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Chapter 1

MTHFR AND OTHER ENZYMES ASSOCIATED WITH THE CIRCULATION OF METHYL IN NEURODEGENERATIVE DISEASES

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ABSTRACT

Homocysteine (Hcy), which originates from methionine (Met), may undergo remethylation, due to involvement of 5,10-methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR), or transsulfuration with cystathionine β–synthase (CBS) to cysteine (Cys).

Elevated levels of Hcy (hyperhomocysteinemia, HHcy) are a known risk factor for many neurological diseases, such as Alzheimer's disease

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(AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and epilepsy after antiepileptic drug (AEDs) therapy.

HHcy can affect the damage of DNA and RNA. It is believed that HHcy may also lead to generation of oxidative stress and induction of apoptosis, and leads to the onset and progression of diseases involving deterioration of neurons.

MicroRNAs (miRNAs) are small, ~22 nucleotides in length, RNA molecules involved in regulatory networks responsible for the fine-tuning of the expression of diverse genes, e.g., genes involved in the synthesis and metabolism of biothiols. It has been shown that the expression of the *MTHFR* gene is regulated by miR-149 and miR-22, and mutation +905A of the *MTR* gene influence the binding of miR-485, miR-608, and miR-1293, as well as Hcy levels.

The pathogenesis of neurodegenerative disorders is most often associated with *MTHFR* C677T and A1298C genotypes, which reduce MTHFR activity and lead to HHcy.

Much data does not support a major role for Hcy transsulfuration in the production of Cys necessary for GSH synthesis in the brain, but it is believed that this metabolic pathway may be involved in the pathological mechanism of some neurodegenerative diseases, such as AD and HD. Although relationship of the *CBS* gene with AD, as well as PD, remains undefined, some reports indicate that the *CBS* mutation may be an independent risk factor for AD.

The pathogenesis of neuronal damage involving HHcy is not completely explained, and requires further study.

Keywords: MTHFR, CBS, CTH, homocysteine, neurodegenerative diseases

INTRODUCTION

Homocysteine (Hcy) was isolated by Vincent du Vigneaud in 1932. Thirty years later, in 1962, Nina Carson and D. Neill [1] described the presence of elevated levels of Hcy (hyperhomocysteinemia, HHcy) in the urine of two siblings from Northern Ireland. Over the next few years, the National Institute of Health (NIH) in the USA demonstrated the association of homocysteinuria (Hcy level between 300-500 μM) with the lack of cystathionine β–synthase (CBS), an enzyme converting Hcy to cysteine (Cys) involving vitamin B6. This was also the first time that the relationship between the B-group vitamins and elevated Hcy levels was demonstrated. In 1969, Kilmer McCully [2] conducted a post-mortem examination of the corpses of two children who died

of complications of homocysteinuria, and concluded that they had extensive atherosclerosis and thrombosis. Kilmer McCully also first drew attention to the possible association between elevated Hcy levels in blood and the occurrence of atherosclerosis. However, the presence of Hcy in blood plasma was detected at the end of the 1970s [3].

Over the years, a connection between HHcy and rare homozygous enzyme defects has been demonstrated, including 5,10-methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR) deficits that are involved in the process of Hcy remethylation to methionine (Met) and depends of folic acid (FA) and vitamin B12 levels [4, 5].

Elevated Hcy levels are currently a known risk factor for many diseases, such as vascular, neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and fetal development defects [6-12].

It is known that Met is supplied by food and it is converted to S-adenosylmethionine (SAM). SAM leads to the formation of S-adenosylhomocysteine (SAH), which is further hydrolyzed to Hcy [13]. Moreover, SAM is a donor of methyl groups for DNA modifications. There is a relationship between DNA methylation and AD pathology.

Other enzymes transforming Hcy to Met include the tri-functional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclo-hydrolase/formyltetrahydrofolate synthetase (MTHFD1) and betaine-homocysteine methyltransferase (BHMT). Significant differences in Cys/Hcy levels have been shown between AD and PD patients that carry the *MTHFD1* GA (G1958) variant. It has also been shown that patients with HHcy and a defective *BHMT* gene respond well to treatment with vitamin B6 [14].

Hcy can also be converted to Cys. Transsulfuration of Hcy to Cys is linked to the enzymes CBS and cystathionine γ-lyase (CTH) [15-17].

Assessing mutations present in patients with HHcy has demonstrated about 17 different *CBS* gene defects. Moreover, it has been shown that mutations in the *CBS* gene may contribute to increasing frequency of seizures in patients with epilepsy via elevated concentrations of Hcy [18]. Additionally, some reports have indicated *CBS* mutations as independent risk factors for AD development in subjects aged 75 years or more. However, the relationship between the *CBS* gene with AD, as well as PD, remains undefined [12, 19].

CTH activity is more than 100-fold lower in the brain than in the liver, although the transsulfuration pathway is active in the central nervous system

(CNS). A major depletion of CTH has been demonstrated in the tissues of HD patients, which may mediate the pathophysiology of HD [20-22].

HHcy can affect damage of DNA and RNA. The microRNAs (miRNAs) are small, ~22 nucleotides in length, RNA molecules involved in the regulatory network responsible for fine-tuning the expression of diverse genes, e.g., genes involved in the synthesis and metabolism of biothiols [23]. It has been shown that the expression of the *MTHFR* gene is regulated by miR-149 and miR-22, and mutation +905A of the *MTR* gene, which influence the binding of miR-485, miR-608, and miR-1293 and Hcy level [24].

It is believed that HHcy in neurological diseases, such as AD, PD, epilepsy, and vascular disease, may also be due to a pharmacological effect of levodopa (L-dopa), antiepileptic drugs (AEDs) and lipid-lowering drug (such as fibrates) therapy [8, 25, 26].

The contribution of Hcy in the pathogenesis of neurological diseases is not fully understood and requires further research.

METABOLISM OF HOMOCYSTEINE TO METHIONINE IN NEURODEGENERATIVE DISEASES

In the body, Hcy (Figure 1) is a point of intersection of two main metabolic pathways: transsulfuration and remethylation. Under physiological conditions, approximately 50% of Hcy is catabolized by transsulfuration and undergoes transformation to Cys, and the remaining 50% of Hcy undergoes remethylation to Met [14].

Met is supplied with food and its transformation to Hcy involves several steps. At the first step, Met is transformed to SAM and then to SAH, and hydrolyzed to Hcy [13]. In the rate-limiting step of the methyl cycle, MTHFR irreversibly reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate is used to convert Hcy (a potentially toxic amino acid) to Met by the MTR enzyme (Figure 2).

The enzyme MTHFR is coded by the *MTHFR* gene on chromosome 1 at p36.3 in humans and consists of 11 exons with a length of 102-432bp, and 10 introns 250-4200bp in length [4]. There are genetic mutations and/or genetic polymorphisms associated with this gene (Table 1). In 2000, a report brought the number of known polymorphisms in the *MTHFR* gene up to 24 [27]. Two of the most investigated are the C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms (SNPs) [28]. The *MTHFR* nucleotide at

position 677 in the gene has two possibilities: C (cytosine) or T (thymine). C at position 677, leading to an alanine at amino acid 222, is the normal allele. The 677T allele, leading to a valine substitution at amino acid 222, encodes a thermo-labile enzyme with reduced activity.

It has been shown that carriers of recessive forms of the irregular *MTHFR* gene may account for up to 40% of the population, while the homozygous genotype can be present in 10-13% of the people [29]. Moreover, the frequency of genetic change in the *MTHFR* gene depends on the population and ranges from 1% to 30% whereas European and North American populations have demonstrated the most common C677T mutation in 5-18% of cases [30]. In the case of mutations in the *MTHFR* gene, indications of a possible hereditary enzyme deficiency are the occurrence of a neurological disorder, including stroke episodes, psychiatric disorders, epilepsy, and vascular diseases [28, 31, 32]. Impaired myelination of the CNS can be clearly seen in magnetic resonance imaging (MRI). However, MTHFR deficiency in a homozygous form may lead to severe homocysteinuria [31, 33].

Literature data show that thermo-labile MTHFR enzyme exhibits a relationship with elevated Hcy levels and the occurrence of mild HHcy (Hcy level between 16-30 μM) [34-37]. It is believed that people with the *MTHFR* C677T mutation respond well to supplementation with FA [4, 38, 39]. It has also been shown that the heat-labile form of the MTHFR occurs in 25% of people with mild HHcy, who have demonstrated the presence of vascular disease. Moreover, it has been shown that HHcy in the elderly relates to 30-40% of the population in the USA and 29% of Polish people over 59 years of age [40].

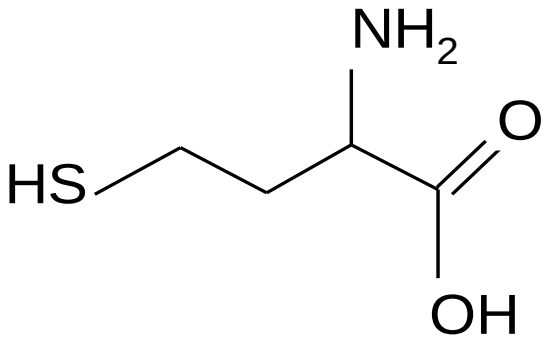

Figure 1. The structural formula of homocysteine.

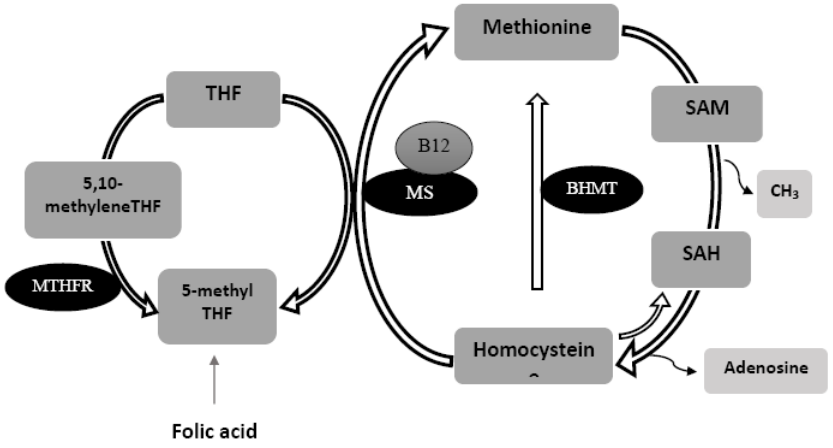

Figure 2. Metabolism of homocysteine to methionine. BHMT- betaine-homocysteine S-methyltransferase; MS – methionine synthase; MTHFR - methylenetetrahydrofolate reductase; THF- tetrahydrofolate; SAM – S-adenosylmethionine; SAH - S-adenosylhomocysteine.

Table 1. Some genetic variations in the *MTHFR* gene

Mutation/ polymorphism	dbSNP	Clinical symptom	References
ARG184TER	rs121434294	**Homocystinuria caused by mutation in *MTHFR***	Goyette et al., 1994 [4]
ARG158GLN	rs121434295	**Homocystinuria caused by mutation in *MTHFR***	Goyette et al., 1994 [4]
677C-T ALA222VAL	rs1801133	Related *MTHFR* polymorphism: vascular diseases neural tube defects hypertension Down's syndrome tumors depression schizophrenia migraine with aura thrombosis glaucoma cleft palate	Frosst et al., 1995 [33]
1298A-C GLU429ALA	rs1801131	**Related *MTHFR* polymorphism: schizophrenia**	van der Put et al., 1998 [28]

Mutation/ polymorphism	dbSNP	Clinical symptom	References
983A-G ASN324SER		**Homocystinuria caused by mutation in *MTHFR***	Hyland et al., 1988 [41]
1027T-G TRP339GLY		**Homocystinuria caused by mutation in *MTHFR***	Kluijtmans et al., 1998 [42]
1084C-T (Arg>stop)		**Homocystinuria caused by mutation in *MTHFR***	Hyland et al., 1988 [43] Kluijtmans et al., 1998 [42]
1711C-T (Arg>stop)		**Homocystinuria caused by mutation in *MTHFR***	Kluijtmans et al., 1998 [42]
1081C-T (Arg>Cys)		**Homocystinuria caused by mutation in *MTHFR***	Tonetti et al., 2000 [44]
MET581ILE	rs45590836	**Homocystinuria caused by mutation in *MTHFR***	Beckman et al., 1987 [45]
ARG377CYS	rs121434296	**Homocystinuria caused by mutation in *MTHFR***	Goyette et al., 1996 [46]
LEU323PRO	rs121434297	**Homocystinuria caused by mutation in *MTHFR***	Goyette et al., 1996 [46]

Agnati et al. [47] showed that Hcy may pass the blood/brain barrier (BBB) and that the level of plasma Hcy corresponds to the Hcy concentration in the brain. Elevated levels of Hcy in the CNS may damage vascular endothelium, deteriorate the functionality of the BBB, lead to disturbed production of nitrogen oxide (NO), and to neurotoxic effects both in the senescent brain and in neurological diseases, such as the neurodegenerative disorders AD, PD, HD, ALS and MS.

MTHFR and Alzheimer's Disease

AD is the most common cause of cognitive disorders and behavior leading to dementia. Both environmental and genetic factors play an important role in the pathogenesis of this disease. HHcy was recognized as an independent risk factor for AD and vascular dementia (VaD) [48,49]. Hogervorst et al. [48] have shown that changes in the white matter of the brain type leukoaraiosis were more frequent in patients with AD than in controls matched for age and gender, and the incidence of white matter changes increased significantly when the Hcy concentration rose to about 5 μM. The literature also describes an increased incidence of white matter changes associated with HHcy in the

temporal lobe of the brain [50]. Age is the most important risk factor for dementia and one of the most important risk factors for HHcy in the general population. Hcy levels remain relatively constant in the first four decades of life and then gradually increase, especially after 70 years of age [51].

It is believed that Hcy may influence the development of AD by generation of oxidative stress [52]. Hcy is subject to autoxidation, producing hydrogen peroxide and other reactive oxygen species (ROS), and reduces the activity of glutathione (GSH), a major antioxidant in the CNS, thereby inducing apoptosis. It may also induce excitotoxicity by the action of the N-methyl-D-aspartate (NMDA) receptors as the agonist [53, 54]. Hcy may also interfere with normal methylation of DNA or proteins and modulate the expression of genes and enzymes involved in the pathogenesis of AD, such as BACE-1, and presenilin 1 (PSEN1) [55]. HHcy leads to vascular brain damage, which can damage the BBB, impairs endothelial function, blocks the synthesis of NO, and increases the degradation of elastin in the intima of vessels, which accelerates the process of fibrosis and calcification of blood vessels. Hcy participates in the formation of β-amyloid (Aβ) and tau protein phosphorylation by GSK3, and the activation of GSK3β (glycogen synthase kinase) and γ-secretase [56]. Moreover, Hcy inhibits neurogenesis and activation of immune processes in the brain [57].

Literature data indicate a relationship of AD pathogenesis with *MTHFR* C677T and A1298C genotypes, which reduce MTHFR activity [6, 58]. It is known that MTHFR is the key FA-dependent enzyme involved in regulating Hcy levels. MTHFR also influences DNA methylation and nucleic acid synthesis [59]. Several hypotheses have suggested that an increased AD risk is due to high plasma Hcy levels and low plasma FA. Hcy plays a key role in the metabolism of SAM and SAH, which regulate the activity of methylation processes and epigenetic modification of genes relevant to the pathogenesis of AD, such as PSEN1 [60]. It is known that FA deficiency fosters a decline in SAM and DNA methylation in AD, and may lead to the development of dementia [61].

Moreover, reports in the literature indicate a correlation between the *MTHFR* 677TT genotype in AD and/or VaD patients with the highest plasma Hcy levels when compared to other genotypes [6, 62-64]. However, the study of Dorszewska et al. [14] has demonstrated that the level of Hcy was higher in AD patients with the *MTHFR* 677CC genotype, was characteristic only for the AD, and that it was not shown to be that high in other degenerative diseases e.g., PD. At the same time, elevated levels of Hcy were related with the occurrence of a significantly higher level of oxidative DNA damage expressed

level of 8-oxo-2'-deoxyguanosine (8-oxo2dG) only in patients with AD. On the other hand, Wakutani et al. [65] suggest that the presence of the *MTHFR* haplotype 677C-1298C-1793G, defined by the authors as haplotype C, may protect from the development of AD.

The literature data have also suggested the *MTHFR* 677CT genotype as a candidate AD risk factor [63, 66-68]. These studies, however, are conflicting and require confirmation [6].

Another frequently occurring in AD is the *MTHFR* polymorphism A1298C. This type of polymorphism has been studied much less frequently in patients with AD and the results of association studies are not conclusive [64, 69, 70].

Several hundreds of genes have been investigated in genetic association studies as possible AD susceptibility or modifier genes, however, only apolipoprotein E (*APOE*) epsilon 4 is a validated AD risk factor [71]. The study of Nishiyama et al. [59] suggests that the *APOE* epsilon 4, genotype and the *MTHFR* mutation are associated with the clinical phenotype and the clinical onset of senile dementia. However, Anello et al. [64] have shown that, in AD, levels of Hcy, *MTHFR* 677TT, and *APOE* epsilon 4 alleles are associated with risk of this disease.

Moreover, the correlation between *MTHFR* C677T and *RFC1* G80A in women who had children with Down's syndrome (DS) at an early age has been studied. In young mothers of children with DS, a higher incidence of genotype *RFC1* 80GG/*MTHFR* 677TT has been observed as compared to control women. It has been shown that individuals with the *MTHFR* 677TT genotype have an increased frequency of micronuclei, including malsegregation of chromosome 21, and the highest levels of chromosome damage that is similar to AD patients [72].

MTHFR and Parkinson's Disease

Recently, there have been many reports in the literature indicating a relationship between PD and elevated levels of Hcy, not only in the blood but also in the cerebrospinal fluid [7, 73].

Furthermore, molecular epidemiological studies have shown a correlation between *MTHFR* C677T genotypes and age at onset or susceptibility to PD and Hcy levels [7, 74]. Although it has been demonstrated that the C677T SNP the *MTHFR* may elevate Hcy levels and increase the risk of PD, these results are conflicting [7, 14, 26, 75, 76].

The study of Zhu et al. [7] carried out on 2690 PD patients demonstrated that *MTHFR* C667T may confer PD susceptibility in Europeans. This meta-analysis also indicated that the T allele may be an independent risk factor for elevated Hcy levels in PD patients. The meta-analysis of Wu et al. [74] supported that the *MTHFR* C677T polymorphism is associated with an increased risk of PD. However, in this study has been shown that the *MTHFR* A1298C polymorphism may not increase the susceptibility to PD.

It is known that elevated Hcy levels can lead to prooxidative activity, most probably through direct interaction with NMDA receptors and *sensitization* of dopaminergic neurons to age-related dysfunction and death. Increased Hcy levels may also have important implications in patients affected by basal ganglia disturbances, generation of oxidative damage, contributing to neurotransmitter imbalance in motor function, and risk of vascular insults. Moreover, in PD, increased Hcy levels may lead to dementia, depression, and progression of this disease. Several studies have shown that higher concentrations of Hcy in PD are related to long-term administration of L-dopa (L-3,4-dihydroxyphenylalanine) [8, 76].

Many reports have indicated that higher Hcy levels in PD patients treated with L-dopa correlate with the *MTHFR* 677TT genotype [77-79]. Furthermore, PD patients with the *MTHFR* 677CC genotype treated with L-dopa have Hcy levels from 10.9 to 14.6 μM, while the *MTHFR* 677TT homozygote Hcy levels range from 11.9 to 29.3 μM [80]. Hence, it seems that the *MTHFR* C677T polymorphism may potentiate the adverse effects of L-dopa involving the generation of Hcy. Moreover, the study of Yasui et al. [77, 80] has shown that the coexistence of additional FA deficiencies may increase the impact of the *MTHFR* C677T polymorphism on therapy with L-dopa in PD patients. Therefore, it appears that supplementation with FA in PD patients treated with L-dopa and with the *MTHFR* 677TT genotype can be beneficial in preventing HHcy.

However, Todorovic et al. [79] have shown that the *MTHFR* C677T polymorphism does not appear to affect the risk of PD, because distribution of individual genetic variants of *MTHFR* C677T between PD patients and controls was similar. This study also showed that the Hcy concentration was significantly higher in both those patients with PD that were treated and those that were not treated with L-dopa, and in individuals with *MTHFR* 677TT homozygotes compared to *MTHFR* 677CT and 677CC genotypes. Additionally, it was shown that an increase in the level of Hcy was associated with increased duration of PD and reached a maximum after 3-6 years of disease.

The authors suggest that in patients with PD the presence of the *MTHFR* 677TT genotype is an important risk factor for HHcy, in both patients treated and not treated with L-dopa. However, it seems that the adverse impact of the *MTHFR* 677TT genotype is more pronounced in PD patients treated with L-dopa [79]. These results have been confirmed by the study of Religa et al. [81]. Both groups of researchers have demonstrated that the dose of L-dopa does not affect the level of Hcy in patients with PD. However, the study of Muller et al. [82] showed that long-term L-dopa therapy in patients with PD may elevate plasma Hcy levels and can lead to atherosclerosis and vascular endothelial dysfunction, and to the development of cognitive disorders and depression.

The study by Dorszewska et al. [14] has shown that the wild-type homozygotes of *MTHFR* C677T and G1783A predominate in PD patients, while higher Hcy levels have been demonstrated in patients with the *MTHFR* 677CT genotype. It has also been shown that heterozygous *MTHFR* 677CT is characterized by higher concentrations of Cys, which may indicate possible disturbances in the process of Hcy transsulfuration in these individuals. However, in the study of Yesui et al. [77] this correlation was not confirmed. Dorszewska et al. [14] showed that in patients with PD and the heterozygous variant of *MTHFR* A1298C there were more disturbances in the remethylation and transsulfuration processes of Hcy.

It seems that the *MTHFR* C677T genetic variant in Caucasians may leads to the development of PD and HHcy. Moreover, the *MTHFR* 677TT genotype may influence age at onset, susceptibility, risk, and progression of PD, especially during L-dopa therapy.

MTHFR and Other Neurodegenerative Diseases

HD is a genetic disorder of the CNS with symptoms like uncontrolled movements and dementia. The severity of this disease progresses over time. The cause of the disease is mutation of the *IT15* gene, encoding the Huntingtin protein and located on the short arm of chromosome 4. Onset of HD is occurs earlier with an increased number of CAG repeat length of the *IT15* gene. It has been shown that the mutant Huntingtin protein affects the metabolism of Hcy. Hcy increases oxidative stress by acting on NMDA receptors. Excitotoxicity also plays an important role in the pathogenesis of HD. It has been shown that there are increased Hcy levels in HD and these may contribute in the neurodegenerative processes of this disease [9].

The level of Hcy is regulated by the MTHFR enzyme. The study of Brune et al. [83] demonstrated that individuals with the *MTHFR* 1298CC genotype experienced HD symptoms earlier than individuals without this genotype.

ALS is a progressive and fatal disease characterized by loss of motor neurons in the cerebral cortex, brain stem, and spinal cord. Genetically determined ALS (FALS) occurs in 5-10% of the cases and about 90% of ALS is sporadic (SALS). It is understood that up to 20% of FALS is caused by mutations in the gene encoding superoxide dismutase (SOD1) on chromosome 21. SOD is involved in the regulation of oxidative stress. It is known that elevated levels of Hcy may lead to damage of macromolecules, depending on the level of ROS.

MTHFR controls the metabolism of Hcy depending on the genotype of the MTHFR gene. The study of Kuhnlein et al. [84] demonstrated that there is a strong correlation between MTHFR C677T and ASL in the German/Swiss population. However, the study of Sazci et al. [10] indicated that the MTHFR C677T and A1298C polymorphisms are genetic risk factors for SALS in women, in a gender-specific manner, and affect the appearance of clinical symptoms. These results need confirmation in other populations.

MS is a neuroinflammatory autoimmune disease characterized by multiple lesions in the CNS. It is believed that the manifestation of MS is affected by environmental and genetic factors. Many studies have shown that patients with MS inherit certain regions on individual genes more frequently than the general population. Currently, research is focused on a region of chromosome 6, which contains the *HLA* gene.

It has been shown that MS belongs to diseases with metabolic disorders of Hcy and there appears to be participation of the MTHFR C677T and A1298C polymorphisms in its pathogenesis [11, 85]. The study of Alatab et al. [11] has shown that, in MS patients, the levels of inflammatory mediators such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are higher in 677TT homozygotes.

Additionally, individuals with the T allele developed MS almost 4 years sooner than those with the other genotypes. However, the study of Mrissa et al. [85] indicated a significant association between the *MTHFR* A1298C polymorphisms and MS. Further studies of MTHFR polymorphisms may explain the mechanism of HHcy in MS.

METABOLISM OF HOMOCYSTEINE TO CYSTEINE IN NEURODEGENERATIVE DISEASES

The transsulfuration is a metabolic pathway involving the interconversion of Hcy to Cys through the intermediate cystathionine. The enzymes included in the transsulfuration are CBS and CTH. Transsulfuration is an irreversible process that in the situation of oversupply of Met is intense, whereas in with normal Met levels metabolisms about 50% Hcy. In this pathway, Hcy conjugates to serine catalyzed by CTH, of which the coenzyme is the active form of vitamin B6 - pyridoxal phosphate. The resulting cystationin disintegrates, involving CTH, to form Cys and α-ketobutyrate which enters the tricarboxylic acid cycle, and ammonia. Further in the cycle, Cys is involved in the synthesis of GSH or is degraded to taurine (Figure 3).

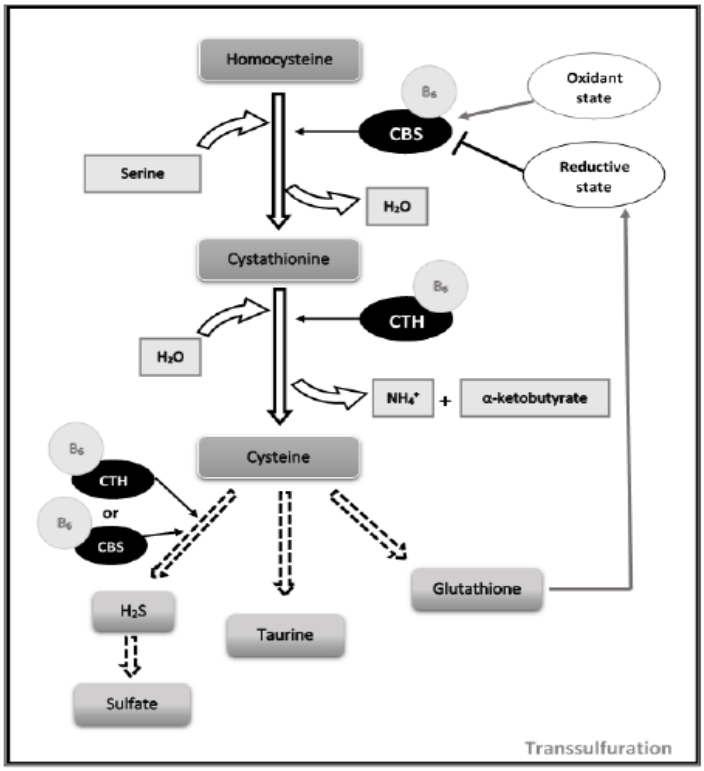

Figure 3. Role of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) enzymes in transsulfuration of homocysteine.

Importantly, the role of transsulfuration in the brain is still unclear. The enzyme CBS has been detected in the human brain, while studies on the presence of CTH in the brain have not been consistent [15-17]. Moreover, there have been few studies that showed large regional variations in CTH activity in the brain [16, 17]. Furthermore, it has been shown that Cys and cystathionine may be utilized by astroglial culture to produce GSH wherein Hcy and Met may not be able to pass the transsulfuration pathway to be converted into GSH in astroglial culture [86].

Although many data do not support a major role for Hcy transsulfuration in the production of Cys necessary for GSH synthesis in the brain, it is believed that this metabolic pathway may be involved in the pathomechanisms of some neurodegenerative diseases [86, 87].

CBS and Selected Neurodegenerative Diseases

CBS catalyzes the first irreversible step of transsulfuration. It has been shown that large amounts of CBS occur in the brain of mammals, particularly in Purkinje cells and the hippocampus [88].

The CBS enzyme is a homotetramer of 63 kDa subunits and requires pyridoxal phosphate and heme for activity. It can also be allosterically regulated by the addition of SAM [89, 90] and TNF-α [91]. The N-terminal segment of CBS contains a regulatory heme binding domain [92, 93]. It has also been noted that the oxidation state of the heme moiety is sensed by the phosphorus nucleus of the enzyme cofactor pyridoxal phosphate, which is bound to the catalytic domain [94]. Oxidation of the heme moiety increases CBS activity, while reduction decreases transsulfuration [95, 96]. Simultaneously, the C-terminal domain exerts an inhibitory effect on the catalytic domain under normal conditions. SAM binds to the C-terminal domain of CBS and abolishes its inhibitory effect on the catalytic site, thereby acting as an allosteric activator of CBS (Figure 3) [97]. Interestingly, it has been shown that the action of CBS might increase levels of the antioxidant GSH [98] in a possible compensatory mechanism that counteracts the potential oxidative damage resulting from increased Hcy [95]. Thus, changes in the levels of expression or functional activity of CBS can affect the levels of Hcy. It has also been suggested that brain CBS activity is both Ca^{2+} and calmodulin dependent, suggesting that in Ca^{2+} influx into neuron cells after depolarization of the cell membrane may be involved in the short-term control of production of neuronal hydrogen sulfide (H_2S) [99, 100]. It has also been observed that

functions of CBS may be affected by genetic changes in the *CBS* gene, which encodes this enzyme.

CBS is located on the long arm of chromosome 21 (21q22.3) and is composed of 19 exons [101]. It has been shown that *CBS* encodes multiple mRNAs, differing in their 5′ untranslated region (5′ UTR) [102]. So far, more than 100 *CBS* mutations have been described in patients with a reduced level of CBS enzyme. Additionally, the majority of these mutations showed a significant reduction in the activity of the CBS enzyme [103]. Mutations of *CBS* are the most common cause of severe homocysteinuria. Homocysteinuria is characterized by elevated levels of Hcy in the blood and its secretion into the urine [104].

The most frequent change in the *CBS* gene is mutation T833C, representing approx. 50% of all the genetic modifications of this gene and replacing isoleucine to threonine at position 278 of the protein. Although the T833C mutation is the most common change within the *CBS* gene, it rarely occurs, about 1/75 to 1/100000 000 births, and the carrier frequency is estimated at 1:150 births. Furthermore, the T833C mutation is the most common mutation causing of homocysteinuria [105, 106].

In the homozygous form of the *CBS* T833C mutation is responsible for the occurrence of severe HHcy. The result of mutation T833C induced CBS enzyme deficiency is an increase in the level of Met in the blood (up to 300-500 mM) and increased levels of Met and Hcy in urine [107-109]. Furthermore, there is a characteristic similar to the phenotype of Marfan syndrome: the appearance of hair is mostly white in patients with the homozygous mutation T833C *CBS* gene, due to disturbances and changes in skeletal osteoporotic patients [50, 110, 111].

Since the 1980s, reports of a link between a heterozygous *CBS* mutation and the presence of mild HHcy (concentration 16-30 μM) started to appear; however, the results presented in the individual studies were often divergent [112, 113]. Presently, it is believed that heterozygous *CBS* mutations are the cause of approx. 10-20% of mild HHcy cases [114]. It is also known that patients with defective CBS respond well to treatment with pyridoxine (vitamin B6) [115-118].

The second most common mutation of *CBS* is the G919A mutation, leading to a substitution of glycine for serine at position 307 of the enzyme CBS [116]. Moreover, the work of Kraus et al. [89] shows that within intron 13 of the *CBS* gene is a polymorphic variable number of tandem repeats (VNTR) region 31bp length. As shown by recent studies, this region is located between exon 13 and intron 13 and may be important for the process of

alternative splicing. It was also shown that the VNTR polymorphism is associated with elevated levels of Hcy when Met is available. Furthermore, it has been demonstrated that an increased number of repeats in the VNTR region and a decreased level of the CBS enzyme [119]. In addition, Yang et al. [120] pointed to lower Hcy levels in the blood after administration of Met in individuals with genotype 17/18 and 18/19 VNTR compared to the most common genotype 18/18.

There have also been reports on the potential relationship of two silent polymorphisms of the *CBS* gene (C699T and C1080T) and the occurrence of mild HHcy. However, the literature reports are divergent: Arras et al. [121] reported a statistically significant correlation between the presences of these polymorphisms with levels of Hcy in the blood after administration of Met but research by De Stefano et al. [122], Kruger et al. [123] or Lievers et al. [124] does not support such a link.

Interestingly, HHcy has been proposed as a possible risk factor for the development of AD. Although the relationship of the *CBS* gene with AD and PD remains undefined, some reports have indicated *CBS* mutation may be independent risk factor for AD. In a prospective study, increased Hcy levels have been detected up to 8 years before AD onset and the study concluded that a strong association exists between total plasma Hcy levels and the risk of AD [49, 56]. Furthermore, the paper by Bayer et al. [12] showed that both the 844ins68 mutation and allele 19 in the VNTR region of the *CBS* gene are independent risk factors for AD in people over 75 years of age. However, the results of the Bi et al. [19] study indicate no significant differences in the incidence of *CBS* gene polymorphisms in patients with AD and control groups. Then again, VNTR allele 21–carrying genotype frequencies were elevated in patients with onset of disease before 64 years of age, increasing AD risk almost twofold compared with control individuals. It is suggested that in these patients, higher Hcy levels (compared with non-VNTR allele 21 carriers) most probably lead to damage and early-onset AD manifestation [125]. On the other hand, related with the allele 19 VNTR, the milder CBS activity reduction may not cause Hcy-mediated damage until a later age. Therefore, the effect of VNTR allele 19 as an AD risk factor may be partial and limited to ages greater than 75 years.

It has also been shown that high Hcy levels in AD patients were associated with activation of hippocampal neuron cell cycle, a mechanism linked to apoptosis and AD pathology [126]. Currently, it is also believed that Hcy acts as a direct neurotoxic agent able to produce DNA damage and leads to apoptosis [125]. However, some authors have suggested that although

plasma Hcy levels in AD patients are probably involved in the pathogenesis of AD, this may be due to environmental factors rather than genetic factors of the CBS mutations [66].

It is also known that HHcy is related with increased risk of seizures. The incidence of seizures is increased up to 20% in patients with homocysteinuria. Moreover, it was also shown that mutations in the *CBS* gene can contribute to increasing the frequency of seizures also in patients with epilepsy, by increasing concentrations of Hcy [18]. However, its relation to epilepsy has not been clearly demonstrated.

Nevertheless, Hcy is known to accumulate in homocysteinuria and is metabolized to homocysteate and homocysteine sulphinate, both known to be powerful excitotoxic amino acids. Moreover, it has been suggested that excitotoxicity is one of the mechanisms involved in neurodegeneration. Furthermore, it is believed that HD involves the action of excitotoxic amino acids. In view of these reports, interaction of Huntingin with *CBS*, demonstrated by Boutell et al. [127] and confirmed with *in vitro* studies, may suggest a mechanism for such excitotoxic damage in HD with participation of CBS.

CTH and Selected Neurodegenerative Diseases

CTH was first isolated in 1958 from rat liver by Matuso and Greenberg [128]. Four years later, Cavallini et al. [129] observed that CTH decomposes cystathionine to pyruvate, ammonia, and tiocysteine, while Chatagner et al. [130] reported an increase in the activity of CTH in rat liver after the administration of Met. Currently, it is known that the presence of CTH in the cell is limited the cytosol [131, 132] and CTH occurs in humans (as well as in rats and mice) in the form of a tetramer (4 x 40 kDa) [133-135]. Each monomer of human CTH has three distinct domains: an N-terminal (the component of the active site formed by the adjacent monomers forming the active dimer), the binding pyridoxal phosphate (PLP) domain, and the C-terminal domain [133, 134].

According to Cooper [136], the main physiological function of CTH is catabolism of cystathionine in the Cys from Met formation pathway.

Possible regulatory control of CTH expression has also been suggested. CTH expression is induced following exposure to lipopolysaccharide (LPS) [137]. It is also suggested that CTH can respond to hypoxia through transcriptional and post-transcriptional regulation, and CTH expression can be

up-regulated by hypoxia to a certain extent. Therefore, the up-regulation of CTH expression during hypoxia may be useful in increasing the production and concentration of H_2S in mammalian cells and indirectly protecting cells from hypoxia [138].

It is also known that the activity of CTH in a mammal is the highest in the liver and kidneys, while in other tissue CTH levels are lower. There is also little CTH activity in the brain [139,140]. It is estimated that CTH activity is more than 100-fold lower in the brain than in the liver, although the transsulfuration pathway is active in the CNS [20, 21].

As shown, CTH occurs in three isoforms with a different number of amino acids (405, 361 and 373 amino acids) resulting from alternative splicing. Although initially the gene encoding the human CTH (*CTH*) was assigned to chromosome 16 [141], currently it is known that this gene is located on the short arm of chromosome 1 (1p31.1), and consists of 13 exons and 12 introns.

To date, approx. 30 *CTH* gene mutations have been described, one of which was frameshift, and the others were point substitutions.

As demonstrated, mutations in the gene encoding CTH affect the proper function of this enzyme and may lead to a reduction in CTH expression, which in turn entails a decrease in the concentration of Cys, GSH, taurine and H_2S in the cells [142, 143]. It has been also reported that *CTH* mutations lead to considerable accumulation of cystathionine in the liver, kidney, brain, and cerebrospinal fluid. Finally, there is the increased concentration of Cys in plasma and the incidence of cystathioninuria [144].

Cystathioninuria is inherited as an autosomal recessive trait, with no pathological characteristics. The disease is characterized by an abnormal accumulation of plasma cystathionine, which leads to an increase its excretion in the urine. Because of its heterogeneity and a wide range of related diseases, cystathioninuria is considered a mild biochemical abnormality [145]. Wang and Hegele [144] identified two nonsense *CTH* mutations (exon 8: c.940-941delCT; exon 11: c.1220delC) and two missense *CTH* mutations (exon 2: c.356C>T (T67I); exon 7: c.874C>G (Q240E)) in unrelated patients with cystathioninuria, wherein the mutations occurred on both the homo- and heterozygous configuration. In addition, a nonsynonymous *CTH* polymorphism in exon 12 - transition c.1364G>T has been identified that leads to the substitution of serine to isoleucine at position 403 (S403I) [144]. Additionally, a cohort study of 496 Caucasian subjects showed a significant association between homozygosity of the *CTH* 1364T/T genotype and elevated plasma Hcy [146].

The presence of H_2S, synthesized by CTH (and CBS), has been demonstrated in the brain and peripheral nerves. Evidence is growing that, similarly to NO, this gas has a role either as a neuromodulator or a neurotransmitter. It has also been determined that deranged biosynthesis of H_2S is a feature not only of animal models of stroke in the rat [147] but the most probably also of AD [12].

Furthermore, it is currently believed that CTH may also be included in the pathogenesis of HD [22, 148].

CTH mutant mice have displayed neurologic abnormalities such as hind limb clasping, which resembled mouse models of HD and suggested the possibility of CTH being involved in the HD pathological pathway. In the mouse model of HD, diminished levels of CTH have been detected. Moreover, an 85-90% decrease in the level of CTH has been observed in the stratium of HD patient, wherein the greater reductions were associated with more severe clinical manifestations of the disease. It has also been shown that this depletion of CTH was selective for the stratium and diminished for the cortex, while in the cerebellum the levels of CTH were unchanged. Interestingly, depletion of CTH has not been observed in other neurodegenerative diseases. Furthermore, it is suggested that CTH deficit is most probably controlled on a transcriptional level. Indeed, a substantial reduction of mRNA for CTH has been demonstrated in Q111 HD cell lines [22]. It is also known that Huntingtin binds and inhibits specificity protein 1 (Sp1), which is a transcription factor for CTH [149]. Moreover, it has been shown that overexpression of Sp1 can reverse the diminished CTH expression in Q111 cells on the level of mRNA and protein. Therefore, it is suggested that CTH depletion in HD is resulting from an inhibition of Sp1 factor by mutant Huntingtin and there may be therapeutic potential of Cys supplementation [22].

MICRORNA AND METABOLISM OF HOMOCYSTEINE

MicroRNAs (miRNAs) are small, ~22 nucleotides in length, RNA regulatory molecules [150]. They are involved in controlling the network responsible for fine-tuning the expression of diverse genes [151]. The mechanism of action depends on specific binding of a 6 nucleotide long "seed sequence" to respective sections of an UTR in the 3' end of the mRNA chain with an assistance of miRNA induced silencing complex (miRISC). This impedes translation, thus reducing the amount of produced protein [152]. Such

a mechanism is responsible for adjusting the expression of roughly 60% of all human genes, e.g., those involved in Hcy synthesis and metabolism [153].

One of these genes is discussed previously, namely *MTHFR* encoding the MTHFR enzyme, which is essential for Hcy metabolism. The expression of this gene has been shown to be regulated by various miRNA, such as miR-149 and miR-22. Moreover, the rare variants in the binding sites of these miRNAs, weakening the miRNA:mRNA interaction, have been shown to be associated with pathology. Wu et al. [154] have demonstrated that the polymorphism rs4846049 (G>T), located in 3'-UTR of *MTHFR*, was associated with increased risk of coronary heart disease (CHD). The authors performed an SNP sweep in 1062 individuals, 618 patients with CHD and 444 non-CHD controls, and observed that the disease was significantly associated with the T allele [OR 1.39 (95% CI, 1.26 to1.90; $p<0.05$)]. Subsequently, the authors performed *in silico* analysis and predicted that the tested polymorphism may affect the binding of miR-149. They used luciferase assay with HEK 239 cells and demonstrated that miR-149 significantly decreases the expression of MTHFR protein; however, this occurs only in the presence of rs4846049 T-allele [154]. Koturbash et al. [155] used a rat model of early hepatocarcinogenesis to find primary changes in the formation of a hepatic neoplasm. They found that the expression of miR-22 increased by a factor of 4.2 ($p<0.05$) in a rat liver after just 24 weeks of dietary supplementation with 2-AAF, a known carcinogen. At the same time, the expression of *MTHFR* was significantly decreased. To validate the miRNA mediated down-regulation, the researcher's transfected TRL1215 cells with pre-miR-22, a predicted modifier of *MTHFR* expression. They found that miR-22 binds to the 3'-UTR of *MTHFR*, and indeed reduces its expression.

Zhao et al. [24] have shown by luciferase assay that further miRNAs, namely *miR-485, miR-608*, and *miR-1293, may bind to the 3'-UTR of MTR – another gene associated with Hcy metabolism, encoding MTR.* Furthermore, the mutation +905A in the 3'-UTR of *MTR* mRNA was shown to increase the binding stability of miR-608 and miR-129. Moreover, the stable complexes of the +905A variant with respective miRNAs *were* associated with a decreased production of MTR protein. Insufficient expression of MTR leads to elevated concentrations of Hcy in plasma – HHcy.

The elevated levels of Hcy were shown to directly influence the expression of several miRNAs. Mishra et al. [156] have shown dramatic down-regulation of miR-188 and other factors e.g., associated with oxidative stress in cardiomyocytes cultured with high concentrations of Hcy. Their findings suggest that the pathogenic influence of Hcy on tissues may be

mediated by miRNA. In the brain, HHcy may be involved in BBB dysregulation. Such pathology may be associated with neurodegeneration. Kalani et al. [157] used a CBS deficient mouse model (*CBS* +/-) of HHcy and to analyze the integrity of the BBB. The researchers observed disruption of the BBB, and overexpression of miR-29b in transgenic mice exhibiting elevated levels of Hcy. Moreover, it was demonstrated by synthetic mimics and inhibitors that miR-29b regulated the activity of BBB destructive metalloproteases via *DNMT3b* and *MMP9*.

PHARMACOTERAPHY IN NEUROLOGY AND HYPERHOMOCYSTEINEMIA

There are literature reports pointing to the occurrence of HHcy during therapy with AEDs, L-dopa and lipid-lowering drugs, including fibrates [8, 40].

The past decade has resulted in many studies on HHcy in epileptic patients treated with AEDs. It has been shown that HHcy occurs in 10-40% of the epileptic patients, and that AEDs (e.g., carbamazepine, valproic acid) pharmacotherapy has a fundamental effect on the levels of Hcy in both children and adults, and in peripheral apoptosis [25, 26, 158].

It has been demonstrated that a specific predisposition to generate circulating Hcy in patients with polymorphisms in both *CBS* and the *MTHFR* C677T and A1298C genes is an important factor in the levels of HHcy in epileptic patients treated with AEDs. It is also known that the three-functional enzyme MTHFD1 is involved in the regulation of circulating Hcy. The study by Sniezawska et al. [26] shows greater increases in Hcy concentration during AEDs treatment of epileptic patients with the *MTHFR* 677CT and *MTHFD1* 1958GG genotypes.

Reports in the literature [8] indicate that plasma Hcy levels in PD have been affected also by pharmacotherapy with L-dopa. It has been shown that L-dopa may induce elevated levels of Hcy during its methylation to 3-O-methyldopa (3-OMD) with involvement of catechol O-methyltransferase (COMT) both in peripheral blood leukocytes and in nigrostriatal neurons. In the course of the reaction, COMT induces, in parallel, the transition of SAM to SAH and further hydrolysis of SAH to Hcy. It appears that the use of combination therapy including L-dopa, a COMT inhibitor, and a DA agonist is the most optimal form of the pharmacological treatment of patients with PD.

It is important to patients with PD and epilepsy chronically treated with L-dopa or AEDs to monitor the levels of FA, vitamins B6 and B12, and to provide proper supplementation. Ensuring the appropriate concentration of cofactors for the remethylation and transsulfuration of Hcy is very important for the efficiency of metabolic processes and can have a significant impact on the formation of HHcy.

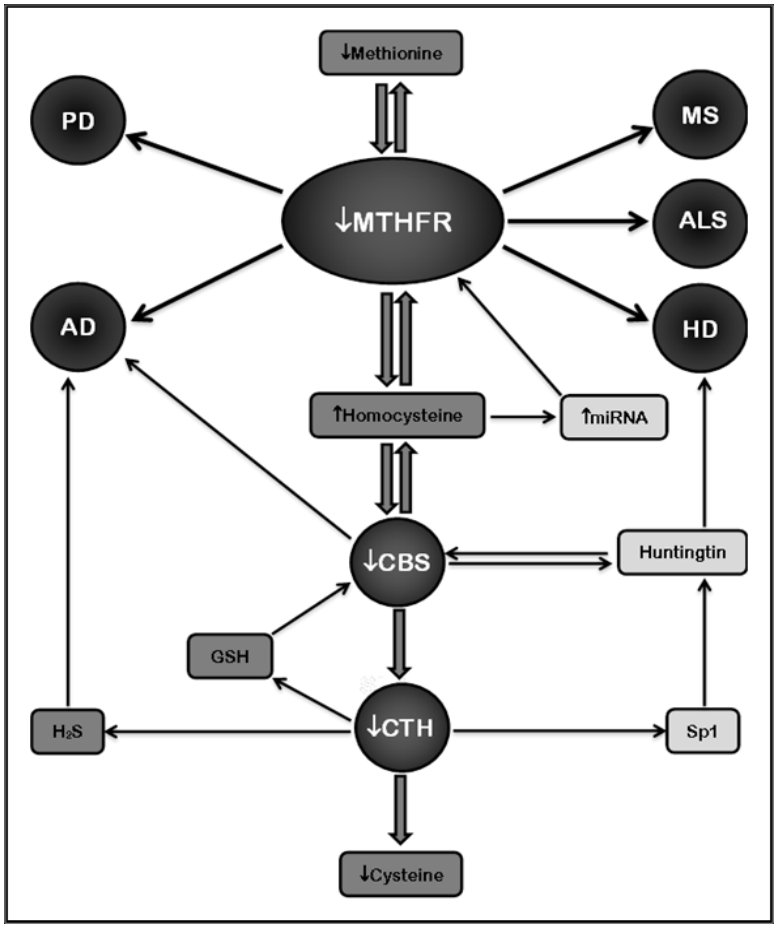

Figure 4. Significance of homocysteine metabolism in neurodegenerative diseases. AD - Alzheimer's disease, ALS - amyotrophic lateral sclerosis, HD – Huntington's disease, MS – multiple sclerosis, CBS - cystathionine β-synthase; CTH - cystathionine γ-lyase; GSH – glutathione; H_2S - hydrogen sulfide; MTHFR - methylenetetrahydrofolate reductase; Sp1 - specificity protein 1.

CONCLUSION

The pathogenesis of neurodegenerative diseases, such as AD, PD, HD, ALS, MS and others, is a complex, multifactorial issue that is still not fully clarified. Effective treatment for these disorders is currently not known, hence the search for new drugs and an effective therapy including potential modifiable risk factors, including HHcy, may have more importance (Figure 4).

It also seems that during treatment with L-dopa in patients with PD and in epileptic patients treated with AEDs, only supplementation with B-vitamins and FA can effectively help regulate the levels of the vascular disease factor Hcy and the metabolism of biothiols (Hcy, Met, Cys, GSH).

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