

Relation of Plasma Selenium and Lipid Peroxidation End Products in Patients With Alzheimer's Disease

Z. CHMATALOVA^{1,3}, M. VYHNALEK^{2,3}, J. LACZO^{2,3}, J. HORT^{2,3}, R. POSPISILOVA¹, M. PECHOVA¹, A. SKOUMALOVA¹

¹Department of Medical Chemistry and Clinical Biochemistry, Second Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czech Republic, ²Memory Clinic, Department of Neurology, Second Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czech Republic, ³International Clinical Research Center, St. Anne's University Hospital Brno, Brno, Czech Republic

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Summary

Increased oxidative stress in the brain during the course of Alzheimer's disease (AD) leads to an imbalance of antioxidants and formation of free radical reaction end-products which may be detected in blood as fluorescent lipofuscin-like pigments (LFPs). The aim of this study was to evaluate and compare LFPs with plasma selenium concentrations representing an integral part of the antioxidant system. Plasma samples from subjects with AD dementia (ADD; n=11), mild cognitive impairment (MCI; n=17) and controls (n=12), were collected. The concentration of selenium was measured using atomic absorption spectroscopy. LFPs were analyzed by fluorescence spectroscopy and quantified for different fluorescent maxima and then correlated with plasma selenium. Lower levels of selenium were detected in MCI and ADD patients than in controls (P=0.003 and P=0.049, respectively). Additionally, higher fluorescence intensities of LFPs were observed in MCI patients than in controls in four fluorescence maxima and higher fluorescence intensities were also observed in MCI patients than in ADD patients in three fluorescence maxima, respectively. A negative correlation between selenium concentrations and LFPs fluorescence was observed in the three fluorescence maxima. This is the first study focused on correlation of plasma selenium with specific lipofuscin-like products of oxidative stress in plasma of patients with Alzheimer's disease and mild cognitive impairment.

Key words

Alzheimer's disease • Mild cognitive impairment • Lipofuscin-like pigments • Oxidative stress • Selenium

Corresponding author

Z. Chmátalová, Department of Medical Chemistry and Clinical Biochemistry, Second Faculty of Medicine, Charles University and Motol University Hospital, Plzeňská 130/221, 150 06 Prague 5, Czech Republic. E-mail: zuzana.chmatalova@seznam.cz

Introduction

Oxidative stress is implicated in a pathophysiology of numerous neurodegenerative disorders including Alzheimer's disease (AD). AD is characterized by the accumulation of amyloid plaques, the formation of neurofibrillary tangles and the loss of synapses followed by atrophy (Braak and Braak 1991) that manifests clinically as a progressive cognitive decline. The dementia stage of AD (ADD) is preceded by mild cognitive impairment (MCI), a prodromal stage of AD, in which brain damage is not severe enough to compromise self-sufficiency. The MCI patients with objective memory impairment are labelled as amnesic MCI (aMCI), their rate of progression to ADD is high: 15 % of them convert to ADD every year (Petersen 2004).

Oxidative stress is considered to be one of important pathogenic factor in the development and progression of AD (Zhao and Zhao 2013). Nervous tissue is particularly vulnerable to the deleterious effects of free radicals for several reasons. The ATP used by neurons is

produced primarily by oxidative phosphorylation, during which free radicals are generated. Moreover, the membranes of neurons are rich in polyunsaturated fatty acids, and the concentration of metal ions is high, which enables lipoperoxidation. In addition, the concentration of antioxidants in the brain is low in comparison to other tissues (Gilkun-Sherky *et al.* 2001). Finally, neurons are post-mitotic cells, in which oxidative damage products accumulate during the life span (Coyle and Puttfarcken 1993).

Oxidative stress markers are increased during all stages of the disease. It was shown that AD patients have increased levels of 4-hydroxynonenal and malondialdehyde in blood, which are markers of free radical-induced lipid peroxidation, as well as increased levels of F2-isoprostane and F4-neuroprostane in the brain tissue (Markesberry *et al.* 2005, Praticó *et al.* 1999). The above-mentioned markers of lipoperoxidation are general markers. More advanced and specialized end products of lipid free radical damage are represented by lipofuscin-like pigments (LFPs) (Skoumalova and Hort 2012). These pigments exhibit native fluorescence due to their cyclic structures and can be detected using fluorescence spectroscopy (Wilhelm and Herget 1999). Increased levels of LFP in the blood were previously found in ADD and MCI patients in comparison to controls (Skoumalova *et al.* 2011, Chmátalová *et al.* 2016).

All aerobic organisms have a very complex and effective antioxidant system, which protects molecules against oxidative damage. Selenium plays a major role in antioxidant defense *via* the redox regulation of key enzymes (Naziroğlu and Yürekli 2013). Selenium is incorporated into proteins such as selenocysteine, which is a part of important antioxidant enzymes, particularly glutathione peroxidase and thioredoxin reductase. Moreover, selenium attenuates amyloid beta production and amyloid beta-induced neuronal death by reducing the activities of beta and gamma secretases (Gwon *et al.* 2010). Several studies of selenium plasma levels were provided in a recent years, but dissonant results of selenium assessment were obtained (Cardoso *et al.* 2014, Krishnan and Rani 2014).

In our previous study we found increased plasma levels of LFPs in patients with ADD and MCI (Chmátalová *et al.* 2016). The aim of this follow-up study was to compare plasma selenium levels in patients with aMCI, patients with ADD and to correlate it with fluorescence intensities of plasma LFPs. We wanted to

evaluate whether lipoperoxidation end product overproduction in plasma, which might be used as a possible marker of neurodegeneration in AD, is accompanied by selenium imbalance. Moreover, selenium measurement could be an additional referent analyte for higher robustness of LFPs measurement.

Materials and Methods

Subjects

All participants were recruited at the Memory Clinic at Motol University Hospital in Prague, Czech Republic, between 2012 and 2014. The group consisted of patients with mild ADD (n=11), patients with aMCI (n=17) and cognitively healthy older adults (controls, n=12). All subjects underwent a complex diagnostic procedure including brain MRI, neuropsychological examination by neuropsychological battery, neurological examination. The diagnosis was made upon agreement between neurologist and neuropsychologist. Patients with clinically defined stroke, other neurological diseases (i.e. Parkinson disease, etc.), subjects with a history of diabetes, impaired glucose tolerance, hyperlipidemia, depression and significant vascular impairment on brain MRI (>2 points on Fazekas scale) were not included (Fazekas *et al.* 1987).

The subjects with mild ADD met the National Institute on Aging – Alzheimer's Association criteria for probable AD without biomarker evidence (McKhan *et al.* 2011).

The subjects with aMCI met clinical criteria for aMCI, including memory complaints reported by a patient or a caregiver, evidence of memory dysfunction on neuropsychological testing, generally intact activities of daily living, and the absence of dementia (Petersen 2004). Memory impairment was established when the patient scored more than 1.5 standard deviations below the mean age- and education-adjusted norms on any memory test (Laczo *et al.* 2011).

The control group of cognitively healthy individuals was recruited from older adults attending the University of the Third Age at Charles University in Prague or from relatives of patients of the Memory Clinic at Motol University Hospital in Prague. Participants who met the DSM IV-TR criteria for dementia, Petersen's criteria for MCI (Petersen 2004) or who scored more than 1.5 SD below age- and education-adjusted norms on neuropsychological examination were not included. Characteristics of the groups are listed in Table 1.

Table 1. Participants' characteristics of the tested groups.

	Men	Age range	Average age \pm SD	MMSE score \pm SD
ADD (n=11)	7 (64 %)	68-85	74.27 \pm 5.14	19.40 \pm 3.17
aMCI (n=17)	13 (76 %)	56-88	71.88 \pm 7.73	25.14 \pm 3.25
Controls (n=12)	3 (25 %)	69-85	75.33 \pm 6.68	28.40 \pm 1.71

MMSE score – Mini Mental State Exam score.

Sample collection

Five milliliters of blood from all subjects were collected into tubes coated with K₃EDTA. Plasma was obtained from fasting morning blood samples. All samples were centrifuged at 4,000 g for 5 min immediately after collection. Separated plasma was removed into a new tube and stored at -80 °C. The tubes were labelled with codes to ensure donor anonymity. All participants signed an informed consent before sample collection. This study was approved by the Ethics Committee of Motol University Hospital.

Selenium assay

The selenium assay was performed in the Department of Medical Chemistry and Clinical Biochemistry of Motol University Hospital in Prague, Czech Republic. The selenium concentration in the plasma was determined using atomic absorption spectrometry (atomic absorption spectrometer VARIAN SpectrAA 220 FS). According to the guidelines of Ministry of Health of the Czech Republic, the physiological range for selenium in plasma is 59.2-146.8 µg/l (Statement of Ministry of Health of the Czech Republic).

LFP assay

LFP from the plasma was extracted into organic solvents using the method described by Goldstein and McDonagh, with modifications (Goldstein and McDonagh 1976). 2 ml of plasma were added into the 6 ml of mixture of organic solvents and LFPs were extracted for 1 h. The chloroform phase was collected after centrifugation for fluorescence analysis using an AMINCO Bowman series 2 spectrophotometer. Fluorescence analyses of LFP in plasma were based on the measurement of synchronous fluorescence spectra. Synchronous spectra were measured for emission in the range of 250-500 nm with the fixed difference for excitation 25 and 50 nm. The relative fluorescence intensity of different fluorescence

maxima characterized by specific excitation and emission wavelengths were analyzed. The fluorescence maxima at 355/380 nm (excitation/emission), 350/400 nm and 360/410 nm were identified. The fluorescence intensity at 260/480 nm excitation/emission was used as a reference for the calculation of relative amounts of LFPs. The LFP measurement procedure and its principles were described in detail in our previous work (Chmátalová *et al.* 2016).

Statistical analysis

All statistical analyses were performed using the statistical programme SigmaPlot. To assess a normality of distribution Shapiro-Wilk test was used. An analysis of variance (ANOVA) with *post hoc* Holm-Sidak method was performed to evaluate differences between groups. Statistical significance was set at 2-tailed alpha of 0.05. Pearson's correlation coefficients were used to estimate the associations between selenium concentrations and LFPs, cognitive measures and age. The results are reported as the mean values \pm standard deviations (SD).

Results

No statistically significant difference in the age of participants was observed between groups in this study.

Selenium concentration

The plasma selenium concentration was significantly higher in the control group than in the ADD (P=0.049) and aMCI (P=0.003) groups. Significant difference between selenium concentration in ADD and aMCI group was not observed. Mean selenium concentration in plasma was 76.07 \pm 18.45 µg/l in ADD group, 69.63 \pm 14.71 µg/l in aMCI and 90.72 \pm 17.56 µg/l in the control group. The comparison of selenium concentrations in patients and controls is shown in Figure 1 and Table 2.

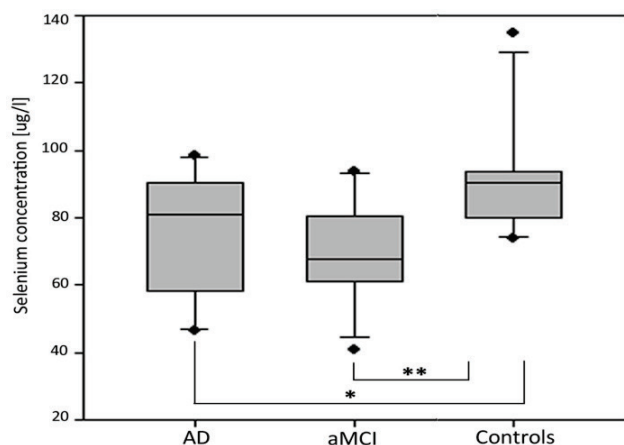


Fig. 1. Box plot representing comparison of selenium concentration in AD and aMCI patients and controls. Statistical significance: * $P<0.05$ and ** $P<0.01$.

Table 2. Selenium concentration (\pm SD) in the plasma in $\mu\text{g/l}$ in ADD patients, aMCI patients and controls.

	Se concentration \pm SD [$\mu\text{g/l}$]
ADD	76.07 ± 18.45
aMCI	69.63 ± 14.71
Controls	90.72 ± 17.56

With respect to the physiological range for plasma selenium, all of our tested groups were in the recommended interval. However, some trends were observed. In the patient groups there were subjects with plasma selenium below the lower limit. In the ADD group 3 out of 11 and in the aMCI group 4 out of 17 subjects had selenium lower than $59.2 \mu\text{g/l}$. Given that age might influence selenium concentrations, a correlation analysis between selenium concentrations and age was performed. However, no significant correlation was found. Likewise, no correlation of selenium levels with Mini Mental State Exam (MMSE) scores was observed.

LFP fluorescence intensity

The fluorescence intensity in chloroform extracts from the plasma of ADD and aMCI patients and controls, which reflects the extent of oxidative damage, was measured. The results showed that fluorescence intensity is significantly higher in the aMCI patients compared to the controls in four specific fluorescence maxima 355/380 nm, 350/400 nm, 360/410 nm and 415/440 nm

excitation/emission wavelength (355/380: $P<0.001$; 350/400: $P<0.001$; 360/410: $P<0.001$; 415/440: $P=0.003$). A significant difference in fluorescence intensity was also observed between ADD and aMCI patients in terms of higher levels of LFP in aMCI compared to ADD. The significant difference was observed in three specific fluorescence maxima 355/380 nm, 350/400 nm and 415/440 nm (355/380: $P=0.008$; 350/400: $P<0.001$; 415/440: $P=0.010$). The comparison of relative fluorescence intensities in specific fluorescence maxima between patients and control group is shown in Figure 2. Similar to our observations concerning selenium concentrations, no significant correlation between LFPs and MMSE and also no significant correlation between LFP levels and age of participants was observed.

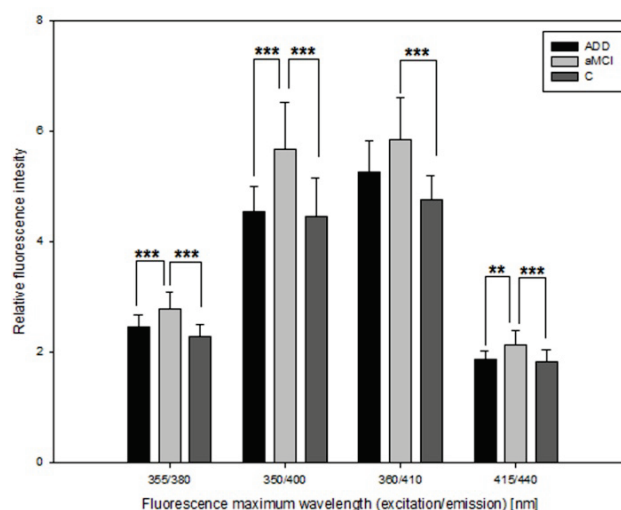


Fig. 2. The comparison of fluorescence intensities between the ADD and aMCI patient and control groups in four significant specific fluorescence maxima at 355/380 nm, 350/400 nm, 360/410 nm and 415/440 nm (excitation/emission). Statistical significance: ** $P<0.01$ and *** $P<0.001$.

Association between selenium and LFP

As selenium represents a part of the antioxidant defense system, its concentration was correlated with LFP levels, which are markers of oxidative stress. Pearson's correlation test showed that selenium concentrations correlated negatively with the amount of LFPs in plasma extracts at three fluorescence maxima: 355/380 nm ($r=-0.420$; $P=0.012$), 350/400 nm ($r=-0.390$; $P=0.015$) and 360/410 nm ($r=-0.398$; $P=0.016$). The correlation between selenium concentration and relative fluorescence intensity in specific fluorescent maxima is shown in Figure 3.

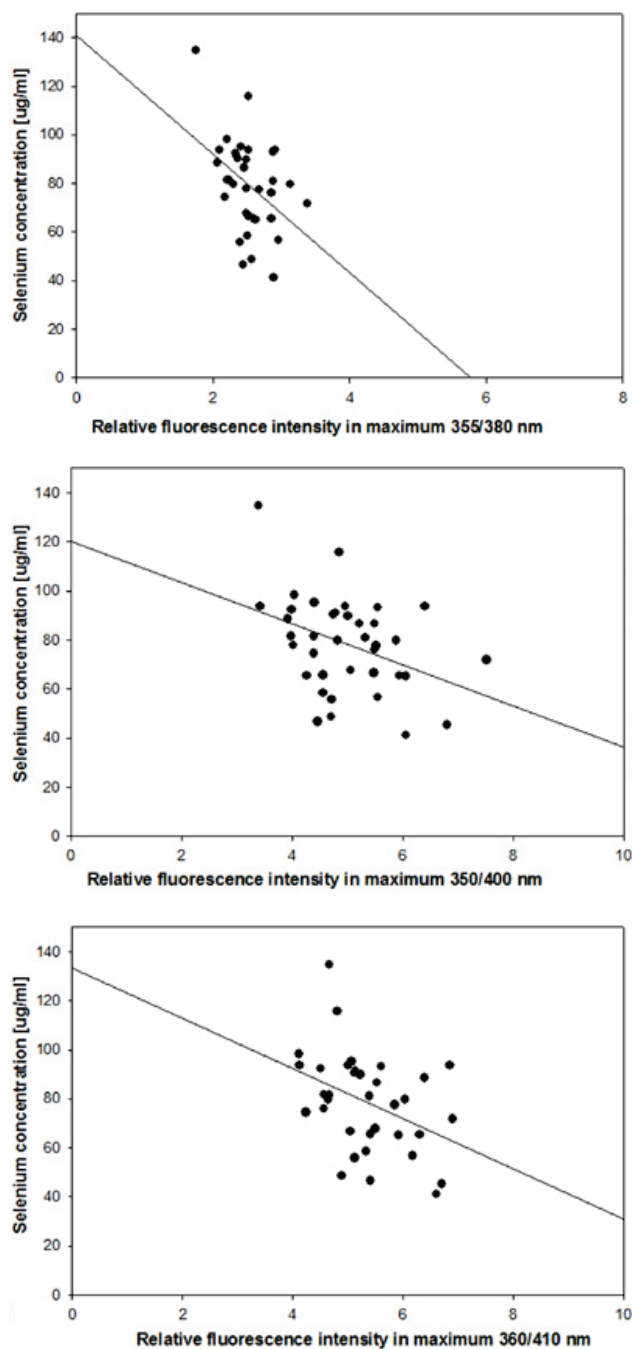


Fig. 3. Correlation graphs illustrating the correlation between selenium concentration and relative fluorescence intensity in three significant fluorescence maxima at 355/380 nm ($r=-0.420$), 350/400 nm ($r=-0.390$) and 360/410 nm ($r=-0.398$) (excitation/emission).

Discussion

Selenium plays an important role in the antioxidant defense and has been studied in many neurodegenerative diseases, including AD (Steinbrenner and Sies 2013). Some of these studies revealed lower selenium concentrations in plasma in AD patients

compared to controls (Cardoso *et al.* 2014, González-Domínguez *et al.* 2014), but couple of them did not proved significantly lower levels (Krishnan and Rani 2014, Koç *et al.* 2015).

In this study, we showed that although the mean selenium plasma concentrations in our tested groups were within the normal reported range (59.2-146.8 µg/l), the levels were significantly lower in patients with ADD and aMCI compared to controls. Moreover, many of patients in the ADD and aMCI groups had values below the normal range. Additionally, the recommended molar selenium concentration, which is required to achieve full glutathione peroxidase expression activity in the plasma, is in the range of 90.01-94.75 µg/l (Thomson *et al.* 1993, Duffield *et al.* 1999). Consequently, our results showed that only the control group had sufficient selenium concentrations to support the maximal effective activity of the antioxidant enzyme glutathione peroxidase in the plasma.

Many studies demonstrated that increased oxidative stress leads to an increased production of free radical end products that arise from native biomolecules. But these commonly known markers of oxidative stress are not specific for particular pathologies. In contrast LFPs may represent such products with probable higher potential specificity for different diseases accompanied by oxidative stress and protein aggregation (Grune *et al.* 2004, Brunk and Terman 2002). Significantly higher levels of specific LFPs characterized by specific fluorescence intensities were detected in this study in plasma of ADD and aMCI patients compared to controls. These findings are consistent with the hypothesis that oxidative stress measured using non-specific markers is elevated during the course of AD, which is even more pronounced during prodromal stages of AD (Nunomura *et al.* 2001, Ansari and Schew 2010). Further, these findings are consistent with results of our previous study showing higher levels of specific LFPs in erythrocytes and plasma among individuals with prodromal AD and ADD (Chmátalová *et al.* 2016).

The findings of the lower selenium levels and higher levels of specific LFPs in plasma among patients aMCI and ADD lead us to search for the associations between selenium levels and fluorescence intensities of LFPs. The correlation analysis revealed that there is a negative association between selenium concentrations and the amount of LFPs in plasma extracts. These results showed that the decreased selenium concentrations in patients with aMCI and ADD were related to higher

detected fluorescence intensities of LFPs in plasma, which represent more pronounced oxidative stress damage. Consequently, decreased selenium concentrations in patients with aMCI and ADD might be the cause of attenuated glutathione peroxidase expression activity, which might lead to a higher rate of oxidative damage and increased formation of LFPs.

Despite relatively sufficient mean selenium levels, which were within a normal range in all three groups, we found significant differences in selenium concentration in patients with ADD and aMCI compared to controls. Moreover, significantly higher levels of oxidative stress markers in plasma among aMCI and ADD patients were observed. Regarding selenium concentration in the plasma, aMCI patients had the lowest levels, ADD patients were in the middle and the control group was found to have the highest selenium concentrations. The opposite findings were observed in terms of fluorescence intensities of LFPs, in aMCI most pronounced, in ADD in the middle and in controls less pronounced. These findings might be explained by increased demand for antioxidants in the course of AD development, in which oxidative stress is increased mainly in the early stages of the disease, including aMCI. Under conditions of increased oxidative stress, an increase in free radical defense is required. Biosynthesis of several antioxidants, such as glutathione peroxidase, thioredoxin reductase, selenoprotein P and methionine sulfoxide reductase B, is dependent on the availability of selenium (Schweizer *et al.* 2004). That might explain the fact that patients with aMCI and ADD had significantly lower selenium in plasma compared to controls even though selenium food intake was sufficient in all tested groups.

The brain represents a high-priority organ that is supplied with selenium from other tissues if needed and that is the reason why the brain is the last organ to experience selenium deficiency (Cardoso *et al.* 2010). Consequently selenium concentration in plasma is rather short-term marker and selenium in blood and other organs might serve as a selenium pool for the brain in case of need in conditions of accelerated oxidative stress. This phenomenon might further explain our findings. The lowest effective concentration of selenium in plasma of aMCI patients correspond to the highest fluorescence intensities of LFP in plasma, which represent the marker of oxidative stress rate. In case of aMCI and ADD patients plasma selenium might be readily consumed for selenoprotein and other antioxidants

synthesis not only in plasma, but preferentially in the brain to protect polyunsaturated fatty acids in the membranes of neurons. This might explain lower effective concentrations of selenium in plasma in patients and higher portion of free radical-damaged biomolecules in plasma. This hypothesis is in agreement with our previous work, in which we detected higher rate of lipoperoxidation of polyunsaturated fatty acids in membranes of erythrocytes in ADD and aMCI patients (Chmátalová *et al.* 2016). The lowest selenium concentrations in plasma of aMCI patients might be also explained by the fact that selenium attenuates the production of amyloid β 42 by reducing the activities of β and γ secretase in neuronal cell lines, which is more pronounced in the early stages including aMCI (Gwon *et al.* 2010).

Based on our results it might be hypothesized that a selenium diet supply as a therapeutic coadjutant approach might slow down the course of the disease or prevent the outcome. On the other hand, interventional studies with selenium supplementation were done with ambiguous results (Loef *et al.* 2011). This might be due to the fact that AD is multifactorial disease with long preclinical period and oxidative stress plays role in all of these stages.

Finally, no correlation between MMSE score and selenium or LFPs fluorescence intensities was found. Thus LFPs levels and selenium plasmatic concentrations seem to be independent markers of AD pathology regardless the stage of the disease.

In conclusion, our results revealed significantly lower plasma selenium concentrations in aMCI and ADD patients than in controls, which may be the consequence of the increased consumption of glutathione peroxidase and other selenium-dependent antioxidants in a course of AD development. These results were completed with measurement of specific end-products of oxidative damage, which proved significantly higher levels in aMCI and ADD patients than in controls.

Conflict of Interest

There is no conflict of interest.

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