

Serum Ferroxidase Activity in Patients with Multiple Sclerosis: A Pilot Study

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Abstract. Emerging evidence suggests that oxidative stress might contribute to demyelination and axonal damage in multiple sclerosis (MS). Ferroxidase (FeOx) activity of ceruloplasmin prevents the formation of free radicals from Fe²⁺ by promoting the incorporation of this pro-oxidant ion to transferrin. The aim of our study was to investigate serum FeOx activity in a cohort of patients with MS and neurological controls. Serum FeOx activity was determined in 69 relapsing-remitting patients with MS and in 62 patients with other inflammatory neurological disorders (OIND) and 52 patients with other non-inflammatory neurological disorders (NIND) as controls. Serum FeOx activity was lower ($p < 0.01$) in MS and OIND than in NIND, without any significant differences among MS patients grouped according to clinical and magnetic resonance evidence of disease activity. A reduced serum FeOx activity, which can potentially lead to a rise in oxidative stress-induced biomolecular damage, seems to be a shared condition in inflammatory disorders of the central nervous system including MS.

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) of supposed autoimmune origin which is currently considered as a de-myelinating and neurodegenerative disease because both de-myelination and axonal damage are observed in MS lesions (1). A growing body of evidence suggest that an excessive generation of reactive oxygen and nitrogen species (ROS and RNS, respectively), leading to oxidative stress-related damage to

biomolecules, is involved in demyelination and axonal damage (2). In particular, the respiratory burst of activated macrophages present in the de-myelinating lesions has been indicated as one of the major causes of these phenomena (3). More generally, besides inflammation, a pathological increase of oxidant species could be mainly ascribed to mitochondrial dysfunction and Fenton reactions (4). In particular, the latter pathway leads to the formation of the most reactive ROS, hydroxyl radical (*OH), via catalysis by ferrous ion. Thus, keeping free Fe²⁺ levels low in blood and the CNS is important for preventing detrimental effects of oxidative stress. In this framework, the ferroxidase (FeOx) activity of ceruloplasmin, a protein expressed in liver as well as in the brain (5), has emerged as a crucial factor (6, 7). Indeed, by promoting the oxidation of Fe²⁺ to Fe³⁺, ceruloplasmin allows the incorporation of the ion into transferrin, the main iron carrier in blood. Through this antioxidant-like activity, ceruloplasmin plays an important role in the flux of iron through the body, influencing the extent of deposition of this metal in the tissues (5). Consistent with this notion, both aceruloplasminemic patients and the ceruloplasmin-deficient mice are characterized by iron dyshomeostasis and an increased accumulation of iron in the brain, in astrocytes and nerve cells in the basal ganglia and thalamus, correlating with motor dysfunction (7, 8). Of note, brain iron overload has been recently observed by magnetic resonance imaging (MRI) scan in patients affected by MS (9). Considering these observations, the aim of our study was to investigate serum FeOx activity in patients with MS and controls.

Materials and Methods

Study design. This study included 69 consecutive untreated patients (47 females and 22 males, mean age 37.4±11.0 years) with relapsing-remitting (RR) definite MS according to the currently accepted criteria (10). All patients were imaged with a 1.5-Tesla MRI unit within 48 h after blood sampling. Evidence of a relapse at admission and lesions showing gadolinium enhancement on T1-weighted scans were considered as clinical and MRI disease

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Table I. Features of patients with multiple sclerosis (MS), other inflammatory neurological diseases (OIND) and non-inflammatory neurological diseases (NIND) at study entry.

Patients	n	Gender		Age (years)	Characteristics
		F	M		
MS	69	47	22	37.4±11.0	Disease duration (mean±SD)=30.7±50.9 months; EDSS=2.0±1.4; EDSS ≤2: n=44; EDSS >2: n=25; Presence of relapse (CA): n=51; Absence of relapse (CS): n=18; Gd ⁺ : n=33; Gd ⁻ : n=36
OIND	63	43	20	36.8±11.3	Chronic inflammatory demyelinating polyneuropathy: n=20; Acute inflammatory demyelinating polyneuropathy: n=15; Viral encephalomyelitis: n=10; Bacterial meningitis: n=7; Optic neuritis: n=6; Eurolupus: n=3; Neurosjogren: n=2
NIND	52	35	17	38.3±10.7	Headache: n=12; Epilepsy: n=8; Brain tumor=7; Ischemic stroke=6; Amyotrophic lateral sclerosis: n=4; Compression neuropathy: n=4; Migraine: n=4; Paresthesia: n=4; Alzheimer's disease=3

EDSS: Expanded disability status scale; SD: standard deviation; CA: clinically active; CS: clinically stable; Gd⁺: magnetic resonance imaging (MRI) appearance of gadolinium-enhancing lesions; Gd⁻: no MRI evidence of gadolinium-enhancing lesion.

activity, respectively. Disease severity was scored at the time of sample collection using the Expanded Disability Status Scale (EDSS) (11). The duration of the disease was expressed in months. At entry, none of the patients had fever nor other symptoms or signs of acute infection. Moreover, at the time of sample collection, none of the patients had received any potential disease-modifying therapies during the six months before the study.

Sixty-three patients with other inflammatory neurological disorders (OIND) and 52 with other non-inflammatory neurological disorders (NIND) were selected as neurological controls who were age- and sex-matched to those with RRMS. Serum samples prospectively collected from patients with MS, OIND and NIND were obtained for purposes of diagnosis and measured under exactly the same conditions. The study was approved by the Regional Committee for Medical Ethics in Research and written informed consent was obtained from all participants in the study.

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FeOx activity assay. Enzymatic activity of FeOx was measured in serum samples by a Tecan infinite M200 Tecan Group Ltd., Männedorf, Switzerland) microplate spectrophotometer according to Erel's method (6) with minor modifications. Briefly, 5 µl of sample was added to 195 µl of acetate buffer (0.45 mol/l,

pH=5.8). After 1 min incubation at 37°C, 43 µl of 370 mM Fe(NH₄)₂SO₄ was added, and the resulting mixture was incubated for a further 3.8 min at 37°C. At the end of the incubation, 20 µl of chromogen {3-(2-pyridyl)-5,6-bis-[2-(5-furylsulfonic acid)]-1,2,4-triazine} was added. The rate of formation of colored complex (formed by the chromogen and ferrous ions) was recorded at 600 nm. The difference in the ferrous ion concentration before and after the enzymatic reaction indicated the amount of oxidized ferrous ion. The amount of enzyme that converted 1 µmol of substrate into product per minute in one liter of sample was defined as 1 U/l. The detection limit and intra-assay coefficient of variation were 25.8 U/l and 2.5%, respectively.

Statistical analysis. Statistical analysis was performed with GraphPad Prism 5 (GraphPad, San Diego, CA, USA) and SPSS 18.0 for Windows (IBM, Chicago, IL, USA). Variables were first analyzed for the normal distribution by the Kolmogorov-Smirnov test. Difference between groups was checked by unpaired *t*-test with Welch's correction and Wilcoxon-Mann-Whitney for normally and non-normally distributed variables, respectively. Correlations between normal variables were checked with Pearson's analysis, while those between non-normal variables with Spearman's test. A two-tailed probability value <0.05 was considered statistically significant.

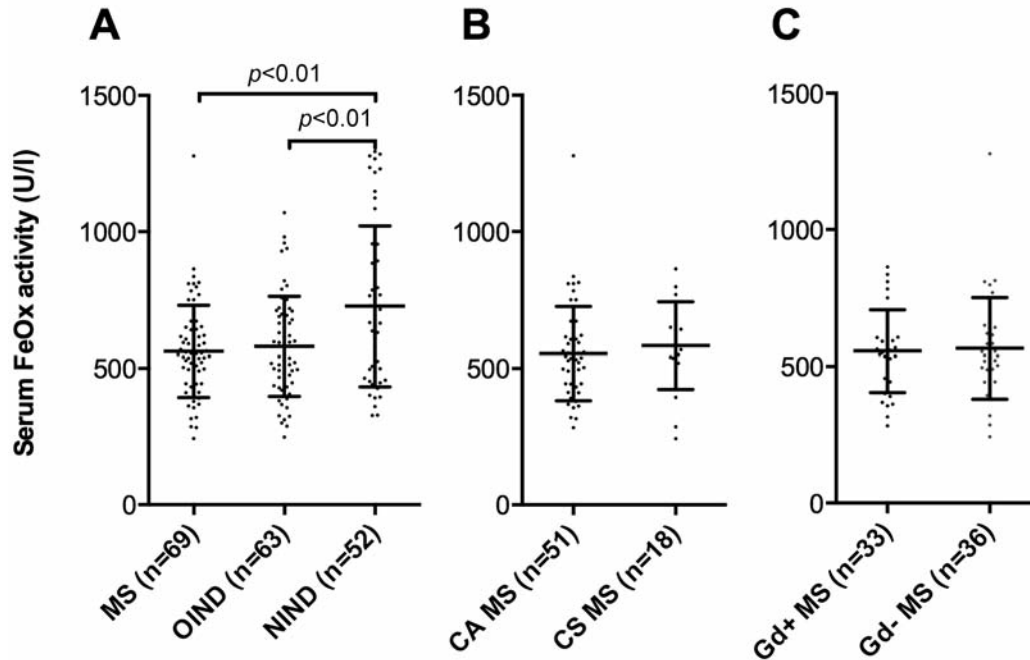


Figure 1. Serum ferroxidase activity was significantly higher in patients with non-inflammatory neurological disorders (NIND) than in those with multiple sclerosis (MS) and other inflammatory neurological disorders (OIND) ($p < 0.01$) (A) without any further statistical differences between patients with MS grouped according to clinical ($p = 0.5277$) (B) and magnetic resonance imaging evidence of disease activity ($p = 0.8010$) (C) by unpaired t-test with Welch's correction. Each point represents a single observation, including outliers. Horizontal bars indicate means and error bars correspond to standard deviations. CA: Clinically active (presence of relapse at entry); CS: clinically stable (absence of relapse at entry); Gd⁺: MRI appearance of gadolinium-enhancing lesions; Gd⁻: no MRI evidence of gadolinium-enhancing lesion; EDSS: expanded disability status scale.

Results

Demographical and clinical findings from patients with MS, OIND and NIND are presented in Table I. Analysis of data with the Kolmogorov-Smirnov test showed a normal distribution of serum FeOx activity levels in MS ($p = 0.0656$) OIND ($p = 0.2000$) and NIND ($p = 0.0841$). As shown in Figure 1A, serum FeOx activity was significantly higher in NIND than in MS and OIND ($p < 0.01$). Differences in FeOx activity did not reach statistical significance level when patients with MS were grouped according to clinical (Figure 1B) and MRI (Figure 1C) evidence of disease activity. Moreover, among patients with MS, serum FeOx activity did not correlate with disease severity by EDSS (Spearman; $p = 0.2363$), duration of the disease in months (Spearman; $p = 0.1975$), nor age (Spearman; $p = 0.3988$) (data not shown).

Discussion

Cumulative evidence suggests that both circulating and brain ceruloplasmin play an important physiological role in neuronal iron homeostasis (5). Indeed, on one hand, the ability to oxidize ferric iron in the bloodstream induces the release of iron from tissue stores. On the other hand, FeOx

activity of brain ceruloplasmin, present both as glycosylphosphatidylinositol-anchored and soluble forms, promotes the binding to transport carriers, transferrin, in cerebrospinal fluid (CSF) and final uptake by neurons (5). Recently, this function, as well as the capability of preventing pro-oxidant Fenton reaction, has been used as a conceptual key for interpreting the decrease in peripheral and CSF FeOx activity observed in patients with neurodegenerative disorders, in particular Parkinson's, Alzheimer's and Huntington's disease, which were characterized by abnormal oxidative stress and excessive iron accumulation in the brain (7, 12). These two features have been also described in patients suffering from inflammatory CNS diseases, such as MS (2, 3, 9). However, despite this intriguing parallel, to our knowledge, this is the first study to investigate a possible relationship between peripheral FeOx activity and MS.

Our main finding was that compared to patients with non-inflammatory CNS disorders, FeOx enzymatic activity is significantly decreased in patients with MS and in patients suffering from other inflammatory diseases of the CNS compared to those with non-inflammatory CNS disorders. It might be speculated that, as observed for other diseases (7, 12), a decrease in serum FeOx activity may reflect a loss of ceruloplasmin activity in the CNS, which

eventually could engender vulnerability to iron toxicity. Indeed, a reduced release of iron from the brain may lead to an excessive increase of free iron deposition in neuronal tissue (5, 7, 13). It is well-recognized that even a low level of non-ferritin- and non-transferrin-bound iron, present as ascorbate-Fe²⁺, citrate-Fe²⁺ *etc.*, is detrimental for brain health because it is capable of catalyzing the formation of free radicals which are highly noxious for this tissue, due to the lack of effective endogenous antioxidant defence (7, 12). The consequent oxidative injury may contribute to exacerbating pre-existing local inflammation, which, in turn, might further increase oxidative stress *e.g.* by activation of microglia and macrophage NADPH oxidase, generating a self-perpetuating cycle (2). The lack of differences between patients with MS and those with OIND and between patients with MS grouped according to clinical and MRI evidence of disease activity could indicate that reduced serum FeOx activity is a shared condition in inflammatory disorders of the CNS, sustained by inflammation itself, and that this phenomenon does not seem correlated to disease exacerbation. Further research is required to evaluate the several issues raised by the present pilot study. Firstly, a longitudinal approach could be valuable in elucidating the cause-effect relationship between FeOx activity in MS and other neuroinflammatory disorders, and in CSF. Secondly, it is important to elucidate if the changes in FeOx activity observed here might be due to one of the 40 or so known mutations of ceruloplasmin gene (14), or rather to post-translational modifications which were seen both to reduce catalytic efficiency of ceruloplasmin and to impair brain iron metabolism in *in vivo* and *in vitro* models of neurodegenerative diseases (7, 8). Moreover, since FeOx activity is highly correlated with serum copper concentration (6), it cannot be ruled out that its decrease may be due to a reduction in the bioavailability of this element, as occurs in inherited disorders such as Wilson's and Menkes' diseases (15).

In conclusion, our preliminary findings of an association between low serum FeOx activity and MS suggest that impairment of iron homeostasis might be implicated in the pathogenesis of this disease, as well as other pathological conditions characterized by neuroinflammation.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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