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Elevated Levels of Carbonyl Proteins in Cerebrospinal Fluid of Patients with Neurodegenerative Diseases

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The importance of oxidative stress in the pathogenesis of neuroimmunological and neurodegenerative diseases, such as multiple sclerosis (MS), has been discussed for a long time. However, markers for oxidative stress in cerebrospinal fluid are hardly detected. The aim of the present study is to assess whether carbonyl proteins as end products of metabolic processes may serve as a marker for oxidative stress in the cerebrospinal fluid (CSF) of patients with neuroimmunological and neurodegenerative diseases. Levels of carbonyl proteins in the CSF were assessed in 15 patients suffering from MS, four patients with neurodegenerative diseases, including one patient with dementia complicated by carcinomatous meningitis due to breast cancer, and four control subjects with no established neurological disease. Levels of carbonyl proteins were measured with a commercially available KIT. A significant difference (P = 0.025) was shown for mean values of various subgroups with highest levels for patients with neurodegenerative diseases (756.1 pmol/mg), followed by the MS (630.8 pmol/mg) and the control group (356.5 pmol/mg). Post-hoc pair wise comparisons showed significant differences between the MS group and healthy controls (P = 0.016) as well as for patients with neurodegenerative diseases and healthy controls (P = 0.02). This pilot trial showed that carbonyl proteins might serve as measure for oxidative stress in the CSF of relapsing as well as progressive MS patients and in patients with neurodegenerative diseases. Larger trials have to show whether they may serve as biomarkers and be helpful in monitoring patients with MS or neurodegenerative diseases.

Keywords: carbonyl proteins; cerebrospinal fluid; multiple sclerosis; neurological disorders; oxidative stress
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Introduction

Multiple sclerosis (MS) is the most common chronic neuroimmunological disease in young adults. Hitherto the pathogenesis of MS is not fully understood; an autoimmune process seems to be the underlying cause (Weinshenker 1998; Weiner 2009). Diagnosis of clinically definite MS (CDMS) is established after new symptoms or progression of disability have occurred after a first clinical event indicative of MS (clinically isolated syndrome [CIS]) (Polman et al. 2011). MS may proceed in attacks (relapses) with full or limited remission (relapsing remitting MS [RRMS]), or it may be characterized by steady worsening of neurological functions either from disease onset (primary progressive MS [PPMS]) or after an initial relapsing remitting course (secondary progressive MS [SPMS]) (Lublin and Reingold 1996). At an early stage, the progression of disease is associated with inflammatory processes, whereas neurodegenerative processes become more important in the later stages of MS (Lassmann et al. 2012).

The importance of oxidative stress (OS) in the etiology of immunological and degenerative diseases has been discussed for a long time (Perry et al. 2002; Greilberger et al. 2008; Drechsel et al. 2012). In MS, for instance, the linkage between inflammation and OS is well established. Additionally, neurodegeneration seems to be driven by oxidative injury and mitochondrial dysfunction (Lassmann et al. 2012).

An imbalance between antioxidants and radical oxygen species (ROS) leads to the development of OS (Gonsette 2008). ROS cause damage to cells by oxidizing proteins, lipids and DNA (Lassmann et al. 2012). Amino acids are modified, resulting in the accumulation of carbonyl- and hydroxyl groups. Higher levels of nitric oxide metabolites could be detected in patients suffering from MS (Rejdak et al. 2004). Lipid peroxidation products and glu-

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tathione peroxidase activity were observed in the serum of RRMS patients (Ortiz et al. 2009). Increased levels of sulfhydryl groups, transferrin, DJ-1 and transthyretin isoforms mirror OS and were assessed in the serum of MS patients (Calabrese et al. 1994, 1998; Zeman et al. 2000; Hirotani et al. 2008; Poulsen et al. 2012).

Carbonyl proteins (CPs) are the end product of metabolic processes (Greilberger et al. 2010; Pennisi et al. 2011) and recent trials suggested CPs as a marker for OS in the plasma of patients with degenerative diseases like Alzheimer's disease (Greilberger et al. 2010).

Markers for OS in the cerebrospinal fluid (CSF) were hardly detected and pose a substantial challenge. Nitrit oxide metabolites were assessed in the CSF of MS patients by Rejdak et al. (2008). Whereas CPs have recently been assessed in the CSF of relapsing MS patients (Pennisi et al. 2011), the relevance in the CSF of patients with diseases characterized by neurodegeneration remains unexplored. The aim of this proof of concept trial is to explore CPs in the CSF of neurodegenerative diseases, and whether they may serve as a marker for OS in the CSF of patients with neurodegenerative diseases.

Methods and Patients

The study was approved by the institutional ethics committee (Medical University of Vienna, Vienna, Austria; EK-NR 588/2009). All patients gave their written and informed consent.

Subgroups were analyzed by Kruskal-Wallis-Test. Kolmogorov-Smirnov-test was performed for checking distribution in various subgroups. In the case of significant results post-hoc pair wise comparisons were performed by Mann-Whitney-*U* test. Statistics were performed with software IBM® SPSS® Statistics Version 20 (Armonk, U.S.A.).

All lumbar punctures were performed after excluding increased intracranial pressure and were performed for diagnostic purposes only. In all cases atraumatic "Sprotte" cannulas (Pajunk Medizintechnologie GmbH, Geisingen, Germany) were used. 200 μl of CSF were collected for further analysis. CSF was immediately held on ice and stored at −80°C. EDTA blood was collected; plasma was separated by centrifugation and stored at −80°C. CP content was measured with the Carbonyl Protein ELISA Kit (Immundiagnostik AG, Germany) as originally described (Greilberger et al. 2008, 2010). Each sample (4 μL) was reacted with 80 μL di-nitro-phenylhydrazine (DNPH) for 45 min at room temperature. DNPH-derivatised proteins were separated by centrifugation from non-protein constituents and unconjugated DNPH reagent using 5,000 Da molecular weight cut-off filters (Amicon ultra-centrifugal filter, Merck, Vienna, Austria). One part of the diluted proteins is adsorbed onto an ELISA plate and incubated with anti-DNPH antibody followed by antibody-linked horseradish peroxidase. The absorbance of the carbonylated protein content of the samples is related to a standard curve prepared with oxidized human serum albumin. The carbonyl protein content is calculated from the estimated carbonyl concentration and the total protein content of the sample. In parallel, a protein determination of the centrifuged samples was measured using a PIERCE 660 nm protein assay (Pierce Biotechnology, Rockford, IL).

Results

In total, 23 patients were included in this study. EDTA blood was collected from 16 patients, whilst CSF was assessed in all 23 patients. List of patients see Table 1. Out of the 23 enrolled patients, 15 patients suffered from MS (either CDMS or CIS), four patients suffered from neurodegenerative diseases. In one of the latter four patients, dementia was initially diagnosed, but after careful diagnostic work-up in the same patient, carcinomatous meningitis due to breast cancer was finally diagnosed. In remaining four patients, lumbar puncture was performed for ruling out neurological diseases like subarachnoid hemorrhage.

The mean value of CPs in EDTA blood was 395.1 pmol/mg in all patients. The mean blood levels of CPs did not show any correlation with clinical parameters: 368.8 pmol/mg for CDMS patients, 278.6 pmol/mg for CIS patients, 366.4 pmol/mg for patients with neurodegenerative diseases, and 389.3 pmol/mg for those without established neurological disease. Thus, only levels of CPs in the CSF are presented.

Average levels of CPs in the CSF of patients with MS (CIS or CDMS) were 596.1 pmol/mg; 756.1 pmol/mg in patients with neurodegenerative diseases, and 356.5 pmol/mg for the healthy controls (without established neurological disease). Kolmogorov-Smirnov-test revealed not normally distributed samples in the various subgroups. Kruskal-Wallis-test showed significant differences between subgroups (P = 0.025); post-hoc pair wise comparisons showed significant differences between the MS group and the healthy controls (P = 0.016) as well as between the neurodegenerative group and the healthy controls (P = 0.020).

Further analyses showed that 12 patients suffered from CDMS according the revised McDonald's criteria (Polman et al. 2011). 8 patients suffered from RRMS, 3 from SPMS and 1 one from PPMS; mean values of CPs were 630.8 pmol/mg (SD 245.1) for all patients, 623.2 pmol/mg (SD 276) for RRMS patients, and 646 pmol/mg (SD 203) for progressive MS (SPMS and PPMS) patients with no significant differences between both groups (P = 0.495). Interestingly, two patients received natalizumab prior to lumbar puncture and showed lower levels (364.6 pmol/mg and 475 pmol/mg, respectively) than the average. 3 patients suffered from CIS: mean value was 457.2 pmol/mg. One of those patients developed CDMS (657.1 pmol/mg), whereas the other 2 patients did not develop MS within follow up of two years (317.9 pmol/mg and 396.7 pmol/mg, respectively).

When analyzing CDMS patients and CIS patients separately Kruskal-Wallis-test showed significant differences between various groups (P = 0.024). Post-hoc pair wise comparisons between subgroups showed significant differences between the CDMS group and healthy controls (P = 0.034), but not for CIS and healthy controls (P = 0.289). Kolmogorov-Smirnov-test revealed no normal distribution for the various samples. Fig. 1 shows the results in detail.

Table 1. List of patients.

patient number	sex	age	Disease	Level of CPs pmol/mg
1	f	21	RRMS acute relapse	513.2
2	f	41	RRMS	384.4
3	f	30	healthy	379.2
4	f	49	progressive MS	478.3
5	m	27	RRMS	430.7
6	m	70	ALS	719.8
7	m	66	Lewy-Body dementia	641.5
8	f	69	dementia carcinomatous meningitis	1,064.2
9	m	32	CIS	657.1
10	m	34	healthy	384
11	f	48	progressive MS	636.3
12	f	27	CIS	317.9
13	f	32	RRMS	364.4
14	f	47	progressive MS	534.9
15	m	41	progressive MS	934.4
16	f	34	RRMS acute relapse	796.7
17	f	35	RRMS	1,083.5
18	f	64	ALS	599.1
19	f	63	healthy	296.7
20	f	25	CIS	396.7
21	f	31	healthy	366
22	m	27	RRMS	937.7
23	f	38	RRMS	475

CIS, clinically isolated syndrome; RRMS, relapsing remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; ALS, amytrophic lateral sclerosis.

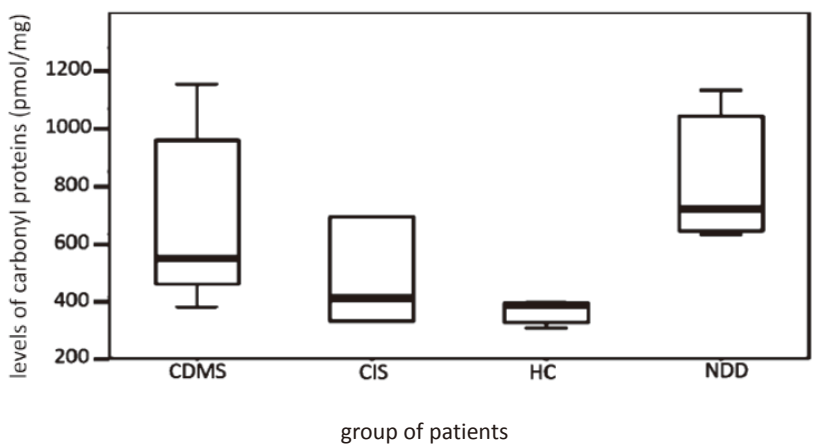

Fig. 1. Levels of carbonyl proteins in the CSF of patient groups and controls.
Shown are the CSF levels of carbonyl proteins (pmol/mg) in patients with clinically definite MS (CDMS), clinically isolated syndrome (CIS) or other neurodegenerative diseases (NDD) and in healthy controls (HC). The levels of CPs in the CSF were highest in NDD, followed by patients with CDMS, CIS, and HC. The respective mean values are 756.1 pmol/mg (standard deviation: SD 211.3), 630.8 pmol/mg (SD 245.1), 457.2 pmol/mg (SD 177.5), and 356.5 pmol/mg (SD 40.6). The levels differed significantly ($P = 0.025$) between the subgroups. Post-hoc pair wise comparisons revealed significant differences between the CDMS and healthy controls ($P = 0.034$), as well as for NDD and healthy control ($P = 0.020$), but not for CIS and healthy control ($P = 0.289$). Levels of CPs in CDMS and NDD did not differ significantly ($P = 0.395$).

Four patients suffered from neurodegenerative diseases (2 patients with amyotrophic lateral sclerosis [ALS], 1 patient with Lewy-Body dementia, 1 patient with unspecified dementia). The fourth patient also suffered from meningeosis carcinomatosa due to breast cancer. All over mean value was 756.1 pmol/mg (SD 211.3). In healthy controls mean value of CPs was 356.5 /mg (SD 40.6).

Discussion

The results of this proof of concept study showed differences in the levels of CPs in patients with CDMS (relapsing and progressive MS) compared to healthy controls. These results suggest that CPs may serve as marker for OS in the CSF of relapsing and progressive MS patients. The significance of the results in MS patients are underlined by the levels of CPs in patients suffering from neurodegenerative diseases (ALS and Lewy-Body dementia) and from malignancies (meningeosis carcinomatosa) with significant higher levels of CPs in the CSF than in controls with no established neuroimmunological or neurodegenerative disorders.

High levels of CPs in immunological and degenerative diseases are explained by the fact that inflammation as well as neurodegeneration leads to OS. This is in accordance with the findings of Petzold (2005) who found higher levels of neurofilament heavy chains phosphoforms (NfH) in neurodegenerative and neuroimmunological diseases. In progressive MS NfH seem to be a predictor of axonal damage (Petzold 2005). Whereas higher levels of CPs in the CSF are associated with inflammatory or degenerative diseases, this does not hold for levels in the EDTA blood. This is in contrast to the findings of Pennisi et al. (2011) who presented significant differences in the plasma of MS patients when compared to controls. Whereas Pennisi et al. (2011) investigated CPs only in RRMS patients, we assessed the levels in relapsing and progressive MS patients as well as in patients with neurodegenerative diseases. In relapsing MS patients, inflammation drives disease activity. In later stages of MS neurodegeneration is driven by inflammation beyond the blood-brain-barriers as well as by mitochondrial dysfunction (Lassmann et al. 2012). Consequently, oxidative reactions may be less detectable in peripheral blood of progressive MS patients. Furthermore, environmental factors – like smoking – will influence plasma levels. 20 of the subjects included in the study were non-smokers, data was not available for three subjects.

A limitation of our trial may be the small number of controls. Moreover, former studies showed that levels of CPs in the CSF of patients with RRMS were higher than in controls (Pennisi et al. 2011), and elevated levels of CPs were detected in the plasma of patients with Alzheimer's disease when compared with controls without dementia (Greilberger et al. 2010). Here we show for the first time that CPs are increased in the CSF of neurodegenerative diseases, such as ALS and progressive MS.

The ultimate goal of biomarkers is the prediction of disease outcome and/or therapy response. Sbardella et al. (2013) demonstrated that isoprostanes might serve as biomarkers for OS in the CSF of MS patients. Interestingly, higher levels in patients with CIS predicted progression to definitive MS (Sbardella et al. 2013). Furthermore, nitric oxide metabolites in the CSF were associated with disease activity and sustained disability progression in MS patients (Rejdak et al. 2008).

The results of our study (detection of elevated CPs in the CSF of patients with neurodegenerative and neuroimmunological diseases compared to controls) may help to design future confirmative trials. Future trials will have to show if levels of CPs may be a useful marker for therapy response or for prognosis.

Conflict of Interest

FL received speaker/consulting honorary from Bayer Schering, Biogen Idec, Genzyme, Merck Serono, Novartis and Teva. Other authors declare no conflict of interest.

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