

OXIDATIVE STRESS BIOMARKERS IN PATIENTS WITH MULTIPLE SCLEROSIS: SEX-RELATED DIFFERENCES AND THE IMPACT OF SMOKING STATUS

Marija Vasić¹, Evica Dinčić², Aleksandra Arsić³

¹Military Medical Academy, Institute of Medical Biochemistry, Belgrade, Serbia

²Military Medical Academy, Neurology Clinic, Belgrade, Serbia

³Military Hospital Novi Sad, Novi Sad, Serbia

Contact: Marija Vasic

17 Crnotravska St., 11040 Belgrade, Serbia

E-mail: marija.vma28@gmail.com

Oxidative stress (OS) plays an important role in the pathogenesis of multiple sclerosis (MS). Sex-related biological differences and exposure to tobacco smoke may influence redox balance. However, their combined effects on oxidative stress biomarkers in MS remain insufficiently investigated. To examine sex-specific differences in OS biomarkers in patients with MS and to assess the impact of smoking status on redox homeostasis. This study included 175 patients with MS and 254 healthy controls. Serum total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI) were determined, while urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) normalized to creatinine was measured as a marker of oxidative DNA damage. Participants were stratified by sex and smoking status. Data were analyzed using

two- and three-factor analysis of variance (ANOVA) with Sidak post hoc testing. Compared with controls, MS patients exhibited significantly higher TOS, OSI, and urinary 8-oxodG/creatinine levels, along with reduced TAS. Female MS patients exhibited lower TAS and higher TOS, OSI, and urinary 8-oxodG/creatinine levels than male MS patients. Smoking status was associated with oxidative DNA damage with passive and former smokers exhibiting markedly elevated 8-oxodG/creatinine levels, with higher levels observed among MS patients. Oxidative DNA damage was more pronounced among female MS patients and individuals exposed to tobacco smoke. Multiple sclerosis is associated with increased systemic OS and impaired antioxidant defense, more pronounced in women and exacerbated by exposure to tobacco smoke. These findings highlight the importance of sex-specific approaches and smoking prevention strategies in MS management.

Keywords: multiple sclerosis; oxidative stress; sex differences; smoking; 8-oxodG/creatinine; antioxidant status

BIOMARKERI OKSIDATIVNOG STRESA KOD PACIJENATA SA MULTIPLOM SKLEROZOM: RAZLIKE POVEZANE SA POLOM I UTICAJ STATUSA PUŠENJA

Marija Vasić¹, Evica Dinčić², Aleksandra Arsić³

¹Vojnomedicinska akademija, Institut za biohemiju, Beograd, Srbija

²Vojnomedicinska akademija, Klinika za neurologiju, Beograd, Srbija

³Vojna bolnica Novi Sad, Novi Sad, Srbija

Kontakt: Marija Vasic

Crnotravska 17, 11040 Beograd, Srbija

E-mail: marija.vma28@gmail.com

Oksidativni stres (OS) igra važnu ulogu u patogenezi multiple skleroze (MS). Biološke razlike povezane sa polom i izloženost duvanskom dimu mogu uticati na redoks ravnotežu, ali njihovi kombinovani efekti na biomarkere oksidativnog stresa kod MS nisu dovoljno istraženi. **Cilj:** Ispitati razlike specifične za pol u biomarkerima oksidativnog stresa kod pacijenata sa MS i proceniti uticaj statusa pušenja na redoks homeostazu. Studija je obuhvatila 175 pacijenata sa MS i 254 zdrave kontrolne osobe. Određeni su ukupni oksidativni status (TOS), ukupni antioksidativni status (TAS) i indeks oksidativnog stresa (OSI) u serumu, dok je 8-okso-7,8-dihidro-2'-deoksiguanozin (8-oksodG) u urinu, normalizovan na kreatinin meren kao marker oksidativnog oštećenja DNK. Učesnici su podeljeni po polu i statusu pušenja. Podaci su

analizirani primenom dvo- i trofaktorske analize varijanse (ANOVA). U poređenju sa kontrolnom grupom, pacijenti sa MS su imali značajno više nivoe TOS, OSI i urinarnog 8-oksodG/kreatinina, uz snižene vrednosti TAS. Kod žena sa MS uočeni su izraženiji poremećaji redoks statusa u odnosu na muškarce. Pušački status je bio povezan sa izraženijim oksidativnim oštećenjem DNK, pri čemu su pasivni i bivši pušači pokazivali izrazito povišene nivoe 8-oksodG/kreatinina, posebno među pacijentima sa MS. Multipla skleroza je povezana sa povećanim sistemskim OS i oslabljenom antioksidativnom odbranom, izraženije kod žena i pogoršano izloženošću duvanskom dimu.

Ključne reči: multipla skleroza; oksidativni stres; polne razlike; pušenje; 8-oksodG/kreatinin; antioksidativni status

INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system, characterized by immune-mediated demyelination, axonal damage, and progressive neurological dysfunction. Although the etiology of MS is multifactorial and not yet fully understood, accumulating evidence indicates that oxidative stress (OS) plays a pivotal role in disease initiation and progression by amplifying inflammatory responses and contributing to neurodegeneration (1-3).

Oxidative stress results from an imbalance between the production of reactive oxygen and nitrogen species and the capacity of antioxidant defense systems to neutralize them. In MS, activated immune cells, mitochondrial dysfunction, and sustained inflammation promote excessive generation of reactive species, leading to lipid peroxidation, protein oxidation, and oxidative damage to nucleic acids (4,5).

Consequently, biomarkers reflecting systemic redox status have gained increasing attention as indicators of disease-related oxidative damage.

Among these biomarkers, total oxidant status (TOS), total antioxidant status (TAS), and the oxidative stress index (OSI) provide an integrated assessment of the pro-oxidant/antioxidant balance, while 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is widely recognized as a sensitive marker of oxidative DNA damage. Elevated levels of these biomarkers have been reported in patients with MS, supporting their relevance in the evaluation of systemic OS associated with the disease (6-8).

Sex-related differences represent an important yet insufficiently explored aspect of MS pathophysiology. The disease occurs more frequently in women, who also exhibit differences in immune responses, clinical course, and disease progression compared with men (9,10). Biological factors such as sex hormones, genetic factors, and variations in antioxidant capacity may contribute to sex-specific regulation of redox homeostasis (11,12). Although several

studies suggest that women may be more susceptible to oxidative damage under pathological conditions, findings regarding sex-specific OS profiles in MS remain inconsistent (13,14).

Smoking exposure is recognized as a significant modulator of MS risk and disease activity. Active smoking is an established risk factor for MS development and progression, while increasing evidence indicates that passive smoking may also contribute substantially to disease susceptibility (15-17). Tobacco smoke contains numerous pro-oxidant compounds capable of inducing OS and DNA damage, potentially exacerbating redox imbalance in individuals with MS (18,19). However, the combined influence of smoking status and biological sex on OS biomarkers in MS has not been sufficiently clarified.

Given the complex interplay between biological sex, smoking-related exposure, and OS, a comprehensive evaluation of redox biomarkers stratified by sex and smoking status may provide valuable insight into MS pathogenesis. Therefore, the aim of this study was to assess the impact of biological sex and smoking status on systemic OS biomarkers in patients with MS, with particular emphasis on oxidative DNA damage.

MATERIALS AND METHODS

Study Design and Participants

This cross-sectional study included a total of 429 participants, comprising 175 patients diagnosed with MS and 254 healthy control subjects. Patients were recruited from the Clinic of Neurology and were diagnosed with MS according to the revised McDonald criteria. Exclusion criteria included the presence of other neurological, inflammatory, or autoimmune disorders, as well as acute systemic illnesses.

Demographic and clinical data were collected for all participants, such as age, sex, body mass index (BMI), disease duration (for MS patients), and smoking status. All participants provided written informed consent prior to inclusion in the study. The study was conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Committee of the Military Medical Academy (MMA) (approval No.4494-1 from 01.04.2016).

Smoking status was assessed using a structured questionnaire. Participants were categorized as non-smokers, former smokers, passive smokers, or active smokers. Smoking status was analyzed as a categorical variable and further stratified according to sex and disease presence.

Sample Collection and Laboratory Procedures

Venous blood samples were collected from all participants after overnight fasting. Serum was separated by centrifugation and stored at -80°C until biochemical analysis. Spot urine samples were obtained from both MS patients and control subjects. Serum TAS and TOS were measured using automated colorimetric methods based on commercially available assay kits (20). TAS values were expressed as mmol Trolox equivalents per liter, while TOS values were expressed as μ mol hydrogen peroxide equivalents per liter. The oxidative stress index (OSI) was calculated as the ratio of TOS to TAS and expressed in arbitrary units. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, was determined using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) and a previously validated method described by Topic et al. (21). Separation was performed on a Thermo Scientific Hypersil GOLD aQ column (3 μ m, 100 x 4.6 mm ID) and acetonitrile as the mobile phase. The mobile phase flow rate was 500 μ L/min, and the column temperature was maintained at 25 °C. Detection was performed using a Thermo TSQ Quantum Access Max triple quadrupole mass spectrometer equipped with a heated electrospray ionization source operating in positive ion mode. Quantification was based on multiple reaction

monitoring of precursor ion m/z 284 and product ion m/z 167. Urinary creatinine concentration was measured using the kinetic Jaffe method, and 8-oxodG levels were normalized to creatinine concentration and expressed as nmol/mmol creatinine.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics for Windows, Version 20.0. Data normality was assessed using the Shapiro-Wilk test. Homogeneity of variances was assessed using Levene's test. Normally distributed, continuous variables are presented as mean $\pm$ standard deviation. When significant main effects were observed, Sidak post hoc testing was performed for multiple comparisons. A p value < 0.05 was considered statistically significant. Binary logistic regression analysis (Enter method) was performed to identify independent factors independently associated with MS status, including smoking status, OSI, and urinary 8-oxodG/creatinine levels. Regression coefficients (β), odds ratio (ORs) with 95% confidence intervals (CIs), and model fit statistics were calculated. For comparisons between two groups, Student's t-test was used for normally distributed continuous variables. Two-factor and three-factor analysis of variance (ANOVA) with Sidak post hoc testing were applied to evaluate the combined effects and interactions of sex, smoking status, and disease presence on oxidative stress biomarkers. Effect sizes were expressed as partial eta squared (η_p^2).

RESULTS

Baseline characteristics of study participants

Demographic and clinical characteristics of patients with MS and healthy control subjects are presented in Table 1. A significant difference in age was observed between MS patients and controls, whereas body mass index did not differ significantly between the groups. Sex

distribution differed significantly between the groups. The distribution of smoking status differed between the groups, with a higher proportion of former and passive smokers observed among MS patients.

Table 1. Demographic and clinical characteristics of study participants

Variable	Controls (n=254)	MS patients (n=175)	p value
Age (years)	36.90 ± 8.78	33.43 ± 9.10	< 0.001
Female, n (%)	173 (68.1)	99 (56.6)	0.015
BMI (kg/m ²)	24.4 ± 3.6	24.7 ± 3.9	ns
Disease duration (years)	-	7.56 ± 5.60	-
Smoking status, n(%)	< 0.001		
Non-smokers, n (%)	140 (55.1)	77 (44.0)	
Former smokers, n (%)	24 (9.4)	4 (2.2)	
Passive smokers, n (%)	6 (2.4)	40 (21.8)	
Active smokers, n (%)	84 (33.1)	56 (32.0)	

Data are presented as mean ± standard deviation (SD) or number (%); BMI, body mass index; MS, multiple sclerosis; ns, not statistically significant.

Oxidative stress biomarkers in MS patients and controls

Comparisons of OS biomarkers between MS patients and healthy controls are summarized in Table 2. MS patients exhibited significantly higher serum TOS and OSI, along with significantly lower TAS, compared with controls. In addition, urinary 8-oxodG/creatinine levels were markedly elevated in MS patients, indicating increased oxidative DNA damage.

Table 2. Oxidative stress biomarkers in MS patients and controls

Oxidative stress biomarker	Controls (n=254)	MS patients (n=175)	p value
TAS (mmol Trolox Equiv/L)	2.02 ± 0.21	2.00 ± 0.20	0.018
TOS(μmol H ₂ O ₂ Equiv/L)	8.67 ± 1.85	9.02 ± 1.76	0.023
OSI (arbitrary units)	0.43 ± 0.10	0.45 ± 0.10	0.023
8-oxodG/creatinine (nmol/mmol)	1.18 ± 0.56	1.58 ± 1.09	< 0.001

Data are presented as mean ± SD. TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine.

Sex -specific differences in oxidative stress biomarkers

Sex-stratified analysis revealed pronounced differences in oxidative stress profiles among MS patients. Female MS patients tended to show lower TAS levels and higher TOS, OSI, and urinary 8-oxodG/creatinine levels compared with male MS patients. These sex-related differences were less pronounced or absent in the control group.

Two-factor ANOVA demonstrated a significant interaction between sex and disease presence for urinary 8-oxodG/creatinine levels ($F(1,301) = 7.987$, $p = 0.005$, $\eta_p^2 = 0.026$). Sidak post hoc analysis showed that female MS patients exhibited significantly higher urinary 8-oxodG/creatinine levels compared with healthy women ($p < 0.001$, $\eta_p^2 = 0.068$). These sex-specific differences are illustrated in Figure 1.

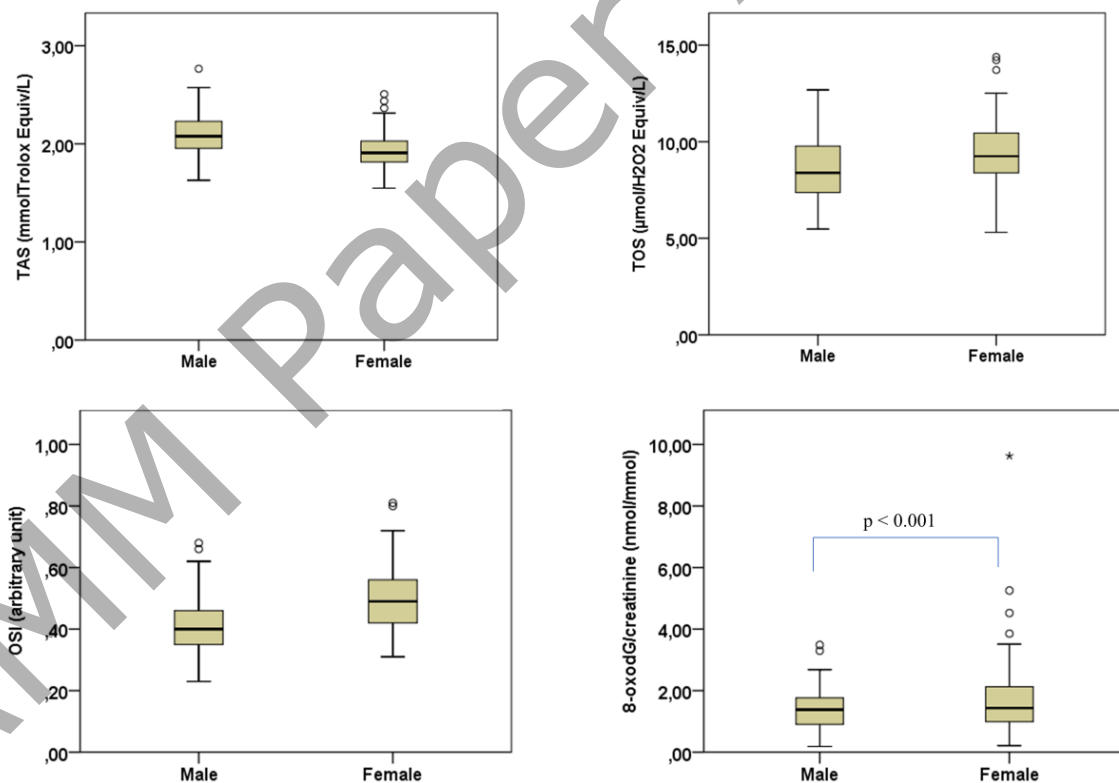


Figure 1. Sex-specific differences in oxidative stress biomarkers in patients with MS.

(A) Total antioxidant status (TAS), (B) total oxidant status (TOS), (C) oxidative stress index (OSI) and (D) urinary 8-oxodG/creatinine levels in male and female MS patients. Female MS patients exhibited significantly higher urinary 8-oxodG/creatinine levels than male MS patients ($p < 0.001$). Asterisks indicate statistical outliers.

Influence of smoking status on oxidative stress DNA damage

Smoking status was associated with differences in oxidative DNA damage. Passive and former smokers tended to exhibit higher urinary 8-oxodG/creatinine levels compared with non-smokers, particularly among MS patients. Active smokers also demonstrated elevated oxidative DNA damage, although the magnitude of this increase was smaller than that observed in passive smokers. The effect of smoking status on oxidative DNA damage in MS patients and healthy controls is shown in Figure 2.

Two-factor ANOVA demonstrated a significant main effect of disease presence on urinary 8-oxodG/creatinine levels ($F(1,301) = 7.951$, $p = 0.005$, $\eta_p^2 = 0.026$), whereas the interaction between smoking status and disease presence was not statistically significant ($F(3,301) = 1.221$, $p = 0.302$, $\eta_p^2 = 0.012$). Post hoc analysis indicated that MS patients generally exhibited higher urinary 8-oxodG/creatinine levels in MS patients compared with controls across smoking categories.

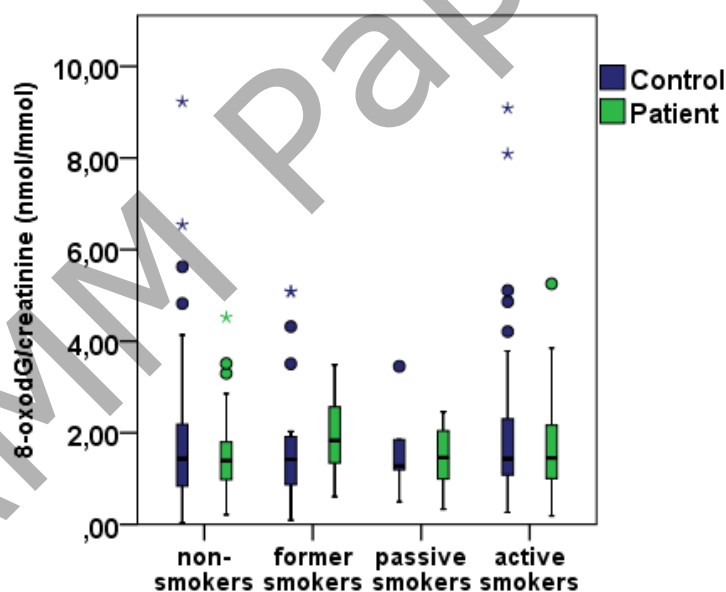


Figure 2. Distribution of urinary 8-oxodG/creatinine levels according to smoking status in MS patients and healthy controls. Asterisks indicate statistical outliers.

Combined effects of sex, smoking status, and disease presence

Three-factor ANOVA did not reveal statistically significant interactions between sex, smoking status, and disease presence on urinary 8-oxodG/creatinine levels, although a trend toward interaction was observed ($F(3,301) = 2.559$, $p = 0.055$, $\eta_p^2 = 0.025$). Female MS patients exposed to tobacco smoke tended to exhibit higher oxidative DNA damage levels.

Association between smoking status, oxidative stress biomarkers, and multiple sclerosis

Binary logistic regression analysis identified passive smoking, OSI, and urinary 8-oxodG/creatinine levels as significant independent predictors of multiple sclerosis (**Table 3**). Passive smoking was significantly associated with MS occurrence (OR = 10.83, 95%CI 3.54-33.12, $p < 0.001$). Increased OSI and higher urinary 8-oxodG/creatinine levels were also significantly associated with increased odds of MS. The overall model was statistically significant ($\chi^2 = 54.37$, $p < 0.001$) and explained part of the variance in MS occurrence.

Table 3. Binary logistic regression model identifying independent predictors of MS

Predictor	β (S.E)	p	OR (95% CI)
Passive smokers exposure	2.38 (0.57)	<0.001	10.83 (3.54-33.12)
8-oxodG/creatinine	0.69 (0.19)	<0.001	1.99 (1.35-2.92)
OSI	3.00 (1.23)	0.014	20.12 (1.82-222.62)
Overall model	χ^2 (df = 5, N = 351) = 54.37, $p < 0.001$		

β , regression coefficient; SE, standard error; OR, odds ratio; CI, confidence interval; OSI, oxidative stress index; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine. The wide confidence intervals suggest variability in the estimated effects.

Exploratory analyses additionally evaluated the potential influence of disease-modifying therapy (DMT) on oxidative stress biomarkers in MS patients. Patients receiving DMT exhibited lower urinary 8-oxodG/creatinine levels and higher TAS values compared with untreated patients, compared with untreated patients.

Subgroup analyses according to disease severity (MSSS) suggested a trend toward increased oxidative imbalance in patients with more severe disease forms, particularly among female smokers, who tended to exhibit higher TOS and OSI levels.

Binary logistic regression analysis identified OSI and urinary 8-oxodG/creatinine as independent predictors associated with MS status, independent predictors associated with MS status.

DISCUSSION

In the present study, we demonstrated a pronounced imbalance in systemic OS biomarkers in patients with MS compared with healthy controls. This imbalance reflects a shift toward a pro-oxidant systemic environment and enhanced oxidative DNA damage, supporting the role of OS in MS-related tissue injury (1-3). These findings further support the concept that OS plays an important role in MS pathophysiology and contributes to disease -related systemic oxidative damage.

OS has been implicated in MS through mechanisms involving mitochondrial dysfunction, chronic inflammation, and excessive production of reactive oxygen species, which can damage lipids, proteins, and nucleic acids (4-6). The modest reduction in antioxidant defense may reflect impaired redox homeostasis in MS, potentially increasing susceptibility to oxidative injury, consistent with previous observations in MS populations (7,8).

A key finding of this study is the presence of significant sex-specific differences in OS biomarkers among MS patients. Sex-specific differences in OS profiles were evident among MS patients, suggesting that biological sex may modulate redox balance and susceptibility to oxidative damage (9-11). Sex-related differences in immune regulation, hormonal influences, and mitochondrial function may contribute to these observations (12-14).

Women with MS, particularly those exposed to tobacco smoke, tended to exhibit a more pronounced oxidative imbalance. These findings support the hypothesis that sex-related biological factors may modulate susceptibility to oxidative stress in MS. Potential mechanisms may include hormonal influences, differences in inflammatory responses, and altered antioxidant defense capacity.

Although estrogens have been reported to exert both antioxidant and pro-oxidant effects depending on inflammatory context, chronic neuroinflammation in MS may shift this balance toward increased oxidative damage in women (15,16).

In addition to hormonal influences, sex-specific differences in mitochondrial activity and antioxidant defense mechanisms may partially contribute to the observed differences to oxidative injury in women with MS. Enhanced immune activation and higher production of pro-inflammatory mediators may amplify reactive oxygen species generation, particularly in the presence of smoking-related oxidative exposure. These mechanisms may partially explain the tendency toward greater oxidative imbalance observed among female MS patients exposed to tobacco smoke.

Smoking status was associated with increased oxidative DNA damage, particularly among MS patients. Both active and passive smoking exposure may be associated with sustained OS in MS, potentially through persistent pro-oxidant effects and impairment of DNA repair mechanisms (17-19). Our findings are consistent with previous studies reporting increased oxidative stress and oxidative DNA damage in patients with MS compared with healthy controls (22). Similar associations between smoking exposure, enhanced reactive oxygen species production, and increased oxidative injury in MS have also been described previously (23). Furthermore, earlier studies suggested that female patients may exhibit greater susceptibility to oxidative imbalance, potentially due to hormonal and immunological

differences (24). The present results extend these observations by demonstrating combined effects of biological sex and smoking exposure on systemic oxidative stress biomarkers.

The combined influence of biological sex, smoking exposure, and disease presence highlights the multifactorial nature of OS in MS, suggesting that oxidative damage arises from interactions between intrinsic susceptibility and environmental exposures rather than disease status alone (25-29).

The findings of the present study should be interpreted in light of several limitations.

LIMITATIONS

The cross-sectional design precludes causal inference. Therefore, it cannot be determined whether altered OS biomarkers represent a cause or a consequence of disease-related processes. Longitudinal studies are needed to clarify whether systemic OS biomarkers are associated with disease progression or response to therapy in MS (30,31). In addition, information on dietary antioxidant intake and physical activity was not available, both of which may influence systemic OS levels and potentially modulate biomarker variability (25). Despite these limitations, the strengths of this study include a relatively large sample size, simultaneous assessment of multiple OS biomarkers, and detailed stratification by sex and smoking exposure. This approach enables a more comprehensive interpretation of OS in MS, emphasizing the interactions between biological susceptibility and modifiable environmental factors rather than disease status alone (28,29). The unexpectedly high oxidative DNA damage observed among passive smokers should be interpreted cautiously. The relatively small number of passive smokers in the control group may have contributed to increased variability and potential overestimation of the observed effect. In addition, exposure misclassification cannot be completely excluded due to the questionnaire-based assessment of smoking status.

Additionally, menopausal status, hormonal profiles, smoking intensity, cumulative smoking exposure, treatment duration, and longitudinal disease activity and follow-up data were unavailable and may have influenced oxidative stress biomarker levels. Exploratory analyses involving disease severity and DMT exposure should therefore be interpreted cautiously and confirmed in larger prospective cohorts.

Exploratory observations from subgroup analyses is provided in Table 4.

Table 4. Summary of exploratory subgroup analyses

Exploratory subgroup analysis	Main observation	Potential biological relevance
Sex-specific analysis	Women with MS exhibited more pronounced oxidative imbalance than men	Female sex may be associated with increased susceptibility to oxidative stress through hormonal and immunological mechanisms
Smoking exposure	Active and passive smoking were associated with increased oxidative DNA damage	Smoking may potentiate oxidative injury and contribute to persistent redox imbalance
Disease severity	More severe disease forms were associated with higher oxidative stress biomarker levels	Oxidative stress may reflect cumulative inflammatory burden and disease activity
DMT exposure	DMT-treated patients showed lower urinary 8-oxodG/creatinine levels and partially preserved antioxidant capacity	DMT exposure may attenuate oxidative and inflammatory damage, although confirmation in longitudinal studies is required

Abbreviations: MS, multiple sclerosis; DMT, disease-modifying therapy; OS, oxidative stress; OSI, oxidative stress index

Exploratory analyses further suggested a potential protective effect of DMT exposure on systemic oxidative stress, reflected by lower urinary 8-oxodG/creatinine levels and partially preserved antioxidant capacity in treated patients. Although these findings should be interpreted cautiously due to the cross-sectional design, they are consistent with previous evidence suggesting that DMT exposure may be associated with attenuation of oxidative and inflammatory damage in MS.

The identification of OSI and urinary 8-oxodG/creatinine as independent predictors associated with MS occurrence further emphasizes the potential clinical relevance of oxidative stress

biomarkers in MS pathophysiology. However, the wide confidence interval observed for OSI suggests variability of the estimate and warrants cautious interpretation. However, these associations should not be interpreted as evidence of causality due to the observational design of the study.

CONCLUSION

In this study, patients with multiple sclerosis exhibited a marked imbalance in systemic oxidative stress, characterized by reduced antioxidant capacity and increased oxidative DNA damage. These alterations were more pronounced in women and among participants exposed to tobacco smoke, particularly passive and former smokers. Urinary 8-oxodG/creatinine may represent a sensitive biomarker reflecting both disease-related and modifiable environmental contributors to oxidative damage. Together, these findings emphasize the importance of considering sex and smoking exposure when evaluating oxidative stress in multiple sclerosis. Further longitudinal studies are needed to clarify the clinical and prognostic utility of oxidative stress biomarkers in MS.

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