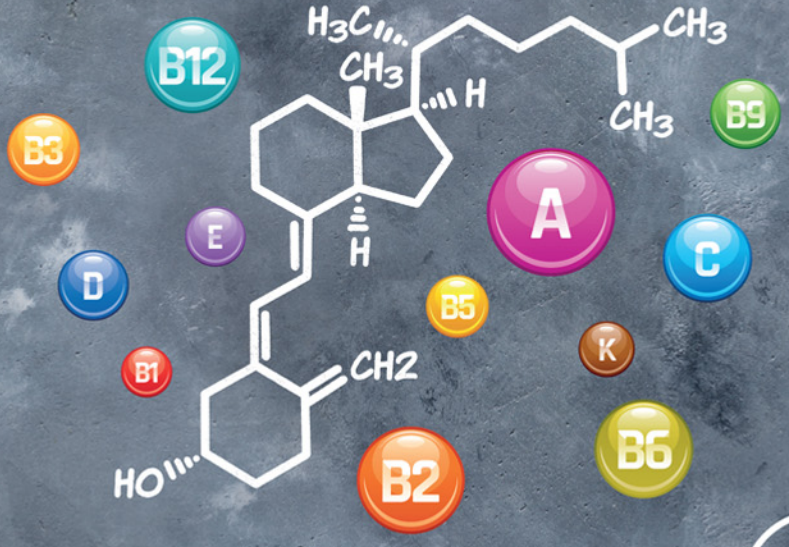
Molecular Nutrition Series

Molecular Nutrition
Vitamins



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CHAPTER 6

A review of vitamin B12

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Contents

Key facts of vitamin B12

- Even if there are different isoforms of cobalamin (Cbl), cofactor forms must follow the endocellular process to be engaged by specific enzymes.
- There is no upper intake level for vitamin B12 because its absorption and retention is limited. Usually the excess vitamin is excreted with urine.
- Only a few microbes can synthesize vitamin B12 but humans can obtain this vitamin through food, due to the bioaccumulation process.
- With senescence, the elevation of gastric pH reduces the ability to digest food efficiently. However, the absorption of the crystalline form of vitamin B12 is not affected.
- Pernicious anemia (PA) is an autoimmune disease that disrupts the gastric mucosa and leads to the incapacity of vitamin B12 absorption.
- Even if vitamin B12 shortage was identified with anemia symptoms, neurological ones can occur without anemia and in an irreversible manner.
- The complexity of the carrier system is well elucidated by modern molecular and cellular techniques; however, there are actually inborn defects that are still unknown.

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Summary points

- Mammals, and so humans, cannot synthesize Cbl.
- Only animal foodstuffs are adequate sources of Cbl.
- If Cbl intake is not sufficient, supplementation is needed.
- Some disease and dysfunction can decrease Cbl absorption.
- Oral Cbl supplementation at a high dosage is as effective as intramuscular injection, as well as in the presence of PA.
- Cbl is crucial for energetic metabolism and in cell replication processes.
- There are only two enzymes that need Cbl for their reactions.
- There is not a gold standard essay for body Cbl status.
- The use of multiple markers can better define Cbl sufficiency than just blood Cbl concentration.
- A wide pool of transporters and endocellular chaperones ensure efficient absorption and utilization of Cbl, minimizing the absorption of useless molecules with no vitamin properties.
- A diffuse Cbl deficiency among the population is a source of concern, in particular among children and pregnant women.
- Among vegetarian people an adequate intake of Cbl can be easily obtained from supplements.

Definitions of words and terms

Corrin: A corrin is a chemical compound formed by a heterocyclic structure. It forms the central ring of vitamin B12 where a cobalt atom is coordinated by four nitrogen atoms, a lateral chain, and a sixth variable ligand for different isoforms.

Cellular trafficking: Endocellular management of molecules through a chaperone system that engages specific elements and escorts them to their cellular fate.

Microbiota: The microbiota is a pool of microorganisms with symbiotic and commensal relationships among strains. Usually the digestive tracts of mammals have specific microbiota associated with various districts with a plethora of functions in food digestion and immunological mechanisms. Sometimes an alteration of these relationships among strains and between host and microbiota can develop negative outcomes.

Bioavailability: The bioavailability of a substance takes into account not only the concentration of it but also some phenomena that alter

physiological capabilities to absorb it. This may depend on the presence of other substances or the presence of different mechanisms of transport.

Pernicious anemia: This is an autoimmune disease that disrupts gastric parietal cells due to the production of specific antibodies. This phenomenon alters the secretion of gastric substances, such as the intrinsic factor, a carrier that mediates the absorption of Cbl.

Food-bound malabsorption: The low gastric pH is required for the release of some vitamins from foodstuffs. With senescence the reduction of gastric barriers with the rise of pH reduces the ability to cleave Cbl from food, with a consequent risk of vitamin shortage.

Enterocyte: Enterocyte is a cytotype of the brush border of absorptive gut epithelia. It is a polarized cell characterized by different membrane organization between the apical side, that mediates absorption from gut lumen, and the basolateral side, that mediates the release of substances to the vessel lumen.

Proton pump inhibitors: They are drugs that target the gastric proton pump. They are used to reduce the acidity of the stomach to protect gastric cells in the case of iatrogenic erosion of parietal tissue or in gastroesophageal reflux disease.

Small intestinal bacterial overgrowth: This is a pathological translocation of some strains from large bowel microbiota to the small intestine with the alteration of physiological digestive functions.

Inflammatory bowel disease: This is a group of correlated degenerative diseases that affect bowel tissues with digestive and systemic dysfunctions.

Enterohepatic circulation: The enterohepatic circulation consists of a process of reuptake of some substances that have been excreted with bile. This phenomenon is very important for the efficient use of some vitamins like Cbl.

Microcytemia: This is a disruption of normal hematopoiesis that leads to a low volume of erythrocytes. It depends on an inadequate availability of iron during the process.

Abbreviations

AdoCbl	Adenosylcobalamin
AMN	Amnionless protein
Cbl	Cobalamin
CNCbl	Cyanocobalamin
CNS	Central nervous system

DMB	5,6-Dimethylbenzimidazole
DRI	Dietary reference intake
HC	Haptocorrin
HCY	Homocysteine
HTCII	Holotranscobalamin II
IF	Intrinsic factor
MCM	Methylmalonyl-CoA mutase
MCV	Mean corpuscular volume
MetCbl	Methylcobalamin
MMA	Methylmalonic acid
MMAA	Methylmalonic aciduria type A
MMAB	Methylmalonic aciduria type B
MMACHC	Methylmalonic aciduria type C and homocystinuria
MMADHC	Methylmalonic aciduria type D and homocystinuria
MRP1	Multidrug resistance protein 1
MS	Methionine synthase
MSR	Methionine synthase reductase
OHCbl	Hydroxocobalamin
PA	Pernicious anemia
TCII	Transcobalamin II

6.1 Introduction

Vitamin B12, also called cobalamin (Cbl), is an indispensable molecule with a very complex structure and an intricate pathway of absorption and cellular trafficking that requires molecular escort proteins in body fluids and intracellular chaperones. After the discovery of this vitamin, a lot of mechanisms were clarified, although to date there are still some aspects that need to be elucidated.

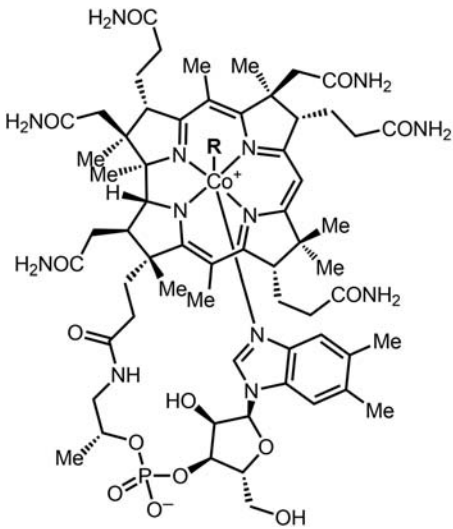
The biosynthetic pathway of Cbl numbers about 30 steps, but only some prokaryotes have the required enzyme pool (Martens et al., 2002). Interestingly this complex biosynthetic capacity is limited to some phyla that are not necessarily interrelated (Zhang et al., 2009).

Mammals, humans included, are not able to synthesize Cbl but a highly modulated absorption ability and transport through body fluids prevents any possible shortage even after many years of no intake (Carmel, 2008). Nevertheless, Cbl deficiency can have devastating and sometimes irreversible complications. So the correct sufficiency must be taken in account, especially with a reduced intake or compromised absorption.

Cbl is an organometallic factor composed of a tetrapyrrolic corrinic ring with a cobalt atom coordinated to four equatorial nitrogen atoms. The molecular scaffold is related to well-known prosthetic groups like the protoporphylinic ring of eme group of hemoglobin or cytochromes p450

of the electron transport chain, using the redox state of the metallic atom to produce conformational changes. The central cobalt atom is bound at the lower side with a 5,6-dimethylbenzimidazole base (DMB), with α-axial conformation. DMB is already linked to a lateral chain of the corrinic structure and its conformation seems to be crucial for the interaction with chaperones and final enzymes that use Cbl as a cofactor. At the upper side of the cobalt atom, there is the sixth ligand of the cobalt atom in the β-axial position. There is a variability of this chemical group with relevant significance in catalytic functions. A methyl group on the β-axial position forms a methylcobalamin variant of vitamin B12 (MetCbl), while a 5-deoxyadenosyl group bound to the cobalt atom forms the adenosylcobalamin isoform (AdoCbl). These two alkylcobalamin isoforms have the cofactorial function but there are also other isoforms such as hydroxocobalamin (OHCbl) with a hydroxyl group or cyanocobalamin (CNCbl) with a β-axial cyano group. The Cbl structure with more common β-axial ligands is displayed in Fig. 6.1.

CNCbl was the first isoform characterized by crystallographic techniques and it is currently called vitamin B12. Nowadays we know that it was an artifact of the extraction procedures, but CNCbl functions in the bloodstream are still far from being fully elucidated: maybe it is a



R	Cobalamin isoform
–OH	Hydroxocobalamin
–CN	Cyanocobalamin
$-CH_4$	Methylcobalamin
$-C_{10}H_{13}N_5O_3$	Deoxyadenosylcobalamin

Figure 6.1 Cobalamin structure and isoforms.
The structure of Cb with characteristic corrinic ring and with the most common β-axial ligands that characterize Cbl isoforms.

by-product of scavenger functions for cyanide. Moreover, it was widely described a cellular decyanase activity that enabled the utilization of CNCbl as a vitamin (Kim et al., 2008).

The need for Cbl arose from its ability of reverting hematological signs of pernicious anemia (PA). Nowadays we know that other B vitamins also have related interconnected functions and they take part in the metabolic pathways needed for the cellular replication and the energy production.

6.2 Cobalamin content in food

Because the biosynthetic ability is restricted to a few microorganisms, mostly anaerobes, humans usually need to take advantage of accumulation through the trophic chain in order to obtain a sufficient amount of Cbl from the diet. Ruminants benefit from the microbic biosynthesis of Cbl during vegetable fermentation by gastric microbiota. Thus ruminants accumulate more of the vitamin in their tissues than monogastric animals, such as poultry and pigs (Watanabe, 2007). Moreover, the bioaccumulation of Cbl in tissues is time-dependent so older cattle display higher concentrations of the vitamin (Williams, 2007). In addition, different cuts show variable concentrations depending on their oxidative or glycolytic metabolism tendency, as can be seen in Table 6.1. Red fibers type I (slow) have more mitochondria in the cytosol with a greater oxidative predisposition: on the one hand, cuts with a prevalence of white fibers type II (fast) have low concentrations of Cbl because of the glycolytic metabolism; on the other hand, lean meat has higher vitamin concentrations, due to the water solubility of Cbl (Ortigues-Marty et al., 2005). Table 6.2 summarizes the variability of Cbl concentrations among different species.

Cbl in food is usually photo- and thermolabile, so cooking oxidizes vitamins with the process enhanced in the presence of vitamin C, sulfites, and iron (Gille and Schmid, 2015).

The bioavailability of Cbl in milk seems to be higher compared to other animal food sources, despite its reduced concentration (Tucker et al., 2000). This phenomenon could depend on the vitamin fraction being bound to specific carriers that enhance absorption. Similarly to other food sources, thermal processing and storage in an oxidative environment decrease vitamin retention in milk (Gille and Schmid, 2015). During fermentation food processing some strains that are used for yogurt production (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) compete with Cbl utilization, thus reducing the final content (Arkbåge et al.,

Table 6.1 Variability of cobalamin (Cbl) (μg) concentration among cuts in different species.

Beef		**Lamb**	
Brisket	2.25	Foreshank	2.34
Rib eye steak	1.73	Leg	2.50
Shoulder top blade steak	4.33	Loin	2.04
Sirloin cap steak	2.64	Rib	2.09
Tenderloin	3.47	Shoulder	2.53
Pork		**Turkey**	
Leg (ham)	0.63	Breast	0.42
Shoulder	0.74	Leg	0.39
Sirloin	0.56	Wing	0.39
Spareribs	0.38		
Tenderloin	0.52		
Chicken		**Veal**	
Back	0.25	Leg (top round)	1.04
Breast	0.34	Loin	2.46
Drumstick	0.53	Rib	1.29
Leg	0.56	Shank	1.89
Thigh	0.62	Shoulder	1.67
Wing	0.25	Sirloin	1.27

Variability of Cbl concentrations in meat depends on species and cuts. Selected cuts (raw) from different species form US Department of Agriculture database are displayed.

Table 6.2 Mean and range of cobalamin (Cbl) concentration (μg) from different foods.

	Beef	**Pork**	**Chicken**	**Lamb**	**Turkey**	**Veal**
Mean	2.66	0.56	0.42	2.3	0.4	1.6
Range	1.73–4.33	0.38–0.74	0.25–0.62	2.04–2.53	0.39–0.42	1.04–2.46

The table summarizes means and range of Cbl concentrations of items from Table 6.1.

2003). However, some strains could be used in biotechnological production to improve Cbl retention in fermented products, and also plant-based products (Gu et al., 2015). In cheese production, the removal of the aqueous phase of whey decreases Cbl content in the final products (Arkbåge et al., 2003).

The highest source of Cbl is the liver, followed by the red meat of ruminants (Gille and Schmid, 2015). However, there is a reverse correlation between the concentration in food and bioavailability (Matte et al., 2012). The best way to absorb the highest fraction of Cbl is to spread discrete intakes of vitamin through the day to avoid the saturation of the absorption system (Allen, 2010).

Although there are official tables about Cbl contents in foods, there could be a wide variability among samples and among detection techniques used to quantify Cbl concentration. Different techniques have variable sensibilities to active and inactive vitamin forms (microbiological, HPLC, or radioisotope) (Gille and Schmid, 2015). Moreover, detailed US Department of Agriculture tables refer to Retail Cuts that could vary from European ones.

Microalgae are inadequate sources of Cbl because of the presence of recurrent inactive analogues (Watanabe, 2007). Inactive corrinoids are not useful for vitamin function and could also block chaperones that transport Cbl, so they cannot be available for active vitamin transport. Some plant foods could show small amounts of Cbl, due to an associated biofilm such as occurs for seaweed or fermented food like tempeh (Watanabe, 2007). Nevertheless, they cannot be considered reliable sources of Cbl because of a missed standardization of products; microorganisms able to perform the biosynthesis of the vitamin could not be represented in the starter pool. Currently, the dietary reference intake (DRI) for Cbl for the general population is 2.4 μg/day (Institute of Medicine, 1998). This requires a normal physiological absorption ability. Moreover, this quantity does not take into account the need of a single ingestion intake, such as in the case of supplementation in the vegetarian diet in the absence of other sources of Cbl or fortified foods. In a single ingestion, the limit of absorption depends on the saturation of transport system, which is capable of binding only 1.5−2 μg (Allen, 2010).

6.3 Absorption and transport through the body

A complex system of carriers escorts Cbl through extracellular fluids from the oral cavity to the final cellular site of utilization. These carriers have high affinity for Cbl but with different specificities that represent the pivotal characteristics of the transport system. Indeed it allows a selection of active molecules, avoiding the absorption of inadequate ones that could show antivitaminic effects.

Except when Cbl is taken as a supplement or by fortified foods (called the crystalline form), it can be found in the cofactorial (MetCbl and AdoCbl) form bound to food proteins or in the dissociated form after cooking or other processing (OHCbl).

The oral mucosa secretes the first carrier R-protein (transcobalamin I). This molecule binds Cbl after gastric dissociation from food. This protein protects Cbl from gastric acidity thanks to a glycosylated structure that is resistant to low pH (Hygum et al., 2011). This carrier has been found in various body fluids such as breast milk and plasma (Morkbak et al., 2007). With the progression to the duodenum, pancreatic proteases promote R-protein degradation with the release of Cbl that is promptly bonded to the second carrier secreted by gastric parietal cells: intrinsic factor (IF) or Castle factor. Also this protein protects the vitamin from enzymatic digestion, a theory that it is supported by the presence of a lot of glycosylation sites in the amino acid sequence (Gordon et al., 1991). In this scenario, different carriers have variable specificity: R-binder is a massive ligand with low specificity that binds also inactive nonvitamin corrinoids, such as a cobinamide that has lost a DMB group; despite IF being secreted in limited quantities, it has a high specificity for vitamin forms with intact DMB groups (Quadros, 2010). The rapid turnover of IF allows the regeneration of the most critical carriers to avoid the persistence of blocked forms by inactive corrinoids. In the terminal ileum, the IF−Cbl complex is internalized by the cubam receptor complex with a receptor-mediated endocytosis process, which is very specific for IF−Cbl, on the luminal side of a polarized enterocyte (Birn et al., 1997).

The cubam complex is located on the brush border of enterocytes and it is composed of a transmembrane domain formed by the amnionless protein (AMN) of 48 kDa that drives internalization, and an extrinsic protein of 460 kDa called cubilin that binds the IF−Cbl complex. Both proteins of the cubam complex are expressed also on the apical membrane of the proximal tubule cells of the kidney and on the visceral side of the yolk sac (Sahali et al., 1988).

After endocytosis, IF is degraded in the lysosome and Cbl crosses the intracellular lysosomial membrane with the help of LMBRD1, a transmembrane protein of 61 kDa (Rutsch et al., 2009). A schematic representation of the absorption process of Cbl is displayed in Fig. 6.2.

In the cytoplasm, Cbl follows cellular utilization or the release from the basolateral side of the enterocyte before entering the blood flow. The gateway for Cbl into the body fluids after digestion is thought to be the

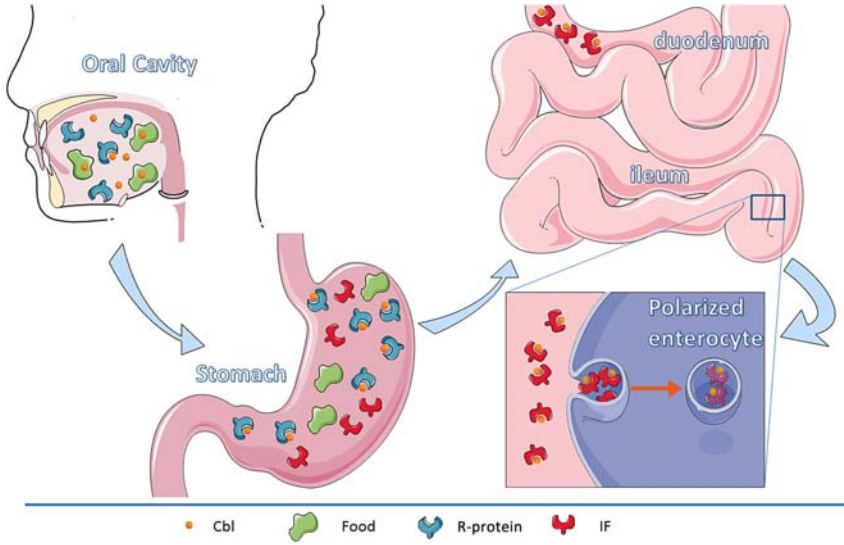


Figure 6.2 Cobalamin uptake.
The absorption process of Cbl from the oral cavity to cellular uptake is displayed. *Modified from Servier Medical Art database by Servier (Creative Commons 3.0).*

multispecific membrane transporter of 190 kDa called multidrug resistance protein 1 (MRP1) (Beedholm-Ebsen et al., 2010). However, the presence of another transporter not yet described or passive diffusion of Cbl in its free form could be possible (Deeley et al., 2006). The mechanism is not well understood, but it is known that Cbl is yielded to the blood carriers transcobalamin II (TCII) and haptocorrin (HC). As occurs for the transporter couple R-binder/IF, these two proteins also have different affinities for the vitamin with a selective significance. TCII is secreted by vascular endothelial cells and binds Cbl with high selectivity to form a complex called holotranscobalamin II (HTCII), which is the circulating bioavailable fraction of the vitamin (Quadros et al., 1989). The remaining Cbl, about 80% of the total blood vitamin, is bound to HC (Seetharam and Yammani, 2003). The function of HC is not well understood, but in light of its low selectivity compared to TCII, it is proposed to have a role in the removal of damaged vitamin molecules or has a vitamin reservoir function (Kanazawa et al., 1983). In fact a reverse transport to the liver is thought because HC receptors are characterized only on hepatocytes' membranes (Mørkbak et al., 2006). However, active molecules captured by reverse transport could be recovered by enterohepatic circulation.

Some microbe strains of gut microbiota are able to biosynthesize Cbl. However, in human feces Cbl is found mostly in the inactive form. Even if the active vitamin could be present in the gut after biosynthesis by symbiotic bacteria, the terminal tract of the bowel is far from the site of IF-mediated absorption. This means that the excess Cbl not bound to IF is lost with the progression to the distal tract of the gut and this phenomenon minimizes acute toxicity events in the case of a higher intake through food or supplements.

Cbl is a water-soluble vitamin, so the excess absorbed is then excreted with urine. There is a reuptake system in the proximal tubule of the kidney that recovers the vitamin through a 600 kDa protein called megalin or low-density lipoprotein receptor-related protein 2. Cbl can follow the cellular storage or bloodstream restitution. It was proposed that there is an interaction with the cubam complex coexpressed in this kidney cytotype. Megalin has high specificity for HTCII that justifies its role in the selection of active forms from urine (Nielsen et al., 2012).

The sophisticated uptake–reuptake process of Cbl allows an efficient economy of vitamin, so if there is a low intake the clinical signs of deficiency need many years to become overt. However, in the case of rapid utilization (such as in infancy or pregnancy) or when absorptive capacity is limited, the manifestation of the shortage speeds up.

The method of delivery to the central nervous system (CNS) is largely unknown, even if the neurological effects of Cbl deficiency have been widely explored.

6.4 Cellular trafficking and metabolism

Cbl enters peripheral cells via a receptor-mediated endocytosis process that involves a 58 kDa protein called CD320 or TCb1R, which belongs to the same receptor family as megalin and the LDL receptor (Quadros and Sequeira, 2013). CD320 is modulated by cellular proliferative signals that suggest the importance of Cbl in cellular replication (Amagasaki et al., 1990). In the lysosome, TCII is degraded and the receptor is recycled to the membrane to make it available again to bind a new Cbl molecule. Leaving the intracellular organelle, Cbl enters the cytoplasm to follow the cellular trafficking that adopts an articulated system of chaperones to deliver activated vitamin cofactor to the final molecular targets. In recent decades the understanding of this pathway has had progressed rapidly due to new molecular techniques that use cell cultures of fibroblasts. Rare inborn

mutations of genetic loci encoding for cellular chaperones of Cbl gave the opportunity to understand the multistep pathway from internalization to utilization.

The passage through the lysosomal membrane is mediated by LMBRD1 and ABCD4 proteins, products of genetic loci cblF and cblJ, respectively (Coelho et al., 2012). Within the cytoplasm, Cbl is promptly engaged by the first cytosolic chaperone MMACHC (methylmalonic aciduria type C and homocystinuria), a product of the cblC locus. This molecule is capable of carrying out the dealkylation of alkylcobalamins MetCbl and AdoCbl, mediating also the decyanation of CNCbl. This wide specificity of MMACHC for Cbl with different β-axial ligands highlights the pivotal role of this chaperone molecule (Kim et al., 2008). In detail, this protein uses a glutathione molecule for the dealkylation reaction while it needs a reduced flavin molecule for decyanation (Kim et al., 2008, 2009). Cbl in its provitaminic form (without an upper ligand) can follow the cytosolic pathway acquiring a methyl group, or the mitochondrial pathway where it will be transformed into AdoCbl. In the cytoplasm the OHCbl is engaged by MMADHC (methylmalonic aciduria type D and homocystinuria), a product of the cblD locus. This protein escorts the provitamin to methionine synthase reductase (MSR), a product of the cblE locus, which is the enzyme responsible for MetCbl formation (Coelho et al., 2008). MSR interacts with methionine synthase (MS), a product of the cblG locus, which catalyzes the transfer of a methyl group from N5-methyltetrahydrofolate to a homocysteine (HCY) molecule. MS is one of the two final molecular targets for the Cbl utilization currently known in mammals (Matthews et al., 2008). The catalytic reaction consists of two steps, during which methionine and Cbl(CoI) are generated as reaction intermediates. In a second step, the MetCbl is regenerated with the transformation of N5-methyltetrahydrofolate to tetrahydrofolate. Cbl (CoI) is one of the most reactive compounds currently known and this is why it is called a "supernucleophile" (Schrauzer and Deutsch, 1969). The role of MSR is to intercept molecules of Cbl occasionally oxidated to Cbl (CoII) in order to catalyze their reactivation through reductive methylation, using *S*-adenosylmethionine as a methyl group donor and NADPH as an electron donor. The methyl transfer is an exothermic reaction and drives the unfavorable coupled reduction (Banerjee et al., 1990).

The access to the mitochondrion could be mediated by a not yet characterized transporter, although passive diffusion from cytoplasm to mitochondrial trafficking machinery is not excluded. In the mitochondrial

matrix, the provitamin without an upper ligand is engaged by MMAB (methylmalonic aciduria type B), the ATP-dependent Cbl adenosyltransferase that is the product of the cblB locus, and that catalyzes the formation of AdoCbl (Leal et al., 2003). MMAB transfers AdoCbl to methylmalonyl-CoA mutase (MCM), the protein product of the *mut* locus, that is the final mitochondrial molecular target of coenzyme Cbl, one of the only two Cbl utilizer proteins known, together with the aforementioned cytosolic MS.

There is another mitochondrial protein involved in the Cbl pathway. Methylmalonic aciduria type A (MMAA) is the product of the cblA locus and has the role of maintaining Cbl in the AdoCbl active form for the catalytic cycle (Froese and Gravel, 2010). MMAA is a GTPase with the function of expelling Cbl(CoII) from the active site of MCM. This happens when occasionally 5′-deoxyadenosine groups escape from Cbl during the catalytic cycle, by blocking the enzyme (Padovani and Banerjee, 2009). Schematic cellular trafficking of Cbl is displayed in Fig. 6.3. Table 6.3 summarizes proteins involved in Cbl transport, trafficking, and metabolism.

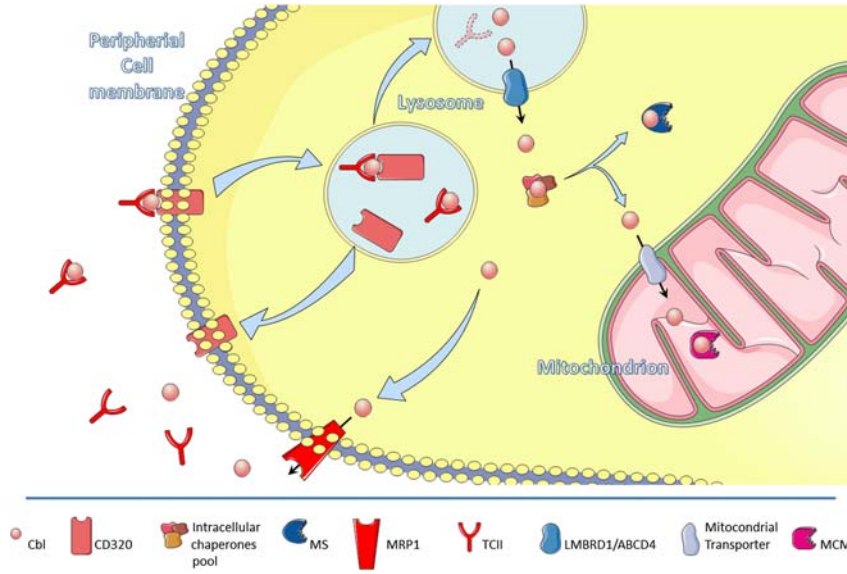


Figure 6.3 Cellular trafficking.
After the cellular uptake of Cbl, a complex pool of chaperones escorts the vitamin to the final enzymes. During these pathways, Cbl is deprived form β-axial ligand and equipped with specific residue to obtain vitamin isoforms. *Modified from Servier Medical Art database by Servier (Creative Commons 3.0).*

Table 6.3 Proteins (and their loci) involved in cobalamin (Cbl) homeostasis and trafficking.

Protein	Locus	Function	Location
R-protein		Binds free Cbl	Oral cavity and stomach
Intrinsic factor		Binds Cbl released from food	Small intestine
AMN	*AMN*	Mediates Cbl-IF internalization (cubam complex)	Enterocyte of the terminal ileum, apical side
Cubilin	*CUMN*	Binds Cbl-IF for the internalization (cubam complex)	Enterocyte of the terminal ileum, apical side
MRP1		Mediates the enter into blood flow	Enterocyte, basolateral side
Transcobalamin II		Transports Cbl through blood flow	Blood
Haptocorrin		Binds Cbl in blood, maybe for an inverse transport to liver	Blood
CD320		Binds Cbl-TCII and mediates its internalization	Peripheral cell membrane
Megalin		Mediates kidney reabsorption	Cell of renal proximal tubule, apical side
MMAA	cblA	Mediates adenosilation of Cbl	Mitochondrion
MMAB	cblB	Catalyzes adenosilation of Cbl	Mitochondrion
MMACHC	cblC	Catalyzes the decyaniation or dealkylation of Cbl	Cytosol
MMADHC	cblD	Routes Cbl to the intracellular destiny (mitochondrion or cytosol)	Cytosol
MSR	cblE	Catalyzes methylation of Cbl	Cytosol
LMBRD1	cblF	Escorts Cbl for exit the lysosome (in a complex with ABCD4)	Lysosomal membrane
MS	cblG	Catalyzes methylation of homocysteine using Cbl as cofactor	Cytosol
ABCD4	cblJ	Escorts Cbl for exit the lysosome (in a complex with LMBRD1)	Lysosomal membrane
MCM	*mut*	Synthesizes succinyl-CoA form methylmalonyl-CoA using Cbl as cofactor	Mitochondrion

Function and tissue location of factors involved in absorption, trafficking, and metabolism of Cbl. Complementation loci are also displayed.

MS has a pivotal role in methyl transfer reactions so it is crucial for nucleic acids synthesis. If one of the cofactors of the enzyme pool (including Cbl) is missing, the metabolic cycle is blocked by the accumulation of by-products of reactions such as HCY.

MCM is an enzyme of odd-chain fatty acids degradation. It is involved also in metabolic pathways of branched-chain amino acids and cholesterol. It catalyzes the conversion of methylmalonyl-CoA into succinyl-CoA, which can enter the Krebs cycle for catabolic utilization. The Krebs cycle accepts only two-carbon molecules so odd-chain fatty acids could not be completely catabolized without this pathway involving the Cbl cofactor. In the absence of AdoCbl, there is an accumulation of methylmalonic acid (MMA) as a by-product.

The central cobalt atom of Cbl structure can exist in three oxidative states (III), (II), and (I), that allow different coordination ligands: six, five, and four, respectively. The conformational state of Cbl is crucial for engagement to chaperones and for adequate intracellular trafficking. In fact in physiological conditions, the DMB group is coordinated to cobalt. This conformation is called "base-on" and protects the molecule from spurious reactivity. The protonation of DMB at low pH allows a change in conformation from "base-on" to "base-off." However, there is another conformational state in which Cbl is bound to a target protein with a "base-off/his-on" in which a histidine residue of the client protein (MS or MCM) is linked to a cobalt atom. This conformation enables the ready utilization of the cofactor in a catalytic process by controlling at the same time its reactivity (Banerjee, 2006). The lability of the β-axial coordination bond is pivotal for cofactor reactivity and the upper ligand shows different bond dissociation energies (Gherasim et al., 2013). IF and TCII bind Cbl only in the base-on conformation, while intracellular carriers MMAB and MMACHC bind vitamin in the base-off conformation, probably because of a higher reactivity of the β-axial position (Gherasim et al., 2013).

6.5 Cobalamin shortage and deficiency processes

If there are animal foods in the diet, the Cbl requirement is easily granted by physiological mechanisms (Allen, 2008). Unfortunately DRI does not take into account the variability in bioavailability of the vitamin in different foods, impairments in absorption capability, increased needs in shortage situations, and the net absorption in the case of a single-dose intake (Allen, 2009). Intakes far above the DRI show improvements in blood markers

with better outcomes among older adults (Dullemeijer et al., 2013). Apart from the shortages in the case of inborn defects on loci encoding transporters and chaperone proteins, there is a diffused deficiency state among the population that depends on malnutrition or a vegetarian diet without correct planning and supplementation. Cbl deficiency is typical of low-income countries and among elderly people worldwide because of absorption disturbances (Dali-Youcef and Andrès, 2009; McLean et al., 2008). There are also some clues that a global vitamin insufficiency has an impact on childhood and among women at reproductive age (Allen, 2009). During pregnancy and breastfeeding the transfer of Cbl to the fetus and the infant is crucial, and when the offspring is female it could establish a transgenerational deficiency which self-implements over time. The massive utilization of Cbl for cellular replication processes rapidly decreases vitamin deposits in the case of an inadequate intake (Green et al., 2017). Vitamin requirements are still important in infancy and adolescence and the shortage in these ages is a source of concern (de Benoist, 2008).

Even if PA, a historical pathology of Cbl deficiency, manifests with hematological and neurological signs, often the former could be absent and only CNS dysfunctions could appear during severe shortage (Lindenbaum et al., 1988). Neurological mechanisms need to be better understood but the role of Cbl is suggested by hyperhomocysteinemia involvement in neurotoxic pathways (Fuso and Scarpa, 2011).

The coexistence of Cbl deficiency and dementia with high frequency in senescence suggests multiple mechanisms of correlation between these two phenomena. The most harmful neurological effect of vitamin shortage is characterized by the demyelination of central and peripheral nerves, mostly irreversibly (Stabler, 2013).

Even if there are commercial isoforms of Cbl with different upper axial ligands, the most used form for fortification and supplement formulation is the crystal one (CNCbl), because of its safety and cheapness. Moreover, this provitaminic isoform has a good stability compared to the thermal- and photolability of alkylcobalamins that easily lose the DMB group because of the weak cobalt−carbon bond (Waddington and Finke, 1993). There is no advantage in using alkylcobalamins in place of CNCbl because, as already described above, the decyanation reaction is efficiently carried out by intracellular enzymatic machinery. The CblC complementation group of inborn errors is responsive only to OHCbl because of the inability of decyanation or dealkylation of the Cbl consumed (Andersson and Shapira, 1998).

The shortage of Cbl is more frequent in low-income countries, mostly due to insufficient nutrition or microbial infections (Yajnik et al., 2006). Vegetarians who do not take Cbl supplements or consume fortified foods show deficiency states with variable severity (Rizzo et al., 2016).

The American Institute of Medicine suggests supplementation with crystal Cbl in older people, because of an impaired absorptive functionality typical of this age, and define a vitamin bioavailability of about 50% in supplements (Institute of Medicine, 1998). Usually, these absorption issues depend on low gastric acidity that causes a food-bound malabsorption with the inability to dissociate Cbl from food proteins but without involving an IF dysfunction. The preserved IF activity allows a responsiveness to a physiological dosage of Cbl in a crystal form via the oral route.

Over 80 years old, the prevalence of Cbl deficiency can reach 23%–25% (Johnson et al., 2010).

In the case of PA, Cbl hypovitaminosis is caused by anti-IF or antiparietal antibodies that disrupt receptor-mediated endocytosis. This pathology can affect all ages but it is more frequent after 65 years old (Carmel, 1996). This clinical picture is coherent with the inability of the physiological oral intake of Cbl to grant the vitamin requirement.

Megadoses of oral Cbl can be still absorbed even with PA, due to passive diffusion of Cbl at very high dosage that concerns 1% of total amount taken in a single dose (Berlin et al., 1968). Oral treatment has advantages for compliance for patients and for logistical aspects compared to treatment with injections that often requires the involvement of professional care. Currently, there is no defined tolerable upper intake level for Cbl (Tucker et al., 2000).

Small intestinal bacterial overgrowth or other infections (*Helicobacter pylori*, *Giardia lamblia*, malaria, or tuberculosis) could promote deficiency through competition with vitamin absorption by microbes. Usually treatments for infection eradication resolve the shortage (Premkumar et al., 2012).

Ileal or gastric resections favor the establishment of clinically evident Cbl deficiency with a spectrum resembling food-bound malabsorption to PA because of reduced absorption, secretive alteration, or disturbance of enterohepatic circulation.

Celiac disease and inflammatory bowel disease can influence absorption processes, especially in the acute phase. Also alcoholism and prolonged pharmacological treatments could interfere with various steps of Cbl absorption. Proton pump inhibitors attenuate the gastric acidity

needed for the release of the vitamin from food and the following engagement by IF, while metformin interferes with Cbl internalization by sequestering calcium that is needed for endocytic cubam receptor process (Bauman et al., 2000). Cholestyramine, colchicine, and some antibiotics can block IF (Green et al., 2017).

Currently, there is no consensus or gold standard for adequate markers of Cbl deficiency. Taking into account that total blood Cbl concentration cannot discriminate between the active and inactive vitamin (bound to HC for reverse transport), low–normal blood concentrations are not preventive for Cbl shortage. Moreover, some hepatic and myeloproliferative diseases can raise blood HC (Arendt and Nexo, 2013). So in the process of evaluation for Cbl adequacy it could be helpful to verify normal hepatic functionality. Occasionally blood concentrations of Cbl show normal levels in diagnosed PA (Carmel and Agrawal, 2012). To better understand the vitamin status, it could be useful to measure HTCII, the only available form for cellular uptake (Nexo and Hoffmann-Lücke, 2011). However, total Cbl and HTCII are not adequate as markers for follow-up improvements during treatments because of their rapid rise after Cbl injection (Carmel, 2008). There are also functional markers, namely HCY and MMA, that are useful to verify cellular sufficiency (Green, 1995). Both markers can rise in the case of renal insufficiency, so an interpretation of their blood levels could be puzzling in older age (Loikas et al., 2007). Moreover, HCY increases also in the case of the shortage of other B vitamins, such as folate (vitamin B9) and pyridoxine (vitamin B6). Conversely, in the case of small intestinal bacterial overgrowth there is an increase of MMA in blood flow that can confuse the diagnosis. The increase in MMA may depend on bacterial propionate production captured by a portal system that acts as an MMA precursor (Leonard, 1997). Translocation of microbes to the upper intestinal tract can compete with Cbl utilization and produce inactive corrinoids that could distort blood Cbl detection by hiding a shortage below a normal apparent concentration (Degnan et al., 2014). Cbl heterotrophic utilization can take place in blind loop syndrome after the creation of surgical anastomosis. An increase of propionate also could derive from odd-chain fatty acids degradation from milk, thus affecting the utility of this marker in nursling infants (Monsen et al., 2003).

Even if not so specific there are other useful hematic markers in the case of Cbl shortage, because of the influence of this vitamin in cell replication. Mean corpuscular volume (MCV) is one of the signs of

macrocytic anemia in the case of hematopoiesis disturbance (Stabler, 2013). However, iron deficiency anemia causes microcytemia so it can mask this marker utility. Cbl deficiency can also disturb hematopoiesis of leukocytes by causing hypersegmented neutrophils. The progression of the shortage causes pancytopenia.

Using various blood markers may have enhanced the comprehension of metabolic pathways that use Cbl for a better prevention of a deficit by identifying the transition from subclinical to shortage conditions (Green et al., 2017). Nevertheless, from another point of view, these tools can cause overdiagnosis and overprescription, in some cases without any clinical relevance (Carmel and Sarrai, 2006).

Herbert proposed the use of multiple markers to define a staging of Cbl deficiency in vegetarians (Herbert, 1994). Even if there are different cutoff values depending on diagnostic method used or clinical reference method, Herrmann proposed a particularly higher threshold for total blood Cbl to define deficiency among vegetarians (Herrmann and Geisel, 2002).

Underestimating deficiency without an adequate program of treatment can lead to serious dysfunctions such as megaloblastic anemia and neurological disorders that can cause death.

There is a good characterization of inherited genetic defects of cellular trafficking but there is still 15%–20% of malabsorption caused by unknown recessive inborn errors that needs to be clarified by the characterization of other pivotal proteins (Shah et al., 2011).

6.6 How other vitamins are affected or behave

The B vitamins are a group of water-soluble vitamins not necessarily linked by structure homology but with interconnected metabolic functions in catabolic and anabolic pathways, in particular regarding one-carbon metabolism (Kennedy, 2016).

Even if the involvement of Cbl, folate, and pyridoxine in the HCY cycle is widely known and explored, the contribution of other B vitamins is often underestimated. For example, riboflavin (vitamin B2) is the coenzyme of methyltetrahydrofolate reductase, while niacin (vitamin B3) is converted to NAD and used as a cofactor for dihydrofolate reductase and *S*-adenosylhomocysteine hydrolase. Moreover, while interconnection among Cbl and folate in dementia has been highlighted (Reynolds, 2006), niacin also seems to have a role in Parkinson disease (Wakade et al., 2015).

Treatments with a pool of B vitamins seem to better improve HCY status than the use of just a single vitamin (Haskell et al., 2010).

The interplay between Cbl and folate is still debated. The folate trap is a phenomenon in which an elevation of serum folate depends on an HCY blockage because of Cbl shortage. Folate accumulates in the methyltetrahydrofolate form without the chance of a methyl transfer (Reynolds, 2006). In this case the high blood folate does not ensure repletion of peripheral cell deposits.

The rise of HCY in high-income countries often reflects a low folate status (Monsen et al., 2003). In countries with mandatory fortification program of flours with folate, HCY better reflects Cbl status (Green and Miller, 2005).

Cbl and folate have other interactions that are still unknown, such as the effect of folate supplementation when there is a Cbl deficiency. There is a higher risk of anemia and cognitive impairment in patients with low blood concentration of Cbl and high concentration of folate than in patients with low Cbl but normal folate (Morris et al., 2007). Moreover, a high intake of folate can mask early hematic signs of a Cbl shortage (MCV and hypersegmented neutrophils), delaying the diagnosis and increasing the risk of neurological damage and therefore of possible irreversible damage (Campbell, 1996). There are some clues suggested with the worsening neurological status in patients with a Cbl shortage after the intake of folate supplements, even with hematological improvement (Brito et al., 2016). These aspects need to be clarified carefully, taking into account that some countries have a fortification policy to prevent congenital malformation but that can cause uncontrolled vitamin intakes. This phenomenon could confuse the differential diagnosis and even worsen some medical aspects. Epidemiological and clinical trial studies focused on a subgroup of B vitamins are still needed, but some clues suggest a wider interdependence among B vitamins and there is the possibility that a more concerted approach to vitamin deficiency could be useful (Kennedy, 2016).

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