COMPARISON OF OREXIN-A AND NEUROFILAMENT LIGHT CHAIN LEVELS IN PATIENTS WITH RELAPSING-REMITTING MULTIPLE SCLEROSIS: A PILOT STUDY

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Background and purpose – Multiple sclerosis is an autoimmune disease of the central nervous system, with myelin degeneration and Relapsing-Remitting Multiple Sclerosis (RRMS) as the most common type. The aim of this study was to determine the levels of Neurofilament Light Chain (NFL) and Orexin-A (OXA) in patients with RRMS and compare it with healthy control subjects' data.
Methods – In this case-control study of 61 subjects, serum and cerebrospinal fluid samples were collected from 23 RRMS patients and 38 healthy control subjects. NFL and OXA levels were determined in cerebrospinal fluid and serum samples using enzyme-linked immunosorbent assay kits. Self-reported questionnaires were also administered to evaluate fatigue severity and impact. Receiver operating characteristic curve analysis was used to determine the optimal cut-off value of NFL and OXA.
Results – The NFL and OXA concentrations in cerebrospinal fluid of RRMS patients were significantly higher than those of the control group ($p < 0.001$), but no significant difference was found in the serum concentrations ($p = 0.842$, $p = 0.597$, respectively). The cut-off values were found to be 1.194 ng/ml for NFL and 77.81 pg/ml for OXA in cerebrospinal fluid. A positive correlation was found between the Expanded Disability Status Scale and Epworth Sleepiness Scale in RRMS patients ($\rho = 0.49$, $p = 0.045$).
Conclusion – These results suggest that increased levels of both NFL and OXA in cerebrospinal fluid reflect neu-

OREXIN-A- ÉS NEUROFILAMENTUM-KÖNNYŰLÁNC FEHÉRJESZINTEK RELAPSZÁLÓ-REMITTÁLÓ SCLEROSIS MULTIPLEXBEN SZENVEDŐKNÉL: PILOT VIZSGÁLAT

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Háttér és cél – A sclerosis multiplex (SM) a központi idegrendszer autoimmun betegsége, aminek leggyakoribb típusa a relapszáló-remittáló forma (RRSM). A tanulmány célja az volt, hogy meghatározza a neurofilamentum-könnyűlánc- (NFL-) és az orexin-A- (OXA-) fehérjeszinteket RRSM-betegekben, és összehasonlítsa azokat egészséges kontrollszemélyek adataival.
Módszerek – Ebben az eset-kontroll vizsgálatban összesen 61 személytől (23 RRSM-beteg és 38 egészséges kontroll) gyűjtöttünk szérum- és cerebrospinalisfolyadék-mintákat. A szérum- és cerebrospinalisfolyadék-minták NFL- és OXA-szintjeit enzimkapcsolt immunszorbens esszékkel határoztuk meg. A vizsgálati alanyok fáradtságszintjük és annak életminőségre kifejtett hatásának meghatározása céljából kérdőíveket is kitöltöttek. Az NFL- és OXA-szintek optimális határértékének meghatározása érdekében ROC-görbe-analízist végeztünk.
Eredmények – A kontrollszemélyekkel összehasonlítva, az RRSM-betegek cerebrospinalis folyadékmintáiban szignifikánsan magasabbak voltak az NFL- és az OXA-koncentrációk ($p < 0{,}001$), de a szérumkoncentrációk között nem találtunk szignifikáns különbséget ($p = 0{,}842$, $p = 0{,}597$). A cerebrospinalis folyadékminták NFL- és OXA-szintjének optimális határértékei a következők voltak: 1,194 ng/ml (NFL) és 77,81 pg/ml (OXA). Az RRSM-betegek esetében pozitív korrelációt találtunk a kiterjesztett rokkantsági skála (EDSS) és az Epworth-féle álmosságskála pontszámai között ($\rho = 0{,}49$, $p = 0{,}045$).

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ronal destruction in RRMS. Further research of neurodegeneration should focus on neuropeptides to determine the possible roles in RRMS pathogenesis.

Keywords: *fatigue, multiple sclerosis, neurofilament light chain, orexin*

Következtetés – Eredményeink alapján a cerebrospinalis folyadék megemelkedett NFL- és OXA-szintjei egyaránt arra utalnak, hogy az RRSM-betegekben neuronalis destrukció zajlik. A neurodegenerációval kapcsolatos további vizsgálatoknak arra kell fókuszálniuk, hogy meghatározzák a neuropeptidek szerepét az RRSM patogenezisében.

Kulcsszavak: *fáradtság, sclerosis multiplex, neurofilamentum-könnyűlánc, orexin*

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), in which myelin degeneration and axonal damage are seen, caused by several inflammatory molecules activated by the autoimmune response[1]. Of the different types of MS based on the course of the disease, Relapsing-Remitting Multiple Sclerosis (RRMS) is the most common, characterized by attacks of neurological symptoms and periods of remission. Although symptoms vary among patients, fatigue is the most frequent symptom in MS patients and can be underdiagnosed[2, 3].

It has been previously shown that peptides can play a role in the pathogenesis of fatigue in patients with MS[4]. Orexin-A (hypocretin-1, OXA) is one of these peptides and the role of OXA has been reported in some studies in literature[5]. OXA is a hypothalamic neuropeptide that regulates energy homeostasis, feeding behavior, sleep-wake cycle; a deficiency of OXA causes narcolepsy[6–8]. *Papuc* et al. found a positive correlation between OXA level and fatigue level[5]. Orexins also have neuroprotective and immune-modulatory properties[9].

Neurofilaments are the structural parts of neurons, which are subdivided into light, medium, and heavy chain ones according to protein size[10]. Neurofilament Light Chain (NFL) is an important neurodegeneration marker and predictor of MS and higher levels in cerebrospinal fluid (CSF) have been found to correlate with disease progression[11–13].

These data suggest that NFL and OXA are good predictors of neurodegeneration and can be used to understand the pathogenesis of MS. While fatigue is a common symptom in MS, the mechanisms of this symptom are not well understood. Therefore, it was hypothesized that RRMS patients with fatigue could have lower OXA and higher NFL levels compared to control subjects. The aim of this study was to determine the relationship of these biomarkers with fatigue and sleepiness, which are the most common symptoms of RRMS. In addition, cut-off values were determined for NFL and OXA parameters in the diagnosis of RRMS, and correlations were examined of serum and CSF concentrations to ascertain if serum levels could be used as non-invasive diagnostic markers instead of CSF levels. These parameters could help to achieve better understanding of the neurochemical mechanisms of fatigue and the pathogenesis of RRMS.

Methods

STUDY DESIGN

A total of 61 subjects were enrolled in the study: 23 treatment-naive patients diagnosed with RRMS according to the 2017 revisions of the McDonald criteria[14] and 38 control subjects with no neurological diseases. All neurological examinations, cranial and spinal magnetic resonance imaging (MRI), and detection of the oligoclonal band in CSF were conducted for a definitive diagnosis of MS. The control group included elective cases who had no systemic or neurological diseases and were scheduled for surgery under spinal anesthesia. Patients were excluded if they had a previous diagnosis of MS, a history of cardiovascular disease, diabetes mellitus, hypertension, sleep disorder, or a body mass index (BMI) > 35 kg/m^2, or were aged under 18 or over 65 years.

Ethical approval for this study was obtained from the Clinical Research Ethics Committee of Mugla Sitki Kocman University (28/06/2018-10/III). This study was conducted in accordance with the ethical standards as laid down in the Helsinki declaration and its later amendments. Informed consent was obtained from all participants included in the study.

ASSESSMENTS

Disability in patients with RRMS was measured using the Expanded Disability Status Scale (EDSS) score[15]. The total EDSS score ranges from 0 to 10 and higher scores represent greater disability in RRMS. Fatigue and sleepiness of participants were assessed using validated questionnaires[16–18]. To evaluate excessive daytime sleepiness, the Epworth Sleepiness Scale (ESS) was applied. This is an 8-item questionnaire, with a possible maximum score ranging from 0 to 24. An ESS score of > 16 indicates greater sleepiness during daily activities[19]. Fatigue in participants was evaluated by using the well-validated scales of the Fatigue Severity Scale (FSS) and the Modified Fatigue Impact Scale (MFIS). FSS measures the severity of fatigue and was developed especially for use in neurological disorders. Each of the nine items in the scale is scored from 1 to 7 and the FSS score is calculated by using the arithmetic mean. A score of > 4.6 is indicative of severe fatigue[20]. The MFIS is a modified questionnaire of the Fatigue Impact Scale, indicating how fatigue affects the daily life of the patient[21]. The 21 items of the MFIS assess the impact of fatigue in respect of physical, cognitive, and psychosocial functioning.

BIOCHEMICAL ANALYSIS

All patients in the control group received standard monitoring with electrocardiography, non-invasive blood pressure, and peripheral oxygen saturation measuring in the operating room. A sedation protocol with 2 mg intravenous midazolam was applied before the spinal anesthesia. Under aseptic conditions, an anesthesiologist performed a lumbar puncture with a 25-gauge spinal needle and 2 ml of CSF were collected into polypropylene tubes. CSF samples of patients with RRMS were collected into 2 ml polypropylene tubes by lumbar puncture with a 25-gauge spinal needle by a neurologist. CSF samples were centrifuged in 1 hour at 1000 x g for 10 minutes. Venous blood samples were collected into blood tubes by venipuncture simultaneously with CSF samples. The blood tubes were centrifuged at 2000 x g for 15 minutes to separate serum. CSF and serum samples were transported to the freezer in one hour and stored at -86 °C until analysis.

CSF protein concentrations were determined by the turbidimetric method on a COBAS c702 analyzer (Roche Diagnostics GmbH; Mannheim, Germany). Neurofilament Light Chain (Cat# E4467Hu) and Orexin-A (Cat# E1296Hu) concentrations were measured in serum and in CSF using human-specific enzyme-linked immunosorbent assays (ELISA) (BT-laboratory, Shanghai, China) according to the instructions of the manufacturer. NFL assay sensitivity was 0.054 ng/mL with inter-assay and intra-assay coefficients of variation less than 10% and 8%, respectively. OXA assay sensitivity was 2.53 pg/mL with inter-assay and intra-assay coefficients of variation less than 10% and 8%, respectively.

STATISTICAL ANALYSIS

To determine whether there was a significant difference between patients and controls in terms of serum and CSF biochemical values, Wilcoxon-Mann-Whitney test was used. Summary statistics were expressed as minimum, maximum, median, first and third quartiles, and mean ± standard deviation. The correlation between variables was explored by using Spearman's correlation analysis. P-values less than 0.05 were considered statistically significant. All data analysis was performed by statistical software R (R Core Team, 2016). Receiver Operating Characteristic (ROC) curve analysis was used to determine the ability of the NFL and OXA in CSF to predict the demyelinating disease. We also used the same analysis to determine the optimal cut-off value based on Youden index. The area under the ROC curve (AUC) was used to determine the accuracy of these biomarkers. Higher AUC values indicate better test performance. A biomarker with AUC value is equal to 1 discriminates individuals perfectly as diseased or healthy. We used DeLong's method to estimate the AUC and its 95% confidence interval (CI). The 95% CIs were computed with 2000 stratified bootstrap replicates for sensitivities and specificities. Post hoc power calculation was performed using GPower 3.1 software.

Results

SUBJECTS

A total of 61 subjects (28 female, 33 male) were included in this study. The mean age of RRMS patients was 36.7 ± 9.7 years (range 22-55 years), while the mean age of the control group was 48.2 ± 14.5 years (range 20-65 years). Post hoc power calculations were applied and the sample size was seen to provide 0.982 power and 1.118 effect size for OXA at α error probability level of 0.05.

Table 1. *Comparison of CSF and serum parameters between RRMS patients and controls*

	Controls (n=38)	RRMS (n=23)	p-value
NFL_{CSF} (ng/mL)	0.78 ± 0.54	1.55 ± 0.40	
	0.72 (0.49, 0.92)	1.53 (1.24, 1.85)	*<0.001**
OXA_{CSF} (pg/mL)	62.68 ± 38.80	105.99 ± 38.64	
	59.79 (42.61, 72.35)	97.7 (76.5, 123.20)	*<0.001**
NFL_{SER} (ng/mL)	5.84 ± 10.68	7.57 ± 12.67	
	1.26 (1.04, 2.20)	1.46 (0.92, 2.77)	0.842
OXA_{SER} (pg/mL)	304.57 ± 488.62	369.18 ± 595.05	
	81.83 (55.64, 226.8)	79.61 (49.05, 385.5)	0.597
$Protein_{CSF}$ (mg/dL)	35.68 ± 13.89	31.98 ± 12.13	
	31.20 (27.55, 41.85)	27.60 (24.95, 37.70)	0.227

RRMS: relapsing and remitting multiple sclerosis, NFL: neurofilament light chain, OXA: orexin-A, CSF: cerebrospinal fluid, SER: serum
Data are presented as mean ± SD, median, and quartiles (25th–75th percentiles).
p-values were obtained from Wilcoxon-Mann-Whitney test.
* An italic p-value indicates a statistically significant difference between groups.

COMPARISON OF BIOMARKERS BETWEEN PATIENTS AND CONTROLS

The NFL and OXA concentrations in the CSF of RRMS patients were significantly higher than those of the control group ($p < 0.001$ for both, Wilcoxon-Mann-Whitney test, **Table 1**, **Figure 1** and **2**), but no significant difference was found in serum concentrations ($p = 0.842$, $p = 0.597$, respectively). No correlation was determined between serum and CSF biomarkers (all p-values > 0.05, Spearman's correlation). There was no evidence to suggest a significant difference in CSF protein concentrations between the groups ($p = 0.227$, Wilcoxon-Mann-Whitney test, **Table 1**).

CUT-OFF VALUES FOR RRMS DIAGNOSIS

ROC analysis was used to measure the diagnostic ability of NFL and OXA in CSF and to define the cut-off values for these biomarkers in predicting RRMS disease. The AUCs were 0.91 and 0.86 respectively for NFL and OXA in CSF. Three cut-off values based on local maxima of the ROC curves were determined for each biomarker to classify the condition. The cut-off values for NFL in CSF in predicting RRMS were 1.034 (sensitivity = 0.96, specificity = 0.79), 1.119 (sensitivity = 0.87, specificity = 0.84), and 1.194 (sensitivity = 0.83, specificity = 0.92). For OXA in CSF, the cut-off values were 72.76 (sensitivity = 0.78, specificity = 0.76), and 77.81 (sensitivity = 0.74, specificity = 0.84). The cut-off values giving the highest Youden Index, or equivalently, the highest Sensitivity + Specificity were 1.194 ng/mL for NFL in CSF and 77.81 pg/mL for OXA in CSF **(Table 2, Figure 3** and **4)**.

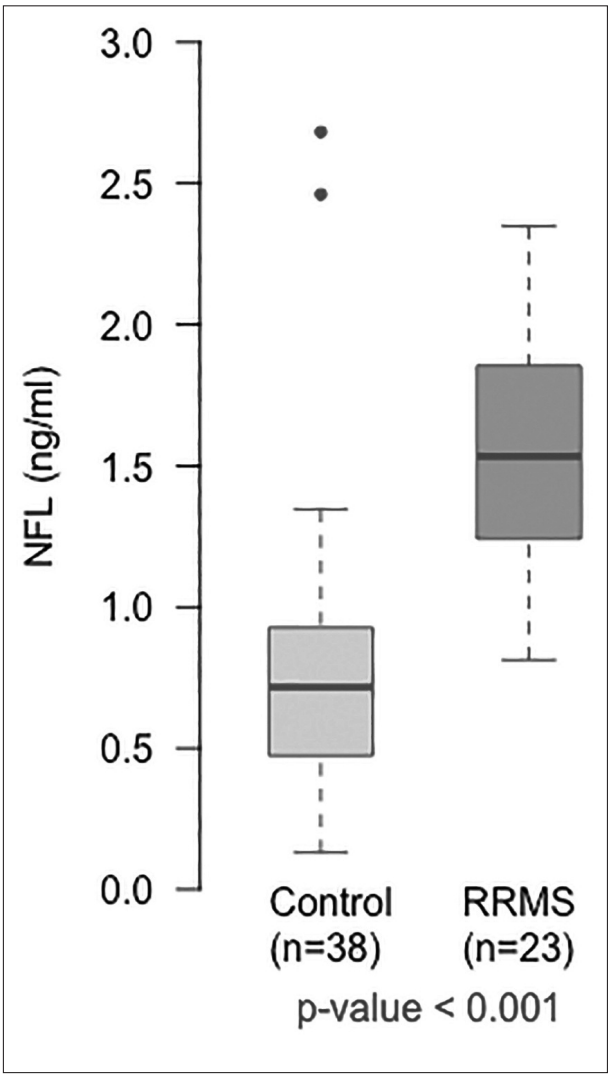

Figure 1. *Comparison of NFL levels in CSF between groups*

Table 2. *Optimal cut-off values that can be used to diagnose RRMS and their corresponding sensitivity and specificity values*

	AUC 95% CI	Cut-off	Sensitivity 95% CI	Specificity 95% CI
NFL (ng/mL)	0.91	1.03	0.96	0.79
	0.83 – 0.99		0.87 – 1.00	0.66 – 0.92
		1.12	0.87	0.84
			0.74 – 1.00	0.71 – 0.95
		1.19*	0.83	0.92
			0.65 – 0.96	0.82 – 1.00
OXA (pg/mL)	0.86	72.76	0.78	0.76
	0.77 – 0.95		0.61 – 0.91	0.61 – 0.89
		77.81*	0.74	0.84
			0.57 – 0.91	0.71 – 0.95

* The cut-off values giving the highest Youden Index, or equivalently, the highest Sensitivity and Specificity.

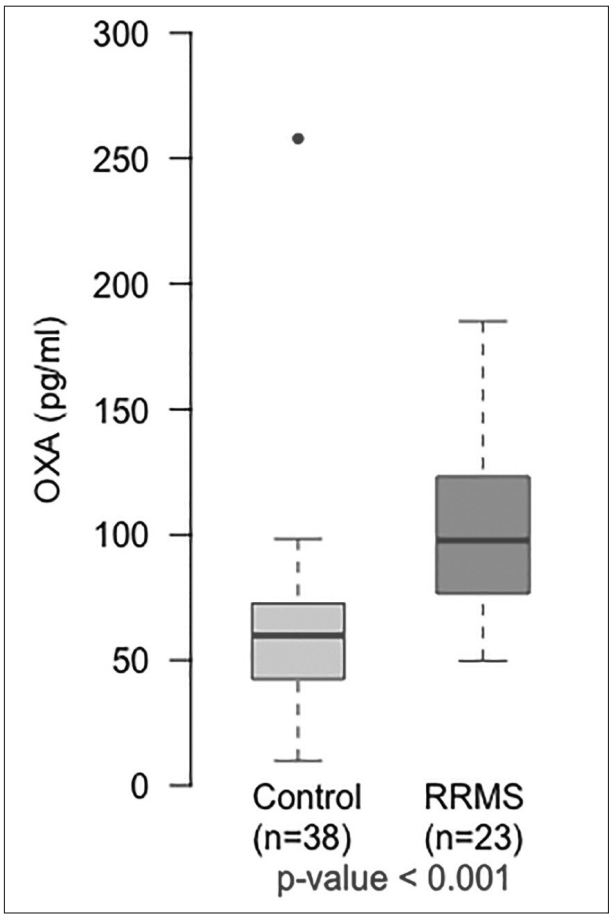

Figure 2. *Comparison of OXA levels in CSF between groups*

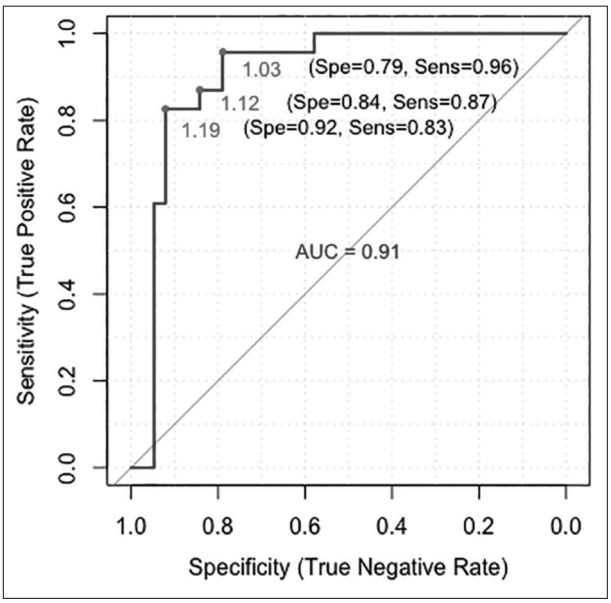

Figure 3. *The ROC plot of the NFL in CSF. Three best cut-off values for the NFL in CSF in predicting RRMS are marked on the graph; 1.034 (sensitivity = 0.96, specificity = 0.79), 1.119 (sensitivity = 0.87, specificity = 0.84), and 1.194 (sensitivity = 0.83, specificity = 0.92)*

FATIGUE AND SLEEPINESS SCALES

A positive correlation was determined between the EDSS and ESS scores in RRMS patients (rho = 0.49, p = 0.045, Spearman's correlation). No correlation was observed between the EDSS and other scales (MFIS and FSS). MFIS, FSSS, and ESS of 17 RRMS patients were determined. Fatigue was seen in 6 of the patients. There was no significant difference between RRMS patients with fatigue and those without, in terms of NFL and OXA concentrations.

MAGNETIC RESONANCE IMAGING

The McDonald criteria for demonstration of dissemination in space on MRI examination were fulfilled by 23 RRMS patients. Dissemination in space is defined as one or more lesions showing hyperin-

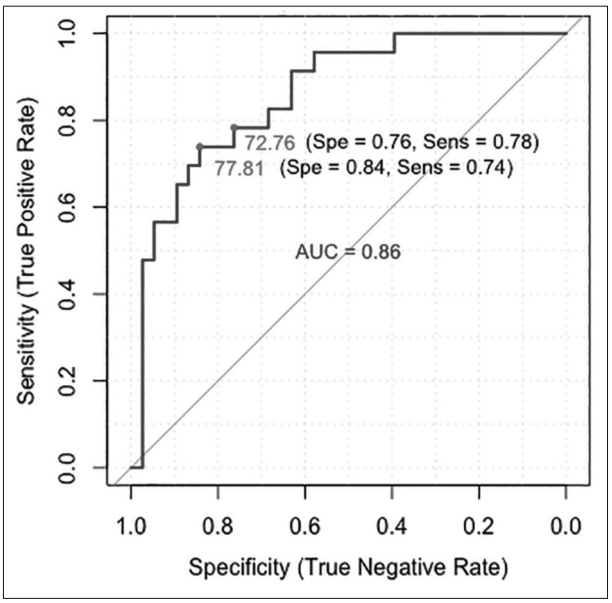

Figure 4. *The ROC plot of the OXA in CSF. Two best cut-off values for the OXA in CSF in predicting RRMS are marked on the graph; 72.76 (sensitivity = 0.78, specificity = 0.76), and 77.81 (sensitivity = 0.74, specificity = 0.84)*

tensity on T2-weighted images, which are characteristic of MS in two or more of four areas of the CNS: periventricular, cortical, or juxtacortical, and infratentorial brain regions, and in the spinal cord. No hypothalamic MS lesions were detected on the T2-weighted images of any patient.

Discussion

In this study, the neurochemical mechanisms of RRMS were evaluated by measuring OXA and NFL concentrations in CSF and serum. The most significant finding of the study was that the levels of both OXA and NFL in CSF were higher in the RRMS patients than in the control group. Axonal degeneration might increase NFL and OXA levels in CSF. No significant difference was found in serum levels. The trace levels of released NFL and OXA in CSF after axonal damage might not affect serum concentrations. Therefore, the use of serum biomarkers is of little value in the diagnosis of RRMS. The CSF levels of NFL and OXA can predict RRMS better than serum biomarkers, and cut-off values were determined in this study as 1.194 ng/ml for NFL and 77.81 pg/ml for OXA in CSF to diagnose RRMS. The low sensitivity of NFL and OXA cut-off values makes them difficult to use as reliable diagnostic markers. NFL and OXA levels have been studied separately in previous studies, but these biomarkers have not been evaluated together before. Further studies on neurodegeneration biomarkers should focus on the correlation of these peptides' levels in serum and CSF to be able to use the serum as a non-invasive diagnostic marker and more reliable cut-off values are needed for RRMS diagnosis.

NFL is considered a relevant biomarker of CNS degeneration and it increases in different neurological diseases such as stroke, dementia, and MS. The increase can be determined not only in the CSF but also in the serum, especially in progressive MS[22], but also in RRMS[23]. NFL levels in CSF and serum may be a good predictor of MS as described in previous studies[24–26]. These studies found that serum and CSF NFL levels correlated with disease severity and activity but serum NFL has been found to be less sensitive. In the current study, the higher NFL levels in CSF were in line with previous studies but no significant differences could be determined in serum NFL levels. This discordance could be attributed to the small sample size of this study and to the ELISA method. *Kuhle* et al. found that correlations between CSF and serum NFL levels were strongest for Simoa method and weaker for ELISA method[27]. In addition to neurodegeneration, metabolic alterations in the turnover of NFL may play a role. A significant correlation was found between serum NFL levels and age in previous studies[23, 28]. Cut-off values determined for the prediction of RRMS in the current study were similar to those reported by *Bhan* et al.[29].

Previous studies have demonstrated a correlation between CSF OXA levels and disease activity. *Gencer* et al. found decreased CSF OXA levels in MS patients compared to a healthy control group, and CSF OXA levels were negatively correlated with the progression index in RRMS[30]. The results of the current study were not in line with those findings. Gencer et al. did not compare RRMS with healthy control subjects and *Knudsen* et al. compared the attack and remission groups of RRMS and found no difference in CSF OXA levels[31]. These conflicting results of the current study and the literature may be attributed to the comparison of healthy control subjects and RRMS patients in the current study. It can be considered that OXA is another product of axonal degeneration which is prominent in the early phase of RRMS, and therefore, higher levels of CSF OXA could be a good predictor of neurodegeneration in RRMS.

OXA regulates the sleep-wake cycle and the association with fatigue in RRMS has been investigated in other studies. Papuc et al. found no difference between MS patients with fatigue and healthy control subjects, but a positive correlation was determined between CSF OXA levels and fatigue

severity in MS patients[5]. Another study of MS patients by *Constantinescu* et al. showed no correlation between fatigue and OXA levels in CSF[32]. In the current study, there was also found to be no difference in CSF OXA levels between fatigued and non-fatigued RRMS patients, but higher levels were found in RRMS patients compared to the healthy control subjects. These findings showed that OXA levels in RRMS patients were not associated with fatigue but could be used as a good predictor of neurodegeneration. Nevertheless, the relationship between OXA and fatigue remains uncertain.

In respect of the correlation of fatigue scores and NFL levels in RRMS, *Hakansson* et al. found no association between fatigue scores and NFL concentrations[33]. In the current study, there was also found to be no correlation between NFL, OXA, and fatigue scores. These results indicated that fatigue in RRMS had different mechanisms beyond the scope of these biomarkers. Further studies should focus on new biomarkers for the pathogenesis of fatigue in RRMS.

The main strength of this study was the well-designed comparison of a healthy control group with treatment-naive RRMS patients. However, there were some limitations to the study, primarily that fatigue and sleepiness were evaluated from the self-reported subjective scales of MFIS, FISS, and ESS in patients, and not in the control group. That fatigue may have been potentially under-reported by the patients may have caused bias in the study. A second limitation that could have affected the results was the relatively small sample size. Especially the number of RRMS patients with fatigue (n = 6) was not sufficient to be able to make a reliable statistical analysis of the biomarkers. Further prospective cohort studies with a larger number of patients are required to investigate the role of NFL and OXA in MS-related fatigue. Third, this was a cross-sectional study, which cannot describe the cause and effect relationship between biomarkers and clinical outcomes. Nevertheless, despite these limitations, this study can be considered of value in respect of the determination of cut-off values and differences between OXA and NFL in a comparison of RRMS patients and healthy control subjects.

Conclusion

Despite neuropeptides having been investigated in many clinical trials, the research on the subject of neurodegeneration in RRMS is still at an early stage. The results of this study demonstrated that increased levels of both NFL and OXA in CSF may reflect axonal degeneration in RRMS. However, NFL and OXA cannot be used as diagnostic markers, because of the low sensitivity of the cut-off values. In addition, relatively little is known about the turnover of these peptides in humans, limiting their potential use as a biomarker. These are markers of neuronal degeneration and certainly cannot be considered as diagnostic biomarkers, especially in a disorder such as MS where plenty of clinical and laboratory data are utilized for the diagnosis, such as clinical history, neurophysiological studies, MRI, and the presence of CSF oligoclonal bands. Further research of neurodegeneration should focus on neuropeptides to determine the possible roles of them in RRMS pathogenesis. These biomarkers may have also therapeutic potential in RRMS. The effect of treatment on these parameters should also be studied to be able to establish new treatment modalities based on these neuropeptides.

DECLARATION OF COMPETING INTEREST
The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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