Review

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Genetic defects in folate and cobalamin pathways affecting the brain

Abstract

Folate and cobalamin are necessary for early brain development and function. Deficiency of folate or cobalamin during pregnancy can cause severe malformation in the central nervous system such as neural tube defects. After birth, folate and cobalamin deficiency can cause anemia, failure to thrive, recurrent infections, psychiatric and neurological symptoms. The folate and the homocysteine metabolic pathways interact at a central step where 5-methyltetrahydrofolate donates its methyl group to homocysteine to produce methionine and tetrahydrofolate. Methyl cobalamin and folate interact at this critical step. Both nutrients have a crucial role in DNA synthesis and in delivering S-adenosylmethionine, the universal methyl donor. Severe and mild inherited disorders in folate and cobalamin pathways have been described. The two groups of disorders share some similarities, but differ in the molecular mechanism, metabolic dysregulation, and disease management. This review summarizes selected disorders, including rare and common mutations that affect folate and cobalamin absorption, transport, or dependent enzymes. When the mutations are discovered early enough, many of the described disorders are easily treatable by B vitamin supplementation, which often prevents or reverses the manifestation of the disease. Therefore, the screening for mutations is recommended and should be carried out as early as possible: after occurrence of the first symptoms or when a certain constellations of the folate and cobalamin related markers are measured, such as elevated homocysteine and/or methylmalonic acid.

Keywords: brain; cobalamin; folate.

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Introduction

It is our great pleasure to congratulate *Clinical Chemistry and Laboratory Medicine* (CCLM) on its 50th anniversary and to contribute this article for the celebration issue. CCLM, an internationally recognized and leading journal in the field of clinical chemistry and laboratory medicine, also plays an important role in the scientific area of hyperhomocysteinemia (HHcy) and B vitamin deficiency. One of the first CCLM publications in this field dates back to the year 1998 [1] and examined the relationship between HHcy and cardiovascular diseases. In the period from 2000 to 2012, CCLM has published four international conferences on HHcy and B vitamin deficiency which have been organized by our group (2nd Conference on Hyperhomocysteinemia [2], 3rd Conference on Hyperhomocysteinemia [3], 4th Conference on Hyperhomocysteinemia [4], and the World Congress on Hyperhomocysteinemia [5]) with more than 100 articles. To date over 200 articles dealing with homocysteine (Hcy) and B vitamin deficiency have been published in CCLM. This reflects the contribution that CCLM has made and underlines the important role of the journal for the scientific community active in that research field. HHcy and B vitamin deficiency are still the focus of medical research but topics have changed during the last decade. Ten years ago the main interest in HHcy research was directed towards its relationship with cardiovascular diseases, however, the current focus lies in its association with neurodegenerative diseases [6, 7]. Large scale intervention has failed to show that B vitamin supplementation improved patients cardiovascular outcome [8]. A very recent meta-analysis with 47,921 participants from 19 studies (plasma Hcy was reduced in all studies) found that B vitamin supplementation has a significant protective effect on stroke, but none on the risk of cardiovascular disease, myocardial infarction, chronic heart disease, cardiovascular death, or all-cause mortality [9]. It has also been shown that HHcy and B vitamin deficiency is correlated to neurological and psychiatric diseases as well as cognitive decline [7, 10, 11]. The important role of biomarkers in neurodegenerative

diseases has been highlighted in a special issue of CCLM (Biomarkers of Neurodegenerative Diseases) [7, 12–21]. Moreover, recent publications provided convincing evidence that B vitamin supplementation significantly slowed brain atrophy and cognitive decline in patients with mild cognitive impairment after 2 years of treatment [22, 23].

Plausible pathomechanisms behind this relationship are, on the one hand, oxidative stress and other neurotoxic the mechanisms and a lowered cellular methylation potential caused by HHcy and B vitamin deficiency [10, 24, 25]. On the other hand, HHcy and B vitamin deficiency result from lifestyle factors but genetic factors also contribute significantly to dysregulated metabolic pathways in this respect. Beside the very common 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) polymorphism that influences remethylation of Hcy to methionine [26] many other genetic mutations affect folate and cobalamin dependent pathways [27] and have disease modifying effects [28]. Beside disturbed DNA synthesis, DNA hypomethylation due to HHcy and B vitamin deficiency is an important epigenetic factor in gene regulation and expression and has impact on disease development [29, 30]. This review is focused on the role of genetic defects in folate and cobalamin metabolism and their impact on brain function.

Genetic defects in folate pathway

Folate metabolism and distribution

Folate is a methyl donor for Hcy methylation to methionine. It participates in the de novo synthesis of purines and thymidylates [deoxythymidine monophosphate (dTMP)]. Therefore, folate cycle links the DNA synthesis with the methyl group metabolism. The universal methyl donor, *S*-adenosylmethionine (SAM), is synthesized from methionine and it regulates the coordination between folate and methionine metabolism. Folate and cobalamin deficiency as well as HHcy at conception may negatively influence the health of the offspring [31] and have been related to early pregnancy loss and other congenital birth defects [32]. Several mutations in genes of folate-catabolizing enzymes and transporting proteins have been described. Many of these disorders affect the central nervous system and are associated with severe clinical symptoms that are manifested at early infancy.

Dietary folates are derivatives of folylpolyglutamates that are enzymatically hydrolyzed upon ingestion into monoglutamates in the brush border cells of the duodenum and jejunum. The hydrolysis of the polyglutamate is mediated by glutamate carboxypeptidase II (GCPII, EC 3.4.17.21) or the exopeptidase γ-glutamyl hydrolase (GGH, EC 3.4.19.9) located in the lysosome [33]. The folylpoly-γ-glutamate synthase (FPGS, EC 6.3.2.17) facilitates folate polyglutamation. The anionic nature of the folate oligomers renders them unable to leave the cell and, therefore, the polyglutamate forms of reduced folates (and certain antifolates) accumulate in the cell. Only the monoglutamates can be transported across cell membranes [34].

Three independent types of membrane systems are responsible for cross membrane transport of folate monoglutamate forms. These are the membrane folate receptor (FR), the reduced folate carrier 1 (RFC1), and the proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1). The FR has high affinity for folate (K_m approx. 1 nmol/L) and conducts the receptor-mediated endocytosis (unidirectional) across the cell membrane at neutral pH [35]. FRα (FOLR1) is expressed in certain epithelial cells, the choroid plexus, the placenta [36], and the kidney. FRβ (FOLR2) is expressed in the fetal brain, kidney, placenta, spleen, and thymus [37]. RFC1 (SLC19A1) has a higher affinity for 5-methyltetrahydrofolate (5-methylTHF) than for folic acid and transports folate at an optimal pH of 7.5 [38]. The PCFT (SLC46A1) system acts at an optimal pH in the acidic range (4.5–5.5) explaining its role as the major intestinal folate transporter [39]. Transport of folates into the cerebrospinal fluid (CSF) occurs in the choroid plexus, where 5-methylTHF is transported across the blood-brain barrier by FRα in adult or FRβ in the fetal brain. PCFT is ubiquitously expressed in the human brain, where it functions in concert with FRα and FRβ or might export folates after FRα-mediated endocytosis [37, 40]. Biomarkers of folate and methionine metabolism in blood are important determinants of CSF levels of the metabolites [24, 41].

Tetrahydrofolate (THF) represents the active form of folate that is formed from dihydrofolate (DHF) by means of dihydrofolate reductase (DHFR, EC 1.5.1.3) (Figure 1). 5,10-MethyleneTHF is converted to 5-methylTHF by MTHFR in an irreversible reaction [42]. 5-MethylTHF donates its methyl group to Hcy and is converted into THF by means of methionine synthase (MTR, EC 2.1.1.13), a cobalamin (Cbl)-dependent enzyme. The enzyme methionine synthase reductase (MTRR, EC 1.16.1.8) is involved in the reductive regeneration of the Cbl-cofactor, which is required for MTR function. 10-FormylTHF is utilized for purine synthesis and 5,10-methyleneTHF for synthesis of dTMP and methionine. Serine hydroxymethyltransferase 1 (SHMT1, EC 2.1.2.1) utilizes serine to form 5,10-methyleneTHF [43]. The conversion

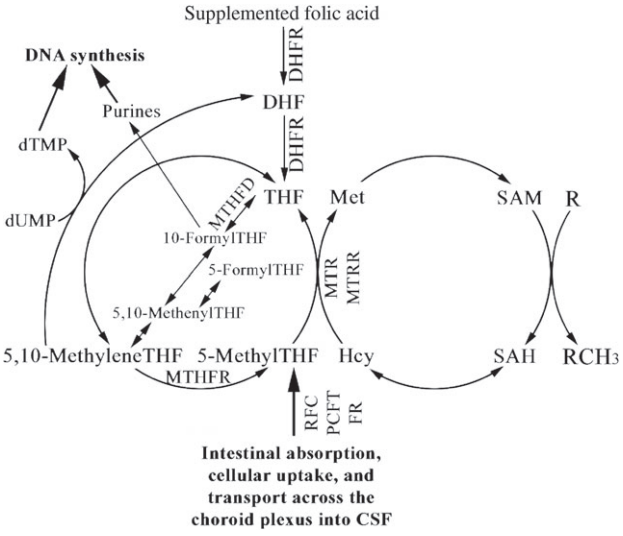

Figure 1 Folate metabolism, related enzymes, and transporters. DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FR, folate receptor; MTHFD, 5,10-methylenetetrahydrofolate dehydrogenase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; PCFT, proton coupled folate transporter; RFC, reduced folate carrier; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; TYMS, thymidylate synthase.

of the different forms of folate is very fast and depends on other factors like Cbl and vitamin B_6 status, SAM, and certain polymorphisms in folate-catabolizing enzymes. The 5-methylTHF is the predominant folate form in plasma that constitutes up to 90% of total folate. Folic acid, the synthetic form of the vitamin, enters the folate cycle after a two-step reduction via DHFR into DHF and then THF.

Inherited disorders of folate metabolism and transport

Several inherited disorders of folate metabolism and transport have been described: MTHFR deficiency, MTR deficiency (either caused by mutations in *MTR* gene or *MTRR* gene), cerebral folate deficiency (CFD) caused by FOLR1 mutation, hereditary folate malabsorption, and glutamate formiminotransferase (FTCD) deficiency [44] (Table 1). In addition, several putative inherited disorders related to folate metabolism are discussed in the literature like DHFR deficiency, cellular uptake defects, and the 5,10-methyleneTHF cyclohydrolase [part of the trifunctional enzyme 5,10-methyleneTHF dehydrogenase 1 (MTHFD1, EC 1.5.1.5)] deficiency [66].

Hereditary folate malabsorption

Hereditary folate malabsorption is a clinical syndrome manifested a few months after birth [67]. The pathophysiology of the disorder is attributed to impaired intestinal folate absorption and impaired folate transport into the central nervous system causing very low serum folate concentrations and CFD. After birth, affected newborns rapidly develop severe folate deficiency with megaloblastic anemia, diarrhea, oral mucositis, and recurrent infections (Table 1). Further symptoms include poor feeding, failure to thrive and neurologic manifestations including seizures and developmental delays [68]. If untreated, patients might develop ataxia and cognitive impairment. The disorder has been described in about 30 patients and mostly females are affected [52, 69].

The molecular bases of the hereditary folate malabsorption might be related to mutations in the *SLC46A1* gene, encoding the PCFT, which is present in the intestine and the choroid plexus [37, 39, 52, 70]. Patients deficient in PCFT develop severe folate deficiency suggesting that RFC (one folate carrier), that is expressed in the intestinal epithelium, does not significantly participate in folate absorption [71]. Treatment with large doses (oral or parenteral) of folinic acid (5-formylTHF) can normalize folate levels. CSF folate is far more difficult to normalize and patients require supraphysiological folate concentrations in blood to be able to increase CSF folate [72]. Monitoring the therapy is important and includes measurements of serum and CSF folate with the aim of achieving normal concentrations of folate in CSF.

Mutations in FRα, FRβ, or RFC1 may be the cause of disturbed folate uptake. Folic acid treatment normalized serum folate and improved the clinical symptoms in one study, but red blood folate remained very low suggesting an impaired cellular uptake [73]. Patients show low uptake of labeled 5-methylTHF by stimulated lymphocytes and bone marrow cells or red cells [73]. The genetic background of these cases is unknown but might be variants of the hereditary folate malabsorption.

Cerebral folate deficiency

The disease is characterized by severe developmental disorders, mental retardation, epilepsy, and movement disorders [74]. Unlike children with hereditary folate malabsorption, those with CFD present with neurological disorders several years after birth. The disease is characterized by impaired folate transport across the choroid

Disorder	Gene Mendelian inheritance in man (MIM) code [45]	Involved enzyme/protein [EC number, cytogenetic location]	Function	References
5,10-Methylenetetrahydrofolate reductase deficiency	607093, #236250	5,10-Methylenetetrahydrofolate reductase (MTHFR) [EC 1.5.1.20, 1p36.3]	Conversion of 5,10-methyleneTHF to 5-methylTHF	[46–48]
Cerebral folate deficiency	136430, #613068	Folate receptor (FOLR1) [11q13.3-q14.1]	High affinity folate transporter, transport of 5-methylTHF across choroid plexus	[37, 49]
Glutamate formiminotransferase deficiency	606806, #229100	Formiminotransferase cyclodeaminase (FTCD) [EC 4.3.1.4, 21q22.3]	Channels 1-carbon units from formiminoglutamate to the folate pool	[50, 51]
Hereditary folate malabsorption	611672, #229050	Proton coupled folate transporter (PCFT or SLC46A1) [17q11.2]	High affinity folate transporter	[39, 52]
Cobalamin A deficiency	607481, #251100	Methylmalonic aciduria type A protein (MMAA) [4q31.21]	Involved in translocation of Cbl into the mitochondrion during adenosylcobalamin synthesis	[53, 54]
Cobalamin B deficiency	607568, #251110	Cobalamin adenosyltransferase (MMAB) [EC 2.5.1.17, 11q24.11]	Involved in adenosylcobalamin synthesis	[53, 54]
Cobalamin C deficiency	609831, #277400	Methylmalonic aciduria and homocystinuria type C protein (MMACHC) [1p34.1]	Involved in the binding and intracellular trafficking of Cbl	[55–57]
Cobalamin D deficiency	611935, #277410	Chromosome 2 open reading frame 25 (C2orf25) [2q23.2]	Protein involved in an early step of cobalamin metabolism	[58, 59]
Cobalamin E deficiency	602568, #236270	Methionine synthase reductase (MTRR) [EC 1.16.1.8, 5p15.2-p15.3]	Reductive regeneration of the Cbl cofactor	[48, 60]
Cobalamin F deficiency	612625, #277380	LMBR1 domain-containing protein 1 (LMBRD1) [6q13]	Probable lysosomal Cbl transporter	[61, 62]
Cobalamin G deficiency	156570, #250940	Methionine synthase (MTR) [EC 2.1.1.13, 1q43]	Transfer of methyl group from 5-methylTHF to homocysteine to form methionine and THF (Cbl-dependent)	[48, 63]
Methylmalonyl-CoA mutase deficiency	609058, #251000	Methylmalonyl-CoA mutase (MUT) [EC 5.4.99.2, 6p12.3]	Mitochondrial enzyme, catalyzes isomerization of methylmalonyl-CoA to succinyl-CoA	[64, 65]

Table 1 Inherited disorders of folate and cobalamin metabolism and transport.
Phenotype MIM number.

plexus, but not in the intestine. Therefore, when dietary folate intake is sufficient, patients show normal concentrations of serum folate, but very low CSF 5-methylTHF [71, 75]. Mutation in the *FOLR1* gene, which encodes the FRα, can cause severe 5-methylTHF deficiency in the CSF [37, 49]. Oral folinic acid can normalize the CSF folate status and lowers frequency and severity of epileptic seizures [37]. Cario et al. reported heterozygous mutations in both *FOLR1 C352T* and *FOLR1 C525A* genes, leading to nonsense mutations (FOLR1 Q118X and C175X) (Table 2) and the loss of FR-specific folate binding in two siblings [49]. Treatment with folinic acid led to improvement of the symptoms, whereas one of the siblings completely recovered. The homozygous missense mutation FOLR1 T313C was also identified in one patient with CFD [76]. Additionally, 12% of children with any neurological disorder (71 of 584 patients) exhibited deficient CSF 5-methylTHF concentration [76]. FRα is the most abundant folate transporter in the choroid plexus, supporting its role as major folate transporter across the blood-brain barrier [37]. Normalizing CSF folate in children affected with CFD or those with hereditary folate malabsorption can be better achieved by administration of high doses of folinic acid or methylfolate [71]. Oral treatment is less effective than intramuscular injections. Using folic acid is not advisable, because it must be reduced first and the unmetabolized folic acid can bind to FRα with a high affinity thus preventing the binding of 5-methylTHF.

Gene	Genetic variant	Amino acid substitution	Reference SNP (rs) number
MTHFR	C677T	A222V	1801133
	A1298C	E429A	1801131
MTR	A2756G	D919G	1805087
MTRR	A66G	I22M	1801394
SHMT1	C1420T	L435F	1979277
MTHFD1	G1958A	R653Q	2236225
DHFR	A458T	D153V	121913223
TYMS	2R/3R (28 bp)	–	–
RFC1	G80A	H27R	1051266
FOLR1	C352T	Q118X	121918405
	C525A	C175X	121918406
	130_147 dup	P49PRKSQAP	121918843

Table 2 Common polymorphisms in folate-metabolizing enzymes.

CFD may be related to causes other than mutations in the *FOLR1* gene such as antibodies against the folate receptor [77, 78]. Since the formation of the antibodies can be downregulated with a milk-free diet, it has been suggested that soluble folate-binding proteins in cow's milk [74] may induce antibody formation [79]. Furthermore, CFD has been found in patients with single nucleotide polymorphisms (SNPs) in the *DHFR* gene in two recent studies [44, 80]. DHFR deficiency caused megaloblastic anemia, normal total Hcy (tHcy), and low tetrahydrobiopterine in the CSF [44]. DNA sequencing revealed a homozygous DHFR C238T (Leu80Phe) missense mutation [44]. Treatment with folinic acid led to improvement of anemia and CSF 5-methylTHF levels, and seizure control. However, the neurodevelopmental improvement was less than that reported in patients with FOLR1 mutations or FR autoantibodies [44]. CFD was also reported in patients with megaloblastic anemia who were heterozygous for the DHFR A458T polymorphism [80].

Other conditions with neurological manifestations that are associated with CFD are Rett syndrome [81], Kearns-Sayre syndrome [82], dihydropteridine reductase deficiency [83], Aicardi-Goutiere's syndrome [84], schizophrenia [85], and hypomyelination with atrophy of the basal ganglion syndrome [86].

Glutamate formiminotransferase deficiency

Glutamate formiminotransferase deficiency is an autosomal recessive disorder caused by mutations in the *FTCD* gene [50]. Symptoms include elevated urinary levels of formiminoglutamic acid (FIGLU) after histidine load, megaloblastic anemia, mental retardation, and developmental delay. Heterozygous missense mutations (C457T and G940C) in the *FTCD* gene were found in the mild form of the disease [51].

Severe 5,10-methylenetetrahydrofolate reductase deficiency

Severe MTHFR deficiency is an inborn disease which is associated with homocystinuria, developmental delay, decreased neurotransmitter levels, or seizures [87, 88]. One patient homozygous for the missense mutation in the MTHFR c.1129C>T was presented with severe psychomotor retardation, generalized cerebral atrophy, and hypomyelination on magnetic resonance imaging examination [89]. Folate (folic acid, 5-methylTHF, or folinic acid), vitamin B_6, Cbl, and methionine supplementations are the basic therapeutics. In addition, supplementation of large doses of betaine seemed to support the metabolic requirements of the brain by increasing SAM and SAM-dependent methyl transferases [87]. Improvement in the neurological signs has been reported, but it depends on the age of starting the betaine supplementation [87].

Common polymorphisms associated with neural tube defects

Common polymorphisms in the *MTHFR* gene have been described. The C>T substitution in exon 4 at bp 677 causes a substitution of valine for alanine and results in a thermolabile variant of the enzyme [90] that has a 50%–70% less activity when folate intake is limited [42, 90, 91] (Table 2). The TT genotype is found in 10%–20% of the European populations and it increases the risk of neural tube defects (NTDs). Another common polymorphism in the *MTHFR* gene is the A>C substitution at bp 1298 leading also to decreased enzyme activity but without marked effect on tHcy or folate plasma levels [92]. The prevalence of this polymorphism ranges from 6%–11% in Europe [93].

Folates are essential for brain development and function. Folate deficiency during pregnancy can cause NTDs in the offspring. NTDs are common severe congenital malformations that arise early in embryogenesis because of the failure of neural tube closure. Depending on the location of the lesion, NTDs are divided into spina bifida and anencephaly. Folate and Cbl deficiencies as well as elevated tHcy during pregnancy increase the risk of having a child with NTDs [94, 95]. Mutations in genes encoding enzymes involved in the folate/Hcy metabolism have been related to the risk of NTDs [96]. Several studies confirmed

the association between the MTHFR 677TT genotype and the risk of NTD [97, 98], but a number of studies failed to find an association [99, 100]. Shaw et al. found no association between the risk of spina bifida and 118 polymorphisms of folate-related genes [101]. However, the association seems to depend on folate and Cbl intake [46]. The effects of the polymorphisms seem to be counterbalanced by higher folate and Cbl intakes. This is the reason for the insignificant effect of theses polymorphisms on NTD risk in countries applying mandatory fortification with folic acid.

In a study on fibroblasts from 41 NTD-affected fetuses, Ou et al. found a 7.2-fold higher risk for NTDs in samples with MTHFR 677TT genotype [102]. Christensen et al. reported an odds ratio (OR) (95% confidence interval) for NTD of 2.2 (0.82–5.99) for affected children, 2.0 (0.75–5.43) for mothers with an NTD child, and 6.0 (1.26–28.53) for mothers and child pairs with MTHFR 677TT [46]. Low red blood cell (RBC) folate further increased the risk of developing NTD, and RBC folate was lower in cases and case mothers compared to controls and control mothers, respectively. The MTHFR 677TT combined with low RBC folate resulted in an OR for NTD of 3.28 (0.84–12.85) for mothers and 13.43 (2.49–72.33) for cases [46].

The association between MTHFR A1298C polymorphism and NTD risk is not consistent. Few studies reported gene-gene interactions with the MTHFR C677T genotype [92, 103], other studies found a protective effect for the MTHFR 1298 C allele [104, 105]. In addition, the MTRR 66GG genotype in the mothers was associated with a 2.1-fold (OR 2.1, 95% CI 1.3–3.3) higher risk for having a child with NTD [106]. The MTR A2756G, the MTRR A66G [107], and the MTHFD1 1958AA polymorphisms [108] were also reported to enhance the maternal risk of having a child with spina bifida [28, 107]. Polymorphisms in RFC1 G80A [109], DHFR [110], and thymidylate synthase (TYMS; EC 2.1.1.45) 28-bp tandem repeat [111], and the presence of autoantibodies against the FR [112] might also be involved in maternal risk of having a child with NTD. Many NTDs can be prevented by improving maternal folate status before the conception. Therefore, it seems that the polymorphisms related to the folate cycle have no independent effect in case of high folate status.

Genetic defects affecting cobalamin-transport or dependent reactions

Cobalamin (Cbl, vitamin B_{12}) is a water soluble vitamin from the B-group. It is essential for cell growth and division. Cbl deficiency can cause severe hematological and/or neurological manifestations. Low serum Cbl levels are associated with pregnancy loss [113, 114]. Inherited defects in Cbl metabolism are mostly associated with failure to thrive, irritation, feeding problems, and neurological or neurodevelopmental disorders. Hematological and neurological symptoms of folate and Cbl deficiency are similar which is consistent with the crosstalk between the folate and Cbl pathways.

Animal-based diet is the only source of Cbl for humans. The daily requirement for Cbl termed as recommended dietary allowance is 2.4 μg/day for adults [115]. Recent studies accessing blood concentrations of modern markers of cobalamin, suggested that the daily requirements for cobalamin should be set at >6 μg [116]. A relatively large amount of Cbl is stored in the body. The depletion of the vitamin takes several years to develop to deficiency when one stops to consume Cbl-containing diet.

Cbl is a cofactor for only two biochemical reactions in humans (Figure 2). Methylcobalamin (MeCbl) is a cofactor for the cytosolic enzyme MTR that transfers a methyl group from 5-methylTHF to Hcy converting it into methionine [117]. Adenosylcobalamin (AdoCbl) is a cofactor for the mitochondrial enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) that converts succinyl-CoA into methylmalonyl-CoA. Classical nutritional Cbl deficiency is associated with low holotranscobalamin (holoTC) and elevated plasma concentration of tHcy and methylmalonic acid (MMA). Patients with defects in Cbl metabolism can show severely elevated MMA and/or tHcy without any evidence of low holoTC. This can lead the diagnoses and indicate that elevated MMA and/or tHcy are not related to transcobalamin (TC) deficiency, but to defects in the Cbl-dependent enzymes or trafficking proteins within the cells.

The absorption, transport, and dissimilation of Cbl are complex processes that require several proteins and cellular receptors. Food Cbl is first released from food proteins by means of salivary amylase and acidic conditions and is then bound to haptocorrin released in the saliva. Haptocorrin protects the vitamin from the acid surroundings in the stomach. In the alkaline environment of the intestine haptocorrin is degraded by pancreatic enzymes, and the vitamin liberated from food is recognized by intrinsic factor (IF). IF, another Cbl transporter, is a glycoprotein synthesized in the parietal cells of the stomach that binds only the forms of the vitamin which are active within the body. In the distal ileum, IF-bound-Cbl is taken up by a specific receptor called cubam. Cubam is composed of two proteins, cubilin (CUBN) and amnionless

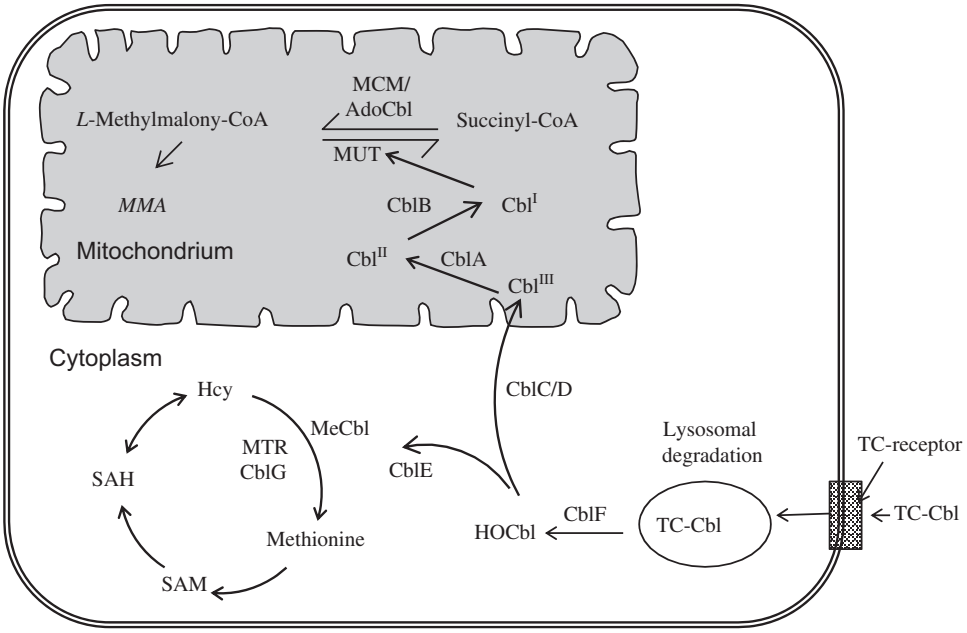

Figure 2 Cobalamin homeostasis and inherited disorders.
AdoCbl, adenosylcobalamin; Cbl, cobalamin; Hcy, homocysteine; HOCbl, hydroxycobalamin; MCM, methylmalonyl-CoA mutase; MeCbl, methylcobalamin; MMA, methylmalonic acid; MTR, methionine synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TC, transcobalamin.

(AMN) [118]. Binding of IF-Cbl to cubam is mediated by CUB domains 5–8 in the CUBN protein. Amnionless is a small transmembrane protein that anchors cubilin to the cell membrane in the enterocytes [119]. In the enterocytes, Cbl is released and transferred to transcobalamin, forming the complex holoTC that is secreted into the blood by a yet unknown mechanism probably involving the multidrug resistance protein (MRP1) [120]. As the enterocytes have a high rate of synthesis of transcobalamin II (TC), it has been a common view that Cbl is secreted from the enterocytes in complex with TC. An alternative hypothesis is that free Cbl is transported from the cytosol across the basolateral cell surface into plasma, where it subsequently forms a complex with circulating TC [120]. In the blood, the major part of Cbl is bound to haptocorrin (70%–90%) which is called holohaptocorrin (metabolically inert fraction). Only 10%–30% of Cbl is bound to TC, which carries the metabolically active Cbl. The cellular uptake of Cbl is receptor mediated endocytosis via the TC-receptor or cubam. TC-bound Cbl is degraded in the lysosome and Cbl is released and directed into the two pathways requiring it as a cofactor. The lysosomal degradation and transport of Cbl within the cell are not fully understood.

Several inherited defects in Cbl absorption, transport, or assembly within the cell have been described (Table 1). Defects in any step involved in converting methylmalonyl-CoA to succinyl-CoA cause methylmalonylacidemia. This can be due to either defective methylmalonyl-CoA mutase (MUT) or impaired synthesis or utilization of AdoCbl (CblA, B, D, and H). There are eight distinct complementation group defects of the intracellular Cbl metabolism (Table 1, Figure 2). The different types were identified by somatic complementation studies applied on fibroblasts isolated from the patients [121]. Moreover, the Cbl genetic defects have been identified on the molecular level [58, 122]. The CblF and CblC defects caused homocystinuria and methylmalonic aciduria [59, 123]. The CblD defect can cause either homocystinuria or methylmalonic aciduria or a combination of homocystinuria and methylmalonic aciduria [59]. The CblA, CblB, and MUT cause only methylmalonic aciduria, and CblE and G cause only homocystinuria. The molecular bases of these disorders have been partly elucidated [124]. The clinical features are similar and the start of the manifestations varies from a few weeks to the adulthood. Lifelong Cbl treatment is required but the prognoses may differ. Clinical symptoms include feeding difficulties, hypotonia, megaloblastic anemia, mental retardation [125], visual loss, or nystagmus. Neuroradiological studies on children with CblC/D defects have shown severe white matter abnormalities like edema, swelling [125], or hydrocephalus [126]. The exact mechanisms behind the neurological manifestations are not known, but may be related to elevated tHcy, lowered SAM causing hypomethylation, or accumulation of MMA that is neurotoxic.

Defects of cobalamin transport and absorption

CblA

The gene responsible for CblA was identified in 2002 by analyses of prokaryotic gene arrangements [53]. This disorder causes isolated methylmalonyluria.

Cobalamin C defect (CblC, MMACHC)

CblC is the most common severe disorder of intracellular Cbl metabolism. CblC is an autosomal recessive disorder of Cbl metabolism related to mutations in *MMACHC* gene [127]. The methylmalonic aciduria and homocystinuria type C protein (MMACHC) is responsible for processing the upper-axial ligands of dietary Cbl before AdoCbl and MeCbl can be synthesized [128, 129]. It causes impaired conversion of Cbl into its two metabolically active forms, MeCbl and AdoCbl. There are several known and recently identified mutations in the *MMACHC* gene [55, 56].

Severe neurological clinical symptoms are manifested in the early onset form during the first year of life. Symptoms include failure to thrive, microcephaly, feeding difficulties, hypotonia, vomiting, developmental delay, seizures, and speech delay. Symptoms are rarely responsive to treatment [130] in the infantile form of the disease. Retinal dysfunction has also been reported in two cases with CblC [131]. Several non-specific hematological symptoms can be seen like megaloblastic anemia and thrombocytopenia.

In 1984 [132] and later reports, a late onset form of CblC which is diagnosed in previously asymptomatic cases at older ages up to adulthood was identified [133, 134]. The first patient identified with CblC had acute onset of dementia, myelopathy, and motor neuron disease [132]. The late onset form has better outcome and response to treatment. A recent study on normal fibroblasts and fibroblasts from early onset CblC disorder identified several proteins that are downregulated in the mutant cells probably explaining some of the neurological manifestations of functional Cbl deficiency [135]. Hydroxycobalamin (HOCbl) did not cause any reduction in the excretion of tHcy from the mutant cells. Interestingly, because of the role of MTR in cellular folate retention, intracellular folate was lower in the CblC cells even after treatment with HOCbl compared to normal fibroblasts [135]. Moreover, protein markers related to brain function were found to be upregulated in fibroblasts from CblC patients and the level of expression was not restored to normal after incubation with HOCbl [135]. This probably explains the neurocognitive manifestations of the disease or effects on brain development during the prenatal life.

There seems to be a genotype-phenotype association with some mutations: the c.271dupA and c.331C>T (R111X) mutations are more prevalent in the early onset disease. Some missense mutations c.394C>T and c.482G>A are associated with late-onset disease [55, 56]. Genotype-phenotype correlations were explained by variations in the levels of MMACHC mRNA being severely lower in the early-onset forms [55].

In several cases with late onset CblC defect (age at onset 16–41 years) thrombotic events were common. Moreover, six of 11 cases described had encephalopathy, two had seizures, six suffered from myelopathy, and six suffered from psychiatric disturbances [133]. Neurological symptoms, as shown by magnetic resonance imaging, dominated with abnormalities in the white matter area, cortical atrophy, or medullar lesions mostly reported [133]. None of the patients suffered from mental retardation and few remained free of neurological or psychiatric illnesses [133]. Death occurred in few cases despite treatment with Cbl. The mechanisms behind the neurological manifestations might be related to impaired methylation causing cerebral perivascular demyelination [136]. Interestingly, one case had recurrent thrombotic events that were prevented by HOCbl injections. Folinic acid and betaine were used as adjacent therapy to lower tHcy. Oral HOCbl was less effective than the injections in preventing the thrombosis [133].

CblD (*MMADHC* gene)

The disorder is caused by mutations in the methylmalonic aciduria and homocystinuria type D protein (*MMADHC*) gene that can result in isolated homocystinuria (variant 1), isolated methylmalonic aciduria (variant 2), or combined homocystinuria and methylmalonic aciduria. Patients unable to synthesize AdoCbl and those unable to produce MeCbl have methylmalonic aciduria or homocystinuria, respectively. The third complementation group are patients unable to synthesize both coenzyme forms and have therefore methylmalonic aciduria and homocystinuria [59]. Depending on the mutation, the clinical, cellular, and molecular phenotype of the CblD disorder is heterogeneous. Mutations affecting the *N*-terminus of *MMADHC* are thought to be associated with methylmalonic aciduria, and mutations affecting the C-terminus are associated with homocystinuria [137].

CblG

This group summarizes disorders in MTR that is related to disorders in the enzyme itself or in the co-factor, MeCbl. CblG is caused by defects in MeCbl synthesis that in turn cause MTR deficiency [138].

CblF (*LMBRD1* gene)

CblF is a disorder in Cbl lysosomal trafficking. This defect has been identified in 1986 as a distinct complementation group [121]. Fibroblasts from CblF patients are able to take Cbl via TC-receptor, but unable to release the vitamin from the lysosome [139]. This defect has been reported in only 13 patients so far [140].

Rutsch et al. recently identified the LMBD1 domain-containing protein (*LMBRD1*) gene on chromosome 6q13, that might be defect in CblF patients and might explain why Cbl is trapped in the lysosome [61]. The gene product is probably lysosomal cobalamin transporter (LMBD1), a lysosomal membrane protein with homology to lipocalin-interacting membrane receptor (LIMR). Five frame shift mutations in LMBRD1 resulting in loss of LMBD1 function were identified. Fibroblasts of individuals with CblF showed improved synthesis of Cbl cofactor after transfection with wild-type LMBD1. Genetics defects on the molecular level have been recently identified in *LMBRD1* gene in one Turkish [140] and three Canadian [141] patients with CblF disorder. The LMBD1 protein is synthesized in the liver and is hypothesized to participate in Cbl uptake and transport in the lysosome [140].

The CblF disorder is manifested at an early age and patients have abnormal newborn screening. Patients are small for gestational age or growth retarded at birth. Beside homocystinuria and methylmalonic aciduria, cerebral seizures, intraventricular hemorrhage [140], failure to thrive, anemia, lethargy, feeding difficulties [61, 142], and developmental delay have been reported [61]. Other disorders have been reported in CblF patients like congenital heart failure, gastritis, ventricular hypertrophy, and hypotonia [61].

CblJ defect (mutations in *ABCD4* gene)

One previously identified peroxisomal ATP-binding cassette (ABC) transporter (ABCD4) has been recently shown to be involved in one inherited defect affecting Cbl metabolism. The ABCD4 protein has been shown to colocalize with the lysosomal proteins LAMP1 and LMBD1 [143]. The last protein is encoded by *LMBRD1* gene and is deficient in patients with CblF defect.

Imerslund-Gräsbeck syndrome (IGS) or juvenile megaloblastic anemia

This selective Cbl malabsorption is a rare autosomal recessive disorder characterized by Cbl deficiency that is responsive to treatment. Megaloblastic anemia is common and mild proteinuria occurs in approximately 50% of the patients [144, 145].

In 1960, the first description of the disease was by Gräsbeck as “selective cobalamin malabsorption with proteinuria” [146]. In 1963, Imerslund described a similar disorder called idiopathic chronic megaloblastic anemia in children [147]. The disease was first diagnosed in Finland and Norway where the estimated prevalence is 1:200,000. Although the exact prevalence worldwide is not known, many new cases have been reported from eastern Mediterranean countries. In contrast to the case of TC deficiency, symptoms do not appear directly after birth but from the age of a few months to 15 years [148]. Symptoms are rather unspecific and include fatigue, failure to grew or thrive, megaloblastic anemia, and mild neurological symptoms [149].

Investigations of the disorder identified several mutations in two different proteins that constitute the functional IF-receptor: CUBN and AMN [150]. CUBN and AMN form a complex called cubam that represents part of the IF receptor responsible for intestinal Cbl uptake and renal protein reabsorption. Imerslund-Gräsbeck syndrome (IGS) can lead to low expression of IF-receptor, increased degradation [151], or decreased affinity of IF-Cbl to the receptor [152]. A recent study identified genetic mutations in AMN causing a premature stop codon and a strong decrease in the luminal receptor activity [153]. Analyses of renal biopsy from a patient with AMN mutation showed no immunologic reaction for CUBN and an abnormal cytoplasmic, vesicular distribution of the receptor partner AMN suggesting that AMN depends on CUBN for correct localization in the human proximal tubule [154].

Defects in CUBN or AMN and those in IF cause similar symptoms and can be mistaken [155]. Life-long treatment with Cbl in IGS is necessary for preventing the symptoms.

The exact mechanism behind the transport of Cbl into the CNS is not clear. An active transport mechanism into the CNS has been proposed for Cbl. In one case of IGS, CSF Cbl was low and Cbl was required in short intervals for the remission of the psychiatric symptoms [156] suggesting

that AMN protein might be involved in Cbl transport into the brain [156].

Pediatric patients present with megaloblastic anemia, funicular myelosis, or benign proteinuria should be tested for IGS syndrome. The definitive test is based on genetic testing of the mutation, and Cbl should be administered lifelong.

Congenital gastric intrinsic factor deficiency

The discovery of new genes involved in Cbl assimilation and metabolism has improved our knowledge of the rare inborn errors of Cbl metabolism [119]. Juvenile Cbl deficiency leads to hematological and neurological disturbances. There is a lack of Cbl in congenital pernicious anemia due to gastrointestinal intrinsic factor (GIF) deficiency and megaloblastic anemia due to selective intestinal malabsorption of Cbl.

Overgaard et al. [157] recently described a case (15-year-old boy) of a compound heterozygous mutation in the *GIF* gene, with a previously described mutation (c.79+1G>A) and a novel mutation (c.290T>C; M97T) leading to a megaloblastic anemia in an adolescent. Serum Cbl was decreased and serum folate was normal. Cbl therapy together with oral iron supplement normalized blood parameters. Ament et al. [158] identified a specific GIF mutation [c.183_186delGAAT frame shift mutation (M61fs)] to be responsible for juvenile Cbl deficiency in cases of West-African origin. A child from Spain with a megaloblastic anemia due to GIF deficiency has been reported by Garcia Jimenez et al. [159]. The patient is a compound heterozygous in *GIF* gene for a splice site mutation inherited from his mother and a missense change inherited from his father. Leunbach et al. [160] reported a 28-month-old boy with pallor and weight stagnation, macrocytic anemia, and pancytopenia due to Cbl deficiency and a homozygous mutation in the intrinsic factor gene. His sister showed similar symptoms at the age of 15 months. All were given monthly cyanocobalamin injections which, however, caused leg cramps. Replacement with monthly HOCbl was successful.

Congenital transcobalamin deficiency

TC is a non-glycoprotein that has a half-life of 90 min [161]. The vascular endothelium is the major cite of the synthesis of TC [162], but many other tissues can also synthesize it. TC deficiency might be caused by the absence of the protein, error in RNA editing [163], or a protein that is not functional either because it can not bind to the TC-receptor or to Cbl [164, 165]. The molecular bases of several TC defects have been identified [163, 166, 167] after cloning the *TC* gene [168].

Newborns with TC deficiency are asymptomatic at birth. Symptoms are developed during the first few weeks of life and include megaloblastic anemia, failure to thrive, vomiting, infections, and neurological symptoms [163, 169]. Cbl treatment must be started as soon as possible and continue for life. Despite Cbl treatment many patients show continuous neurological symptoms like seizures, cerebral disturbances, and impaired visual abilities [163]. As approximately 80% of serum Cbl is bound to haptocorrin, total serum Cbl might be normal in patients with TC deficiency. This may give the wrong impression that Cbl status is normal and may thus delay the diagnosis of severe Cbl deficiency. Patients have severely increased concentrations of MMA and tHcy in blood and urine.

Haptocorrin (TC I) deficiency produces low serum Cbl levels similar to Cbl deficiency. Diagnosis is especially difficult when TC I deficiency is mild. The phenotype is asymptomatic. A prospective study found severe TC I deficiency with absence of TC I in 0.6% of 537 patients with low Cbl levels [170]. The low Cbl levels of TC I deficiency are usually misattributed to Cbl deficiency. Severe homozygous TC I deficiency features virtually undetectable TC I in plasma and secretions, and serum Cbl is usually<100 pmol/L but heterozygous have mild to moderate lowering of plasma TC I, and mildly lowered (100–150 pmol/L) or low-normal serum Cbl [170, 171].

The *TCN1* gene [Mendelian inheritance in man (MIM) 189905] is located on chromosome 11q11-q12.3 [172], has 9 exons of 59 to 191 bp and 8 introns of 160 bp to 3.2 kb, and encodes TC I, a protein of 433 amino acids [173]. Two mutations have been described, both are located in exon 2 of the 9-exon *TCN1* gene [171]; a 315C > T nonsense mutation and a G deletion at position 270 that causes a frame shift leading to a premature stop codon. These mutations lead to degradation of the transcripts via nonsense-mediated mRNA decay.

Conclusions and final remarks

Disorders in the folate and cobalamin transport or metabolism that affect folate and Cbl absorption, transport, or dependent enzymes, cause severe neurological symptoms that are in some instances reversible after supplementing

the co-enzyme form of the vitamin. Severe elevation in plasma or urine concentrations of tHcy and/or MMA can be considered as a screening test for these disorders. However, it is crucial to identify the defective pathway in order to supplement the proper form of the vitamin as early as possible. The neurological and neurodevelopmental complications in patients with defects in the folate or Cbl related reactions underline the importance of the vitamins in the pre- and post-natal development of the central nervous systems.

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