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Oxidative stress parameters in plasma of Huntington's disease patients, asymptomatic Huntington's disease gene carriers and healthy subjects: a cross-sectional study

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Abstract *Background* Animal data and postmortem studies suggest a role of oxidative stress in the Huntington's disease (HD), but *in vivo* human studies have been scarce. *Aim* To assess the presence of oxidative stress in HD patients and its occurrence relative to clinical symptoms. *Methods* Oxidative stress markers were determined in plasma of HD patients (n = 19), asymptomatic HD gene carriers (with > 38 CAG repeats) (n = 11) and their respective sex and age-matched healthy controls (n = 47 and n = 22) in a cross-sectional study. *Results* With adjustment for age and sex, HD patients had higher plasma lipid peroxidation (LP) levels (ratio 1.20, 95 % CI 1.09 to 1.32, p < 0.001) and lower reduced glutathione (GSH) levels (ratio 0.72, CI 0.55 to 0.94, p = 0.011) than their age and sex-matched controls. Although considerably younger, HD gene carriers did not differ from HD patients regarding LP and GSH levels, and had higher plasma LP (ratio 1.16, CI 1.02 to 1.32, p = 0.016) and lower GSH than their matched controls (ratio 0.73, CI 0.5 to 1.05). They had higher LP (ratio 1.18, CI 1.02 to 1.34, p = 0.019) and lower GSH (ratio 0.75, CI 0.51 to 1.11) than the healthy subjects matched to HD patients. *Conclusions* Oxidative stress is more pronounced in HD patients and asymptomatic HD gene carriers than in healthy subjects. Differences in plasma LP and GSH are in line with the brain findings in animal models of HD. Data suggest that oxidative stress occurs before the onset of the HD symptoms.

Key words Huntington's disease · oxidative stress · Unified Huntington's Disease Rating Scale (UHDRS) · lipid peroxidation · reduced glutathione

Introduction

Huntington's disease (HD) is a fatal neurodegenerative disorder with autosomal dominant inheritance. It leads to progressive dementia, psychiatric disturbances and incapacitating choreiform symptoms culminating in premature death [12]. HD is caused by expansion of CAG trinucleotide repeats on chromosome 4 in exon 1 of the gene coding for a protein of unknown function named huntingtin [15]. The CAG expansion results in a series of uninterrupted glutamine residues and when it exceeds 38 repeats the pathological variant of huntingtin is produced, which causes the disease [24, 33]. The neurological symptoms are due to selective neurodegeneration that predominantly affects the basal ganglia [3]. The exact cause of neuronal death in HD is unknown; however oxidative stress may play an important role [22]. Excessively increased levels of a number of markers of oxidative stress have been reported in the areas of degeneration in the brain affected by HD. These include oxidative damage products such as malondi-

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aldehyde, 8-hydroxydeoxyguanosine, 3-nitrotyrosine and heme oxygenase [4]. Whether oxidative stress is a primary event or merely a secondary constituent of the cell death remains to be established.

The occurrence of oxidative stress in HD is supported by postmortem studies, while studies monitoring in vivo parameters of oxidative stress in HD patients have been scarce. We aimed to assess the presence of oxidative stress in HD patients and its occurrence relative to the occurrence of clinical symptoms of the disease. For this purpose, we determined markers of oxidative stress in the blood of healthy subjects, asymptomatic HD gene carriers and symptomatic HD patients. We also assessed the relationship between the number of CAG repeats and oxidative stress markers in HD patients and asymptomatic HD gene carriers, and the relationship between the number of CAG repeats or oxidative stress markers and symptom severity in HD patients.

Patients and methods

This was a monocentric cross-sectional study approved by the local Ethics Committee at the Zagreb University School of Medicine Clinical Hospital Center.

Patients

Eligible for inclusion were symptomatic HD patients, asymptomatic HD gene carriers and healthy volunteers (HV). Two groups of HVs were planned: one matched by sex and age (± 5 years) to HD patients, and the other one matched to HD gene carriers. A common inclusion criterion was a written informed consent, while subjects suffering from any kind of a chronic inflammatory disease, diabetes mellitus, chronic heart failure, anemia or a malignant disease were not included in the study. HD was diagnosed by a senior neurologist (M.R. or T.B.) based on specific clinical features in persons with a positive family history. Asymptomatic HD gene carriers were defined as subjects with a proven increased number of CAG repeats (≥ 38) in the huntingtin gene with positively excluded presence of clinical symptoms of HD, and were recruited among the HD patients' family members. Healthy volunteers were defined as subjects not related to HD patients or HD gene carriers, and were enrolled based on personal and family medical history and neurological and psychiatric assessment proving a lack of neurological/psychiatric disorders.

A total of 19 symptomatic HD patients and 11 asymptomatic HD gene carriers were enrolled. Their respective age and sex-matched control groups comprised 47 (10 patients had 2 matches and 9 had 3 matches) and 22 healthy volunteers (each HD carrier had 2 matches).

Assessment of the symptom severity and functional testing in HD patients

HD symptoms were evaluated using the Unified Huntington's Disease Rating Scale (UHDRS) [16]. The scale assesses primary features of HD yielding several scores: motor, cognitive, behavioral and a score of functional abilities. The motor subscale rates clinical features of the disease: chorea, dystonia, gait, speech, oculomotor movements, rigor, bradykinesia and postural stability. It allows for assessment of a total motor score and a total chorea score. The behavioral subscale monitors severity and frequency of behavioral problems. The functional part is based on three scales: independence scale, total functional capacity scale and a functional checklist. The cognitive part consists of three tests: digit symbol modality test, verbal fluency and Stroop interference test. All tests were scored using the raw scores. The scales were administered during a.m., always by the same investigator (N.K.).

Blood sampling and sample handling

For a period of at least 10 days before the blood sampling, the subjects were to be free of any acute disease and were not to take any medication apart from that prescribed for treatment of HD. Blood samples (5 ml) were taken between 7:00 and 8:00 a.m., after a nights rest. EDTA was used as an anticoagulant. Plasma was separated by centrifugation (1000 × g, 10 minutes at + 4 °C). Both plasma and the cellular pellet were immediately frozen at –50 °C until the analysis.

DNA analysis

Amplification of the CAG triplet repeat region in the HD gene was performed by Expand Long PCR system (Roche Diagnostics, Mannheim Germany) as previously reported [13]. To determine the precise number of the CAG triplets primers HD1 and HD3 were used that exclude adjacent CCG repeat region in the HD gene [32]. PCR products containing CAG repeats were resolved through 10 % polyacrylamide gels and the precise CAG triplet number was determined by comparison to the products of the known CAG size.

Determination of the plasma markers of oxidative stress

All chemicals used for determination of the oxidative stress markers were purchased from Sigma (Taufkirchen, Germany), if not specified otherwise. Superoxide dismutase (SOD), superoxide anion (O_2*), thiobarbituric acid reactive substances as indicators of lipid peroxidation (LP), carbonyl proteins (CarbP), catalase activity (CAT) and the reduced glutathione (GSH) were determined spectrophotometrically (Ultraspec® 1000 E Pharmacy Biotech spectrophotometer). Plasma proteins were determined by the Lowry method [21].

Plasma SOD was determined as described by Marklund [23]. The method is based on inhibition of the O_2*-mediated autooxidation of 0.2 mmol/L pyrogallol by SOD at pH 8.2 and 20 % O_2. Pyrogallol autooxidation results in increased absorbance at 420 nm (A_{420}) over several minutes, at a rate of 0.02 min^{-1}. Inhibition of autooxidation results in inhibition of A_{420} increase. One unit of SOD is defined as an amount required to inhibit the reaction by 50 %. Briefly, the assay mixture to which 100 µL of plasma sample was added (total volume 1.0 mL, pH 8.2, aerated 20 % O_2) contained 50 mmol/L Tris-HCl buffer, 0.2 mmol/L pyrogallol and 1 mmol/L diethylenetriaminopentaacetic acid (Fe^{2+} chelator). All samples were processed in the same run. Intra-assay variability assessed as relative standard deviation for 10 samples of 100 ng/mL of bovine (Zn-Cu) SOD (Merck, Darmstadt, Germany) was 8 %. SOD in the samples (U/mL) was determined against a standard curve and is expressed as U/mg plasma protein.

Plasma level of O_2* was determined based on reduction of ferricytochrome c [17]. Briefly, 100 µL of 20 µmol/L ferricytochrome c solution was added to 50 µL of the plasma sample and absorbance at 550 nm was measured immediately and after 3 minutes. Absorbance was corrected for absorbance determined in the presence or 50 U/mL SOD, and superoxide-specific reduction of cytochrome c was quantified based on the absorbance difference between the first and the second measurement using an extinction coefficient of 2.1×10^4 $(mol/L)^{-1} \cdot cm^{-1}$. The O_2* level is expressed as $\mu mol/L \cdot mL^{-1} \cdot min^{-1}$. All samples were assayed in the same run.

Plasma LP was determined by measuring thiobarbituric acid reactive substances [25]. The method is based on the fact that malondialdehyde, a specific secondary product of lipid peroxidation, reacts with thiobarbituric acid at pH 3.5 to form a colored complex with a

maximum absorbance at 532 nm. Briefly, 500 µL of 0.8 % thiobarbituric acid in water (w/v) and 1.25 mL of 20 % trichloracetic acid (w/v) were added to 200 µL of the plasma sample, and the mixture was incubated in a water bath at 100 °C for 1 hour. After cooling, samples were washed once with 4.0 mL of *n*-butanol/pyridine (1:1, v/v). The *n*-butanol layer was separated by centrifugation (3000 × g, 20 minutes), and used for spectrophotometric measurements. The amount of pigment reflecting the amount of thiobarbituric acid reactive substance was determined using an extinction coefficient of 1.56×10^5 $(mol/L)^{-1} \cdot cm^{-1}$. The level of LP is expressed as µmol/mL plasma. All samples were assayed in the same run.

Plasma CarbP were estimated as described by Levine [20]. Briefly, 500 µL of 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl (w/v), or 500 µL of 2 N HCl as a blank control, was added to 50 µL of a plasma sample. The mixture was incubated for 1 hour at room temperature. Proteins were precipitated with an equal volume of 20 % trichloroacetic acid and were washed three times with ethanol/ethyl acetate (1:1, v/v). The final precipitate was redissolved in 1 mL of 6 mol/L guanidine hydrochloride and 20 mmol/L potassium phosphate buffer (pH 2.3). The absorbance of the DNPH derivatives was measured at 360 nm. The carbonyl concentration was determined using an extinction coefficient of 21.5 $(nmol/L)^{-1} \cdot cm^{-1}$, and is expressed as nmol/mg plasma proteins. All samples were assayed in the same run.

Plasma CAT (E.C.1.11.1.6.) was determined as described by Johansson and Borg [18]. The method is based on reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide, which results in formation of formaldehyde. In reaction with Purpald, formaldehyde forms a chromophore, which is quantified spectrophotometrically at 540 nm. Briefly, 100 µL of phosphate buffer (pH 7.0), 40 µL of methanol and 20 µL of hydrogen peroxide were added to 50 µL of the plasma sample. The mixture was incubated at room temperature for 20 minutes. Reaction was stopped by addition of 50 µL of phosphate buffer and 100 µL of 34.2 mmol/L Purpald. Quantification of the chromophore was carried out by comparing the sample absorbance with those obtained with formaldehyde calibrators. CAT is expressed as µmol/mg plasma protein. All samples were assayed in the same run. Intra-assay variability (as relative standard deviation for 10 determinations of 0.1 nmol/L of formaldehyde) was 11 %.

Plasma GSH was assessed as described by Lang [19]. In brief, proteins in 100 µL of a plasma sample were precipitated with 50 µL of 20 % trichloracetic acid and removed by centrifugation (4000 × g, 10 minutes), while 100 µL of 0.5 mmol/L 5.5'-dithiobis-2-nitro benzoic acid (color reagent) was added to the resulting supernatant. Absorbance at 412 nm was determined, GSH was quantified through a comparison with GSH standards and expressed as µmol/mL plasma. All samples were assayed in the same run. Intra-assay variability (as relative standard deviation for 10 samples of a 0.1 µmol/mL GSH standard) was 10 %.

Statistics

Summary statistics is reported for the measured variables. In the preliminary analysis, HD patients and asymptomatic HD gene carriers were compared to their respective matched healthy controls. Mann-Whitney test or Chi^2 test were used as appropriate. Differences are reported with 95 % confidence intervals (CIs), which in the case of median differences were exact 95.2 % CIs. Oxidative stress parameters found significantly different for both HD patients and asymptomatic HD gene carriers as compared to their respective controls in the preliminary analysis were analyzed further taking into consideration all four groups of subjects simultaneously. A non-parametric one-way analysis of variance followed by Kruskal-Wallis z-test with Bonferroni adjustment for multiple comparisons was implemented. To adjust for the effects of sex and age, the analysis was repeated on logarithmically transformed (base e) data by applying analysis of variance (group, sex, age). Least square means were used to determine differences between the groups expressed as geometric means ratios with Tukey-Kramer simultaneous 95 % CI. Spearman rank correlation coefficients based on pooled data for HD gene carriers and HD patients were determined between the number of CAG repeats and each of the oxidative stress parameters. Correlations between the number of CAG repeats or oxidative stress parameters and each of the motor, cognitive, functional and behavioral tests results were also estimated in the group of symptomatic patients. We used SAS for Windows System release 9.1 (SAS Inc., Cary, NC, USA).

Results

HD patients were slightly older (median difference 4 years) and asymptomatic HD gene carriers were slightly younger (median difference –4 years) than their respective matched healthy volunteer controls (see Tables 1 and 2). As compared to their respective controls, both HD patients and HD gene carriers had higher plasma lipid peroxidation ($p < 0.001$ and $p = 0.009$, respectively) and lower plasma GSH levels ($p = 0.006$ and $p = 0.012$, re-

Table 1 Demographics and oxidative stress parameters in plasma: Huntington's disease patients (HD patients) and age and sex-matched healthy volunteers (matched HV). Data are counts (percentages) or medians (ranges). Differences between groups are given with 95 % confidence interval (CI)

	HD patients	Matched HV	HD – HV (95 % CI)	P-value*
N	19	47	–	–
Males	14 (74)	31 (66)	8 (–18.1 to 29.0)	0.578
Age (years)	46 (18–58)	41 (21–58)	4 (–2 to 10)	0.195
HD duration (years)	5 (0.5–14)	NA	–	–
CAG repeats (number)	45 (40–66)	NA	–	–
O_2* ($\mu mol/L \cdot mL^{-1} \cdot min^{-1}$)	0.71 (0.10–3.79)	0.58 (0.08–2.36)	0.16 (–0.21 to 0.47)	0.430
SOD (U/mg plasma protein)	0.96 (0.09–2.68)	0.96 (0.02–3.95)	0.04 (–0.34 to 0.72)	0.392
CAT (µmol/mg plasma protein)	35.3 (19.4–56.3)	29.9 (19.2–68.1)	2.51 (–1.67 to 9.52)	0.326
LP (µmol/mL plasma)	13.3 (8.95–15.9)	10.2 (7.30–13.7)	2.77 (1.7 to 3.43)	< 0.001
GSH (µmol/mL plasma)	8.21 (3.54–13.6)	11.4 (6.1–19.2)	–2.92 (–5.56 to –1.13)	0.006
CarbP (nmol/mg plasma protein)	2.30 (0.90–3.92)	1.98 (0.04–5.34)	0.27 (–0.25 to 0.99)	0.266

* From Chi^2 test for proportions or Mann-Whitney test for medians
NA not applicable; *HD* Huntington disease; *O_2** superoxide anion; *SOD* superoxide dismutase; *CAT* catalase; *LP* lipid peroxidation; *GSH* reduced glutathione; *CarbP* protein carbonyls

Table 2 Demographics and oxidative stress parameters in plasma: asymptomatic Huntington's disease gene carriers (HD carriers) and age and sex-matched healthy volunteers (matched HV). Data are counts (percentages) or medians (ranges). Differences between groups are given with 95 % confidence interval (CI)

	HD carriers	Matched HV	HD-HV (95 %CI)	P-value*
N	11	22	–	–
Males	6 (55)	12 (55)	0	1.000
Age (years)	23 (17–30)	25.5 (17–31)	–4 (–8 to 0)	0.083
CAG repeats (number)	46 (42–65)	NA	–	–
O_2* (μmol/L · mL^{-1} · min^{-1})	0.47 (0.12–4.73)	0.49 (0.10–2.32)	–0.017 (–0.55 to 0.47)	0.858
SOD (U/mg plasma protein)	0.89 (0.24–2.89)	1.29 (0.02–4.91)	–0.32 (–0.88 to 0.66)	0.553
CAT (μmol/mg plasma protein)	40.4 (19.2–54.8)	27.0 (22.0–55.0)	6.32 (–1.94 to 15.5)	0.105
LP (μmol/mL plasma)	13.0 (9.20–14.2)	10.3 (8.41–12.1)	1.80 (0.60 to 2.97)	0.009
GSH (μmol/mL plasma)	8.48 (7.01–14.1)	13.8 (6.1–18.5)	–4.20 (–6.59 to –0.66)	0.012
CarbP (nmol/mg plasma protein)	2.88 (0.40–4.80)	1.72 (0.88–4.83)	0.76 (0 to 1.44)	0.050

* From Fischer exact test for proportions or Mann-Whitney test for medians
NA not applicable; *HD* Huntington disease; *O_2** superoxide anion; *SOD* superoxide dismutase; *CAT* catalase; *LP* lipid peroxidation; *GSH* reduced glutathione; *CarbP* protein carbonyls

spectively). Within the current sample, other oxidative stress markers did not significantly differ between HD patients or HD gene carriers and their respective controls (see Tables 1 and 2). However, when data for HD patients and asymptomatic HD gene carriers were pooled (n = 30) and compared to pooled data for healthy volunteers (n = 67), plasma CAT and carbonyl protein levels were significantly higher in HD patients/carriers: median 35.7 vs. 27.9 μmol/mg plasma protein, median difference 4.48, 95 % 0.23 to 10.1, p = 0.031 for plasma CAT; and median 2.33 vs. 1.88 nmol/mg plasma protein, median difference 0.46, 95 % CI 0.01 to 1.0, p = 0.043 for plasma carbonyl proteins.

To further evaluate lipid peroxidation and GSH levels the four groups of subjects were considered simultaneously, in an unadjusted (non-parametric analysis of variance) and adjusted analysis (analysis of variance on ln-transformed data with adjustment for age and sex). The two analyses yielded similar results for either of the two parameters, with significant "group" effects (see Fig. 1, Table 3).

Lipid peroxidation was higher in HD patients than in their matched controls (p < 0.001 in both analyses), and the adjusted analysis indicated a difference of around 20 % (see Fig. 1, Fig. 2). Although younger, HD carriers were comparable to HD patients regarding lipid peroxidation. HD carriers had higher lipid peroxidation than their matched controls (p = 0.012 and p = 0.016 in the unadjusted and adjusted analysis, respectively), and the adjusted analysis indicated a difference of around 16 % (see Figs. 1 and 2). Although younger, HD carriers had higher lipid peroxidation than healthy volunteers matched to HD patients, as well. The difference did not attain statistical significance in the unadjusted analysis (p = 0.016 vs. the statistical significance limit 0.0125) (see Fig. 1), but was significant in the adjusted analysis (p = 0.019) and was estimated at around 18 % (see Fig. 2).

Table 3 Summary of analysis of variance on logarithmically transformed values of plasma lipid peroxidation (Ln LP) and reduced glutathione (Ln GSH) in Huntington's disease patients (n = 19), asymptomatic Huntington's disease gene carriers (n = 11) and their respective matched healthy controls (n = 47 and n = 22)

		Ln LP		Ln GSH	
	Df	F ratio	P-value	F ratio	P-value
Model	4	7.82	< 0.001	4.18	0.002
Age	1	0.83	0.365	2.53	0.115
Sex	1	0.30	0.585	0.12	0.727
Group	2	12.02	< 0.001	5.14	0.003

Plasma GSH level was lower in HD patients than in their matched controls (p = 0.005 and p = 0.011 in the unadjusted and adjusted analysis, respectively). The difference was estimated at around –28 % (see Fig. 1, Fig. 2). HD gene carriers were comparable to HD patients regarding GSH. They had lower GSH levels than either of the two control groups, but the differences failed (by small amounts) to attain statistical significance. In the adjusted analysis, the difference between HD carriers and their matched controls was estimated at around –27 %, and the difference between HD carriers and controls matched to HD patients was estimated at around –25 % (see Fig. 1, Fig. 2).

After adjustment for age and sex, plasma CAT levels in HD patients and HD gene carriers (taken together) remained significantly higher than in healthy subjects (geometric means ratio 1.15, 95 % CI 1.01 to 1.31, p = 0.031), while plasma carbonyl proteins remained higher, but the difference was not statistically significant any more (p = 0.107).

The number of CAG repeats did not appear correlated to any of the oxidative stress markers (pooled data on HD patients and asymptomatic HD carriers, n = 30) or to any of the clinical features in HD patients (n = 19). Correlation analysis of oxidative stress parameters and clinical features in HD patients indicated the following associations: higher plasma lipid peroxidation and

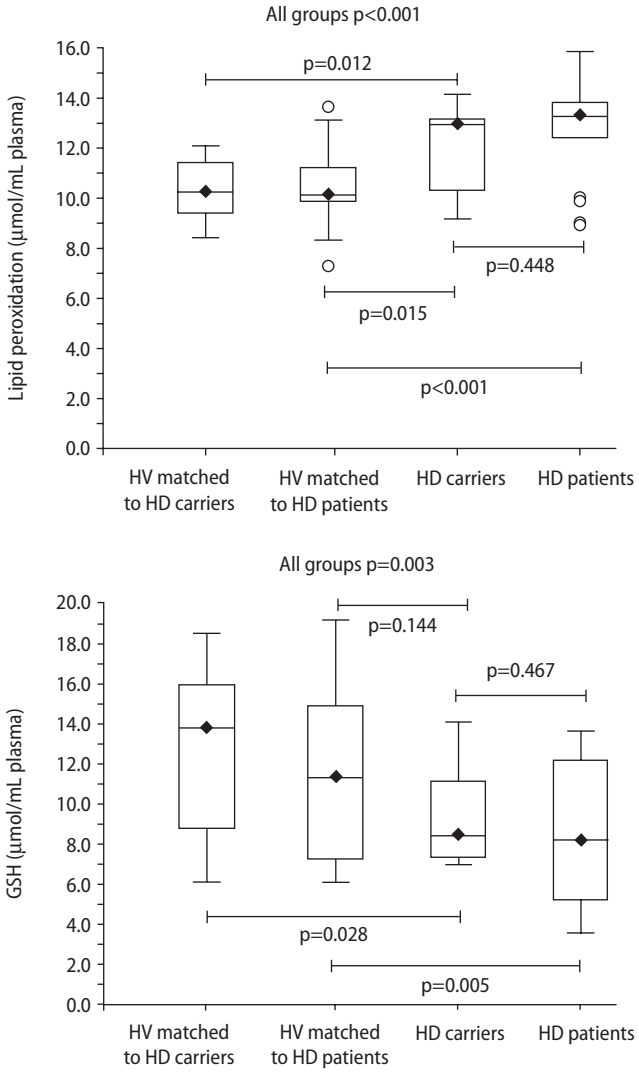

Fig. 1 Plasma lipid peroxidation and reduced glutathione levels in HD patients (n = 19), asymptomatic HD gene carriers (n = 11) and their respective sex and age-matched healthy controls (HV) (n = 47 and n = 22). Box plots display medians (diamonds), quartiles (boxes), inner fences (bars) and outliers (circles). P-values are from one-way non-parametric analysis of variance ("all groups"), followed by Kruskal-Wallis z-test for multiple pairwise comparisons. Four comparisons of interest were carried out with Bonferroni adjustment, so that p-values < 0.0125 were considered statistically significant

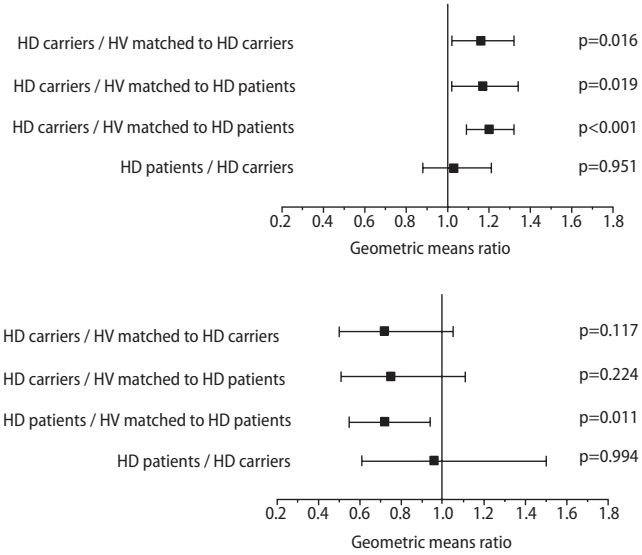

Fig. 2 Adjusted pairwise differences in plasma lipid peroxidation and reduced glutathione levels for HD patients (n = 19), HD gene carriers (n = 11) and their respective age and sex-matched healthy volunteer controls (HV) (n = 47 and n = 22). Least-square mean differences from analysis of variance on ln-tranformed parameters with factors age, sex and group (main effects given in Table 3) are shown as geometric means ratios (exponents) with 95 % simultaneous Tukey-Kramer confidence intervals

worse outcomes in the verbal fluency test (p = 0.012), Stroop word test (p = 0.05) and a worse total chorea score (p = 0.036) (see Fig. 3).

Discussion

Oxidative stress, defined as an imbalance between oxidant production and antioxidant protection, has been linked to many neurological disorders [2]. High lipid concentration and high energy requirements make the brain particularly susceptible to free radical and oxidant-mediated insults. A growing body of evidence suggests that HD may have components of the free radical and oxidative stress-induced injury [11]. The most compelling evidence comes from animal studies. For example, systemic administration of 3-nitropropionic acid produces lesions that closely resemble the neuropathological features of HD. The lesions are associated with increased markers of free-radical damage and are significantly attenuated in mice overexpressing superoxide dismutase, an enzyme that protects against the free radicals [6]. Due to a high content of polyunsaturated fatty acids and transition metals like iron and copper, the brain is particularly vulnerable to lipid peroxidation and accumulating evidence substantiates extensive interactions between the reactive oxygen species and neuronal lipids in the brain areas directly involved in the HD process [1, 5]. Recently, higher levels of lipid peroxidation products in plasma were reported in a sample of patients with advanced HD as compared to healthy subjects [8, 29]. This finding was associated with alterations of tryptophan metabolism in the kynurenin pathway in HD patients, and tryptophan metabolites (kynurenines) are assumed to modulate reactive oxygen species generation (and oxidative stress) either directly, or through interactions with the NMDA glutamate receptor [29]. It was concluded that the changes detected in the peripheral blood did, although likely to a limited extent, reflect the processes going on in the brain [29]. In line with those observations, in the present study we found significantly higher plasma levels of lipid peroxidation in

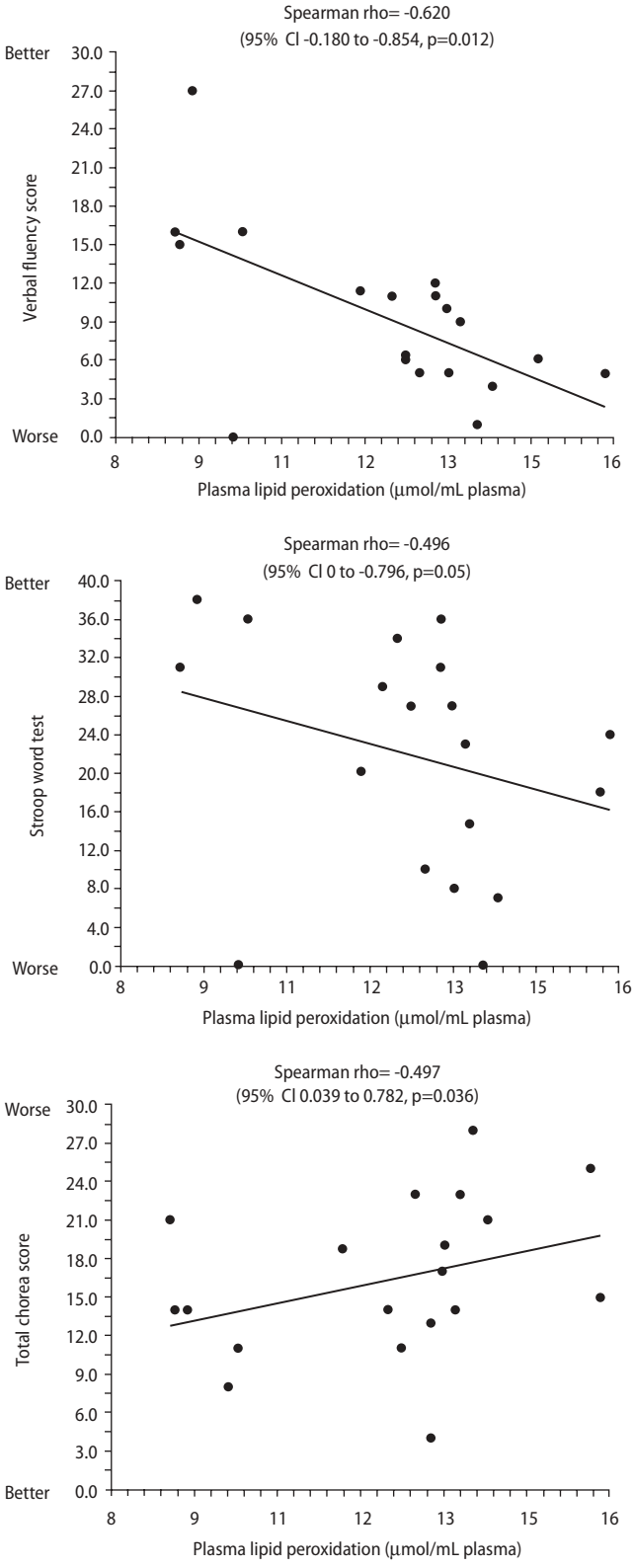

Fig. 3 Correlation between plasma lipid peroxidation and verbal fluency score, Stroop word test score and total chorea score in HD patients (n = 19)

HD patients than in the age and sex-matched healthy controls. The observed difference of around 20 % is lower than the one reported by others [8, 29]. This could be due to two facts: first, our HD patients were mostly outpatients with a moderately advanced disease, while those considered by others (n = 11) suffered from a very advanced disease and were permanently disabled [8, 29]; second, in the present study HD patients were only slightly older than the control subjects (median difference 4 years), while the mean difference in age between HD patients and healthy controls in the previous reports was 17 years [8, 29]. Furthermore, we found significantly higher (by around 16 %) levels of plasma lipid peroxidation in asymptomatic HD gene carriers (> 38 CAG repeats in the huntingtin gene) than in their age and sex-matched healthy controls. Although HD gene carriers were considerably younger than HD patients, their lipid peroxidation levels were comparable to those found in HD patients and were significantly higher (by around 18 %) than the levels in healthy subjects matched by age to HD patients. Despite the limitation of a cross-sectional design (as opposed to a prospective longitudinal follow-up), the results suggest that an increase in lipid peroxidation precedes the occurrence of the clinical symptoms of HD. To the extent to which plasma levels of lipid peroxides may be considered illustrative of the processes going on in the brain, the current observations appear to be in line with data directly relating progression of lipid peroxidation in the brain and progression of the neurological phenotype in mice transgenic for the HD mutation [26]. Correlation between plasma lipid peroxides and clinical features in HD patients (higher the levels, worse the total chorea score, verbal fluency score and Stroop word test score) further supports, albeit only circumstantially, a role of lipid peroxides in clinical symptoms of HD and suggests that, once they are present, more severe symptoms are associated with higher lipid peroxidation levels. The observed lack of correlation between the plasma levels of oxidative stress markers or clinical features of HD and the number of CAG repeats is in agreement with a recent report showing no association between the number of CAG repeats and oxidative stress markers or activity of mitochondrial respiratory chain enzymes in cultured fibroblasts of HD patients [14].

Reduced glutathione is an important defense mechanism that protects cellular constituents from the damaging effects of peroxides. Decreased GSH levels are indicative of oxidative stress and are associated with cell damage, progression of aging and certain neurodegenerative disorders [9, 10]. A single post-mortem study found no difference in basal ganglia GSH levels between HD patients and subjects without neurodegenerative disorders, while the level of oxidized glutathione was reduced in the HD patients' caudate nucleus [28]. On the other hand, the rat model of HD with lesions induced by

3-nitropropionic acid is characterized by a marked depletion of the striatal GSH [30]. In parallel with higher plasma lipid peroxidation levels, HD patients in the present study had significantly lower (by around 28 %) plasma GSH levels than their age and sex-matched controls. Similarly, HD gene carriers were comparable to HD patients regarding GSH, and had lower GSH levels than their age and sex-matched controls (by around 27 %) or healthy subjects matched by age to HD patients (by around 25 %). Although these differences failed to attain statistical significance (by small amounts), the patterns of differences in plasma GSH levels among the four groups paralleled the pattern of differences in lipid peroxidation levels as a "mirror image", and appeared to be in line with the animal data on reduced basal ganglia GSH in experimental HD [30].

The fact that the plasma O_2*, SOD, CarbP and CAT levels did not significantly differ between HD patients or HD gene carriers and their respective controls within the current sample could be due to one or more of the following reasons: i) true (population) lack of difference; ii) insufficient power of the study and/or iii) methodological flaws (e.g., sampling, measurement methodology). In respect to this last possibility it should be noted that we included consecutive subjects who met predefined inclusion/exclusion criteria and were free of comorbidities or treatments that could substantially affect the measured oxidative stress markers, blood was sampled under standardized conditions and we used established analytical methodology. Hence, we believe that the study achieved a fair level of internal validity and provided reliable difference estimates. However, as with any study aimed at testing a null hypothesis of no difference, lack of significant difference (between groups) is difficult to interpret since it could simply be due to inadequate power. In the present study, both HD patients and HD gene carriers had somewhat higher plasma CAT and CarbP levels than their respective controls, but the differences attained statistical significance only in the analysis of pooled data (HD patients/HD gene carriers vs. all healthy controls). The finding of higher plasma CAT levels in HD patients/HD gene carriers appears plausible in biological terms, since CAT acts synergistically with GSH in scavenging free radicals [11] and higher levels could indicate that this antioxidant enzyme might have been stimulated by an enhanced free radical production. On the other hand, the finding of higher CarbP levels (as markers of oxidative damage to proteins) appears to be in agreement with the reportedly increased brain CarbP levels in neurodegenerative disorders like Parkinson's or Alzheimer's diseases [7]. Plasma O_2* levels and total SOD activity appeared fully comparable across the analyzed groups. In the case of O_2*, this was in a way expected considering its extremely short half-life [31]. As for SOD, the cross-sectional design of the study might not have been too informative considering its age(time)-related oscillations described in HD-transgenic mice: striatal total SOD activity was found increased (by around 10 % vs. the wild-type animals) on day 19 of life (presumably as a reaction to increased H_2O_2 production) and decreased (by around 10 % vs. the wild-type animals) on day 35 of life [27].

In conclusion, current results demonstrate presence of oxidative stress in HD patients illustrated by a higher plasma level of lipid peroxidation products and a lower level of oxidant-protective GSH as compared to healthy controls. The observed differences may be reasonably viewed as indicative of the processes going on in the brain [8, 29] and are in line with the brain findings in animal models of HD. The same pattern of differences found in asymptomatic HD gene carriers (with > 38 CAG repeats) as compared to age and sex-matched controls and the lack of differences between HD gene carriers and HD patients suggest that oxidative stress occurs before the onset of HD symptoms and support a role of oxidative stress in pathophysiology of HD.

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