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Isolated and Combined Remethylation Disorders: Biochemical and Genetic Diagnosis and Pathophysiology

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Abstract

Genetic defects affecting the remethylation pathway cause hyperhomocysteinemia. Isolated remethylation defects are caused by mutations of the 5, *10-methylenetetrahydrofolate reductase (MTHFR)*, *methionine synthase reductase (MTRR)*, *methionine synthase (MTR)*, and *MMADHC* genes, and combined remethylation defects are the result of mutations in genes involved in the synthesis of either methylcobalamin or adenosylcobalamin, that is, the active cofactors of MTRR and methylmalonyl-CoA mutase. Diagnosis is based on the biochemical analysis of amino acids, homocysteine, propionylcarnitine, methylmalonic acid, S-adenosylmethionine, and 5-methylentetrahydrofolate in physiological fluids. Gene-by-gene Sanger sequencing has long been the gold standard genetic analysis for confirming the disorder and identifying the gene involved, but massive parallel sequencing is now being used to examine all those potentially involved in one go. Early treatment to rescue metabolic homeostasis is based on the following of an appropriate diet, betaine administration, and, in some cases, oral or intramuscular administration of vitamin B_{12} or folate. Elevated ROS levels, apoptosis, endoplasmic reticulum (ER) stress, the activation of autophagy, and alterations in Ca^{2+} homeostasis may all contribute toward the pathogenesis of the disease. Pharmacological agents to restore the function of the ER and mitochondria and/or to reduce oxidative stress-induced apoptosis might provide novel ways of treating patients with remethylation disorders.

Keywords

remethylation disorders, oxidative stress, massive parallel sequencing, homocysteine, vitamin B_{12}

Introduction

Dietary folate and cobalamin (vitamin B_{12}) play essential roles in maintaining the blood homocysteine (Hcy) balance via the latter's conversion into methionine (Met) via the remethylation pathway. Low dietary intake of folate or vitamin B_{12} may lead to hyperhomocysteinemia (HHcys; Figure 1), which has been associated with neural tube defects during fetal development and early-onset cardiovascular disease.[1-3] Genetically induced defects in the enzymes involved in the remethylation pathway also lead to HHcys with a range of severe consequences.

Homocysteine is maintained at nontoxic levels via the degradation to cysteine in the transsulfuration pathway. Additionally, the donation of a methyl group from the folate derivative 5-methyltetrahydrofolate (5-MTHF; synthesized from tetrahydrofolate by 5, 10-methylenetetrahydrofolate reductase [MTHFR]) to cob[I]alamin (forming methylcobalamin [MeCbl]

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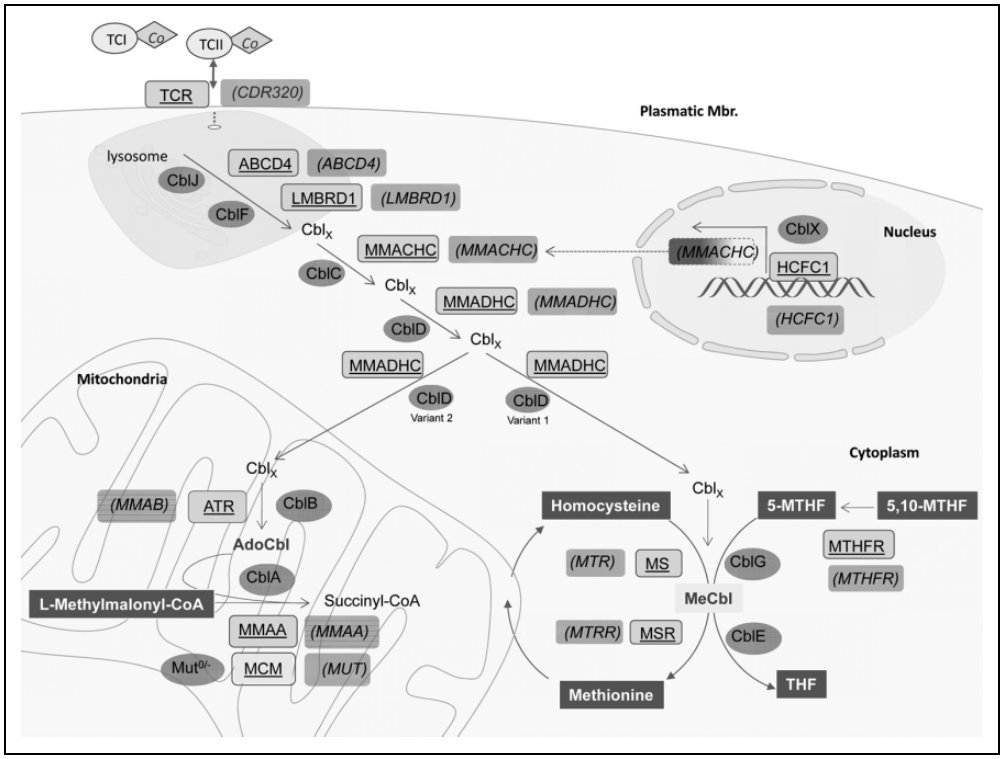

Figure 1. Remethylation disorders causing hyperhomocysteinemia (HHcys). Cobalamin enters cells by endocytosis after cobalamin-bound transcobalamin attaches to its cellular receptor. Cobalamin leaves the lysosome to be processed by MMACHC and MMADHC and thus produce methylcobalamin (MeCbl), the active cofactor of methionine synthase (MTR). The MTR is activated by methionine synthase reductase (MTRR), a reaction that needs 5-methyltetrahydrofolate (MTHF), which is generated by 5, 10-methylenetetrahydrofolate reductase (MTHFR). The complementation groups are in circles; the proteins (underlined) and genes (italics) are in squares.

through the action of the enzyme methionine synthase [MTR]) also maintains Hcys at nontoxic levels in the remethylation pathway. The same enzyme then passes this methyl group from MeCbl to Hcys, thus forming Met. Methionine synthase reductase undertakes the reductive reactivation of the cob[I]alamin.[4] Defects in the genes *MTR*, *MTRR*, or *MTHFR* all lead to isolated remethylation disorders, in which HHcys concentrations become elevated.

In combined remethylation disorders, Hcys concentrations become elevated along with those of methylmalonic acid (MMA). The latter becomes elevated because the synthesis of adenosylcobalamin (AdoCbl) is affected; this might occur if any of the enzymes involved in its synthesis are defective (Figure 1). Adenosylcobalamin is necessary for the activity of mitochondrial methylmalonyl-CoA mutase (EC 5.4.99.2), the enzyme responsible for the transformation of L-methylmalonyl-CoA into succinyl-CoA (which enters the citric acid cycle; Figure 1).[4] This review summarizes the genetic basis, diagnostic clues, treatment, and pathophysiology associated with isolated and combined remethylation disorders.

Genetic Basis of Isolated Remethylation Disorders

Isolated remethylation disorders are caused by defects in *MTRR* (complementation group *cblE*), *MTR* (complementation group *cblG*), *MTHFR*, and *MMADHC* (complementation group *cblD-Hcy*; Table 1).

Methionine synthase is a cytosolic modular enzyme that catalyzes the transfer of a methyl group from 5-MTHF to Hcys, forming Met. During each turnover cycle, MTR catalyzes 2 consecutive methyl transfer reactions, from 5-MTHF to cob(I)alamin and then from MeCbl to Hcys to form Met and regenerated cob(I)alamin.[5-7] Inactive MTR is returned to the catalytic cycle via a coupled methylation reaction in which an electron derived from nicotinamide adenine dinucleotide phosphate (NADPH) is transferred by MTRR and S-adenosylmethionine (SAM) serves as methylating agent. The reaction is vital since it ensures the provision of Met for the production of SAM, the so-called universal donor of methyl groups. The MTR protein consists of 4 functional modules distributed in a linear fashion, connected by a single interdomain. Each of the subreactions is catalyzed by a different substrate-binding domain. The *N*-terminal module binds and activates Hcys; the second module activates 5-MTHF for methyl transfer, and the third module binds cobalamin. The fourth, *C*-terminal module, binds SAM and is involved in MTR reactivation. Patients with the complementation defect *cblG* present megaloblastic anemia and developmental delay (MIM#156570) and show mutations in the *MTR* gene, which is located in chromosome 1q42.3-43 and consists of a 3795 base pair (bp) open reading frame (ORF) spanning 33 exons encoding a 140.3 kDa protein.[8,9] To date, 39 mutations have been described in *MTR*, 35 of which are associated with a disease phenotype and 1 associated with disease-associated polymorphism (HGMD Professional 2015.4).

Table 1. Combined and Isolated Remethylation Disorders.

Defects	MIM	Gene Location	Clinical Symptoms	Plasma B_{12}	Biochemical Findings
Isolated remethylation disorders					
Homocystinuria cblD-Hcy	611935	*MMADHC* 2q22.11-23.3	Development delay, megaloblastic anemia	N	↑ tHcy (pl + ur)
Methionine synthase *cblG* type	156570	*MTR* 1q43	Development delay, megaloblastic anemia	N	↑ tHcy (pl + ur); ↓Met (pl + ur)
Methionine synthase reductase, *cblE* type	602568	*MTRR* 5p15.2-p15.3	Development delay, megaloblastic anemia	N	↑ tHcy (pl + ur); ↓Met (pl + ur)
5,10-methylenetetrahydrofolate reductase	607093	*MTHFR 1p36.22*	Encephalopathy, neurocognitive impairment, epilepsy	N	↓Met (pl); ±↑tHcy (pl); ↓↓5-MTHF (CSF)
Combined remethylation disorders					
MMA and homocystinuria *cblF* type	612625	*LMBRD1* 6q13	Development delay, megaloblastic anemia	N	↑MMA + tHcy (pl + ur)
MMA and homocystinuria *cblJ* type	603214	*ABCD4* 14q24	Development delay, megaloblastic anemia	N	↑MMA + tHcy (pl + ur)
MMA and homocystinuria *cblC* type	609831	*MMACHC* 1p32.2	Development delay, megaloblastic anemia	N	↑MMA + tHcy (pl + ur)
MMA and homocystinuria *cblD* type	611935	*MMADHC* 2q22.11-23.3	Development delay, megaloblastic anemia	N	↑MMA + tHcy (pl + ur)
MMA and homocystinuria *cblX* type	309541	HCFC1 Xq28	Intractable epilepsy, severe cognitive retardation, and development		↑MMA (pl + ur); ±↑tHcy (pl)

Abbreviations: CSF, cerebrospinal fluid; Hcy, homocysteines; Met, methionine; MMA, methylmalonic acid; ; 5-MTHF, 5-methyltetrahydrofolate *MTHFR*, 5, 10-methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; pl, plasma; tHcy, total homocysteine; ur, urine.

Methionine synthase reductase plays an auxiliary role in the remethylation of Hcys to Met by maintaining MTR in its functional, reduced status.[10,11] Studies have shown that although MTR can be reduced by 2 other redox proteins under physiological conditions, MTRR represents the choice for MTR reductive activation.[12] Severe deficiency of MTR activity leads to megaloblastic anemia and developmental delay. The *MTRR* (MIM #602568) is composed of 15 exons that encode a 698 amino acid protein with a predicted molecular weight of 77.7 kDa. The MTRR belongs to the ferredoxin-nicotinamide adenine dinucleotide phosphate ($NADP^+$) reductases, which include Flavin mononucleotide (FMN), Flavin adenine dinucleotide (FAD), and NADPH. Mutations affecting *MTRR* are responsible for the *cblE* complementation defect.[13]

To date, 35 mutations have been identified in *MTRR*, 29 of which are associated with disease, 3 with disease-associated polymorphism, 1 with functional polymorphism (c.66A>G), and 1 which has no defined effect (HGMD Professional 2015.4). Individuals with the AA genotype for c.66A>G have mild HHcys.[14] The most prevalent mutation is a deep intronic T>C transition located in intron 6. It has been reported that antisense therapy can rescue the normal splicing process.[15]

Methylenetetrahydrofolate reductase deficiency (MTHFR, MIM#607093) is the most common of the genetic disorders of folate metabolism. The MTHFR is a cytosolic enzyme that catalyzes the irreversible reduction of 5, 10-methyleneTHF to 5-methylTHF, a reaction that requires NADPH as an electron donor and FAD as a cofactor. The clinical manifestation of the disorder is variable, including severe disease in infancy leading to death, developmental delay, neurological and psychiatric disease, and epilepsy.[2,11] Some patients present with a late childhood onset form, often with developmental delay and varying neurological manifestations. This autosomal recessive disorder is owed to mutations in the *MTHFR* gene which is located on chromosome 1p36.3. Over 126 disease-causing mutations have been reported to date.[16]

In addition to the severe deficiency, a number of functional polymorphisms have been described, which increase the risk of developing cardiovascular disease, Alzheimer disease, diabetes mellitus, neural tube defects, and some forms of cancer. Two of these functional polymorphisms, c.677C>T and c.1298A>C, are associated with reduced MTHFR activity. With respect to c.677C>T, 1 copy of allele T reduces the enzyme's activity by 40%, whereas 2 copies result in about a 70% reduction. The frequency of the minor allele T is variable among populations. Genetic analyses of both polymorphisms have some predictive value of the risk of increased HHcys. The presence of a T allele in homozygosity is associated with HHcys, and the presence of low folate levels is associated with cardiovascular disease, neural tube defects, and so on.[3,17]

Genetic Basis of Combined Remethylation Disorders

Combined remethylation disorders are a heterogeneous group of diseases caused by defects in the synthesis of MeCbl and AdoCbl, leading to methylmalonic aciduria combined with HHcys (MMAHC).[18] Genes are summarized in Table 1.

In mammals, cobalamin is an essential nutrient. When bound to transcobalamin II, it enters the cell through receptor-mediated endocytosis and the transporter is degraded

in the lysosome by lysosomal proteases. Cobalamin is then released into the cytosol. However, the latter step is impaired in patients in complementation group *cblF* (MIM #277380).[19,20] This defect was first described in 1986 by Watkins and Rosenblatt,[21] although the gene responsible—*LMBRD1*—was only discovered in 2009 by Rutsch et al.[22] LMBD1, a lysosomal membrane protein of 61.4 kDa and consisting of 540 amino acids, is predicted to consist of 9 transmembrane helices and a cytoplasmic C terminus.[19] LMBD1 shares homology with lipocalin membrane receptors, which are responsible for the internalization of lipocalins by receptor-mediated endocytosis. Despite LMBD1 being a possible lysosomal exporter of cobalamin, the presence of an additional adaptor protein in the lysosome was postulated by Rutsch et al.[19,22] Patients in the *cblF* group present a range of clinical abnormalities, failure to thrive, developmental delay, macrocytic anemia, neutropenia, thrombocytopenia, and pancytopenia.[20] To date, 9 mutations have been described in *LMBRD1* (HGMD Professional 2015.4), the majority being small deletions (66.7%).

The *cblJ* complementation defect is caused by mutations in the gene *ATP Binding Cassette Subfamily D Member 4 (ABCD4)* (MIM #603214). This encodes ABCD4, an adenosine triphosphate-binding cassette (ABC) transporter. The ABCD4 interacts with LMBD1.[23] Although the specific role of each in the transport of cobalamin across the lysosomal membrane is not known, studies in bacteria support the idea that ABCD4 might be the actual cobalamin transporter, whereas LMBD1 functions as an accessory or regulator protein.[23] To date, 5 mutations have been described in *ABCD4* (HGMD Professional 2015.4).[24]

The *cblC* complementation defect—or methylmalonic aciduria and homocystinuria *cblC* type (MIM #277400)—is the most common of cobalamin defects and is caused by mutations affecting *MMACHC*.[25] *MMACHC*, which contains an 846-bp ORF, encodes a protein of 282 amino acids with decyanase (reductive decyanation of cyanocobalamin into cob(II)alamin) and dealkylase properties.[26,27] Several patients have been described with this complementation defect. A total of 85 disease-causing mutations have been reported (HGMD Professional 2015.4), the majority being of the missense type (53%), followed by small deletions (23%). To date, the most prevalent mutation is c.271dupA, which accounts for 80% of the mutant alleles in the Spanish population.[28]

The *cblX* complementation defect, a phenocopy of the *cblC* complementation group, was only recently identified. It is caused by mutations in *host cell factor C1 (HCFC1)* (MIM #300019), which is located on the X chromosome and is a regulator of the zinc finger transcription factor THAP domain containing 11 (THAP11), also known as RONIN.[29] This is the first complementation defect described in which the gene affected does not encode a protein directly involved in the transport and/or metabolism of cobalamin. Instead, the HCFC1-THAP11 complex binds to consensus sequence motifs in genes such as *MMACHC*, *MTR*, and *ABCD4*, which are directly involved in cobalamin metabolism.

Patients in this complementation group—all of which are males—present a phenotype very similar to that of patients with the *cblC* complementation defect, but with more severe neurological involvement. Of the 15 mutations described for this gene, only 5 are responsible for an methylmalonic aciduria phenotype combined with homocystinuria, and all 5 affect conserved amino acids located in the Kelch domain (responsible for protein–protein interactions, catalytic activity, and transportations). Although these mutations do not seem to affect *MMACHC* expression, the role of HCFC1 (joined to THAP11) as a transcriptional factor in *MMACHC* activation is compromised.[29] A number of explanations exist for the severe neurological presentations observed in patients with the *cblX* complementation defect compared to those with the *cblC* complementation defect—(1) HCFC1 is involved in several processes, including the cell cycle, proliferation, and transcription, (2) *HCFC1* defects have been involved in brain development and function,[30] and (3) 6 of the 12 mutations described in the literature are responsible for an intellectual disability phenotype (HGMD Professional 2015.4).

Cellular cobalamin processing can be divided into 2 major pathways, cytosolic and mitochondrial. The protein thought to be responsible for cobalamin sorting with respect to these pathways is MMADHC (*cblD* complementation group). *MMADHC* complementary cDNA has an ORF of 891 bp that is translated into a 296-amino acid protein with a predicted molecular mass of 32.8 kDa.[31] The protein has a mitochondrial leader sequence (residues 1-11), a weakly conserved putative B_{12}-binding motif (GXXXHXD, residues 81-86), and a region homologous to the adenosine triphosphatase component of a bacterial ABC transporter (residues 78-168). The *MMADHC* is located on chromosome 2q23.2, and mutations affecting its function are responsible for the *cblD* complementation defect. This complementation group is the most complex of all because patients show biochemical heterogeneity ranging from isolated homocystinuria (*cblD*-Hcy), or isolated methylmalonic aciduria (*cblD-MMA*), to methylmalonic aciduria combined with homocystinuria (*cblD*-HcyMMA). This heterogeneity provides strong evidence for a dual cobalamin processing and assimilation function, one in the cytoplasmic pathway and another in the mitochondrial pathway. Problems in this dual function are related to the location and type of mutation affecting the gene (Figure 2). Mutations causing the appearance of a premature translation stop codon affecting the *N*-terminal region of the protein are found in patients with *cblD*-MMA, whereas missense mutations affecting the *C*-terminal of the protein are related to *cblD*-Hcy, and nonsense and missense mutations affecting the middle and the *C*-terminal of the protein are related to the *cblD*-combined phenotype.[31,32] The 3 biochemical phenotypes are explained by the existence of 2 additional initiation codons in the protein's messenger RNA (mRNA), involving positions 62 (Met 62) and 116 (Met 116). Therefore, mutations affecting residues located after Met 62 or even Met 116 only affect the protein region responsible for AdoCbl synthesis, leaving a protein fully capable of MeCbl synthesis. Mutations downstream of Met116 abolish MeCbl synthesis capacity but leave AdoCbl synthesis intact. Mutations affecting patients with the combined phenotype involve a short stretch of

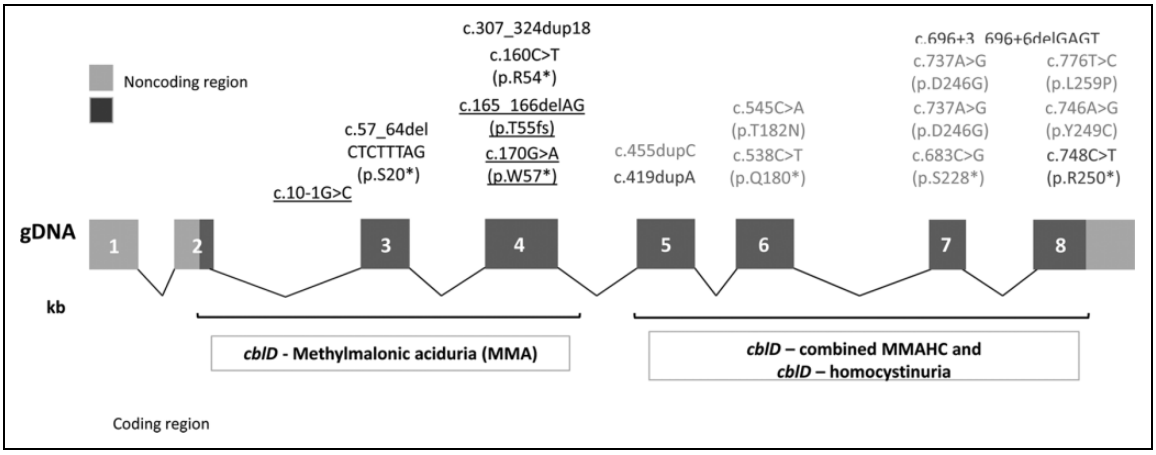

Figure 2. Mutations described in MMADHC causing the methylmalonic aciduria (MMA), methylmalonic aciduria combined with hyperhomocysteinemia (MMAHC), and HC phenotypes. Mutations giving rise to a premature translation stop codon, or that disrupt the normal splicing profile toward the *N*-terminal of the protein, are seen in patients with *cblD*–MMA, causing MMA (black). Missense, nonsense, and small duplication mutations affecting the *C*-terminal of the protein are related to *cblD*–homocysteines (Hcy) causing HC (light gray). Nonsense, small deletions and small duplication mutations affecting the middle and *C*-terminal of the protein are related to the *cblD*–HcyMMA (gray). Mutations highlighted in bold are described for the first time in this work and are not collected in HGMD (Professional 2015.4).

14 amino acids toward the *C*-terminal of the protein. According to recent studies, this small region is 1 of the 5 putative sites of interaction between MMADHC and MMACHC.[32]

It has been shown that MMACHC interacts mainly with the *C*-terminal domain of MMADHC in the cytosol, indirectly assisting the transfer of cob(II)alamin to MTR.[33] A recent study has shown that MMADHC and MMACHC are close structural relatives and that MMADHC enhances the oxidation of cob(II)alamin bound to MMACHC. Mutations affecting MMADHC thus impair this reaction.[34]

Patients with the *cblD*-combined phenotype show developmental delay, seizures, hypotonia, lethargy, and megaloblastic anemia, those with the *cblD*-Hcy show developmental delay, ataxia, and megaloblastic anemia, and those with the *cblD*-MMA have respiratory distress, cranial hemorrhage, seizures, and abnormal electroencephalograms.[35] To date, only 17 patients with *cblD* have been described in the literature.[32]

*Diagnosis of Isolated (*cblD-Hcy, MTHFR, cblE, cblG*) and Combined Remethylation Disorders (*cblC, cblD-HcyMMA, cblF, cblJ, cblX*)*

The biochemical diagnosis of both isolated and combined remethylation disorders is based on the detection of following key metabolites in plasma and/or urine—total Hcys (tHcy), MMA, Met, propionylcarnitine (C3), and 5-methyltetrahydrofolate (5-MTHF) in cerebrospinal fluid (CSF).

Low plasma Met, increased plasma tHcy, and C3, with increased urinary excretion of MMA, 3-hydroxypropionate, and methylcitrate, are diagnostic of combined remethylation disorders. Low plasma Met with increased tHcy, but with normal C3 and MMA levels, is diagnostic of isolated defects. The additional detection of much reduced CSF levels of 5-MTHF points to an MTHFR defect. The presence or absence of megaloblastic anemia may also help in making a diagnosis. Figure 3 shows the workflow for making clinical and biochemical diagnoses.

Patients with isolated remethylation disorders (*cblD-Hcy, cblE*, and *cblG*) show reduced [5-^{14}C] methyl-THF incorporation into cultured fibroblasts and impaired MeCbl synthesis. The MTHFR deficiency can be confirmed by the determination of the enzyme's activity in the same kind of cells.[36]

Patients with combined remethylation disorders (*cblC, cblD-HcyMMA, cblF, cblJ*, and *cblX*) show reduced conversion of propionate to succinate along with reduced MTR function, respectively, measured by [1-^{14}C] propionate and [5-^{14}C] methyl-THF incorporation into the protein of cultured fibroblasts grown in basal and hydroxocobalamin-supplemented media. In addition, they show impaired synthesis of AdoCbl and MeCbl. Somatic cell complementation analysis is sometimes used to categorize the different genetic defects.[37]

The identification of disease-causing mutations facilitates accurate prenatal diagnosis, the detection of carrier status in family members, genetic counseling, preimplantation decisions, and in some cases, genotype–phenotype correlations. Following biochemical studies, genetic analysis by conventional Sanger sequencing has for many years been the gold standard for identifying the gene affected in remethylation disorders. However, this method can only examine 1 gene at a time, exon by exon, which is not always cost-effective. Fortunately, recent developments in high-throughput sequence capture have made massive parallel sequencing routine in genetic diagnosis. This very cost-effective technology is particularly appropriate for screening mutations in disorders of highly heterogeneous genetic background, including remethylation disorders.[38-43] The strength of this technology lies in its ability to generate large amounts of sequence data. Furthermore, the possibility of adding specific sequence tags (DNA bar codes)

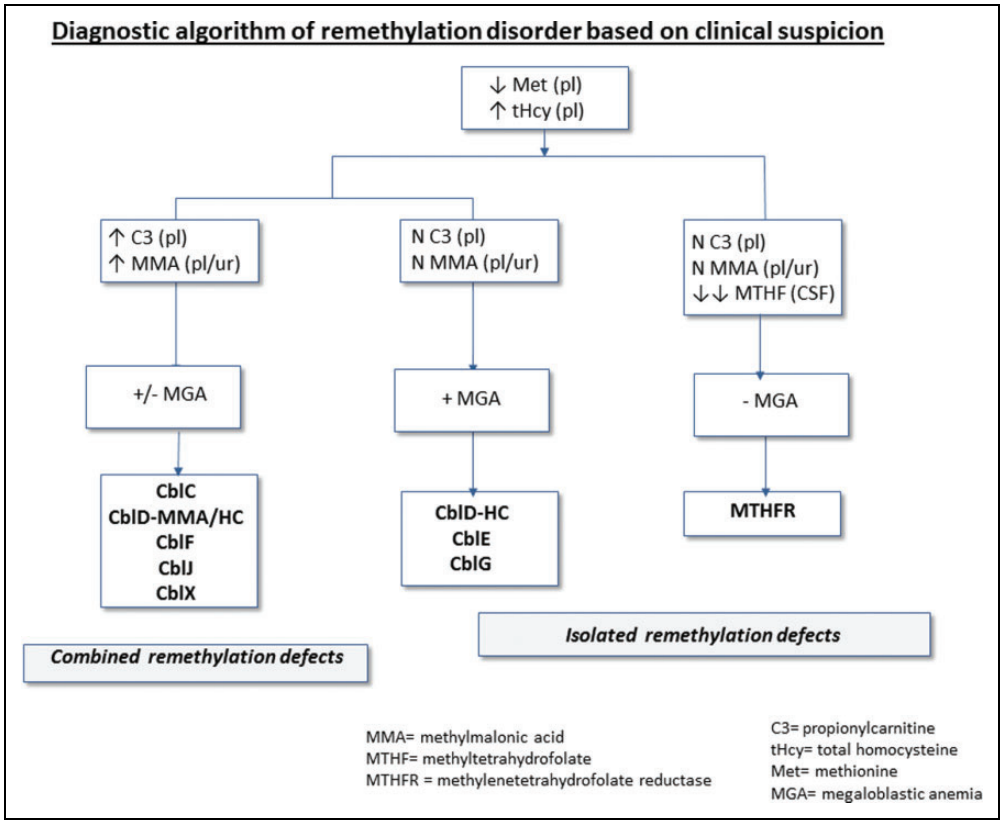

Figure 3. Clinical and biochemical workflow for identifying combined and isolated remethylation. The figure illustrates the steps to diagnose remethylation disorders.

to each sample allows the testing of pooled DNA from different patients, further reducing costs and time requirements. In clinical diagnosis, disease-associated gene panels with fewer genes than used in whole-exome sequencing, but with better base pair coverage, can be used. Also, a large panel such as the TruSight One exome panel (with its comprehensive coverage of >4800 clinically important genes), can be used. After gene capture, specific subsets of genes (based on information from biochemical tests) can be focused upon (Figure 4).

Expanded Neonatal Screening

The primary markers used in expanded newborn screening involving tandem mass spectrometry (MS/MS) for the diagnosis of combined remethylation disorders are C3, the propionylcarnitine to acetylcarnitine (C3:C2) ratio, Met, and the Met to phenylalanine (Met:Phe) ratio. An increase in C3 and C3:C2 provides a sensitive means of detecting these combined disorders, but they are not very specific since they are also altered in maternal vitamin B_{12} deficiency and propionic acidemia (PA). The measurement of MMA and tHcy in dried blood spots (DBSs) is therefore recommended as a second-tier test. Recently, heptanodecanoyl carnitine (C17) has been identified as a primary marker that improves the sensitivity of the first-tier test but also is detected in patients with PA.[44] The diagnosis of isolated remethylation defects is sometimes possible via the detection of reduced concentrations of Met and smaller Met to Phe ratios in DBS, followed by the determination of Hcys as a second-tier test.[45]

The advent and refinement of massive parallel sequencing has resulted in both cost and time reductions, and its use has been proposed as a first test for newborn screening.[46,47] The technology is robust enough to replace the MS/MS used in such screening, but for a number of reasons, this is not yet the best option. For example, the detection of variants of unknown clinical significance and the detection of changes that might not cause a significant disease are currently serious problems that only future information can solve. Indeed, massive parallel sequencing has identified children as "affected" (with all the personal, family, and treatment consequences implied) that in fact were probably quite healthy. Currently, the National Institute of Child Health and Development is funding four 5-year research projects to examine the use of massive parallel sequencing in newborn screening (http://www.nih.gov/news/health/sep2013/nhgri-04.htm, Accessed January 29, 2014).[47]

Prenatal Studies

The prenatal diagnosis of both isolated and combined remethylation diseases is feasible. It is desirable that the index case first be confirmed biochemically and genetically and that the carrier status of the parents be confirmed by mutation analysis.

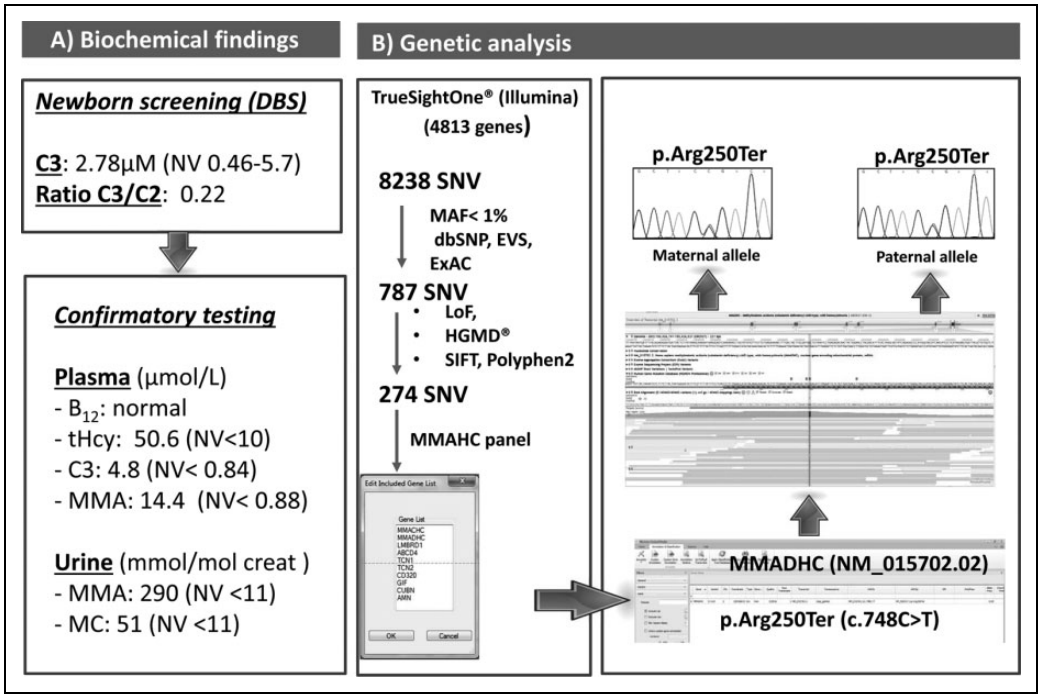

Figure 4. Biochemical and genetic analysis of one patient with methylmalonic aciduria combined with hyperhomocysteinemia (MMAHC). The figure shows the biochemical findings in dried blood spots at 48 hours for a patient detected in a newborn screening program, plus the results of confirmatory testing (A). Genetic analysis by massive parallel sequencing focused on a primary list of genes based on the biochemical phenotype. This allowed the detection of an already described homozygous mutation in *MMADHC* (B). The mutation was confirmed by Sanger sequencing in the parents' blood samples (dried blood spots).

Mutation analysis of the DNA from chorionic villi is the most used method in making a prenatal diagnosis. If mutation analysis is not available, analysis of the amniotic fluid for tHcy, C3, MMA, and methylcitrate should be performed. In addition, MTHFR activity in cultured chorionic villus cells and amniocytes can be assayed.[36,48]

Treatment of Remethylation Disorders

Early recognition of remethylation disorders, followed by aggressive treatment, may lead to a favorable clinical outcome.[49] Treatment should be personalized, although no consensus exists regarding the design of diets, doses of cobalamin, folate/folinic acid or betaine, and the assessment of treatment effectiveness.[45,50] The aims of treatment are to bypass the defects present by correcting the patient's hematological and biochemical abnormalities and to avoid neurological deterioration. The correction of Met levels and the reduction of tHcy (to below 50 µmol/L) in all defect types, as well as MMA levels only in combined defect types, should help correct hematological abnormalities. Treatment usually requires large doses of parenteral hydroxocobalamin (which reduce elevated metabolite concentrations, although not to normal levels), the oral administration of betaine (the substrate of betaine-Hcys methyltransferase, which helps to decrease the tHcy levels),[51] and supplementation with folinic acid (to avoid folate depletion). Some patients seem to benefit from a reduction in dietary protein and from oral Met supplementation.

Pathophysiology of Remethylation Disorders

The HHcys and defective Met synthesis are the major pathophysiological mechanisms underlying remethylation defects. Methylation disturbances in the hippocampus have been related to short-term memory impairment in MTRR knockout mice.[52] In addition, MTHFR-deficient mice have been found to have abnormalities in the size and/or structure of the cerebellum, cortex, and hippocampus, to exhibit memory impairment and to show behavioral anomalies.[53,54] When tHcy is increased, it competes with SAM for the binding site on DNA methyltransferase, leading to DNA hypomethylation with consequences for epigenetic programming.[55] Neural tube defects may be associated with abnormal Hcys levels, folate metabolism, and methylation during embryogenesis.[3]

Even though the pathophysiology of combined remethylation disorders such as type C cobalamin deficiency is not the same as the isolated remethylation defects, several hypotheses have been proposed for explaining the pathophysiology of HHcys, such as alterations in signal transduction pathways, activation of inflammatory factors, oxidative stress, perturbations in calcium homeostasis, and endoplasmic reticulum (ER) stress.[56] Homocysteines is readily oxidized in plasma to form Hcys and Hcys-mixed disulfides (the predominant forms of this amino acid in circulation).[56] It can also undergo autoxidation causing the disruption of redox homeostasis in vascular and neuronal cells.[57] This oxidation has been correlated with reactive oxygen species (ROS) generation. Indeed, data from our

laboratory support the involvement of oxidative stress in the pathophysiology of HHcys. In 1 study, fibroblasts derived from patients with combined remethylation disorder *cblC* or isolated disorder *cblE* (among others) showed a significant increase in intracellular ROS, an increased expression of the antioxidant manganese superoxide dismutase (MnSOD), and a higher rate of apoptosis than healthy fibroblasts. The overexpression of MnSOD is probably due to a compensatory mechanism that counteracts the consequences of elevated ROS, as reported in other disorders.[58-61] In general, the *cblC* fibroblasts had the highest intracellular ROS content of all these patient-derived fibroblasts.[28] In addition, the examination of F-2 isoprostanes and dityrosine as markers of oxidative damage showed that patients with *cblC* disorder consistently had the highest levels of oxidative damage in urinary oxidative stress profiling.[62]

In other work, the study of apoptosis revealed an activation of stress-sensing pathways. p38 and c-Jun N-terminal (JNK) kinases were particularly activated in patients with *cblC* or isolated *cblE*—the patients with highest intracellular ROS levels. In fibroblasts from the former patients, the induction of apoptosis was essentially maintained by the activation of the death receptor-mediated apoptotic pathway. Functional rescue by MMACHC overexpression induced via retroviral infection showed that this defect is at least in part responsible for the increased ROS and apoptosis levels observed.[63]

Extending the study of ROS overproduction, the analysis of the overproduction of MnSOD, and the activation of p38- and JNK kinase-induced apoptosis to other patients-derived fibroblasts with isolated remethylation defects (MTRR, MTR, and MTHFR deficiencies) confirmed that Hcys may play an important role in the increase of ROS and apoptosis.[64] In fact, experiments in cellular models generated by stable *MTRR*-silencing and *MTRR* gene correction in mutant cells lines have suggested that defects in *MTRR* might participate, at least in part, in the oxidative stress of patients with homocystinuria.

Perturbations in calcium homeostasis and ER stress have also been proposed to explain the pathophysiology of HHcys.[56] The ER is a unique cellular compartment involved in protein synthesis and Ca^{2+} homeostasis. Oxidative and metabolic stress and Ca^{2+} overload can interfere with the function of this organelle, leading to the accumulation of misfolded proteins.[65] The ER unfolded protein response consists of 3 main signaling systems initiated by the stress sensors PERK, IRE-1, and ATF6. These sense ER stress through Grp78 binding/release via their respective luminal domains.[66] Recently, increased upregulation of several mRNAs and proteins involved in ER stress, that is, Grp78, IP_3R1, pPERK, ATF4, CHOP, asparagine synthase, and GADD45, has been described in patient-derived fibroblasts with isolated remethylation defects, suggesting that these cells have ER stress and calcium perturbations.[67] The same has been suggested by mRNA differential expression and cDNA microarray analysis of cells exposed to supraphysiological concentrations of Hcys.[56] Nevertheless, ER stress has never been detected in animal models, neither does the mouse model of Cystathionine-β-synthase (Cbs) deficient homocystinuria show hepatopathy due to the induction of such stress (probably due to the protective effects of cystathionine[68]) nor do MTHFR model mice seem to have oxidative or ER stress.[69,70]

In addition to the modification of the redox environment in ER, Hcys upregulates Hcys-inducible ER stress protein (Herp), a membrane protein of this organelle. Increased Herp appears to be essential for the resolution of ER stress via the maintenance of Ca^{2+} homeostasis and protein degradation.[65,71] We have recently reported an increase in cell death after Herp knockdown,[67] which suggests that the upregulation of this protein protects against death under conditions associated with ER stress. Herp is also markedly increased in neurons subjected to ER stress, especially in the substantia nigra in patients with Parkinson disease.[65,72] In addition, our results showed increased IP_3R1 levels, suggesting the aberrant accumulation of ER Ca^{2+} channels that perhaps disrupt Ca^{2+} homeostasis, as previously described in cellular models of neuronal degeneration.[73]

The ER and mitochondria are tubular organelles that together show a characteristic ''network structure'' that facilitates the formation of connections between them. The ER and mitochondria join together at multiple contact sites to form mitochondria–ER-associated membranes (MAMs) where intracellular lipid rafts regulate Ca^{2+} homeostasis, the metabolism of glucose, phospholipids, and cholesterol.[74] An increase in several MAM-associated proteins (Grp75, σ-1 R, and Mfn2) has been observed in cells from patients with remethylation defects that might result in mitochondrial calcium overload and increased oxidative stress.[67] The upregulation of these 3 MAM-associated proteins may indicate altered ER–mitochondrial communication and therefore aberrant calcium homeostasis in fibroblasts derived from patients with isolated remethylation disorders.

Recently, it has also been shown that MAMs are important for autophagy via the regulation of autophagosome formation; the ER–mitochondria interface provides membranes for autophagy.[75] In fibroblasts derived from patients with isolated remethylation defects, increased protein levels of LAMP1 (lysosomal-associated membrane protein) have been observed, along with an increased number of autophagosomes per unit area, the colocalization of cytochrome C and lysotracker, a reduced number of mitochondria, and a greater presence of laminar bodies compared to controls. Together, these observations suggest that autophagy and mitophagy are activated in *cblE*, *cblG*, and MTHFR fibroblasts.[67]

In conclusion, patient-derived fibroblasts showing isolated or combined remethylation defects show elevated ROS and apoptosis. Moreover, p38 and JNK might be activated in an ROS-dependent manner in these fibroblasts, which might activate apoptosis. The ER stress, the activation of autophagy, and alterations in Ca^{2+} homeostasis also occur and may contribute to the pathogenesis of these inherited metabolic disorders. In light of these observations, agents that stabilize calcium homeostasis restore the proper function of ER–mitochondria communications and diminish intracellular ROS, and oxidative stress-induced apoptosis might provide new ways of treating these devastating diseases.

In summary, given the important role of Hcys in cell metabolism, the involvement of HHcys in apoptosis activated by increased ROS, in ER stress, in the activation of autophagy, and in alterations of Ca^{2+} homeostasis, the early diagnosis of isolated and combined remethylation disorders is imperative. In the era of precision medicine, the challenge of metabolic disorders lies in providing early treatment. Mass spectrometry is a powerful tool for detecting remethylation defects in the first days of life, but a second-tier metabolic test is needed for a differential diagnosis to be made, and genetic analysis should be performed to identify the specific gene defect involved. Massive parallel sequencing will undoubtedly be of great clinical use by offering confirmatory diagnostic testing for the subset of newborns that screen positive in traditional biochemical tests.

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