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Biological properties of vitamin B_{12}

Monika Moravcová[1], Tomáš Siatka[2], Lenka Kujovská Krčmová[3,4], Kateřina Matoušová[3], Přemysl Mladěnka[1]* and on behalf of the OEMONOM[+]

[1]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic*
[2]*Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic*
[3]*Department of Clinical Biochemistry and Diagnostics, University Hospital Hradec Králové, Hradec Králové, Czech Republic*
[4]*Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic*

Abstract

Vitamin B_{12}, cobalamin, is indispensable for humans owing to its participation in two biochemical reactions: the conversion of L-methylmalonyl coenzyme A to succinyl coenzyme A, and the formation of methionine by methylation of homocysteine. Eukaryotes, encompassing plants, fungi, animals and humans, do not synthesise vitamin B_{12}, in contrast to prokaryotes. Humans must consume it in their diet. The most important sources include meat, milk and dairy products, fish, shellfish and eggs. Due to this, vegetarians are at risk to develop a vitamin B_{12} deficiency and it is recommended that they consume fortified food. Vitamin B_{12} behaves differently to most vitamins of the B complex in several aspects, e.g. it is more stable, has a very specific mechanism of absorption and is stored in large amounts in the organism. This review summarises all its biological aspects (including its structure and natural sources as well as its stability in food, pharmacokinetics and physiological function) as well as causes, symptoms, diagnosis (with a summary of analytical methods for its measurement), prevention and treatment of its deficiency, and its pharmacological use and potential toxicity.

Keywords: **cobalamin: cobalt: megaloblastic anaemia: neuropathy: physiological function**

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1. Introduction

Vitamins of the B complex are water-soluble molecules with essential roles in humans. Our previous paper comprehensively summarised the biological properties of vitamins B_1, B_2, B_3 and B_5[(1)]. Although there is a recent practical paper on vitamin B_{12}[(2)], a comprehensive paper on vitamin B_{12} is missing.

Vitamin B_{12}, cobalamin, with a molecular weight of 1355.4 Da, is indispensable for humans as an integral part of two biochemical reactions: the conversion of L-methylmalonyl coenzyme A to succinyl coenzyme A, and the formation of methionine by methylation of homocysteine. Humans, as well as animals and plants, are unable to synthesise it. Therefore, we have to obtain vitamin B_{12} from food[(3–6)].

The compound was first identified as a nutrient or external factor in the 1920s thanks to the research efforts of Minot, Murphy and Whipple, who showed that the symptoms of pernicious anaemia can be overcome by adding liver to the diet. The structure of the compound was solved by Dorothy Hodgkin's pioneering X-ray crystallography, which revealed that the vitamin was cyanolated, amidated, tetrapyrrole-containing cobalt. As a result, it was called cyanocobalamin[(6,7)].

The structure of vitamin B_{12} is shown in Figure 1. The cobalt is located in the middle of a circular contraction of a modified tetrapyrrole macrocycle coordinated by four nitrogen atoms. This centre of the molecule is known as the corrin ring and is similar to, although quite different from, the tetrapyrrole-derived ring systems found in haem and chlorophylls. The lower nucleotide loop is bound to the corrin ring by a side chain attached to a macrocyclic ring that contains an unusual natural base 5,6-dimethylbenzimidazole, which also coordinates the cobalt ion. Thus, in cyanocobalamin, the cobalt ion is ligated not only by the four pyrrole nitrogens of the central ring, but also by the upper (β) and lower (α) ligands. The β-ligand in the molecule of B_{12} is a cyano group, while the α is nitrogen from the mentioned dimethylbenzimidazole. In biological systems, the upper cyano ligand is usually replaced by an adenosyl group to form adenosylcobalamin (more precisely, 5′-deoxyadenosylcobalamin, AdoCbl), or a methyl group to form methylcobalamin, or a hydroxyl group to form hydroxocobalamin. Similarly, some species use a different lower base where dimethylbenzimidazole is replaced by bases such as adenine, substituted

Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin (adenosylcobalamin); CoA, coenzyme A; EFSA, European Food Safety Authority; IF, intrinsic factor; holo TC, serum holotranscobalamin; MMA, methylmalonic acid; TC, transcobalamin; TCA, tricarboxylic acid; tHcy, total serum homocysteine.

* **Corresponding author:** Přemysl Mladěnka, email: mladenkap@faf.cuni.cz
[+] https://portal.faf.cuni.cz/OEMONOM/EN/Home/

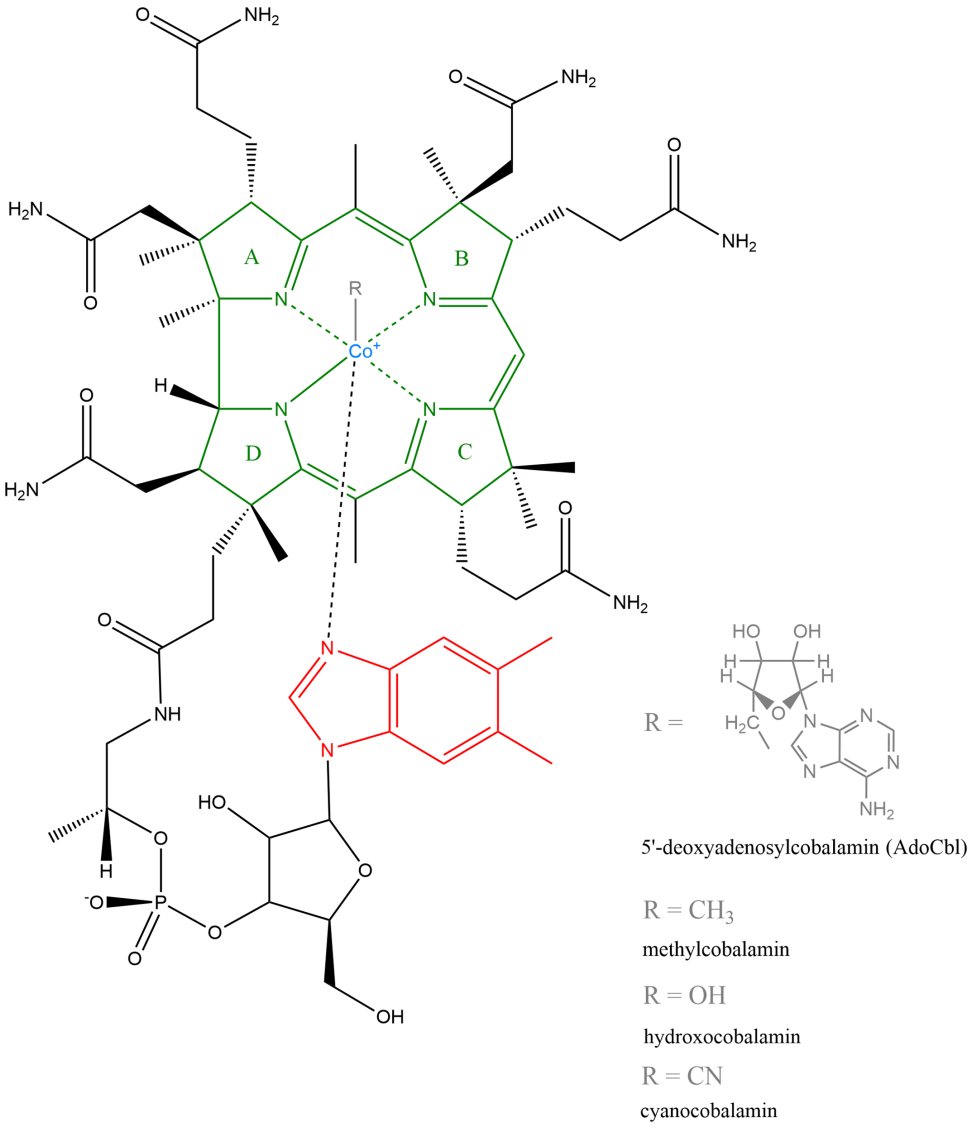

Fig. 1. Structure of vitamin B_{12}: Natural forms include 5′-deoxyadenosylcobalamin (AdoCbl), methylcobalamin and hydroxocobalamin, industrially produced is cyanocobalamin[7]. Structure was created by ChemDraw, version 20.0.

benzimidazoles, which include hydroxy- or methoxybenzimidazole, or phenolic compounds, including phenol or cresol[6,7].

2. Sources of vitamin B_{12}

Eukaryotes, encompassing plants, algae, protists, fungi, animals and humans, do not synthesise vitamin B_{12}[6–22]. Vitamin B_{12} is biosynthesised exclusively in prokaryotes. However, only about a third of all bacteria and archaea species are able to synthesise it[8,9,23–38]. These species provide it for other cobalamin non-producing bacteria and archaea[30,39–51] as well as for eukaryotes, except plants and fungi which do not use vitamin B_{12} as an enzyme cofactor[6,7,17,18,30,52–58].

Vitamin B_{12} is also produced by microbiota in the large intestine of humans, but it is not utilisable by the human body as it is not spatially bioavailable since the intrinsic factor-mediated absorption of cobalamin occurs in the upper part of the gastrointestinal tract (i.e. upstream of the location of its synthesis) as will be described in the following text related to pharmacokinetics[6,59–69]. Moreover, a major proportion of the microbially produced vitamin B_{12} is utilised by other non-vitamin-producing microbes, further limiting its possible availability for the human host[60,69–76]. The latest observational study in adult humans reveals a slight colonic absorption of cobalamin (approximately 7%) and speculates about the potential involvement of the colonic microbiome in the body's vitamin B_{12} homoeostasis[77]. However, the mechanism of vitamin B_{12} uptake from the colon and the overall contribution of the 'endogenously produced' colonic vitamin B_{12} to the maintenance of vitamin B_{12} status remain unknown[78]. Based on the current knowledge, it seems that humans are mostly dependent on vitamin B_{12} from exogenous sources.

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In animals, the vitamin B_{12} from prokaryotes is obtained and stored in tissues through microbial interactions in the natural food chain. A good example are ruminants, such as cattle and sheep. They are herbivores, i.e. they feed on pasture that does not contain cobalamin, but they obtain the essential vitamin B_{12} through symbiotic relationship with microbes inside their body. They have a specialised digestive organ positioned upstream of the small intestine – a rumen that is heavily colonised with various micro-organisms, including cobalamin-synthesising ones, and that allows fermentation of the ingested feed. The synthesised cobalamin is later absorbed in the small intestine, incorporated into organs and muscles, and secreted into milk[49,52,60,73,79–81]. Monogastric, non-ruminant herbivores, e.g. rabbits, receive the benefit of microbial cobalamin production in their own large intestine by consuming their faeces. This coprophagy enables microbial cobalamin absorption in the upper part of the digestive tract[52,60,73,82]. Omnivores, such as pigs and poultry, as well as carnivores acquire cobalamin from feed of animal origin[80,81]. In aquatic environments, cobalamin is produced only by certain bacteria and archaea. It is taken up by cobalamin-requiring bacteria as well as eukaryotic plankton, transferred to fish bodies via plankton, and concentrated in the larger piscivorous fishes. Similarly, the vitamin concentrates in the bodies of shellfish feeding on plankton[80,83–85].

Accordingly, foods of animal origin are the principal sources of vitamin B_{12} for humans[63,80,86,87]. The most important ones include meat, milk and dairy products, fish, shellfish and eggs[64,68,83,84,88–136]. The highest levels of cobalamin are found in offal, especially the liver and kidney[64,68,70,93,111,137–141]. Shellfish, such as mussels, oysters, clams and shrimps, are rich in cobalamin. However, certain types of edible shellfish (herbivorous sea snails such as abalone and turban shell) are not suitable as a source of vitamin B_{12} because they contain substantial amounts of pseudocobalamin[80,85,142–147]. Pseudocobalamin (or pseudovitamin B_{12}) is an analogue of cobalamin, in which adenine substitutes for 5,6-dimethylbenzimidazole as the lower axial ligand. It is biologically very likely inactive in humans because the intrinsic factor is very specific for binding cobalamin. Prevention of the absorption of cobalamin analogues might protect humans from their potential deleterious effects[6,74,79,142,148–152]. Interestingly, edible insects, such as mealworms, grasshoppers and cockroaches, might also be a source of vitamin B_{12} for humans. Crickets, however, do not suit this purpose because they contain pseudocobalamin as the predominant corrinoid compound[153–156].

Fungi, as mentioned above, neither produce nor utilise vitamin B_{12}. Therefore, mushrooms and yeasts, e.g. black morels (*Morchella conica*), oyster mushrooms (*Pleurotus ostreatus*), parasol mushrooms (*Macrolepiota procera*), porcini mushrooms (*Boletus* sp.) and yeasts (*Yarrowia lipolytica*), generally contain none or very low amounts of this vitamin, which is presumably derived from the substrate on which they grow or from cobalamin-synthesising bacteria that live on the mushroom surface. Among edible mushrooms, the highest vitamin B_{12} contents were found in truffles (*Tuber* sp.), black trumpet (*Craterellus cornucopioides*), golden chanterelle (*Cantharellus cibarius*) and shiitake (*Lentinula edodes*)[14,80,85,157–167].

Algae, like other eukaryotic organisms, are not capable of synthesising vitamin B_{12} *de novo*. Over one half of all algal species require vitamin B_{12}. Those algae, as well as cobalamin-independent ones, which can, however, also accumulate exogenous cobalamin, acquire the vitamin from prokaryotic producers that are in symbiotic relationships with algae or reside on algal surfaces[11,12,53,56,80,83,158,168–179]. Changes in the character and magnitude of the epiphytic prokaryotic communities related to the region or algal physiological state (e.g. growing conditions and harvesting period) may contribute to variation in the vitamin content, but these factors are currently poorly quantified[180]. Contents of vitamin B_{12} vary interspecifically and intraspecifically in edible algae ranging from traces, e.g. in Irish moss (*Chondrus crispus*) and hijiki (*Sargassum fusiforme*), to substantial amounts, e.g. in green laver (*Ulva* spp.), purple laver or nori (*Porphyra/Pyropia* spp.) and *Chlorella* spp., especially the *Chlorella* grown non-aseptically under open culture conditions[80,83,144,150,158,172,176,181–193].

Plants, like fungi, neither synthesise nor use vitamin B_{12} in their metabolism[18,20]. A few exceptions of plants containing some cobalamin have been reported: edible duckweed *Wolffia globosa* called mankai[194,195], sea buckthorn (*Hippophae rhamnoides*), elecampane (*Inula helenium*), couch grass (*Elymus repens*)[21,61] and tea plant (*Camellia sinensis*)[196]. Cobalamin in those plants is, like in other eukaryotes, of prokaryotic origin and is presumably produced by symbiotic endophytic microbes[21,194,195] or taken up from soil containing some organic fertilisers such as fishmeal or manure[82,196,197]. Therefore, cobalamin is not a normal constituent of commonly eaten plant foods unless they are contaminated with cobalamin-producing microbes (e.g. from soil or manure), contain yeasts or have been exposed to microbial fermentation that have produced the vitamin, or have been fortified with cobalamin (e.g. fortified ready-to-eat breakfast cereals and bread)[6,21,81,82,84,88–90,104,137,152,198–202].

Thus, individuals who consume diets completely free of animal products (vegans) and even lacto-ovo vegetarians are at risk of vitamin B_{12} deficiency compared with omnivores. Indeed, different national nutritional societies quote a need to ensure a reliable source of vitamin B_{12} in persons on plant-based diets[63,66,81,102,104,108,163,203–244]. Some dietary ingredients or food supplements of non-animal origin may be useful for vegetarians to partly contribute to the supply of vitamin B_{12}. Microalgae *Chlorella* and *Spirulina* (*Arthrospira*) are the commercially most produced microalgal genera as dietary supplements [245,246]. In particular, the green alga *Chlorella* containing cobalamin is a relevant source of it. On the contrary, microalgae, e.g. *Spirulina*, *Aphanizomenon* and *Nostoc*, i.e. cyanobacteria, contain predominantly pseudocobalamin that they synthesise, and only minor amounts of cobalamin acquired from the environment; therefore, they are not suitable sources of vitamin B_{12}. It should also be emphasised that nutrient labels on products often do not differentiate between forms of vitamin B_{12} – i.e. they do not specify that pseudocobalamin is present there instead of cobalamin[13,23,24,84,85,102,142,163,171,172,180,182,184,189,191,192,247–253]. Macroalgae (seaweeds) green laver and purple laver, the most widely consumed edible algae, belong to the best non-animal sources of cobalamin; consumption of approximately 4 g of

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Table 1. Cobalamin contents in selected foodstuffs

Food	Cobalamin content (μg/100 g)	References
Oat, wheat, maize, rye, barley, millet, sorghum	0	(697)
Rice, both brown and white	0	(697)
Soybean, lentil	0	(697)
Macadamia nut, peanut, pistachio nut, hazelnut walnut almond	0	(697)
Potato, carrot, cabbage, tomato, garlic	0	(697)
Broccoli, cauliflower, spinach	0	(697)
Orange, strawberry, apple, pear	0	(697)
Avocado	0	(697)
White and brown bread	0	(697)
Pork	0.3–2	(116–118,126,138,521,698,699)
Beef	1.09–3.17	(111,116–118,138,378,395,398,400,521,698,699)
Chicken breast	0.23–0.40	(118,401,521,698,699)
Liver, beef	59.3–110	(138,697)
Liver, pork	24.8–59.7	(123,138,697)
Tuna	0.5–2.21	(698,700)
Sardines	8.31	(700)
Baker's yeasts	0.01	(697)
Oyster mushroom	0.05	(164)
Button mushroom	0.05–0.06	(164)
Milk	0.4–0.51	(120,133,521,701)
Yoghurt	0.2–0.25	(133,701)
Cheese, cheddar	1.06	(697)
Eggs	0.89–2.7	(521,702)

dried purple laver could supply the US recommended dietary allowance of 2.4 μg/d[80,85,115,150,158,181,182,184,185,187,188,193,254–260]. Shiitake can serve as a source of cobalamin. Nevertheless, although about 50 g of dried fruiting bodies could be adequate to achieve the daily cobalamin requirement, ingestion of such large amounts would not be feasible daily[14,80,158,259]. Sea buckthorn berries as well as mankai contain acceptable quantities of cobalamin; approximately 18 g of sea buckthorn jam (about 1.5 tablespoons), 6.5 g of sea buckthorn dried berries or circa 100 g of dried mankai (equivalent to 500 g of frozen mankai for making five cups of green shakes) may cover the daily US recommended amount of the vitamin specified above[21,195,261]. However, the cobalamin contents in all these alternative sources fluctuate greatly[14,21,61,80,158,160,163,172,189]. Moreover, safety hazards posed by some constituents of those dietary products should not be underestimated. Continuous intake of large amounts of those products might adversely affect human health owing to the presence of potentially harmful substances, whose content should be monitored during the quality control[262–267]. High consumption of algae is associated with higher risks of deleterious effects due to excessive intake of some minerals that algae naturally accumulate, e.g. iodine, cancerogenic arsenic and toxic heavy metals[150,180,193,218,228,249,262,263,265,268–284]. In addition, these products might be detrimental to human health because they may be contaminated with toxic cyanotoxins produced by some cyanobacteria[262,264–267,270,285] and with cancerogenic polycyclic aromatic hydrocarbons from the environment[265,266,280]. Similarly to algae, excessive ingestion of mushrooms may threaten human health as a consequence of exceeding dietary exposure limits of heavy metals, arsenic and radionuclides[286–299]. Consumption of *Wolffia globosa* (mankai) is of safety concern because it leads to an increase in manganese intake which could represent a risk of adverse health effects[300].

Fermented plant-based foods, such as kimchi, sauerkraut, injera, kombucha, tempeh and miso, are generally poor dietary sources of vitamin B_{12} for vegetarians mainly because there is simply not enough vitamin B_{12} produced by these fermentations[21,23,65,85,114,115,158,163,184,205,214,255,261,301–303]. Taking everything into account, all these alternative sources of cobalamin for vegetarians are unreliable, insufficient or impractical to meet cobalamin needs of the human body in the long term. They may improve vitamin B_{12} status in vegetarians but cannot replenish the total body store of the vitamin[85,158,184,192–195,205,304–309].

Plant-based diets, except those rich in ultra-processed plant-based food products[310–319], are considered potentially superior to a traditional omnivorous diet for reducing the risk of chronic diseases, such as metabolic syndrome with type 2 diabetes mellitus, hypertension, cardiovascular diseases in general, and several types of cancer[205,217,222,227,241,320–344]. Meanwhile, compared with omnivorous diets, plant-based ones are deficient in some nutrients, which could have detrimental health implications as well. Vitamin B_{12} is of particular relevance[204,222,227,231,241–244,305,345,346]. The proposed benefit of plant-based-diets is conditioned by the balance and incorporation of all missing constituents so that nutritional quality is not compromised. Regular intake of cobalamin-fortified foods (mostly ready-to-eat-cereals, bread, nutritional yeast, meat analogues, such as tofu, and milk substitutes, e.g. soy, almond and rice milk) and/or cobalamin-containing supplements is recommended for people on plant-based diets to prevent vitamin B_{12} deficiency[70,82,85,93,141,163,184,205,218–222,224–233,235,236,238,241–243,256,259,305–308,320–326,345–373]. The vitamin B_{12} status should also be monitored regularly[102,141,218,223,224,227,233,235,242,256,306,307,320,325,326,347,360–362,367,374].

Contents of cobalamin in some selected foodstuffs are presented in Table 1.

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The stability of vitamin B_{12} in food products during processing, preparation and storage is an important parameter affecting the supply of the vitamin to consumers[70,85,375–378]. Cyanocobalamin is chemically more stable than adenosylcobalamin, methylcobalamin and hydroxocobalamin. In neutral and weakly acid aqueous solutions, it is stable at room temperature with highest stability at pH 4.5–5 and it is relatively stable to the thermal processing. Loss of vitamin activity may occur due to heat treatments under alkaline and strong acid conditions or due to light or via contact with reducing agents, such as ascorbic acid, polyphenols, nitrous oxide, sulphite and iron(II) salts, but also with oxidising ones, such as atmospheric oxygen, hypochlorous acid and chloramine-T[14,21,68,70,124,142,160,198,375–377,379–393]. On the one hand, degradation of vitamin B_{12} caused by ascorbic acid might have little practical importance because foods containing vitamin B_{12} generally do not contain significant amounts of vitamin C[376], but on the other hand, it should be taken into account, e.g. in vitamin fortified beverages[388,390].

In food matrices, vitamin B_{12} is generally considered to be rather heat stable compared with other water-soluble vitamins. Reported losses depend on the type of food and processing conditions[68,70,134,378,394–406]. In milk, vitamin B_{12} is affected by heat processing; the more severe the process, the greater the loss is. Vitamin losses are generally less than 10% after pasteurisation, 10–20% after ultra-high temperature treatment, up to 20% following sterilisation and 20–35% during spray drying[84,375,396,407–411]. The vitamin B_{12} amount decreased three times more in heated chocolate milk (by about 33%) than in the unflavoured one because cocoa powder used for milk flavouring contains polyphenols, which are mainly responsible for the decomposition of the vitamin[383]. In meat, vitamin B_{12} is stable during the cooking, if the vitamin content in the cooking liquids, gravy and drippings is taken into consideration[375,403]. Indeed, most vitamin B_{12} losses result from leaching into water, e.g. during cooking or freezing/thawing[399,403,412–415]. This process is essentially similar to that of other water-soluble B vitamins[1]. Boiling, stewing and frying lead to vitamin B_{12} losses of 20–40%, 10–40%, 30–50% and 10–20% in pork, beef, chicken and fish, respectively[403]. The highest reduction in the vitamin content occurs during boiling[403–406]. The best method for cooking fish was vacuum-packed pouch cooking with no loss of vitamin B_{12}, compared with steaming, boiling, grilling, frying and microwaving[399]. Scrambled, fried and hard-cooked eggs lose during cooking 5%, 5–15% and 20% vitamin B_{12}, respectively[403,406].

Treatment of foods with penetrating waves such as microwaves was shown to promote the degradation of vitamin B_{12}[70,399,416–418]. For instance, decreases by 17%, 14% and 48% were estimated in beef, pork and milk, respectively, treated with microwave heating for 6 min, which is a common time used for reheating of foods[416].

Puffed rice extrudates could be used as a palatable vehicle for fortification with vitamin B_{12}; losses of added vitamin ranged from 19% to 64% depending on extrusion processing parameters[419]. The stability of the added and *in situ*-produced vitamin B_{12} in breadmaking varied according to the chosen process (straight-, sponge- and sourdough processes)[386,420]. Converning baking from fortified whole wheat, the vitamin amounts were reduced by 9%, 20%, 66% and 76% in chapattis (unleavened flatbread), bread, cake and cookie, respectively. The lowest vitamin B_{12} retention was recorded in pooris prepared from fortified whole wheat flour by frying; the vitamin loss was 86%[421].

Vitamin B_{12} is sensitive to light and ultraviolet (UV) radiation[70,84,375,376,381,382,384,422,423]. Sunlight at a brightness of 8000-foot candles (approximately 86 000 lux) caused a 10% loss of cyanocobalamin for each 30 min of exposure in neutral aqueous solutions, but exposures to levels of brightness below 300-foot candles (approximately 3200 lux) had little effect[375]. Photodegradation of cyanocobalamin in aqueous solutions may be accelerated by riboflavin, which acts as a sensitiser[376,422]. The vitamin B_{12} concentration in milk exposed to the light for 24 h decreased by 1–27%, depending on the type of milk tested[424], while no changes in the vitamin B_{12} content occurred in pasteurised milk packed in a clear polyethylene terephthalate bottle exposed to fluorescent light (1700 lux) for 10 d[425]. The photostability of vitamin B_{12} in foods may be increased due to matrix effects, such as binding to proteins[70,121,426,427]. Furthermore, light penetrates only slightly below the surface of foods, which would suggest that vitamin B_{12} photosensitivity is not a serious issue in most foods[70].

Vitamin B_{12} content decreases in fermented milk products[397,410]. Fermentation of milk resulted in vitamin B_{12} losses of 25% in yoghurt and 15% in Filmjölk. Storage of an unopened package of the final product at 4°C for 14 d, until the 'use by date', reduced the vitamin concentrations further by 33% and 26% for yoghurt and Filmjölk, respectively, so that they contained 40–60% of vitamin B_{12} originally present in the milk. This is most likely attributed to the consumption of the vitamin by starter cultures of lactic acid bacteria, which are metabolically active not only during fermentation but also at lower temperatures during storage[410]. During the cheese-making process, the whey fraction is removed, leading to a considerable loss of vitamin B_{12} (on average about 50% of the vitamin originally present in the milk) due to its water solubility. Meanwhile, the vitamin content in final products (especially in hard cheeses) is higher relative to the starting milk owing to the milk thickening during cheese production (for instance, about 10 litres of milk is required to produce 1 kg of hard cheese). Ripening and storage of cheeses do not alter the vitamin B_{12} content, except in mold cheeses, in which the content may decline[68,410,428]. Swiss-type cheeses (e.g. Emmentaler and Gruyère) contain higher amounts of vitamin B_{12} than other ones owing to the application of propionibacteria as adjunct starter cultures for ripening (responsible for the characteristic flavor and opening formation) that are able to produce vitamin B_{12}[68,99,410,429,430].

Maturation of meat between the time of slaughtering and consumption for up to 14 d does not affect the vitamin B_{12} content in beef[398].

Vitamin B_{12} is fairly stable to ionising radiation, which is used as a food preservation method to control foodborne pathogens and extend product shelf life; no losses of the vitamin were found in irradiated pork, chicken, clam and haddock[431–433]. Hypochlorous acid water (used to sanitise food products, e.g. vegetables, fruits and meat) as well as sodium metabisulfite and sodium sulfite (used to prevent black discoloration of shrimps) destroy vitamin B_{12} in aqueous solutions but do not reduce its

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content in shrimps. Similarly, no significant changes in vitamin B_{12} amounts occur in beef treated with hypochlorous acid water. This is explained by the fact that most vitamin B_{12} present in foods is in protein-bound form rather than free[385].

Some food ingredients have been shown to influence positively the stability of vitamin B_{12}. Sorbitol, a sweetener, protects cyanocobalamin from degradation by heat, ascorbic acid, thiamine, UV light, and low or high pH values [379,384]. Whey proteins enhance thermal stability of vitamin B_{12} by 20% and could be useful as protective agents against the physical destruction of the vitamin during food processing[427]. Carnosine, a dipeptide naturally present in meat, prevents the destruction of cyanocobalamin by vitamin C in the presence of copper ions and may be useful as an additive to multivitamin–mineral food supplements. Carnosine has been shown to possess antioxidant and metal chelating activity, which could be responsible for the observed protection[21,434].

Storage may influence the rate of vitamin B_{12} decomposition[376,388,390,435,436]. For instance, storage of ultra-high-temperature milk at room temperature for 18 weeks resulted in the complete disappearance of vitamin B_{12}, probably due to exposure to dissolved oxygen in the container, while low temperature (7°C) did not alter the vitamin content for up to 18 weeks[68,131,376,396,407]. No appreciable losses of vitamin B_{12} were found in pasteurised milk during storage in a refrigerator for 9 d, regardless of how long it had been since the packages had been opened[409]. Effects of storage conditions (time, temperature, moisture content, oxygen and nitrogen) on the vitamin B_{12} amount in milk powders have also been studied[435]. No remarkable changes in the vitamin B_{12} content occurred in vacuum-packaged salmon stored for 880 d at room temperature either on the Earth or exposed to spaceflight[437]. Cyanocobalamin loss reached up to 63% in fortified wheat flour packed in permeable paper bags, whereas no significant reduction of vitamin amounts occurred, when the flour was packed in multilayer aluminium/polyethylene bags (non-permeable to oxygen and humidity)[436]. When whole wheat flour fortified with cyanocobalamin was stored in air-tight plastic containers in the dark under different combinations of temperature (25°C and 45°C) and relative humidity (33%, 63% and 93%) to mimic the effects of various climatic conditions, the highest and lowest vitamin losses of 51% and 15% were recorded at 45°C/93% and at 25°C/all, respectively, after 120 d of storage, suggesting that wheat flour may be effectively fortified with vitamin B_{12}[421]. The degradation kinetics of vitamin B_{12} in fortified co-crystallised sugar cubes was studied under different storage conditions as for temperature and humidity; a half-life of 23 months was achieved at 25°C and 33% relative humidity[438]. Vitamin B_{12} was stable in a salt fortified with multiple micronutrients, including microencapsulated vitamins, during 6 months of storage[439]. Different storage temperatures of fortified juices from carrot (pH 6) and lime (pH 2, richer in vitamin C promoting vitamin B_{12} degradation) showed losses of vitamin B_{12} of 8%, 15% and 19% in carrot juice and 82%, 95% and 100% in lime juice, respectively after 28 d at 4°C, 25°C and 37°C. Carrot juice is therefore more suitable for vitamin B_{12} fortification owing to its mild acidic character[388].

Production of vitamin B_{12} and biofortification with B_{12}

Vitamin B_{12}, mainly in the most stable form cyanocobalamin, is commercially produced for use in fortified foods, dietary supplements, pharmaceuticals and animal feeds[67,81,88,93,102,141,152,199,214,256,260,304,348,351,379,440–465]. The total synthesis of vitamin B_{12} comprising about seventy reactions was achieved in 1972[466–470]. Due to the vitamin's enormous structural complexity, its chemical synthesis is highly complicated and not economically feasible on an industrial scale. Therefore, industrial production of vitamin B_{12} is exclusively based on microbial fermentation. Currently, the most commonly employed micro-organisms are high-producing bacterial strains of *Pseudomonas denitrificans* and *Propionibacterium freudenreichii*, developed by means of random mutagenesis and selection, as well as genetic engineering from wild strains with high natural production ability[6,7,48,52,67,70,175,450,471–502]. Fermentations produce a mixture of hydroxocobalamin, adenosylcobalamin and methylcobalamin; they are then converted to cyanocobalamin by the addition of potassium cyanide[7,48,70,450,471,474,476,478,479]. Current industrial biotechnological processes for the production of vitamin B_{12} are suboptimal. Accordingly, further possible micro-organisms suitable for large-scale production have been widely studied in recent years, e.g. natural producers *Bacillus megaterium* and *Sinorhizobium meliloti*, and even *Escherichia coli*, which is not able to synthesise vitamin B_{12} *de novo* in nature but can do so after genetic modifications (heterologous expression of the whole biosynthetic pathway). However, the reported yields are not yet competitive with those achieved in present-day manufacturing bioprocesses[7,48,450,471,472,503–517].

Biofortification aims to make crop plants naturally more nutritive rather than adding nutrient supplements to the foods during food processing. Biofortification can be achieved through three main approaches including plant breeding, transgenic techniques and agronomic practices[518–522]. No efforts are being made to biofortify crops with vitamin B_{12} through conventional breeding or genetic engineering due to the fact that the biosynthetic pathway is present exclusively in some bacteria and archaea[521,523]. As for the agronomic approach, which requires physical application of cobalamin to plants for enriching them with this vitamin, some biofortification attempts have been reported, e.g. in wheat and spinach[524] and green tea[196] by the addition of organic fertilisers (naturally rich in vitamin B_{12}) to the soil, in garden cress seedlings by growing on an agar medium containing cobalamin[197], in Japanese radish sprout (kaiware daikon) by soaking its seeds in cobalamin solution[415], in lettuce[423] and spinach[418] in hydroponic culture by treatment with cobalamin, and in the recombinant alga *Chlamydomonas* (expressing human intrinsic factor) by culturing in a medium supplemented with cobalamin[525]. Milk concentrations of vitamin B_{12} are influenced by the genotype of the cow. Genomic regions associated with vitamin B_{12} concentrations in milk have been identified, which offer an interesting potential for marker-assisted genetic selection and breeding to increase the content of vitamin B_{12} in cow milk. However, feeding composition management could help optimise vitamin B_{12} amounts in milk only to a limited extent as there are adequate

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cobalt levels in the cow's diet required by ruminal microbiota for cobalamin biosynthesis[86,97,99,526–531]. The introduction of vitamin B_{12} into foods may also be achieved via the *in situ* production by micro-organisms naturally capable of synthesising cobalamin. The propionibacterium *Propionibacterium freudenreichii* has been most often investigated (rarely other bacteria, e.g. *Bacillus megaterium*[61] and *Acetobacter pasteurianus*[532]) for fortification by fermentation during food processing; and various substrates have been fermented, including cereals (e.g. wheat, barley, rye, oat, rice, sorghum and millet), pseudocereals (e.g. buckwheat, quinoa and amaranth), legumes (e.g. faba bean, soy bean and lupin beans), whey, soy milk, sunflower seed milk, cabbage, ground elder and black tea[61,386,420,450,533–550]. The usefulness of lactic acid bacteria, such as *Lactobacillus plantarum*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus*, for *in situ* fortification with vitamin B_{12} is questionable. It is not clear which, if any, of these strains synthesises true cobalamin and not merely pseudocobalamin. The ability of lactic acid bacteria to produce vitamin B_{12} is, in fact, usually evidenced by a microbiological assay that does not distinguish between cobalamin and pseudocobalamin and is not verified by a reliable analytical method for structure elucidation and/or by a genetic analysis confirming the presence of the whole biosynthetic pathway in the genome of a particular micro-organism[79,303,372,536,539,545,547,551–563]. The practical importance of vitamin B_{12} biofortification strategies, including *in situ* methods, is so far low, if any, compared with fortification (i.e. external addition of cobalamin to foods). However, it is a promising way to provide the vitamin to consumers and requires further research.

Pharmacokinetics and homoeostasis

Absorption

In humans, the selective absorption of vitamin B_{12} is a multi-step process (Figure 2). The bioavailability depends on the individual's gastrointestinal absorption capacity and, in terms of food sources, on the amount and type of protein consumed. Indeed, vitamin B_{12} ingested through food appears to have varying rates of absorption[5,564]. In general, the bioavailability of the vitamin from the usual diet is assumed to be about 50% (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), but lower from sources containing high amounts, e.g. from liver, due to saturation of the active absorption process[63,66,81,84,88,92,93,98,152,565].

Vitamin B_{12} is bound to proteins in food and is available for absorption only after releasing by pepsin and hydrochloric acid produced by the gastric mucosa. Subsequently, it binds to transcobalamin I (TCI) belonging to haptocorrins (HC)/R binders. Due to this classification, it is sometimes simply referred as haptocorrin. It is a glycoprotein that is found in saliva and gastric fluids and, *inter alia*, in blood serum. TCI has a high affinity for both B_{12} and for its analogues. In the duodenum, TCI is degraded by pancreatic proteases and free cobalamin binds to intrinsic factor (IF), a glycoprotein that is secreted by gastric

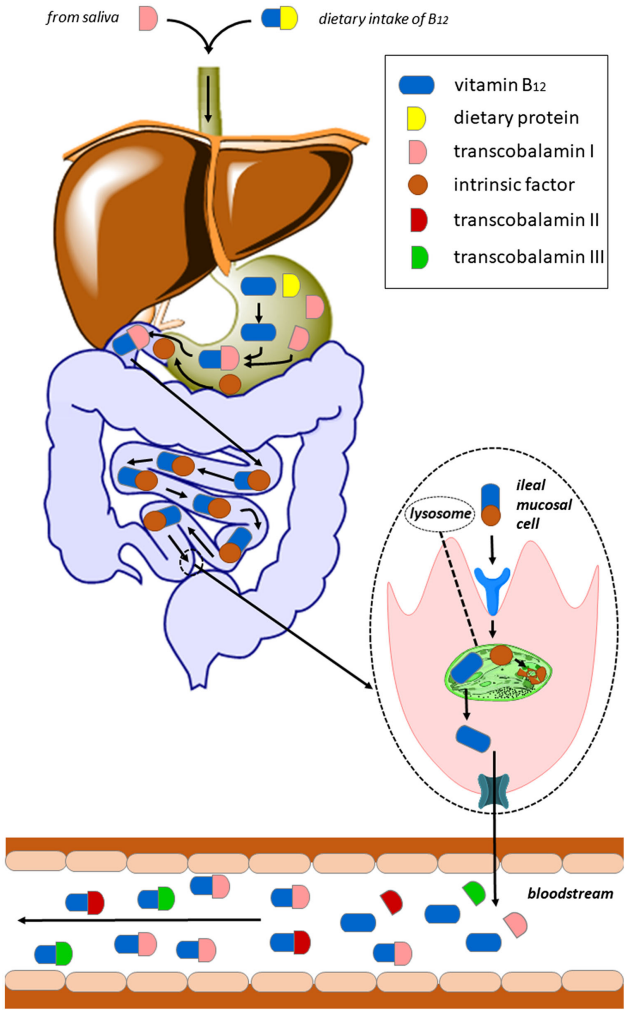

Fig. 2. Absorption of vitamin B_{12} via the IF pathway: Dietary protein-bound vitamin B_{12} can bind to transcobalamin I (TCI) only after its release mediated by pepsin and hydrochloric acid produced by the gastric mucosa. In the duodenum, TCI is degraded by pancreatic proteases and free cobalamin binds to intrinsic factor (IF). The IF–cobalamin complex is absorbed in the distal ileum by receptor-mediated endocytosis enabled by cubilin with participation of other protein(s), e.g. amnionless (AMN). IF is degraded in the lysosome and released cobalamin enters the cytoplasm likely by use of the transmembrane protein LMBD1. The precise mechanism of vitamin B_{12} efflux from enterocytes into the circulation is not yet well described. It appears to be mediated by several exporters; one of them is multidrug resistance protein 1 (MRP1, shown in teal colour).

parietal cells after a meal. There is high homology between IF and TCI, and both of them bind one molecule of vitamin B_{12}[566]. Cobalamin binds to IF with a higher affinity in a more alkaline environment; hence, in the stomach, where the pH is acidic, IF has a very low affinity for vitamin. This glycoprotein is much more specific for B_{12} binding than TCI and has limited affinity for cobalamin analogues. The IF–cobalamin complex is absorbed in the distal ileum by receptor-mediated endocytosis enabled by cubilin with participation of other protein(s), e.g. amnionless (AMN). IF is degraded in the lysosome, and released B_{12} enters the cytoplasm likely by use of the transmembrane protein LMBD1. The precise mechanism of vitamin B_{12} efflux from enterocytes into the circulation is not yet well described. It

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appears to be mediated by several exporters; one of them is multidrug resistance protein 1 (MRP1)[5,260,566].

The normal mechanism of absorption of orally administered vitamin B_{12}, via the IF pathway, is readily saturated. While approximately 70% of vitamin B_{12} is absorbed from doses of 0.1–0.5 μg, it decreases to 56% at 1 μg, to 16% at 10 μg and to 3% for doses 25–50 μg[6]. High oral doses (100–100 000 μg) are absorbed passively, but the extent reaches only about 1% of the ingested dose[567].

Transport

In the bloodstream, the majority of B_{12} (80%) and all cobalamin analogues are bound to TCI, which, thanks to its relatively long biological half-life of 10 d, forms a circulating supply of vitamins in the body[568]. Of the total B_{12}, 20–30% is carried by transcobalamin II (TCII), a non-glycosylated protein[6]. TCII binds physiological forms of vitamin B_{12}, while TCI also binds B_{12} analogues. TCII and TCI deliver vitamin B_{12} to peripheral tissues and liver, respectively[569]. The TCII–cobalamin complex binds in the presence of calcium to its receptor, a transmembrane, highly glycosylated protein CD320 (8D6A) containing two low-density lipoprotein-receptor class A domains. The receptor is selective to cobalamin–TCII, and neither TCI nor IF binds to it[570]. After endocytosis, the complex enters the lysosome, where TCII is degraded and free B_{12} is exported to the cytosol by use of the ATP-binding cassette transporter ABCD4. Presence of another membrane lysosomal protein LMBD1 is also necessary [6,571]. A third specific vitamin B_{12} transport protein found in human serum, transcobalamin III (TCIII), is also reported in the literature[572–576]. Like TCI, TCIII is a glycoprotein and, in addition, these two transcobalamins are immunologically identical [573,574].

Reabsorption and excretion

Vitamin B_{12} is secreted into the bile, and a part is reabsorbed by the enterohepatic circulation through ileal receptors that require IF[566,577]. Cobalamin is excreted in the faeces, which consists of unabsorbed biliary vitamin B_{12}, vitamin B_{12} from gastrointestinal cells and secretions, and that synthesised by bacteria in the colon. When the vitamin B_{12} is found in the excess in the circulation, it outreaches the binding capacity of TCII, and it is also excreted in the urine[5]. However, it is partially reabsorbed in the kidney by the transcobalamin II receptor megalin (Lrp2)[566]. Further losses of vitamin B_{12} occur through the skin and metabolic reactions[564].

Storage of cobalamin in the human body

Cobalamin, unlike other water-soluble vitamins, is stored in the human body. Most adults have stores of up to 5 mg. The liver is the main reservoir of this vitamin, and it stores normally up to one half of the total amount of the vitamin[577,578]. Smaller amounts of accumulated cobalamin can also be found in the kidneys and brain, and the circulating supply of TCI-bound vitamin B_{12} in plasma cannot be neglected[577].

Physiological function

Vitamin B_{12} is essential for human metabolism, production and regeneration of carbohydrates, fats and proteins, as well as for the proper development of erythrocytes and the central nervous system. Only two forms are biologically active: methylcobalamin and adenosylcobalamin[564,566]. The former is a cofactor for methionine synthase and the latter for L-methylmalonyl-CoA mutase (Figure 3). These B_{12}-mediated reactions are facilitated by the ability of the cobalt ion to change its oxidation states, Co (I), Co (II) and Co (III) (Figures 4 and 5)[579,580]. Co (I) is unstable and acts as a supernucleophile. The corrin ring helps to stabilise this form[6].

Methionine synthase

Methionine synthase is a cytoplasmic enzyme requiring both vitamin B_{12} and vitamin B_9. It converts homocysteine into methionine by transfer of a methyl group. The donor of the methyl group is methylcobalamin, which is subsequently recovered by transfer of one carbon unit from methylenetetrahydrofolate (vitamin B_9). It need not be emphasised that mutation in methionine synthase or derangement in vitamin B_{12} physiology leads to hyperhomocysteinaemia[581]. High plasma homocysteine levels are considered vasculotoxic and neurotoxic, but the relationship between human diseases, homocysteine levels and supplementation by B vitamins to decrease the homocysteine levels it is still a matter of debate[582,583]. Furthermore, the formation of the essential amino acid methionine allows several methylation reactions necessary for the synthesis of nucleotides for DNA/RNA and proteins. Failure of this step can be observed especially in rapidly multiplying cells, such as erythrocytes or enterocytes, and can affect several processes including the growth of vascular endothelial cells or the production of noradrenaline, which is involved in both stress response and cardiovascular system function[93,566,584].

Methylmalonyl-coenzyme A mutase

The mitochondrial enzyme methylmalonyl-coenzyme A mutase is involved in the catabolism of odd-chain fatty acids, some branched-chain amino acids and cholesterol to form succinyl-coenzyme A. A defect in this response is thought to be involved in several neurological manifestations of vitamin B_{12} deficiency, including movement disorders, seizures and mental retardation[93,566,584].

Laboratory assessment of vitamin B_{12} status

Vitamin B_{12} status can be assessed by measuring the serum/plasma B_{12} level, which is the sum of B_{12} TCI-bound (holohaprocorrin) and TCII-bound (holotranscobalamin); serum holotranscobalamin (holoTC) concentration; serum/plasma methylmalonic acid (MMA) concentration; and total serum homocysteine (tHcy) concentration[4,585]. However, no single laboratory marker is suitable for assessing B_{12} status in all patients. Combinations, such as multiple markers or sequential assay selection algorithms[585] or the calculations[586], that combine the single B_{12} diagnostic indicators, such as combined

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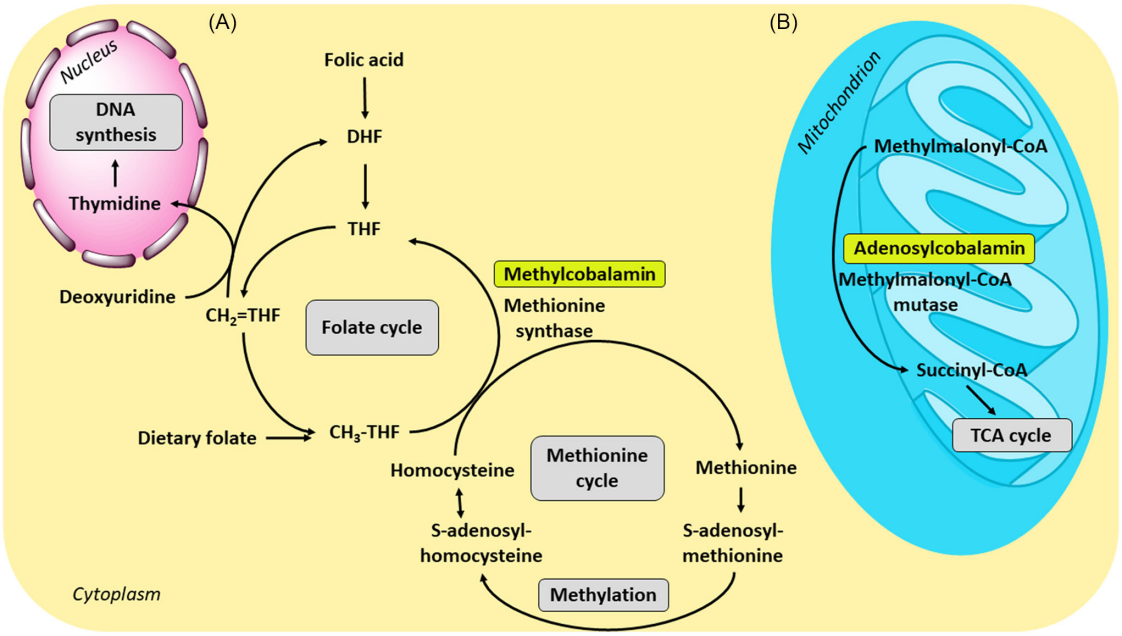

Fig. 3. Physiological function of vitamin B_{12} and its connection with folate metabolism: (A) Together with folic acid (vitamin B_9), methylcobalamin as a cofactor for the enzyme methionine synthase is necessary for the formation of methionine. During the reaction, the methyl group is transferred from methyltetrahydrofolate (CH_3-THF) to homocysteine by the enzyme; the resulting tetrahydrofolate can be then converted to methylenetetrahydrofolate (CH_2=THF), the form required for *de novo* thymidine synthesis. (B) In the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A, B_{12} is involved in its active form adenosylcobalamin as a cofactor of the enzyme methylmalonyl-coenzyme A mutase. The resulting succinyl-coenzyme A is a major mediator of the tricarboxylic acid (TCA) cycle; CoA, coenzyme A; DHF, dihydrofolate; THF, tetrahydrofolate.

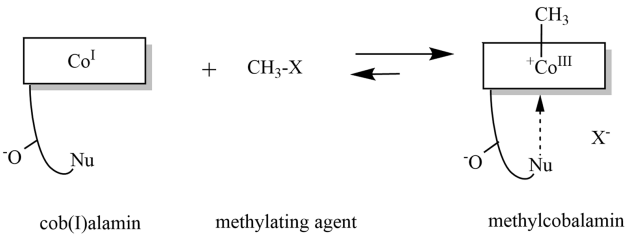

Fig. 4. Formation of methylcobalamin: The highly nucleophilic cob(I)alamin reacts with a methylating agent to form methylcobalamin. Modified in ChemDraw, version 20.0 on the basis of publication of Kräutler[579].

indicator (cB_{12}), which uses all four most commonly available markers: $cB_{12} = \log_{10}[(\text{holoTC} \times \text{total serum } B_{12} \text{ level})/(\text{MMA} \times \text{tHcy})] - (\text{age factor})$, are usually employed for more precise determination of vitamin B_{12} levels[585,587]. Analytical methods used for the measurement of vitamin B_{12} in serum/plasma and other biological fluids are summarised in Table 2.

Determination of total serum/plasma cobalamin

Serum or plasma B_{12} levels provide information on the long-term B_{12} status and liver stores. Recent intake has no particular effect. This marker is not very sensitive or specific, leading to a false positive and negative diagnosis. A serum level of vitamin B_{12} below 148 pM with symptoms is a strong indicator of deficiency, but symptoms might be present even with serum levels above this value[2]. Conditions that increase TCI levels such as chronic granulocytic leukaemia, autoimmune lymphoproliferative syndrome, alcoholism, liver disease and cancer will also elevate vitamin B_{12} levels[93].

Determination of holotranscobalamin

Serum holoTC levels determine the amount of the physiologically active form of cobalamin bound to TCII. As aforementioned, holoTC accounts for 20–30% of total B_{12} serum levels. This method is the most sensitive to recent intake with a response within few hours. Hence, this marker can be increased even when body reserves are low. HoloTC levels are, however, also increased in patients with renal impairment[93].

Determination of methylmalonic acid

Serum MMA is the most sensitive biomarker of B_{12} status. It is a good indicator of liver stores, and it reflects the utility of vitamin B_{12} for metabolic functions, i.e. methylmalonyl CoA mutase activity. MMA as a by-product of methylmalonyl CoA metabolism increases in B_{12} deficiency. When total serum B_{12} is <287 pM, its concentrations increase. MMA metabolite is affected neither by folate nor other B vitamins, but it increases with renal dysfunction, so serum creatinine should be measured, especially in the elderly. MMA levels increase with aging, especially after age 70, and neither lower intake nor impaired renal function fully explains why this occurs. Higher serum concentrations of MMA occur also with an overgrowth of intestinal bacteria that produce propionic acid. Conversely, antibiotic treatment may lower the level[93].

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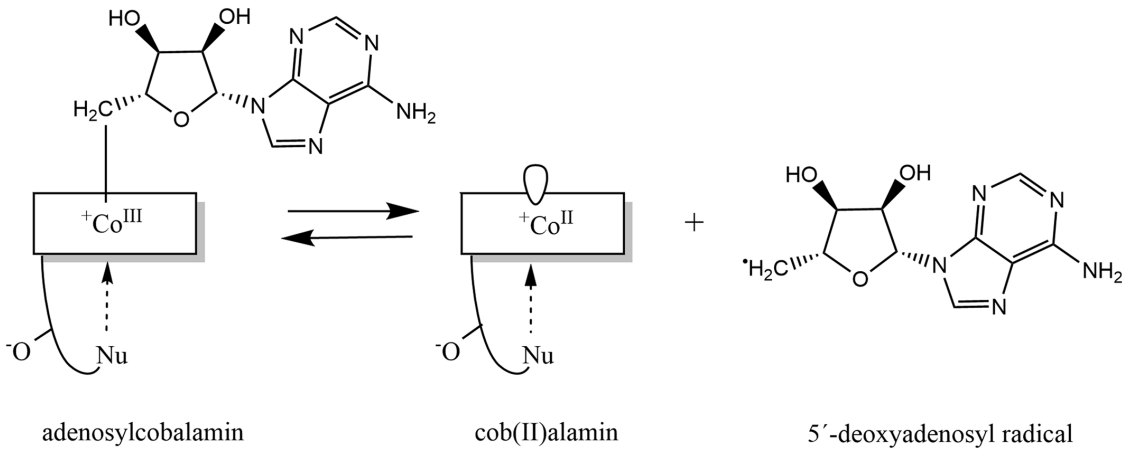

Fig. 5. Formation of adenosylcobalamin: Adenosylcobalamin functions as a reversible source of the 5′-deoxyadenosyl radical, this reaction produces cob(II)alamin. Modified in ChemDraw, version 20.0 on the basis of publication of Kräutler[579].

Determination of total serum homocysteine

The biomarker tHcy is not specific for B_{12} levels. It reflects the availability of B_{12} for metabolic functions, i.e. methionine synthase activity. Concentrations increase when total serum B_{12} is <300 pM, but also in deficiency of folates, riboflavin and vitamin B_6, as well as with renal insufficiency and hypothyroidism[93].

Combination of biomarkers

The combination of all four biomarker values (serum B_{12} levels, holoTC, tHcy and MMA) or the cB_{12} mathematical model using 'four-biomarker' analysis appears to be the most accurate for determining B_{12} levels, but in some cases is unbearably expensive[93]. A study by Fedosov *et al.*[587] showed that the cB_{12} model using analysis of 'three-biomarker'or 'two-biomarker' estimates the level of B_{12} within acceptable error limits compared with the analysis of 'four-biomarker'. The combination of holoTC, MMA and total serum B_{12} levels appears to be the best in the 'three-biomarker' analysis. Further quality analysis is achieved by omitting total serum B_{12} or holoTC from the 'four-biomarker' analysis. In terms of 'two-biomarker' analysis, the smallest error was observed when holoTC was used in conjunction with the MMA assay. The article by Fedosov *et al.*[587] also provides formulas for cB_{12} for cases where one or two biomarkers are missing.

Identification of aetiology of vitamin B_{12} deficiency

A formerly used Schilling test was withdrawn mainly due to concerns of bovine spongiform encephalopathy transmission via its animal-derived intrinsic factor[585,588]. Another way to detect a deficiency caused by impaired absorption is the CobaSorb test, which uses holoTC as a sensitive marker of the recent intake of B_{12}. The test consists of taking blood samples before and 1–2 d after taking an oral dose of 9 µg of cyanocobalamin three times a day and measuring the increase in holoTC[589]. This method can detect holoTC elevation of ≥10 pM only if the holoTC baseline was <75 pM. Under these conditions, both sensitivity and specificity are considered excellent[590]. Hardlei *et al.* improved this method. After stating that a major part of the oral test dose of cyanocobalamin is absorbed without modification, it was suggested that the capacity to absorb vitamin B_{12} can be evaluated by measuring TC-cyanocobalamin before and after administration of the test dose of cyanocobalamin. C-Cobasorb, as this method is named, has a higher specificity than the previously mentioned CobaSorb[591]. Therefore, if holoTC baseline is >65 pM, it is recommended to use the C-CobaSorb assay for the assessment[592]. Other laboratory tests usable for determination of the cause of B_{12} deficiency are tests based on plasma IF antibodies, plasma gastrin and pepsinogen I, and plasma parietal cell antibodies[585,593].

Cobalamin deficiency

Subclinical and clinical cobalamin deficiency

Low levels of vitamin B_{12} can be divided into four stages. The first two stages represent depletion, while the second two represent deficiency. Stage I is low serum vitamin B_{12}; stage II is low stores in cells; stage III is biochemical deficiency; and stage IV is clinical deficiency with overt manifestations[594]. Other classification divides vitamin B_{12} deficiency into clinical and subclinical forms. Subclinical cobalamin deficiency involves mild biochemical changes without clinical manifestations. Clinical deficiency usually results from severe, persistent malabsorption, while dietary insufficiency, intermittent or partial malabsorption can usually merely induce only subclinical cobalamin deficiency[595].

At the cellular and molecular level, vitamin B_{12} deficiency manifests itself in one or both forms of coenzyme B_{12} (methylcobalamin and adenosylcobalamin). Methylcobalamin deficiency leads to impaired nucleotide synthesis and methylation, and adenosylcobalamin deficiency disrupts the metabolism of methylmalonate, which is derived from the catabolism of odd-chain fatty acids and ketogenic amino acids[93,260]. This can also be the key to understanding the mechanisms responsible for clinical manifestations.

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Table 2. Summary of analytical methods for the assessment of vitamin B_{12} in biological fluids

Technique	Sensitivity nmol/L	Analytes	Matrix	Advantages	Disadvantages	Ref.	Published
LC–MS	LLOQ 7-11	B_{12} and its metabolites (9 analytes)	o plasma o milk o mice brain samples	o small sample volume (30 µl plasma, 20 mg milk or brain tissue) o short analysis time o various matrices		(703)	2015
	LLOQ 0.03	B_{12} and its metabolites (4 analytes)	o rat plasma	o online SPE o short analysis time o small sample volume (100 µl)		(704)	2022
	LOD 0.22	B_{12} and 7 others	o whole blood	o simple sample preparation	o relatively long analysis time	(705)	2022
	LLOQ 1000	B_{12}	o pig plasma	o MRM o online SPE o short analysis time	o low sensitivity	(706)	2012
HPLC-PDA	LOD 29.51	B_{12} and 1 other	o urine	o simple sample preparation	o long analysis time	(707)	2009
	LLOQ 6×10^{-5}	B_{12}	o saliva	o FPSE membrane o reusable	o long sample preparation time o long analysis time (25 min)	(708)	2023
Electrochemical sensor	LOD 4.13×10^{-3}	B_{12}	o urine	o selective o sensitive	o not available in the market	(709)	2018
Nanoparticles and nanosensor	LOD 3	B_{12}	o serum	o small sample volume (200 µl) o no sample preparation	o not available in the market	(710)	2018
Nanosensor FLD		B_{12}	o cell lines	o *in vivo*, *in vitro*, prokaryotic, eukaryotic cells	o not available in the market	(711)	2018
Nanoparticles FLD	LOD 0.0216	B_{12}	o plasma o urine		o large sample (5 ml) and solvent volume	(712)	2017
MIP – nanoparticles (magnetic)		B_{12}	o breast milk	o reusable (5 times)	o not available in the market	(713)	2018
ELISA kits	LOD 91.09×10^{-3}	B_{12}	o serum o plasma o tissue	o small sample volume (250 µl) o one kit for various matrices o sensitive	o for research only o cross-reactivity with analogues o time and money consuming for small sample series	(714)	2021
	LOD 0.2814	B_{12}	o serum o plasma o biological fluids o tissue homogenate	o small sample volume (50 µl) o one kit for various matrices o sensitive	o for research only o cross-reactivity with analogues o time and money consuming for small sample series	(715)	2021
	LOD 0.034	B_{12}	o serum o plasma o biological fluids o tissue homogenate	o small sample volume (50 µl) o one kit for various matrices o sensitive	o for research only o cross-reactivity with analogues o time and money consuming for small sample series	(716)	2022
CLIA kit	LOD 1.8×10^{-8}	B_{12}	o serum	o small sample volume (34 µl) o sensitive o short analysis time (15 min)	o for research only	(717)	2022
	LOD $0.83\text{-}2.99 \times 10^{-3}$	B_{12}	o serum	o small sample volume (50 µl) o sensitive	o results guaranteed only with the use of the defined analyser	(718)	2020

Causes of vitamin B_{12} deficiency and diagnosis

B_{12} deficiency can be caused by several pathophysiological processes. They can affect both B_{12} supply and demand, and they can occur at any time during human life. Specifically, cellular B_{12} deficiency could be due to insufficient intake and/or bioavailability including malabsorption, chemical inactivation[3] or disruption of B_{12} transport in the blood or intracellular uptake and metabolism. Also, some diseases, medications and bacterial overgrowth are related to B_{12} deficiency[5,260,577]. It can also occur in people with increased demands, such as during major bleeding, and in pregnant or breastfeeding women, and this can impact their infants as well[260,577].

People from developing countries and those who do not voluntarily consume animal products because of their religion, culture or personal attitude may suffer from B_{12} deficiency[260,577]. In terms of new vegans, the symptoms of vitamin B_{12} deficiency may not appear for several years owing to the large reserves in the human body and reabsorption of the vitamin[569,577]. Lacto- and lacto-ovo-vegetarian diet could be sufficient thanks to milk and eggs that contain a low amount of cobalamin[577]. Metabolic and clinical signs of cobalamin deficiency have been reported in neonates from strict vegetarian mothers or in breastfed infants by cobalamin-deficient mothers[566,596,597]. To avoid the risk of deficiency, they can obtain cobalamin from fortified foods (see section Prevention by fortification of food) or food supplements[5,260].

Furthermore, the deficiency occurs because of cobalamin malabsorption. In the case of pernicious anaemia (chronic atrophic gastritis type A), cobalamin cannot be absorbed because of the lack of IF. It is a consequence of autoimmune gastritis when the parietal cells producing IF and hydrochloric acid in the stomach are destroyed. Chronic atrophic gastritis type B, which is related to persistent infection with *Helicobacter pylori*, may also impair vitamin B_{12} absorption. Both are due to disturbance of the pH of the stomach and the inability to release vitamin B_{12} from food proteins[260,566,577]. Proton pump inhibitors, H_2-receptor antagonists and antacids also suppress the gastric acidity and, hence, similarly impair vitamin B_{12} absorption[260,569,577,578]. Diseases affecting the small intestine such as coeliac disease, Crohn's disease or ulcerative colitis are connected with B_{12} malabsorption due to villous atrophy and mucosal injury or recurrent diarrhoea[5,260,569,577]. Last but not least, cobalamin malabsorption is associated with gastric, post-gastric or ileal resection, or pancreatic insufficiency or pancreatectomy that cause inability of B_{12} release from HC binding[5,260,569]. Another disease associated with low levels of cobalamin is HIV infection. Plasma concentrations decrease with disease progression[66]. In addition, older people are at risk of malabsorption not only because of the various diseases and medications they take, but also because of the natural aging process. Several age-related physiological factors can adversely affect the absorption of the vitamin from the intestine[577].

In addition to the above-mentioned drugs, low levels of vitamin B_{12} are also associated with oral contraceptives[598], metformin, cholestyramine, colchicine, several antibiotics, and drugs such as *p*-aminosalicylic acid[260,578] and in elderly patients also with ACE inhibitors[599]. In some, the mechanism is known as with cholestyramine that may bind intrinsic factor, while colchicine, several antibiotics, and antituberculotic drug *p*-aminosalicylic acid, may act as inhibitors of intrinsic factor-B_{12} endocytosis[260].

In terms of the chemical inactivation, the anaesthetic gas nitrous oxide irreversibly oxidates cobalt. This oxidation is, however, relevant solely the enzyme methionine synthase[3,260,600]. On the other hand, adenosylcobalamin is not changed. The reason is that mechanisms of the catalytic reaction differ between methionine synthase and methylmalonyl-coenzyme A mutase particularly in relation to changes in the oxidation state of the central cobalt ion. During methionine synthesis, cob(I)alamin intermediate, which is susceptible to oxidation, is formed, whereas during methylmalonyl-succinyl coenzyme A transformation, cobalt is never reduced to Co(I) and, thus, not susceptible to N_2O-induced oxidation. [580,600].

Moreover, there are some rare genetic defects causing impairment in absorption, transport, metabolism or utilisation of cobalamin. Hereditary IF deficiency caused by recessive mutations in the IF gene (*CBLIF*) is an inherited disorders of vitamin B_{12} absorption associated with congenital pernicious anaemia. Imerslund–Gräsbeck syndromes 1 and 2 (also known as hereditary megaloblastic anaemia 1 and 2) are associated with selective vitamin B_{12} malabsorption in the ileum; there is a defect in transport of cobalamin into the enterocytes due to mutation in cubilin (gene *CUBN*) and amnionless (gene *AMN*), respectively. With respect to the physiological function of TCI (gene *TCN1*) and TCII (gene *TCN2*), it is clear that congenital deficiency of at least one of them leads to defective absorption as well as transport into cells. In addition, mutations in TCII receptor CD320 are known. There is also a group of historically so-called cobalamin mutant diseases cblA, cblB, cblC, cblD, cblE, cblF, cblG, cblJ, cblK and cblX. These diseases affect different processes associated with vitamin B_{12}: (1) efflux from lysosomes to cytosol (cblF – gene *LMBRD1*, cblJ – gene *ABCD4*); (2) intracellular vitamin B_{12} trafficking via chaperone MMACHC (cblC, /gene *MMACHC*/), silencing its gene ('epi-cbl C', gene *PRDX1*), or proteins regulating its expression – cblX /gene *HCFC1* on X chromosome/ and cblK /gene *ZNF143*/; (3) likely transport to methionine synthase – cblD /gene *MMADHC*/; (4) metabolism by methionine synthase (cblG, methione synthase, gene *MTR*) or methionine synthase reductase (cblE, gene *MTRR*); (5) synthesis of adenosylcobalamin by ATP:cobalamin adenosyltransferase /cblB, gene *MMAB*/ and transport of produced adenosylcobalamin to methylmalonyl CoA mutase /cblA, gene *MMAA*/. Methylmalonyl CoA mutase deficiency (gene *MMUT*) is also known[566,571,577,581,601].

The reference intervals of the individual biomarkers as well as their values indicating B_{12} deficiency are given in Table 3[260,587]. To determine the aetiology of vitamin B_{12} deficiency, the (C-) CobaSorb assay or plasma IF antibodies, plasma gastrin and pepsinogen I, and plasma parietal cell antibodies [585,593] can be used, as mentioned above.

Clinical manifestations

Clinical deficiency is manifested by haematological, neurological and neuropsychiatric symptoms[2,260,441,569,577,602–604]. No specific

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Table 3. The reference intervals of the individual biomarkers, values indicating transitional status and B_{12} deficiency

Biomarker; unit	B_{12} reference interval		Transitional B_{12} status	B_{12} deficiency	
References:	(587)	(260)	(587)	(587)	(260)
B_{12} (pM)	186–650	200–600	119–186	<119	<148
holoTC (pM)	37–190	40–100	20–37	<20	<35
tHcy (μM)	8–13.6	8–15	13.6–19.2	>51	>15
MMA (μM)	0.11–0.35	0.04–0.37	0.35–1.7	>1.7	>0.37
cB_{12}	−0.5 to +1.5	−2.5 to +1.5	−2.5 to −0.5	<−2.5	<−0.5

B_{12}, total serum B_{12} concentrations; cB_{12}, combined indicator of vitamin B_{12} status.

correlation between haematological and neurological symptoms has been demonstrated, so patients with neurological manifestations may not have any haematological abnormalities and vice versa[93,605].

Haematological manifestations are usually but not always connected with macrocytic or megaloblastic anaemia that is characterised by enlarged but less numerous erythrocytes, and by hypersegmented neutrophils. It is the result of disruption of DNA synthesis. In B_{12} deficiency, the recovery of tetrahydrofolate is disrupted. This limits the supply of folate for the synthesis of thymidylate, purine nucleotides and, subsequently, DNA. The haematopoietic system is particularly affected because blood cells are cells with a rapid turnover. DNA synthesis in bone marrow blood cell precursors is stopped, which prevents mitosis but allows cytoplasmic maturation[93,260]. Because other haematopoietic cells are also affected, it can lead to isolated thrombocytopenia and neutropenia and even pancytopenia with impairment of cellular and humoral immunity[93,260,441,569,602].

Cobalamin deficiency appears to be more common in patients with a number of chronic neurological and neuropsychiatric diseases. Clinical manifestations of vitamin B_{12} deficiency can include several CNS symptoms (peripheral neuropathy, subacute combined degeneration of the spinal cord, paraesthesia, ataxia, abnormal reflexes, bowel and bladder incontinence, erectile dysfunction, stroke, optic atrophy, orthostatic hypotension, dementia, multiple sclerosis, Alzheimer's disease, parkinsonian syndromes, depression, mania, irritability, paranoia, delusions, psychosis, delirium), but also atherosclerosis. However, it is still not clear whether there is a direct causality[441,569,577,602,603]. There are several theories that link cobalamin deficiency with neurological and neuropsychiatric problems such as (a) disorders of the formation of monoamine neurotransmitters, because cobalamin and folate are essential for the production of tetrahydrobiopterin that is required for monoamine synthesis[606]; (b) DNA synthesis disturbances due to deficiency of methyl donors[604]; (c) hyperhomocysteinaemia and its discussed vasculotoxic and neurotoxic effects[583,604,607,608]; (d) demyelination due to a lack of *S*-adenosylmethionine as a consequence of inhibition of homocysteine methylation to methionine[604,608,609]; (e) inhibition of the methylmalonyl-succinyl CoA pathway causes disruption of odd–chain fatty acid metabolism and may also lead to demyelination[604,609].

In addition, there is a higher risk for infants born to B_{12}-deficient women to develop neural tube defects with impairment of psychomotor function and brain development, anaemia and growth disorders. Neural disturbances may be irreversible[244,610].

Prevention by fortification of food

Fortification of foods with vitamin B_{12} is primarily intended for populations at risk for its deficiency. The highest prevalence of deficiency occurs in people who have a low dietary intake of the vitamin and in those, primarily the elderly, suffering from malabsorption of food-bound cobalamin, whose vitamin B_{12} status can be poor despite intakes often appearing to be adequate. As already discussed, vegetarian diets also represent a clear risk factor for vitamin B_{12} deficiency, as those diets have become very popular in the past few decades, especially in developed countries, because of their potential health benefits but also due to ethical and environmental issues. The diets of populations in low- and middle-income countries are typically low in animal-source foods because of their relatively high cost, lack of availability, and/or cultural and religious reasons[66,81,611–613]. Reduced acid production in the stomach associated with atrophic gastritis does not interfere with the absorption of the free crystalline vitamin present in fortified foods or supplements because intrinsic factor is still secreted[63,66,81,85,93,137,152,199,200,205,260,455,565,612,614–623]. Fortification with cobalamin has also been recommended in foods fortified with folate because of concerns that excess intake of folic acid alone by people with a low vitamin B_{12} status, particularly in the elderly, may delay the diagnosis of cobalamin deficiency. High levels of folate may mitigate ('mask') symptoms of anaemia caused by cobalamin deficiency and cannot correct nervous system damage, allowing or even accelerating its progression in persons with unrecognised and untreated cobalamin deficiency. Significance of this phenomenon has been an issue of persistent debate[63,200,242,453,615–619,623–656]. Whether the concerns are substantiated or not[108,456,612,627,639,657–666], cobalamin deficiency should be prevented in any case, and fortification with both folate and cobalamin would reduce possible risks. As of 2022, there are twenty-five countries in Africa, Central and South America, and Asia having mandatory fortification of wheat flour, maize flour or rice with vitamin B_{12} (Burundi, Cameroon, Chad, Ethiopia, Ghana, Kenya, Liberia, Malawi, Mozambique, Nigeria, Rwanda, Tanzania, Uganda, Zimbabwe, Costa Rica, Cuba, Guatemala, Nicaragua, Panama, Peru, Afghanistan, Jordan, Palestine, Uzbekistan and Vietnam)[667–670]. In addition, food is fortified with the vitamin on a voluntary basis in many other countries including, e.g., the United States, Brazil, India,

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Bangladesh, Myanmar, Indonesia, Sudan, Sierra Leone and countries of the European Union[224,444,449,450,461,521,667,668,671–674]. Foods fortified with vitamin B_{12} include cereal-based foods, meat substitute products (such as tofu), milk substitutes (such as soy-based milk and yoghurt alternatives), nutritional yeast, fruit juices, multivitamin sweets and milk powder infant foods[85,93,152,199,216,224,256,259,444,449,612,613,636,669,671,672,675–679]. Attractive strategies for fortification have been investigated, such as fortification of milk [616,621], yoghurt[680], tea [681,682], fruit and vegetable juices[388], ready-to-blend fresh-cut fruit/vegetable mix[683], mineral water[684], sugar cubes[438], salt [391,439], dried soup[621] and toothpaste[685,686].

Therapy

The first-line treatment in patients with risk factors for the vitamin deficiency is prevention of this deficiency and/or supplementation with vitamin B_{12}. Treatment should be started as soon as possible[260]. Parenteral therapy or high-dose oral administration can be used to treat vitamin B_{12} deficiency if due to inadequate dietary intake. In diagnosed vitamin B_{12} deficiency, intramuscular administration should be preferred as the effect or oral treatment is lower based on current knowledge[2]. Intramuscular injections of 1000 μg of cyanocobalamin or hydroxocobalamin are given daily or every other day for at least one week followed by weekly injections for at least one month. Thereafter, they are reduced to a dose of 1000 μg of cyanocobalamin monthly. Injections of hydroxocobalamin can be given each 3 months after the initial intensive therapy. Alternatively, subcutaneous administration is recommended in cases of contraindications of intramuscular application, e.g. when a patient is treated with anticoagulants. Oral treatment consists of 1000–2000 μg of cyanocobalamin orally per day. In some countries, also sublingual and intranasal vitamin B_{12} is available, but their clinical profit has not yet been clearly established[2,260,441,602]. The development of nanoparticles for oral vitamin B_{12} administration able to overcome the IF-absorption pathway can enrich the palette of current treatment modalities in the future[452].

The duration of treatment depends on the cause and clinical manifestations of the deficit. Blood count usually recovers within 2 months, and neurological signs correct or improve within 6 months[441]. However, in patients with severe neurological disorders, improvement usually will be weak after 1 year of adequate therapy. These patients may suffer from permanent impairments[260]. In case of malabsorption, treatment continues even after the symptoms have disappeared; it is usually a life-long therapy. Both routes of administration (including self-injection at home) can be used[2]. Selection of the route of administration usually depends on patient preferences and the compliance. As mentioned above, other less studied routes of administration (sublingual, nasal or even transdermal) are available, but they are also more expensive[441,455]. Regarding dietary deficiency, it is recommended to take at least 6 μg/d after the symptoms have disappeared and the body's vitamin B_{12} levels have been restored. In infants, treatment is usually started with intramuscular injections of 250–1000 μg cyanocobalamin or hydroxocobalamin daily, then until recovery once a week, followed by an oral administration of 1–2 μg daily using various B_{12}-containing formulas. Treatment of the mothers is also indicated to adjust breast milk vitamin levels[260].

There is dose–response relationship between oral B_{12} dose and serum/plasma B_{12} levels[687]. Doubling vitamin B_{12} increases serum/plasma levels approximately by 11% and slightly more in the elderly (13%) than in adults (8%).

Pharmacological use

Vitamin B_{12} is mainly used, as mentioned above, to supplement at-risk people and to treat deficiency. In particular, older people who are more susceptible to food–cobalamin malabsorption should consume vitamin B_{12} in crystalline form, i.e. from supplements or fortified foods, as this form is likely to be better absorbed[93].

It is speculated that vitamin B_{12} could be used as an adjunctive or integrative treatment for painful conditions such as various types of neuralgia and neuropathy, low back pain and aphthous stomatitis. Further studies are needed in this area[688,689]. Vitamin B_{12} could have a positive effect on sperm quality. It primarily increased sperm count and secondarily elevated sperm motility and reduced sperm DNA damage[690]. However, in both cases, further studies are needed as well.

Toxicity

To date, there are minimal claims on cobalamin toxicity. Vitamin B_{12} is usually well tolerated with rare incidence of adverse effects. Allergic reactions are unusual but may be anaphylactic[691,692]. Injectable forms appear to be more allergenic than pills, but this could be associated rather with some preservatives (e.g. benzylalcohol) in injections than by the vitamin itself[2]. High oral dose supplementation seems to be better tolerated, but there are known exceptions[693]. Although there is a cross-reaction between hydroxocobalamin and cyanocobalamin, it is possible to reintroduce vitamin B_{12} with concomitant administration of glucocorticoids or antihistamines or with desensitisation therapy[691,692]. There are also cases of acneiform eruptions after intramuscular or high oral doses of vitamin B_{12}, which easily disappear after discontinuation[694,695]. Nausea, dry mouth and blurred vision were reported after administration of vitamin B_{12} as well[696]. Other rare documented side effects of vitamin B_{12} include discolouration of the skin and urine, mild arterial hypertension, hypokalaemia, congestive heart failure, pulmonary oedema and local pain at the injection site in case of parenteral administration[578].

Conclusion

This review summarised all critical aspects of vitamin B_{12} biology. It emphasised that animal-based diets are almost the sole source of this vitamin for humans and, hence, vegetarians are at risk of developing its deficiency, which can progress to very severe and irreversible stages. At present, many countries have mandatory or voluntary fortification of food by this vitamin, but the risk of vitamin B_{12} deficiency is still not negligible

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worldwide. Moreover, there are many diseases, drugs and surgical procedures which can cause its deficiency, which is mostly delayed by several years as humans have relatively large stores of this vitamin. Prevention or treatment should be ideally case specific due to the complicated absorption mechanism of this vitamin. There are no convincing data on the pharmacological administration of vitamin B_{12} with exception of its deficiency. Its administration is considered safe in the majority of patients, but hypersensitive reactions can occur particularly after its parenteral administration.

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Competing interests

None.

Authorship

M.M. wrote the initial draft of the whole paper. T.S. prepared the parts related to sources of the vitamin, while L.K.K. and K.M. prepared that in relation to the detection of the vitamin. P.M. prepared the concept and revised the prepared paper. All authors revised the paper before submission and as a part of the peer-review process.

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