

NORMAL-APPEARING WHITE MATTER
N-ACETYLASPARTATE CHANGES IN MULTIPLE
SCLEROSIS AS A POTENTIAL BIOMARKER

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Abstract

The use of an accessible and quantifiable biomarker provides an opportunity for research and monitoring of multiple sclerosis. The aim of the present study was to assess potential changes in N-acetylaspartate, creatine and choline levels in normal-appearing white matter and to investigate the sex-related distribution of these metabolites in relapsing remitting multiple sclerosis.

A total of 50 patients and 28 healthy controls were studied. Corresponding normal-appearing regions of white matter were examined by proton magnetic resonance spectroscopy in all participants. We were able to establish elevated levels of N-acetylaspartate, choline and creatine in the patients' group. The statistical analysis showed that there were significant differences in creatine levels between the two groups, whereas N-acetylaspartate demonstrated significantly higher PrMRS peaks in male RRMS subjects.

The results obtained during our study by PrMRS established metabolic changes in NAWM. We need to look at the underlying pathological process which is leading to demyelinating plaques. The gender impact also requires further investigation, and it can be inferred that PrMRS provides quantifiable measurement with characteristics of a potential biomarker.

Key words: biomarker, spectroscopy, metabolite, multiple sclerosis, cognitive impairment

Introduction. The use of an accessible and quantifiable biomarker provides an opportunity for research and monitoring of multiple sclerosis (MS). The disease affects predominantly young people in the age between 30–34 years. Females

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are twice as likely to develop the disease [1]. Conventional magnetic resonance imaging (MRI) is utilized currently in diagnosing MS [2, 3]. It detects scattered lesions in the brain tissue that have the characteristics of demyelination. Conventional imaging is not sensitive to the parenchymal changes outside the plaques of demyelination. There is microglia and astrocyte activation, and low intensity inflammation accompanied by axonal damage without markers of demyelination in normal-appearing white matter (NAWM). These pathological changes require the exploration of alternative imaging techniques. The National Institute of Health defines biomarkers as objectively measured and evaluated characteristics [3]. Various approaches have been used for the investigation of different biological processes and potential biomarkers which are representative of the pathogenesis of MS. Biomarkers that predict the disability progression, monitor the ongoing disease activity, and assess the treatment response are fundamental when it comes to making important decisions regarding the treatment of MS [4]. Proton Magnetic Resonance Spectroscopy (PrMRS) captures brain metabolites and changes in their concentration levels. It is the only method used specifically for capturing the damage of certain cell types [5]. Changes in brain metabolite concentrations might turn out to be a potential biomarker.

Methods and study population. We recruited subjects (in the age range between 20 and 40 years' old), with relapsing remitting multiple sclerosis (RRMS). The diagnosis was determined in accord with the revised criteria of McDonald [5]. The selection criteria focused on patients with a disease history of less than 10 years. The patients' group studied was compared to a group of 28 healthy individuals, selected according to the corresponding demographic criteria (Table 1). All participants signed a written informed consent to be enrolled in the study. We performed all the physical, neurological and PrMRS tests for each patient on a single day.

Proton Magnetic Resonance Spectroscopy. We applied PrMRS for measuring choline (Cho), creatin (Cr) and N-acetylaspartate (NAA) in parts per million (ppm). The Protocol for PrMRS was conducted, used in the Imaging Diagnostics Department of "St. Ivan Rilski" University Multiprofile Hospital for Active Treatment. The imaging was performed on a 1.5 Tesla machine (GE Signa HDxt). We studied frontoparietal zones of normal-appearing white matter, free of demyelinating lesions on conventional magnetic resonance T2-normal images in the patient's group. We selected corresponding subcortical zones of interest in the group of healthy controls. Proton magnetic resonance spectroscopy imaging data were acquired using a Point Resolved Spectroscopy localization sequence (echo time [TE] 104 milliseconds; repetition time [TR] 5000 milliseconds; number of excitations 2; section thickness [ST] 5 mm) with outer volume suppression bands.

Statistical analysis. The data obtained by means of PrMRS was analyzed with the Independent Samples *T*-test and The Mann–Whitney test. We used Pearson correlation analysis to evaluate the relationships between the metric vari-

T a b l e 1
Demographic data

Demographic data	Group*	Group**
Patients with clinically proven RRMS	N/A	+
Age between 20–40 years	33 ± 5	33 ± 7
Time elapsed since specifying the diagnosis up to 10 years [1, 5, 6]	N/A	4.85 ± 2.68
Treatment with DMT Interferon β -1a (years)	N/A	2.40 ± 2.21
Evidence of relapse over the last 6 months [7]	N/A	–
Corticosteroid administration at least 2 months prior to the study	N/A	–
Other autoimmune diseases	–	–
History of specified diagnosis depression, severe depressive episode and/or treatment with antidepressants for symptoms of depression found	–	–
Active inflammatory process	–	–
Treatment with another disease-modifying therapy	–	–
Motor deficit, visual or auditory disorders	–	–
Alcohol or psychotropic medications and substances abuse	–	–
Clinically healthy individuals	+	+
Participants	28	50
male	14	21
female	14	29
EDSS up to 3.5	N/A	1.75 ± 0.680
male		1.69 ± 0.715
female		1.79 ± 0.662
Education higher	21	30
Education secondary	7	20
Working activity full time	+	+

Group* – healthy controls; Group** – RRMS patients; Data are means $\pm$ Standard Deviation. RRMS – Relapsing Remitting Multiple Sclerosis, DMT – Disease modifying treatment, EDSS – Expanded Disability Status Score

ables, which include the Cho, Cr and NAA levels. For the sake of the statistical analysis the statistical software IBM SPSS Statistics v.23 was used. We considered a p value less than 0.05 statistically significant.

Results. We enrolled 50 patients with relapsing remitting multiple sclerosis in clinically stable condition (age 33 ± 7 years) and 28 healthy controls (age 33 ± 5 years). The average disease duration in RRMS group was 4.85 ± 2.68 years. The evaluation of the Expanded Disability Status Scale (EDSS) varied between 1.0–3.5. The disability assessment of the female subjects showed an EDSS of 1.793 ± 0.661 and the male subjects showed an EDSS of 1.690 ± 0.715 ($p = 0.6033$, $\alpha = 0.05$).

RRMS patients had DMT for 2.40 ± 2.21 years. The peripheral blood cell counts and the levels of C reactive protein (CRP) that were examined demonstrated an absence of an inflammatory condition.

The analysis of the metabolite data indicated a distinct change in metabolite peaks with higher concentrations of Cho, Cr and NAA in the RRMS group (Table 2).

T a b l e 2

Comparison between healthy controls and RRMS patients for Cho, Cr, and NAA mean values (Man-Whitney test)

Metabolite	Status	<i>N</i>	Mean	SD	<i>p</i> -value
Cho	Group*	28	78196.43	15798.724	0.122
	Group**	50	85682.92	20018.070	
Cr	Group*	28	61056.96	14334.289	0.025
	Group**	50	70227.98	17326.469	
NAA	Group*	28	153609.43	23048.165	0.151
	Group**	50	160673.88	28647.211	

Group* – healthy controls; Group** – RRMS patients; *N* – number of subjects, SD – Standard Deviation

There was a significant difference in the mean levels of Cr ($p = 0.025$, $\alpha = 0.05$). The morbidity risk doubles the frequency in female subjects according to MILANOV [5]. We evaluated for sex related differences in the mean values of N-acetylaspartate, choline and creatine (Table 3).

In this respect no significant difference between male and female subjects in both groups was established. In terms of statistical significance, we found increased levels of NAA ($p = 0.0195$, $\alpha = 0.05$) and of Cho ($p = 0.0428$, $\alpha = 0.05$) in male RRMS group. As for the remaining variables, there was no significant difference between healthy controls and patients with MS.

The analysed parameters play a significant role in metabolite homeostasis in the central nervous system. We assessed the correlation between the metabolite levels (Table 4). We were able to establish a moderate relation between NAA and Cho ($r = +0.472$, $*p < 0.05$) and a significant relation between NAA and Cr ($r = +0.563$, $*p < 0.05$) in the healthy controls group. Furthermore, we found significant relation between NAA and Cr also in the RRMS group ($r = +0.665$, $***p < 0.001$).

Discussion. The aim of our study was to explore NAA, Cr and Cho metabolite levels in NAWM in RRMS patients with a short course of disease in comparison with the same in healthy controls. The analysis of data revealed elevated levels of all the metabolites evaluated, where the study revealed a significant elevation of Cr. Our results are not fully in line with the findings of metabolite changes reported in other studies. ABOUL-ENEIN et al. [8] studied 27 RRMS

T a b l e 3

Comparison of the metabolite levels between male and female subjects in the groups and between the groups

Variable (ppm)	Mean	SD	<i>p</i> -value
NAA			
female**	157433.4	31393.91	0.3525
male**	165148.9	24383.65	
Cr			
female**	68298.24	17273.74	0.3601
male**	72892.86	17461.81	
Cho			
female**	82748.93	18962.71	0.2269
male**	89734.62	21180.99	
NAA			
female*	161989.7	21555.82	0.0524
male*	145229.1	22076.74	
Cr			
female*	60445.57	12671.71	0.8263
male*	61668.36	16290.24	
Cho			
Female*	81482.07	11398.83	0.2793
male*	74910.79	19119.58	
NAA			
female**	157433.4	31393.91	0.6276
female*	161989.7	21555.82	
Cr			
female**	68298.24	17273.74	0.1382
female*	60445.57	12671.71	
Cho			
female**	82748.93	18962.71	0.8193
female*	81482.07	11398.83	
NAA			
male**	165148.9	24383.65	0.0195
male*	145229.1	22076.74	
Cr			
male**	72892.86	17461.81	0.0645
male*	61668.36	16290.24	
Cho			
male**	89734.62	21180.99	0.0428
male*	74910.79	19110.58	

Group* – healthy controls; Group** – RRMS patients; SD – Standard Deviation

patients and compared them to 8 controls by assessing the metabolic pattern in the fronto-parietal NAWM. There was no significant difference of absolute NAA and Cr concentrations between RRMS patients and controls. No differences of Cho concentrations were found either. In a longer term study (up to 34 months)

T a b l e 4

Correlation between metabolites in healthy control and RRMS groups

		Healthy controls			RRMS patients		
		Cho	Cr	NAA	Cho	Cr	NAA
Cho	Pearson correlation	1	.302	.472*	1	.194	.254
	Significance level		.199	.011		.177	.075
	Number	28	28	28	50	50	50
Cr	Pearson correlation	.302	1	.563**	.194	1	.665**
	Significance level	.119		.002	.177		.000
	Number	28	28	28	50	50	50
NAA	Pearson correlation	.472	.563**	1	.254	.665***	1
	Significance level	.011	.002		.075	.000	
	Number	28	28	28	50	50	50

*** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$

SCHUBERT et al. [9] assessed 10 RRMS patients, treated with interferon β -1b. N-acetylaspartate, total creatine and choline-containing compounds were evaluated in unenhanced lesions and contralateral NAWM using MRS. The concentrations of Cho and Cr were higher in the MS patients than in the healthy controls. Besides a lower (insignificant) NAA concentration in lesions than in NAWM, there were no concentration differences between lesions and NAWM. The statistical analysis revealed stability of the metabolite concentrations over time, although they exhibited variability. The authors speculated it might be a result from the interferon β therapy. The processes preceding the lesion formation in NAWM according to the literature references stipulate an increase in Cho metabolites levels [5, 10]. Cho is essential for the structural integrity and the signal function of membranes. It is a vital component of the myelin sheath within the nerve fibres [5, 10, 11]. Months before the formation of a new plaque of demyelination PrMRS can detect a local increase in NAWM Cho level [5]. Elevated choline peak represents heightened cell-membrane turnover, as seen in demyelination, remyelination, inflammation, or gliosis [12]. Cr is a marker for energy homeostasis. Its normal level is accepted as stable. Further increase in Cho, Cr and lipids levels and decrease in NAA occurs after lesion formation [5]. N-acetylaspartate is considered a marker of viable neurons [13]. A diminished N-acetylaspartate peak signals a dysfunction or loss of neurons and/or of axons [5]. N-acetylaspartate synthesis takes place in the mitochondria. The metabolite is predominantly active as a neuronal osmolyte, a source of acetate for lipid and myelin synthesis, and a contributor to energy production from glutamate in neuronal mitochondria [13, 14]. The redistribution of NAA leads to the establishment of an osmotic environment, but it also passively removes water [13]. Elevated levels of NAA might reflect its molecular water pump importance for removal of neuronal metabolic water [15]. We enrolled RRMS patients, treated with Interferon β -1a. The study population had

no registered clinical activity for six months prior to the study. The positive effect of the DMT should impact the extent of inflammation in the NAWM. According to Schubert et al. [9] there should be no significant difference in metabolite concentrations, and especially in NAA levels. We found elevated concentrations of Cho and Cr in RRMS patients. N-acetylaspartate concentration was decreased in female RRMS patients. The findings correspond to references [5, 10, 12]. They represent ongoing structural impairment. There was an elevated concentration of NAA in RRMS group, due to male subjects. We could speculate about the role of elevated NAA. Structural changes in the neuron membrane in NAWM lead to changes in the ion homeostasis, the neuron cell osmolarity and the polarization of the membrane with consequent deterioration of neuron conductivity. Impaired electrical membrane properties demand subsequent increase in energy utilization. The elevated Cr and NAA levels reflect the increased cell demands for energy resources and activity. The point of exhaustion of compensating mechanisms marks the cell damage and activation of immune mechanisms. We might presume that the increased level of NAA signals a mechanism of adaptation and an attempt to overcome unfavourable structural changes leading to virtual hypoxia paired with active metabolite synthesis. There was not a significant difference in EDSS scores between male and female RRMS patients ($p = 0.6033$, $\alpha = 0.05$). Female subjects' disability assessment presented a slightly higher mean Expanded Disability Status Scale (EDSS) score. The data analysis revealed gender dependent difference in metabolite variations with significant increase in NAA and Cho concentrations in male RRMS patients. We might speculate that there is a gender determined mechanism in the pathogenesis of the structural changes. It needs further investigation. It interferes with metabolite concentrations and the relation between NAA and Cho. The PrMRS examinations detected changes in the metabolite pattern in NAWM. These changes imply a measurable indicator for early energy and structural variations leading to MRI detectable lesions and neuron-neuron communication predicament and disconnection [16]. N-acetylaspartate has the potential to evolve to a biomarker. A sensitive metabolite biomarker will remodel the diagnostic approach in MS [2]. It will allow early diagnosis and potentially better disability outcomes based on early changes in lifestyle [2]. Well-timed DMT will reduce brain atrophy and neurological deterioration [2, 17].

We present an original study on the NAWM metabolite pattern in RRMS patients treated with DMT, compared to healthy controls. The study applied a highly technological method to determine the metabolite profile of brain tissue without evidence from MRI of pathological changes. This is one of the large-scale studies on RRMS and HC according to the literature references examined. PrMRS provides a readily available non-invasive and safe method for a repeated quantitative brain tissue investigation in larger groups of MS patients. The identification of a model for the changes of metabolites with the potential for early diagnosis, disease follow-up and therapy efficacy evaluation will contribute to achieve lower

disability ratio and a better quality of life. In due course of time these benefits lead inevitably also to a positive economic effect in terms of patients being able to accept employments, having lower expenses for medical treatment, and needing less care from people in their immediate surroundings.

The main limitation of our work is the small number of participants. We did not perform morphometric magnetic resonance imaging for the evaluation of brain atrophy. However, we should emphasize the strengths of our study. We evaluated fifty MS patients on DMT with similar disease phenotype in a stable clinical state with at least six months relapse free history. The adjustment for confounding factors such as age, educational background and work type excluded the impact of these variables on the metabolite elevation, found in the RRMS group.

In conclusion, the PrMRS was successful in capturing metabolite variations in NAWM. The elevated levels of Cho, Cr, and especially of NAA signalled significant differences between the sexes in the subjects that were considered in the study. PrMRS offers an available, non-invasive, and safe method for exploring gender specific characteristics of parallel processes prior to demyelination. Longitudinal studies with neuroimaging investigation of metabolites together with morphometry designed to explore gender differences may help to elucidate mechanisms leading to demyelination.

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