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Original article

Serum NOXs and Iron Metabolism Biomarkers in RRMS Patients

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Abstract:

Background: Multiple sclerosis (MS) is an inflammatory and neurodegenerative disorder. Relapsing-remitting MS (RRMS) is the most common MS pattern characterized by relapses or exacerbations. This study aimed to investigate the status of serum endothelial NOXs (NOX5 and NOX4) and iron metabolism biomarkers in RRMS patients. **Methods:** The study was carried out on 40 RRMS patients and 40 control subjects. All the participants were subjected to complete history taking, general and full neurological examination, Serum NOX4,5, ferritin, iron, iron binding capacity, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) levels were measured in all the study subjects. **Results:** MS cases showed significantly lower Nox4, significantly higher Nox5 when compared to control group (median =0.081 versus 0.395, $p<0.001$; median=3.5 versus 0.225, $p<0.001$ respectively). High accuracy AUC was found regarding Nox 4 and Nox 5 (AUC=0.928, 0.988 respectively). At best cut off value of Nox-4 (=0.24 Mmol/L), sensitivity was 90%, specificity was 95%, PPV was 95%, NPV was 90% and accuracy was 92.4%. At best cut off value of Nox-5 (=1.67 Mmol/L), sensitivity was 100%, specificity was 97.5%, PPV was 97.6%, NPV was 100% and accuracy was 98.8%. **Conclusion:** Our data suggests that increased NOX5 expression and decreased levels of NOX4 might be related with oxidative stress related vascular changes and BBB disruption in MS patients.

Keywords: NADPH Oxidase; Iron; Metabolism; Relapsing; Multiple Sclerosis.

Introduction

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disorder. Relapsing-remitting MS (RRMS) is the most common MS pattern characterized by relapses or exacerbations. Although the etiology of the disease is still unknown, it has been proposed that reactive oxygen species (ROS), which are small, oxygen-derived molecules, contribute to MS pathology [(1)].

Dysregulation of iron metabolism in the brain is contributing to OS within the pathogenesis of MS. During myelin breakdown and subsequent phagocytosis of myelin debris which occur at active MS lesions, iron is liberated into the extracellular space. Subsequently, it undergoes conversion to the divalent ferrous form, which can increase the toxicity of ROS [(2)].

In Fenton reaction, Fe2+ is oxidized to Fe3+, while hydrogen peroxide (H2O2) is converted to highly toxic hydroxyl radical (•OH) and hydroxyl anion (OH-). Liberated iron is taken up by macrophages and microglia, which release and deposit iron during phagocytosis of damaged neurons [(3)].

High iron load may even induce cell death, after which iron is further released initiating a second wave of OS. Additionally, activated macrophages release NO that can promote other iron release from ferritin [(3)]. Abnormal iron depositions have been associated with the production of ROS [(4)].

Elevated nicotinamide adenine dinucleotide phosphate (NADPH) oxidases activations and concentrations are one of the main enzymatic source of ROS including superoxide anion and its derivatives [(5)]. The NADPH oxidases (NOX) family consists of seven catalytic homologues. Four NOX isoforms including NOX1, NOX2, NOX4 and NOX5 are expressed from endothelial cells [(6)].

Increased expressions of NOX1, 2, and 5 have been related with endothelial dysfunction and vascular inflammation However, NOX4 exerts protective effects on the vessel wall [(6)].

There has been growing evidence implicating the role of NOX isoforms in the pathogenesis of several neurodegenerative diseases including Amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease**;** However, little is known about the status of serum NOX1, NOX4 and NOX5 in RRMS patients [(7)]**.**

Several previous studies have focused on the interaction between MS and nutritional intake to reduce the symptoms such as decreased cognitive, sensory and physical functions [(8)]. One of the important parts of the diet component are micronutrients such as trace elements [(9)].

Abnormal iron depositions have been associated with the production of ROS. Alterations in iron deposition and serum biomarkers of iron metabolism have been consistently reported in patients with MS. However, uncertainty still exists about the relationship between iron metabolism and oxidative stress in MS [(10)].

This study aimed to investigate the status of serum endothelial NOXs (NOX5 and NOX4) and iron metabolism biomarkers in RRMS patients.

Patients and Methods

This case control study included 80 participants, who were recruited from the Neuropsychiatry Department, Benha University Hospital and outpatient clinic of Benha Insurance Hospital, during the period from January 2021 to December

2021. Laboratory work was performed in Clinical Pathology Department, Benha University Hospital.

Study groups;

- **Patient group:** included 40 patients presented by relapsing remitting multiple sclerosis.
- **Control group:** included 40 age and sex matched healthy individuals.

Inclusion criteria

1. Age: 20-45 years.
2. Patients diagnosed with relapsing remitting multiple sclerosis according to the 2017 McDonald criteria.

Exclusion criteria

1. Probable multiple sclerosis or clinically isolated syndrome.
2. Pregnancy and breast feeding.
3. Any gastrointestinal or hematologic disease.
4. Severe concomitant medical condition (e.g., metastatic cancer, AIDS, renal failure, liver failure…etc.).
5. Patients consumed iron compounds, nutritional supplements or anti-oxidants.
6. Patients had corticosteroid therapy during the last 3 months.

All procedures were revised and approved by Research Ethics Committee in Faculty of Medicine, Benha University. An informed written consent was obtained from patients and control subjects before their participation in the current study. An official permission was obtained from Benha University and Benha Insurance Hospitals managers to conduct this study.

All patients were subjected to medical history taking, full general and neurological examination, and laboratory investigations as Erythrocyte Sedimentation Rate (ESR), C-reactive protein (CRP), Serum iron, TIBC, serum ferritin, and NADPH oxidase (NOX) by ELISA

NOX4 and NOX5 were measured in serum sample using Human NADPH oxidase 4 peptide (NOX4) and Human NADPH oxidase 5 peptide (NOX5) ELISA kits (cata.No.: In-Hu 1940 , In-Hu 1041 respectively, NOVA, China), these ELISA kits use Sandwich-ELISA as the method.

Blood Sampling:

Ten milliliters of venous blood were drawn under complete aseptic conditions from each patient and distributed as follows: Two milliliters in an EDTA (ethylene diamine tetra-acetic salt (1.2mg/mL) vacutainer as an anticoagulant for complete blood count (CBC). Two milliliter in citrated tube (1:9) for ESR. Six milliliters in plain tube for serum separation. Samples were allowed to clot for 30 minutes at room temperature, then centrifuged (at 1500 rpm for 15 minutes). Serum was separated and divided into two aliquots; one for CRP, iron, ferritin and TIBC testing and the other stored at -80ºC for subsequent NOX4 and NOX5 assay by ELISA.

Statistical analysis

The collected data was revised, coded, tabulated using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. Normality of data; Shapiro test was done to test the normality of data distribution. Descriptive statistics: Mean, Standard deviation (± SD) for parametric numerical data, while Median and range for non-parametric numerical data. Frequency and percentage of non-numerical data. Analytical statistics: Student T Test was used to

assess the statistical significance of the difference between two study group means. For the comparison of more than two groups' means, one way analysis of variance (ANOVA) was used. Mann Whitney Test (U test) was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. The Kruskal-Wallis test was used to assess the statistical significance of the difference between more than two study group non parametric variables.Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Correlation analysis: To assess the strength of association between two quantitative variables. The correlation coefficient defines the strength and direction of the linear relationship between two variables. The ROC Curve (receiver operating characteristic) provides a useful way to evaluate the sensitivity and specificity for quantitative diagnostic measures that categorize cases into one of two groups. The optimum cut off point was defined as that which maximized the AUC value. The area under the ROC curve (AUC) results were considered excellent for AUC values between 0.9-1, good for AUC values between 0.8-0.9, fair for AUC values between 0.7-0.8, poor for AUC values between 0.6-0.7 and failed for AUC values between 0.5-0.6. All reported p values were two-tailed and p <0.05 was considered to be significant.

Results

The present study was conducted on 40 cases with RRMS. Their mean age was 32.2 years. They were 6 males (15%) and 34 females (85%). In addition, 40 healthy subject of matched age and gender were added as a control group. Table 1

Comparing cases and control groups revealed that MS showed significantly higher ESR, ferritin, significantly higher frequency of high CRP, significantly lower iron, TIBC when compared to control group (p<0.001 for each). table 2

MS cases showed significantly lower Nox4, significantly higher Nox5 when compared to control group (median =0.081 versus 0.395, p<0.001; median=3.5 versus 0.225, p<0.001 respectively). Figure 1&2.

Table (1): Comparison of demographic data between studied groups.

		Control N=40	**Case N=40**	***P***
Age (years)	mean± SD	29.9±5.5	32.2±7.7	0.118
Males	N (%)	5 (12.5%)	6 (15.0%)	0.745
Females	N (%)	35 (87.5%)	34 (85.0%)	

*Significant; < 0.05, ** High significant; < 0.01, *** Very high significant< 0.001. SD, standard deviation.

Table (2): Comparison of laboratory data between studied groups.

			Control N=40	Case N=40	*P*
ESR (mm/hour)		mean± SD	27.4±8.1	54.3±17.2	<0.001***
CRP(mg/l)	**Low**	N (%)	33 (82.5%)	13 (32.5%)	<0.001***
	High	N (%)	7 (17.5%)	27 (67.5%)	
Iron (µg/dl)		median (range)	65 (38-139)	34 (22-119)	<0.001***
Ferritin (ng/ml)		median (range)	70 (17-213)	165 (43.5-450)	<0.001***
TIBC (µg/dl)		median (range)	310 (220-430)	244.5 (160-450)	<0.001***

*Significant; < 0.05, ** High significant; < 0.01, *** Very high significant< 0.001. SD, standard deviation.

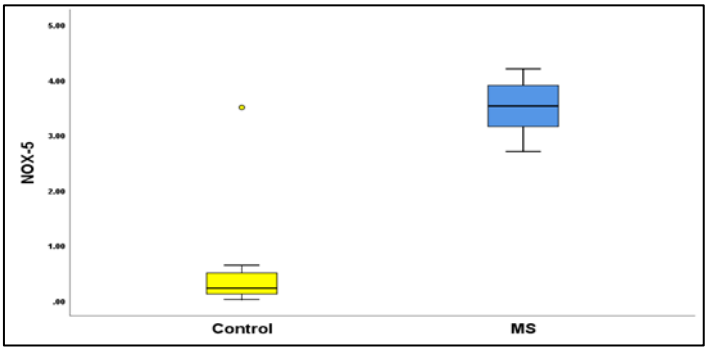

Figure (1): Nox5 level among studied groups.

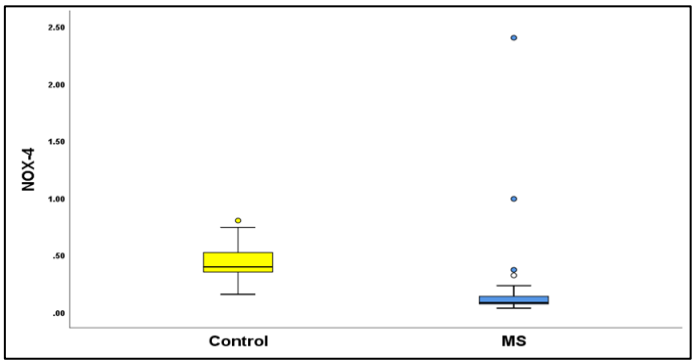

Figure (2): Nox4 level among studied groups.

Receiver operating characteristic curve (ROC) of Nox-4, Nox-5, iron, ferritin and TIBC was conducted for discrimination between MS cases and control groups. High accuracy AUC was found regarding Nox 4 and Nox 5 (AUC=0.928, 0.988 respectively). At best cut off value of Nox-4 (=0.24 Mmol/L), sensitivity was 90%, specificity was 95%, PPV was 95%, NPV was 90% and accuracy was 92.4%. At best cut off value of Nox-5 (=1.67 Mmol/L), sensitivity was 100%, specificity was 97.5%, PPV was 97.6%, NPV was 100% and accuracy was 98.8%. Moderate accuracy AUC was found regarding iron, ferritin and TIBC (AUC=850, 816, 0.748 respectively). At best cut off value of iron (=52.5 µg/dL), sensitivity was 77.5%, specificity was 85%, PPV was 83.8%, NPV was 79.1% and accuracy was 81.3%. At best cut off value of ferritin (=108.5 ng/mL), sensitivity was 65%, specificity was 90%, PPV was 86.7%, NPV was 72% and accuracy was 77.5%. At best cut off value of TIBC (=272.5 µg/dL), sensitivity was 65%, specificity was 77.5%, PPV was

74.3%, NPV was 68.9% and accuracy was 71.3%. Table 3 & figure 3

No significant correlation of NOX-4 level was found with NOX-5 among control as well as MS groups (p>0.05 for each group). Table 4

Table (3): Validity of Nox4, Nox5, iron, ferritin and TBC for prediction of multiple sclerosis.

	Nox-4	Nox-5	Iron	Ferritin	TIBC
AUC	0.928	0.988	0.850	0.816	0.748
Cut off	0.24 µmol/L	1.67 µmol/L	<52.5 µg/dl	108.5 ng/ml	<272.5 µg/dl
Sensitivity (%)	90%	100%	77.5%	65%	65%
Specificity (%)	95%	97.5%	85%	90%	77.5%
PPV (%)	95%	97.6%	83.8%	86.7%	74.3%
NPV (%)	90%	100%	79.1%	72%	68.9%
Accuracy (%)	92.4%	98.8%	81.3%	77.5%	71.3%

AUC, area under ROC curve; ROC, receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value.

Table (4): Correlation of NOX-4 with NOX-5 among control and MS groups.

	NOX-5 Control		MS	
	Rs	***P***	***Rs***	***P***
NOX-4	0.083	0.610	-0.244	0.129

*Significant; < 0.05, ** High significant; < 0.01, *** Very high significant< 0.001 rs, correlation coefficient.

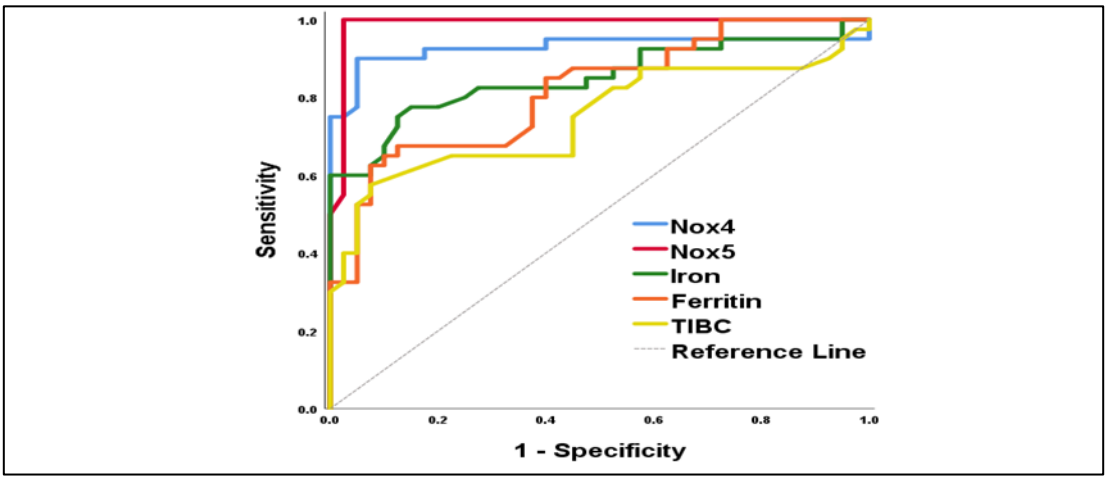

Figure (3): ROC curve for discrimination between MS cases and control groups.

Discussion

The present study showed that MS cases have significantly higher ESR, ferritin, significantly higher frequency of high CRP, significantly lower iron and TIBC when compared to control group. By Comparing level of Nox4 and Nox5 between the studied groups, the current study reported that MS cases showed significantly lower NOX4, significantly higher NOX5 when compared to control

group. These finding agreed with other studies that showed that higher serum NOX5, CRP titer, ferritin and lower serum NOX4, iron concentrations were found in the patients than in controls [11,12].

Our results were in concordance with other studies found that serum iron concentrations were significantly lower in MS patients than the matched controls [8,13]. While another study restricted the decrease in iron to female MS patients than the matched controls [14]. However, no differences have been reported between control groups and MS patients in terms of serum iron concentrations [10,15].

It is thought that the discrepancy in the results of serum iron concentrations might be related with the differences in the nutritional status of patients as well as differences in the experimental models between studies, presence of other chronic diseases and different ethnic backgrounds.

Some authors suggested that the decreased blood levels of iron in MS patients are associated with longer disease duration, when iron begins to accumulate more in the brain [16].

Iron is essential for maintaining the health and development of the myelination and remyelination process. Decreased availability of iron in the diet has been associated with hypomyelination [17].

Other researchers [15] were observed elevated serum ferritin concentrations in MS patients compared to healthy controls**.** However, another report has shown no differences between MS patients and healthy controls in terms of ferritin levels [12]**.** Discordant ferritin results might be due to differences of iron status and neuroinflammation degree of patients between studies.

Findings from studies carried out by Other researchers [18,19] failed to show any differences in iron content, but elevated transferrin levels were observed in MS patients.

The higher CRP level, a well-known positive acute phase reactant, was observed in at the current study in MS patients than in controls, that was not inconsistent with the study done by Other researchers[11]. It is thought that both increased ferritin and CRP levels could be related to the acute phase reactions owing to the increased CNS inflammation in patients [20].

For the prediction of MS susceptibility in the present study, a receiver operating characteristic curve (ROC) of Nox4, Nox5, iron, ferritin, and TIBC was conducted. High accuracy AUC was found regarding NOX 4 and NOX 5. While, moderate accuracy AUC was found regarding iron, ferritin and TIBC.

These results agreed with other studies that showed that oxidative status may play a role in chronic inflammation and neurodegeneration which are considered critical etiopathogenetic factors in Multiple Sclerosis (MS), both in the early phase of the disease and in the progressive one [21].

Other studies have measured total hydroperoxides and total antioxidant status (TAS), along with the iron metabolism biomarkers including iron, ferritin, transferrin, transferrin saturation, and ceruloplasmin panel biomarkers, found that lower TAS levels in MS patients than in healthy controls and normal reference level and higher ROS and Ceruloplasmin: transferrin ratio in MS than in healthy controls, while biomarkers of iron metabolism were not different between patients and controls [22].

Previous studies demonstrated the role of different NOX isoforms in

neurodegenerative diseases including Alzheimer and Parkinson's disease [23]. Upregulated NOX expressions have been also associated with MS pathogenesis in the experimental and clinical studies [24].

Abnormal iron homeostasis may contribute to neurodegeneration which is relevant to the pathology of MS. Iron may lead to neuronal damage through triggering oxidative stress [25]. Iron analyses showed heterogeneity within different geographical subgroups. In this regard, Asians showed significantly lower circulating levels of Fe in comparison with other regions [26].

Conclusion

Our data suggest that increased NOX5 expression and decreased levels of NOX4 might be related with oxidative stress related vascular changes and BBB disruption in MS patients. We also demonstrated that lower concentrations of iron and TIBC in RRMS patients. Because of the importance of iron on myelination and oligodendrocytes functions, serum iron levels should be closely monitored in MS patients.

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Conflicts of interest

No conflicts of interest

References

1. Gonsette RE. Oxidative stress and excitotoxicity: a therapeutic issue in multiple sclerosis? Mult Scler J. 2008;14(1):22–34.
2. Mahad DH, Trapp BD, Lassmann H. Pathological mechanisms in progressive multiple sclerosis. Lancet Neurol. 2015;14(2):183–93.
3. Neher JJ, Neniskyte U, Zhao J-W, Bal-Price A, Tolkovsky AM, Brown GC. Inhibition of microglial phagocytosis is sufficient to prevent inflammatory neuronal death. J Immunol. 2011;186(8):4973–83.
4. Galaris D, Pantopoulos K. Oxidative stress and iron homeostasis: mechanistic and health aspects. Crit Rev Clin Lab Sci. 2008;45(1):1–23.
5. Panday A, Sahoo MK, Osorio D, Batra S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. Cell Mol Immunol. 2015;12(1):5–23.
6. Drummond GR, Sobey CG. Endothelial NADPH oxidases: which NOX to target in vascular disease? Trends Endocrinol Metab. 2014;25(9):452–63.
7. Ajayi A, Yu X, Ström A-L. The role of NADPH oxidase (NOX) enzymes in neurodegenerative disease. Front Biol (Beijing). 2013;8(2):175–88.
8. Armon-Omer A, Waldman C, Simaan N, Neuman H, Tamir S, Shahien R. New insights on the nutrition status and antioxidant capacity in multiple sclerosis patients. Nutrients. 2019;11(2):427.
9. Bredholt M, Frederiksen JL. Zinc in multiple sclerosis: A systematic review and meta-analysis. ASN Neuro. 2016;8(3):1759091416651511.
10. Bergsland N, Agostini S, Laganà MM, Mancuso R, Mendozzi L, Tavazzi E, et al. Serum iron concentration is associated with subcortical deep gray matter iron levels in multiple sclerosis patients. Neuroreport. 2017;28(11):645–8.
11. Doğan HO, Yildiz ÖK. Serum NADPH oxidase concentrations and the associations with iron metabolism in relapsing remitting multiple sclerosis. J Trace Elem Med Biol. 2019;55:39–43.
12. Siotto M, Filippi MM, Simonelli I, Landi D, Ghazaryan A, Vollaro S, et al. Oxidative stress related to iron metabolism in relapsing remitting multiple sclerosis patients with low disability. Front Neurosci. 2019;86.
13. Forte G, Visconti A, Santucci S, Ghazaryan A, Figà-Talamanca L, Cannoni S, et al. Quantification of chemical elements in blood of patients affected by multiple sclerosis. Ann Ist Super Sanita. 2005;41(2):213–6.
14. Van Rensburg SJ, Kotze MJ, Hon D, Haug P, Kuyler J, Hendricks M, et al. Iron and the folate-vitamin B12-methylation pathway in multiple sclerosis. Metab Brain Dis. 2006;21(2):117–33.
15. Oliveira SR, Kallaur AP, Lopes J, Simão ANC, Reiche EM, de Almeida ERD, et al. Insulin resistance, atherogenicity, and iron metabolism in multiple sclerosis with and without depression: associations with inflammatory and oxidative stress biomarkers and uric acid. Psychiatry Res. 2017;250:113–20.

16. Duck KA, Connor JR. Iron uptake and transport across physiological barriers. Biometals. 2016;29(4):573–91.
17. Todorich B, Pasquini JM, Garcia CI, Paez PM, Connor JR. Oligodendrocytes and myelination: the role of iron. Glia. 2009;57(5):467–78.
18. Sfagos C, Makis AC, Chaidos A, Hatzimichael EC, Dalamaga A, Kosma K, et al. Serum ferritin, transferrin and soluble transferrin receptor levels in multiple sclerosis patients. Mult Scler J. 2005;11(3):272–5.
19. Abo-Krysha N, Rashed L. The role of iron dysregulation in the pathogenesis of multiple sclerosis: an Egyptian study. Mult Scler J. 2008;14(5):602–8.
20. Olsson A, Gustavsen S, Gisselø Lauridsen K, Chenoufi Hasselbalch I, Sellebjerg F, Bach Søndergaard H, et al. Neutrophil-to-lymphocyte ratio and CRP as biomarkers in multiple sclerosis: A systematic review. Acta Neurol Scand. 2021;143(6):577–86.
21. Sheykhansari S, Kozielski K, Bill J, Sitti M, Gemmati D, Zamboni P, et al. Redox metals homeostasis in multiple sclerosis and amyotrophic lateral sclerosis: a review. Cell Death Dis. 2018;9(3):1–16.
22. Zivadinov R, Tavazzi E, Bergsland N, Hagemeier J, Lin F, Dwyer MG, et al. Brain iron at quantitative MRI is associated with disability in multiple sclerosis. Radiology. 2018;289(2):487–96.
23. Ganguly U, Kaur U, Chakrabarti SS, Sharma P, Agrawal BK, Saso L, et al. Oxidative stress, neuroinflammation, and NADPH oxidase: implications in the pathogenesis and treatment of Alzheimer's disease. Oxid Med Cell Longev. 2021;2021.
24. Seo J-E, Hasan M, Rahaman KA, Kang M-J, Jung B-H, Kwon O-S. A leading role for NADPH oxidase in an in-vitro study of experimental autoimmune encephalomyelitis. Mol Immunol. 2016;72:19–27.
25. Ndayisaba A, Kaindlstorfer C, Wenning GK. Iron in neurodegeneration–cause or consequence? Front Neurosci. 2019;13:180.
26. Nirooei E, Kashani SMA, Owrangi S, Malekpour F, Niknam M, Moazzen F, et al. Blood trace element status in multiple sclerosis: a systematic review and meta-analysis. Biol Trace Elem Res. 2022;200(1):13–26.

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