake were milk and dairy products in infants and children and to a lesser extent meat and meat products. In some infant groups, special food products for the young population were significant contributors to cobalamin intake. Meat and meat products as well as milk and dairy products were the main contributors to cobalamin intake in children aged 10 to < 18 years and in adults. Fish and fish products contributed to cobalamin intake in the older population groups (Appendices D and E). Offal-containing dishes contributed to the cobalamin intake in Finland, France and Sweden. Differences in main contributors to cobalamin intake between males and females were small.

When EFSA cobalamin intake estimates were compared with published intake estimates from the same national dietary surveys, the EFSA estimates deviated in all countries and population groups between 0–13 % from the published data, except for the Swedish adult population (18–80 years), the French children and adolescents (3–17 years) and the Finnish adolescents (13–15 years) (Appendix F). In these population groups, the EFSA cobalamin intake estimates exceeded the published intake estimates by 13–39 %. The differences between the EFSA estimates and the results from national surveys in the Netherlands and the UK may partly be due to the use of weighing factors in national report calculations, while this was not applied in EFSA intake estimates. In addition, published data for the UK are based on two years of data collection, while EFSA intake estimates include three years of collection. The large differences between the cobalamin intake estimates for the Swedish adult population and French children and adolescents may be due to a large variation in the cobalamin content of foods reported in food composition databases. As an example, offal was an important source of cobalamin in both France and Sweden. In the composition databases, twofold differences were observed in the cobalamin concentration of liver, ranging from 50 to 100 µg/100 g. Further uncertainties in the estimates may be caused by differences in disaggregating data for composite dishes before intake estimations, inaccuracies in mapping food consumption data according to the FoodEx2 classification, analytical errors or errors in estimating the cobalamin content of foods in the food composition tables, the use of borrowed cobalamin values from other countries or the replacement of missing cobalamin values by values of similar foods or food groups in the cobalamin intake estimation process. These uncertainties may, in principle, cause both under- and overestimation of cobalamin intake. Cobalamin losses during food processing can be up to 50 % (e.g. in stewing or frying meat or poultry) but cobalamin retention factors usually vary between 65 and 100 % (Bergström, 1994). It is not possible to conclude which of the intake estimates (i.e. those by EFSA or the relevant country) would be closer to the actual cobalamin intake.

4. Overview of Dietary Reference Values and recommendations

4.1. Adults

D-A-CH (2015) stated that by measuring plasma cobalamin concentration and haematological parameters it can be assessed whether the cobalamin requirement of an individual is met (Stewart et al., 1970; Herbert, 1987; Narayanan et al., 1991). D-A-CH considered the AR for cobalamin of a healthy adult to be 2 µg/day and set the recommended intake at 3 µg/day for adults considering that the absorption efficiency from a mixed diet is 50 %.

In their review of the Nordic Nutrition Recommendations (NNR), the Nordic countries considered that there were no additional scientific data to update their recommendations from 2004 (Nordic Council of Ministers, 2014). The requirement for cobalamin was based on studies of patients with pernicious anaemia. In a study in 20 patients, an intramuscular dose of 0.5–2.0 µg/day was needed for normalising and maintaining haematological status, with 0.5–1.0 µg being sufficient for most subjects (Darby et al., 1958). As these patients are unable to reabsorb cobalamin secreted into bile, the physiological requirement of healthy individuals was considered to be somewhat lower. An average physiological requirement of cobalamin was set at 0.7 µg/day, based on this study and other studies (Herbert, 1987, 1988). With correction for absorption efficiency (50 %) the AR was set at 1.4 µg/day for adults. By assuming a CV of 15 % and adding two SD to allow for individual variation, the recommended intake for adults was set at 2 µg/day. It was considered that results from intervention and epidemiological studies do not support benefits of higher intakes for the prevention of common diet-related diseases such as cancer, cardiovascular disease or cognitive impairment. It was also considered that there was insufficient evidence for an association between subnormal blood concentrations of cobalamin and anaemia among older adults. It was also noted that results from cross-sectional population studies have shown that biomarkers of cobalamin status are stabilised at dietary intakes of about 4–10 µg/day among adults (Bor et al., 2006; Vogiatzoglou et al., 2009a; Bor et al., 2010) but it was considered unclear whether intakes in the above range were associated with long-term benefits.

WHO/FAO (2004) adopted the approach of the IOM (1998) for deriving its recommended nutrient intake for cobalamin for adults.

The Health Council of the Netherlands (2003) estimated that the minimum required body store of cobalamin should be approximately 500 µg, based on research carried out on individuals who did not yet show any haematological symptoms of cobalamin deficiency (WHO/FAO, 1970). Considering a (maximum) daily loss of 0.2 % of this minimum body reserve and an absorption efficiency of 50 %, a requirement of 2 µg/day was set for adults aged 19 years and older. Using a CV of 20 %, a PRI of 2.8 µg/day was derived for men and women from 19 years of age. Although it was acknowledged that some older adults may experience a reduction in absorption efficiency, it was stated that an increase in cobalamin intake from food does not lead to an improvement in vitamin cobalamin status in these subjects. Thus, the same AR and PRI were set as for younger adults.

Afssa (2001) proposed a daily intake of 2.4 µg/day for adults, based on an estimated average requirement of 2.0 µg/day and assuming a CV of 10 %. This was estimated from an average loss of biliary cobalamin in the faeces of 0.8 µg/day, considering a mean secretion of cobalamin into bile of 1.3 µg/day (el Kholty et al., 1991) and a re-absorption of 50 % (Adams et al., 1971). A correction factor for cobalamin absorption from foods of 40 % was applied.

The US Institute of Medicine (IOM, 1998) calculated an Estimated Average Requirement (EAR) of 2.0 µg/day and set a Recommended Dietary Allowance (RDA) of 2.4 µg/day assuming a CV of 10 %. The EAR was based on the amount of intramuscular cobalamin required daily to maintain haematological status and serum cobalamin concentration in patients with pernicious anaemia in remission (approximately 1.5 µg/day (Darby et al., 1958)), after adjusting for the additional cobalamin losses in faeces in these patients compared with healthy individuals (0.5 µg/day), and correcting for

absorption efficiency from the diet (50 %). The evidence available from studies which assessed the required dose to maintain haematological status and serum cobalamin concentration in vegetarians or subjects with low cobalamin intake (Jadhav et al., 1962; Winawer et al., 1967; Stewart et al., 1970; Baker and Mathan, 1981; Narayanan et al., 1991) supported an intake of at least 1.5 µg/day. The Committee also noted that studies have indicated losses of 0.1–0.2 % per day of the cobalamin pool regardless of the size of the pool (Heyssel et al., 1966; Reizenstein et al., 1966; Boddy and Adams, 1972; Amin et al., 1980). A person with a pool of 1 000 µg would excrete 1 µg/day, while a person with a pool of 3 000 µg would excrete 3 µg/day, and the amounts required daily to replenish these pools would be 2 µg and 6 µg of cobalamin, respectively, assuming that 50 % of dietary cobalamin is absorbed. Considering that the lowest pool size compatible with health is 300 µg (derived from Bozian et al. (1963)), stores of 1–3 mg allow maintaining adequate body stores for a few years. Insufficient data were available to use serum MMA concentrations.

The SCF (1993) derived an AR of 1.0 µg/day and set a PRI of 1.4 µg/day assuming a CV of 20 %. SCF took into consideration the fact that in subjects on strict vegetarian diets there was no evidence of haematological or neurological dysfunction at an intake of 0.5 µg/day (Armstrong et al., 1974; Jathar et al., 1975; Abdulla et al., 1981), but there was some evidence of biochemical abnormality (elevated urinary MMA concentration) (Specker et al., 1990; Miller et al., 1991). The SCF also considered evidence that people with apparently normal haematology were developing irreversible neurological damage (Karnaze and Carmel, 1990). SCF referred to two studies on vitamin turnover which estimated mean daily requirements of cobalamin of between 0.25 µg/day and 1.0 µg/day (Anderson, 1965) and of 1.3 µg/day (Herbert, 1987).

The UK COMA (DH, 1991) considered that the amount to prevent or cure megaloblastic anaemia of cobalamin deficiency in adults appeared to be less than 1 µg/day, based on evidence from studies in vegetarians (Armstrong et al., 1974; Abdulla et al., 1981), subjects with diet-related cobalamin deficiency anaemia (Baker and Mathan, 1981) and subjects with pernicious anaemia (Sullivan and Herbert, 1965; Cooper and Lowenstein, 1966). The Committee set the Lower Reference Nutrient Intake (LRNI) for cobalamin at 1 µg/day, and proposed an AR of 1.25 µg/day and a Reference Nutrient Intake (RNI) of 1.5 µg/day.

An overview of DRVs for cobalamin for adults is presented in Table 1.

Table 1: Overview of Dietary Reference Values for cobalamin for adults

	D-A-CH (2015)	NCM (2014)	WHO/FAO (2004)	NL (2003)	Afssa (2001)	IOM (1998)	SCF (1993)	DH (1991)
Age (years)	≥ 19	≥ 18	≥ 19	≥ 19	≥ 20	≥ 19	≥ 19	≥ 19
PRI (µg/day)	3.0	2.0	2.4	2.8	2.4	2.4	1.4	1.5

NCM, Nordic Council of Ministers; NL, Health Council of the Netherlands; PRI, Population Reference Intake.

4.2. Infants and children

D-A-CH (2015) derived an Adequate Intake (AI) of 0.4 µg/day for infants younger than four months (i.e. 0.06 µg/kg body weight per day) from the average intake with breast milk (Souci et al., 2008). Recommended intakes for older infants and children were derived from the reference value for younger infants considering the increase in weight, and varied from 0.8 µg/day for infants aged 4 to < 12 months to 3.0 µg/day for adolescents aged 13 to < 19 years.

For children, the Nordic countries maintained their approach of NNR 1996, which was based on an AR for cobalamin of 0.05 µg/kg body weight per day (Nordic Council of Ministers, 2014). This value was derived by the US (National Research Council, 1989) from a proposed RDA of 0.3 µg/day for infants aged 0–6 months, based on the consideration that a dose of 0.1 µg cobalamin/day had been shown to provide full therapeutic response in cobalamin-deficient infants and allowing for some storage.

Based on the assumption that breast milk contains sufficient cobalamin for optimum health, WHO/FAO (2004) estimated an AR of between 0.3 and 0.6 µg/day for infants and a recommended nutrient intake of between 0.4 and 0.7 µg/day. WHO/FAO proposed to use the lower figure of 0.4 µg/day for infants aged 0–6 months and 0.7 µg/day for infants aged 7–12 months. For children, WHO/FAO adopted the same approach as the IOM.

The Health Council of the Netherlands (2003) proposed an AI of 0.4 µg/day for infants up to five months of age based on an estimated breast milk intake of 800 mL/day and a breast milk cobalamin concentration of 0.45 µg/L. For older infants and children, studies on cobalamin intake in macrobiotic children were considered and it was stated that a daily cobalamin intake of 1.8 µg is insufficient and may have contributed to an impairment in cognitive function observed in some children on a macrobiotic diet (Louwman et al., 2000). It was, however, considered that evidence on the cobalamin requirement is lacking, and AIs for infants older than 6 months and children were interpolated based on the AI for infants aged 0–5 months and the AR for adults.

Afssa (2001) proposed a daily intake of 0.5 µg/day for infants up to one year of age, based on the average cobalamin concentration and daily intake of maternal milk. For children, the recommended intakes were derived by scaling down from the reference value for adults using square height as an extrapolation factor.

IOM (1998) set an AI of 0.4 µg/day for infants aged 0–6 months based on an average cobalamin concentration in milk of mothers with adequate cobalamin status. An AI of 0.5 µg/day for infants from 7–12 months was proposed, which was extrapolated from the AI for younger infants. It was noted that these intakes were above the intake level associated with increased urinary MMA concentrations in infants of vegan mothers. Supplementation with cobalamin at the AI was recommended for infants of vegan mothers on the basis of evidence that their stores at birth are low and that their mother's milk would supply only small amounts of the vitamin. Owing to lack of data in children, the EARs were extrapolated down from adult values using allometric scaling and applying a growth factor.

SCF (1993) proposed a PRI of 0.5 µg/day for infants aged 6–11 months, based on evidence that infants born with very poor stores of cobalamin needed 0.37 µg/day to cure cobalamin insufficiency as evidenced by increased urinary MMA excretion (Specker et al., 1990). In the absence of specific studies, SCF extrapolated values for children from those for adults on the basis of energy expenditure.

The UK COMA (DH, 1991) set a LRNI of 0.1 µg/day for infants, as this dose was shown to cure megaloblastic anaemia in infants receiving less than 60 ng/day from breast milk (Jadhav et al., 1962). The RNI was set at 0.4 µg/day which was the intake required to normalise elevated urinary MMA excretion (Specker et al., 1990). Amounts for children were interpolated between these and the values for adults.

An overview of DRVs for cobalamin for infants and children is presented in Table 2.

Table 2:   Overview of Dietary Reference Values for cobalamin for infants and children

	D-A-CH (2015)	NCM (2014)	WHO/FAO (2004)	NL (2003)	Afssa (2001)	IOM (1998)	SCF (1993)	DH (1991)
Age (months)	4–< 12	6–11	7–12	6–11	0–12	7–12	6–11	7–12
PRI (µg/day)	0.8	0.5	0.7 [(a)]	0.5 [(a)]	0.5 [(a)]	0.5 [(a)]	0.5	0.4
Age (years)	1–< 4	1–< 2	1–3	1–3	1–3	1–3	1–3	1–3
PRI (µg/day)	1.0	0.6	0.9	0.7 [(a)]	0.8	0.9	0.7	0.5
Age (years)	4–< 7	2–5	4–6	4–8	4–6	4–8	4–6	4–6
PRI (µg/day)	4.5	0.8	1.2	1.3 [(a)]	1.1	1.2	0.9	0.8
Age (years)	7–< 10	6–9	7–9	9–13	7–9	9–13	7–10	7–10
PRI (µg/day)	1.8	1.3	1.8	2.0 [(a)]	1.4	1.8	1.0	1.0
Age (years)	10–< 13	10–17	10–18	14–18	10–12	14–18	11–14	11–14
PRI (µg/day)	2.0	2.0	2.4	2.8 [(a)]	1.9	2.4	1.3	1.2
Age (years)	13–< 19				13–15		15–17	15–18
PRI (µg/day)	3.0				2.3		1.4	1.5
Age (years)					16–19			
PRI (µg/day)					2.4			

NCM, Nordic Council of Ministers; NL, Health Council of the Netherlands

(a):   Adequate Intake.

4.3.   Pregnancy

D-A-CH (2015) considered that 0.1–0.2 µg/day of cobalamin is transferred to the fetus during pregnancy, but that a cobalamin deficiency of the mother or the newborn can be excluded in cases of normal body stores prior to pregnancy. The additional recommended intake of 0.5 µg/day during pregnancy was set as a precaution in case the extent of pre-existing body stores was unknown and also in order to maintain a high nutrient density of the diet. Hence, the recommended intake during pregnancy was set at 3.5 µg/day.

The Nordic countries considered that there were no additional data to update their recommendations from 2004 for pregnant women (Nordic Council of Ministers, 2014). Considering that pregnant women have adequate stores to cover the additional requirement of 0.1–0.2 µg/day (Herbert, 1987), it was stated that the same recommendations apply as for non-pregnant women.

On the basis of a fetal deposition of 0.1–0.2 µg/day throughout pregnancy and evidence that maternal absorption of the vitamin becomes more efficient during pregnancy, the IOM (1998) proposed to increase the EAR by 0.2 µg/day during pregnancy. The RDA was set at 2.6 µg/day. Based on similar considerations, Afssa (2001) and WHO/FAO (2004) also proposed a daily intake of 2.6 µg/day for pregnant women. A fetal deposition of 0.16 µg cobalamin/day during the second and third trimester of pregnancy was estimated, or 0.11 µg/day over the whole pregnancy (Health Council of the Netherlands, 2003). Assuming an absorption efficiency of 50 %, it was estimated that the additional requirement in pregnancy is 0.3 µg/day. Adding this to the AR for non-pregnant women and using a CV of 20 %, the Health Council of the Netherlands (2003) derived a PRI of 3.2 µg/day for pregnant women.

SCF (1993) recommended an additional intake of cobalamin of 0.2 µg/day for pregnant women, in order to prevent the risk of an inadequate supply for the developing fetus that could impair growth rate and lead to neurological damage, as had been observed in children from women on strict vegetarian diets.

The UK COMA (DH, 1991) considered the RNI of 1.5 µg/day for adults to be also sufficient for pregnant women, on the assumption that their body stores would not be depleted at the beginning of pregnancy.

An overview of DRVs for cobalamin for pregnant women is presented in Table 3.

Table 3: Overview of Dietary Reference Values for cobalamin for pregnant women

	D-A-CH (2015)	NCM (2014)	WHO/FAO (2004)	NL (2003)	Afssa (2001)	IOM (1998)	SCF (1993)	DH (1991)
Age (years)						14–50		
PRI (µg/day)	3.5	2.0	2.6	3.2	2.6	2.6	1.6	1.5

NCM, Nordic Council of Ministers; NL, Health Council of the Netherlands

4.4. Lactation

D-A-CH (2015) recommended an additional intake of 1.0 µg/day for lactating women (after rounding), taking into account the daily secretion of 0.4 µg/day in milk of mothers fully breastfeeding, and an average absorption efficiency of 50 %. Hence, the recommended intake during lactation was set at 4.0 µg/day.

The NNR maintained its earlier recommendation from 2004 and considered that an additional 0.6 µg/day is needed to compensate for the secretion of cobalamin in breast milk (Nordic Council of Ministers, 2014).

The Health Council of the Netherlands (2003) assumed that an additional intake of 0.7 µg/day is needed to replace the amount of cobalamin secreted daily with breast milk (0.36 µg), thus considering that absorption efficiency is about 50 %. Adding this to the AR for non-pregnant women and using a CV of 20 %, a PRI of 3.8 µg/day was derived for lactating women.

For lactating women, several organisations proposed an increment in cobalamin intake of 0.4 µg/day (IOM, 1998; Afssa, 2001; WHO/FAO, 2004) to 0.5 µg/day (DH, 1991), in order to cover the amount of cobalamin secreted in breast milk. SCF (1993) recommended an increment of 0.5 µg cobalamin/day in order to replace an amount of cobalamin secreted in breast milk of 0.37 µg/day, which is the level of intake below which cobalamin insufficiency may occur in breast-fed infants (Specker et al., 1990).

An overview of DRVs for cobalamin for lactating women is presented in Table 4.

Table 4: Overview of Dietary Reference Values for cobalamin for lactating women

	D-A-CH (2015)	NCM (2014)	WHO/FAO (2004)	NL (2003)	Afssa (2001)	IOM (1998)	SCF (1993)	DH (1991)
Age (years)						14–50		
PRI (µg/day)	4.0	2.6	2.8	3.8	2.8	2.8	1.9	2.0

NCM, Nordic Council of Ministers; NL, Health Council of the Netherlands

5. Criteria (endpoints) on which to base Dietary Reference Values

5.1. Indicators of cobalamin requirement

5.1.1. Adults

5.1.1.1. Maintenance of haematological status

Some expert bodies established reference values for cobalamin intake based on the relationship between cobalamin intake and maintenance of an adequate haematological status (IOM, 1998; WHO/FAO, 2004; Nordic Council of Ministers, 2014; D-A-CH, 2015) (Section 4). In this approach, the amount of daily cobalamin required to maintain a normal haematological status (i.e. stable haemoglobin concentration, normal MCV and reticulocyte response) in individuals with pernicious anaemia in remission was considered. The requirement of healthy subjects was then derived by subtracting the extra biliary loss of cobalamin due to pernicious anaemia and correcting for percentage absorption, and estimated to be 1.5–2 µg/day. Data on the cobalamin dose required to maintain normal haematological status and serum cobalamin concentrations in vegetarians or subjects with low

cobalamin intake were also considered by some expert bodies (DH, 1991; IOM, 1998; WHO/FAO, 2004; D-A-CH, 2015).

The Panel notes that daily losses of 0.1–0.2 % of body stores have been observed irrespective of the size of the body pool (see Section 2.3.6.1). Individuals with pernicious anaemia in remission are expected to have depleted body stores, and thus lower absolute daily losses than healthy individuals. An intake of 1.5–2 µg/day represents a minimum requirement for maintenance of a normal haematological status, associated with low body stores of 1–2 mg. Higher body stores (typically between 2 and 3 mg) are usually observed in healthy people (see Section 2.3.4), whose maintenance would require higher levels of intake.

5.1.1.2. Factorial approach

The factorial approach estimates daily obligatory losses of cobalamin in healthy subjects, which need to be replaced by dietary intake. Considering cobalamin stores of 2–3 mg (see Section 2.3.4) and a rate of daily losses of 0.1–0.2 % of stores (see Section 2.3.6.1), total cobalamin losses would range between 2 and 6 µg/day. With an absorption of dietary cobalamin of 40 % (see Section 2.3.1), the dietary cobalamin intake needed to compensate daily losses would range from 5 to 15 µg/day (Table 5).

Table 5: Estimated daily obligatory losses of cobalamin and associated estimated requirements

Cobalamin body stores (mg)	2 mg		3 mg	
% Losses	0.1 %	0.2 %	0.1 %	0.2 %
Daily losses [a] (µg)	2.0	4.0	3.0	6.0
Requirement [b] (µg)	5.0	10.0	7.5	15.0

(a): Daily losses = cobalamin body stores × % losses.
(b): Estimated cobalamin intake required to compensate daily cobalamin losses. Requirement = daily losses/absorption. An absorption of 40 % was considered.

When considering other values for absorption, e.g. 30 or 50 % (see Section 2.3.1), the range of associated requirements becomes even wider (4–20 µg/day), underlining the uncertainty associated with this approach. The Panel notes that the factorial approach has limitations in that it relies heavily on assumptions as regards absorption, body stores and losses, for which reported values are limited and based on relatively old studies with few subjects using isotopes and invasive methods that cannot be updated for ethical reasons. The inherent uncertainty of this approach is reflected in the wide range of cobalamin intake calculated to compensate for estimated daily losses, depending on the assumptions taken.

5.1.1.3. Serum/plasma biomarkers of cobalamin status

Total serum cobalamin, holoTC, MMA or plasma tHcy concentrations, when used in isolation, lack sufficient sensitivity and/or specificity to define adequate cobalamin status (Section 2.4.6). The Panel concludes that the limitations of all biomarkers make a combination of biomarkers necessary to assess cobalamin status. Serum holoTC carries the functional fraction of cobalamin that can be taken up by tissues. The Panel considers that serum holoTC is the most specific biomarker to characterise adequate cobalamin status. The Panel considers serum cobalamin as a less specific but supportive biomarker. Serum MMA and plasma tHcy reflect cobalamin coenzymatic functions (i.e. activities of methionine synthase and methylmalonyl-CoA mutase) and are considered as supportive biomarkers for the assessment of cobalamin status.

The Panel considers that serum concentrations of holoTC and cobalamin within the reference ranges for healthy adults, together with serum MMA and plasma tHcy concentrations below cut-off values for cobalamin insufficiency and hyperhomocysteinaemia, which have been proposed in the literature, are indicative of an adequate cobalamin status. In adults, lower limits of available reference ranges for serum holoTC and cobalamin range between 11 and 48 pmol/L (Section 2.4.3) and between 134 and 178 pmol/L (Section 2.4.2), respectively. Proposed cut-off values range from 210 to 450 nmol/L for

serum MMA concentrations (Section 2.4.4). A plasma tHcy concentration above 15 μmol/L in adults can be considered as indicative of hyperhomocysteinaemia (Section 2.4.5).

Only a few studies report simultaneously on the relationships between cobalamin intake and serum cobalamin, holoTC, MMA and plasma tHcy concentrations.

One observational study included 98 postmenopausal women aged 41–75 years from Denmark (Bor et al., 2006) with established osteoporosis or risk factors for osteoporosis and who were participating in a clinical trial testing soymilk or progesterone for prevention of bone loss (Lydeking-Olsen et al., 2004). Impaired renal function was not part of the exclusion criteria and 36 % of the subjects had a gastric pH $\geq$ 3 or a prolonged alkali challenge test, indicating impaired gastric function. The Panel considers that no conclusions can be drawn from this study on the relationships between cobalamin intake and biomarkers of cobalamin status in the healthy general population.

One observational study included 299 healthy women and men aged 18–50 years in the USA (Bor et al., 2010). Cobalamin intake was assessed using an FFQ that included foods fortified with cobalamin; subjects taking supplements were excluded from the study. IF antibodies were not found in any subject, while antibodies against *H. pylori* were detected in 12 % of the subjects. Significantly lower serum cobalamin concentrations were detected in subjects with antibodies against *H. pylori* (n = 35) than in the other subjects (n = 264) (p = 0.011). No differences between groups were found for the other biomarkers. The relationship between quintiles of cobalamin intake (median intake for each quintile) and serum concentrations of holoTC, cobalamin, MMA and plasma tHcy was assessed (Figure 3).
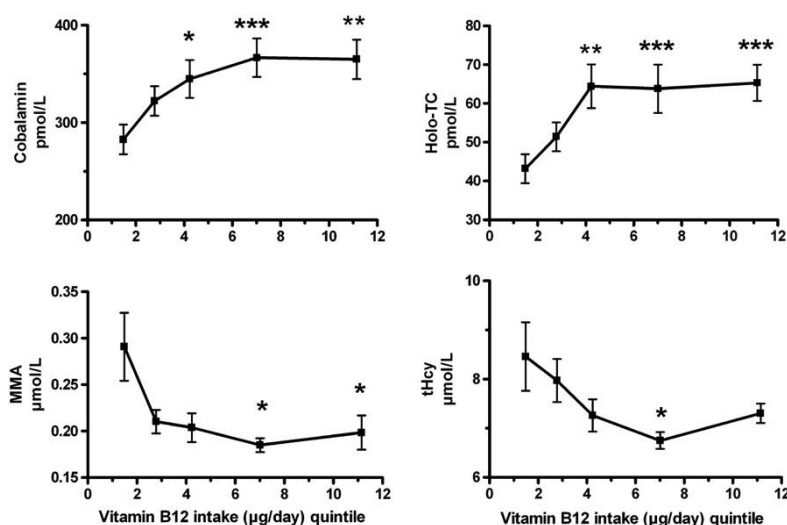
Mean ($\pm$ standard error of the mean (SEM)) concentrations are plotted against the median intake for each quintile (each with n = 60, except for the fifth quintile, n = 59). Quintiles cover the following median (range) daily cobalamin intakes: quintile 1, 1.5 μg (0.4–2.1 μg); quintile 2, 2.8 μg (2.1–3.4 μg); quintile 3, 4.2 μg (3.4–5.3 μg), quintile 4, 7.0 μg (5.4–8.6 μg) and quintile 5, 11.2 μg (8.7–22.7 μg). Concentrations of holoTC (p < 0.0001), cobalamin (p = 0.0003), tHcy (p = 0.017) and MMA (p = 0.009) differed significantly between quintiles for cobalamin intake (one-factor analysis of variance (ANOVA), after log transformation). *, **, *** Cobalamin intakes in the lowest quintile differed significantly from other quintiles as labelled: *p < 0.05, **p < 0.01, ***p < 0.001 (all by Tukey's multiple-comparisons test). American Journal of Clinical Nutrition 2010;91:571–7. © 2010 American Society for Nutrition.

Figure 3:   Relation between cobalamin intake and cobalamin biomarkers (n = 299, women and men aged 18–50 years)

The Panel notes that at a cobalamin intake of 2.8 μg/day (median of quintile 2), mean serum holoTC concentration was approximately 50 pmol/L, which is close to the lower limit of available reference

ranges and the proposed cut-off values for cobalamin insufficiency (Section 2.4.3). At this level of cobalamin intake, mean serum cobalamin, MMA and plasma tHcy concentrations were around 325 pmol/L, 210 nmol/L and 8 µmol/L, respectively. At a cobalamin intake of 4.2 µg/day (median of quintile 3), mean serum holoTC concentration was approximately 65 pmol/L, which is well above lower limits of available reference ranges. The Panel notes that a further increase in cobalamin intake did not result in higher serum holoTC concentration in this population. At this level of cobalamin intake, mean serum MMA concentration was around 200 nmol/L. Mean plasma tHcy and mean serum cobalamin concentrations were 7 µmol/L and 350 pmol/L, respectively. This falls within the range of concentrations considered adequate for these biomarkers. Concentrations of the biomarkers levelled off at cobalamin intakes between 4 and 7 µg/day. The Panel notes that information on the distribution of the individual data for each biomarker in the different quintiles has not been reported.

An RCT in 231 healthy adults in the UK investigated the response of cobalamin biomarkers to supplementation with various doses of cobalamin (3.4, 12.7 and 46.1 µg/day) for 16 weeks, after folate repletion (400 µg folic acid/day for 11 weeks). An average background dietary cobalamin intake of 4 µg/day was estimated in this cohort. At the end of the study, mean ($\pm$ SD) serum MMA, plasma tHcy and serum cobalamin concentrations were 220 nmol/L, 8.4 µmol/L and 300 pmol/L, respectively, in the placebo group. Minimum serum MMA concentration (around 190 nmol/L) was achieved with the supplemental cobalamin dose of 3.4 µg/day, i.e. a total cobalamin intake of ~ 7 µg/day; no further decrease in serum MMA concentration was observed with the higher supplemental doses. No significant effect of supplementation was observed on plasma tHcy (mean concentrations between 8 and 8.4 µmol/L) (Pentieva et al., 2012) (Figure 4).
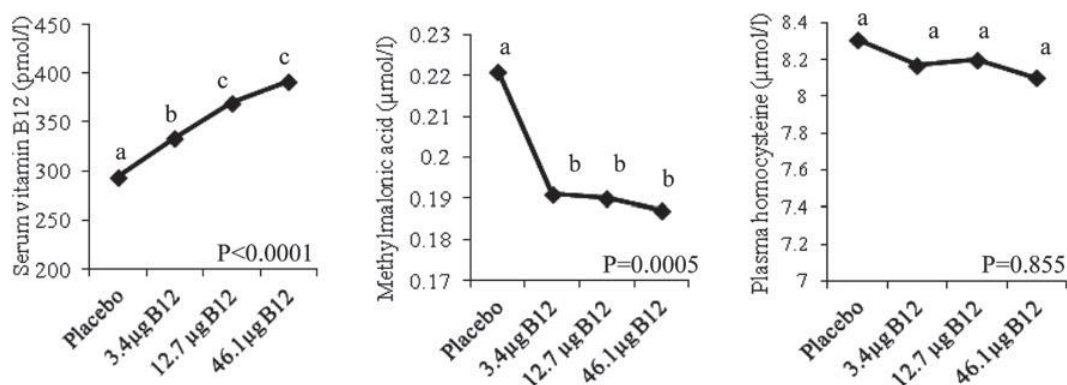
Values are geometric means. Differences between responses were assessed by ANOVA with Tukey post hoc test.

Figure 4: Responses of vitamin B12 biomarkers to supplementation with vitamin B12 for 16 weeks. ©Pentieva et al. (2012). An intervention trial to determine the response of vitamin B12 biomarkers to chronic supplementation with low dose vitamin B12 after folate repletion. Proceedings of the Nutrition Society. Cambridge University Press.

The Panel notes that at a cobalamin intake of 4 µg/day mean serum cobalamin concentration was well above the lower limits of available reference ranges and both biomarkers of cobalamin function, MMA and tHcy, were close to or below proposed cut-off values. No information is available from this study on concentrations of biomarkers of cobalamin status at intakes below 4 µg/day.

In a large population-based study in 5 937 middle-aged (47–49 years) and older (71–74 years) men and women in Norway, cobalamin intake, assessed by FFQ, and cobalamin biomarkers were assessed (Vogiatzoglou et al., 2009a). Mean daily cobalamin intakes were 5.5 and 5.1 µg for middle-aged and older women, respectively, and 7.3 and 6.9 µg for middle-aged and older men, respectively. Mean

plasma holoTC concentration was available for older subjects only and was 86 and 93 pmol/L for men and women, respectively. Mean plasma cobalamin concentrations were 353 and 358 pmol/L for the middle-aged men and women, respectively, and 335 and 352 pmol/L for older men and women, respectively. Mean plasma MMA concentrations were 160 nmol/L for the middle-aged men and women and 200 nmol/L for the older men and women. Mean plasma tHcy concentrations were 10.4 and 8.8 µmol/L for the middle-aged men and women, respectively, and 12.5 and 10.8 µmol/L for the older men and women, respectively.

The Panel notes that at intakes between 5 and 7 µg/day, both plasma holoTC and cobalamin were well above the lower limits of available reference ranges and both biomarkers of cobalamin function, MMA and tHcy, were below proposed cut-off values. No information is available from this study on the dose-response relationship between cobalamin intake and biomarkers of cobalamin status at lower intakes.

5.1.1.4. Conclusions on indicators of cobalamin requirement in adults

The Panel notes that intakes of 1.5–2 µg/day seem to represent a minimum requirement for maintenance of a normal haematological status, associated with low body stores of 1–2 mg (Section 5.1.1.1). The factorial approach results in a range of estimated requirement between 5 and 15 µg/day (Section 5.1.1.2), which reflects the large uncertainties associated with this approach.

The Panel considers the approach based on cobalamin biomarkers of status as the most suitable approach to derive DRVs for cobalamin for adults. The Panel notes that there is consistent evidence from observational and intervention studies that a cobalamin intake of 4 µg/day and greater is associated with serum concentrations of holoTC and cobalamin within the reference ranges derived from healthy adults together with serum MMA and plasma tHcy concentrations below proposed cut-off values, which is assumed to be indicative of an adequate cobalamin status.

5.1.2. Infants and children

During the first weeks of life, a decrease in serum cobalamin concentration accompanied by an increase in serum MMA and plasma tHcy concentrations has been observed in several studies (Bjorke Monsen and Ueland, 2011), being lowest (cobalamin) and highest (tHcy and MMA) in infants six weeks to six months of age. In a study involving 700 children aged 4 days to 19 years in Norway, serum cobalamin was observed to increase and achieve a maximum at three to seven years of age and then decreased, median plasma tHcy remained low (< 6 µmol/L) and increased from the age of seven years, whereas median plasma MMA remained low throughout childhood (< 0.26 µmol/L) (Monsen et al., 2003). Cobalamin intake was not reported in these studies.

In a systematic review on the associations between cobalamin intake and biomarkers in children (Iglesia et al., 2013), one cross-sectional study was identified which examined dietary intake of cobalamin and serum cobalamin, holoTC, MMA and tHcy concentrations in 155 healthy, non-breast-fed Norwegian toddlers at 24 months of age (Hay et al., 2011). Seven-day weighed food records were used for cobalamin intake assessment. Median cobalamin intake was 3.1 µg/day and none of the children had a cobalamin intake below 0.8 µg/day. Median (25th–75th percentile) concentrations were 410 pmol/L (334–521 pmol/L) for serum cobalamin, 94 pmol/L (67–121 pmol/L) for serum holoTC, 160 nmol/L (130–200 nmol/L) for serum MMA and 5.0 µmol/L (4.2–5.7 µmol/L) for plasma tHcy. Cobalamin and holoTC concentrations were plotted against the corresponding median of each quartile of cobalamin intake (Q1 ~ 2 µg/day, Q2 ~ 3 µg/day, Q3 ~ 3.5 µg/day, Q4 ~ 5 µg/day). A positive association between intake of cobalamin and serum holoTC (Spearman's correlation $\rho = 0.21$, $p < 0.05$) was observed. Mean serum cobalamin and holoTC concentrations were > 350 pmol/L and > 75 pmol/L across quartiles. A plateau in serum cobalamin and holoTC was reached at a cobalamin intake of ~ 3 µg/day. Neither MMA nor tHcy concentrations decreased with increasing cobalamin intakes. HoloTC was negatively associated with MMA concentrations ($r = -0.41$, $p < 0.001$). Neither MMA nor tHcy concentrations correlated with serum cobalamin concentration. No conclusion could

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be drawn from the selected papers on the relationship between biomarkers of cobalamin status and cobalamin intake in older children.

The Panel notes that results from one study in children at 24 months of age indicate that, at intakes above 3 µg/day, holoTC in serum does not rise further. Mean serum holoTC and cobalamin concentrations were well within the reference ranges proposed for this age group (Section 2.4) for all quartiles of intake (median intake range across quartiles: 2–5 µg/day). No decrease in MMA or tHcy concentration was apparent with increasing cobalamin intakes, which may be indicative of an adequate cobalamin status at these levels of intake. However, no information is available on cobalamin status at cobalamin intakes below 2 µg/day.

5.1.3. Pregnant women

IOM (1998) and WHO/FAO (2004) estimated that the fetus accumulates an average of 0.1–0.2 µg/day of cobalamin, based on three studies of the liver content of infants born to women considered to have an adequate cobalamin status (Baker et al., 1962; Vaz Pinto et al., 1975; Loría et al., 1977) and assuming that liver contains half of the total body cobalamin content. The placental cobalamin content was considered negligible (Muir and Landon, 1985).

In cohorts of healthy pregnant women, a steady fall in serum cobalamin concentration is observed during pregnancy (Section 2.4.2), accompanied by increases in serum MMA (Section 2.4.4) and plasma tHcy concentrations (Section 2.4.5) , while serum holoTC concentration remained unchanged (Section 2.4.3). Cobalamin intake was not reported in these studies.

Lower maternal cobalamin or holoTC concentrations were associated with lower cobalamin and higher MMA and tHcy concentrations in infants at birth (Bjorke Monsen et al., 2001; Hay et al., 2010).

In the study by Koebnick et al. (2002) in 39 pregnant women in Germany (Section 2.4.2), cobalamin intake (mean ± SD, 5.6 ± 2.0 µg/day) was not associated with serum cobalamin concentration. Serum cobalamin concentration and percentage of saturation of cobalamin-binding proteins decreased steadily throughout pregnancy. Significant reductions in haemoglobin concentration and red blood cell counts and increases in MCV and neutrophil segmentation were observed during the course of pregnancy. Plasma tHcy concentration significantly decreased between the first and second trimester and then came back to the initial value in the third trimester. Results of an ANOVA showed that these changes were not affected by cobalamin status but rather by folate and iron status.

The Panel notes that data on the relationship between cobalamin intake and biomarkers of cobalamin status in pregnancy are limited. At present, the causes (e.g. physiological changes, other determinants) and significance of the changes in cobalamin biomarkers of status observed in pregnancy are unknown. The Panel considers that available data cannot be used for deriving DRVs for cobalamin for pregnancy.

5.1.4. Lactating women

In a longitudinal study in 60 mother–child matched pairs in Denmark, the majority of whom took a daily multivitamin supplement (1.0–4.5 µg cobalamin), maternal plasma cobalamin concentration did not change during lactation (measured at two weeks, four months and nine months post partum), total haptocorrin concentration slightly increased, while the concentrations of holoTC and MMA declined over time (Greibe et al., 2013). In the four-month-old children, plasma concentrations of cobalamin and holoTC were significantly lower, while concentrations of MMA were significantly higher than the concentrations found at two weeks or nine months. In a subgroup of 25 mothers, a decline in breast milk cobalamin concentration was observed between two weeks and four months post partum followed by an increase at nine months post partum (Section 2.3.6.2). In a longitudinal study in 89 Danish lactating women, most of whom reported to take cobalamin supplements (1–18 µg/day, median 1 µg/day), no change in serum cobalamin concentration was observed from three weeks to nine

months post partum, whereas a significant decrease in serum holoTC and an increase in haptocorrin concentrations were reported (Mørkbak et al., 2007a). Serum cobalamin, holoTC and haptocorrin concentrations after nine months showed no statistical difference between the supplemented (n = 23) and unsupplemented (n = 25) mothers. Total cobalamin intake was not reported in these studies.

The Panel notes that there are no data on the relationships between total cobalamin intake and biomarkers of cobalamin status in lactating women and their infants. At present, the causes (e.g. physiological changes, other determinants) and significance of the changes in cobalamin biomarkers of status observed in lactating women and their infants are unknown. The Panel considers that available data cannot be used for deriving DRVs for cobalamin for lactation.

5.2. Cobalamin intake and health consequences

Some observational studies and intervention studies have examined the association between cobalamin intake and health outcomes. RCTs and prospective (cohort and nested case–control) studies are considered in this section. The Panel notes that such relationships have mostly been investigated in observational studies, where associations between cobalamin intake and health outcomes may be confounded by the effect of dietary, sociodemographic, environmental, lifestyle, genetic or other factors.

Doets et al. (2013b) conducted a systematic literature review on the relationship between cobalamin intake and cognitive function in healthy adults. Four categories of cognitive outcomes were defined: incident dementia, incident Alzheimer's disease, global cognition and domain-specific cognition. Six prospective cohort and nested case–control studies and two RTCs which assessed cobalamin intake were included in the review. The RCTs involved older adults (~ 80 years) and used 10 or 50 µg cobalamin/day for four weeks and 1 000 µg cobalamin/day for 24 weeks, respectively. The two RCTs showed no effect of supplementation on measures of cognitive function. The cohort studies involved older adults ($\geq$ 60 years), with sample sizes between 122 and 3 718 subjects and follow-up duration between 3 and 9.3 years. Three cohort studies assessed the association between cobalamin intake and various measures of cognitive function, with inconsistent results. Three cohort studies assessed the incidence of Alzheimer's disease. The pooled estimate of these three studies showed no association between cobalamin intake and incidence of Alzheimer's disease (relative risk (RR) = 0.99, 95 % confidence interval (CI) = 0.99–1.00; I^2 = 0 %, p = 0.92).

Intake of cobalamin was inversely associated with the risk of ischaemic stroke (455 cases) in a prospective study in 43 732 men (40–75 years; 14 years of follow-up) in the USA, after adjustment for body mass index, physical activity, history of hypertension and hypercholesterolaemia, smoking status, aspirin use, alcohol, total calorie and intakes of fibre, potassium and vitamin E (Q5 vs. Q1: RR = 0.73, 95 % 0.73 (0.53–0.99) , p for trend = 0.04) (He et al., 2004). No association was observed with haemorrhagic stroke (125 cases). In a prospective study in 26 556 male Finnish smokers (50–69 years; mean follow-up 13.6 years), no association was found between cobalamin intake and risk of stroke subtypes (cerebral infarction (2 702 cases), intracerebral haemorrhage (383 cases) and subarachnoid haemorrhage (196 cases)) (Larsson et al., 2008). No association was found between cobalamin intake and mortality from stroke, coronary heart disease and total cardiovascular disease in a prospective cohort study in Japan (n = 58 730, 40–79 years; median follow-up 14 years) (Cui et al., 2010).

Cobalamin intake was not associated with hip fracture risk in a prospective cohort study in Chinese adults (n = 63 257, 45–74 years; follow-up 13.8 years) (Dai et al., 2013).

The Panel considers that available data on cobalamin intake and health outcomes are inconsistent or limited and cannot be used for deriving DRVs for cobalamin.

6. Data on which to base Dietary Reference Values

In consideration of the available data on the relationship between cobalamin intake and its status, functions and health consequences (Section 5), the Panel considers the combination of biomarkers, i.e. holoTC, MMA, tHcy and cobalamin in serum/plasma, as the most suitable criterion for deriving DRVs for cobalamin.

6.1. Adults

The Panel notes that data on the dose–response relationships between cobalamin intake and biomarkers of cobalamin status, considered together, are limited (Section 5.1). One cross-sectional study (Bor et al., 2010) provides information on the dose–response relationship between quintiles of estimated cobalamin intake and different biomarkers of cobalamin status in one population group in the USA (Section 5.1.1.3). One intervention study (Pentieva et al., 2012) provides data for a range of cobalamin intakes ≥ 4 µg/day; the adequacy of cobalamin intake below 4 µg/day cannot be assessed from this study. In addition to the limited data available, the Panel notes the uncertainties with respect to the cut-off values of the indicators for cobalamin insufficiency and considers that an AR, i.e. the level of cobalamin intake that meets the requirement of half of the healthy individuals in a group, cannot be determined from the available evidence. However, there is consistent evidence from observational and intervention studies that a cobalamin intake of 4 µg/day and greater is associated with serum concentrations of holoTC and cobalamin within the reference ranges derived from healthy subjects, together with MMA and tHcy concentrations below the cut-off values for adults (Section 5.1.1.3), which indicates an adequate cobalamin status.

Therefore, the Panel sets an AI for cobalamin for adults at 4 µg/day based on data on different biomarkers of cobalamin status and in consideration of observed intakes in several EU countries, which in adults range between 4.2 and 8.6 µg/day (Appendices B and C).

6.2. Infants and children

The Panel notes the limited number of studies using an accurate method to estimate the breast milk concentration of cobalamin, in addition to their small sample sizes and the inclusion of mothers receiving cobalamin supplements (Section 2.3.6.2). Furthermore, there is a lack of data from which an AR could be derived for infants aged 7–11 months (Section 5.1.2). Therefore, the Panel decides to set an AI for infants aged 7–11 months.

Downwards extrapolation from the AI for adults, using allometric scaling on the assumption that cobalamin requirement is related to metabolically active body mass, was carried out as follows:

$$AI_{child} = AI_{adult} \times (\text{body weight of child/body weight of adult})^{0.75} \times (1 + \text{growth factor}).$$

For infants, the mean of the median weight-for-age of male and female children aged 9 months (8.6 kg) according to the WHO Growth Standard (WHO Multicentre Growth Reference Study Group, 2006) was used. For adults, the mean (63.3 kg) of median body weights of 18- to 79-year-old men (68.1 kg) and women (58.5 kg), respectively, based on measured body heights and assuming a body mass index of 22 kg/m^2 (see Appendix 11 in EFSA NDA Panel (2013b)) was used. A growth factor of 0.57 was applied (see Appendix G of EFSA NDA Panel (2014a)), which was calculated as the proportional increase in protein requirement for growth relative to the maintenance requirement at the different ages (EFSA NDA Panel, 2012). Thus, a value of 1.4 µg/day was calculated.

In comparison, upwards extrapolation from the estimated cobalamin intake of fully breast-fed infants during the first six months of life of 0.4 µg/day (Section 2.3.6.2), using allometric scaling and reference body weights of 6.1 kg for an infant aged 3 months and 8.6 kg for an infant aged 9 months (WHO Multicentre Growth Reference Study Group, 2006), results in an estimated cobalamin intake of 0.5 µg/day.

$$AI_{infants\ 7–11\ months} = \text{cobalamin intake}_{infants\ 0–6\ months} \times (\text{weight}_{infants\ 9\ months}/\text{weight}_{infants\ 3\ months})^{0.75}$$

Owing to uncertainties in estimating breast milk cobalamin concentration (Section 2.3.6.2) used for upwards extrapolation from the cobalamin intake in exclusively breast-fed infants aged 0–6 months, and considering that the approach of scaling down from adults used, as a basis, an intake consistent with biomarker data, the Panel decides to set an AI for infants aged 7–11 months at 1.5 µg/day, after rounding to the closest 0.5.

For children, the Panel also considers that there are insufficient data to derive an AR (Section 5.1.2). Therefore, the Panel decides to set AIs for children by extrapolation from the AI for adults based on allometric scaling and application of a growth factor.

There are no data indicating that sex should be considered for cobalamin requirement for children. As a consequence, the same values are given for boys and girls.

For children, rounded mean values of the median weight-for-age of boys and girls aged 24 months, (according to the WHO Growth Standard (WHO Multicentre Growth Reference Study Group, 2006)), and aged 5, 8.5, 12.5 and 16 years (according to (van Buuren et al., 2012)) were used. The following growth factors were applied: 0.25 for boys and girls aged 1–3 years, 0.06 for boys and girls aged 4–6 years, 0.13 for boys and girls aged 7–10 years, 0.11 for boys and 0.08 for girls aged 11–14 years and 0.08 for boys and 0.03 for girls aged 15–17 years. These growth factors were calculated as the proportional increase in protein requirement for growth relative to the maintenance requirement at the different ages (EFSA NDA Panel, 2012). The value for each age group corresponds to the mean of values for the years included (see Appendix G of EFSA NDA Panel (2014a)).

Table 6: Reference body weights and Adequate Intakes of cobalamin for infants from seven months and children

Age	Reference body weight [a] (kg)	AI (µg/day) [b]
7–11 months	8.6	1.5
1–3 years	11.9	1.5
4–6 years	19.0	1.5
7–10 years	28.7	2.5
11–14 years	44.6	3.5
15–17 years	60.3	4

(a): Rounded mean of median weight-for-age of boys and girls aged 24 months, according to the WHO Growth Standard (WHO Multicentre Growth Reference Study Group, 2006), and aged 5, 8.5, 12.5 and 16 years, according to van Buuren et al. (van Buuren et al., 2012).

(b): Adequate Intakes were derived from the unrounded AIs for adults after adjustment on the basis of differences in reference body weight, then rounded to the closest 0.5.

The Panel notes that the calculated values from the scaling approach are within the range of observed intakes in infants and children in the EU (Appendices B and C).

6.3. Pregnancy

Some studies have investigated cobalamin status of infants at birth in relation to maternal cobalamin status during pregnancy. Lower maternal cobalamin or holoTC concentrations were associated with lower cobalamin and higher MMA and tHcy concentrations in infants at birth in two studies (Bjorke Monsen et al., 2001; Hay et al., 2010) that did not report on cobalamin intakes, but point to the importance of maintaining cobalamin status in pregnancy (Section 5.1.3).

As no new data on the cobalamin requirement during pregnancy could be retrieved, in this opinion estimations of the extra requirement for pregnancy rely on data indicating a fetal cobalamin deposition of 0.1–0.2 µg/day (Section 5.1.3).

Based on these data, the Panel proposes a cobalamin intake of 0.5 µg/day in addition to the AI for non-pregnant women in consideration of a fetal accumulation of 0.2 µg cobalamin/day and of an

absorption of 40 % (Section 2.3.1). The Panel sets an AI for cobalamin for pregnant women of 4.5 µg/day.

6.4. Lactation

For lactating women, an additional intake of cobalamin is necessary to balance cobalamin losses with breast milk. There is a wide range of cobalamin concentrations reported in breast milk, partly because of the differences in the methods of analysis and partly because of differences in maternal cobalamin intake and status (Section 2.3.6.2). A mean breast milk concentration of cobalamin of 0.38 nmol/L (0.51 µg/L) was observed in 10 studies in unsupplemented women from Western countries using analytical methods with uncertain accuracy. Recent data based on a more accurate analytical method report a mean/median concentration of 0.57 nmol/L (0.8 µg/L) in a group of Californian women and 0.29–0.76 nmol/L (0.4–1.0 µg/L), depending on the lactation stage, in a group of Danish women.

The Panel notes the limitations and uncertainties inherent in the available data (Section 2.3.6.2). The Panel decides to assume a cobalamin concentration of 0.5 µg/L as representative of the mean cobalamin concentration of breast milk in healthy women and a mean milk transfer of 0.8 L/day (Butte et al., 2002; FAO/WHO/UNU, 2004; EFSA NDA Panel, 2009) during the first six months of lactation in exclusively breastfeeding women. From this, an average quantity of 0.4 µg/day of cobalamin is estimated to be secreted with breast milk. Taking into account an absorption of 40 % (Section 2.3.1), a mean cobalamin intake of 1.0 µg/day is required to balance cobalamin secretion in milk for exclusively breastfeeding women during the first six months of lactation, in addition to the AI for non-lactating women. The Panel sets an AI for cobalamin for lactating women of 5 µg/day.

CONCLUSIONS

The Panel concludes that there is insufficient evidence to derive an AR and a PRI for cobalamin.

The Panel also concludes that there is consistent evidence from observational and intervention studies that a cobalamin intake of 4 µg/day and greater is associated with serum concentrations of holoTC and cobalamin within the reference ranges derived from healthy subjects, together with MMA and tHcy concentrations below cut-off values in adults, proposed to indicate an adequate cobalamin status. Based on these data on different biomarkers of cobalamin status and in consideration of observed cobalamin intakes, which range between 4.2 and 8.6 µg/day in adults, in several EU countries, the Panel sets an AI of 4 µg/day for adults. The estimated amount of cobalamin deposited in the fetus over the course of pregnancy was used as a basis to increase the AI for pregnant women. For lactating women, an increase in the AI was estimated based on the estimated amount of cobalamin secreted in breast milk. In infants over six months of age and children, AIs were proposed based on extrapolation from the AI for adults, using allometric scaling, reference body weights, and applying a growth factor.

Table 7: Summary of Dietary Reference Values for cobalamin

Age	AI (µg/day)
7–11 months	1.5
1–3 years	1.5
4–6 years	1.5
7–10 years	2.5
11–14 years	3.5
15–17 years	4
≥ 18 years	4
Pregnancy	4.5
Lactation	5

RECOMMENDATIONS FOR RESEARCH

The Panel recommends:

- to pursue studies on cobalamin biomarkers as a function of habitual intake in infants, children and adults, including during pregnancy and lactation;

- to further investigate the relationships between cobalamin intake, cobalamin biomarkers and health outcomes;

- to further characterise the bioavailability of cobalamin from various foods and dietary intake pattern in relation to age and physiological states (e.g. pregnancy, lactation).

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APPENDICES

Appendix A.   Dietary surveys in the EFSA Comprehensive European Food Consumption Database included in the nutrient intake calculation

Country	Dietary survey (year)	Year	Method	Days	Number of subjects [a]						
					Infants < 1 year	Children 1–< 3 years	Children 3–< 10 years	Children 10–< 18 years	Adults 18–< 65 years	Adults 65–< 75 years	Adults ≥ 75 years
Finland/1	DIPP	2000–2010	Dietary record	3	499	500	750				
Finland/2	NWSSP	2007–2008	48-hour dietary recall [b]	2 × 2 [b]				306			
Finland/3	FINDIET2012	2012	48-hour dietary recall [b]	2 [b]					1 295	413	
France	INCA2	2006–2007	Dietary record	7			482	973	2 276	264	84
Germany/1	EsKiMo	2006	Dietary record	3			835	393			
Germany/2	VELS	2001–2002	Dietary record	6	159	347	299				
Ireland	NANS	2008–2010	Dietary record	4					1 274	149	77
Italy	INRAN-SCAI	2005–2006	Dietary record	3	16 [a]	36 [a]	193	247	2 313	290	228
Latvia	FC_PREGNANTWOMEN	2011	24-hour dietary recall	2				12 [a]	991 [c]		
Netherlands	DNFCS	2007–2010	24-hour dietary recall	2			447	1 142	2 057	173	
Sweden	Riksmaten	2010–2011	Dietary record (web)	4					1 430	295	72
United Kingdom/1	DNSIYC	2011	Dietary record	4	1 369	1 314					
United Kingdom/2	NDNS RollingProgrammeYears1–3	2008–2011	Dietary record	4		185	651	666	1 266	166	139

DIPP, type 1 Diabetes Prediction and Prevention survey; DNFCS, Dutch National Food Consumption Survey; DNSIYC, Diet and nutrition survey of infants and young children; EsKiMo, Ernährungsstudie als KIGGS-Modul; FC_PREGNANTWOMEN, food consumption of pregnant women in Latvia; FINDIET, the national dietary survey of Finland; INCA, étude Individuelle Nationale de Consommations Alimentaires; INRAN-SCAI, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione – Studio sui Consumi Alimentari in Italia; NANS, National Adult Nutrition Survey; NDNS, National Diet and Nutrition Survey; NWSSP, Nutrition and Wellbeing of Secondary School Pupils; VELS, Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern für die Abschätzung eines akuten Toxizitätsrisikos durch Rückstände von Pflanzenschutzmitteln.

(a): 5th or 95th percentile intakes calculated from fewer than 60 subjects require cautious interpretation as the results may not be statistically robust (EFSA, 2011b) and, therefore, for these dietary surveys/age classes, the 5th and 95th percentile estimates will not be presented in the intake results.

(b): A 48-hour dietary recall comprises two consecutive days.

(c): One subject was excluded from the dataset due to the fact that only one 24-hour dietary recall day was available, i.e. final n = 990.

Appendix B.   Cobalamin intake in males in different surveys according to age classes and country (µg/day)

Age class	Country	Survey	n	Average	P5	P50	P95
< 1 year [a]	Finland	DIPP_2001_2009	247	1.0	0.0	1.0	2.2
	Germany	VELS	84	1.1	0.3	0.9	2.7
	Italy	INRAN_SCAI_2005_06	9	0.8	[b]	0.5	[b]
	United Kingdom	DNSIYC_2011	699	2.1	0.5	2.1	3.9
1 to < 3 years	Finland	DIPP_2001_2009	245	2.7	0.5	2.6	5.0
	Germany	VELS	174	2.4	0.9	2.4	4.0
	Italy	INRAN_SCAI_2005_06	20	3.2	[b]	3.3	[b]
	United Kingdom	DNSIYC_2011	663	3.5	1.1	3.5	6.2
	United Kingdom	NDNS-RollingProgrammeYears1–3	107	4.0	1.6	3.9	7.4
3 to < 10 years	Finland	DIPP_2001_2009	381	4.8	2.1	4.4	8.2
	France	INCA2	239	4.8	2.3	4.3	9.3
	Germany	EsKiMo	426	4.2	2.0	3.8	7.3
	Germany	VELS	146	2.8	1.5	2.8	4.6
	Italy	INRAN_SCAI_2005_06	94	5.7	2.3	5.1	11.3
	Netherlands	DNFCS 2007–2010	231	3.6	1.7	3.3	6.8
	United Kingdom	NDNS-RollingProgrammeYears1–3	326	3.7	1.8	3.3	6.3
10 to < 18 years	Finland	NWSSP07_08	136	6.2	3.1	5.5	11.7
	France	INCA2	449	6.5	2.9	5.8	12.8
	Germany	EsKiMo	197	4.6	2.2	4.2	8.2
	Italy	INRAN_SCAI_2005_06	108	6.6	3.6	6.3	11.8
	Netherlands	DNFCS 2007–2010	566	4.6	1.9	4.3	8.5
	United Kingdom	NDNS-RollingProgrammeYears1–3	340	4.2	2.0	3.9	7.6
18 to < 65 years	Finland	FINDIET2012	585	6.8	2.3	5.9	13.6
	France	INCA2	936	6.8	2.9	6.0	14.0
	Ireland	NANS_2012	634	6.4	3.0	5.9	11.5
	Italy	INRAN_SCAI_2005_06	1 068	6.2	2.7	5.6	10.7
	Netherlands	DNFCS 2007–2010	1 023	5.8	2.3	5.2	10.8
	Sweden	Riksmaten 2010	623	8.2	3.0	7.4	15.8
	United Kingdom	NDNS-RollingProgrammeYears1–3	560	5.3	2.2	4.6	9.7
65 to < 75 years	Finland	FINDIET2012	210	6.1	1.9	5.1	12.3
	France	INCA2	111	6.3	2.8	5.7	13.3
	Ireland	NANS_2012	72	6.9	2.4	6.1	14.2
	Italy	INRAN_SCAI_2005_06	133	6.0	2.4	5.5	10.0
	Netherlands	DNFCS 2007–2010	91	5.6	2.0	4.9	14.8
	Sweden	Riksmaten 2010	127	8.6	3.3	7.9	15.2

Age class	Country	Survey	n	Average	P5	P50	P95
≥ 75 years	United Kingdom	NDNS-RollingProgrammeYears1–3	75	6.6	2.6	5.5	15.1
	France	INCA2	40	5.4	(b)	4.7	(b)
	Ireland	NANS_2012	34	5.9	(a)	5.4	(b)
	Italy	INRAN_SCAI_2005_06	69	5.5	2.6	5.1	9.1
	Sweden	Riksmaten 2010	42	8.6	(b)	7.5	(b)
	United Kingdom	NDNS-RollingProgrammeYears1–3	56	6.4	(b)	5.1	(b)

n, number of individuals; P5, 5th percentile; P50, 50th percentile; P95, 95th percentile.

DIPP, type 1 Diabetes Prediction and Prevention survey; DNFCS, Dutch National Food Consumption Survey; DNSIYC, Diet and nutrition survey of infants and young children; EsKiMo, Ernährungsstudie als KIGGS-Modul; FINDIET, the national dietary survey of Finland; INCA, étude Individuelle Nationale de Consommations Alimentaires; INRAN-SCAI, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione – Studio sui Consumi Alimentari in Italia; NANS, National Adult Nutrition Survey; NDNS, National Diet and Nutrition Survey; NWSSP, Nutrition and Wellbeing of Secondary School Pupils; VELS, Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern für die Abschätzung eines akuten Toxizitätsrisikos durch Rückstände von Pflanzenschutzmitteln.

(a): The proportions of breast-fed infants were 58 % in the Finnish survey, 40 % in the German survey, 44 % in the Italian survey and 21 % in the UK survey. Most infants were partially breast-fed. For the Italian and German surveys, breast milk intake estimates were derived from the number of breastfeeding events recorded per day multiplied by standard breast milk amounts consumed on an eating occasion at different ages. For the UK survey, the amount of breast milk consumed was either directly quantified by the mother (expressed breast milk) or extrapolated from the duration of each breastfeeding event. As no information on the breastfeeding events were reported in the Finnish survey, breast milk intake was not taken into consideration in the intake estimates of Finnish infants.

(b): 5th or 95th percentile intakes calculated from fewer than 60 subjects require cautious interpretation as the results may not be statistically robust (EFSA, 2011b) and, therefore, for these dietary surveys/age classes, the 5th and 95th percentile estimates will not be presented in the intake results.

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Appendix C. Cobalamin intake in females in different surveys according to age classes and country (μg/day)

Age class	Country	Survey	n	Average	P5	P50	P95
< 1 year [a]	Finland	DIPP_2001_2009	253	0.9	0.0	0.8	2.2
	Germany	VELS	75	0.8	0.2	0.7	1.7
	Italy	INRAN_SCAI_2005_06	7	1.3	[b]	0.6	[b]
	United Kingdom	DNSIYC_2011	670	1.9	0.4	1.9	3.5
1 to < 3 years	Finland	DIPP_2001_2009	255	2.7	0.5	2.5	4.7
	Germany	VELS	174	2.2	0.9	2.1	3.9
	Italy	INRAN_SCAI_2005_06	16	2.9	[b]	2.7	[b]
	United Kingdom	DNSIYC_2011	651	3.3	0.9	3.2	5.8
	United Kingdom	NDNS-RollingProgrammeYears1−3	78	3.3	1.5	3.2	5.5
3 to < 10 years	Finland	DIPP_2001_2009	369	4.4	2.1	4.0	8.3
	France	INCA2	243	4.2	2.2	4.0	7.1
	Germany	EsKiMo	409	3.6	1.7	3.4	6.6
	Germany	VELS	147	2.6	1.3	2.5	4.0
	Italy	INRAN_SCAI_2005_06	99	4.8	2.0	4.7	7.7
	Netherlands	DNFCS 2007−2010	216	3.7	1.3	3.4	6.8
	United Kingdom	NDNS-RollingProgrammeYears1−3	325	3.4	1.5	3.2	5.8
10 to < 18 years	Finland	NWSSP07_08	170	4.4	1.7	4.1	7.6
	France	INCA2	524	4.8	2.2	4.3	8.9
	Germany	EsKiMo	196	4.0	2.1	3.7	6.9
	Italy	INRAN_SCAI_2005_06	139	5.8	2.4	5.1	9.9
	Latvia [c]	FC_PREGNANTWOMEN_2011	12	4.9	[b]	4.4	[b]
	Netherlands	DNFCS 2007−2010	576	3.7	1.6	3.4	6.8
	United Kingdom	NDNS-RollingProgrammeYears1−3	326	3.3	1.5	3.1	5.9
18 to < 65 years	Finland	FINDIET2012	710	4.9	1.8	4.4	9.7
	France	INCA2	1 340	5.2	2.0	4.5	11.6
	Ireland	NANS_2012	640	4.4	1.9	4.1	7.9
	Italy	INRAN_SCAI_2005_06	1 245	5.1	2.1	4.7	9.2
	Latvia [c]	FC_PREGNANTWOMEN_2011	990	5.2	2.3	4.2	11.0
	Netherlands	DNFCS 2007−2010	1 034	4.4	1.7	3.9	8.6
	Sweden	Riksmaten 2010	807	6.1	2.1	5.7	11.5
	United Kingdom	NDNS-RollingProgrammeYears1−3	706	4.3	1.5	3.8	8.9
65 to < 75 years	Finland	FINDIET2012	203	4.6	1.6	3.9	9.3
	France	INCA2	153	5.0	1.9	4.3	12.7
	Ireland	NANS_2012	77	5.2	2.4	4.7	9.6

Age class	Country	Survey	n	Average	P5	P50	P95
	Italy	INRAN_SCAI_2005_06	157	4.6	1.5	4.3	8.8
	Netherlands	DNFCS 2007–2010	82	4.3	1.5	3.5	8.7
	Sweden	Riksmaten 2010	168	7.3	3.1	6.2	16.8
	United Kingdom	NDNS-RollingProgrammeYears1–3	91	5.2	1.9	4.1	15.4
≥ 75 years	France	INCA2	44	5.5	(b)	4.3	(b)
	Ireland	NANS_2012	43	4.9	(b)	4.9	(b)
	Italy	INRAN_SCAI_2005_06	159	4.2	1.6	3.9	7.6
	Sweden	Riksmaten 2010	30	6.6	(b)	6.5	(b)
	United Kingdom	NDNS-RollingProgrammeYears1–3	83	5.0	2.4	4.4	9.2

n, number of individuals; P5, 5th percentile; P50, 50th percentile; P95, 95th percentile.

DIPP, type 1 Diabetes Prediction and Prevention survey; DNFCS, Dutch National Food Consumption Survey; DNSIYC, Diet and nutrition survey of infants and young children; EsKiMo, Ernährungsstudie als KIGGS-Modul; FC_PREGNANTWOMEN, food consumption of pregnant women in Latvia; FINDIET, the national dietary survey of Finland; INCA, étude Individuelle Nationale de Consommations Alimentaires; INRAN-SCAI, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione - Studio sui Consumi Alimentari in Italia; NANS, National Adult Nutrition Survey; NDNS, National Diet and Nutrition Survey; NWSSP, Nutrition and Wellbeing of Secondary School Pupils; VELS, Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern für die Abschätzung eines akuten Toxizitätsrisikos durch Rückstände von Pflanzenschutzmitteln.

(a): The proportions of breast-fed infants were 58 % in the Finnish survey, 40 % in the German survey, 44 % in the Italian survey and 21 % in the UK survey. Most infants were partially breast-fed. For the Italian and German surveys, breast milk intake estimates were derived from the number of breastfeeding events recorded per day multiplied by standard breast milk amounts consumed on an eating occasion at different ages. For the UK survey, the amount of breast milk consumed was either directly quantified by the mother (expressed breast milk) or extrapolated from the duration of each breastfeeding event. As no information on the breastfeeding events were reported in the Finnish survey, breast milk intake was not taken into consideration in the intake estimates of Finnish infants.

(b): 5th or 95th percentile intakes calculated from fewer than 60 subjects require cautious interpretation, as the results may not be statistically robust (EFSA, 2011b) and, therefore, for these dietary surveys/age classes, the 5th and 95th percentile estimates are be presented in the intake results.

(c): Pregnant women only.

Appendix D.   Minimum and maximum percentage contribution of different food groups to cobalamin intake in males

Food groups	Age						
	1 month to < 1 year	**1 to < 3 years**	**3 to < 10 years**	**10 to < 18 years**	**18 to < 65 years**	**65 to < 75 years**	**≥ 75 years**
Additives, flavours, baking and processing aids	< 1	< 1	0	0	0	0	0
Alcoholic beverages	0	0	0	0	< 1–3	0–3	0–1
Animal and vegetable fats and oils	0–1	< 1–1	< 1–2	< 1–2	< 1–2	< 1–2	< 1–1
Coffee, cocoa, tea and infusions	0	0	< 1–1	< 1–1	< 1–2	< 1–1	0–2
Composite dishes	< 1–3	< 1–9	< 1–13	< 1–19	< 1–16	< 1–11	1–10
Eggs and egg products	< 1–1	1–5	< 1–8	< 1–8	< 1–5	< 1–5	< 1–5
Fish, seafood, amphibians, reptiles and invertebrates	1–5	4–11	4–17	5–20	10–25	19–38	19–39
Food products for young population	21–53	2–13	< 1–1	< 1	< 1	–	–
Fruit and fruit products	0	0	0	0	0	0	0
Fruit and vegetable juices and nectars	0–2	0–2	< 1–3	0–2	0–1	0–1	0–1
Grains and grain-based products	< 1–4	3–8	1–11	< 1–11	3–10	3–11	3–13
Human milk	< 1–21	< 1	–	–	–	–	–
Legumes, nuts, oilseeds and spices	0	0	0	< 1	< 1	0	0
Meat and meat products	2–21	8–26	16–40	24–43	29–48	27–52	26–48
Milk and dairy products	23–50	48–65	25–51	17–48	14–35	15–29	16–27
Products for non-standard diets, food imitates and food supplements or fortifying agents	0–1	0	0–1	0	< 1	0	0
Seasoning, sauces and condiments	< 1–2	< 1	< 1–1	< 1–1	< 1–1	< 1–1	< 1–2
Starchy roots or tubers and products thereof, sugar plants	0	0	0	0	0	0	0
Sugar, confectionery and water-based sweet desserts	0	< 1–2	< 1–3	< 1–3	< 1–1	< 1	< 1
Vegetables and vegetable products	0	< 1	< 1	< 1	< 1	< 1	< 1
Water and water-based beverages	0	0	0–12	0–19	< 1–12	0–1	0

"–" means that there was no consumption event of the food group for the age and sex group considered, while "0" means that there were some consumption events, but that the food group does not contribute to the intake of the nutrient considered, for the age and sex group considered.

Appendix E.   Minimum and maximum percentage contribution of different food groups (FoodEx2 level 1) to cobalamin intake in females

Food groups	Age 1 month to < 1 year	1 to < 3 years	3 to < 10 years	10 to < 18 years	18 to < 65 years	65 to < 75 years	≥ 75 years
Additives, flavours, baking and processing aids	0	0	0	0	0	0	0
Alcoholic beverages	0	0	0	0	< 1–1	0	0
Animal and vegetable fats and oils	< 1–1	< 1–1	< 1–2	< 1–2	< 1–2	< 1–1	< 1–2
Coffee, cocoa, tea and infusions	0	0	< 1–1	0–1	< 1–2	< 1–1	0–1
Composite dishes	0–3	< 1–8	< 1–14	1–19	1–16	< 1–11	1–10
Eggs and egg products	< 1–1	1–5	< 1–8	< 1–8	< 1–5	< 1–5	< 1–6
Fish, seafood, amphibians, reptiles and invertebrates	2–10	5–14	2–17	5–25	13–27	17–39	17–35
Food products for young population	14–51	2–11	< 1–1	< 1	0	–	< 1
Fruit and fruit products	0	0	0	0	0	0	0
Fruit and vegetable juices and nectars	0–2	0–3	< 1–3	0–2	0–1	0–1	0–1
Grains and grain-based products	0–5	2–9	1–11	< 1–11	3–12	3–12	4–13
Human milk	< 1–7	< 1	–	–	–	–	–
Legumes, nuts, oilseeds and spices	0	0	0	0	< 1	< 1	< 1
Meat and meat products	8–16	8–24	15–40	20–44	27–46	26–51	24–53
Milk and dairy products	20–51	48–62	26–54	19–50	16–36	17–33	18–32
Products for non-standard diets, food imitates and food supplements or fortifying agents	0	0	0–1	0–1	< 1–1	0–1	0–1
Seasoning, sauces and condiments	< 1–1	< 1–1	< 1–1	< 1–2	< 1–2	< 1–1	< 1–1
Starchy roots or tubers and products thereof, sugar plants	0	0	0	0	0	0	0
Sugar, confectionery and water-based sweet desserts	0–1	< 1–1	< 1–3	< 1–3	< 1–1	< 1	< 1–1
Vegetables and vegetable products	0	0	< 1	< 1	< 1	< 1	< 1
Water and water-based beverages	0	0	0–10	0–13	0–9	0	0

"–"   means that there was no consumption event of the food group for the age and sex group considered, while "0" means that there were some consumption events, but that the food group does not contribute to the intake of the nutrient considered, for the age and sex group considered.

Appendix F.   Comparison between EFSA intake estimates and published estimates from the same surveys

Country	Survey (age range)	Reference	% of published intake estimates [a]
Finland	DIPP (6 months–6 years)	Kyttälä et al. (2008)	91–102 %
	NWSSP (13–15 years)	Hoppu et al. (2010)	113–117 %
	FINDIET 2012 (25–74 years)	Helldán et al. (2013)	89–97 %
France	INCA 2 (3–17 years)	Afssa (2009)	132–138 %
	INCA 2 (≥ 18 years)		102–104 %
Germany	EsKiMo (6–11 years)	Mensink et al. (2007)	103–110 %
Ireland	NANS (18–90 years)	IUNA (2011)	98–104 %
Italy	INRAN-SCAI (1 month–98 years)	Sette et al. (2011)	90–100 %
The Netherlands	DNFCS 2007_2010 (7–69 years)	van Rossum et al. (2011)	100–113 %
Sweden	Riksmaten (18–80 years)	Amcoff et al. (2012)	112–139 %
The United Kingdom	NDNS, Years 1–3 (3–94 years)	Bates et al. (2012)	87–95 %

DIPP, type 1 Diabetes Prediction and Prevention survey; DNFCS, Dutch National Food Consumption Survey; EsKiMo, Ernährungsstudie als KIGGS-Modul; FINDIET, the national dietary survey of Finland; INCA, étude Individuelle Nationale de Consommations Alimentaires; INRAN-SCAI, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione – Studio sui Consumi Alimentari in Italia; NANS, National Adult Nutrition Survey; NDNS, National Diet and Nutrition Survey; NWSSP, Nutrition and Wellbeing of Secondary School Pupils.

(a): Range over different age groups in a specific survey.

ABBREVIATIONS

AdoCbl	5′-deoxyadenosylcobalamin
Afssa	Agence française de sécurité sanitaire des aliments
AI	Adequate Intake
ANOVA	Analysis of variance
AR	Average Requirement
CI	Confidence interval
CNCbl	Cyanocobalamin
CoA	Coenzyme A
cobalamin$_n$	Normalised cobalamin concentration
COMA	Committee on Medical Aspects of Food Policy
CV	Coefficient of variation
Da	Dalton
D-A-CH	Deutschland–Austria–Confoederatio Helvetica
DH	UK Department of Health
DIPP	Type 1 Diabetes Prediction and Prevention survey
DMG	Dimethylglycine
DNA	Deoxyribonucleic acid
DNFCS	Dutch National Food Consumption Survey
DNSIYC	Diet and nutrition survey of infants and young children
DRV	Dietary Reference Values
EAR	Estimated Average Requirement
EC	European Commission
EsKiMo	Ernährungsstudie als KIGGS-Modul
EU	European Union
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization
FC_PREGNANTWOMEN	Food consumption of pregnant women in Latvia
FFQ	Food frequency questionnaire
FINDIET	National dietary survey of Finland
HELENA	Healthy Lifestyle in Europe by Nutrition and Adolescence study
holoTC	Holotranscobalamin
holoTC$_n$	Normalised holotranscobalamin concentration
I^2	Heterogeneity index
IF	Intrinsic factor
INCA	Etude Individuelle Nationale de Consommations Alimentaires

INRAN-SCAI	Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione – Studio sui Consumi Alimentari in Italia
IOM	US Institute of Medicine of the National Academy of Sciences
LOD	Limit of detection
LRNI	Lower Reference Nutrient Intake
LTI	Lowest Threshold Intake
MCV	Mean corpuscular volume
MeCbl	Methylcobalamin
5-methyl-THF	5-methyl-tetrahydrofolate
MMA	Methylmalonic acid
MMA_n	Normalised methylmalonic acid concentration
mRNA	Messenger ribonucleic acid
n	Sample size
NANS	National Adult Nutrition Survey
NDNS	National Diet and Nutrition Survey
NHANES	National Health and Nutrition Examination Survey
NNR	Nordic Nutrition Recommendations
NWSSP	Nutrition and Wellbeing of Secondary School Pupils
OHCbl	Hydroxocobalamin
PLP	Pyridoxal 5′-phosphate
PRI	Population Reference Intake
Q1	1st quintile
Q5	5th quintile
r	Correlation coefficient
RCT	Randomised controlled trial
RDA	Recommended Dietary Allowance
RNI	Reference Nutrient Intake
ROC	Receiver operating characteristics
RR	Relative risk
SAM	S-adenosyl-methionine
SCF	Scientific Committee for Food
SD	Standard deviation
SEM	Standard error of the mean
TCblR	Transcobalamin receptor
TC	Transcobalamin
THF	Tetrahydrofolate
tHcy	Total homocysteine

$tHcy_n$	Normalised total homocysteine concentration
TMP	Thymidine monophosphate
UK	United Kingdom
UL	Tolerable Upper Intake Level
UMP	Uridine monophosphate
USA	United States of America
VELS	Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern für die Abschätzung eines akuten Toxizitätsrisikos durch Rückstände von Pflanzenschutzmitteln
w	Wellness parameter
	$w = \log_{10}(holoTC_n \times cobalamin_n) - \log_{10}(MMA_n \times tHcy_n)$
WHO	World Health Organization