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# Malondialdehyde, carbonyl proteins and albumin-disulphide as useful oxidative markers in mild cognitive impairment and Alzheimer's disease

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**Abstract**
The question arises as to whether oxidative stress has a primary role in neurodegeneration or is a secondary end-stage epiphenomenon. The aim of the present study was to determine oxidative stress parameters like malondialdehyde (MDA), carbonyl proteins (CP) and Albumin-disulphide (Alb-SSR) and relate these parameters to the immune parameter neopterin, folic acid and vitamin B12 as vitamins and homocysteine in patients with neuro-degenerative diseases (NDD), namely mild cognitive impairment (MCI) and Alzheimer's disease (AD) compared to an aged matched control group. MDA, CP and Alb-SSR were significantly increased in the NDD group compared to controls, but not vitamin B12, folic acid and neopterin. Significant correlations were found between CP and Alb-SSR, CP and MDA and between MDA and Alb-SSR including patients with NDD and the control group. These results support the hypothesis that oxidative damage to lipids and proteins is an important early event in the pathogenesis of neurodegenerative diseases.

**Keywords:** *Neuro-degenerative diseases, mild cognitive impairment (MCI), Alzheimer's disease (AD), reactive oxygen and nitrogen species (RONS), malondialdehyde (MDA), carbonyl proteins (CP), sulphydryl-albumin (Alb-SH), albumin-disulphide (Alb-SSR)*

## Introduction

Neurodegenerative diseases like Alzheimer's disease (AD) and mild cognitive impairment (MCI) are age-associated neurodegenerative disorders resulting in the loss of memory and cognition [1]. The aetiology and pathogenesis of these diseases are still unclear. There exist several aetiologic and pathogenetic hypotheses for the development of AD such as: genetic defects in the APP gene on chromosome 21, latent virus disorders, deficits in the energy metabolism resulting in mitochondrial defects, deficits in neurotrophic factors and trace element neurotoxicity [2]. In the last decades oxidative stress has become of increased interest in neurologic disorders like AD or other neuro-degenerative diseases [3,4]. On one hand, oxidative stress in the brain is thought to be central, by the mechanism of A$\beta$ (1–42)-associated oxidation in neurons [5]. On the other hand, trace

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amounts of free metals, lipid peroxidation products like malondialdehyde (MDA) and hydroxynonenal (HNE) as well as lipid peroxidation end products like carbonyl proteins (CP) and isoprostanes are increased in AD, MCI and the neurodegenerative brain [6–8]. Estimation of increased reactive carbonyls and carbonyl proteins, especially in the brains of patients with AD but also in other neurodegenerative diseases, are demonstrated in numerous reports [3,4,9].

Recent studies demonstrated that oxidative damage of proteins in MCI and AD subjects were significantly higher than of age-matched control subjects, suggesting that oxidation stress is an early event in MCI and early AD [10,11]. Furthermore, this situation is often combined with a reduction of enzymatic, like glutathione peroxidise, glutathione S-transferase and superoxide dismutase, non-enzymatic antioxidative defences [12] and on protein synthesis and protein degradation [13,14]. New approaches to investigate oxidative stress in MCI and AD such as proteomics of CP, $F_2$-IsoP levels and damage of DNA and RNA showed that oxidative damage is involved in a variety of cellular targets which occur early in the progression of AD [5,15–18]. Reactive aldehydes, like MDA and HNE, but also CP seemed to be involved in the pathogenesis of neurodegenerative diseases in the brain and also in the peripheral blood system [19–21]. Questions arise about the origin of free radicals or reactive oxygen and nitrogen substances (RONS), which are definitely connected with the pathogenesis of this disease [22,23]. An end product of membrane lipid peroxidation, MDA is one of the most widely used markers for free radical mediated damage [24]. It has been reported that MDA increases in patients with neurodegenerative diseases [8], but also in elderly healthy persons compared to a young healthy control group [25,26]. Oxidized forms of albumin are also becoming a more interesting field in neurodegenerative disorders, especially acrolein-albumin [27]. Less is known about the oxidation of cystein 34 of albumin to the disulphide form albumin (Alb-SSR). Cys-34 of albumin may contain a free sulphydryl group which can easily be oxidized to a mixed disulphide (Alb-SSR) or higher to sulphinic or sulphonic acid. Oxidized albumin was reported to be a parameter for ageing and different types of diseases like uremia [28]. The thiol group of albumin is discussed to contribute the anti-oxidative capacity in plasma.

The present study shows for the first time the estimation of a set of oxidative stress blood parameters of patients with neurodegenerative diseases in comparison to an age matched control group to gain more insight into the 'diverse and controversial role of oxidants in neurodegeneration' [29].

## Materials and methods

### *Patients characteristics*

The initial population of 31 subjects consisted of 15 healthy volunteers (11 females and four males; age: 60.8 years $\pm$ 4.7) and 16 patients with neurodegenerative diseases (NDD): six females and four males with AD, three females and three males with MCI; average age: 67.6 years $\pm$ 5.2). Mini-Mental State Examination (MMSE) of NDD patients (MMSE: 23.5 $\pm$ 2.2) was evaluated. AD patients fulfilled the NINCDS ADRDA criteria [30], whereas MCI diagnosis followed the criteria of Petersen et al. [31] when there was evidence of memory impairment, preservation of general cognitive and functional abilities and absence of diagnosed dementia. NDD patients were recruited from the Wagner-Jauregg-Hospital in Linz, Austria. The patients did not receive vitamin supplementation within their treatment regimen. Patients or one of their relatives gave informed consent to participate in this study, which was approved by the local ethics committee. For the patients without cognitive impairment, Mini-Mental State Examination was carried out (MMSE: 30 $\pm$ 0). Furthermore, healthy volunteers did not receive any vitamin supplements within 6 weeks before blood collection. Subjects with additionally diseases like malignant diseases or clinical relevant gastrointestinal, renal, hepatic, cardiorespiratoric, hematological, as well as patients with metabolic disorders or chronic infections were excluded from the study.

### *Determination of oxidative stress parameter*

Blood samples were collected after an overnight fast, allowed to clot and centrifuged immediately. Sera were aliquoted and stored at $-70^{\circ}$C until measurement. Total homocysteine concentrations were measured by HPLC as described previously [32]. Determination of neopterin concentrations was performed by ELISA (BRAHMS Diagnostica, Berlin, Germany). For the measurement of folate and vitamin B12 concentrations a double-labelled radioimmunoassay (Chiron Diagnostic Corp., Walpole, MA) was used.

Malondialdehyde was quantitated by a HPLC method after reaction with thiobarbituric acid as described elsewhere [33].

The redox state of human serum albumin was followed by HPLC separation of albumin giving a peak representing the sulphydryl form (Alb-SH) and another representing the mixed disulphide form (Alb-SSR), according to Hayashi et al. [34]. Separation was carried out using a Shodex Asahipak ES-502N 7C anion exchange column (7.6 $\times$ 100 mm) with 50 mm Na-acetate, 400 mm Na-sulphate, pH 4.85 as mobile phase. Elution was carried out with a gradient of 0–6% ethanol and a flow rate of 1 mL/min. The column was kept at 35°C. Fluorescence detection was

carried out at 280/340 nm. Samples were diluted 1:100 with 0.1 M Na-phosphate, 0.3 m NaCl, pH 6.87, filtered through a 0.45 μm nylon filter and 20 μL were injected into the HPLC system. Data are expressed as the percentage of Alb-SSR.

For the determination of carbonyl proteins, oxidized bovine serum albumin (BSA) was prepared for standardization as described elsewhere [35]. Serum samples and standards were diluted to give a final protein concentration of 4 mg/mL. Measurement of CP was performed after derivatization with 2,4-dinitrophenyl-hydrazine (DNPH) by a chemiluminescence technique on a chemiluminescence reader (Lumistar,BMG, Germany) after addition of 200 μL/well Super Signal Maximum Sensitivity substrate (Pierce, Rockford, USA). Serum protein was measured with the bicinchoninic assay (BCA; Pierce, Rockford, USA).

### *Statistical analysis*

Statistical analysis was performed by SPSS software 14.0. Data are presented as means ± standard deviation (SD). Significance was set at $p < 0.05$. Mean values of NDD and control group were compared using *t*-test for unpaired samples. Further, we pooled data for gender and age and compared concentrations of all parameters by one-way-ANOVA. Pearson's correlation coefficient and regression analysis were used to evaluate bivariate relationships of gender or age with all biochemical markers of NDD patients as well as healthy volunteers.

## Results

Figure 1A shows the significant difference ($p < 1.0 \cdot 10^{-3}$) in the content of MDA between the control group ($n = 15$; $1.15 \pm 0.32$ μm) and the NDD group ($n = 16$; $2.62 \pm 1.27$ μm). Concentration of CP of the control group ($n = 15$; $0.30 \pm 0.12$ nmol/mg protein) were significantly lower ($p < 4.9 \cdot 10^{-6}$) than in the NDD group ($n = 16$; $0.90 \pm 0.29$ nmol/mg protein), as shown in Figure 1B. The fraction of Albumin-SSR was significantly increased ($p = 1.7 \cdot 10^{-7}$) in the NDD group ($n = 16$; $49.2 \pm 9.2\%$) compared to the control group ($n = 15$; $24.7 \pm 4.0\%$), as shown in Figure 1C. Comparing the amount of neopterin, folic acid, vitamin B12 and age, no significant differences were found between the control and the NDD group, as described in Table I.

Figure 2A–C show the Pearson correlations between the different oxidative stress markers including data of patients and controls. The best correlation was found between CP and Albumin-SSR ($n = 31$; $r = 0.906$; $p = 4 \cdot 10^{-10}$; Figure 2A), then between CP and MDA ($n = 31$; $r = 0.85$; $p = 3.8 \cdot 10^{-8}$; Figure 2B) and least between MDA and Albumin-SSR ($n = 31$; $r = 0.786$; $p < 1.9 \cdot 10^{-6}$; Figure 2C).

When correlating CP with Alb-SSR, within the control group alone no significant correlation was found ($n = 15$; $r = 0.037$; $p = 0.895$). However, a significant correlation was found within the patient group ($n = 16$; $r = 0.736$; $p = 2.3 \cdot 10^{-6}$). This situation was similar for the correlation of CP and MDA: while we found no significance in the controls ($n = 15$; $r = 0.3$; $p = 0.3$), there was a good correlation in the NDD group with high significance ($n = 16$; $r = 0.83$; $p < 0.001$). The correlation between MDA vs Albumin-SSR in the control group showed a trend toward significance ($n = 15$; $r = 0.489$; $p = 0.064$), while a significant correlation was found in the NDD group ($n = 16$; $r = 0.723$; $p < 0.01$).

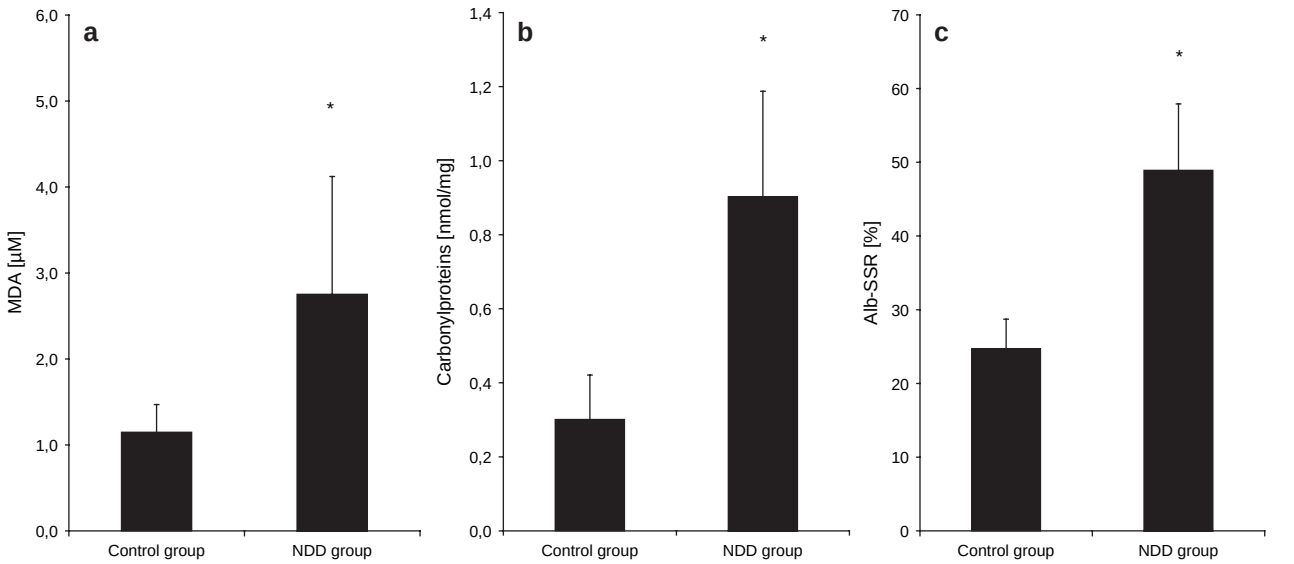


Figure 1. Oxidative stress parameters in serum from a control and a NDD group. MDA content (A), CP content (B) and the amount of Albumin-SSR (C) in serum were determined. Bars indicated with * are significantly different from the control value ($p < 0.05$).

Table I. Clinical parameters in patients with neurodegenerative diseases (NDD: includes MCI and AD group) and the control group.

| | Control ($n=15$) | NDD ($n=16$) |
|---|---|---|
| Age | 60.8±4.7 | 67.6±5.2 |
| Neopterin (nm) | 6.8±1.5 | 7.6±3.8 |
| Folic acid (µg/L) | 8.2±4.2 | 8.9±4.6 |
| Vitamin B12 (ng/L) | 541.8±418.2 | 465.4±204.6 |
| Homocysteine (µm) | 13.9±3.3 | 14.1±6.8 |

Comparison of age ($<63.0$ years and $\geq 63.0$ years) and gender with CP, Alb-SSR and MDA showed no significant differences in any parameter ($p>0.1$). Neither age nor gender affected the statistical relationship between CP, Alb-SSR and MDA.

## Discussion

The usage of peripheral markers to show oxidative stress in neurodegenerative diseases was not convincing. This is mostly attributed to the main opinion that the blood–brain-barrier is intact in neurodegenerative disorders and therefore oxidative stress markers are not present in the peripheral circulation. However, there is growing evidence that abnormal small-vessel structures could affect the blood–brain-barrier in AD.

Lipid peroxidation is a central feature of oxidative stress and can be assessed by a number of methods including the quantification of peroxidation end products like MDA. MDA was shown to be increased with age in human brain, but several studies failed to detect any increase in serum in AD or other neurodegenerative diseases [36,37]. We have found that MDA levels are higher in the NDD group compared to controls. McGrath et al. [52] reported an increase of 4-hydroxynonenal but not of MDA in patients with AD. This disagreement could be ascribed to the commonly used spectrophotometric assay of MDA, while we used the more specific HPLC technique.

Lipid peroxidation end products play an important role in the modification of proteins beside a direct attack of RONS. The determination of CP is a widely used marker for oxidative stress for the investigation of samples from patients with neurodegenerative diseases [3,9,38]. An increase of CP in hippocampus and inferior parietal lobule regions of AD patients compared to age-matched controls was found by Hensley et al. [39]. In blood samples no difference was found concerning the amount of CP between AD patients and control subjects [40]. Our data demonstrate a severe increase in the generation of carbonyl proteins in the NDD group compared to the control group which is in good agreement with a recently published study [41]. Both markers MDA and carbonyl proteins correlated well in the NDD group [8,42].

We report for the first time an increase of Alb-SSR in neurodegenerative patients compared to an age-matched control group. As albumin is quickly distributed between blood and the extravascular compartment it serves as a global marker for the redox state in the body. The disulphide fraction of albumin is increased during ageing and in different kinds of diseases like senile cataract, diabetes mellitus or in hemodialysis patients [34,43–45]. Therefore, Alb-SSR is not a specific marker for neurodegenerative disease. In addition, the age of patients as well as controls has to be taken into account. Peroxynitrite and hydrogen peroxide are well known RONS which oxidize Alb-SH to Alb-SSR [29]. As peroxynitrite is initiating both lipid peroxidation and oxidation of

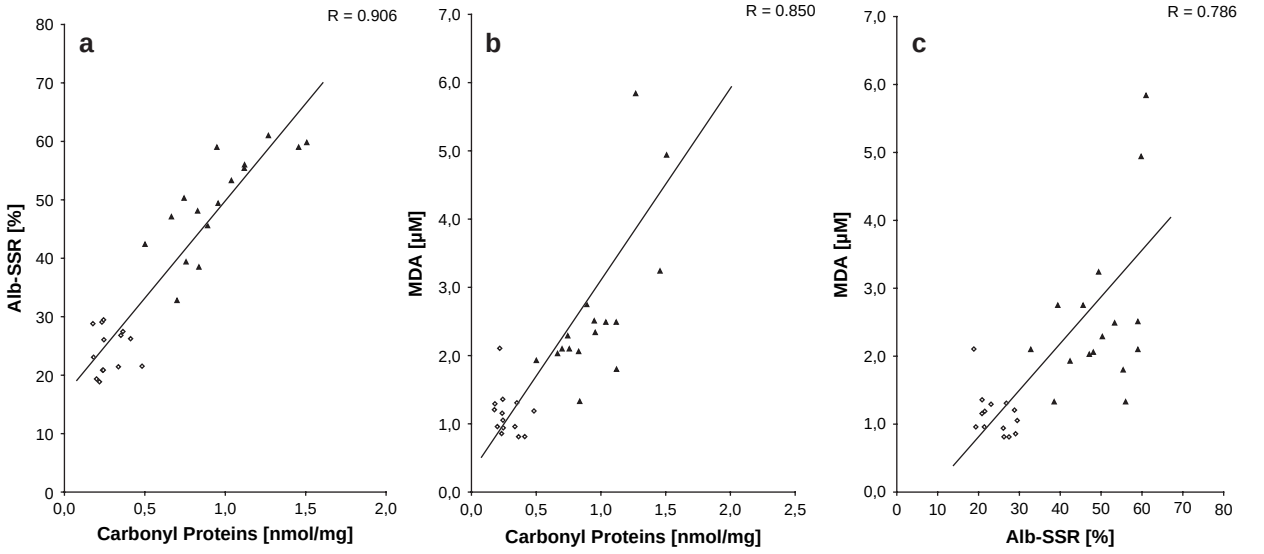


Figure 2. Correlation of oxidative stress parameters of serum from control (◇) and NDD group (▲). Albumin-SSR vs CP (A), MDA vs CP (B) and MDA vs Albumin-SSR (C) were analysed by linear regression.

proteins directly an increased peroxynitrite formation in neurodegenerative disease patients could explain the increased levels of MDA, CP and Alb-SSR [23].

Elevated plasma total homocysteine concentration is discussed as a risk factor for cognitive decline or AD [46]. Mild elevations of plasma total homocysteine are associated with increased AD independent of folate and vitamin B12 [47]. These findings differ from ours, where the content of plasma homocysteine in NDD was essentially equal to the age-matched control group. Our results are in good agreement with the Rotterdam and MacArthur studies showing homocysteine not as a risk factor for cognitive impairment of elderly subjects [48,49]. Finally, Ravaglia et al. [50] found an even negative association between homocysteine concentration and cognitive impairment. Formation of Alb-SSR may therefore not be attributed to an increased homocysteine.

High homocysteine plasma levels in MCI and AD have been associated with vitamin deficiency, especially folate and vitamin B12. We found no association of folate with cognitive function or vitamin B12. Furthermore, folate and vitamin B12 levels of patients with AD were within the normal range [51]. It is expected that folate is associated with vitamin B12 by the folate dependent methylation pathway. We found no difference in plasma levels between NDD patients and the control group for neither folate nor vitamin B12, which is in agreement with a recently published study [48].

We examined the immune status of the NDD and the control group by estimating neopterin. No difference in the amount of neopterin between NDD and the control group was found, suggesting that cellular immune defence is not activated during the pathogenesis of NDD.

It is well known that CP, Alb-SSR and MDA are sensible markers for age. Bivariate regression analysis showed no significant effect of age or gender on oxidative stress markers used in this study.

Summarizing all data from our study, we suggest that in patients with NDD oxidative stress parameters (namely MDA, carbonyl proteins and Albumin-SSR) although not specific are more useful markers for neurodegenerative disorders than plasma homocysteine, vitamin B12 or folate.

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