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Elevated Levels of Methylmalonate and Homocysteine in Parkinson's Disease, Progressive Supranuclear Palsy and Amyotrophic Lateral Sclerosis

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Key Words
Parkinson's disease · Progressive supranuclear palsy · Amyotrophic lateral sclerosis · Levodopa · Homocysteine · Methylmalonate · Vitamin

Abstract
Background/Aims: Increasing evidence suggests that elevated levels of homocysteine (Hcy) and methylmalonate (MMA) may be involved in the pathogenesis of neurodegenerative diseases. ***Methods:*** The urine levels of MMA and serum levels of Hcy as well as folic acid and vitamin B_{12} were measured in patients suffering from the distinct neurodegenerative diseases progressive supranuclear palsy (PSP), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD), and compared to age- and gender-matched control subjects. ***Results:*** We found significantly elevated concentrations of Hcy (PD 15.1, PSP 15.8, ALS 13.9, control 11.2 μmol/l) and MMA (PD 3.7, PSP 3.1, ALS 3.7, control 1.8 mg/g) in all patient groups in comparison with controls. Levels of Hcy and MMA did not differ significantly between the neurodegenerative diseases. ***Conclusion:*** Our findings might imply that Hcy and MMA are released as a consequence of neurodegeneration regardless of the underlying cause and serve as surrogate markers of neurodegeneration. Alternatively they might be directly implicated in the pathogenesis of these diseases. Since elevated levels of both Hcy and MMA are neurotoxic, further studies might investigate the effect of vitamin therapy on disease progression.

Introduction

Amyotrophic lateral sclerosis (ALS), progressive supranuclear palsy (PSP) and Parkinson's disease (PD) are debilitating neurodegenerative disorders that are caused by progressive loss of neurons in the central nervous system. PD and PSP mainly affect patients between 50 and 70 years of age [1, 2], but ALS can affect individuals as young as 20 years of age [3]. Homocysteine (Hcy) is a neuro- and vasculotoxic, sulfur-containing intermediary product in the methionine cycle. Hcy can be transsulfurated to cystathionine and, subsequently, to cysteine, which is a component of glutathione. Hcy can be remethylated to methionine by the addition of a methyl group from 5-methyltetrahydrofolate. Remethylation can be

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catalyzed by methionine synthase, which requires vitamin B_{12} in the form of methylcobalamin as cofactor.

Hcy has been found to be elevated in several neurodegenerative diseases such as Alzheimer's disease and PD [4–6]. In PD, Hcy has been associated with levodopa treatment [7–9]. The Hcy elevation might be explained by the methylation of levodopa via catechol-O-methyltransferase. Catechol-O-methyltransferase, which is present in brain and peripheral tissues, releases S-adenosylhomocysteine from levodopa which is subsequently hydrolyzed by S-adenosylhomocysteine hydrolase to Hcy (fig. 1a). This pathway is supported by data that show a normalization of Hcy levels secondary to entacapone treatment [10–12]. Metabolism of elevated Hcy may take two pathways. One possibility involves the methionine synthase which catalyzes Hcy in a vitamin B_{12}- and methyltetrahydrofolate-dependent reaction to methionine. Methionine is converted to S-adenosyl methionine, which will be a substrate for methylation reactions such as the methylation of levodopa. The other possible metabolic pathway is the catalyzation of Hcy by the cystathionine β-synthase which leads via cysteine to methylmalonate (MMA) and succinyl-CoA (fig. 1a). Surprisingly, in several recent articles, a relationship between levodopa treatment in PD patients and elevated Hcy levels could not be found [13–15]. In one study, a deficit in folic acid has been found to explain Hcy elevation in levodopa-treated PD patients [14]. Therefore, the underlying cause of elevated Hcy in PD patients is still under debate. Very recently, it has been shown that Hcy is elevated in plasma and cerebral spinal fluid of ALS patients [16, 17]. The levels of Hcy in the atypical Parkinson syndrome PSP have not been studied before.

Hcy serum concentrations increase with age as does the incidence of neurodegenerative diseases [18]. Elevated levels of Hcy correlate with cognitive impairment and finally with dementia [19]. Additionally, a relationship between Hcy and brain atrophy in elderly individuals has been found [20]. It was demonstrated only recently that the reduction of Hcy with folic acid supplementation effectively improved domains of cognitive function that tend to decline with age [21]. Genetic studies, animal and cell culture experiments also support a possible direct involvement of Hcy in neuronal cell death in neurodegenerative diseases [22–24]. Several possible modes of action have been discussed [25, 26].

MMA is closely linked to Hcy metabolism (fig. 1a). It has been demonstrated in vitro that MMA is neurotoxic [27, 28]. Therefore, MMA might be of importance in neurodegenerative diseases in general. Due to its link to Hcy, MMA might contribute to the understanding of the role of Hcy metabolism disturbances in these diseases. To date, little is known about MMA in patients with neurodegenerative diseases.

To shed more light on the role of Hcy and its metabolite MMA in neurodegenerative diseases, we set out to study the Hcy and MMA serum concentrations in several groups of patients with neurodegenerative diseases and in control subjects. To exclude possible confounding by vitamin depletion, we checked the levels of vitamin B_{12} and folic acid.

Materials and Methods

Patients

Samples of serum and urine from PD patients [n = 41, mean age = 66.29 years (range 51–84)], PSP patients [n = 22, mean age = 66.95 years (range 57–80)], ALS patients [n = 27, mean age = 66.04 years (range 50–88)] and control subjects [n = 30, mean age = 63.79 years (range 42–81)] were collected at the Department of Neurology of the Ludwig Maximilian University Munich between February 2005 and June 2008. Diagnosis of ALS was based on clinical and electrophysiological criteria, diagnosis of PSP was based on clinical and PET diagnostics [29], and diagnosis of PD was based on UK Brain Bank criteria [30]. PD patients were treated with steady doses of levodopa ranging from 150 to 750 mg per day (table 1). None of the PSP patients or the ALS patients received levodopa. Control subjects were free of neurological illness. Patients and controls did not take supplementary vitamins.

Following informed consent, 10 ml of blood were collected by venipuncture and 10 ml of urine were collected as well. The patients had no evidence of urinary tract infections at the time of sampling. Blood and urine samples were taken on working days between 9 a.m. and 12 p.m. from fasting patients. The samples were immediately sent on ice to the laboratory and analyzed at once at the Department of Clinical Chemistry. All experimental procedures were performed by individuals blinded to sample identity and diagnosis. This study followed the rules of the Declaration of Helsinki.

Analytical Methods

Serum concentrations of vitamin B_{12} and folic acid, and plasma concentrations of Hcy were determined using automated ligand-binding assays [Roche Elecsys for vitamin B_{12} and folic acid (Roche Diagnostics, Mannheim, Germany); Bayer Centaur for Hcy (Bayer Vital, Fernwald, Germany)]. Urinary MMA was determined using isotope dilution gas chromatography/mass spectrometry after extraction and derivatization with threefold deuterated MMA as the internal standard [31, 32]. For the quantification of Hcy and urinary MMA, respectively, intra- and interassay coefficients of variation below 10% were observed for quality control samples in the clinically relevant concentration range. MMA concentrations in urine are higher than in serum, which supports analysis in urine [31]. MMA in urine is largely dependent on creatinine levels in urine [31, 32]. Urinary creatinine was analyzed using the photometric Jaffé method in order to calculate the uri-

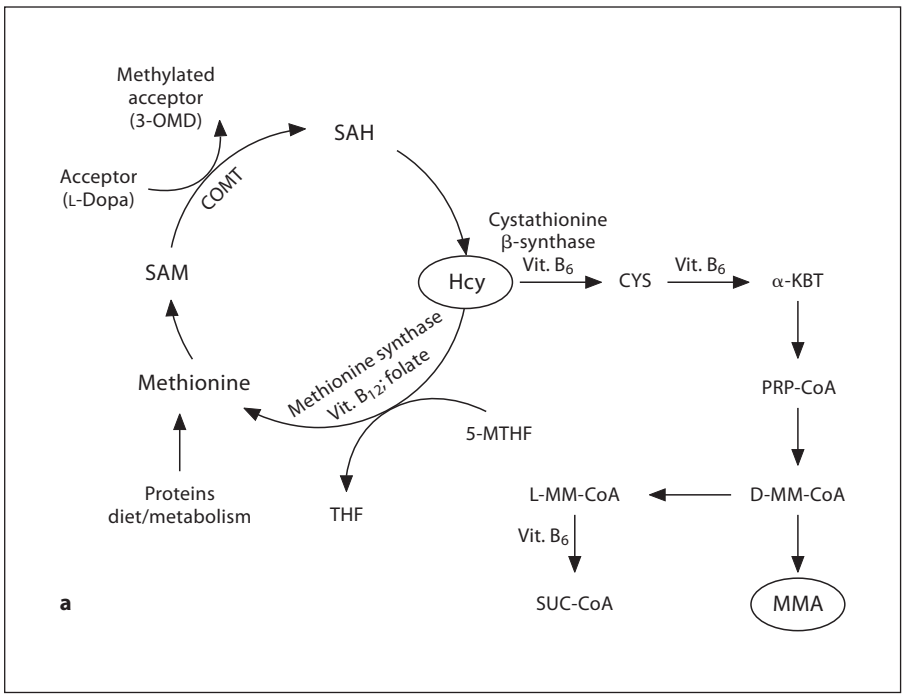

Fig. 1. **a** Hcy metabolism and its connection to levodopa (L-dopa) and MMA. **b** Concentrations of folic acid, vitamin B_{12} and Hcy in serum and MMA/creatinine ratio in urine. The patient groups of PD, PSP and ALS had significantly higher Hcy serum concentrations and higher MMA/creatinine ratio in urine compared to control subjects. COMT = Catechol-O-methyltransferase.

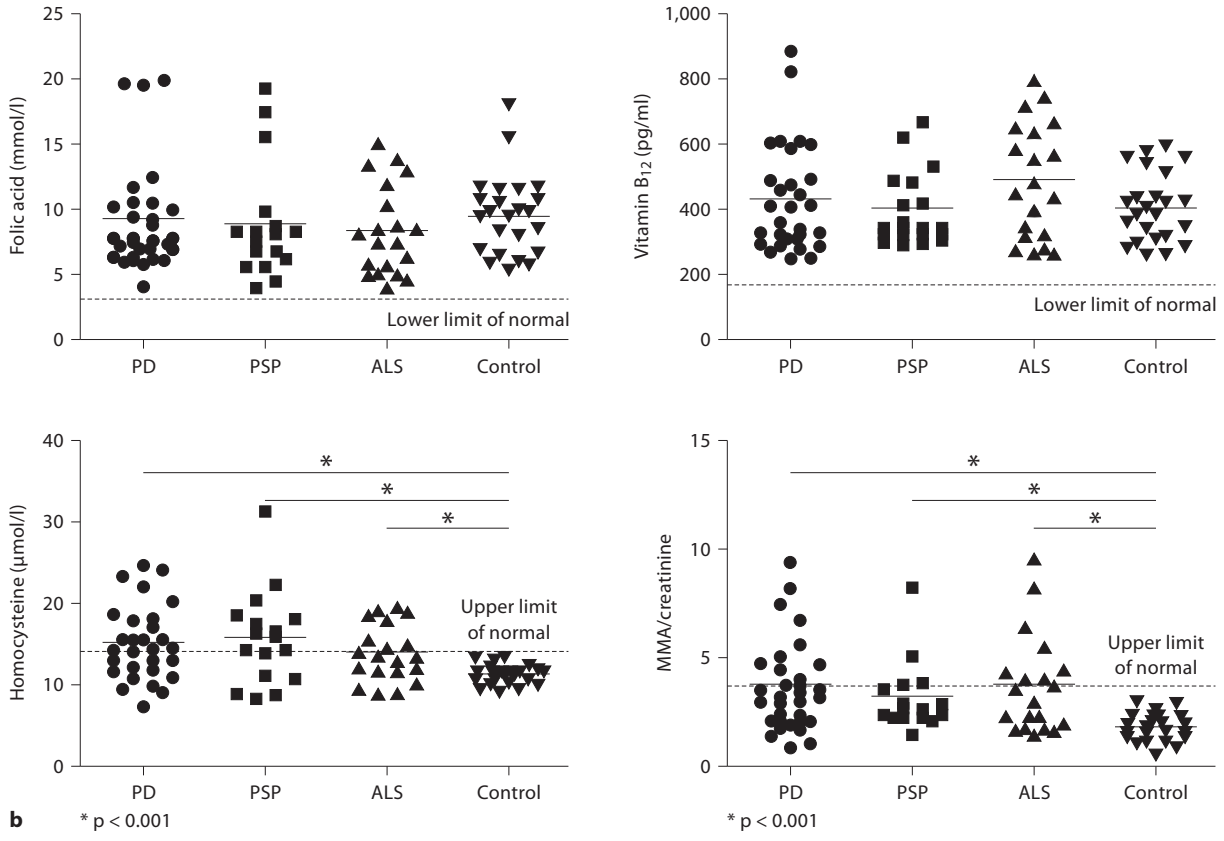

Table 1. Demographic and treatment characteristics of patient and control groups

	PD patients	PSP patients	ALS patients	Control subjects
Number (analyzed)	41 (30)	22 (18)	27 (20)	30 (24)
Age, years	66.29 ± 7.69; 51–84	66.95 ± 5.71; 57–80	66.04 ± 7.45; 50–88	63.62 ± 11.32; 42–81
Women/men	14/17	10/12	8/19	12/13
Levodopa, mg/day	466 ± 173; 150–900	–	–	–
Creatinine in urine, g/l	0.93 ± 0.48; 0.3–1.5	0.87 ± 0.42; 0.3–1.4	0.88 ± 0.49; 0.2–1.7	0.77 ± 0.41; 0.2–1.5

Values are given as means ± SD and ranges where applicable.

nary MMA/creatinine ratio. Whereas serum or plasma MMA concentrations are increased in the case of impaired renal function, the urinary MMA/creatinine ratio is a more robust parameter for the assessment of the cobalamin status on the tissue level which is less dependent on changes in renal function [33]. Furthermore, measurement of MMA in urine is more robust compared to measurement in serum because the analytic concentrations are several-fold higher.

Statistical Analysis

Statistical analysis of laboratory data such as folic acid, vitamin B_{12} and Hcy serum levels as well as the MMA/creatinine ratio in urine was performed by ANOVA using SigmaStat 3.5 (Systat Software, Chicago, Ill., USA). Possible differences between the two groups (age, levodopa doses, duration of disease and urine creatinine concentration) were checked for significance. Prior to testing, normal distribution of data was confirmed using Lilliefors testing. Data are indicated as mean values ± standard deviation.

Results

Patients

Prior to data analysis, 6 PD patients and 5 control subjects were excluded due to incomplete data collection. Furthermore, elevated creatinine levels (>2.1 g/l) led to the exclusion of 4 PD patients and 1 control subject because these levels indicated limited renal function which interferes with levels of MMA and Hcy [33]. One PD patient, 4 PSP patients and 7 ALS patients were excluded due to folic acid or vitamin B_{12} deficiency. Folic acid deficiency and vitamin B_{12} deficiency were defined as values below the lower level of normal (fig. 1b). However, it has to be stated that neither the Hcy nor the MMA levels of the excluded patients significantly exceeded the levels of the tested patient population (online suppl. figure 1, www.karger.com/doi/10.1159/000314841).

The basic characteristics of the participants are summarized in table 1. There were no statistical differences between the patient groups and control subjects with regard to age and urine creatinine concentrations ($p = 0.32$, $p = 0.22$, $p = 0.37$; $p = 0.18$, $p = 0.44$, $p = 0.37$). The mean dose of levodopa in the PD patient group was 466 mg/day.

Levels of Vitamin B_{12} and Folic Acid

The vitamin B_{12} and folic acid serum concentrations did not differ significantly between the patients and control subjects (vitamin B_{12}: $p = 0.12$; folic acid: $p = 0.41$; fig. 1b).

Determination of Hcy in Serum

Hcy measurements in serum showed that control subjects had a mean level of 11.19 ± 1.21 μmol/l. PD patients showed significantly increased concentrations of Hcy (15.05 ± 4.63 μmol/l) in comparison with the control subjects ($p < 0.001$; fig. 1b). Levels of Hcy were also significantly ($p < 0.001$) elevated in PSP (15.75 ± 5.75 μmol/l) and ALS patients (13.94 ± 3.47 μmol/l). There was no statistical difference between Hcy values of levodopa-treated PD patients and the patient groups with other neurodegenerative diseases.

Determination of MMA in Urine

Although we generally found the same differences for all groups at similar levels of significance using determination of MMA concentrations in urine, in this work we present data for the more robust MMA/creatinine ratio. The MMA/creatinine ratio was significantly increased in the urine of PD patients as compared to the control subjects (3.71 ± 2.30 mg/g vs. 1.76 ± 0.73 mg/g; $p < 0.001$; fig. 1b). The MMA/creatinine ratio was also found to be significantly elevated in the PSP and ALS patients. The mean values of MMA for PSP and ALS patients were 3.19 ± 1.66 mg/g and 3.70 ± 2.25 mg/g, respectively. No statistically significant differences were observed in the MMA/creatinine ratio between levodopa-treated PD patients and the other patient groups.

Discussion

Previous studies have analyzed single neurodegenerative diseases and have found elevated levels of Hcy [7, 9, 12, 16, 17, 34]. Some data suggest that MMA may also be elevated and these data are mainly available for PD patients [34]. The present study investigated Hcy and MMA levels in PD, PSP and ALS patients without vitamin B_{12} and folic acid deficiency and shows that both are elevated in the three distinct neurodegenerative disorders without obvious differences between the diseases. This might imply that Hcy and MMA are either directly involved in the neurodegenerative process or that both might be a surrogate marker for neurodegenerative processes (e.g. released from dying cells). However, it must be noted that the determination of Hcy and MMA in serum and urine, respectively, does not necessarily allow direct conclusions to be drawn with regard to the brain levels of MMA and Hcy as both can be generated outside the central nervous system as well.

The role of Hcy in neurodegenerative diseases is supported by various findings. It has been shown that elevated serum levels increase the risk for neurodegenerative diseases [35]. Furthermore, an association of more rapid disease progression for PD patients with increased Hcy levels [6] and an association of Hcy with the progression of mild cognitive impairment to dementia have been shown [19]. Hcy is also elevated in patients with manifest Alzheimer's disease [9]. Very recently, Hcy has also been found to be elevated in plasma and cerebral spinal fluid of ALS patients. The authors speculated about a possible implication in the pathogenesis of ALS [16, 17].

Genetic, animal and in vitro studies support the potential relevance of Hcy in neurodegenerative diseases. Mutations in the methylenetetrahydrofolate reductase gene (T/T genotype) encoding for enzymes of the Hcy metabolism have been identified as a risk factor for PD [22]. Some findings suggest that Hcy may play an active role in neurodegeneration. Administration of Hcy in a mouse model of PD resulted in the death of dopaminergic cells [23]. Elevated levels of Hcy increase the vulnerability of hippocampal neurons to excitotoxic and oxidative injury in cell culture and in an animal model [28]. Direct neurotoxicity of Hcy to neuronal cells has been demonstrated [24]. Several mechanisms by which Hcy induces neurotoxicity like excitotoxicity by stimulating NMDA receptors [25], increased production of β-amyloid aggregates, promotion of neuronal apoptosis [26] and an increase of oxidative stress [36] have been suggested. Regardless, elevated serum levels of Hcy expose patients to an increased risk of arteriosclerosis [35].

Not only Hcy but also levels of MMA and MMA/creatinine ratio in the urine of PD, PSP and ALS patients are significantly elevated (fig. 1b). Taken together with elevated Hcy this might imply that the whole metabolic cycle involving S-adenosylhomocysteine, Hcy, cysteine, and MMA is altered in neurodegenerative diseases (fig. 1a). In subjects with normal renal function, elevated levels of MMA usually indicate vitamin B_{12} deficiency [33], because MMA is released in a vitamin B_{12}-dependent reaction from cysteine in the transsulfuration pathway [37]. This also applies to vitamin B_{12}-deficient, levodopa-treated PD patients [34]. Because of the important influence of vitamin B_{12} on the metabolism of MMA we monitored the levels of these vitamins in all groups and excluded patients with vitamin B_{12} and folic acid values below the normal range. We furthermore checked renal function as a possible interfering factor and excluded patients with indications of renal impairment (table 1). According to well-known metabolic pathways (fig. 1a), a causal connection between Hcy and MMA elevations is likely. Hcy accumulation provides an increased amount of substrate for the cystathionine β-synthase, consequently leading to elevated MMA (fig. 1a). MMA has been shown to be an endogenous cellular mitochondrial inhibitor and neurotoxin. When injected into the basal ganglia of the adult rat brain it produced dose-related cell death in the substantia nigra [27]. The neuronal damage caused by MMA has been suggested to involve inhibition of complex II and the mitochondrial creatine kinase [27, 38]. Recently, a relationship between MMA and markers of methylation and pathological findings of the substantia nigra and neurodegeneration was also found [39]. Whether it is sufficient to reduce Hcy in order to achieve reduced MMA levels, or whether a direct intervention in the MMA metabolism, for example by vitamin B_{12}, is necessary, remains to be determined.

Although the neurodegenerative disorders which were investigated in our study have different pathological mechanisms, a significant number of patients will develop dementia during the course of these diseases. Since Hcy has even been shown to be elevated in Alzheimer's disease patients [9, 21, 25], it might be a risk factor for development of dementia regardless of the underlying disease.

Several studies show that elevated levels of Hcy in PD are related to levodopa treatment [6, 8, 10–12]. We found elevated levels of Hcy and MMA in a similar range in all patients investigated regardless of levodopa treatment.

Further studies are needed to determine if the elevation of Hcy and MMA as observed in Alzheimer's disease, PSP and ALS can also be found in levodopa-naïve PD patients at some stages of the disease.

Although vitamin levels are normal in neurodegenerative diseases, vitamin treatment of patients might lead to normal Hcy and MMA levels if additional vitamin B_{12} and folic acid enabled the methionine synthase to catalyze the reaction of Hcy to methionine (fig. 1a). An additional need for B vitamins has been reported previously for PD patients [40]. Further studies are needed to investigate this effect and whether vitamin treatment might be able to reduce elevated levels of Hcy and MMA in all the studied neurodegenerative disorders. Further studies might address the question whether vitamin supplementation may alter the disease progression with a special focus on cognitive impairment.

In summary, we found elevated levels of Hcy and MMA in patients suffering from three different neurodegenerative diseases. There was no difference in the levels of Hcy and MMA in these diseases. This could imply that Hcy might be implicated in the cause or might be a consequence of neurodegeneration. However, further studies are needed to investigate the role of Hcy and MMA in neurodegenerative diseases and to determine if they are directly implicated or if their elevation is due to a surrogate effect. Treatment studies with vitamins on the effect of Hcy and MMA and on clinical outcome of the patients are particularly needed.

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