Biocybernetics and Biomedical Engineering
2013, Volume 33, Number 1, pp. 21–32

NMR-based Metabonomics of Cerebrospinal Fluid Applied to Amyotrophic Lateral Sclerosis

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The aim of this study was applications of cerebrospinal fluid (CSF) NMR-based metabolic fingerprinting to amyotrophic lateral sclerosis (ALS) as possible early diagnostic tool. Two CSF sample categories were collected: 9 ALS patients and 13 age-matched control patients (without neurological disease). Metabolic profile of the CSF was determined by high resolution proton NMR spectroscopy. For statistical analysis magnitudes of 33 signals of the NMR spectrum were selected. Partial least square discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) modeling were used to find potential biomarkers of the disease. Those analyses showed that it was possible to distinguish the ALS patients from the control ones on the basis of the CSF metabolic profile. Significantly higher levels of metabolites observed in the patients with ALS may represent the state of anaerobic metabolism and excitotoxicity.

K e y w o r d s: cerebrospinal fluid, amyotrophic lateral sclerosis, NMR spectroscopy, metabolomics, discriminant analysis

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common type of the motor neuron disease in adults, affecting lower motor neurons and corticospinal tracts. Multiple mechanisms have been involved in the pathogenesis of motor neuron death in ALS. These include glutamate toxicity, mitochondrial dysfunction, protein misfolding, apoptosis, and other mechanisms [1]. Up to date, no biochemical marker was found to

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Received 10 April 2012; accepted 11 September 2012

diagnose ALS with required level of sensitivity and specificity [2–4]. In our research we looked for global biochemical differences that might distinguish the ALS patients from the control subjects [5–7].

Compared to other analytical techniques, NMR spectroscopy has special characteristics which make it uniquely suitable for the analysis of metabolite mixtures. NMR allows reliable detection and quantification of a wide range of metabolites, containing hydrogen present in complex biological fluids at micromolar concentrations. NMR is considered to be a non-destructive technique with low handling and slow preprocessing times. There are multiple examples in the literature of application of the NMR metabolomics to study central nervous system disorders, including a murine model of Huntington disease [8], studies of tissue and plasma in the rodent model of traumatic brain injury [9], and clinical studies of patients with schizophrenia [10].

The composition of CSF is directly dependent upon production rates of various metabolites in the brain; therefore, analysis of the CSF metabolome can offer biochemical insights into central nervous system (CNS) disorders, such as brain injury [11] or others [12].

The present study was carried out to assess the metabolic differences between the CSF samples of the patients with definite ALS, and the age-matched control subjects. In our study we used proton NMR spectroscopy combined with discriminant analyses, PLS-DA (Partial Least Square Discriminant Analysis) and OPLS-DA (Orthogonal Partial Least Square Discriminant Analysis) to evaluate potential use of this technique as an additional tool for diagnostic assessment [13].

2. Patients and Methods

2.1. Patients

Two groups of patients were analyzed: ALS patients (n = 9, 5 female and 4 male patients at mean age of 53±12, 11±5 months after diagnosis) and control subjects (n = 13). The diagnosis of definite ALS patients was made according to diagnostic criteria for ALS based on the El Escorial World Federation [14]. The study protocol was approved by the Medical University of Warsaw Ethics Committee and the informed consent form was obtained in accordance with the Declaration of Helsinki. The control group comprised of patients without neurological diseases who underwent vascular or inguinal hernia surgery under spinal anesthesia. The controls were matched by age with the ALS patients group.

2.2. Sample Preparation and Spectrum Acquisition

The CSF samples used for the examination were collected during diagnostic lumbar puncture or spinal anesthesia. The samples were centrifuged at room temperature at 15000 rpm for 5 min, and the supernatant was frozen at –80°C until the NMR

analysis was performed. The pH of the CSF was 7.8–8.2. The ^{1}H NMR spectra were acquired at 25ºC on a Varian Unity+ 500 NMR spectrometer (Varian Inc., USA) with operating frequency 500.6 MHz. In the NMR examination, standard pulse sequence was used with presaturation of the water signal. Each measurement consisted of 512 scans and 12s pulse repetition. To achieve stable lock signal, 100µl of D_2O was added to each sample and 3-trimethylsilyl propionate (TSP) was used as a reference signal (0 ppm and 1mM) for normalization of all spectra. The signals were assigned according to our own reference database and from literature [15–17].

2.3. Data Analysis

Quantities of metabolites were expressed as relative intensity (based on the magnitude of spectral peaks, and relative to the internal standard TSP). The measured signal magnitudes correspond to concentration of the compounds. All signals used were at least three times greater than the signal-to-noise ratio calculated for each spectrum.

Data reduction technique was applied to each NMR spectrum, using the targeted profiling method. Targeted profiling is an approach to data reduction that involves comparison to the NMR spectral signatures of individual metabolites found in a reference database. This technique works by reducing spectral data to quantified metabolites, which can then be used as input variables in pattern recognition tools such as projection to latent structures. Data normalization is an important step for any statistical analysis. The objective of data normalization is to allow meaningful comparisons of samples within a dataset.

Partial least squares (PLS) are a wide class of methods for modeling relations between sets of observed variables by means of latent variables. It comprises of regression and classification tasks as well as dimension reduction techniques and modeling tools. The underlying assumption of all PLS methods were that the observed data are generated by a system or process driven by a small number of latent (not directly observed or measured) variables. In this pattern recognition technique, a regression model is formed between the biochemical and class membership, allowing for selective removal of variables that do not contribute to class distinction. As part of this routine, the class membership of every seventh sample was iteratively predicted using jack-knifing and the results from this were used to generate a goodness of fit measure Q2 for the overall model: Q2 = (1–PRESS/SS), where PRESS is the predicted squared sum of error and SS is the residual sum of squares of the previous dimension. The goodness of fit is reported as the cumulative score across all of the components – Q2cum. This was used to determine whether the model had any predictive power and the predicted class membership better than chance. This jack-knifing routine was used if the data sets were too small to split the data into training and test sets – as in our case. The theoretical maximum is 1 for a perfect prediction. In order for a PLS component to be considered significant, Q2cum must be significantly larger than zero and is generally considered as good when equal or greater than 0.5. Mean-centering

was performed column-wise to remove the offsets. All the measured biochemical data were treated on an equal level with autoscaling (unit variance scaling), which is commonly applied and uses standard deviation as the scaling factor. After the autoscaling, all metabolites have standard deviation of one, and therefore the data is analyzed on the basis of correlations instead of covariances, as is in the case using the centering method [18]. Correlation coefficients from the PLS-DA were used to rank importance of each variable to further describe the differentially expressed biochemical data accountable for the separation between groups [19, 20].

Orthogonal signal correction (OSC) was the method initially developed for spectral data re-processing by Wold et al. [22]. Employing information in the response matrix **Y** (containing, in our case, class assignment – confirmed diagnosis), the strong systematic variation in the descriptor matrix **X** (containing, in our case spectral data) that is orthogonal (non-correlated) to **Y** can be identified. This variation, denoted as Y-orthogonal variation, can subsequently be studied and, depending on the problem at hand, discarded or retained. Despite the fairly unambiguous concept of OSC, a multitude of implementations occur in the literature [21–23]. OPLS [21] is an extension to the supervised PLS regression method featuring an integrated OSC-filter. In simple terms, the OPLS uses information in the **Y** matrix to decompose the **X** matrix into blocks of structured variation correlated to, and orthogonal to **Y**, respectively. The main benefit of interpretation using the OPLS-DA compared to the PLS-DA lies in the ability of the OPLS-DA to separate predictive from non-predictive (orthogonal) variation [24]. The OPLS-DA technique is capable of removing information unrelated to the response matrix **Y** (descriptor, e.g., 0/1) from an input matrix **X** (NMR spectral data) Thus, the resulting differential metabolites accountable for the discrimination between the two groups are most likely to be concentrated in the first predictive component.

The variable importance in the projection (VIP) value of each variable in the model was calculated to indicate its contribution of the X variables to the classification. Those variables with the VIP value greater than 1.0 are considered significantly different, and the larger VIP value of variable represents higher contribution to the discrimination between two groups.

In our study we use supervised methods of the PLS-DA and OPLS-DA analyses. For the statistical analysis 33 signals of the NMR spectrum (Fig. 1, Table 1) were selected. Since we could not distinguish in the NMR spectrum between Gln and Glu compounds because of the overlap between the signals, both signals were analyzed as one Glx. For univariate data analysis non-parametric Mann-Whitney U test was carried out. The statistical analysis was performed using the Statistica software (STATISTICA version 7.1, StatSoft, Inc., 2005.). A p-value of less than 0.05 was considered to be statistically significant. For multivariate analysis Bonferroni corrections for correlated variables was applied. The PLS-DA and OPLS-DA analyses were performed using the software package SIMCA-P (Version 12, Umetrics AB, Sweden) [25].

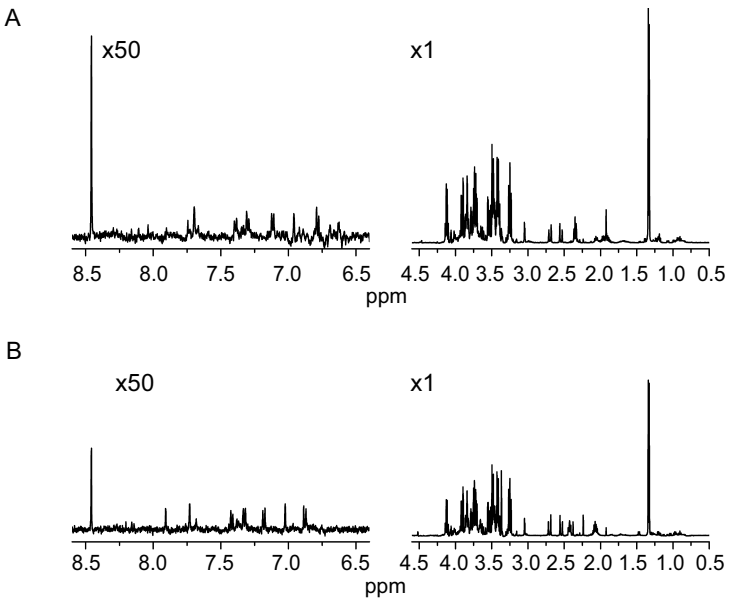

Fig. 1. Normalized proton NMR spectra of CSF samples – ALS patient (A) and control patient (B). Two parts of each spectrum were presented – aromatic region from 8.6 to 6.4 ppm (left) and aliphatic region from 4.6 to 0.5 ppm (right)

Analyzed signals: formate (8.47 ppm), histidine (7.73 ppm), phenylalanine (7.39 ppm), tyrosine (6.86 ppm), myo-inositol (4.07 ppm), glycine (3.52 ppm), scyllo-inositol (3.36 ppm), glucose (3.25ppm), choline (3.21 ppm), citrulline (3.16 ppm), creatinine (3.05 ppm), creatine (3.04 ppm), citrate (2.68 ppm), Glx (2.42 ppm, 2.14 ppm), pyruvate (2.38 ppm), acetoacetate (2.28 ppm), acetone (2.24 ppm), acetate (1.92 ppm), GABA (1.84 ppm), lysine (1.72 ppm), alanine (1.46 ppm), lactate (4.12 ppm and 1.34 ppm), γ-OH-butyrate (1.21 ppm), valine (1.03 ppm), isoleucine (0.96 ppm), leucine/α-OH-n-butyrate (0.90 ppm) and unassigned signals 7.68 ppm, 4.04 ppm, 4.01 ppm, 3.30 ppm and 1.28 ppm

3. Results

Concentrations of lactate, Glx and acetate were significantly higher in the ALS patients group as compared to the control group (Mann-Whitney test, $p < 0.01$) (Table 1).

In order to enhance identification of potential differences in biochemical composition of the CSF the PLS-DA and OPLS-DA modeling were employed, based on the unblinded patient's classification. The best results obtained by the PLS-DA (Fig. 2) consisted of two components (R2cum = 0.714, Q2cum = 0.462). The most important parameters (VIP > 1) that contributed to the class separation were the signals from acetate, GABA, Glx (both Glu and Gln signals), creatinine, myo-inositol, lactate, formate, choline and unassigned signals at 1.28 ppm, 4.04 ppm and 7.68 ppm. Mean concentrations of acetate, Glx, creatinine, myo-inositol, lactate, formate choline and

unassigned signal at 7.68 ppm were higher while mean concentrations of GABA and unassigned signal at 4.04 ppm and 1.28 ppm were lower in the ALS patients group as compared to the control group (Table 1).

Table 1. Concentrations of compounds presented in arbitrary units in mean ± SEM values, measured as signal magnitudes from normalized NMR spectra

Compound	Mean ± SEM [arbitrary units]			
	ALS	Control	p-value	VIP>1
formate	4683 ± 781	2687 ± 245	0.051	PLS-DA, OPLS-DA
histidine	1253 ± 321	905 ± 90	0.431	
unassigned 7.68ppm	343 ± 128	292 ± 48	0.917	PLS-DA
phenylalanie	1280 ± 249	742 ± 60	0.043	OPLS-DA
tyrosine	1115 ± 229	676 ± 74	0.021	OPLS-DA
lactate	**97154 ± 13143**	**53557 ± 6312**	**0.007**	PLS-DA, OPLS-DA
myo-inositol	15019 ± 2006	13605 ± 1610	0.431	PLS-DA
unassigned 4.04ppm	5573 ± 968	6843 ± 911	0.345	PLS-DA
unassigned 4.01ppm	12475 ± 1693	9050 ± 999	0.129	
glycine	65710 ± 9646	42578 ± 5397	0.030	
scyllo-inositol	21967 ± 6285	15709 ± 7360	0.110	
unassigned 3.30ppm	7254 ± 1500	5622 ± 521	0.896	
glucose	130624 ± 19108	83395 ± 9699	0.043	OPLS-DA
choline	3338 ± 644	1646 ± 175	0.021	PLS-DA, OPLS-DA
citrulline	7780 ± 1124	7687 ± 1200	0.794	
creatinine	25913 ± 375	22866 ± 2764	0.471	PLSA-DA
creatine	29678 ± 5264	18275 ± 1858	0.017	OPLS-DA
citrate	37510 ± 7547	26146 ± 2992	0.292	
Glx	**46873 ± 8948**	**21766 ± 2424**	**0.004**	PLS-DA, OPLS-DA
pyruvate	15830 ± 7216	19228 ± 2941	0.179	
acetoacetate	37510 ± 7547	26146 ± 2992	0.471	PLS-DA
acetone	10596 ± 6553	21545 ± 6079	0.025	
Glx	15356 ± 2878	16532 ± 1846	0.695	PLS-DA
acetate	**32400 ± 5456**	**11578 ± 2062**	**0.002**	PLS-DA, OPLS-DA
GABA	955 ± 614	1380 ± 96	0.350	PLS-DA
lysine	3633 ± 892	2046 ± 176	0.164	OPLS-DA
alanine	2115 ± 305	1445 ± 166	0.038	OPLS-DA
lactate	413868 ± 56611	239412 ± 25984	0.014	PLS-DA, OPLS-DA
unassigned 1.28ppm	2063 ± 309	2357 ± 134	0.110	
γ-OH-butyrate	9757 ± 4033	15817 ± 4982	0.357	
valine	2898 ± 337	2023 ± 225	0.060	OPLS-DA
isoleucine	4283 ± 541	3383 ± 317	0.209	
leucine/α-OH-n-butyrate	6959 ± 856	5797 ± 630	0.209	

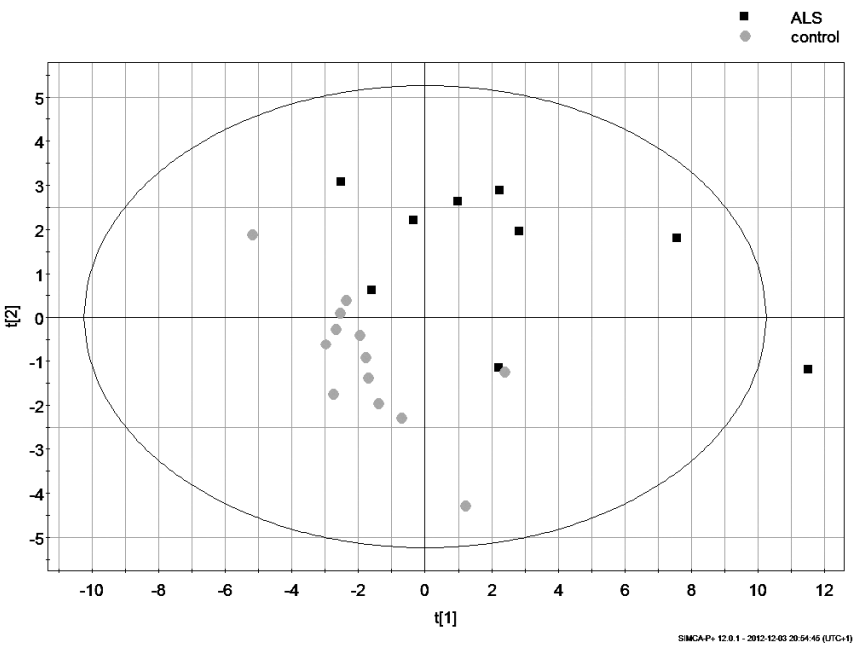

Fig. 2. The score plot of the PLS-DA model of the first two principal components t[1] and t[2]; t[1] represents the greatest amount of correlated variation in the data set, whereas t[2] represents the second greatest amount of correlated variation. Ellipse represents Hotelling T2 with 95% confidence in the score plots

Result of applying the PLS-DA in discrimination of the ALS samples from the control samples is shown in Fig. 2. In this model, 90.91% of all patients were assigned correctly to their groups – 7 out of 9 patients from the ALS group (77.78%) and all patients from the control group (100%).

The best result obtained by the OPLS-DA (Fig. 3) consisted of one predictive and one Y-orthogonal component (R2cum = 0.705, Q2cum = 0.428). The most important parameters (VIP > 1) that contributed to the class separation were signals from acetate, lactate, Glx, choline, formate, phenylalanine, glucose, creatine, valine, tyrosine, lysine and alanine. The concentrations of all those compounds were higher in the ALS patients group as compared to the control group (Table 1).

Both the PLS-DA and the OPLS-DA showed the same ability to distinguish the ALS patients from the control group. The OPLS-DA showed that 90.91% of the patients were assigned correctly to their groups, namely 7 out of 9 patients from the ALS group (77.78%) and all the patients from the control group (100%).

Validity of the models (PLS-DA and OPLS-DA) was tested for both groups of the patients using analysis of variance of cross-validated predictive residuals. Both presented models were valid for two groups ($p < 0.001$).

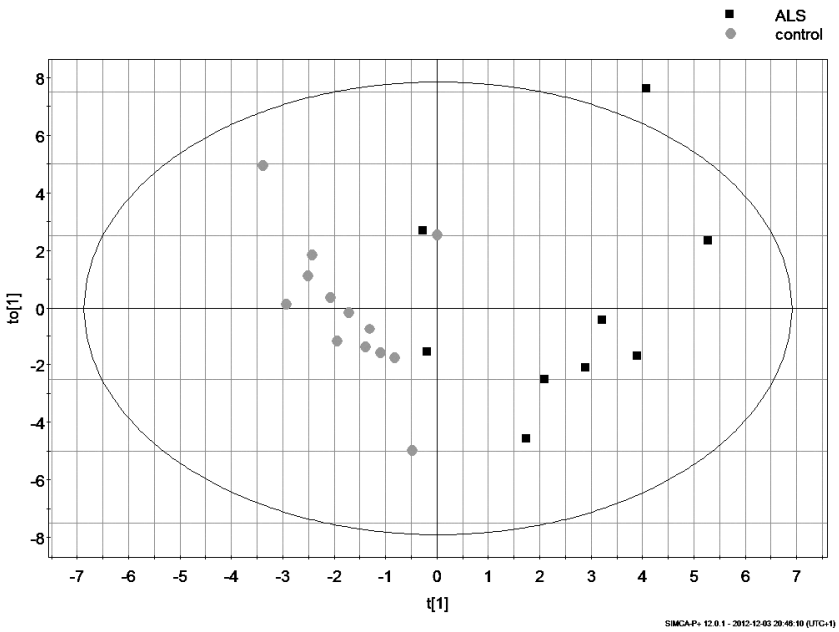

Fig. 3. The OPLS-DA score plot. t[1] is the predictive component and t_0[1] is the first orthogonal component. The ellipse shows the Hotellinger T^2-range (significance level 0.05)

4. Discussion

Non-parametric Mann-Whitney test revealed significant differences in lactate, Glx and acetate concentrations between the ALS patients and the control subjects. Further, the PLS-DA and OPLS-DA models to the CSF biochemical compounds distinguish the ALS and control subjects were applied. The most important compounds that contributed to the group separation using the PLS-DA model were: acetoacetate, acetate, GABA, Glx, creatinine, myo-inositol, lactate, formate and choline, and unassigned signal at 4.04 ppm, and 7.68 ppm. For the OPLS-DA model those compounds were: acetate, lactate, Glx, choline, formate, phenylalanine, glucose, creatine, valine, tyrosine, lysine and alanine.

Both statistical analysis methods (Mann-Whitney test and the PLS-DA/OPLS-DA) confirmed that presence and concentrations of acetate compound be the most important in distinguishing the ALS and control group. Acetate, lactate, Glx, choline, and formate play the "separating" role in both discriminant analysis models.

Significant increase in lactate concentration in the ALS patients group as compared to the control subjects may indicate lack of oxygen that leads to anaerobic respiration. Also, energetic insufficiency leads to anaerobic respiration [26]. Lactate level in CSF largely reflects its production by the brain. Abnormally low

glucose level causes rise in CSF lactate level, as the brain is forced to increase its anaerobic glycolysis in order to meet its metabolic demand [27]. In our study we observed significantly higher levels of both CSF lactate and glucose. Vijayalakshmi et al. [28] studied cells exposed to CSF obtained from ALS patients and observed increased anaerobic glycolysis and lactate production. Their studies show that the CSF from the ALS patients may induce enhanced anaerobic glycolysis or necrotic cell death. Our study confirmed their observation that is an increased lactate production.

Increase in acetate concentration in CSF leads not only to altered NAA metabolism but also to oligodendrocyte dysfunction. Since a major portion of acetate in the brain is utilized in fatty acid and lipid synthesis, the increased acetate concentration may suggest a decreased synthesis or increased degradation of myelin-related fatty acids and lipids in the brain. Elevated concentration of acetate could suggest a disturbance of carbohydrate metabolism. Increased acetate concentration in the CSF has been observed in patients with Creutzfeldt-Jakob disease [10]. Blasco et al. [29], who studied CSF from patients with early ALS, indicated a significantly lower level of acetate and higher level of pyruvate in the ALS patients group as compared to the group of patients with other neurological diseases. Kumar et al. [30] observed increased levels of pyruvate and acetate in the serum of ALS patients as compared to control group. Our study showed a significant increase in acetate concentration and non significant decrease of pyruvate level in the CSF of the ALS patients as compared to the control group.

Acetylcholine formation is limited by the intracellular concentration of choline, which is determined by active transport of choline into nerve endings. Choline is supplied to the neurons either from plasma or by metabolism of choline-containing compounds [31]. In our study, choline level was measured to be significantly higher in the ALS patients versus the control group. Choline is the main component of phosphatidylcholine and sphingomyelin, the two classes of phospholipids which are abundant in cell membranes. Choline is also the main metabolite of one of the neurotransmitters – acetylcholine, which reflects the activity of the central nervous system. Elble et al. [32] studied Alzheimer disease and found deficiency of acetylcholinesterase activity. They suggested that rise of choline level was related to neuronal membrane breakdown and reduced choline uptake by cholinergic neurons, and that the reduction in CSF acetylcholinesterase activity is consistent with the depletion of cholinergic neurons.

Results of our study show that significant increase of glucose and alanine levels indicate insufficiency of the glycolytic cycle. In the ALS patients group the citrate concentration was at the same level as in the control group. This observation suggests that normal concentration of acetyl coenzyme A (Acetyl-CoA) may be compensatory and no TCA (Tricarboxylic Acid) cycle perturbation occurred [33]. Results obtained by other authors [34] who studied CSF composition indicated that reduction in glycolytic energy supply can contribute to ALS pathogenesis. They also

suggested that this reduction can be the overriding cause of ALS related to neuronal cell death and depletion of intracellular ATP levels and may have resulted from the combined reduction of the glycolysis, TCA cycle and oxidative phosphorylation. Our study confirmed most of their findings.

Result of our study show that an impaired glycolysis process was the main biochemical abnormality. However, variables which lead to the differentiation of the groups in multivariate discriminant analyses included also amino acids that play crucial role in neurotransmission i.e. Glx and GABA.

Acute or chronic stimulation by the excitatory amino acids of the glutamate receptors may lead to neuronal death. This enhanced activation could be intensive in a short time period or subliminal in long period, and can be evoked by chronic stress, toxicity, electrolyte imbalance or micro stroke. Excitotoxic index is defined in terms of concentrations of glutamate × glycine/GABA [35]. We found increased Glx concentration that can be caused by elevated Gln and/or Glu concentrations. Glycine concentration was significantly higher and GABA concentration was lower in the ALS group as compared to the control group, which increased the excitotoxic index. In general, the results of our study support the hypothesis of the excitotoxic mechanism in ALS pathogenesis [36–39].

Studies of CSF by Shaw et al. [40] and previous studies have shown inconsistent results. The reasons for these discrepancies may have been partly caused by methodological factors – sample storage and preparation, detection methods – chromatography, and selection of the control group e.g. non neurological patients, healthy volunteers or non motor neuron disease neurological patients. In our study the NMR spectroscopy was used to determine differences between groups of the patients. In many of those studies only amino acids were measured except Blasco et al. [29] who also used NMR spectroscopy but the control group was different – patients with various neurological diseases as compared to our non-neurological patients.

5. Conclusions

We concluded that applying multivariate statistics to ^{1}H NMR data obtained from the CSF samples provide a useful screening tool. The PLS-DA and OPLS-DA models tested in this study were valid for the analyzed data and showed the feasibility of creating fingerprints for initial analysis of the motor neuron disease patients. The OPLS-DA and PLS-DA models allowed distinguishing the ALS patients group and the control group with 90.91% correct classification. Furthermore the analysis of biochemical compound contributed to the class separation show that significantly higher levels of metabolites observed in the patients with ALS may represent the state of anaerobic metabolism and excitotoxicity. Those processes were expected to be involved in motor neuron diseases.

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