SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Functional studies to assess the prognosis of amyotrophic lateral sclerosis

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1. INTRODUCTION AND BACKGROUND

Amyotrophic lateral sclerosis (*ALS*) is the most common adult-onset motorneuron disease, currently incurable, characterized by progressive motorneuron degeneration and consequent muscle atrophy, ultimately leading to patients being bedridden and eventually resulting in death. A distinctive feature of *ALS* is that the muscles involved in facial expression and eye movement remain unaffected throughout the disease course, and autonomic functions are also largely preserved, even in the later stages of the disease.

The incidence of *ALS* in Europe is approximately 2-3 cases per 100,000 people per year, while the prevalence is estimated at 5-7 per 100,000. There are significant regional differences in *ALS* occurrence, which are explained by multifactorial influences. The etiology of *ALS* is multifactorial, involving both genetic and environmental factors. There are two main types of *ALS*: *sporadic ALS (sALS) and familial ALS (fALS*). The pathogenesis of *ALS* is complex, involving multiple steps and affecting various cellular components. Known factors include mutant SOD1 proteins, mitochondrial dysfunction, and elevated glutamate levels, but the trigger that initiates the cascade leading to neuron degeneration remains unknown. Two main hypotheses exist regarding *ALS* progression: the "dying-back" theory suggests that cell death begins in the peripheral axons, whereas the "dying-forward" theory posits that degeneration starts in the cell body and spreads to the axons.

The diagnosis of *ALS* is often delayed due to the nonspecific nature of early symptoms, although certain neurological signs may raise suspicion of the disease. *ALS* can be classified into several subtypes based on the initial site and dominant characteristics of symptoms. The two most common forms are limb-onset (spinal) *ALS*, and less commonly, bulbar-onset *ALS*. A clinical diagnosis of *ALS* requires evidence of simultaneous, progressive damage to both upper and lower motor neurons. Neurophysiological examinations are essential in the diagnostic algorithm for *ALS*, particularly because clinical symptoms of upper or, more frequently, lower motor neuron damage may remain undetected during physical examination. *Electroneurography (ENG), electromyography (EMG),* and *motor evoked potentials (MEPs)* obtained by *transcranial magnetic stimulation (TMS)* play crucial roles. Additionally, other neurophysiological tests, such as *blink reflex* (*BR*) studies and single-fiber electromyography (*SFEMG*), are available for assessing motor neuron integrity, although their roles have not been emphasized in the investigation of motor neuron diseases.

Currently, no curative treatment is available for *ALS*; however, early diagnosis may provide an opportunity to alleviate symptoms and slow disease progression. Given these

considerations, the prompt diagnosis of *ALS* and determination of individual prognosis are particularly important, as they enable the timely initiation of appropriate supportive therapies and psycho-social support for patients and their families. In our current study, we investigated the significance of the *blink reflex* and *SFEMG* in *ALS*.

2. AIM OF THE STUDY

I. First, we aimed to analyze whether the ***blink reflex (BR)*** test is suitable for detecting **subclinical neurological impairments** that occur during the course of *ALS*, and whether it can contribute to a more precise mapping of the **pathomechanisms underlying the neurodegenerative processes** in *ALS*.

II. The second goal of our research was to **explore the correlation between the parameters of the response potenti*ALS*** recorded during ***BR*** testing and the **functional scales used** in *ALS*. We sought to identify *BR* parameters that could assist in **evaluating the prognosis of *ALS*.**

III. Thirdly, we aimed to identify and **analyze the reinnervation-denervation** processes involved in the development of *ALS* using ***single-fiber electromyography (SFEMG),*** with a particular focus on clinically unaffected muscles. This would facilitate early diagnosis and provide a more accurate assessment of the disease's pathomechanisms.

IV. Finally, we intended to investigate the correlation between ***SFEMG*** parameters and the functional scales used in *ALS*, searching for **biomarkers** that could aid in the prognostic assessment of *ALS*.

3. METHODS AND PATIENTS I. Blink reflex examination

3.1.1 Patients, databases

Our prospective studies were conducted at the Neurophysiology Laboratory of the Department of Neurology, Clinical Center, University of De*BR*ecen, between June 1, 2018, and June 31, 2022. During this period, *blink reflex* (*BR*) testing was performed on 29 patients diagnosed with definitive *ALS*.

Prior to the *BR* tests, we analyzed the demographic data and clinical characteristics of the patients. We assessed the patients' age, gender, and recorded the onset and localization of the first clinical symptoms indicative of *ALS*. A detailed neurological examination was performed on all patients before the *BR* tests. To evaluate the *BR* test parameters in the context of the patients' clinical condition, we used the revised Amyotrophic Lateral Sclerosis Functional

Rating Scale (*ALSFRS-R*) score and the Medical Research Council (MRC) muscle strength scale, specifically targeting the extensor digitorum muscle (ED), to assess the functional status of *ALS* patients.

Patients with any additional neurological or non-neurological conditions that could potentially cause central or peripheral nervous system damage, thus influencing the results of the *BR* tests, were excluded from the analysis.

During the tests, patients were seated in a reclined position in a chair or lying on their backs on the examination table. The tests were conducted in a quiet, electrically shielded, temperature-controlled room in the Neurophysiology Laboratory of the Department of Neurology.

Ethical approval was obtained from the Regional and Institutional Ethics Committee (DE KK RKEB/IKEB: 5036-2018).

3.1.2.Blink reflex examination

BR tests were performed with a dual-channel Nicolet Viking Quest EMG device (Nicolet Biomedical Inc., Madison, WI, USA). The reference electrodes used were surface plate electrodes, while rod-mounted surface electrodes were used for stimulation. The active reference electrode was placed on the lower portion of the orbicularis oculi muscle, just above the lower eyelid. The indifferent electrodes were positioned on the lateral side of the nose on both sides of the face. The supraorbital *BR*anch of the trigeminal nerve was stimulated transcutaneously with a cathode, separately on each side of the face, approximately 1–2 cm from the midline of the eye. To avoid artifacts caused by the spread of stimulation, the anode was attached at an oblique angle and laterally displaced from the contralateral supraorbital nerve.

To minimize habituation, a supramaximal stimulus of 0.2 ms duration and 0.5 Hz frequency was applied. The stimulation intensity was gradually increased in 1 mA increments until a reproducible and stable *R1* wave appeared. At least 8–10 *BR* responses were recorded from both sides of the face, determining the shortest response latencies. During the *BR* test, both the early *R1* and late *R2* waves were recorded from both sides of the face; the *R2* waves were recorded ipsilaterally and contralaterally relative to the stimulation when they could be elicited.

3.1.3. Statistical analysis

For statistical analyses, we used GraphPad Prism 8.2.1 and Microsoft Office Excel 2019 software. Initially, normality tests were performed; if the data followed a normal distribution, an ANOVA (analysis of variance) test was conducted. If the data did not show a normal

distribution, a Kruskal–Wallis test with multiple comparisons was performed. When comparing two groups, the Mann–Whitney test was used for non-parametric variables, while the t-test was applied for parametric variables. For age comparisons, either the t-test or
ANOVA was utilized, while other parameters were assessed using Kruskal–Wallis or Mann–Whitney tests. Results were considered statistically significant at $p < 0.05$.

II. SFEMG examination

3.2.1 Patients, databases

Our prospective studies were conducted at the Neurophysiology Laboratory of the Department of Neurology, Clinical Center, University of Debrecen, between June 1, 2018, and June 31, 2022. During this period, single- fiber EMG (*SFEMG*) testing was performed on 26 patients diagnosed with definitive *ALS*.

Prior to the *SFEMG* tests, we analyzed the demographic data and clinical characteristics of the patients. We assessed the patients' age, gender, and recorded the onset and localization of the first clinical symptoms indicative of *ALS*. A detailed neurological examination was performed on all patients before the *SFEMG* tests. To evaluate the *SFEMG* test parameters in the context of the patients' clinical condition, we used the revised Amyotrophic Lateral Sclerosis Functional Rating Scale (*ALSFRS-R*) score and the Medical Research Council (MRC) muscle strength scale, specifically targeting the extensor digitorum muscle (ED), to assess the functional status of *ALS* patients. Patients with any additional neurological or nonneurological conditions that could potentially cause central or peripheral nervous system damage, thus influencing the results of the *SFEMG* tests, were excluded from the analysis. During the tests, patients were lying on their backs on the examination table. The tests were conducted in a quiet, electrically shielded, temperature-controlled room in the Neurophysiology Laboratory of the Department of Neurology.

Ethical approval was obtained from the Regional and Institutional Ethics Committee (DE KK RKEB/IKEB: 5036-2018).

3.2.2 SFEMG examination

In all cases, a 25 μm diameter disposable SF EMG recording needle electrode (Spes Medica, Genova, Italy 0.45 (26G) x 37mm) was used, and the responses obtained during the study were evaluated using the 9031A006401 Keypoint Clinical System (Natus Medical, Pleasanton, CA, USA). During *SFEMG*, in each case, *jitter* was recorded, 20 AP potential pairs were measured

and recorded from different parts of the muscles under study, using at least 3-4 punctures, and the percentage of elongated *jitter* was determined, the percentage of any block was recorded, and the *fiber density* (*FD*) was also recorded. Taking into account that the mimic muscles were spared during the course of *ALS*, *SFEMG* was performed in the musculus frontalis of 8 patients and in the musculus orbicularis oculi of 7 additional patients. The limited number of subjects was due to the fact that needle examination of the facial muscles is considered to be particularly painful, and as a consequence, some of the patients did not agree to undergo needle examination of the musculus frontalis and orbicularis oculi.

3.2.3 Statistical analysis

Statistical analyses were performed using the SPSS for Windows 19.0" software package (SPSS Inc. Chicago, USA) to evaluate the results of the *SFEMG* studies. Multivariate ANOVA tests and pairwise comparisons were used to examine the associations between age, sex and disease duration, and *ALSFRS-R* and MRC score in different subgroups of *ALS*. Data obtained from the *SFEMG* study were compared with those in the normal range using the Mann-Whitney test. Where a normal distribution was found, the Student's t-test was used. In addition, the correlation between the results of the functional tests and the *SFEMG* parameters was evaluated using Spearman's rank correlation test. The results obtained were considered significant at $p < 0.05$

4.Results

I. The role of BR testing in the diagnosis and prognosis of *ALS*

4.1.1. General characteristics of patients included in the BR study

Prior to the study of *ALS* patients, a total of 50 subjects, 23 women and 27 healthy male volunteers (22-75 years old, mean age 50 years), were tested with *BR* in order to create a laboratory-specific control group. In addition, patients with Bell's p*ALS*y (N = 27), myasthenia gravis (ocular or generalized form) (N = 9) and diabetic polyneuropathy (N = 25) were also studied to compare results (total N = 61).

Of the 29 *ALS* patients studied, 12 were female with a median age of 65 years (47-74 years) and 17 were male with a median age of 63 years (36-84 years). Two groups were created based on the localization of symptom onset. Of the patients studied, 6 patients had *ALS* symptoms with bulbar onset, while 19 patients had *ALS* with limb onset. The time from disease onset to the time of the study was 7 months in the bulbar group and 6.79 months in the group with limb

onset. A separate group included 4 patients with severe disease in whom the localization of the initial symptoms could not be clearly determined at the time of *BR* examination. When considering the different subgroups of *ALS* (bulbar onset, limb onset), significant differences were observed with respect to age (p=0.004). In all *ALS* patients, progression of neurological symptoms was detectable during the study period.

On neurological examination, the facial muscles were intact in all *ALS* patients during the study period. During the study period, 8 patients died. *ALSFRS-R* scores of the *ALS* patients were recorded prior to the *BR* study. There was no significant difference in *ALSFRS-R* scores between the bulbar and limb onset groups. In the four severe cases, *ALSFRS-R* scores were worse and the duration of disease before the study was significantly longer ($p<0.0001$).

4.1.2. BR test results in ALS and other peripheral neurological diseases

In all *ALS* patients studied, statistically significantly prolonged ipsilateral and contralateral *R2* response latencies were observed compared to healthy controls ($p <0.001$). In contrast, there was no statistically significant difference in *R1* response latencies (p left side = 0.12, p right side = 0.18) in *ALS* patients compared to healthy controls. The healthy controls were significantly younger, so the healthy control group was further subdivided by age and analysed: there was no significant difference in the results between patients under 65 years and those over 65 years ($p = 0.2$). In four cases, the diagnosis of *ALS* was confirmed at an advanced stage of the disease; during *BR* examination of these patients, their contralateral *R2* waves were completely absent and could not be quantified, but it is also important to record and interpret the missing responses for the diagnosis of *ALS*, as the missing *R2*c responses indicate the importance of *BR*ainstem interneurons. With respect to other neurological pathologies, the latency of *R1*, ipsilateral *R2* and contralateral *R2*c responses increased in Bell's p*ALS*y when stimulated on the same side as the lesion compared to the control group.

All R-wave latencies were longer in myasthenia gravis and diabetic polyneuropathy, but in *ALS* only *R2*i and *R2*c differed significantly compared to the control group. Therefore, the difference between *ALS* and the other diseases studied was in terms of *R1* response.

4.1.3. Examination of the correlation between BR parameters and ALSFRS-R

Spearman rank correlation was calculated to evaluate the relationship between *ALSFRS-R* and *BR* latencies. We found a negative correlation between the variables. For all patients with *ALS*, the following results were obtained: *R1* left latency: $\rho = -0.95$, $p < 0.0001$, *R2*i left latency: $\rho =$

-0.61, p = 0.001, *R2*c left latency: ρ = 0.82, p < 0.0001, *R1* right latency: ρ = 0.90, p < 0.0001, *R2*i right latency: ρ = 0.83, p < 0.0001, *R2*c right latency: ρ = 0.80, p < 0.0001, the results show that there is a very strong negative correlation between the left *R1* latency and *ALSFRS-R*, while strong positive correlations are observed for the right latencies. For patients with bulbar onset, the following results were obtained: *R1* left latency: ρ = 0.90, p = 0.005, *R2*i left latency: ρ = 0.93, p = 0.002, *R2*c left latency: ρ = 0.84, p = 0.017, *R1* right latency: ρ = 0.96, p = 0.0008, *R2*i right latency: ρ = 0.94, p = 0.002, *R2*c right latency: ρ = 0.97, p = 0.0004, the results suggest that there is a strong positive correlation between each of these latencies and the *ALSFRS-R* for *ALS* patients with bulbar onset. The results for patients with limb onset were as follows: left latency *R1*: ρ = 0.99, p < 0.0001, left latency *R2*i: ρ = 0.96, p < 0.0001, left latency *R2*c: ρ = 0.98, p < 0.0001, right latency *R1*: ρ = 0.98, p < 0.0001, *R2*i right latency: ρ = 0.97, p < 0.0001, *R2*c right latency: ρ = 0.97, p < 0.0001, these results suggest that there is a very strong positive correlation between each of these latencies and the *ALSFRS-R* for patients with limb-onset *ALS*.

II. The role of SFEMG in the diagnosis and prognosis of ALS

4.2.1. General characteristics of patients included in the SFEMG study

Of the 26 *ALS* patients included in the *SFEMG* study, 12 were women with a median age of 62 years (45-78 years) and 14 were men with a median age of 60 years (39-84 years). The healthy control group (N = 26) consisted of 12 women and 14 men, matched by age and sex to the study sample. Two groups were formed based on the first clinical signs of *ALS*. Of the patients studied, clinical symptoms started in the bulbar region in 8 cases, while in 18 patients clinical symptoms first appeared in the limbs. The average time from the first symptoms to the time of examination was 6.5 months in the bulbar group and 5.9 months in the limb group. No statistically significant differences were found between subgroups in terms of gender distribution and duration of disease. On average, patients in the bulbar group were more than 10 years younger than those in the limb-onset *ALS* group.

Considering that *ALS* symptoms are typically asymmetric, we created additional subgroups within the limb onset category to compare our data. These subgroups included patients whose right upper limb (RUL), left upper limb (LUL), right lower limb (RLL) and left lower limb (LLL) were affected first during the clinical presentation of *ALS*. All our study patients were right-handed, so we did not create dominant - and non-dominant limb groups. Based on *ALSFRS-R*, no statistically significant difference was found when comparing LUL

and RUL groups. However, significant differences were observed when comparing the LUL group with all other subgroups. Similarly significant differences were found between the RUL groups. Based on our results, *ALS* patients in the LUL subgroup showed the mildest clinical symptoms, while patients in the bulbar subgroup had the most severe clinical symptoms of *ALS*.

Subgroups were also compared on the basis of ED muscle MRC strength scores, both left and right, with no significant differences between subgroups in terms of m. ED MRC scores

4.2.2. Results of the SFEMG study in different groups of ALS patients

The first muscle examined was the ED (extensor digitorum), where the healthy control group had an average *jitter* of 32.5 ± 2.8 ms, no prolonged *jitter* or block was detected, and the *FD* was 1.3 ± 0.1. In contrast, the 26 *ALS* patients had a higher mean *jitter* of 54.4 ± 0.4 ms, an elongated *jitter* rate of 30.9% ± 14.2%, a block rate of 15.4% ± 10.7, and an *FD* value of 2.6 ± 0.45. The groups of *ALS* patients were further subdivided into bulbar and limb-onset groups. For the bulbar-onset group (N = 8), the mean *jitter* was 56.74 ± 4.42 ms, the elongated *jitter* % was 39.38% ± 16.69%, the block % was 16.88% ± 11.93%, and the *FD* value was 2.38 ± 0.35. The mean *jitter* value of the limb-initiation group (N = 18) was 53.34 ± 3.4 ms, the elongated *jitter* % was 27.11% ± 11.59%, the block % was 14.72% ± 10.36%, and the *FD* value was 2.66 ± 0.47. The p-values were <0001 in all cases, indicating statistically significant differences.

The second muscle examined was the orbicularis oculi, where the healthy control group (N = 26) had an average *jitter* of 35.7 ± 2.8 ms, no prolonged *jitter* or block, and a *FD* of 1.2 ± 0.1. For *ALS* patients (N = 7), the mean *jitter* was 51.5 ± 3.0 ms, the elongated *jitter* rate was 23.6% ± 11.1%, the block rate was 15.7% ± 37, and the *FD* value was 2.2 ± 0.23. For the bulbar-onset group (N = 2), the mean *jitter* was 53.4 ± 33.9 ms, the elongated *jitter* rate was 32.5% ± 10.61%, the block rate was 22.5% ± 17.68%, and the *FD* value was 2.1 ± 0.28. The mean *jitter* value of the limb-initiation group (N = 5) was 42.68 ± 13.86 ms, the elongated *jitter* rate was 26.67% ± 5.77%, the block rate was 13.0% ± 12.04%, and the *FD* value was 2.2 ± 0.23. The p-values were <0001 in all three cases, indicating statistically significant differences.

The third muscle tested was the frontal muscle, where the mean *jitter* of the healthy control group (N = 26) was 37.9 ± 2.6 ms, no prolonged *jitter* or block was recorded, and the *FD* was 1.3 ± 0.13. For *ALS* patients (N = 8), the mean *jitter* was 51.24 ± 2.4 ms, the elongated *jitter* rate was 23.75% ± 6.94%, the block rate was 23.13% ± 8%, and the *FD* value was 2.14 ± 0.15. For the bulbar-onset group (N = 4), the mean *jitter* was 51.78 ± 11.6 ms, the elongated *jitter* rate was 26.25% ± 4.79%, the block rate was 27.5% ± 5.0%, and the *FD* was 2.18 ± 0.13. The

limb-initiation group (N = 4) had a mean *jitter* of 45.7 ± 13.2 ms, an elongated *jitter* rate of 25.0% ± 5.0%, a block rate of 18.75% ± 8.54%, and an *FD* value of 2.1 ± 0.18. Pvalues were <0001, indicating significant differences.

4.2.3. Investigating the correlation between SFEMG parameters and functional scales

Comparing the results of the *SFEMG* test of *ALSFRS-R* and m. ED, we found a significant negative correlation for both elongated *jitter* percentage (r = -0.953) and block percentage (r = -0.829) (p <0.001 in all cases). *FD* was positively correlated with *ALSFRS-R* (r = 0.919, p <0.001). All results showed a very strong correlation. We also observed a significant correlation between elongated *jitter* percentage and *FD* (r = 0.89, p <0.001). Since m. ED, the *SFEMG* was performed on the right side in all cases, we also performed the analysis of *SFEMG* parameters without the RUL onset *ALS* patient group, we found a significant correlation (p<0.0001) for elongated *jitter* percentage (r= -0.96), block percentage (r= -0.824) with *ALSFRS-R* scores. *FD* was positively correlated with *ALSFRS-R* (r=0.901, p<0.001). Significant negative correlations were found between the following indicators obtained from the *SFEMG* study of the frontal muscle and the *ALSFRS-R*: r = -0.805 for average *jitter*; p <0.01; r = -0.798 for elongated *jitter* percentage; p = 0.02 (Figure 8); and r = -0.877 for block percentage; p = 0.004. *FD* was positively correlated with the *ALSFRS-R* (r = 0.798; p = 0.02) Significant negative correlations were found between *ALSFRS-R* and *SFEMG* test indicators of the orbicularis oculi muscle for mean *jitter* (r = -0.964), and for elongated *jitter* percentage (r = -0.954) and block percentage (r = -0.963), all p <0.001. These results show a very strong correlation. Similar to what was observed for m. ED and m. frontalis, *FD* in this case also showed a positive correlation with *ALSFRS-R* (r = 0.918; p <0.01), also indicating a very strong correlation.

As the ED was assessed on the right side for all patients, their results were compared with the right side MRC score of the ED. The ED muscle strength and the following three *SFEMG* parameters showed significant negative correlations: mean *jitter* (r = -0.441; p = 0.02, moderate correlation), elongated *jitter* percentage (r = -0.469; p = 0.02, moderate correlation) and block percentage (r = -0.729; p <0.001, strong correlation). Excluding the RUL onset group from the analysis yielded results with similar trends, although the values did not reach the significance threshold. While in the orbicularis oculi muscle there was a significant relationship with the MRC score of the ED muscle for all measured indices, in the frontalis muscle there was only such a relationship for elongated *jitter* percentage, but not for *FD* and block percentage. Disease duration showed no significant correlation with *ALSFRS-R* in the cohort studied.

5. Discussion

I. Blink reflex examination

5.1.1. General characteristics of patients included in the BR study

Most of the *ALS* patients we studied had mild to moderate neurological symptoms, but their overall functional status was more in the intermediate to advanced stage according to the international *ALSFRS-R* scale. No significant differences in *ALSFRS-R* status were found between groups of *ALS* patients with bulbar onset and end-stage onset. Unfortunately, neurological and neurophysiological examination of four patients with severe *ALS* was performed at a later stage of the disease; they were diagnosed definitively only at the onset of severe neurological symptoms. When analysing the subgroups of *ALS*, the patients in the bulbar onset group were younger and of working age, also the other two groups did not have an abnormally high age.

5.1.2. BR test results in ALS and other peripheral neurological diseases

Compared with the healthy control group, abnormally prolonged R response latencies were recorded in all our *ALS* patients during *BR* testing, detailed analysis of which revealed neurological damage at the level of the lower *BR*ainstem formatio reticularis and lateral tegmentum, despite the absence of structural abnormalities detected by imaging techniques (e.g., cranial and cervical MRI) in our *ALS* patients. Among our *ALS* patients we found 6 by whom increased latency, but measurable *R2*i responses *R2*c could not be detected. This might emphasize the importance of *BR*ainstem interneurons in the pathogenezis of *ALS*, since these interneurons are crucial in generating and mediating the *R2* answers.

In our physical examinations, 76% of *ALS* patients had no bulbar or neurological symptoms suggestive of involvement of *BR*ainstem structures. Nevertheless, in these patients, as in those with bulbar symptoms, we observed a gradual elongation of the latency of ipsi and contralateral *R2* waves and, in advanced cases, a disappearance of response potenti*ALS*, suggestive of involvement of brainstem interneurons in *ALS*. Our *BR* studies point to a loss of interneuronal function in the pathogenesis of *ALS*, which is supported by the results of international TMS and TST studies. In these, the reduction of RMT, SICI and LICI indicates hyperexcitability of the motor cortex, explained by the loss of inhibitory interneurons. A better understanding of these processes may help to unravel the neuropathological underpinnings of *ALS* and other neurodegenerative diseases. In the pathogenesis of *ALS*, several factors contribute to the damage to motor neurons, which, although occurring in parallel, may be at different stages. The loss of

function of inhibitory interneurons plays a key role in the development of increased cortical excitability, as supported by the 'dying back' and 'dying forward' theories, which suggest that degeneration starts from the axon termin*ALS* towards the cell body or, conversely, from the CNS towards the periphary. When clinical symptoms appear and a diagnosis can be made, we do not know exactly at what stage these neuropathological processes are.

In comparing the *BR* response potenti*ALS* of patients with *ALS* to those with other peripheral nervous system disorders, a distinctive pattern emerges that is characteristic of *ALS* and different from other neurological conditions. The *R1* responses in *ALS* patients, although recorded with prolonged latency, did not show a statistically significant difference when compared to a healthy control group. In contrast, in myasthenia gravis, all R response latencies were significantly increased, and in Bell's p*ALS*y, the latencies of the *R1* and *R2* responses on the side ipsilateral to the lesion also exhibited significant prolongation.

The anatomical structures responsible for the generation of *R1* responses are located within the *BR*ainstem, particularly in the pons and medulla oblongata regions. However, higher cortical centers, including the sensory cortex and basal ganglia, also play a critical role in the modulation of *R1* latencies. Considering these findings, along with the results from *BR* studies in stroke and other neurological conditions involving central damage, the preservation of *R1* response latencies in *ALS* is crucial for understanding the pathogenesis of the disease. This is particularly important given that even in patients with severe or advanced-stage *ALS*, the *R1* latencies showed only a moderate increase. In *ALS*, the relative preservation of *R1* latencies, combined with the progressive prolongation and eventual disappearance of *R2* responses, creates a unique pattern that distinguishes *ALS* from other neurological disorders.

5.1.3. Investigating the correlation between BR parameters and ALSFRS-R

By analysing the *R2* response latencies of the *BR* tests and the functional status of *ALS* patients, we can see that the ipsi and contralateral *R2* responses correlate with the *ALSFRS-R* scores, making the *BR* test a potential tool for monitoring disease progression and individual assessment of disease prognosis. When analysed across subgroups of *ALS* patients, *BR* response potential latencies were most elevated in the *ALS* subgroup with bulbar onset of symptoms. When further subdividing *ALS* patients with terminal onset into subgroups, we found significant differences between subgroups with RUL and RLL onset in the relationship between *BR* latencies and *ALSFRS-R* values. In the RLL group, the elongation of *BR* response latencies was more pronounced compared to the RUL group, and patients in the RLL subgroup also had lower *ALSFRS-R* values. This observation has relevance for the pathogenesis of *ALS*, as the

asymmetric distribution of clinical symptoms in *ALS* is important and endothelial dominance may play a significant role in neuropathological processes because it may indicate different degrees of neuronal vulnerability. International clinical tri*ALS* on the clinical symptoms and prognosis of *ALS* have shown that the prognosis of *ALS* is worse in patients with lower limb dominant symptoms, which previous research has explained by an increased risk of thromboembolic disease and infections due to loss of motility. The results of our *BR* studies suggest that the worse prognosis of *ALS* patients with lower limb onset may not be solely due to secondary complications, but may be explained by complex neuropathological processes that require further investigation.

II. The role of SFEMG in the diagnosis and prognosis of ALS

5.2.1. General characteristics of patients included in the SFEMG study

Looking at the demographic data of the *ALS* patients in our study, the only significant difference between the bulbar onset and limb onset *ALS* subgroups was the mean age. Patients in the bulbar onset subgroup were significantly younger. In the different subgroups of *ALS* patients, the time between the first symptoms of the disease and the referral of the patients to the neurophysiology laboratory was not significantly different. In the subgroups of *ALS* patients, disease duration did not show a significant correlation with functional status, but this may be due to the sample size, as four patients in the subgroup with bulbar onset had a very rapid progression. *ALS* is an asymmetric disease and therefore the subgroups with a bulbar onset were further subdivided into RUL, RLL, LUL and LLL groups for comparison of demographic, clinical and *SFEMG* parameters. Symptom severity showed no difference between the two sites. Lower limb symptoms had the longest time from presentation to referral, but this was not significant. The *ALSFRS-R* results showed low scores indicating the worst clinical outcome in the two lower limb groups (LLL, RLL), which were similar to those of *ALS* patients with bulbar onset.

5.2.2 Results of the SFEMG study in different groups of ALS patients

Despite the fact that the facial muscles were not clinically affected, significant differences ($p<0,005$) were observed in the mean *jitter*, the percentage of elongated *jitter*, the percentage of blocks and the *FD* of *ALS* patients compared to the *SFEMG* parameters of healthy controls. These parameters may be particularly useful in the early stages of *ALS* and when the diagnosis is uncertain, for example because the patient has sensory neuropathy from other aetiological causes, such as diabetes mellitus, in association with clinical signs suspicious for *ALS*. The

inclusion of *SFEMG* examination of the mimic muscles in the *ALS* screening protocol may be a logical consideration, as it is not expected that, for example, *FD* in the facial muscles would be elongated, for example in MMN or CIDP. The comparison of *SFEMG* parameters detailed in the three muscles studied separately between the bulbar and limb onset subgroups is not clearly meaningful due to the small sample size

5.2.2. Investigating the correlation between SFEMG parameters and functional scales

We also correlated the parameters of the *SFEMG* scans of *ALS* patients with the *ALSFRS-R* and the MRC score for m. ED. Our results showed a significant negative correlation between functional status and mean *jitter*, percentage of elongated *jitter* and percentage of blocks. In contrast, *FD* showed a significant positive correlation with functional scales, indicating a gradual decrease in *FD* values as the disease progressed. We hypothesize that the parameters of *SFEMG* tests in a given patient at a given time point may contribute to the estimation of the individual prognosis of the disease, as they correlate with *ALSFRS-R*, which is considered a prognostic factor.

An increase in *FD* may indicate reinnervation before the histological results of muscle biopsy confirm this. This may be interpreted as a consequence of possible ephaptic activation of adjacent muscle fibres, resulting in increased *FD*, which at this point in the neuropathogenesis of *ALS* may not yet be confirmed in histological samples. Taking all these into consideration, our results suggest that *FD* and the percentage of elongated *jitter* should always be analysed in combination, as these two parameters together serve as better prognostic biomarkers. All correlations between these two parameters and *ALSFRS-R* were significant in ED as well as in orbicularis oculi muscles. For the frontalis muscle, the trend was similar, but correlation did not reach the level of significance, presumably explained by the low number of cases.

In examining the correlation between *SFEMG* parameters and *ALSFRS-R*, we observed a critical point in the neuropathology of *ALS* at the *ALSFRS-R* score of 30. At this point, the percentage of increased *jitter* is high, but *FD* is already low at this stage of the disease. This phenomenon may indicate a neuropathological process where reinnervation capacities are exhausted, resulting in a decrease in *FD*, while the percentage of elongated *jitter* indicates further progression of denervation processes. As denervation processes dominate at this stage, severe instability in the neuromuscular transition is observed, as indicated by the *ALSFRS-R* score. At this stage of the disease, the positive effects of supportive therapies are also likely to be lost compared to the previous functional status. This observation from our *SFEMG* studies suggests that remodelling of neuromuscular junction is dynamic in the neuropathomechanism

of *ALS*, as reflected by changes in *SFEMG* parameters. *SFEMG* can therefore help to establish the prognosis of *ALS*, especially when used in conjunction with functional clinical scales.

SFEMG of facial muscles and evaluation of the results are important for understanding the pathomechanism of *ALS*. In contrast to the limb muscles, the extraocular muscles are spared during the course of *ALS* due to the difference in Wnts expression between facial muscles and muscles of other regions of the body. The Wnt protein family plays a role in the development and regeneration of neuromuscular junctions in both extraocular and limb muscles. However, in the pathomechanism of *ALS*, Wnts proteins in extraocular muscles will only be involved in advanced stages of the disease. Presumably, the "dying back" mechanism as well as microdamage of Piezo2 proteins may be the underlying pathomechanisms. In our study, the results of *SFEMG* analysis in *ALS* patients suggest that combined *jitter* and *FD* analysis can detect pathological lesions in the subclinical phase before the onset of clinical symptoms. In particular, the orbicularis oculi muscle, which shows significant correlation with functional scales used in *ALS*, may be an ideal indicator muscle to confirm early neurophysiological abnormalities and to determine individual prognosis in *ALS* patients.

6. Summary

ALS is a progressive, currently incurable disease. Therefore, any diagnostic test that brings us closer to the knowledge of the pathomechanism of the disease and early diagnosis is important. In our current study, we investigated the significance of the *blink reflex* and *SFEMG*. According to our results, due to its correlation with the *ALSFRS-R*, the *BR* examination is a promising neurophysiological diagnostic tool in *ALS* from both a prognostic and pathophysiological point of view, and can also serve as a biomarker. The results of the *BR* study highlight the role of brainstem interneurons in the pathogenesis of *ALS*. Damage to brainstem interneurons, manifested in the prolongation of the latencies of *BR* responses and the disappearance of *R2* responses, probably contributes to the development and progression of the clinical symptoms of *ALS*. Based on the studies of *BR* latencies in various neurological diseases, it can be concluded that in the case of *ALS*, the relative preservation of the latencies of *R1* responses, the progressive lengthening of the latencies of *R2* responses, and the disappearance of *R2* responses show a characteristic pattern that helps to distinguish *ALS* from other neurological diseases. *SFEMG* enables the exploration of denervation-reinnervation processes in the neuropathogenesis of *ALS*, as well as the analysis of correlations between examined parameters and *ALS* functional scales. During the *SFEMG* examinations, the combined analysis of data

jitter and *FD* gives the best correlation with the clinical scales, thus it can be a suitable method for determining the individual prognosis. In addition to the examination of the ED muscle, the examination of the orbicularis oculi muscle is promising, as changes can be detected here even in a subclinical state, and the results showed a close correlation with the functional scales.

Overall, the *blink reflex* and the *SFEMG* tests contribute to a better understanding of the pathomechanism of *ALS* and can help in the early detection of the disease, even before the appearance of clinical symptoms.

7. New findings

1. *BR* testing may be useful for detecting early subclinical lesions in regions not yet clinically affected in *ALS* before clinical symptoms appear.
2. The latency of *R2* responses correlates well with the *ALSFRS-R* scale, *BR* testing may be useful for monitoring disease progression and assessing individual prognosis.
3. The role of *BR*ainstem interneurons is crucial in the pathogenesis of *ALS*, as evidenced by the elongation of *BR* response latencies and the disappearance of *R2* responses.
4. The pattern of *BR* abnormalities may strengthen the suspicion for ASL diagnosis.
5. *SFEMG* testing is a valuable tool to support the diagnosis of *ALS* and to better assess the neuropathology of *ALS*, becasue it gives important information on denervation and reinnervation capacity.
6. The combined analysis of *jitter* and *FD* data in *SFEMG* studies gives the best correlation with clinical scales, thus it may be a suitable tool to determine individual prognosis.
7. In addition to m. ED, m. orbicularis oculi may be an ideal indicator muscle to support the early neruophysiological abnormalities and also to assess the individual prognosis in patients with *ALS* since it shows correlation with the functional scales.

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8. Publications

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Registry number: DEENK/431/2024.PL
Subject: PhD Publication List

Candidate: Róbert Rostás
Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Rostás, R.**, Fekete, I., Horváth, L., Márton, S., Fekete, K.: Correlation of single-fiber electromyography studies and functional status in patients with amyotrophic lateral sclerosis. *Open Med. 19* (1), 1-13, 2024.
   DOI: http://dx.doi.org/10.1515/med-2024-0990
   IF: 1.7 (2023)
2. **Rostás, R.**, Fekete, I., Horváth, L., Fekete, K.: Blink Reflex Examination in Patients with Amyotrophic Lateral Sclerosis Compared to Diseases Affecting the Peripheral Nervous System and Healthy Controls.
   Brain Sci. 13 (10), 1-16, 2023.
   DOI: http://dx.doi.org/10.3390/brainsci13101384
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List of other publications

3. Csikai, E., Andrejkovics, M., Balajthy-Hidegh, B., Hofgárt, G., Kardos, L., Diószegi, Á., **Rostás, R.**, Czuriga-Kovács, K. R., Csongrádi, É., Csiba, L.: Influence of angiotensin-converting enzyme inhibition on reversibility of alterations in arterial wall and cognitive performance associated with early hypertension: a follow-up study.
Medicine (Baltimore). 98 (34), 1-9, 2019.
DOI: http://dx.doi.org/10.1097/MD.0000000000016966
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