

## ORIGINAL ARTICLE

# Elevated plasma levels of advanced oxidation protein products in Slovak multiple sclerosis patients: possible association with different disability states

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## Abstract

**OBJECTIVES:** Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by neuroinflammation and neurodegeneration. These pathological conditions are coupled with phenomenon named oxidative stress. Excessive production of reactive oxygen and nitrogen species results inter alia in enhanced formation of advanced oxidation protein products (AOPP) detectable in blood plasma. Aim of our study was to analyse AOPP plasma levels in experimental groups and find possible association with multiple sclerosis and different disease forms.

**METHODS:** Our study included 50 control subjects and 61 MS patients, all of them with Central European origin. Patients were additionally divided into subgroups according to the selected criteria, which were disease form and Expanded Disability Status Scale (EDSS). Plasma levels of advanced oxidation protein products were measured by using a biochemical method based on their altered spectroscopic characteristics.

**RESULTS:** In our study we found significantly higher values of AOPP in MS patients compared to control group ( $p < 0.001$ ), both in women and men. The study of the association with the other criteria (disease form and disability status) did not show statistically significant results.

**CONCLUSION:** AOPP can be the candidate marker for the inclusion in the panel of biomarkers characteristic for MS, but its use for the discrimination of MS forms has the limitations. Further study will be needed to verify our results.

## INTRODUCTION

Multiple sclerosis (MS) as a complex disease is characterized by several pathophysiological mechanisms occurring in the central nervous system (CNS), such as autoimmune inflammatory reactions, axon demyelination, neuronal degenerative damage balanced by the partial recovery, gliosis, blood-brain barrier disruption, excitotoxicity and oxidative stress (Bielekova & Martin 2004; Čierny *et al* 2016). Destruction of white and grey matter consequently leads to a variable clinical course with the impairment of motor, autonomic, visual and cognitive abilities (Siffrin *et al* 2010). Multiple sclerosis is the most frequent neurological disease among young adults, affecting women with higher prevalence than men. The four main types of MS, relapsing-remitting (RR), secondary-progressive (SP), primary-progressive (PP) and progressive-relapsing (PR) are generally recognized (Polman *et al* 2005). Degree and extent of neurological impairment is rated by EDSS (Expanded Disability Status Scale) panel (Kurtzke 1983). According to a new concept, MS tends to be considered in general as a biphasic disease – first phase is active and inflammatory with the relapses and remissions. Second phase is progressive and degenerative, with massive demyelination and neuronal destruction. The principles of disease onset and development are still not fully understood. Nevertheless, it is known that the essential role in driving of the MS pathology play the immune system and improper autoimmune reactions leading to the myelin sheaths destruction (Gajdošechová *et al* 2017). Both the innate and acquired immunity are involved. Two hypotheses can clarify the MS onset. Outside-in hypothesis takes account of autoreactive T cells penetration through disrupted blood-brain barrier into the CNS from peripheral tissues. On the other hand, inside-out hypothesis suggests that MS can be caused by the infection with primary origin within CNS (Mahad *et al* 2015).

Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), continuously produced by oxidative metabolism, are finely balanced by antioxidant defense. CNS is notably susceptible to oxidative damage because of intensive mitochondrial metabolism, low level of antioxidant enzymes in oligodendrocytes and high content of unsaturated fatty acids, particularly in myelin sheaths. Oxidation of myelin lipids, subsequent demyelination and axonal loss typical for MS result from excessive production of proinflammatory cytokines and

ROS by activated immune cells (Glass *et al* 2010; Li *et al* 2013; Ortiz *et al* 2016). Neuroinflammatory processes are coupled with changes in mitochondrial activity. There are observed disruptions of electron transport chain with electron leakage, formation of ROS leading to virtual hypoxia and cell apoptosis due to cytochrome C release (Lin & Beal 2006; Reynolds *et al* 2007).

Amino acids, peptides and plasma proteins are also being influenced by oxidation. Excessive protein oxidation results in adverse effects, such as loss of enzymatic activity, aggregation, fragmentation or cross-linking. Advanced oxidation protein products (AOPP) are dityrosine-containing and cross-linking protein products primarily formed by the reaction with chlorinated oxidants (Witko-Sarsat *et al* 1996). These are dominantly released by myeloperoxidase and participate in neutrophil and macrophage defense mechanism. AOPP are intensively studied as a novel marker of oxidative damage (Xie *et al* 2014). Their elevation in numerous pathological conditions was proved in hemodialysis patients, patients with chronic kidney disease (Witko-Sarsat *et al* 1998), diabetes mellitus (Kalousová *et al* 2002; Piwowar *et al* 2009; Reynolds *et al* 2007), pneumonia (Muravlyova *et al* 2014) and chronic hepatitis C (Ozenirler *et al* 2011). Only a few data about AOPP and MS have been reported (Ljubisavljevic *et al* 2013; Kallaur *et al* 2017; Karlík *et al* 2015; Pasquali *et al* 2015a,b; Sadowska-Bartosz *et al* 2013). We hypothesized that an elevation of oxidative stress in MS can be reflected to the higher levels of AOPP in plasma and may be associated with multiple sclerosis onset and disability states.

## MATERIAL AND METHODS

### *Subjects (clinical and demographic data)*

Participants of our study were 50 healthy control individuals and 61 patients with confirmed MS diagnosis (Table 1). The study was approved by the Ethical Committee of the Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin. All patients and healthy subjects provided a written informed consent before the inclusion the study. MS diagnosis was determined according to the McDonald's criteria in the Center for Demyelinating Diseases, Department of Neurology, Jessenius Faculty of Medicine in Martin and Martin University Hospital, Slovak Republic, so as current EDSS score. For the purposes of our study, we stratified the MS patients to relapsing-remitting (RR-MS) and secondary progressive (SP-MS) groups. Cor-

**Tab. 1.** Study group characteristics.

Factor	Controls (CTL, n = 50)		Patients (MS, n = 61)	
Sex	34 Women (68%)	16 Men (32%)	49 Women (80.33%)	12 Men (19.67%)
Mean age (years)	44.53±12.77	36.19±10.94	45.29±10.74	40.33±9.93
	41.86±12.73		44.31±10.69	

**Tab. 2.** Characteristic of MS group.

Factor	Patients (n = 61)		RR-MS (n = 52)		SP-MS (n = 9)	
Sex	49 Women	12 Men	44 Women (84.62%)	8 Men (15.38%)	5 Women (55.56%)	4 Men (44.44%)
Mean age (years)	44.31±10.69		43.06±10.20		51.56±11.20	
EDSS (points)	3.58±1.65		3.09±1.13		6.44±1.18	

**Tab. 3.** Characteristic of RR-MS subgroup.

Factor	RR-MS (n = 52)			
	EDSS<3 (n = 22)		EDSS≥3 (n = 30)	
Sex	18 Women (81.82%)		4 Men (18.18%)	
Mean age	37.41±8.47		47.20±9.43	
EDSS (points)	1.95±0.46		3.92±0.64	

**Tab. 4.** Plasma AOPP levels ( $\mu\text{mol.l}^{-1}$  of chloramine-T equivalents).

	Control group		MS group	
		Total	RR-MS	SP-MS
AOPP	113.74±5.33 <sup>a</sup>	149.19±7.22 <sup>a</sup>	146.13±7.59 <sup>d</sup>	166.83±21.96 <sup>d</sup>
			EDSS<3:	NA
			EDSS≥3:	NA
Women	110.16±6.47 <sup>b</sup>	144.91±6.98 <sup>b</sup>		
Men	121.35±9.39 <sup>c</sup>	166.67±23.27 <sup>c</sup>		

Values are presented as means  $\pm$  SEM of control group and MS patients. The clinical data were available for all MS patients, measuring EDSS in each patient. Student-Neuman-Keuls test was done.

<sup>a</sup>  $p < 0.001$  MS group vs control group

<sup>b</sup>  $p < 0.01$  MS women group vs control women group

<sup>c</sup>  $p < 0.05$  MS men group vs control men group

<sup>d</sup>  $p > 0.05$  RR-MS vs SP-MS

<sup>e</sup>  $p > 0.05$  RR-MS (EDSS<3) vs RR-MS (EDSS≥3)

respond to estimated EDSS score, relapsing-remitting MS group was additionally divided to subgroups with median EDSS value 3. All characteristics are shown in Table 2 and Table 3.

#### Analytical methods

**Blood collection and processing.** Whole blood drawn by venous puncture was collected into appropriate test tubes, than were centrifuged (7000 rpm, 4°C, 5 minutes) and plasma fraction was taken. All plasma samples were stored at  $-80^{\circ}\text{C}$  until examination.

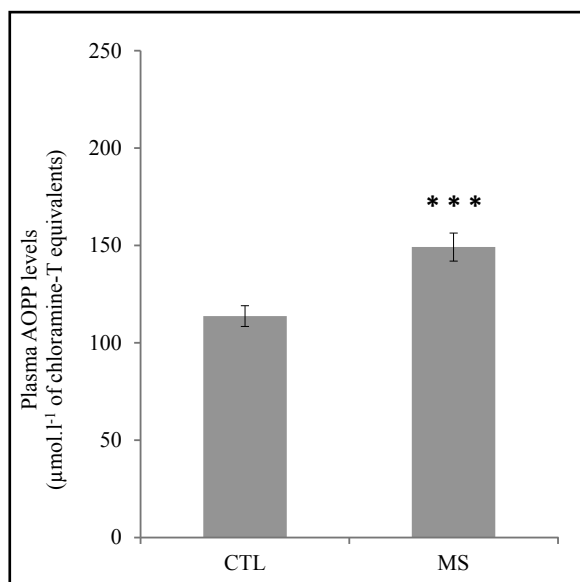
**Principle of AOPP determination.** AOPP show altered spectroscopic characteristics. Their concentrations in plasma samples were assessed with use of OxiSelect™ AOPP Assay Kit (Cell Biolabs, INC., San Diego, USA) according to product manual. Procedure is based on spectrophotometric method (Witko-Sarsat *et al* 1996). AOPP concentrations were expressed as  $\mu\text{mol.l}^{-1}$  of chloramine-T equivalents.

#### Statistical analysis

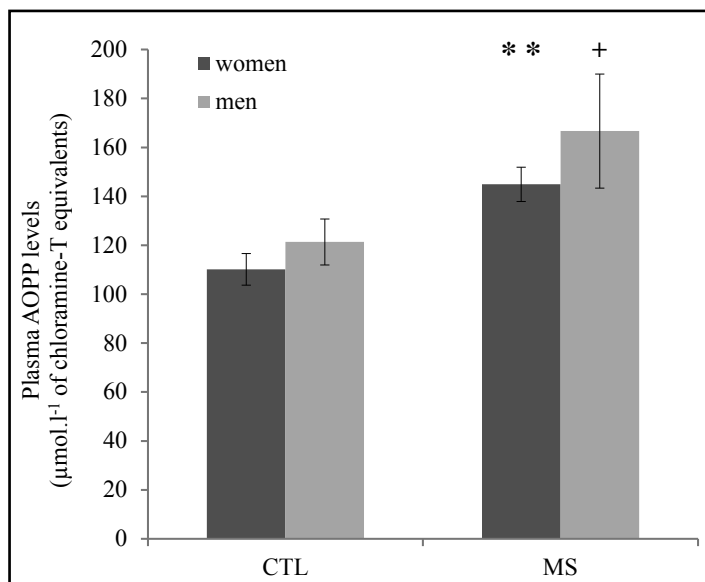
All acquired data were expressed as mean  $\pm$  standard error of the mean (SEM) or their percentage value. The results were statistically evaluated using Graph-Pad INSTAT V3.01 and Student-Neuman-Keuls test was first carried out to test for differences between all groups. Value of  $p < 0.05$  was considered to be statistically significant. Individual plots were created in Microsoft Excel 2013. Error bars reflect the standard error of the mean.

#### RESULTS

Demographic data are listed in Table 1, clinical data in Table 2 and Table 3. Our results are presented in Table 4. AOPP plasma values in MS patients were significantly higher than those in the control subjects ( $p < 0.001$ ) (Figure 1). These results analysed separately for each gender showed significantly elevated AOPP plasma levels in both women ( $p < 0.01$ ) and men ( $p < 0.05$ ) (Figure 2). When we used the other different criteria of stratification for MS patients (RR-MS in comparison to SP-MS, EDSS value less than 3 and equal or higher than 3 in RR-MS group), we did not detect any significant changes between individual groups ( $p > 0.05$ ). Nevertheless the AOPP plasma values tended



**Fig. 1.** Plasma AOPP values in control group and MS group ( $p < 0.001$ ).



**Fig. 2.** Plasma AOPP levels – gender differences between control group and MS group. Significantly different when compared women  $**p < 0.01$  and men  $+p < 0.05$ .

to be increased in SP-MS in comparison with RR-MS and in RR-MS group with EDSS equal or higher than 3 compared to RR-MS group with lower EDSS. Relation between AOPP plasma values and age did not reveal a significant correlation (data not shown).

## DISCUSSION

Oxidative stress is being considered as an important contribution factor of multiple sclerosis pathology going hand in hand with the autoimmune reactions acting through cellular factors and breaking down the immunological tolerance. Pivotal role in neuroinflammation and neurodegeneration play microglial cells. In response to certain molecular inflammatory factors, microglia become activated and express proinflammatory cytokines and neurotoxic molecules. Activated microglia also cause disruption of blood-brain barrier by changing its selectivity and permeability. Infiltration of autoreactive CD4+ T cells into CNS can thus promote additional imbalance of CNS homeostasis and neuroinflammation (González *et al* 2014). Both plasma and memory B cells participate in MS pathology after penetration the CNS, but B cells also promote inflammatory processes at peripheral level. By-product of all immune reactions are reactive oxygen and nitrogen species with potentially harmful impact (Fraussen *et al* 2014; Macchi *et al* 2015; Reindl *et al* 2010). Advanced oxidation protein products are referred as a stable, although nonspecific marker of oxidative damage, arising by reaction of the protein with reactive oxygen species, including superoxide anion, hydrogen peroxide, hydroxyl radical or hypochlorous acid (Witko-Sarsat *et al* 1996). On the basis of occurrence of oxidative stress in MS, the aim of our study

was to find out the association between plasma AOPP levels and multiple sclerosis in Slovak MS patients. We measured the AOPP values in healthy control subjects and MS patients and find out their association with the various disease forms and disability degree.

Our findings are in accordance with previous studies, where the elevated plasma levels of AOPP of MS patients have been also found. In the study (Pasquali *et al* 2015b), examination of 60 MS patients and 81 healthy individuals showed significantly higher levels in MS patients in general, but there was found no significant correlation between AOPP plasma levels and disease disability. Patients were divided to active and not active groups consistent with described criteria (Lublin *et al* 2014) defined by relapse occurrence and MRI lesions activity. According to this, active MS patients had higher AOPP plasma levels than not active, but these results were not statistically relevant. On the contrary, another study (Kalousová *et al* 2002) indicates considerable association between disease activity and AOPP plasma levels. Examination of 50 patients with clinical-isolated syndrome (CIS-MS), which is typical by acute or subacute disease attack indicating a possibility of conversion to the definite MS, revealed higher plasma levels of AOPP than in a group of 57 RR-MS patients ( $p < 0.05$ ). Similar results were observed in MS patients with higher EDSS score ( $p < 0.05$ ). In line with our results, this study showed increased values of plasma AOPP in MS patients compared to healthy control subjects. These data confirm the role of oxidative stress in MS and suggest the additional usability of plasma AOPP in definition of disease state.

The results of a large study (Kallaur *et al* 2017) with inclusion of 212 MS patients and 249 healthy control

subjects showed that plasma AOPP levels were significantly lower in patients with lower EDSS in comparison to MS patients with higher EDSS. Study reveals that neither the applied therapy nor the type of treatment (no drugs, IFN- $\beta$  or glatirameracetate) are significantly related to plasma AOPP values. Increased AOPP levels indicate the presence of chronic oxidative stress in MS due to continual progressive changes. On the other side, research (Sadowska-Bartosz *et al* 2013) indicates higher plasma levels of AOPP in patients with RR-MS without treatment than in patients with treatment. Patients with RR-MS without treatment and in clinical relapse had significantly higher plasma AOPP levels than patients with clinically stable MS and control group. Limitation of that study could be the fact, that it included smaller groups of 14 RR-MS patients without treatment, 18 patients treated with IFN- $\beta$ 1a, 19 with IFN- $\beta$ 1b and 9 with glatiramer acetate.

In contrast to our results, another study did not reveal any statistically significant differences between AOPP plasma values in MS patients with various forms of multiple sclerosis (Pasquali *et al* 2015a). This study involved 51 patients (41 with CIS-MS or RR-MS, 10 with SP-MS) and suggested no association of disease type with plasma AOPP values. Research group (Karlík *et al* 2015) from Central European region focused on possible usefulness of saliva for diagnostic process of MS and the plasma and salivary AOPP values were compared. Interestingly, the examination of 29 patients and 29 healthy controls showed significantly higher AOPP levels in plasma ( $p < 0.01$ ) of MS patients, but not in saliva. Similar salivary concentrations of AOPP in MS patients and controls can be explained by tedious release of these high molecules in saliva (Karlík *et al* 2015).

In our study, a significantly higher levels of plasma AOPP were found in MS patients, both in men and women. In accordance with previously mentioned results of the other studies (Kallaur *et al* 2017; Karlík *et al* 2015; Ljubisavljevic *et al* 2013; Pasquali *et al* 2015b; Sadowska-Bartosz *et al* 2013), we confirmed the association of AOPP plasma levels as a biomarker with multiple sclerosis. Correlation of plasma AOPP values and disease disability progression also seems to be applicable. Our results showed the tendency of positive correlation of EDSS score with plasma AOPP values, but without statistical significance.

In summary, the level of advanced oxidation protein products is easily detectable plasma biomarker of oxidative stress. Increased levels of plasma AOPP found in MS patients demonstrate the oxidative changes of plasma proteins and reflect whole-body oxidative stress due to the autoimmune proinflammatory conditions. Plasma AOPP can be used as additional diagnostic and prognostic marker for MS, but the association with disability status is limited. Further studies are needed to confirm these findings, especially for estimation of disease disability status.

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