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EFFECT OF MELATONIN SUPPLEMENTATION ON PLASMA LIPID HYDROPEROXIDES, HOMOCYSTEINE CONCENTRATION AND CHRONIC FATIGUE SYNDROME IN MULTIPLE SCLEROSIS PATIENTS TREATED WITH INTERFERONS-BETA AND MITOXANTRONE

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Multiple sclerosis (MS) prevalence is higher in geographic regions with less sunlight exposure. Melatonin participates in the effects of sunlight in healthy individuals and could play a role in MS pathophysiology. Melatonin crosses the blood-brain barrier and exerts antioxidative, immunomodulatory, and anti-inflammatory effects. Chronic fatigue syndrome concerns 80 – 90% MS patients. The pathophysiology of chronic fatigue syndrome is unknown, however activation of immune, inflammatory, oxidative and nitrosative stress mechanisms and plasma lipid peroxide elevation was reported. Homocysteine increases plasma lipid hydroperoxides levels. The aim was to determine the effect of melatonin supplementation on chronic fatigue syndrome in MS patients and evaluate plasma lipid hydroxyperoxides (LHP) and homocysteine concentrations as a potential biochemical fatigue biomarkers. Into a case-control prospective study 102 MS patients divided according receiving immunomodifying MS treatment into groups: RRMS-pretreated, RRMS-INF-beta, SP/PPMS-mitoxantrone, RRMS-relapse were enrolled. Patients were supplemented with melatonin over 90 days. Plasma LHP, homocysteine concentration, brain MRI and fatigue score were examined. Results show that LHP concentrations were significantly higher in all studied MS groups vs. controls. In all MS patient groups melatonin application resulted in significant decrease in plasma LHP concentrations. Plasma homocysteine concentration was similar in healthy people, RRMS-pretreated, RRMS-INF-beta and SP/PP-MS-mitoxantrone groups. However, in the RRMS-relapse group plasma levels of homocysteine were significantly higher compared to the RRMS-pretreated group. There were no significant differences in plasma homocysteine concentration in the studied groups before and after melatonin application. The fatigue score was significantly lower in RRMS pretreated group compared to RRMS-INF-beta and SP/PP MS-mitoxantrone treated patients. Plasma lipid hydroxyperoxides could be potential biochemical chronic fatigue syndrome biomarker in MS patients and homocysteine could be a potential marker of acute phase of MS. Melatonin exerts beneficial effects in MS patients based on its' proved antioxidative properties.

Key words: *multiple sclerosis, melatonin, chronic fatigue syndrome, lipid hydroxyperoxides, homocysteine, Modified Fatigue Impact Scale, mitoxantrone, mitoxantrone, interferons beta*

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease that damages myelin and axons. Its prevalence is estimated at about 2.5 million people worldwide (1). MS is the main cause of neurological disability in young adults. Apart from physical dysfunction, cognitive functions impairment, depression and chronic fatigue syndrome in MS patients are often observed (2-10). Chronic fatigue syndrome occurs in about 80 – 90% of MS patients (1, 8, 9, 11, 12). It can be present at any stage of the disease and it affects many aspects of life, such as social functioning, employment, driving safety, physical independence, rehabilitation gains, disease management, treatment adherence, mood, and finally, reduced quality of life (8, 9, 13). Definition of chronic fatigue syndrome consists of exhibiting persistent fatigue which is substantially unrelieved by rest, and accompanied by other symptoms such as circadian rhythm sleep disorders and cognitive dysfunction for a minimum of 6 months (14). The pathogenesis of chronic fatigue remains incompletely understood. Fatigue could be evaluated by Modified Fatigue Impact Scale (MFIS) (15-17). Sleep disturbances could be the cause or result of depression in MS subjects, and both might also be associated with fatigue in these patients. It was also found that fatigue and daytime sleepiness are frequent in MS patients. Moreover, it was reported that anxiety and pain are the most common causes of initial insomnia, and nocturia is the most common cause of middle insomnia (11).

MS prevalence is higher in geographic regions with less sunlight exposure. Melatonin and vitamin D participate in the effects of sunlight in healthy individuals, and both could play a role in MS pathophysiology (18, 19). Melatonin (*N*-acetyl-5-methoxytryptamine) is a natural hormone mainly produced in the mammalian pineal gland during the dark phase. Maximal peak of melatonin's release is observed at darkness and minimum at lightness (20). However, in MS patients, increased serum proinflammatory cytokines and impaired immune cells circulating may disrupt circadian clock through decreased melatonin's production in dark phase (20, 21). With its lipophilic and hydrophilic character, melatonin freely crosses the blood-brain barrier and enters all cells (22). Melatonin and its metabolites have been demonstrated to possess multiple functions, including antioxidative, immunomodulatory, and anti-inflammatory (23, 24). The important role of melatonin as a modulator of sleep is well documented. Melatonin treatment can also entrain the circardian clock (25).

Lack of sunlight in the high altitudes cause melatonin level increase and triggering immune system. Produced during the night melatonin releases into retinal ganglion cells and stimulates Th17 cells and increase circulating levels of IL-17. It could be related with MS worsening (26). But, on the other hand this conflicts the other found result that melatonin can be neuroprotective and antinflamatory. Many papers proved that melatonin play a role in MS as an antioxidant, lipid metabolism regulator also, takes part in neurogenesis, immunomodulation and neuroprotection (23-25, 27-29) Interesting paper published by Farez *et al*. showed that melatonin shifts the immune response toward an anti-inflammatory state and lulling Th17 cells to sleep. It may explain the seasonal variability of multiple sclerosis disease activity (30). Moreover, positive melatonin effect on frequent MS clinical symptoms like fatigue, mood disorder, fluctuation of symptoms and clinical neurological state improvement was observed (27, 31, 32).

Recent studies have suggested that oxidative stress can play a crucial role in the pathogenic traits of MS. Processes responsible for generation of reactive oxygen species (ROS) and lipid peroxidation, hallmarks of oxidative stress, have been studied in attempt to develop therapies that can diminish or stop central nervous system (CNS) damage (33). When generation of ROS exceeds the ability of the endogenous antioxidant system to remove them, the oxidative stress in the brain occurs, subsequently leading to cellular damage.

Pathophysiology of chronic fatigue syndrome is completely unknown. Some previous papers documented that myalgic encephalomyelitis/chronic fatigue syndrome is characterized by activation of immune, inflammatory, oxidative and nitrosative stress mechanisms (34, 35). It was proved that lipid peroxidation is elevated in females with chronic fatigue syndrome (36). Moreover, myalgic encephalomyelitis/chronic fatigue syndrome is characterized by increased oxidative stress through plasma peroxide concentrations elevation (37).

Homocysteine is a sulfur-containing amino acid that is generated from an essential amino acid methionine. Elevated levels of homocysteine unrelated to immune activation, oxidative stress, or a deficiency in vitamin B_6, B_{12}, or folate were observed in patients with MS. On the other hand, unchanged homocysteine levels in MS patients compared to controls were also reported (38). Homocysteine causes production of free radicals and hydrogen peroxide, which could conceivably lead to increased plasma lipid hydroperoxides levels (39). Mental fatigue loading in humans leads to a decrease in blood levels of branched-chain amino acids inter alia homocysteine precursor - methionine (40).

The aim of the study was to determine the effect of melatonin supplementation - a substance regulating circadian rhythms, including sleep, on chronic fatigue syndrome in multiple sclerosis patients. Moreover, the aim of this work was to evaluate plasma lipid hydroxyperoxides (LHP) and homocysteine concentrations as a potential biochemical fatigue biomarkers.

MATERIAL AND METHODS

Patients

Into a case-control prospective study 102 MS patients and 20 healthy subjects matched for age and sex, observed in 2014 in the Department of Neurology in Zabrze, Medical University of Silesia, Poland were enrolled. The following studied groups were created:

- C group (control group): 20 healthy controls observed in our Department due to undiagnosed headaches. Controls were matched for age and sex with the study group.
- RRMS pretreated group: 21 patients with *de novo* diagnosed, according to the McDonald criteria (41), relapsing-remitting form of MS (RRMS), immunomodifying pretreated, without any immunomodifying MS treatment.
- RRMS INF-beta group: 52 patients with RRMS, diagnosed according to the McDonald criteria (41). All of them received interferons beta-1a applied once per week as an intramuscular injection (Avonex) or interferon beta-1b (Betaferon), injected subcutaneously every other day.
- SP/PP MS mitoxantrone group: 17 patients with secondarily progressive (SP) or primary progressive (PP) form of MS, diagnosed according to the McDonald criteria (41). All of them received 5 doses of mitoxantrone i.v. (12 mg/m^2/dose) administered quarterly.
- RRMS relapse group: 12 RRMS patients diagnosed according to the McDonald criteria (41) receiving INF-beta as immunomodifying MS treatment during relapse period, before conventional relapse steroid-therapy.

At least 3 months before the study patients did not use any vitamins, diet supplements, antioxidative substances, hormonal treatment and sleeping pills two weeks prior to the study.

Study protocol

The study was approved by the local Ethics Committee of the Medical University of Silesia (KNW/0022/KB1/130/12).

Informed consent was obtained from all individual participants included in the study. After obtaining informed consent, demographic data, and Kurtzke's Expanded Disability Status Scale (EDSS) (42), MRI examinations were performed in all MS patients at the beginning of the study, in accordance with standard clinical protocols. Neurological examination was performed by a qualified neurologist using the EDSS before the therapy and after its completion. Then, all MS patients were supplemented orally with melatonin, 5 mg per day, over a period of 90 days. Neurological examination (conducted by a qualified neurologist using the EDSS) and biochemical assays were performed before and after the melatonin supplementation.

Enzymatic assays

The blood samples were collected between 6.00 and 7.00 a.m., centrifuged and frozen until laboratory measurements.

Plasma lipid hydroxyperoxides LHP concentration was measured according to Sodergren (43) using ferrous oxidation in xylenol orange assay coupled with triphenylphosphine. Results were expressed as μmol/L.

Plasma homocysteine concentration was estimated according to enzyme immunoassay for the determination of homocysteine in serum or plasma. The peroxidase activity was measured after addition of substrate and the absorbance was inversely related to the concentration of homocysteine in the sample (Axis-Shield Homocysteine EIA, UK). Results were expressed as μmol/L.

Questionnaire

Fatigue was assessed by the Polish validated version of the Modified Fatigue Impact Scale (MFIS) (15). The MFIS is a self-reported questionnaire, assessing the level of symptomatic fatigue in the MS study groups. It consists of 21-item questionnaire tasks describing the effects of fatigue within physical (9-items), psychosocial (2-items) and cognitive (10-items) domains over a period of last month. Patients rated the 21 items on a 5-point Likert-type scale, ranging from never (0 points) to almost always (4 points). The overall score ranges from 0 to 84, and the higher the scores are, the higher the levels of fatigue are indicated. The MFIS evaluates the impact of fatigue on three dimensions of quality of life: physical, cognitive and psychosocial. Previous studies have reported that a total score of 38 demarcates fatigued from non-fatigued individuals (16, 17).

MRI examination

Head magnetic resonance imaging (MRI) was performed in all MS patients at the beginning of the study. The 1,5T scanner imaging (General Electric HDx USA) and standard head protocol for MS patients (multiple planes, slice thickness 5 mm, contrast media: Gadovist (Gd)) and additional postcontrast 3DT1 sequences (1 mm slice thickness) was used. Supratentorial, infratentorial and number of enhancing T1 plaques were evaluated.

Statistics

The results were expressed as means ± S.E.M. The Kolmogorov-Smirnov test was used for normal data distribution. Comparisons between groups were performed using the Mann-Whitney U-test and Wilcoxon test. Differences between means were considered statistically significant at $P < 0.05$. Correlations were calculated using Spearman's test. Results were statistically analyzed using STATISTICA v. 8.0 (StatSoft, Poland).

RESULTS

Demographic and clinical characteristics of studied subjects were presented in *Table 1*.

Results from our study show that LHP concentrations were significantly higher in all studied MS groups vs. controls (6.72 ± 3.26, $P = 0.002$; 11.6 ± 3.84; $P = 0.006$, 9.71 ± 3.49, $P = 0.000003$ versus 1.7 ± 1.9 μmol/L, in RRMS pretreated, RRMS INF-beta and SP/PP MS Mitoxantrone groups, respectively). In all MS patient groups melatonin application resulted in significant decrease in plasma LHP concentrations (*Fig. 1*). In RRMS relapse group plasma LHP concentrations were inconsistent and difficult to interpret (data not shown).

Plasma homocysteine concentration was similar in healthy people, RRMS pretreated, RRMS INF-beta and SP/PP MS Mitoxantrone groups (10.01 ± 1.55, 9.96 ± 2.76, 10.35 ± 3.23, 9.26 ± 2.12 μmol/L, $P > 0.05$, respectively). However, in the RRMS relapse group plasma levels of homocysteine were significantly higher compared to the RRMS pretreated group (12.03 ± 2.05 versus 9.96 ± 2.73 μmol/L, $P = 0.02$) and statistically higher compared to the control group (12.03 ± 2.05 versus 10.01 ± 1.55 μmol/L, $P = 0.02$) (*Fig. 2*). There were no significant differences in plasma homocysteine concentration in the studied groups before and after melatonin application.

Table 1. Demographical, clinical and brain MRI data of studied groups.

Group	Control	RRMS pretreated	RRMS INF-beta	SP/PP MS Mitoxantrone	RRMS Relapse
Subject numer (n) Total n = 122	20	21	52	17	12
Age (years) mean = S.D.	34.10±11.6	39.86±10.28	40.42±10.06	54.15±7.01	41.90±7.13
Female/Male Numer (n) (ratio)	13/7 (1.86)	15/6 (2.5)	33/19 (1.74)	11/6 (1.83)	8/4 (2)
EDSS mean ± S.D.	NA	1.85±0.95	2.92±1.24	5.68±1.51	3.96±1.98
Disease duration (years) Mean ± S.D.	NA	1.85±1.21	6.27±2.97	20.88±13.65	6.53±5.13
Treatment duration (months) mean ± S.D.	NA	NA	24.5±5.33	19.00±12.00	14.74±11.96
Number of brain MRI T2 lesions mean ± S.D.	NA	21.71±13.54	23.88±16.6	31.11±12.44	32.93±5.11
Number of brain MRI T1Gd(+) lesions mean ± S.D.	NA	0.5±07	1.66±1.15	0	1.96±0.83

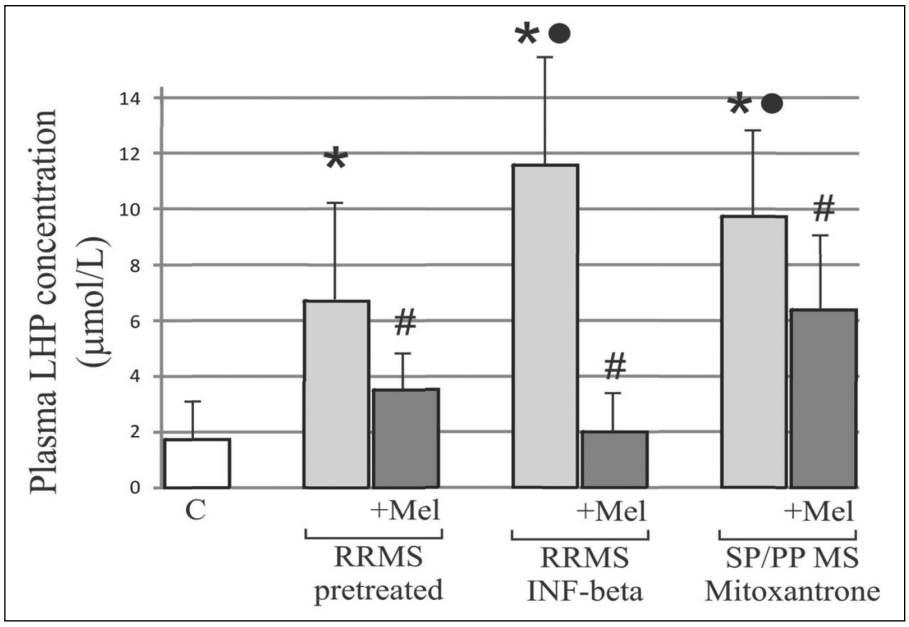

Fig. 1. Plasma lipid hydroxyperoxides (LHP) concentration (μmol/L) in control group (C), immunomodifying pretreated relapsing-remitting MS group (RRMS pretreated), interferons beta treated relapsing-remitting MS group (RRMS INF-beta), mitoxantrone treated secondary progressive/primary progressive MS group (SP/PP MS Mitoxantrone) before and after three months of melatonin treatment (+ Mel). Data presented as mean ± S.D.; * $P < 0.05$ vs. control group; # $P < 0.05$ vs. before Mel; • $P < 0.05$ vs. RRMS pretreated.

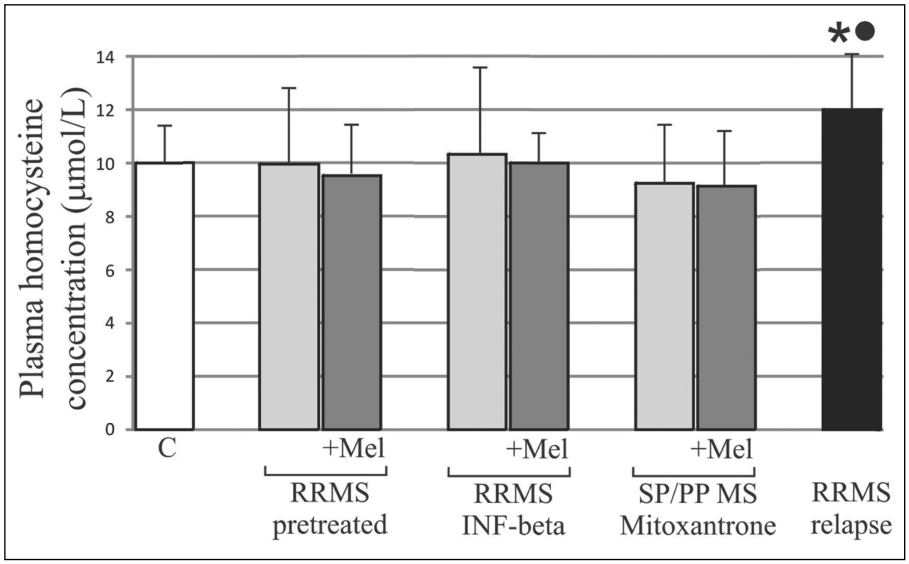

Fig. 2. Plasma homocysteine concentration (μmol/L) in control group (C), immunomodifying pretreated relapsing-remitting MS group (RRMS pretreated), interferons beta treated relapsing-remitting MS group (RRMS INF-beta), mitoxantrone treated secondary progressive/primary progressive MS group (SP/PP MS Mitoxantrone) before and after three months of melatonin treatment (+ Mel) and during relapse in relapsing-remitting MS group (RRMS relapse). Data presented as mean ± S.D.; * $P < 0.05$ vs. control group; • $P < 0.05$ vs. RRMS pretreated.

The degree of fatigue presented as MFIS score was significantly lower in RRMS pretreated group compared to RRMS INF-beta and SP/PP MS mitoxantrone treated patients (27.30 ± 12.31 versus 36.87 ± 15.94 and 49.77 ± 13.42 points, respectively). No statistical changes were found in the studied groups after melatonin treatment (*Fig. 3*). MFIS score was not evaluated during relapses in RRMS relapse group from obvious reasons.

We found no correlations between serum LHP concentration, plasma homocysteine level, MFIS score, MRI changes and EDSS.

DISCUSSION

Results from our study suggest that all MS patient both immunomodifying untreated and receiving interferons beta or mitoxantrone have significantly higher plasma lipid hydroxyperoxides levels compared to healthy subjects. Similarly, our previous papers documented that lipid peroxidation marker malodialdehyde concentration in serum and the cerebrospinal fluid are significantly higher in MS patients, in both *de novo* diagnosed RRMS and in the worsening MS form than in the controls (44, 45). Moreover, other studies reported a significant increase in lipid peroxidation products in the plasma, brain and cerebrospinal fluid in MS patients (46, 47). It is known that oxidative stress products, including lipid peroxidation products are neurotoxic, have proinflammatory properties, and could be involved in demyelination and axonal injury in MS (46). In our present study melatonin supplementation caused a significant decrease in plasma lipid hydroxyperoxides levels in all MS patients, arguing for melatonin's strong antioxidative properties and its beneficial, protective effects in MS subjects. Antioxidative properties of melatonin are well-documented. This indolamine could act indirectly by stimulating antioxidative enzymes, enhancing the activities of other antioxidants, and directly inactivating reactive oxidant species (23, 44, 45). Increase in plasma LHP levels in immunomodifying drugs - interferons and mitoxantrone results from oxidative stress mechanisms presence in this disease what could be confirmed by LHP increase in

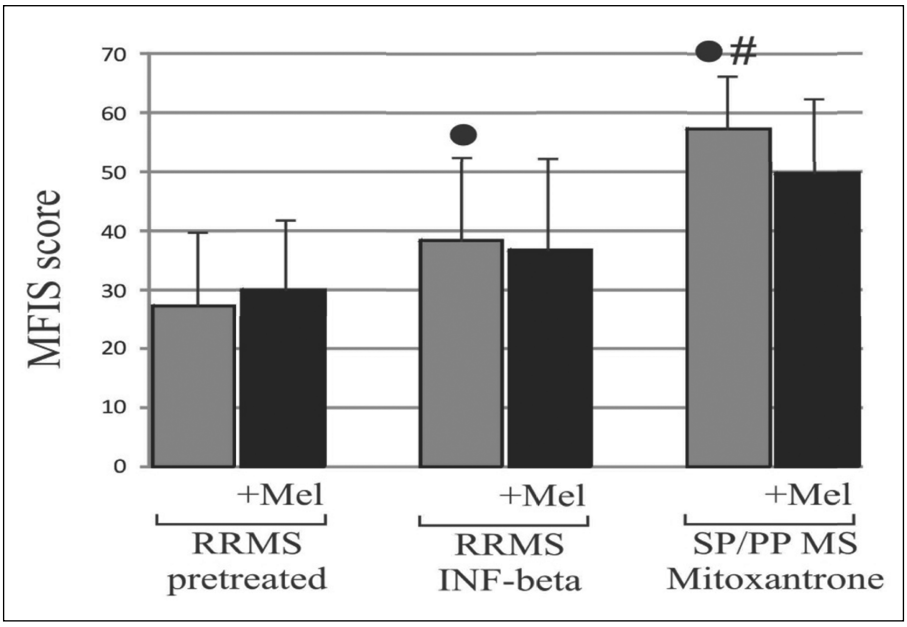

Fig. 3. Modified Fatigue Impact Scale (MFIS) score in immunomodifying pretreated relapsing-remitting MS group (RRMS pretreated), interferons beta treated relapsing-remitting MS group (RRMS INF-beta), mitoxantrone treated secondary progressive/primary progressive MS group (SP/PP MS Mitoxantrone) before and after three months of melatonin treatment (+ Mel). Data presented as mean ± S.D.; • $P < 0.05$ vs. RRMS pretreated; $^{\#}$ $P < 0.05$ vs. RRMS INF-beta.

RRMS pretreated group. Moreover, disease duration period could be connected with LHP level increase in interferons and mitoxantrone group. Furthermore, oxidative stress marker LHP could reflects inflammatory MS phase during RRMS, but not progressive forms what could be confirmed by active (Gd^{+}) brain MRI lesions in RRMS versus SP/PP MS group.

Previously, it was reported that lipid peroxidation is elevated in females with chronic fatigue syndrome (36). Moreover, it was proved that myalgic encephalomyelitis/chronic fatigue syndrome is characterized by increased oxidative stress through plasma peroxide concentrations elevation (37). These observations show increased ROS and oxidative stress in myalgic encephalomyelitis/chronic fatigue syndrome. Additional papers presented that myalgic encephalomyelitis/chronic fatigue syndrome is accompanied by lowered antioxidant serum/plasma levels like zinc, coenzyme-Q10 and vitamin E (48-50). In our study we noticed higher plasma lipid hydroxyperoxides levels and high fatigue level in MS patients compared to controls, but these values did not correlate with the fatigue score.

Fatigue is one of the most annoying symptoms reported by MS-affected people. There are several scales designed to estimate fatigue in MS, but it still seems to be difficult and too subjective to evaluate (16, 17). Many of the instruments have been developed as the 'golden standard' and the final choice depends on the aim of the research. According to our needs we used the MFIS tool. The results of our study present higher fatigue scores in immunomodifying MS patients than in immunomodifying pretreated group, but the highest fatigue scores were observed in the mitoxantrone administered SP/PP MS group. In other research conducted on a Polish group of MS patients the fatigue mean score was higher, than in the control group (51). It was in accordance with the MS patients' results obtained by Jamroz-Wisniewska *et al*. (52). Performed by Gruszczak *et al*., a Polish validation of the MFIS evaluated a fatigue mean score in general as 40.71 ± 14.94 (15). Flachenecker *et al*. reported a Fatigue Severity Scale (FSS) and MFIS as the most discriminative scales for fatigue (53). Results of the mentioned publications indicated a higher level of fatigue among MS-affected patients, than in control group, which confirms the findings of authors from other countries (54, 55). Significantly higher FSS scores occurred in patients with progressive MS, than in those with relapsing-remitting MS with median levels of 6.0 (4.8 – 6.6) and 4.9 (3.8 – 6.1), respectively (53). A similar connection was also confirmed by Kroencke *et al.* (56) and Bergamaschi *et al.* (57), while Pittion-Vouyovitch *et al.* showed that fatigue occurred independently of clinical forms of MS (58). In our research we reported that the highest fatigue score concern progressive forms of MS (SP/PPMS). It is probably associated not with direct drug effects, but with long disease duration and higher EDSS score in these patients. In our study there were no significant differences in mean MFIS scores in the studied groups before and after melatonin therapy.

Pathophysiology of chronic fatigue syndrome is completely unknown. Some previous papers documented that myalgic encephalomyelitis/chronic fatigue syndrome is characterized by activation of immune, inflammatory, oxidative and nitrosative stress mechanisms (34, 35).

Chronic fatigue syndrome is characterized by intense fatigue not improving by rest, neurocognitive dysfunction and poor exercise tolerance. Very frequent symptoms are muscle pain, as well as sleep and cognitive disturbances. Chronic fatigue syndrome occurs in about 80 – 90% of MS patients (1, 8, 9, 11, 12). It can be present at any stage of the disease and it affects many aspects of life, such as social functioning, employment, driving safety, physical independence, rehabilitation gains, disease management, treatment adherence, mood, and finally, a reduced quality of life (8, 9, 13). It was reported that melatonin supplementation improve mood, sleep and body mass in postmenopausal women with increased appetite (45, 59).

In our work we reported similar homocysteine levels in all MS patients groups compared to controls. Interestingly, we observed significantly higher homocysteine levels in the relapse MS group. This data suggests that homocysteine could be a potential marker of acute phase of MS. Recent studies showed elevated levels of homocysteine observed in patients with MS (60, 61). On the other hand, unchanged homocysteine levels in MS patients compared to controls were also reported (38). Homocysteine causes production of free radicals and hydrogen peroxide and, interestingly, this could conceivably lead to increased plasma lipid hydroperoxides levels (39). Mental fatigue loading in humans leads to a decrease in blood levels of branched-chain amino acids inter alia homocysteine precursor -

methionine (40). Neurons could be susceptible to homocysteine-induced excitotoxicity. Elevated homocysteine levels compromise methionine availability, which in turn interferes with methyl group donor. Myelin protein hypomethylation could result in less stable myelin structure susceptible to neurodegeneration (60, 61). Homocysteine as an aliphatic sulfhydryl compound could cause lipid peroxidation thanks to the ability to reduce atmospheric oxygen to hydrogen peroxide. Elevated plasma homocysteine level associated with increased plasma lipid hydroperoxides might trigger proliferation of smooth-muscle cells, promote oxidation of low-density lipoprotein, increase collagen synthesis and procoagulant activity, leading to development of vascular disease (39, 62).

Many studies have proved that melatonin and its' precursors act in immune response modulating, but specified mechanism of it is still unknown (63-65). This pineal hormone can have either suppressing or activating immune response, because it regulates Th1/Th2 balance (20, 21). Finally, interferons as well as melatonin are immunomodulators. Dysregulated melatonin secretion in MS patients may be influenced by interferons β. Even many years of interferons beta presence in MS treatment detailed mechanisms of their therapeutic actions are still unknown. It has been reported that interferons beta, has potential modulatory effects on costimulatory molecules present on dendritic and other cells, decreases antigen presentation, suppresses proliferation of the Th1 cells, increases expression of anti-inflammatory IL-10, change the inflammatory environment from proinflammatory to anti-inflammatory (66). Melamud *et al*. showed significantly decreased levels of melatonin secretion in MS and disrupted circadian clock, which were increased after interferons β therapy in relationship with improved fatigue (31). Moreover, other effective MS drug natalizumab caused an increase in serum melatonin concentrations in RRMS patients confirming the role of this monoclonal antibody in increase of antioxidants and a reduction in oxidative stress biomarkers (67).

Taking into account the hypothesis that serum/plasma lipid hydroxyperoxides and homocysteine concentrations could be potential biochemical chronic fatigue syndrome biomarkers we reported only increased plasma lipid hydroxyperoxides levels in all MS patients. What is important is that we noticed for the first time that plasma homocysteine level was elevated in the relapse patient group, suggesting that homocysteine could be a potential marker of acute phase of MS. Based on the decreased serum/plasma lipid hydroxyperoxides after melatonin supplementation, we proved it antioxidative actions in MS patients. On the other hand, melatonin had no effect on the serum/plasma levels of homocysteine and also on fatigue assessed by MFIS. Results from our study indicate that melatonin supplementation could be potentially beneficial in MS patients. This issue of melatonin is intriguing one which could be further explored in. Also, additional studies to test the biomarkers of chronic fatigue syndrome are required.

Conflict of interests: None declared.

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