

Vitamin B₁₂ deficiency, beyond megaloblastic anemia: the role of alternative pathways for cellular uptake of vitamin B₁₂

La deficiencia de vitamina B₁₂, más allá de la anemia megaloblástica: papel de las vías alternativas de captación celular de vitamina B₁₂

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Abstract

Vitamin B₁₂ deficiency (cobalamin [Cbl]) causes megaloblastic anemia with/without neurological alterations. Cbl binds to transcobalamin (TC), giving rise to holotranscobalamin (holoTC), which is taken up by its receptor, TCblR or CD320; this is the canonical uptake pathway for all cells, but there are alternative pathways. Fetuses with congenital disorders of TC and TCblR/CD320 are viable; B₁₂ uptake is mediated by megalin/LRP2. At birth, its expression decreases markedly and these deficiencies become clinically apparent. However, the TC disorder causes severe hematological and neurological manifestations, while the TCblR/CD320 disorder causes mild symptoms and limited to the nervous system. This difference is attributed to the uptake pathway mediated by the LDLR (low-density lipoprotein receptor), present in all cells except in neurological ones. This function was discovered in the study of autoimmune central vitamin B₁₂ deficiency, as these patients present anti-TCbl/CD320 autoantibodies and neurological symptoms without hematological ones. All three are receptors of the LDLR family, and their involvement in the cellular uptake of vitamin B₁₂ opens new perspectives related to neurological symptoms or thrombosis. Finally, the holoTC-TCblR/CD320 uptake pathway is implicated in multiple sclerosis and in the uptake of drugs active against this disease, where Cbl plays an anti-neuroinflammatory role.

Keywords: Cobalamin. Vitamin B₁₂. Transcobalamin. TCblR. CD320. Megalin. LRP2. LDLR. Multiple sclerosis. Pernicious anemia.

Resumen

El déficit de vitamina B₁₂ (cobalamina [Cbl]) provoca una anemia megaloblástica con o sin alteraciones neurológicas. La Cbl se une a la transcobalamina (TC) dando lugar a la holotranscobalamina (holoTC), que es captada por su receptor, el TCblR o CD320; esta es la vía canónica de captación de todas las células, pero hay vías alternativas. Los fetos con trastornos congénitos de TC y TCblR/CD320 son viables; la captación de B₁₂ es mediada por la megalina/LRP2. Con el nacimiento se restringe su expresión y se manifiestan clínicamente las deficiencias, pero el trastorno de TC provoca manifestaciones graves hematológicas y neurológicas, mientras que en el de TCblR/CD320 son leves y se limitan al sistema nervioso. Esta diferencia se atribuye a la vía de captación mediada por el receptor LDLR (low-density lipoprotein receptor), presente en todas las células excepto en las neurológicas. Al hallazgo de este receptor se llegó a partir de la deficiencia central autoinmunitaria de vitamina B₁₂, ya que estos pacientes presentan autoanticuerpos anti-TCbl/CD320 y clínica neurológica sin

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afectación hematológica. Los tres son receptores de la familia LDLR y su participación en la captación celular de vitamina B₁₂ abre nuevas vías de investigación en relación con la clínica neurológica o la trombosis. Finalmente, la vía de captación holoTC-TCbIR/CD320 está implicada en la esclerosis múltiple y en la captación de fármacos activos frente a la enfermedad, donde la Cbl tiene un papel antineuroinflamatorio.

Palabras clave: Cobalamina. Vitamina B₁₂. Transcobalamina. TCbIR. CD320. Megalina. LRP2. LDLR. Esclerosis múltiple. Anemia perniciosa.

Introduction

The common feature of megaloblastic anemias is a defect in deoxyribonucleic acid (DNA) synthesis, which can result from a deficiency of vitamin B₉ (folate) or vitamin B₁₂ (cobalamin [Cbl]), or have other causes. Although it affects any cell, it particularly affects those with the fastest turnover. Regarding hematopoiesis, the consequence is characteristic cytological disorders in all series, indistinguishable in both deficiencies; the final result is ineffective hematopoiesis¹⁻³.

In addition to the typical hematological syndrome, other manifestations are observed in Cbl deficiency, particularly epithelial and neurological ones, the latter being rare in acquired folate deficiency but frequent in Cbl deficiency¹⁻³.

Regarding neurological manifestations, Cbl deficiency can produce highly variable symptoms, notably bilateral peripheral neuropathy, degeneration (demyelination) of the posterior and pyramidal tracts of the spinal cord (subacute combined degeneration), optic atrophy, and encephalopathy or dementia due to Cbl deficiency. The patient may experience paresthesias, muscle weakness, gait disturbances, dementia, psychotic disorders, or visual impairment. Prolonged Cbl deficiency in infancy can cause poor brain development. The clarification of the pathogenesis of neuropathy due to Cbl deficiency remains a challenge, despite the efforts of several research groups¹⁻³.

However, a notable fact has been known for years: most cases with neurological symptoms show hardly any hematological symptoms; that is, they do not present anemia or macrocytosis, or these are very mild, making it a diagnostic and pathogenic challenge^{1,2}. Such clinical discrepancy between hematological and non-hematological manifestations also occurs in other presentations of Cbl deficiency, which will be reviewed below³.

This leads us to the different forms of presentation of vitamin B₁₂ deficiency. For more comprehensive information, readers are referred to the chapters in the book *Eritropatología*³ by the Spanish Group of Erythropathology. In summary, the following are distinguished:

- The classic form of megaloblastosis, observed in pernicious anemia or folate deficiency associated with alcoholism.
 - Megaloblastosis masked by another anemia, such as iron deficiency, by inappropriate treatment, or by falsely normal or elevated vitamin B₁₂ levels.
 - Acute presentation forms, characterized by severe acute cytopenias, especially thrombocytopenias, whose prototype would be megaloblastosis associated with methotrexate that has not been properly rescued with folinic acid.
 - Atypical or subclinical forms of presentation⁴, which do not have typical symptoms, but laboratory study of metabolites demonstrates a deficiency. The prevalence of these atypical or subclinical forms is high and increases with age, and they have been associated with recurrent miscarriages and neural tube defects, dementia, and thrombotic events. This category can also include the so-called autoimmune central vitamin B₁₂ deficiency and cerebral folate deficiency syndrome, which are characterized by exclusively neurological clinical manifestations.
- Recently, a group consisting mostly of rheumatologists and neurologists, in a pioneering work⁵, described a new condition: autoimmune vitamin B₁₂ central deficiency (ABCD). Their findings are reminiscent of cerebral folate deficiency syndrome, in which autoantibodies directed at folate receptors in the choroid plexus are associated with low levels of 5-methyltetrahydrofolate in the cerebrospinal fluid (CSF) even when blood levels are normal⁶.

Although studies on vitamin B₁₂ began as early as the 1960s, the full extent of the biochemical alterations remains not completely elucidated. It has been known for decades that Cbl is an essential water-soluble vitamin for two metabolic reactions: it acts as a cofactor for methionine synthase and L-methylmalonyl-CoA mutase. However, other metabolic aspects are less well known but of great clinical interest; to mention a few, its role in Cbl-dependent photoreceptors, in the quality control processes of proteins in the ubiquitin-proteasome system, anti-inflammatory effects in macrophage

populations (microglia, ileal macrophages), control of oxidative stress, modulation of gene expression through epigenetic modifications, etc. A review of the different pathogenic mechanisms involved is beyond the scope of this work, so readers are referred to references 1 and 3.

Here, two important aspects will be reviewed: how Cbl uptake occurs in different tissues and different developmental periods, as new aspects have been discovered in recent years, and their clinical repercussion on the pathogenesis of Cbl deficiency.

Mechanisms of cellular cobalamin uptake: role of cellular receptors

Knowledge of Cbl metabolism has increased considerably, especially thanks to studies on the mechanisms by which cells take up Cbl and, consequently, their involvement in the pathogenesis of Cbl deficiency. These studies have demonstrated that Cbl receptors present redundant functions in their uptake of Cbl, but at the same time have differentiated tissue specificity in Cbl transport. This leads to the hypothesis that the different tissue specificity of receptors could contribute to the highly variable clinical symptoms of Cbl deficiency.

Transcobalamin and its receptor

The uptake of cellular Cbl mediated by holotranscobalamin (holoTC) and its receptor (TCblR or CD320) is considered the canonical pathway for cellular Cbl uptake. HoloTC is the result of the binding of Cbl with the transcobalamin (TC) transporter, which occurs after Cbl absorption in the terminal ileum. This circulating holoTC binds to the extracellular domain of its receptor, TCblR/CD320, which results in the cytoplasmic internalization of the holoTC-TCblR/CD320 complex in an endocytic vesicle. The vesicle fuses with a lysosome, and TC and TCblR/CD320 dissociate and degrade. Free Cbl is exported out of the lysosome for use in Cbl-dependent reactions. Since it is not recycled, the appearance of new TCblR receptors on the cell surface depends entirely on its *de novo* synthesis and, subsequently, on the localization of new receptors in the plasma membrane (Fig. 1).

TCblR/CD320 belongs to the large family of low-density lipoprotein receptors (LDLR). This complex system plays an essential role in regulating plasma and intracellular cholesterol homeostasis, but is also involved in the transport of other essential substances. TCblR/CD320 contains in its extracellular N-terminal fragment an epidermal

growth factor-like (EGF-like) domain and two LDLR type A sites, where holoTC binding occurs⁷. O-glycosylation of the receptor⁸ and LDLR-A domains are essential for its proper functioning^{9,10}, while the cytoplasmic tail is fundamental for holoTC internalization^{1,7,10}.

This receptor is present in all cells, but the amount present on the cell membrane is closely related to the cell division cycle, and its presence on the cell surface is high in rapidly growing cells, including cancer cells^{1,11}. It is interesting to consider its expression on the renal and intestinal apical surface, although its function in these organs remains unknown^{6,12}. It can be speculated as a possible complementary alternative mechanism for intestinal Cbl absorption and renal Cbl reabsorption.

To understand its pathology, it is very important to consider that in these same cells there are other receptors of the same family capable of transporting Cbl (redundancy), although not all receptors are present in all tissues.

Polymorphisms in TCblR/CD320 have been identified that are associated with low serum Cbl levels¹³ and others with high holoTC levels¹⁴. In a study of newborns, several cases with the deletion of a single codon c.262_264GAG (p.E88del) and elevated methylmalonic acid concentration were observed¹⁵. However, in another population study with the same mutation p.E88del, analytical data suggest that Cbl metabolism was not affected¹⁶. Current knowledge suggests that the clinical consequences of abnormalities of this receptor are minimal.

However, in view of the scarce and contradictory data, more studies are required to clarify the natural evolution of TCblR/CD320 variants, without being able to rule out mutations with a more severe effect on the protein that result in lethality¹⁶.

Knockout (KO) mouse models have been created, and recently, the presence of anti-TCblR/CD320 antibodies has been detected⁵. These topics will be discussed later.

Embryonic period

High expression of TCblR/CD320 has been observed during the embryonic period in the placenta and other embryonic tissues, although cellular Cbl uptake during this period involves other cellular receptor-mediated transport systems of the same family. This conclusion was reached because the genetic defects of TC and TCblR/CD320, the canonical pathway for cellular Cbl uptake, do not produce embryonic lethality^{1,11}, and therefore other Cbl uptake pathways must exist.

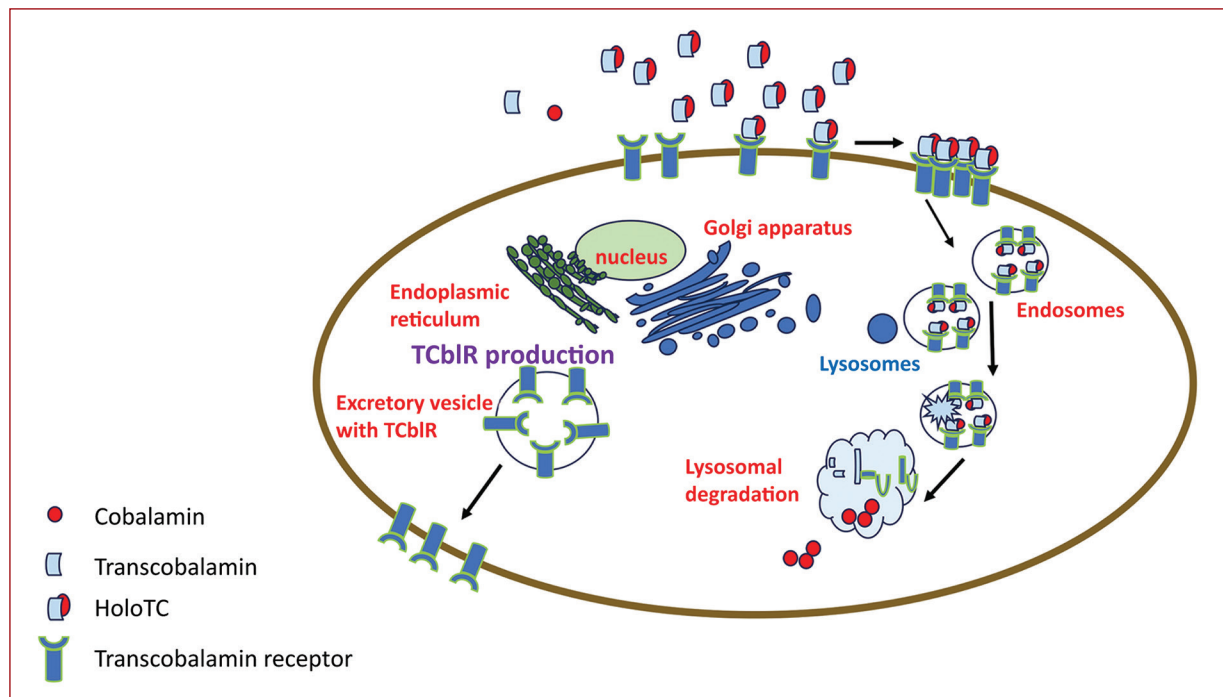


Figure 1. Uptake and utilization of cobalamin (Cbl) in the cell. This figure shows the canonical pathway of Cbl uptake by cells. Cbl absorbed in the intestine binds to transcobalamin (TC) (holoTC), and is thus transported by the blood to its cellular receptor, TCblR or CD320, present on the cell surface of all cells. The holoTC complex binds to the extracellular domain of the receptor. This results in the internalization of holoTC and the receptor into an endocytic vesicle. The vesicle fuses with a lysosome, and holoTC and TCblR/CD320 dissociate and degrade. The appearance of new receptors depends completely on the synthesis, processing, and localization of new receptors in the plasma membrane. Free Cbl is exported out of the lysosome, and once in the cytoplasm, a part is converted to methyl-Cbl and participates as a cofactor in numerous methylation reactions (homocysteine synthesis, DNA synthesis). An additional amount of Cbl is transported to the mitochondria and converted to 5'-deoxyadenosyl Cbl for use in the conversion of methylmalonyl coenzyme A (CoA) to succinyl-CoA. The production of succinyl-CoA feeds the tricarboxylic acid cycle, necessary for optimal mitochondrial function and for the production of glycolipids and sphingomyelin (adapted from Gick et al.¹⁷ and Arora et al.³⁰).

Congenital disorders of TC and TCblR/CD320 are classified within the cluster of Cbl bioavailability disorders, along with congenital deficiencies of gastric intrinsic factor, ileal receptor (or Imerslund-Gräsbeck syndrome types 1 and 2), and haptocorrin (or cobalophilin), and CblF and CblJ disorders related to Cbl exit from the lysosome¹⁷.

TC deficiency causes severe hematological and neurological complications that could be fatal if left untreated. Clinical symptoms appear in the first weeks after birth, not during gestation. In contrast, defects in the *TCblR/CD320* gene produce very mild neurological changes restricted to the nervous system after gestation. This clear clinical difference between the two congenital disorders suggests the existence of alternative pathways to the canonical Cbl uptake pathway^{3,17}. Studying this transport, it was found that another

receptor of the LDLR family, megalin (LRP2, LDL receptor related protein 2)¹⁸⁻²¹, acted as an alternative mechanism for Cbl transport during embryonic development, as has been verified using a KO model of TCblR/CD320²², although this does not exclude other possible mechanisms besides megalin that are not yet known.

Megalyn/LRP2, which also has the ability to bind to holoTC, shows broad tissue expression during the embryonic period. However, at birth, this broad tissue expression becomes very restricted and, therefore, ceases to be an alternative mechanism for Cbl uptake in most tissues, including hematopoietic tissue. This new tissue redistribution means that in the newborn, there is a lack of Cbl uptake through this pathway, which was functional during the embryonic period. As a consequence, in congenital TC deficiencies, symptoms

appear within a few weeks of birth^{3,17,22}. In adults, megalin/LRP2 is expressed primarily in the brain and kidney, where it is involved in the reabsorption of Cbl and multiple other ligands¹⁸⁻²¹.

Megalyn/LRP2 is a receptor for more than 75 putative ligands, including Cbl. It is a member of the LDLR family and combines three types of modules in its ectodomains, including several LDLR-A type repeats, like TCbIR/CD320¹⁸⁻²¹. In adults, megalyn/LRP2 was originally identified in the kidney as an autoantigen and was called megalyn due to its large size. It is expressed particularly in the apical membrane of the proximal tubules of the kidney and in other epithelia (inner ear, thyroid, parathyroid, and alveoli). Megalyn/LRP2 is also expressed with lower intensity in the central nervous system (CNS), where it performs key signaling functions for its development. Megalyn/LRP2 recovers low molecular weight proteins that escape the glomerular filtration barriers, a crucial component of normal renal function. This function of megalyn/LRP2 has been implicated in the Cbl deficiency that can be observed in nephrotic syndrome¹⁸⁻²¹.

Loss of function of megalyn/LRP2 in humans leads to Donnai-Barrow syndrome (OMIM #222448), characterized by low molecular weight proteinuria and CNS disorders. This syndrome is consistent with a role for megalyn/LRP2 as an essential endocytic receptor for capturing ligands in both the kidney and the brain²³.

Other mechanisms of Cbl uptake

Pluvinae et al.⁵ have described a vitamin B₁₂ deficiency restricted to the CNS. In its pathogenesis, an autoantibody directed against TCbIR/CD320 was found. Patients show significant neurological symptoms but without hematological signs of Cbl deficiency. In view of this clinical picture, the hypothesis of other alternative Cbl uptake pathways that compensate for the systemic deficiency of TCbIR/CD320 is raised.

To identify the possible alternative pathway, a high-throughput genetic screen using CRISPR interference was performed²⁴. Thanks to this study, another member of the LDLR family was identified as a receptor for Cbl uptake: the LDLR. To study this pathway of cellular holoTC uptake by LDLR, functional studies were performed using hematological (K562 line), renal (HEK293T line), and primary cerebral endothelial cells. In hematopoietic and renal cells, inhibition of LDLR or TCbIR/CD320 decreased holoTC uptake; however, combined inhibition of both was necessary to eliminate holoTC uptake. In contrast, inhibition of TCbIR/CD320 alone

was sufficient to eliminate uptake in cerebral endothelial cells, which do not express LDLR appreciably. The findings suggest that LDLR contributes to holoTC internalization in hematopoietic and renal cells, providing an alternative pathway for Cbl uptake when TCbIR/CD320 is altered. It would also explain the striking clinical differences between the severe TC deficiency and the mildness of TCbIR/CD320 deficiency. Together, these findings proposed LDLR as a candidate for non-canonical Cbl uptake outside the CNS, at least in adults⁵.

The uptake of cobalamin: role of the low-density lipoprotein receptor gene family (Table 1 and Fig. 2)

The cellular receptors of the LDLR family constitute a complex group of receptors. In addition to being involved in lipoprotein metabolism, they have multiple functions, some poorly studied^{5,25-28}.

This family includes highly specific LDLRs and LDL receptor-related proteins or LRPs, such as LRP1, LRP1b, LRP2 (megalyn), LRP4, LRP5/6, and LRP8 (ApoER2). TCbIR/CD320 should also be added to this group. Members of the LDLR family are divided by size into small (LDLR and TCbIR/CD320) and large (LRP2/megalyn)²⁵.

These receptors, present on the surface of all cells, participate in the endocytosis of their specific ligands. They are a class of multifunctional transmembrane receptors closely related to lipid metabolism and cardiovascular homeostasis, but with diverse functions ranging from embryonic development to cancer²⁵⁻²⁸. This receptor family also has the function of Cbl uptake, in which at least three would be involved: TCbIR/CD320, LRP2/megalyn, and LDLR^{5,18,19,21}.

As has been discussed, regarding uptake, these receptors show differentiated tissue distributions. Some cells present several at once and, in these cases, their function is, at least partially, redundant. It remains to be clarified what the differentiated function of each one is in Cbl uptake when several are present, their role in tissues such as the kidney (Cbl reabsorption) or the intestine (dietary Cbl absorption), and the role of the rest of the members of this family^{5,21}.

As for LDLR, it is a promiscuous cell surface receptor whose main function is the cellular uptake of cholesterol-rich lipids. The normally encoded protein is bound to the cell membrane, where it binds to LDL cholesterol, and the lipoprotein is absorbed by the cell. Lysosomes release cholesterol into the cytoplasm, which is then

Table 1. Pathways of cellular cobalamin uptake. Role of the LDLR receptor family

Variable	Function	Distribution	Pathophysiology
TCbIR/CD320	Canonical holoTC uptake pathway	All cells	Autoantibodies: ABCD Congenital deficiency (mild) Multiple sclerosis High B ₁₂ doses in neuropathies?
Megalín/LRP2	Alternative uptake pathway of holoTC Cbl?	Fetus: all cells After birth: kidney and brain	Ensures fetal viability in congenital deficiencies of TC and TCbIR/CD320
LDLR	Alternative uptake pathway of holoTC Cbl?	All cells, except brain cells	No hematological manifestations in ABCD and congenital TCbIR/CD320 deficiency Thrombosis and B ₁₂ deficiency?
Other LDLR family	HoloTC uptake? Cbl?	?	Thrombosis and B ₁₂ deficiency?

Cbl: cobalamin (vitamin B₁₂); ABCD: autoimmune B₁₂ central deficiency; holoTC: holotranscobalamin; LDLR: low-density lipoprotein receptor; LRP2: lipid-related protein 2; TC: transcobalamin; TCbIR: transcobalamin receptor.

used in cellular lipid metabolism^{25,28}. While TCbIR/CD320 is relatively enriched in the blood-brain barrier, LDLR is poorly represented⁵. Additionally, it is found at the hematopoietic, intestinal, renal, and placental levels⁵ (Fig. 2). Mutations in the *LDLR* gene cause the autosomal dominant disorder called familial hypercholesterolemia²⁸.

Autoimmune central vitamin B₁₂ deficiency: role of anti-TCbIR/CD320 antibodies

In the CNS, the essential role in Cbl uptake of the holoTC-TCbIR/CD320 pathway has been demonstrated from studies in patients with congenital abnormalities of TCbIR/CD320, KO models for TCbIR/CD320, and, more recently, the presence of anti-TCbIR/CD320 antibodies^{5,16,21,22,29-32}.

As mentioned earlier, from the screening of methylmalonic acidurias in newborns, several cases with congenital abnormalities of TCbIR/CD320 have been described. Unlike the severe congenital TC deficiency, the cases currently known with congenital deficiencies of TCbIR/CD320 present few abnormalities³¹, have no hematological involvement, and neurological involvement is of little importance (it has been related to speech disorders)^{15,16,31}.

In murine KO models of TCbIR/CD320, with a normal Cbl diet, hardly any abnormalities are observed; however, with a Cbl-poor diet, they present Cbl deficiency in the brain, with minimal hematological repercussion³⁰.

In other words, humans with TCbIR/CD320 mutations and KO models present normal hematology despite methylmalonic aciduria^{15,16,29-32}. With a Cbl-poor diet, a

selective cerebral deficiency will appear³⁰. Additionally, as already mentioned, some people with acquired systemic Cbl deficiency may present isolated neuropsychiatric symptoms without anemia^{2,3}.

ABCD was described in the course of an immunological investigation, carried out in cases with rheumatological or neurological pathology by American rheumatologists, immunologists, and neurologists⁵. The researchers used a massive autoantibody evaluation (phage immunoprecipitation sequencing [PhIP-seq])³³. As a result, they found eight cases with an autoimmune vitamin B₁₂ deficiency restricted to the CNS, which they called ABCD. In the pathogenic studies of ABCD, they discovered the alternative pathway for vitamin B₁₂ uptake in hematopoietic cells mediated by LDLR, which was discussed earlier.

The pathogenic model proposed for ABCD (Fig. 2) involves an alteration of vitamin B₁₂ transport across the blood-brain barrier due to the presence of anti-TCbIR/CD320 autoantibodies. Autoantibodies in the blood alter the uptake function of TCbIR/CD320 by cerebral endothelial cells.

HoloTC must cross multiple cell membranes on its way to the CSF, including those of endothelial cells, pericytes, and astrocytes. Anti-TCbIR/CD320 antibodies in the CNS can interfere with holoTC uptake by metabolically very demanding cell types (neurons, oligodendrocytes), causing neurological sequelae. These effects may depend on antibody titer, duration of antibody seropositivity, and host vulnerability.

In the eight cases reported⁵, neurological and neuroimaging clinical findings compatible with Cbl deficiency are observed. Brain and spinal cord magnetic

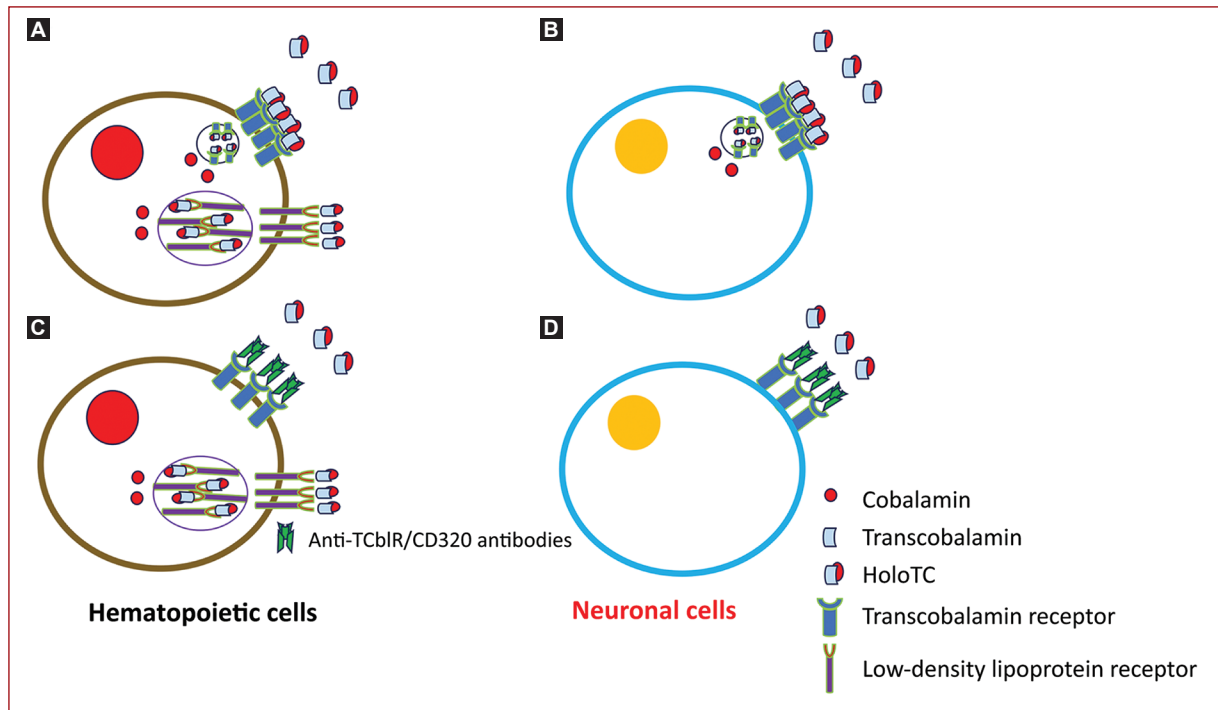


Figure 2. Alternative low-density lipoprotein receptor (LDLR) pathway in cobalamin (Cbl) uptake. LDLR is an alternative receptor for Cbl uptake. Individuals with autoantibodies against the transcobalamin receptor (TCbIR/CD320) or with congenital TCbIR/CD320 disorders do not present hematological manifestations of Cbl deficiency, but do present neurological ones. Therefore, there are alternative Cbl uptake pathways capable of compensating for TCbIR/CD320 deficiency or malfunction. The alternative pathway is LDLR, a receptor whose main function is the cellular uptake of cholesterol-rich lipids. LDLR is capable of internalizing holoTC (**A**, **B**, and **C**). While TCbIR/CD320 is present in the blood-brain barrier, LDLR is not (**C** and **D**). TCbIR/CD320 binds to holoTC through two extracellular LDLR-A domains. The role of LDLR in non-canonical Cbl uptake, outside the central nervous system, was evidenced by inactivating LDLR, alone or in combination with TCbIR/CD320, in granulocytic lineage cells (K562 leukemic line), primary cerebral endothelial cells, and renal cells (HEK293T line). Inactivation of LDLR or TCbIR/CD320 in hematopoietic and renal cells resulted in a decrease in holoTC uptake. However, combined inactivation of both receptors is necessary to eliminate holoTC uptake (**A** and **C**). In contrast, suppression of TCbIR/CD320 alone was sufficient to eliminate holoTC uptake in cerebral endothelial cells, which do not express LDLR appreciably (**D**). These findings suggested that LDLR contributes to holoTC internalization in hematopoietic cells (**A** and **C**), providing an alternative pathway for Cbl uptake when TCbIR/CD320 is altered. The identification of tissue-specific Cbl uptake pathways provides a possible explanation for the isolated neuropsychiatric manifestations of systemic Cbl deficiency. TCbIR/CD320 autoantibodies impair Cbl transport to the brain (**D**), while LDLR can still transport Cbl to blood cells (**C**) (adapted from Jonnalagadda et al.⁴⁴).

resonance imaging showed atrophy and asymmetric and multifocal hyperdense lesions in the cerebral white matter and in the dorsal posterior columns of the spinal cord. In contrast, the cases did not present hematological abnormalities (blood count, normal B₁₂ and metabolite levels). When studying the CSF, vitamin B₁₂ and holoTC were decreased, and methylmalonic acid was elevated. The authors report that immunosuppressive treatment and systemic supplementation with high doses of B₁₂ were associated with an increase in CSF vitamin B₁₂ and clinical improvement, but only two

patients received treatment with Cbl, both orally, and only one of these received parenteral Cbl for 1 week.

Continuing with this interesting study, it is striking that patients with neurological symptoms related to Cbl did not receive adequate treatment with parenteral Cbl, which is the recommended approach³⁴⁻³⁶. However, the increase in CSF Cbl concentration after treatment in the case that received parenteral Cbl suggests that high serum B₁₂ concentrations might be sufficient to compensate for blood-brain barrier transport defects caused by anti-TCbIR/CD320 antibodies⁵.

For diagnosis purposes, it would have been interesting to know the homocysteine levels in the CSF³⁷, which would very likely be elevated like methylmalonic acid. However, they were not evaluated in any of the eight cases with ABCD. Currently, homocysteine determination, despite its pre-analytical limitations, is available in many laboratories, while methylmalonic acid determination is not. Another diagnostic drawback is that the vitamin B₁₂ levels detected in the CSF are well below the detection threshold of the automated methodologies used in clinical laboratories. Vitamin B₁₂ measurement in CSF requires special methodologies, such as a competitive enzyme-linked immunosorbent assay commercial kit⁵. In any case, in the appropriate clinical context, the elevation of methylmalonic acid in the CSF would suggest the diagnosis of ABCD.

In addition to the eight cases, in the same study, autoantibodies directed at the same TCbIR/CD320 epitope were identified in 6% of healthy controls and in 4% of samples from patients with neurological diseases⁵. The relationship between multiple sclerosis and the canonical Cbl uptake pathway will be discussed below.

Of special interest are the results obtained when comparing the seropositivity of anti-TCbIR/CD320 in 408 patients with non-neurological systemic lupus erythematosus (SLE) versus 28 patients with SLE with neurological symptoms⁵. The seropositivity rate in SLE without symptoms was 5.6%, concordant with the seropositivity rate in healthy controls (6%). In contrast, anti-TCbIR/CD320 positivity was four times more prevalent in cases with neurological symptoms (21.4%; odds ratio: 3.97). These findings suggest that anti-TCbIR/CD320 could contribute to neurological deficits in this subset of patients with SLE⁵. Obviously, these patients require a special study to assess the usefulness of Cbl treatment, extendable to other autoimmune rheumatological pathologies with neurological alterations.

A development model for ABCD has been proposed, which has been divided into three stages: 1) seropositive without clinical symptoms or metabolic signs; 2) subclinical without clinical symptoms, but with metabolic signs; and 3) symptomatic with clinical symptoms and metabolic signs⁵. The transition from seropositive to subclinical ABCD may depend on the serum concentration of vitamin B₁₂ and the autoantibody titer. The transition from subclinical to symptomatic may depend on the degree and duration of seropositivity, and on the underlying comorbidity that predisposes to neurological fragility.

In the same vein as the previous study⁵, a dermatological study in cutaneous arteritis, also using massive autoantibody screening methods, found eight positive cases for anti-TCbIR/CD320 among 34 patients (24%)³⁸. Cutaneous arteritis is a vasculitis exclusively of the skin. In that study, animal models with cutaneous arteritis, both *in vitro* and *in vivo*, demonstrated a beneficial effect of methylcobalamin treatment³⁸.

Multiple sclerosis: role of cobalamin and TCbIR/CD320

This is a topic that has been under investigation in recent years, given the evident similarity between the neurological symptoms of multiple sclerosis and those of Cbl deficiency.

It is worth recalling the anti-inflammatory effect of vitamin B₁₂ and other B vitamins, such as B₁, B₂, B₃, B₅, and B₆. Studies with Cbl in macrophage cells (microglial cells and intestinal macrophages) have demonstrated changes in the phenotypic profile from M1 to M2 type. Vitamin B₁₂ functions as an anti-inflammatory agent by binding to CD14 and, therefore, preventing activation of Toll-like receptor 4. This evidence supports the idea that Cbl administration may alleviate inflammation associated with brain pathologies, adding a new layer of complexity to understanding the role of vitamin B₁₂ in neuropathology^{1,39-42}.

In contrast, vitamin B₁₂ deficiency causes an increase in free radicals, tumor necrosis factor alpha, and other pro-inflammatory interleukins (such as interleukin 1β), and a decrease in EGF synthesis in the CNS. This imbalance contributes significantly to myelin damage and vacuolization. Additionally, pro-inflammatory cytokines can activate N-methyl-D-aspartate receptors, causing excessive calcium entry that triggers cytotoxicity. Also, in this deficiency, homocysteine accumulates, which causes an excessive accumulation of S-adenosylhomocysteine, which in turn inhibits methyltransferases.

Several studies have verified a relationship between vitamin B₁₂ and multiple sclerosis. Thus, supplementation with vitamin B₁₂ at high doses may be effective in improving some visual parameters in multiple sclerosis and other neurodegenerative diseases^{1,39}. Also, in relapsing-remitting multiple sclerosis, higher homocysteine values and lower vitamin B₁₂ levels have been observed than in control groups^{1,39,40}, although without observing a relationship between genetic polymorphisms of several enzymes of Cbl metabolism and the risk of multiple sclerosis⁴⁰.

It is a well-established fact that acquired Cbl deficiency usually manifests with neurological symptoms that can simulate multiple sclerosis, and that these will respond to replacement treatment with this vitamin, generally at high doses and parenterally. Special mention deserves a rare congenital error, although it is the most common of Cbl metabolism: methylmalonic acidemia with homocystinuria type CblC. Patients with late-onset CblC usually show symptoms similar to those of multiple sclerosis, although with very broad clinical variability. An adequate diagnosis and treatment with Cbl at high doses can lead to pronounced improvement or disappearance of symptoms^{41,42}. Cbl-related diseases are so chameleon-like that they should always be considered in the differential diagnosis of demyelinating diseases^{5,41,42}.

Focusing on multiple sclerosis, this is a neuroinflammatory disease that produces demyelination and neurodegeneration in the CNS. As already mentioned, its clinical spectrum is similar to the neurological sequelae observed in Cbl deficiency⁴³, suggesting a possible overlapping disease mechanism; in fact, Cbl, as mentioned in the introduction, has an anti-neuroinflammatory effect^{1,50}.

In this sense, the lack of Cbl worsens the symptoms of multiple sclerosis, while its presence has a drug-enhancing effect in the treatment of this and other neurodegenerative diseases⁴⁴. A reduced expression of TCbIR/CD320 has been observed in multiple sclerosis plaques⁴⁵. As mentioned earlier, TCbIR/CD320 mediates holoTC uptake in the CNS⁴³. Pharmacological studies with drugs used in multiple sclerosis, such as FTY720 (fingolimod), have allowed studying the relationship between holoTC-TCbIR/CD320 and multiple sclerosis⁴⁴.

Studies with FTY720 are of special interest in relation to Cbl metabolism in the brain. This drug was the first disease-modifying therapy approved by the Food and Drug Administration for the treatment of relapsing multiple sclerosis. FTY720 functionally antagonizes the sphingosine 1-phosphate receptor (S1P₁) and, as a consequence, suppresses neuroinflammation^{43,44}. The mechanism of action of FTY720 is functionally and physically related to CNS Cbl uptake pathways. In this sense, it has been verified that genetic and pharmacological inhibition of S1P₁ positively regulates the expression of the holoTC receptor, TCbIR/CD320, at the neurological level^{44,45}.

It has been shown that holoTC acts as a chaperone for sphingosine and its analog, the drug FTY720, with both forming a complex with holoTC that is internalized via TCbIR/CD320, suggesting a mechanism of entry of

FTY720/sphingosine via this receptor. FTY720, after its phosphorylation in the cytoplasm of the astrocyte, functionally antagonizes S1P₁, which results in positive regulation of the *TCbIR/CD320* gene and an increase in the production of this receptor in astrocytes⁴⁴. In this experimental pharmacological model, TCbIR/CD320 deficiency or dietary vitamin B₁₂ restriction worsens multiple sclerosis and suppresses the efficacy of drugs such as FTY720, while decreasing type I interferon β signaling⁴⁴.

Therefore, it should be noted that the physical binding of FTY720 to holoTC and Cbl signaling in the CNS provides metabolic links for the recognized similarities between multiple sclerosis and Cbl deficiency^{43,44} (Fig. 3).

In the work by Pluvinage et al.⁵, anti-TCbIR/CD320 antibodies were observed in 4% (5 of 132 patients) of samples from multiple sclerosis or other neurological diseases. In this group of 132 patients, when paired blood and CSF samples were studied, the detection of anti-TCbIR/CD320 in blood predicted B₁₂ deficiency in the brain. However, none showed hematological signs of Cbl deficiency. Anti-TCbIR/CD320 titers in healthy controls and neurological patients, in general, were low.

These experimental data emphasize the pathogenic, metabolic, and clinical differences in neurological and hematological involvement of vitamin B₁₂ deficiency, without ruling out that they may sometimes overlap⁴⁴.

Future perspectives

Considering the complexity of the pathogenesis of Cbl deficiency, in which different pathogenic mechanisms are involved^{1,3}, the new data related to cellular Cbl uptake, including the detection of anti-TCbIR/CD320, ABCD, and alternative pathways for cellular Cbl uptake, open research perspectives related to clinical, diagnostic, or pharmacological aspects of Cbl deficiency. Without being exhaustive in all their pathogenic implications, some aspects will be discussed.

Recent findings raise the question of whether megalin or other LDLRs play any role in vitamin B₁₂ absorption by passive diffusion, since megalin is expressed in the intestine and is capable of binding to the vitamin.

Pernicious anemia, the main acquired cause of vitamin B₁₂ deficiency, is of autoimmune etiology and has been associated with numerous autoimmune diseases mediated by autoantibodies. Data on the presence of anti-TCbIR/CD320 autoantibodies in pernicious anemia have not yet been published, which will probably be positive in some patients. What relationship will these

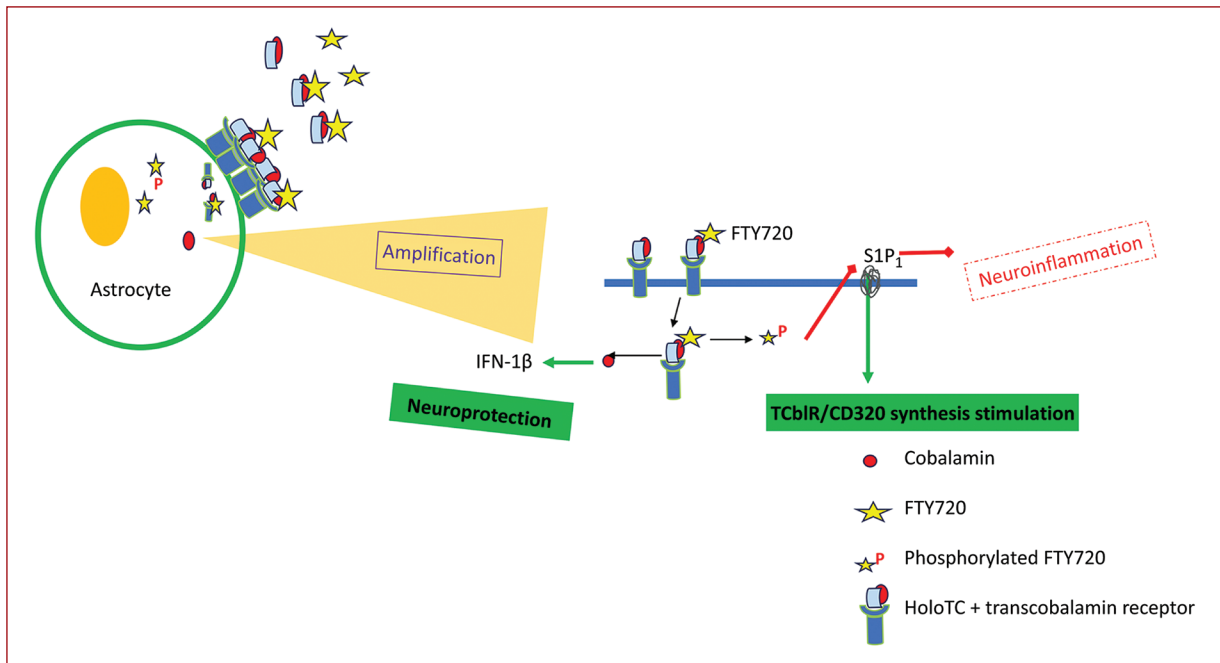


Figure 3. The mechanism of action of FTY720 (fingolimod) in the central nervous system (CNS) is mediated by cobalamin (Cbl) and by interferon beta (IFN-1 β). FTY720 is a beneficial drug authorized for the treatment of multiple sclerosis, a disease with great clinical similarities to the neurological deficit of Cbl. This drug antagonizes the sphingosine 1-phosphate receptor (S1P₁) in astrocytes and peripheral lymphocytes, being the proposed mechanism of action of the drug for its action in the CNS. To exert its function, FTY720 forms a complex with holotranscobalamin (holoTC), and this new holoTC-FTY720 complex is taken up by astrocytes through their cellular receptor TCbIR/CD320. In the cytoplasm, it dissociates into Cbl and FTY720. Cbl is essential for the sensitivity of astrocytes to interferon and the production of IFN-1 β in microglia. In the astrocyte, FTY720 is phosphorylated (FTY720-P), and the resulting FTY720-P functionally antagonizes S1P₁ and negatively regulates its expression on the cell surface. Inhibition of S1P₁ in astrocytes increases the expression of TCbIR/CD320, which allows greater interaction and internalization of the holoTC-FTY720 complex by astrocytes (*adapted from Jonnalagadda et al.⁴⁴*).

autoantibodies have with the neurological symptoms sometimes present in pernicious anemia? It is also unknown whether these autoantibodies or other cellular Cbl uptake pathways may be involved in the epithelial symptoms (e.g., oral ulcers) associated with pernicious anemia.

Another aspect is the treatment of these patients. Up to 25% of cases with pernicious anemia present non-hematological symptoms (oral ulcers, asthenia, depression, paresthesias, neuropathies, etc.). To prevent their reappearance, intramuscular cyanocobalamin contributions higher than usual are required, every 4, 3, or 2 weeks, or even switch to the more potent hydroxocobalamin^{35,36,46}. In some patients, the symptoms improve, but do not disappear^{2,3,35,36,46}. If it were verified that anti-TCbIR/CD320 antibodies are involved, an immunosuppressive treatment could be helpful, and even drugs such as FTY720. Another aspect regarding treatment is to study whether there are differences in

Cbl uptake by receptors other than CD320 (megalin, LDLR), depending on whether it is cyanocobalamin or hydroxocobalamin, which would have implications for treatment.

Cbl has also been related to thromboembolic disease⁴⁷⁻⁵⁰. Hyperhomocysteinemia is a recognized cardiovascular risk factor, but when its cause is studied, surprisingly, it is almost always a Cbl deficiency due to malabsorption. In fact, these patients also present, in addition to hyperhomocysteinemia, an elevation of methylmalonic acid⁴⁷. If Cbl is added to the treatment of thrombosis, patients do not present a new thrombosis, while it is frequent if replacement treatment with the vitamin is stopped^{48,49}. The new findings relating cellular Cbl uptake to lipoprotein receptors open new perspectives for evaluating the relationship between Cbl and thromboembolic disease.

Without ruling out a possible, though uncertain, role of anti-TCbIR/CD320, it is striking that cellular Cbl

uptake is performed by receptors of the LDLR family, implicated in lipoprotein alterations and cardiovascular pathology. The three mentioned above are known, but others of the same family could very well be involved. In this sense, as with neurological pathology, many of these patients with Cbl deficiency and thromboembolism have hardly any hematological alterations. It should be remembered that Cbl, in its 5'-deoxyadenosyl Cbl (AdoCbl) form, is the cofactor for the conversion of methylmalonyl coenzyme A (CoA) to succinyl-CoA. The production of succinyl-CoA feeds the tricarboxylic acid cycle, necessary for optimal mitochondrial function and for the production of glycolipids and sphingomyelin.

Considering the above, it would be important to study the interaction of Cbl and LDLR (or other receptors of its family) with thromboembolic disease.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical considerations

Protection of human subjects and animals. The authors declare that no experiments on humans or animals were performed for this research.

Confidentiality, informed consent, and ethical approval. This study does not involve personal patient data, medical records, or biological samples, and does not require ethical approval. SAGER guidelines do not apply.

Declaration on the use of artificial intelligence (AI). The authors declare that no generative artificial intelligence was used in the writing or creation of the content of this manuscript.

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