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Oxidative DNA damage and level of thiols as related to polymorphisms of *MTHFR, MTR, MTHFD1* in Alzheimer's and Parkinson's diseases

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Abstract. Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are accompanied by increased levels of 8-oxo-2'-deoxyguanosine (8-oxo2dG) and alterations in levels of homocysteine (Hcy), methionine (Met), and cysteine (Cys). Hcy may undergo remethylation due to involvement of MTHFR, MTR and MTHFD1 proteins. Present studies are aimed at determination of 8-oxo2dG, Hcy, Met, and Cys in AD and PD patients as well as in control groups, using HPLC/EC/UV, as well as estimation, by restriction analysis, frequency of following gene polymorphisms: *MTHFR* (C677T, A1298C, G1793A), *MTHFD1* (G1958A), and *MTR* (A2756G). In AD there were significant differences of the levels of only Cys (GG, MTHFR, G1793A) and Met/Hcy (AA, *MTHFD1*, G1958A) whereas in PD there were more significant differences of the levels of thiols: Hcy [*MTHFR*: CT (C677T) and GG (G1793A); *MTR*, AG (A2756G)], Met [*MTR*, AA (A2756G)], Cys [*MTR*, AG (A2756G)], and Met/Hcy [*MTHFR*: CC, CT (C677T) and AA (A1298C), and GG (G1793A); *MTHFD1* AA (G1958A); *MTR* AA (A2756G)]. Significant differences in the levels of Cys/Hcy, *MTHFD1* GA (G1958) were varied between AD and PD groups. The results indicate that of the enzymes studied only polymorphisms of folate-dependent enzyme *MTHFD1* have pointed to significant differences in intensity of turnover of circulating thiols between AD and PD patients.

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INTRODUCTION

Significant progress in medicine and techniques in the second half of the 20th century has been accompanied by elongation of human survival. This, in turn, was associated with an increased incidence of diseases typical for an elderly age, such as Alzheimer's disease (AD) and Parkinson's disease (PD). The common trait of AD (Lustbader et al. 2004) and PD (Tatton 2000) involves degradation of neurons by apoptosis in specific structures of the central nervous system (CNS). Apoptosis can be induced by various physical, chemical or biological factors. These factors include, among others, reactive forms of oxygen (RFO). In the course of AD (Mecocci et al. 2002) and PD (Blake et al. 1997), RFO activate processes leading to the damage of DNA (Kikuchi et al. 2002) proteins (Alvarez et al. 2003, Butterfield and Lauderback 2002) and lipids (Palmer and Burn 1994), and to a low level of antioxidants.

As indicated by literature reports (Mecocci et al. 1998), interaction of reactive oxygen with nucleic acids leads to oxidation of guanine and formation of 8-oxo-2'-deoxyguanosine (8-oxo2dG). Augmented levels of 8-oxo2dG were demonstrated in brain cells and in lymphocytes of patients with AD (Dorszewska et al. 2005, Mecocci et al. 1994, 1998, Morocz et al. 2002) and PD (Alam et al. 1997, Kikuchi et al. 2002, Zhang et al. 1999). This indicates a gradual increase of nucleic acid damage during development of these diseases, and high level of oxidized guanine in DNA is considered a risk factor for senescence and neurodegenerative diseases (e.g., AD and PD).

Increased oxidative stress, observed in AD, reflects deposition of insoluble forms of β-amyloid (Aβ) in the brain (Veurink et al. 2003). In PD, oxidative stress follows accumulation of the degradation products in the gray matter compact part of mesencephalon, and is accompanied by a high level of ferrous ions, decreased level of glutathione, malfunction of the respiratory chain complex I (Schapira et al. 1990, Sian et al. 1994), and excessive oxidation processes in patients treated with L-dopa (Spencer et al. 1994).

The study of Jara-Prado and coauthors (2003) indicates that the excitotoxicity in AD and PD is caused not only by pathological proteins (Aβ in AD, α-synuclein in PD) but also by excessive interaction of homocysteine (Hcy) with NMDA receptors in CNS. Because of this, Hcy is regarded as a risk factor for both vascular and degenerative diseases.

In the body, Hcy is a point of intersection of two main metabolic pathways: transsulfuration and transmethylation. Under physiological conditions, around 50% of Hcy is catabolized by transsulfuration and undergoes transformation to cystathionine and then to cysteine (Cys). The remaining 50% of Hcy undergos methylation to methionine (Met).

Methionine is supplied by diet and its transformation to Hcy involves several steps. At the first step, Met is transformed to SAM (S-adenosylmethionine) and is then demethylated to SAH (S-adenosylHcy) and hydrolyzed to Hcy. SAM is the main donor of methyl groups in many reactions. In AD, decreased level of SAM was documented (Morrison et al. 1996) and was paralleled by a decreased methylation of DNA and augmented levels of Aβ (Fuso et al. 2005). A decreased amount of SAM was also demonstrated in the course of PD (Cheng et al. 1997).

The level of Hcy undergoes control, depending upon the concentration of its metabolites: Cys and Met. In the case of methionine deficit and low concentration of SAM, most Hcy undergoes remethylation to Met, catalyzed by methionine synthase (MTR). MTR is a vitamin B_{12}-dependent enzyme responsible for transfer of methyl groups from N-methyltetrahydrofolate to Hcy, leading to formation of Met (Jarrett et al. 1996). Mutations in the MTR gene are responsible for decreased methylcobalamine level, and result in homocysteinuria, hyperhomocysteinemia and hypomethioninemia (Watkins et al. 2002). The study of Beyer and colleagues (2004) showed that A2756G polymorphism of the *MTR* is linked to the pathogenesis of AD. 5,10-methylenetetrahydrofolate reductase (MTHFR) represents another enzyme involved in remethylation of Hcy to Met. The C667T transition in *MTHFR* results in Ala>Val substitution in position 226 and, as a consequence, in 50% decrease in the enzyme activity, and thus in an increased concentration of Hcy (Frosst et al. 1995). In the studies of Anello and others (2004), McIlroy and others (2002), and Religa and others (2003) most pronounced increase in plasma Hcy was demonstrated in the AD patients with a TT genotype of the C667T polymorphism. In turn, the study of Yasui and coauthors (2000) indicated that the TT genotype might also be linked to pathogenesis of PD, particularly when the level of folates is low.

The tri-functional enzyme, methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (MTHFD1) represents another enzyme linked to transformation of Hcy to Met. Homozygotes of both *MTHFR* and *MTHFD1* are at

risk of cardiovascular diseases connected with elevated levels of Hcy, or folate level-related neural tube hypoplasia (Hol et al. 1998). In the literature, however, less numerous data are available on the involvement of MTHFD1 in the pathogenesis of degenerative diseases.

Under normal conditions, in the presence of a positive methionine balance, most of Hcy undergos transsulfuration catalyzed by cystathionine β-synthetase (CBS), which requires the vitamin B_6, pyridoxal phosphate derivative. Beyer and colleagues (2004) demonstrated that AD was accompanied by low activity of cystathionine β-synthase and by accumulation of Hcy, due to a mutation in *CBS*.

Our study aimed at determining the incidence and genotype frequencies of C677T, A1298C, G1793A, polymorphisms of *MTHFR*, G1958A polymorphism of *MTHFD1*, and A2756G polymorphism of *MTR*, in the AD and the PD patients, and in the controls. Results of the analysis of Hcy, Met, Cys levels, with parallel evaluation of 8-oxo2dG was compared with the genotypes of the polymorphisms of genes involved in Hcy metabolism.

METHODS

Patients

The studies were conducted on 38 patients with AD, including 23 women and 15 men aging 36–85 years (mean age: 66.3 ± 12.2 years) and on 98 patients with PD, including 37 women and 61 men aging 34–81 years (mean age: 60.8 ± 10.7 years).

Control group included 50 individuals, 34 women and 16 men, 22–76 years of age (mean age: 44.6 ± 16.2 years).

Patients with AD were diagnosed using established criteria provided by the National Institute of Neurological and Communicative Disorders – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al. 1984).

Patients with PD, on the other hand, were diagnosed using the criteria of UK Parkinson's Disease Society Brain Bank (Litvan et al. 2003).

None of the control subjects had verifiable symptoms of dementia or any other neurological disorders; smoking and drinking habits were also not present.

A Local Ethical Committee approved the study and the written consent of all patients or their caregivers was obtained.

Determination of 8-oxo2dG

ISOLATION OF DNA

DNA was isolated from peripheral blood lymphocytes by fivefold centrifugation in a lytic buffer, containing 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4, in the presence of buffer containing 75 mM NaCl, 9 mM Na_2EDTA , pH 8.0, and sodium dodecyl sulfate and proteinase K (Sigma, St. Louis, MO) (Leadon and Cerutti 1982). Subsequently, NaCl was added, the lysate was centrifuged, and DNA present in the upper layer was precipitated with 98% ethanol.

ENZYMATIC HYDROLYSIS OF DNA TO NUCLEOSIDES

DNA was hydrolyzed to nucleosides using P1 nuclease (Sigma), for 2 h at 37°C in 10 mM NaOAc, pH 4.5. The solution was buffered with 100 mM Tris-HCl, pH 7.5. Subsequently, the DNA was hydrolyzed with alkaline phosphatase (1U/µl; Roche, Germany) for 1 h at 37°C (Barciszewski et al. 1995) and the obtained nucleosides mixture was applied to a high-pressure liquid chromatography system with both electrochemical and UV detection (HPLC/EC/UV).

ESTIMATION OF 8-OXO2DG

To determine 8-oxo2dG level, the nucleosides mixture was applied to the HPLC/UV system (P580A; Dionex, Germany) coupled to an electrochemical detector (CoulArray 5600; ESA, USA). Nucleosides were separated in a Termo Hypersil BDS C18 (250 × 4.6 × 5 µm) column (Germany). The system was controlled, and the data was collected and processed using Chromeleon software (Dionex, Germany). The results were expressed as a ratio of oxidized nucleosides in the form of 8-oxo2dG to unmodified 2'dG (Olsen et al. 1999).

Analysis of Hcy, methionine, and cysteine concentrations

PREPARATION OF SAMPLES

The analyzed plasma thiol compounds (Hcy, Fluka Germany; Met, Cys; Sigma, USA) were diluted with water at 2:1 ratio and reduced using 1% TCEP (Tris-(2-carboxyethyl)-phosphin-hydrochloride; Applichem,

Germany) at 1:9 ratio. Subsequently, the sample was deproteinized using 1M $HClO_4$ (at 2:1 ratio) and applied to the HPLC/EC system.

DETERMINATION OF THIOL CONCENTRATION

The samples were fed to the HPLC system (P580A; Dionex, Germany) coupled to an electrochemical detector (CoulArray 5600; ESA, USA). The analysis was performed in Termo Hypersil BDS C18 column (250 × 4.6 × 5 µm) (Germany) in isocratic conditions, using the mobile phase of 0.15 M phosphate buffer, pH 2.9, supplemented with 12.5–17% acetonitrile for estimation of Hcy and Met and 0.15 M phosphate buffer, pH 2.8 supplemented with 8–10% acetonitrile for estimation of Cys (Accinni et al. 2000).

The system was controlled, and the data was collected and processed using Chromeleon software (Dionex, Germany).

Genotyping by RFLP

Genotyping for polymorphisms in *MTHFR* (C677T, A1298C and G1793A), MTR (A2756G) (Mostowska et al. 2006), and *MTHFD1* (G1958A) was conducted using PCR–RFLP technique. PCR products were generated by using 10 ng of genomic DNA in 25 µl volume of the reaction mixture containing 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.11 µM each dNTP, 0.3 µM of each primer (Table I) and 1 U *Taq* DNA polymerase (Sigma-Aldrich, USA). The PCR products were digested with an appropriate restriction endonuclease (Fermentas, Vilnius, Lithuania), which recognizes and cuts either wild-type or variant sequence, at 37°C for at least 3 h. The digested fragments were resolved on a 2% agarose gel, in 0.5 × TBE buffer, and were stained with ethidium bromide for visualization under UV light. The results were confirmed by direct sequencing of the amplified fragments.

Statistical analysis of results

The obtained results were analyzed using the non-parametric Mann-Whitney's test and Kruskal-Wallis test, and parametric one-way ANOVA test for unlinked variables.

RESULTS

The level of DNA damage, reflected by oxidized guanine (8-oxo2dG) in lymphocytes, as well as plasma

Table I

Primers and restriction enzymes used for genotyping various polymorphisms in the patients with AD, PD, and in the control group

Gene	Polymorphism	Restriction enzyme	Primers		Annealing temp. (°C)	Fragment size (bp)
			Type	Sequence, 5'–3'		
MTHFR	*C677T*	*HinfI*	Sense Antisense	AGG CTG TGC TGT GCT GTT G CGC TGT GCA AGT TCT GGA C	66	477
	A1298C	*MboII*	Sense Antisense	GGA GCT GCT GAA GAT GTG CTG GGA GAG ACG GTG AG	62	371
	G1793A	*MbiI*	Sense Antisense	CTC TGT GTG TGT GTG CAT GTG TGC G GGG ACA GGA GTG GCT CCA ACG CAG G	66	310
MTR	*A2756G*	*HaeIII*	Sense Antisense	GTT GGT GAA GGG AGA AGA AAT G CTG AAG AAT GGG GGT CTG TG	56	583
MTHFD1	*G1958A*	*MspI*	Sense Antisense	TTC TTC TCA TTC TTC CTC ACA CC TCT GCT CCA AAT CCT GCT TC	60	416

Table II

Levels of DNA oxidative damage (8-oxo2dG/dG × 10^{-5}), and homocysteine (µM), methionine (µM), and cysteine (µM) concentrations in control groups

Parameter	below 60 years of age (22–60 years)	above 60 years of age (63–76 years)
8-oxo2dG	13.1 ± 7.8	16.2 ± 6.7
Hcy	11.7 ± 4.1	16.7 ± 2.6**
Met	24.6 ± 6.0	22.2 ± 9.8
Met/Hcy	2.3 ± 0.9	1.3 ± 0.6**
Cys	213.7 ± 42.3	253.8 ± 55.1*
Cys/Hcy	20.1 ± 7.0	15.5 ± 4.0*

Results are expressed as mean ± SD. The nonparametric test of Mann-Whitney was used. Differences significant at *$P<0.05$, **$P<0.01$, as compared to the control below 60 years of age.

levels of Hcy, Met and Cys were investigated in the AD and the PD patients, and in the control group. The results were correlated with polymorphisms of genes whose protein products are involved in Hcy metabolism [*MTHFR* (C677T, A1298C, and G1793A) *MTHFD1* (G1958A), and *MTR* (A2756G)].

Studies in the controls group demonstrated that after the age of 60, elevated plasma levels of Hcy ($P<0.01$) and cysteine ($P<0.05$) were noted and concentration of methionine was lowered. The Met/Hcy ratio ($P<0.01$) and Cys/Hcy ratio ($P<0.05$) were also lowered and no significant increase was found in the level of 8-oxo2dG in the DNA (Table II). On the other hand, in AD and PD groups of patients (Table III), the levels of 8-oxo2dG in peripheral blood lymphocytes were increased significantly in the AD ($P<0.01$) and insignificantly in the PD patients as compared to the control. In the patient groups, an increase in 8-oxo2dG was accompanied by a rise in plasma concentration of Hcy, ($P<0.01$ in the PD patients). An increase in plasma Hcy was accompanied by lower levels of circulating Met (PD patients, $P<0.05$) and augmented levels of circulating Cys in the AD and the PD patients ($P<0.05$ in PD). These results were reflected by lower ratios of Met/Hcy ($P<0.01$ in the AD and $P<0.001$ in the PD patients) and Cys/Hcy.

Polymorphisms of genes whose protein products are involved in metabolism of Hcy and Met were also investigated. The analysis included three *MTHFR* polymorphisms (C677T, A1298C and G1793A), one *MTHFD1* (G1958A), and one *MTR* (A2756G) (Table IV).

As far as C677T polymorphism of the *MTHFR* is concerned, in the control group most numerous were CC and CT with only 5% of TT genotypes. On the other hand, among patients with AD or PD, more CT and TT genotypes were found. However, there was no difference in the frequency of C677T polymorphism between the AD and the PD groups. The A1298C polymorphism AC, CC seemed to be a little less frequent in the control group than in the AD or the PD patients, but the results were not statistically significant. The CC genotype was nearly 10-times more frequent in the AD patients, and over 5 times more frequent in the PD patients, than in the control group. There was no apparent difference, however, in the

Table III

Levels of DNA oxidative damage (8-oxo2dG/dG × 10^{-5}) and homocysteine (µM), methionine (µM) and cysteine (µM) concentrations in the patients with AD, PD, and in control group

Parameter	Control (22–76 years)	Patients with AD (36–85 years)	Patients with PD (34–81 years)
8-oxo2dG	13.7 ± 7.6	23.7 ± 16.0**	21.8 ± 17.8
Hcy	12.6 ± 4.3	18.8 ± 12.3	18.6 ± 13.6**
Met	24.2 ± 6.7	21.3 ± 5.5	20.1 ± 8.0*
Met/Hcy	2.2 ± 0.9	1.4 ± 0.5**	1.3 ± 0.7***
Cys	220.7 ± 46.6	261.0 ± 58.1	250.6 ± 49.6*
Cys/Hcy	19.3 ± 6.7	17.9 ± 9.5	16.3 ± 6.5

Results are expressed as a mean ± SD. The nonparametric test of Kruskal-Wallis was used. Differences significant at *$P<0.05$, **$P<0.01$, ***$P<0.001$, as compared to the controls.

Table IV

Location and incidence of *MTHFR, MTHFD1,* and *MTR* genotypes in patients with AD or PD and in the control group

Gene Location	Genotype	Control	Patients with AD	Patients with PD
MTHFR C677T	CC	55%	38%	49%
	CT	40%	53%	43%
	TT	5%	9%	8%
MTHFR A1298C	AA	43%	34%	34%
	AC	45%	47%	55%
	CC	2%	19%	11%
MTHFR G1793A	GG	95%	91%	87%
	GA	5%	6%	12%
	AA	0%	3%	1%
MTHFD1 G1958A	GG	23%	33%	31%
	GA	64%	45%	52%
	AA	13%	22%	17%
MTR A2756G	AA	64%	48%	60%
	AG	36%	48%	34%
	GG	0%	4%	6%

frequency of genotypes between the two groups of patients. The frequency of the rare G1793A variant was almost identical in the control group and in AD patients, and no difference in the genotype distribution was discerned between the AD and the PD patients. However, when the G1958A polymorphism of *MTHFD1* was analyzed, showed that the number of heterozygous GA genotypes exceeded 50% in both the control group and the PD patients and reached nearly 50% in the AD patients. The frequency of A2756G polymorphism of *MTR* was the same in the control and in the PD group, and this variant seemed to be

Table V

Levels of oxidative DNA (8-oxo2dG/dG $\times 10^{-5}$) damage and homocysteine (µM), methionine (µM), and cysteine (µM) levels as related to genotypes of C677T polymorphism of *MTHFR* in patients with AD, PD, and in the control group

Parameter	Control		Patients with AD		Patients with PD	
	CC	CT	CC	CT	CC	CT
8-oxo2dG	12.9 ± 8.3	15.0 ± 7.4	19.1 ± 11.2	24.7 ± 15.7	16.1 ± 11.3	23.9 ± 21.8
Hcy	13.0 ± 4.7	11.7 ± 4.7	18.7 ± 6.1	16.1 ± 2.2	14.7 ± 4.3	17.4 ± 6.4*
Met	23.3 ± 7.0	23.1 ± 5.7	24.4 ± 6.7	20.5 ± 2.4	19.1 ± 6.5	19.9 ± 8.3
Met/Hcy	2.1 ± 1.0	2.3 ± 1.1	1.4 ± 0.4	1.2 ± 0.1	1.4 ± 0.5*	1.3 ± 0.9*
Cys	211.7 ± 45.7	227.5 ± 51.6	248.0 ± 74.4	292.3 ± 23.1	237.9 ± 54.5	257.8 ± 42.3
Cys/Hcy	18.3 ± 7.6	21.5 ± 7.2	14.5 ± 6.3	18.3 ± 2.4	17.5 ± 6.4	16.7 ± 6.7

Results are expressed as means ± SD. The parametric test of one-way ANOVA was used. Differences significant at *$P<0.05$, as compared to the controls.

Table VI

Levels of DNA oxidative damage (8-oxo2dG/dG × 10^{-5}), homocysteine (μM), methionine (μM), and cysteine (μM) levels as related to genotypes of A1298C polymorphism of *MTHFR* in patients with AD, PD, and in the control group

Parameter	Control			Patients with AD			Patients with PD		
	AA	AC	CC	AA	AC	CC	AA	AC	CC
8-oxo2dG	13.0 ± 7.1	14.6 ± 9.0	17.5 ± 11.9	22.8 ± 16.5	22.2 ± 14.6	26.8 ± 18.1	23.4 ± 18.1	19.7 ± 18.4	14.4 ± 6.3
Hcy	12.4 ± 4.6	12.8 ± 5.0	12.3 ± 4.7	12.9 ± 5.0	17.7 ± 5.4	19.1 ± 5.6	16.6 ± 5.0	17.7 ± 7.9	13.5 ± 2.6
Met	21.3 ± 6.1	24.2 ± 6.9	27.1 ± 2.6	20.1 ± 4.2	19.7 ± 3.4	25.2 ± 8.2	17.3 ± 7.4	19.5 ± 6.8	23.8 ± 11.9
Met/Hcy	1.9 ± 0.8	2.2 ± 1.1	2.5 ± 1.1	1.7 ± 0.5	1.2 ± 0.2	1.3 ± 0.1	1.1 ± 0.5**	1.4 ± 0.9	1.7 ± 0.7
Cys	229.3 ± 50.6	215.6 ± 46.3	220.7 ± 66.8	284.0 ± 45.3	271.0 ± 98.8	255.3 ± 42.4	246.1 ± 30.2	244.2 ± 40.7	241.2 ± 98.4
Cys/Hcy	20.4 ± 7.9	19.2 ± 7.2	18.9 ± 6.4	24.6 ± 9.8	13.2 ± 3.7	14.4 ± 5.8	15.9 ± 4.7	16.2 ± 7.4	18.3 ± 7.9

Results are expressed as means ± SD. The parametric test of one-way ANOVA was used. Differences significant at $^{**}P<0.01$, as compared to the controls.

a little more frequent in the AD patients, in which the frequency of AA and AG genotypes was equal.

The analysis also included assessment of the levels of 8-oxo2dG and Hcy and its metabolites in relation to *MTHFR* polymorphisms (Figs 1–4). The AD and PD (without PD patients with *MTHFR*, A1298C, CC), showed no significant increase in 8-oxo2dG, which was associated with *MTHFR*, C677T (CC and CT), and A1298C (AA, AC, CC), as well as G1793A (GG), as compared to the control individuals (Tables V–VII). On the other hand, among the PD patients more significant differences were demonstrated in thiol compounds than in AD. Concentration of Hcy was significantly increased in the PD patients, and was associated with the following genotypes: CT (C677T) ($P<0.05$), and GG (G1793A) ($P<0.05$), as compared to the controls.

The most decreased levels of Met were also found in the PD patients with the normal, CC (C677T), and AA (A1298C) genotypes, and in the patients with the heterozygous AC (A1298C) genotype. The product of its transsulfuration (Cys) demonstrated a significant ($P<0.05$) increase in the AD patients with the heterozygous GG (G1793A), as compared to the controls. However, the highest Cys concentration increase was observed in the AD patients with CT (C677T), AA (A1298C), and GG (G1793A) genotypes.

In the patients with AD and PD, the Met/Hcy ratio was low and was relatively independent of the analyzed *MTHFR* polymorphisms but significantly only in the PD patients [C677T, CC genotype ($P<0.05$), CT genotype ($P<0.05$); AA genotype ($P<0.01$) of A1298G, and GG genotype of G1793A variant ($P<0.01$), as compared to the controls]. Conversely Cys/Hcy ratio manifested a tendency to decrease in all patients with only C677T polymorphism. Among the remaining patients with the diagnosis of these neurodegenerative diseases (AD, PD) with A1298C or G1793A polymorphism in the *MTHFR*, only AA and GG genotypes in AD demonstrated tendency for increase in the Cys/Hcy ratio (Tables V–VII).

Table VII

Levels of oxidative DNA damage (8-oxo2dG/dG × 10^{-5}), homocysteine (μM), methionine (μM), and cysteine (μM) levels as related to genotypes of G1793A polymorphism of *MTHFR* in patients with AD, PD, and in the control group

Parameter	Control	AD patients	PD patients
	GG	GG	GG
8-oxo2dG	13.6 ± 8.1	21.5 ± 14.2	19.8 ± 18.7
Hcy	12.9 ± 4.6	16.4 ± 6.4	16.6 ± 6.8*
Met	23.7 ± 6.4	22.7 ± 5.9	20.6 ± 8.0
Met/Hcy	2.1 ± 1.0	1.5 ± 0.5	1.4 ± 0.7**
Cys	224.9 ± 49.5	277.6 ± 45.3*	243.3 ± 49.4
Cys/Hcy	19.3 ± 7.2	20.0 ± 9.9	16.7 ± 6.9

Results are expressed as means ± SD. The parametric test of one-way ANOVA was used. Differences significant at $^{*}P<0.05$, $^{**}P<0.01$, as compared to the controls.

Table VIII

Levels of oxidative DNA damage (8-oxo2dG/dG × 10^{-5}), homocysteine (μM), methionine (μM), and cysteine (μM) as related to genotypes of G1958A polymorphism of *MTHFD1* in patients with AD, PD, and in the control group

Parameter	Control			Patients with AD			Patients with PD		
	GG	GA	AA	GG	GA	AA	GG	GA	AA
8-oxo2dG	15.2 ± 6.9	12.6 ± 9.0	10.9 ± 4.8	14.2 ± 5.0	28.2 ± 11.7	30.2 ± 21.5	19.5 ± 15.5	22.4 ± 19.9	17.1 ± 11.5
Hcy	12.2 ± 5.5	14.6 ± 4.5	11.3 ± 4.7	15.6 ± 5.2	11.9 ± 5.2	17.7 ± 1.5	16.3 ± 5.5	17.5 ± 5.8	15.8 ± 7.8
Met	22.6 ± 5.8	21.5 ± 5.2	22.9 ± 6.8	23.1 ± 4.0	17.1 ± 1.1	23.4 ± 3.5	19.8 ± 9.5	20.0 ± 9.1	18.0 ± 2.8
Met/Hcy	2.4 ± 1.5	1.7 ± 0.8	2.1 ± 0.3	1.5 ± 0.4	1.7 ± 0.7	1.3 ± 0.1*	1.3 ± 0.6	1.3 ± 0.7	1.3 ± 0.4**
Cys	231.7 ± 55.7	213.8 ± 45.6	218.2 ± 66.3	284.0 ± 57.0	256.0 ± 93.7	314.7 ± 27.1	261.9 ± 55.0	246.6 ± 53.3	244.8 ± 42.6
Cys/Hcy	21.0 ± 5.6	15.5 ± 5.0	20.6 ± 7.5	19.1 ± 4.1	26.1 ± 16.1	17.9 ± 2.2	17.3 ± 5.5	15.5 ± 6.1*	17.4 ± 5.8

Results are expressed as means ± SD. The parametric test of one-way ANOVA was used. Differences significant at *P<0.05, **P<0.01, as compared to the controls. Differences significant at *P<0.05, as compared to patients with AD.

Due to the small number of patients with the TT genotype of the C677T polymorphism (two control individuals, three AD patients and six PD patients), no statistical analysis could be performed to compare levels of Hcy and its metabolites between the patients and the controls.

Similarly to the analysis of *MTHFR* polymorphisms, in cases of *MTHFD1* and *MTR* polymorphisms (Tables VIII and IX, Figs 1–4) there were no significant differences in the levels 8-oxo2dG were noted in the AD, and PD patients, as compared to the controls.

Analysis of thiol compounds, in relation to the *MTR* polymorphism (Table IX), revealed significant changes of their levels in the PD patients: AA genotype of A2756G polymorphism (P<0.01 for Met and P<0.05 for Met/Hcy, as compared to the controls), AG genotype of the same polymorphism (P<0.05 for Hcy and Cys, as compared to the controls).

The analysis of G1958A polymorphism of *MTHFD1* (Table VIII) indicated significant alterations in Met/Hcy levels in both diseases, as compared to the controls (AD, AA genotype, P<0.05 and PD, AA genotype, P<0.01).

In parallel, only the G1958A polymorphism of *MTHFD1* coincided with significant differences between AD and PD in the levels of analyzed thiols. In the patients with PD as compared to AD group, GA *MTHFD1* exhibited a significantly lower level of Cys/Hcy (P<0.05) (Table VIII).

Table IX

Levels of oxidative DNA damage (8-oxo2dG × 10^{-5}), homocysteine (μM), methionine (μM), and cysteine (μM) levels as related to genotypes of A2756G polymorphism of *MTR* in patients with AD, PD, and in the control group

Parameter	Control		Patients with AD		Patients with PD	
	AA	AG	AA	AG	AA	AG
8-oxo2dG	14.3 ± 8.8	15.8 ± 11.4	25.2 ± 12.7	21.7 ± 14.4	20.4 ± 17.8	21.3 ± 18.5
Hcy	14.0 ± 4.8	10.8 ± 2.8	13.6 ± 4.6	19.4 ± 8.5	16.3 ± 6.5	16.6 ± 6.4*
Met	23.0 ± 6.5	23.0 ± 4.2	18.9 ± 2.7	25.7 ± 7.8	16.2 ± 6.6**	25.0 ± 10.0
Met/Hcy	1.9 ± 1.0	2.3 ± 0.9	1.5 ± 0.6	1.5 ± 0.5	1.1 ± 0.6*	1.7 ± 0.9
Cys	232.9 ± 49.8	217.9 ± 38.6	245.4 ± 50.5	247.0 ± 53.7	232.3 ± 41.6	275.9 ± 40.3*
Cys/Hcy	18.1 ± 5.4	21.0 ± 4.5	21.1 ± 12.2	14.9 ± 7.8	15.8 ± 5.7	18.8 ± 7.0

Results are expressed as means ± SD. The parametric test of one-way ANOVA was used. Differences significant at *P<0.05, **P<0.01, as compared to the controls.

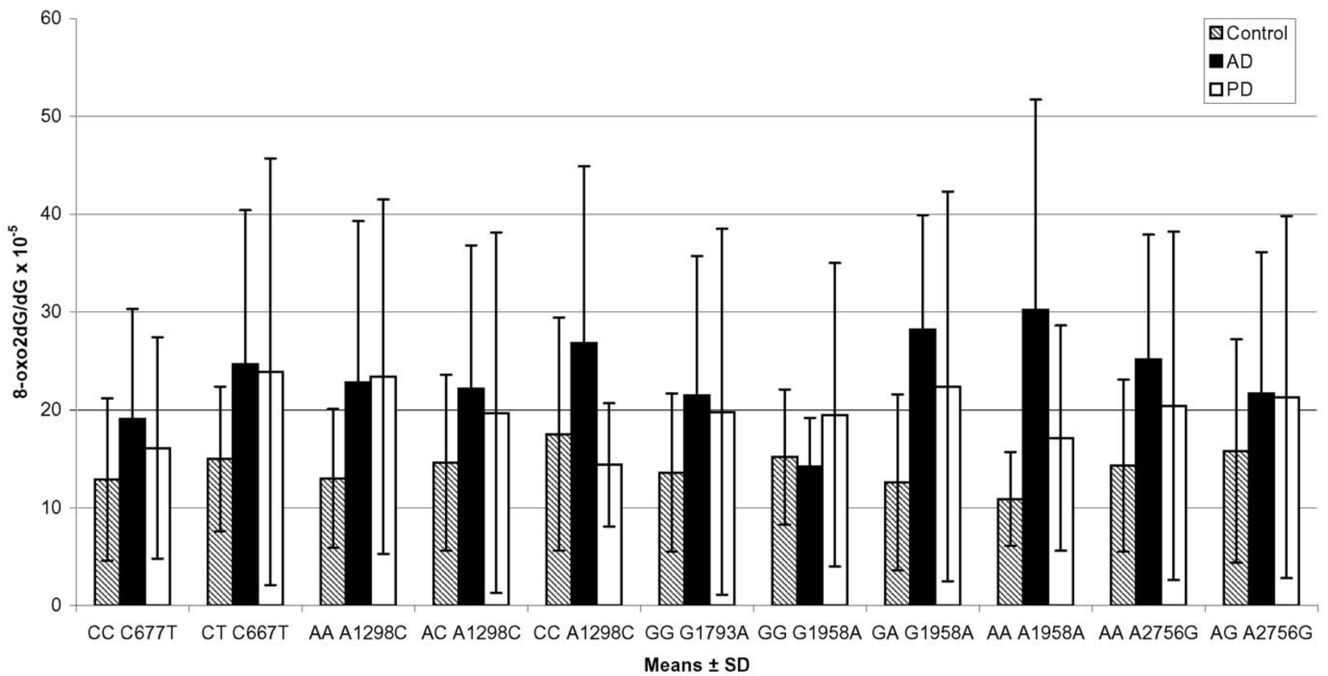

Fig. 1. Levels of oxidative DNA damage as related to genotypes of C677T, A1298C, G1793A polymorphisms of MTHFR, and G1958A polymorphism of MTHFD1, and A2756G polymorphism of MTR in AD, PD, and in controls

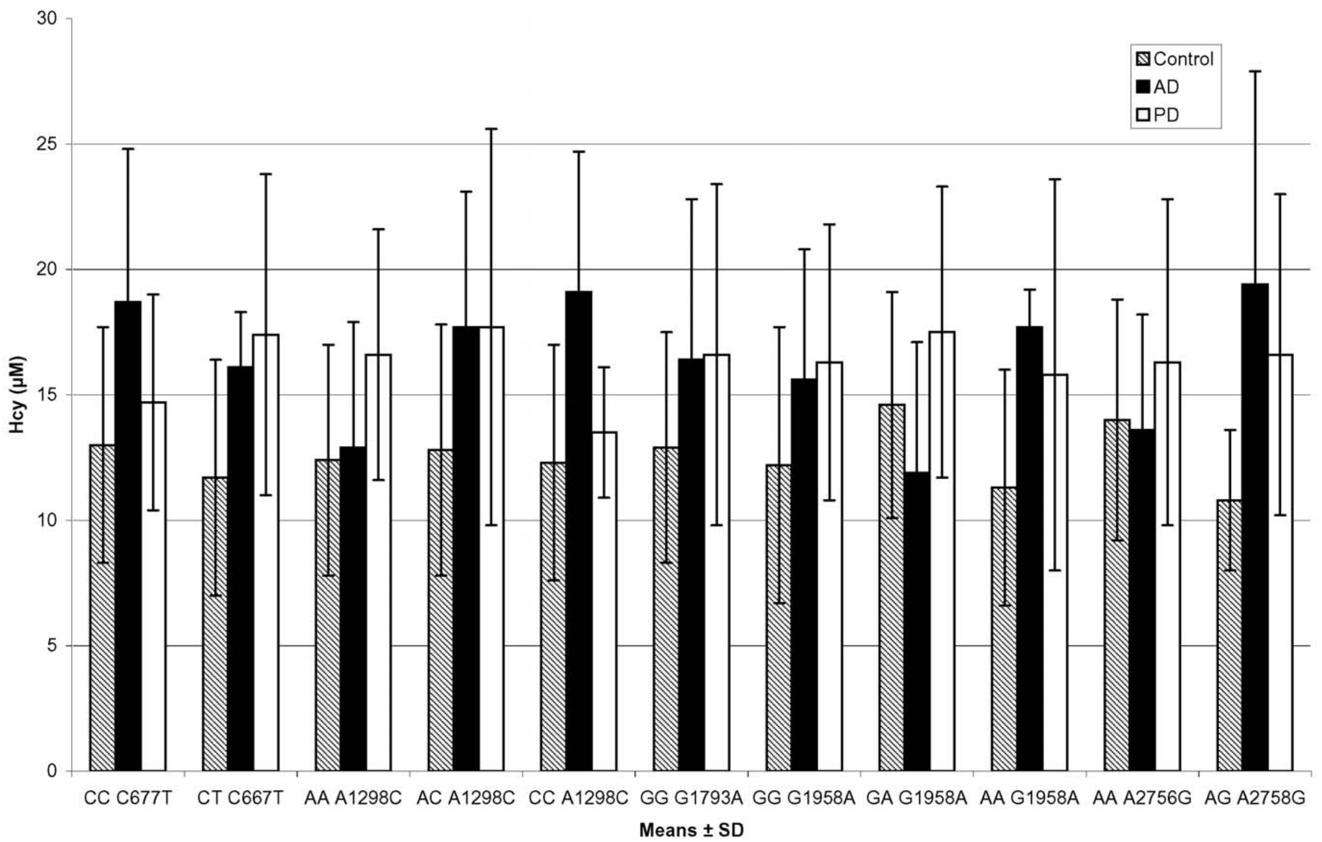

Fig. 2. Homocysteine concentrations as related to genotypes of C667T, A1298C, G1793A polymorphisms of MTHFR, and G1958A polymorphism of MTHFD1, and A2756G polymorphism of MTR in AD, PD, and in controls

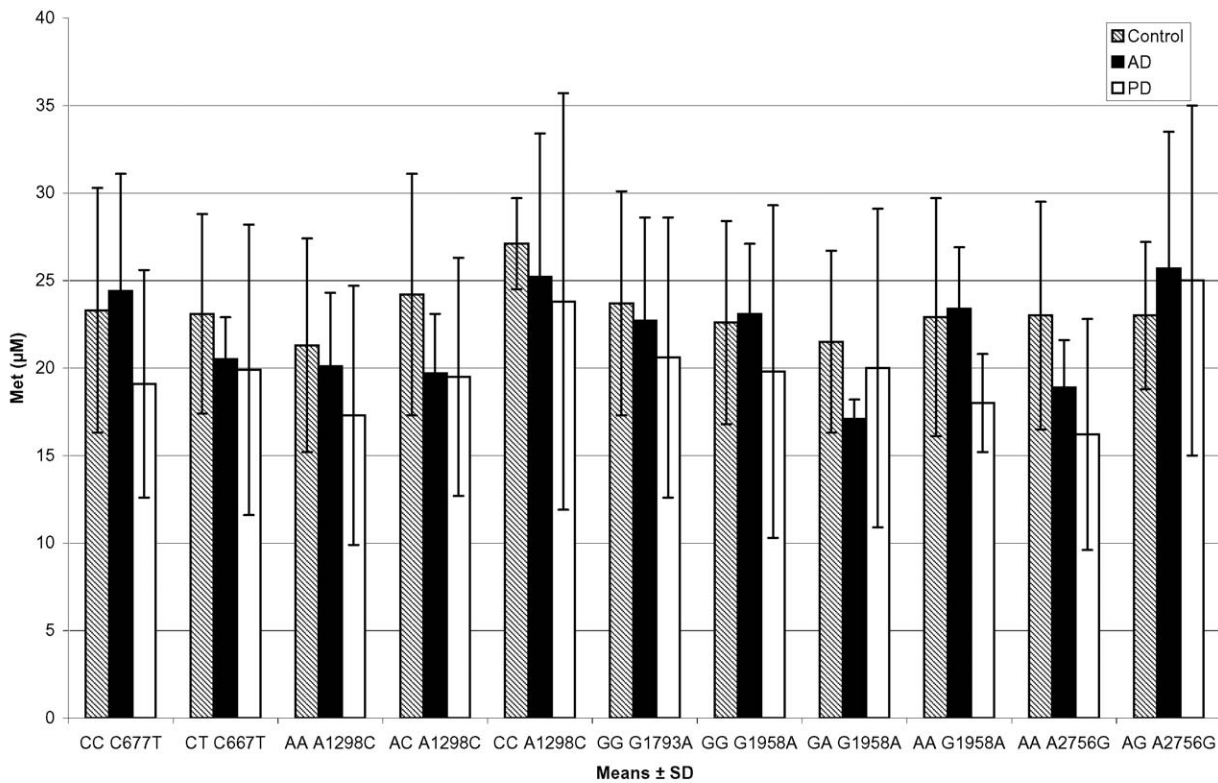

Fig. 3. Methionine concentrations as related to genotypes of C677T, A1298C, G1793A polymorphisms of MTHFR, and G1958A polymorphism of MTHFD1, and A2756G polymorphism of MTR in AD, PD, and in controls

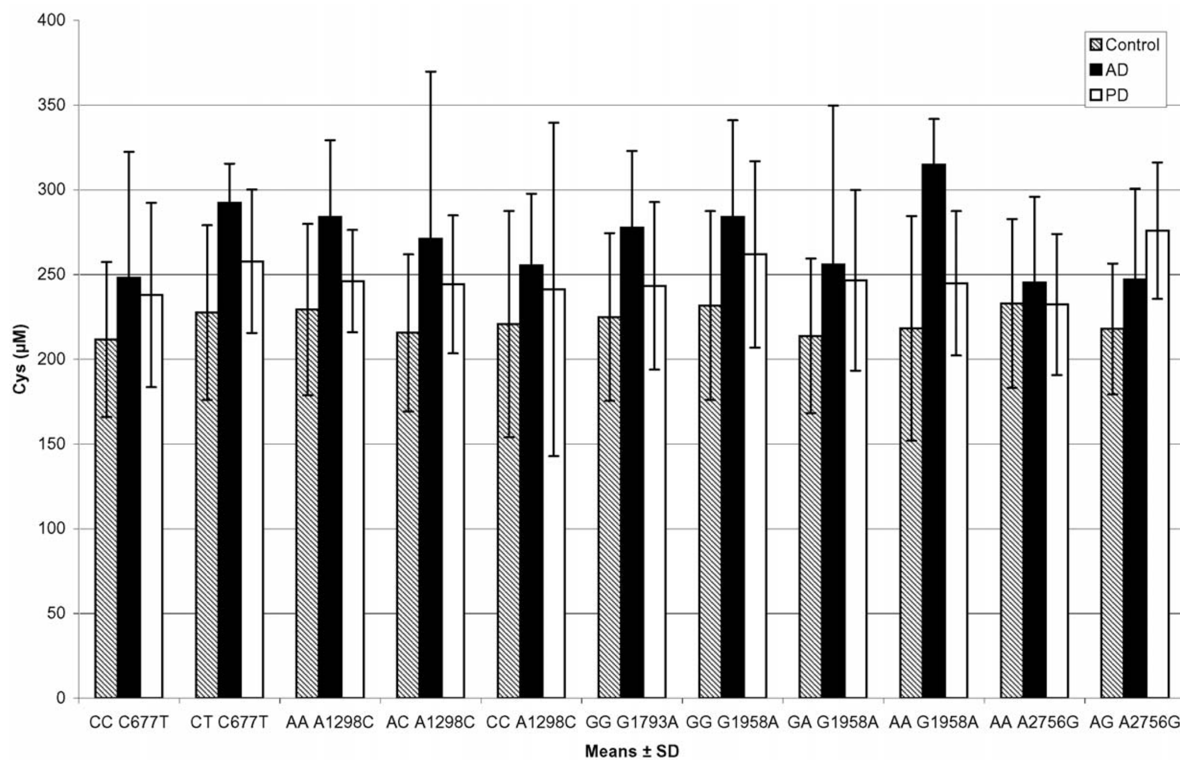

Fig. 4. Cysteine concentrations as related to genotypes of C677T, A1298C, G1793A polymorphisms of MTHFR, and G1958A polymorphism of MTHFD1, and A2756G polymorphism of MTR in AD, PD, and in controls

DISCUSSION

In numerous neurodegenerative syndromes, including AD and PD, pathological forms of proteins deposit in cells and tissues. In familial forms of the diseases, these proteins arise from mutations of the respective genes, while in sporadic forms problems stem from post-translational modification of protein products. In AD, the pathological protein is 42-amino acid form of β-amyloid (Aβ), which deposits in the hippocampus and in entorhinal cortex (Gouras et al. 2000). In the patients suffering from PD, dopamine level-controlled deposition of ubiquitin- and α-synuclein-positive inclusion bodies (Lewy's bodies) takes place in the cytoplasm of dopaminergic neurons (Spillantini et al. 1997). Deposition of pathological proteins in brains of patients affected by the neurodegenerative diseases results in pronounced neurotoxic effects on the CNS. In AD, $A\beta_{1-42}$ induces neuron degradation by decreasing activities of several key mitochondrial enzymes, damage of mitochondrial structure, decreasing efficiency of the endogenous antioxidant system and intensifying oxidative stress (Kim et al. 2003, Parks et al. 2001). In PD, augmented expression of α-synuclein may intensify oxidative stress (Hsu et al. 2000). Oxidative stress and excitotoxicity play an important role in the pathogenesis of PD (Sherer et al. 2001). Bergman and coauthors (1998) demonstrated that in the PD patients, dopaminergic neurons undergo oxidative damage of the compact portion of substantia nigra and dopamine levels decrease in the putamen, a region of the caudate nucleus. Ferrous ions released from damaged substantia nigra may provide an important substrate for oxidative reactions and for production of oxygen reactive forms (ORF) (Jenner 2003), which damage DNA, proteins and lipids (Butterfield and Landerback 2002, Mecocci et al. 2002, Shibata et al. 2001). Several reports indicate that both in AD (Mullaart et al. 1990) and in PD (Migliore et al. 2002), DNA damage is due to oxidation of guanine to 8-oxo2dG (Helbock et al. 1998) and thymine to thymine glycol (Wagner et al. 1992). Studies of Mecocci and colleagues (1998) indicate that in aging organisms levels of 8-oxo2dG significantly increases in peripheral blood lymphocytes, but our studies indicate that their level is insignificant between 22nd and 76th year of age. Alam and others (1997) also found no significant differences in the level of 8-oxo2dG in DNA isolated from substantia nigra of 43 and 91 year-old individuals.

The oxidative damage to both nuclear and mitochondrial DNA can produce mutations leading to lowered efficiency of several organs and to development of morbid symptoms, including neurodegenerative diseases (Ames and Gold 1991). In AD and PD, DNA lesions may develop in the CNS (Alam et al. 1997, Mecocci et al. 1994) and in peripheral blood lymphocytes (Dorszewska et al. 2005, Mecocci et al. 1998, Migliore et al. 2002, Morocz et al. 2002).

In the present study, 8-oxo2dG level was significantly increased in the patients with AD but not significantly in PD. In both AD and PD the reason for increasing levels of oxidatively altered nucleic acids is thought to involve overproduction of free radicals (Christen 2000) as well as decreased levels of enzymatic and non-enzymatic antioxidants (Migliore et al. 2001, Repetto et al. 1999) and less effective repair mechanisms. Brains of AD patients have been found to contain lowered activity of specific 8-oxoguanine glycosylase (Lovell et al. 2000).

Genedani and colleagues (2004) demonstrated that the augmented oxidative stress in neurodegenerative syndromes may also follow the elevated level of the sulfuric amino acid, homocysteine (Hcy). In AD and PD, Hcy is thought to exhibit its pro-oxidative activity most probably through its direct interaction with NMDA receptors (which represents an agonist of NMDA receptor). Agnati and others (2005) have shown that Hcy may pass the blood/brain barrier and that level of plasma Hcy corresponds to Hcy concentration in the brain. Furthermore, elevated levels of Hcy in central nervous system may damage vascular endothelium, deteriorate function of the blood/brain barrier, lead to disturbed production of nitrogen oxide, to neurotoxic effects both in senescent brain and in neurological diseases (Ho et al. 2002). Hcy is thought to represent a factor associated with risk of vascular and degenerative diseases, such as AD (Seshadri et al. 2002) and PD (Duan et al. 2002).

Changes in Hcy level have been demonstrated also in senescent organisms (Mizrahi et al. 2003). The studies indicate that the elevated concentrations appear following the 60th year of life. According to Miner and others (1997), the increase may reflect lowered levels of cofactors of Hcy metabolism due to physiological senescence. Elevation of Hcy level above 14 μM has been shown (Prins et al. 2002) to result in lowered cognitive functions in 25% of elderly persons without traits of dementia.

Studies of Clarke and colleagues (1998) indicate that patients with threefold higher Hcy concentration in serum have a doubled risk of developing AD. In turn, Seshadri and others (2002) have shown that elevation of Hcy level by 5 μM augmented risk of AD by 40%.

Literature reports (Clarke et al. 1998, Kuhn et al. 1998) and present results indicate that development of degenerative diseases (AD and PD) has been accompanied by increased plasma Hcy levels. According to Fuso and coauthors (2005), the elevated concentrations of Hcy in AD are linked to severity of the disease, reflecting lack of control over activity of presenilin 1 (PS1) and BACE, through the lowered level of SAM (S-adenozylmethionine, the controller of DNA methylation). In turn, in PD the high Hcy concentration may augment risk of the disease through its direct toxic effect on dopaminergic neurons. Studies *in vitro* of human dopaminergic neurons have documented a significant increase in neurotoxicity accompanying high Hcy levels (Duan et al. 2002). In parallel, elevated Hcy levels in PD have been shown to carry potential for deterioration of cognitive and motor functions, for depression and elevated risk for developing vascular diseases (Kuhn et al. 1998).

Present studies have shown that augmented plasma levels of Hcy in AD and PD could have also developed due to altered processes of Hcy remethylation to methionine (Met) and transsulfuration to cysteine (Cys). Both in individuals over 60 years of age and in the two analyzed degenerative diseases a decreased concentration of Met has been observed, paralleled by elevated levels of Cys and lowered ratio of Met and Cys to Hcy. The presently demonstrated decrease in Met to Hcy ratio may be linked to transformation of Hcy to thiolactone in endothelial cells. According to one of the more recent hypotheses, sulfonic sulfur of thiol compounds may be involved in development of Hcy-induced arteriosclerotic lesions (Toohey 2001). At the same time, the currently observed increased plasma Cys level in AD, PD and in individuals older than 60 years of age may result from intensified release of the amino acid from proteins due to substitution by the circulating Hcy or due to diminished transformation of Cys to glutathione, important for maintenance of redox homeostasis in the body. In culture of human hepatocytes 50% cysteine has been demonstrated to transform into GSH (Mosharov et al. 2000). It seems also that intensity of dementive disease in particular disturbs transsulfuration of Hcy and leads to decreased levels of the agent (Cys), which provides the natural antioxidant, GSH.

Reports in the literature indicate (Engbersen et al. 1995), that efficiency of Hcy to Met remethylation is indirectly but effectively influenced by the thermolabile form of N^5-N^{10}-methylenetetrahydrofolate reductase (MTHFR).

Manifestation of this form is linked to the C677T point mutation and to haplotypes A1298C, A1793G of *MTHFR*. MTHFR is potentially expected to be involved in pathomechanisms of degenerative diseases through its influence on Hcy homeostasis (Anello et al. 2004). Literature reports and the obtained results indicate that in Polish population (Religa et al. 2003, 2006, and our present results), as well as in populations of Northern Ireland (McIlroy et al. 2002), Italy (Brunelli et al. 2001), Japan (Wakutani et al. 2004 a,b), Brazil (da Silva et al. 2006), and Iran (Keikhaee et al. 2006), the genotypes TT (C677T), CC (A1298C) and AA (G1793A) of *MTHFR* have been the least frequent but their incidence has been slightly increased in the degenerative diseases. Polymorphisms of *MTHFR* are thought to impoverish Hcy remethylation to Met and to increase Hcy level due to a decrease in folate-dependent activity of the enzyme. The C→T transition in MTHFR (C677T) has been shown to decrease activity of the enzyme by 50% (Kang et al. 1988). In parallel, studies of Frosst and coauthors (1995) have shown that homozygotic forms decrease activity of MTHFR (C677T) by 30% and may promote an increase in plasma Hcy level in individuals with these polymorphisms. In present studies, Hcy level was higher in CC genotype of the C677T of *MTHFR* only in AD patients while in PD patients Hcy reached higher levels only in patients with CT genotype of MTHFR C677T. Only in AD the higher levels of the exported to blood Hcy have been paralleled also by a higher level of DNA oxidative damage. It seems that, particularly in persons with CT genotype of the C677T polymorphism of *MTHFR*, processes of Hcy transsulfuration to Cys become disturbed. In present study control individuals as well as patients with diagnosis of AD or PD who carried CT genotype of MTHFR C677T have manifested higher Cys levels even if in Japanese population (Yasui et al. 2000). In Japanese population Cys levels have not been elevated in PD patients, independently of the polymorphism. The most evident alterations in plasma Cys patterns in AD patients with CT genotype of the C677T and AA genotype of the A1298C polymorphisms of *MTHFR*.

The frequently manifested A1298C polymorphism also demonstrates effect on MTHFR activity (Weisberg et al. 1998). Most probably, an interaction develops between the mutation and C677T polymorphism of *MTHFR*. Present results indicate that in control individuals A1298C polymorphism of the *MTHFR* have exerted no effect on Hcy level. Nevertheless, in PD the most pronounced alterations in Hcy levels in cases of A1298C polymorphism of *MTHFR* have been manifested in both genotypes AA and AC, even if this has not been expressed by the increase in parallel levels of oxidized guanine in DNA. Similarly to the cases of AD patients with C677T polymorphism of *MTHFR*, AD patients with A1298C and G1793A polymorphisms of this gene demonstrate the most pronounced disturbances that have been noted in levels of Hcy transsulfuration product (Cys). Wakutani and colleagues (2004b) have indicated that details of effects of A1298C and G1793A polymorphisms on MTHFR activity have not been recognized till now. Nevertheless, parallel effects of C677T, A1298C, A1793C haplotypes may provide protection against development of AD with late origin. Moreover, a lowered activity of MTHFR may lead to decrease in methylation of Hcy to Met due to the lowered activity of MTR.

MTR is responsible for transfer of methyl groups from methyltetrahydrofolate to Hcy with involvement of methylcobalamin as the cofactor. Studies of Watkins and coauthors (2002) have shown that mutation in MTR coding gene may lead to hyperhomocystinemia. The AG genotype of the A2756G polymorphism of *MTR* is probably linked to augmented levels of Hcy. The increase most probably results from lowered activity of MTR, induced by excessive oxidation of cobalamin (McCaddon et al. 2002) due to a more pronounced oxidative stress in senescence and in degenerative syndromes. In parallel, the studies of Matsuo and others (2001) indicate that MTR AG leads also to hypomethylation of DNA and to inactivation of several genes (low levels of SAM).

It seems also that the polymorphism G1958A of the gene coding for MTHFD1 enzyme may be involved in pathogenesis of AD and PD, and that heterozygote and homozygote (GA, AA) genotypes are thought to be responsible for increased levels of Hcy. MTHFD1 represents another folate-dependent enzyme, which catalyzes transformation of tetrahydrofolate to 10-formyl, 5,10-methenyl and 5,10-methylene derivatives. 10-Formyltetrahydrofolate and 5,10-methylenetetrahydrofolate are regarded as serving as donors of methyl groups in DNA biosynthesis (Barlowe and Appling 1990). Studies of Hol and others (1998) indicate that MTHFD1 participates in development of neural tube defects due to metabolic disturbances of Hcy. Its role in disturbances in thiols turnover in developing degenerative diseases has not been fully elucidated.

According to our results, GA, AA genotypes of *MTHFD1* G1958A might be linked to generation of oxidative lesions of DNA in AD. The AA genotype, is also linked to an increase in Cys level in AD, and a decrease in Met/Hcy in AD and PD patients. Most significant alterations in the pattern of circulating thiols between AD and PD were linked to GA genotype for Cys/Hcy ratio.

Present results also indicate that during development of these neurodegenerative diseases cellular Hcy metabolism is disturbed, and its export to peripheral blood augmented. Therefore in AD and PD, monitoring of thiols compound levels in particular Hcy, is recommended.

In the patients with AD or PD, administration of vitamins B_6, B_{12}, and folates may cause a decrease in Hcy level due to increased efficiency of remethylation and transsulfuration processes.

CONCLUSIONS

The findings of the present study indicate that in the control individuals, the least frequent genotypes were: *MTHFR*, TT (C677T), CC (A1298C), AA (G1793A), and *MTHFD1*, AA (G1958A) and *MTR*, GG (A2756G), with a tendency for increased frequency in AD and PD patients. Moreover, the levels of 8-oxo2dG were not significantly increased in AD and PD patients harboring the following genotypes: *MTHFR*, CC and CT (C677T), AA and AC (A1298C), as well as GG (G1793A); *MTR*, AA and AG (A2756G); and *MTHFD1*, GA and AA (G1958A).

In AD, there were significant differences of the levels of only Cys (GG, *MTHFR*, G1793A) and Met/Hcy (AA, *MTHFD1*, G1958A) but in PD there were more significant differences of the levels of thiols: Hcy [*MTHFR*: CT (C677T) and GG (G1793A); *MTR*, AG (A2756G)], Met [*MTR*, AA (A2756G)], Cys [*MTR*, AG (A2756G)], and Met/Hcy [*MTHFR*: CC, CT (C677T) and AA (A1298C), and GG (G1793A); *MTHFD1* AA (G1958A); *MTR* AA (A2756G)]. Only significant differences of the levels of Cys/Hcy ratio, *MTHFD1* GA

(G1958) were shared between AD, and PD groups.

The results indicate that only polymorphisms of folate-dependent enzyme *MTHFD1* have pointed to significant differences in intensity of turnover of circulating thiols between AD and PD patients, which differ in the localization of neurotoxic lesions in the CNS.

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