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Original scientific article

C9orf72 GENETIC SCREENING IN AMYOTROPHIC LATERAL SCLEROSIS PATIENTS FROM SERBIA

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Hexanucleotide repeats expansion in the *C9orf72* gene is the most common cause of familial and sporadic amyotrophic lateral sclerosis (ALS) cases in Europe. In this study we aimed to determine the size and distribution of *C9orf72* alleles, and investigate the possible association of the repeat size with several clinical parameters in ALS patients from Serbia. Patients were recruited from 2011-2021 and analysed using fragment length analysis and Southern blot. Out of 383 ALS patients, we have detected 31 (8.09%) patients with repeat expansion. In the total ALS cohort, clinical overlap with frontotemporal dementia (FTD) was registered in 17 (4.44%) patients, and among them, 5 (29.41%) were expansion carriers. There was no difference in the age of onset, age at the examination or disease duration, gender, and the frequency of spinal and bulbar onset between patients with and without *C9orf72* expansion. The presence of positive family history (34.48% *vs*. 15.65%) and FTD (16.13% *vs*. 3.41%) was more frequent in expansion-positive *vs*. expansion-negative patients. In expansion-positive patients, significantly higher values of the largest detected repeat were found in patients with ALS

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in the family, and in expansion-negative, a higher median value of the smaller allele was noted in patients with a positive family history of ALS, dementia, and both in comparison to the rest of the group. A correlation of the repeat size was not found with the age of onset in both patients with and without the expansion. This is the first detailed study of *C9orf72* sizing in ALS patients from Serbia. Our results emphasize the need for *C9orf72* genetic screening in ALS patients with/without FTD.

Key words: C9orf72, repeat expansion, ALS, ALS/FTD, executive dysfunction

INTRODUCTION

Amyotrophic lateral sclerosis, a neurodegenerative disorder with a progressive course, is characterized by degeneration of motor neurons in the brain and spinal cord that inevitably leads to a fatal outcome 3-5 years after disease onset (TAYLOR *et al*., 2016). Clinically it can be presented as spinal, bulbar, and very rare as a respiratory form of the disease depending on the affected motor neuron (WIJESEKERA AND LEIGH, 2009). There are probably multiple factors contributing to the disease, and considering the many genes and cellular processes involved, several ALS disease mechanisms are proposed and reviewed (MEJZINI *et al*., 2019).

For many years now, data are indicating the clinical association between ALS and FTD, overlap within their pathological features (LILLO AND HODGES, 2009), and a recent connection at the genetic level has been proven (RENTON *et al*., 2011, DEJESUS-HERNANDEZ *et al*., 2011). The common pathological characteristic of these two disorders is the presence of TAR DNA-binding protein (TDP-43) in ubiquitinated inclusions in the brain (ARAI *et al*., 2006, NEUMANN *et al*., 2006). Based on this finding, these diseases are defined as TDP-43 proteinopathies (ARAI *et al*., 2006). In general, ALS is considered a sporadic disease (sporadic ALS, SALS) in 90-95% of the cases, while in 5-10% it can be presented as familial ALS (FALS) (TALBOTT *et al*., 2016). Besides motor symptoms, 50% of the patients with sporadic ALS exhibit some form of cognitive impairment (RINGHOLZ *et al*., 2005). Comorbidity with FTD is present in around 15% of ALS patients (RINGHOLZ *et al*., 2005, PHUKAN *et al*., 2012) while in around 2%, dementia of Alzheimer's type can be present (PHUKAN *et al*., 2012).

More than 30 genes have been discovered to be associated with ALS (MATHIS *et al*., 2019). Expansion of hexanucleotide repeats (GGGGCC) in the *C9orf72* gene, discovered in 2011, (RENTON *et al*., 2011, DEJESUS-HERNANDEZ *et al*., 2011) is the most common cause present in 33.7% of FALS and 5.1% of SALS cases in Europe (ZOU *et al*., 2017). In the USA the *C9orf72* expansion frequency is similar to the European (FALS 36.2%, SALS 5.5%) (MAJOUNIE *et al*., 2012) while in Asia, the frequency is much lower (FALS 2.3%, SALS 0.3%) (ZOU *et al*., 2017). Furthermore, this expansion is the most common cause of FTD (RENTON *et al*., 2011, DEJESUS-HERNANDEZ *et al*., 2011), and ~30% of the patients with the overlapping clinical syndrome, ALS/FTD, have the expansion in *C9orf72* (MAROGIANNI *et al*., 2019).

The hexanucleotide repeats are located in the promoter region or intron 1 of the *C9orf72* gene depending on the transcript variant (RENTON *et al*., 2011, DEJESUS-HERNANDEZ *et al*., 2011). The two protein isoforms are formed (DEJESUS-HERNANDEZ *et al*., 2011) that have a role in autophagy and endosomal trafficking (FARG *et al*., 2014). The commonly used cut-off for the pathological number of repeats is more than 30 (RENTON *et al*., 2011) and very often alleles

carrying 20-30 repeats are referred to as intermediate-size alleles (MAJOUNIE *et al*., 2012, NUYTEMANS *et al*., 2013). With the use of the Southern blot method, repeats up to more than 4000 were detected (BECK *et al*., 2013, DOLS-ICARDO *et al*., 2014). Also, these expansions are characterized by somatic instability (dejesus-hernandez *et al*., 2011) and age-related penetrance which is almost full at the age of 80 (MAJOUNIE *et al*., 2012).

Here we aimed to determine the size and distribution of *C9orf72* alleles, confirm the presence of the pathological expansion, investigate demographic as well as clinical characteristics of *C9orf72* positive patients, and analyze the correlation between clinical and genetic data in *C9orf72* positive and negative patients diagnosed with ALS in the largest tertiary ALS clinic in Serbia.

MATERIALS AND METHODS

All the patients included were informed about the genetic analysis and gave their informed written consent. The study was approved by the Ethics Committee of the University Clinical Center of Serbia (UCCS).

Study patients

In this study, 383 patients with the diagnosis of probable or definite ALS from 2011-2021 according to the revised El Escorial criteria (BROOKS *et al*., 2000) were recruited at the Neurology Clinic, UCCS. The clinical data were obtained through the structured questionnaire that included the following items: age, gender, personal history, and family history (ALS, Parkinson's disease, frontotemporal dementia, Alzheimer's disease, psychiatric disturbances, and suicide) age of onset, age at the time of the examination, disease duration at the time of the examination, form at onset (spinal, bulbar, respiratory or dementia), clinical presentation (upper + lower extremities (UE+LE), predominant UE, predominant LE, predominant right extremities or predominant left extremities). Patients with mutations in other analyzed genes (*SOD1*, *ANG*, and *FUS*) were not excluded from this study. Diagnosis of the frontotemporal dysfunction in ALS patients was made based on the revised diagnostic ALS-FTSD criteria (STRONG *et al*., 2017). Several neuropsychological tests were performed (MARJANOVIC *et al*., 2017). For this study, as familial cases (FALS) we have considered the presence of ALS in the family (FALLIS AND HARDIMAN, 2009). Furthermore, in positive family history data, we have also included extended phenotypes (O'BRIEN *et al*., 2017, RYAN *et al*., 2018). Patients with negative family histories were considered as sporadic ALS (SALS). The patients that did not meet the revised El Escorial criteria were excluded from the study. Also, regarding family history, if the diagnoses of family members were not confirmed, family history was not taken into account.

Genetic analysis

Extraction of DNA from peripheral blood was performed with PureLink™ Genomic DNA Mini Kit (*Life Technologies*, USA) following the manufacturer's protocol, and for the Southern blot method, DNA was salted-out using a modified method (MILLER *et al*., 1988). *C9orf72* normal allele sizing was performed using previously designed primers (available at request) and protocol (available at request), and repeat primed PCR (RP-PCR) was performed

for all the samples presented with a single allele using two different sets of primers (RENTON *et al*., 2011, DEJESUS-HERNANDEZ *et al*., 2011) and protocols (available at request), followed by fragment analysis by capillary electrophoresis on ABI 3500 Genetic analyzer (*Applied Biosystems*, Foster City, CA, USA). For DNA sizing and allele calling GeneMapper software version, 4.1 (*Applied Biosystems*, Foster City, CA, USA) was used. Alleles that carry more than 30 repeats were considered expanded (RENTON *et al*., 2011, CANNAS *et al*., 2015), alleles with 20-29 repeats as intermediate (CANNAS *et al*., 2015), and alleles with less than 20 repeats as wild-type (RENTON *et al*., 2011, CANNAS *et al*., 2015). RP-PCR samples presented with a characteristic saw-tooth pattern with 6bp periodicity characterized as expanded were further analyzed with Southern Blot. DNA obtained with the salting-out method was digested overnight with EcoRI (*Thermo Fisher Scientific*, USA) and diluted. Small pool PCR (SP-PCR) amplification of the region containing repeats was performed with in-house designed primers (available at request). PCR reaction mix and thermal cycling profile are available at request. SP-PCR products were separated by size on 1.2% agarose gel and transferred to nylon membrane Amersham™ Hybond™ -N+ (*GE Healthcare*, UK) by capillary blotting. Probe with complementary repeats $(C_4G_2)_4$ was labeled with (DIG-11-ddUTP) (*Roche*, Germany) and incubated with the membrane for 2h at 65°C. CDP-star (*Roche*, Germany) was used as a chemiluminescent substrate, and blots were exposed to Amersham Hyperfilm TM ECL (*GE Healthcare*, UK) overnight. The size of the bands was determined using VisionCapt software (*Vilber Lourmat*, France) based on the size of Molecular Weight Marker X (*Roche*, Germany). The size of the repeats was calculated using the formula (size of base pairs-162)/6. The given numbers were rounded.

The samples were analyzed 1-3 times (some of them in more than one different dilution) on Southern blot depending on the quality of blots and the obtained bands.

Statistics

In the *C9orf72* negative patients, repeats were considered as normally distributed data and four variables (smaller repeat, larger repeat, difference between smaller and larger repeat, and sum of smaller and larger repeat) for repeats pair on both alleles for each patient were correlated with continuous variables using the Pearson correlation coefficient. The analysis of categorical variables and the number of repeats was done with a t-test for independent samples. For the larger allele, the value of 26 repeats was treated as missing for the analysis of normal (wild-type) repeats. For positive patients, the mean value of the obtained number of repeats was calculated and used for statistical analysis, and one additional value for repeats analysis was added (Blot max) which presented the maximum value of obtained repeats. The values were skewed towards higher values and were analyzed using non-parametrical alternatives compared to the negative group. Correlation analysis of the expanded repeats was done with the Spearman correlation coefficient. The analysis of categorical variables and the number of repeats was done with the median test and Mann-Whitney test. Due to the disbalance of groups and complex family history coding due to different manifestations of the disease, family history coding was started differently for each disease with multiple levels (between 3 and 6). Using visual guidance, multiple levels were aggregated to several combinations of binary coding that made biological sense to avoid using the underpowered Kruskal-Wallis test.

RESULTS

The C9orf72 expansion frequency in the ALS cohort

Of the total number of 383 patients with ALS, 210 patients (54.83%) were males and 173 patients (45.17%) were females. We have detected 31 (8.09%) patients with heterozygous G_4C_2 repeat expansion. Regarding the shorter allele size, among the patients with repeat expansion, one patient (3.23%) had an intermediate repeat number (22 repeats) while the rest of the patients had less than 20 repeats. Among the patients without the expansion, we have detected only one patient (0.26%) with heterozygous intermediate and normal alleles carrying 26 and 4 repeats (Picture 1). The clinical-demographic characteristics of our ALS cohort are presented in Tables 1 and 2. For 4 patients (1.04%) clinical data were incomplete, and they were without expansion.

Table 1. Disease onset frequency in the total ALS cohort, C9orf72 positive, and negative patients

	Total ALS cohort	*C9orf72* positive	*C9orf72* negative
Spinal onset	303 (79.11%)	24 (77.42%)	279 (79.26%)
Sex (male %)	60.07%	54.17%	60.57%
G_4C_2 expansion	24 (7.92)	/	/
ALS/FTD	9 (2.97%)	2 (8.33%)	7 (2.51%)
UE	141 (46.53%)	15 (62.50%)	126 (45.16%)
UE-left	47 (33.33%)	5 (33.33%)	42 (33.33%)
UE-right	55 (39.01%)	5 (33.33%)	50 (39.68%)
UE-both	39 (27.66%)	5 (33.33%)	34 (26.98%)
LE	160 (52.81%)	9 (37.50%)	151 (54.12%)
LE-left	70 (43.75%)	4 (44.44%)	66 (43.71%)
LE-right	46 (28.75%)	2 (22.22%)	44 (29.14%)
LE-both	44 (27.50%)	3 (33.33)	41 (27.15%)
UE+LE	2 (0.67%)	/	2 (0.72%)
UE+LE-left	/	/	/
UE+LE-right	1 (50%)	/	1 (50%)
UE+LE-both	1 (50%)	/	1 (50%)
Bulbar	77 (20.10%)	7 (22.58%)	70 (19.89%)
Sex (male %)	32.47%	42.86%	31.43%
G_4C_2 expansion	7 (9.09%)	/	/
ALS/FTD	8 (10.39%)	3 (42.86%)	5 (7.14%)

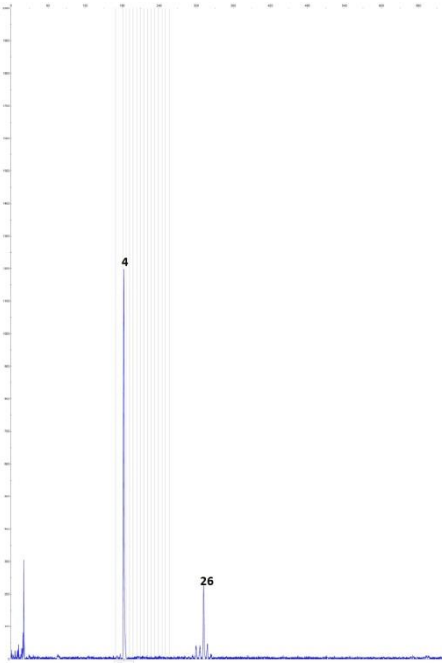
Table 2. Clinical-demographic characteristics of ALS cohort, and comparison of C9orf72 positive and negative patients data

	Total ALS cohort	***C9orf72* positive patients**	***C9orf72* negative patients**	**p value**
Gender				
male	210 (54.83)	16 (51.61%)	194 (55.16%)	p=0.707
female	173 (45.17%)	15 (48.39%)	158 (44.88%)	
ALS/FTD	17 (4.44%)	5 (16.13%)	12 (3.41%)	**p=0.001**
Onset				
spinal	303 (79.11%)	24 (77.42%)	279 (79.94%)	p=0.738
bulbar	77 (20.10%)	7 (22.58%)	70 (20.66%)	
Family history (positive %)	56 (14.62%)	10 (34.48%)	46 (15.65%)	**p=0.011**
Age at disease onset (years): Mean (CI)±SD; min-max	58.33 (95% CI:57.23-59.43)± 10.94; 15.0-81.0	59.29 (95% CI:56.21-62.37)± 8.39; 34.0-73.0	58.25 (95% CI:57.07-59.42)±11.14; 15.0-81.0	p=0.611
Age at the time of the examination (blood draw): Mean (CI)±SD; min-max	59.93 (95% CI:58.81-61.05)± 11.12; 15.0-84.0	60.61 (95% CI:57.54-63.69)± 8.39; 34.0-75.0	59.87 (95% CI:58.68-61.06)±11.34; 15.0-84.0	p=0.723
Disease duration* (years): Mean (CI)±SD; min-max	1.89 (95% CI:1.66-2.11)± 2.23; 0.08-20.0	1.42 (95% CI:0.79-2.05)± 1.71; 0.20-10.0	1.93 (95% CI:1.69-2.17)± 2.27; 0.08-20.0	p=0.223

*disease duration is defined as a period from the age of onset to the time of the examination when the blood is drawn for genetic analysis; statistically significant value p<0.05

Positive family history (ALS, dementia, PD, and psychiatric problems including suicide) was present in 56 (14.62%) patients of the whole ALS group, negative family history in 267 (69.71%), and for 60 (15.67%) data were not available. In the total ALS cohort, 32 (8.36%) patients had FALS based on their family history of ALS, and 5 (15.62%) out of these 32 carried the *C9orf72* expansion (3 patients with expansion (30%) had ALS in the family and 2 (20%) had ALS and dementia in the family). Considering the whole group with positive family history (with extended phenotypes), 10 (17.86%) patients were positive for the *C9orf72* repeat expansion. Among SALS, 19 (7.12%) were *C9orf72* expansion positive. In the total cohort of ALS patients, 17 (4.44%) had ALS/FTD and 5 of them (29.41%) had the expansion.

In the normal *C9orf72* repeat range, the most frequent allele was the allele carrying 2 repeats (51.96%) followed by the allele with 5 and 8 repeats with almost the same frequency (12.14% and 12.01%, respectively). In 120 (31.33%) patients *C9orf72* allele sizes were homozygous. Allele size distribution in the ALS cohort is presented in Figure 1.



Picture 1. Electropherogram of the *C9orf72* fluorescent sizing PCR in ALS patient carrying normal allele (4 repeats) and intermediate allele (26 repeats).

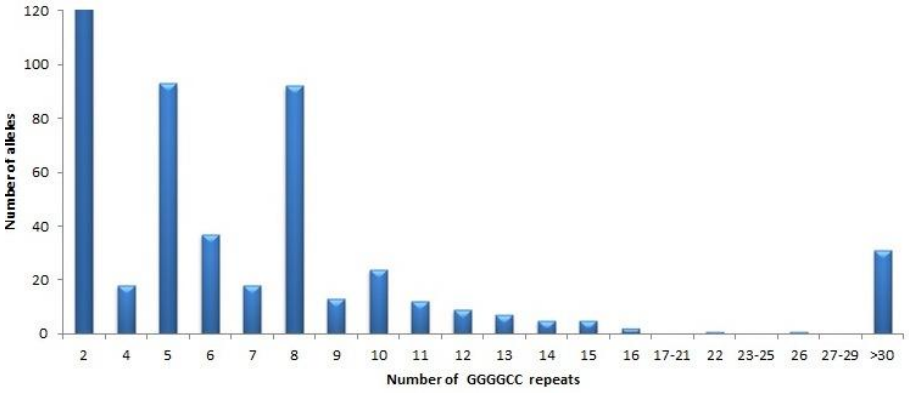
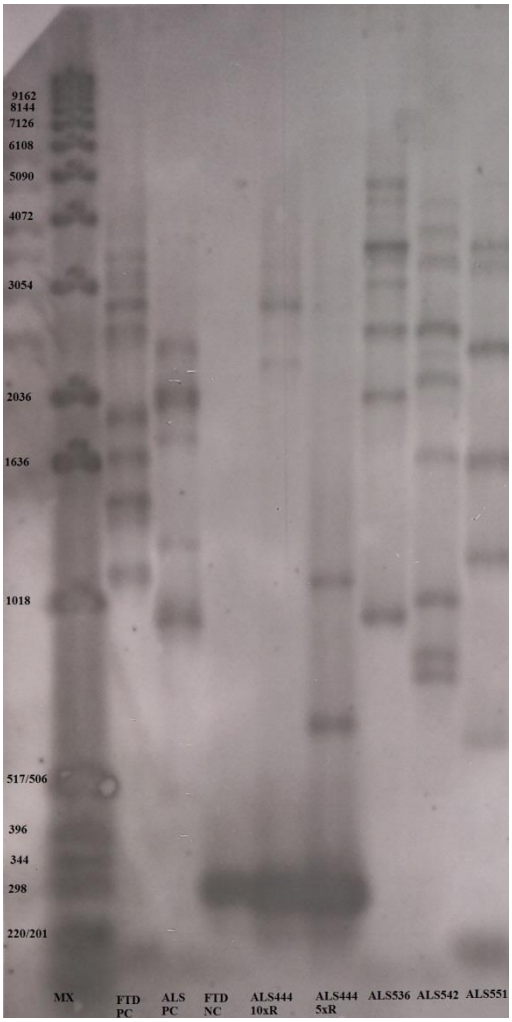


Figure 1. Distribution of *C9orf72* allele sizes in ALS patients.

All expansion carriers were confirmed with Southern blot. The representative Southern blot is shown in Picture 2. The mean repeat size of the expanded repeats obtained with Southern blot was ~247±149 range (32-706) repeats.



Picture 2. Representative Southern blot with four ALS expansion carriers. MX- Molecular Weight Marker X (*Roche*, Germany); PC-positive control (FTD PC-FTD sample; ALS PC-ALS sample); NC-negative control with 24 repeats; ALS marks- *C9orf72* expansion positive ALS patients. ALS444-patient carrying the expansion and the intermediate (22 repeat) on shorter allele presented as 10 times (10xR) and 5 times (5xR) diluted digestion.

Clinical data of C9orf72 expansion positive and negative patients

Out of the total number of 31 patients with *C9orf72* repeat expansion, 16 (51.61%) were males and 15 (48.39%) were females. Spinal disease onset was present in 24 (77.42%) and bulbar in 7 (22.58%) patients (Table 1). Between the males and females, as well as between spinal and bulbar onset patients with *C9orf72* repeat expansion, there was no difference in age of onset, age at the time of the examination, and disease duration ($p>0.05$).

Positive family history with extended phenotypes was more frequent in expansion-positive patients (34.48% *vs*. 15.65%, $p=0.011$) as well as FTD (16.13% *vs*. 3.41%, $p=0.001$) compared to patients without the expansion (Table 2). Basic clinical-demographic characteristics of *C9orf72* expansion positive and negative patients and a comparison of clinical data are shown in Table 2.

Of the total number of *C9orf72*-positive patients, FTD was diagnosed in 5 (16.13%) patients. In 14 (45.16%) patients dementia criteria were not fulfilled (STRONG *et al*., 2017) and they were diagnosed with mild cognitive impairment within the executive domain, and for 6 (19.35%) patients cognitive function was preserved. Also, for 6 patients (19.35%) testing was not performed.

Correlation analysis

Among *C9orf72* expansion negative patients, the higher median value of the smaller allele was noted in the group of patients who had a positive family history of ALS, dementia, and both (median test, $p=0.033$). Regarding patients with *C9orf72* repeat expansion, the group of patients with a positive family history of ALS had significantly higher values of Blot max (Mann-Whitney U test, $p=0.037$) compared to the rest of the group.

We did not find a significant correlation between the examined variables of the size of repeats in patients with and without the expansion with the age of onset, age at the time of the examination, or disease duration. Also, association with the disease onset or the presence of FTD was not found in both groups (data not shown).

DISCUSSION

The *C9orf72* genetic studies so far are showing variable geographic distribution of the expansion with the highest frequency in Europe and the smallest in Asia (MAJOUNIE *et al*., 2012, ZOU *et al*., 2017). In Europe, a very high percentage of expansion carriers (28%) was reported in the Finish population (46.4% of FALS and 21.1% of SALS) (RENTON *et al*., 2011). Of the total number of our tested ALS patients, the frequency of *C9orf72* expansion carriers was 8.09%, similar to the frequency in some European studies: 8.97% in Ireland (BYRNE *et al*., 2012), 7.56% in Germany (MAJOUNIE *et al*., 2012), 9.55% in Italy (MAJOUNIE *et al*., 2012), 8.06% in the UK (BECK *et al*., 2013). In our total ALS cohort, the highest *C9orf72* mutation frequency was found in ALS/FTD patients, as expected, considering the recently published rate of ~ 30% (MAROGIANNI *et al*., 2019).

In our study, the rate of 15.62% *C9orf72* expansion carriers in FALS cases is smaller than the previously published rate of 33.7% in the European populations (ZOU *et al*., 2017) probably due to a lack of some family history data and medical files from missing family members. Also, one of the possible reasons for the lower *C9orf72* frequency reported in our study could be based

on the previous report from our population, where mutations in *SOD1* are the most common, and represent 73% of all genetically positive ALS patients (MARJANOVIC *et al*., 2017). When we included extended family phenotypes (Parkinson's disease, frontotemporal dementia, Alzheimer's disease, psychiatric disturbances, and suicide), our rate of FALS patients increased to 14.62% of ALS patients with 17.86% expansion carriers similar to the previous overall reported rate of 16% (MAROGIANNI *et al*., 2019). The mutation frequency of 7.12% among our SALS patients is the line with the overall mutation frequency of 6-8% (MAROGIANNI *et al*., 2019).

In the study which included European and North American participants, XI *et al*. reported a significantly higher presence of familial cases in expansion carriers (50% *vs*. 8.2%) (XI *et al*., 2012). In comparison to *C9orf72* negative patients, expansion carriers from our study had a positive family history with extended family phenotypes more frequently (34.48% *vs*. 15.65%).

So far, several studies showed that in ALS *C9orf72* expansion carriers disease onset starts earlier compared to the patients without the expansion (BYRNE *et al*., 2012, VAN RHEENEN *et al.*, 2012, GARCIA-REDONDO *et al*., 2013). However, the other studies did not report those results (RATTI *et al*., 2012, UMOH *et al*., 2016, XI *et al*., 2012) which is also the case in our research. It is worth mentioning that our group of *C9orf72* negative patients also included patients with known mutations in *SOD1, ANG*, and *FUS* which are known to have an earlier age of onset (CONNOLLY *et al*., 2020), and this may have had an impact on our result.

The clinical picture of FTD among our ALS patients was registered in 4.44% of the total cohort, while among expansion carriers, FTD was more common (16.13%) than in those who do not carry the expansion (3.41%) which is supported by the literature data (BYRNE *et al*., 2012, GARCIA-REDONDO *et al*., 2013, UMOH *et al*., 2016). Also, in expansion carriers, executive dysfunction was registered in nearly one-half of the patients. In total, 61.29% of the expansion carriers had cognitive impairment, and this high percentage is in accordance with the previous data (BYRNE *et al*., 2012).

All ALS patients from our study in whom *C9orf72* expansion was detected were heterozygous carriers. We have also detected one expansion carrier who had an intermediate repeat number (22 repeats) on a shorter allele, and one patient (0.26%) carrying 4 and 26 repeats characterized as an intermediate repeat carrier. In the patient with 22 repeats on the shorter allele, the disease occurs at the age of 56 as the spinal phenotype on the left lower extremity. Neuropsychological testing showed the presence of mild cognitive impairment within the executive domain. The patient's father had dementia, while her cousin had FTD/ALS and the presence of the *C9orf72* repeat expansion. Our patient with 26 repeats had disease onset at 72 years, with bulbar symptomatology and negative family history. In the population-based cohort, a possible association of repeats of 20-30 and ALS was suggested (BYRNE *et al*., 2014), and in support of that finding, the data from the recent meta-analysis are showing an association of 24-30 repeats with ALS, suggesting lowering pathogenicity cut-off to ≥ 24 repeats (IACOANGELI *et al*., 2019).

Regarding family history, in our patients without the expansion, the higher median value of the smaller allele was noted in the group who had a positive family history of ALS, dementia, and both. Additionally, opposite to the report of Dols-Icardo et al. where no correlation was found with family history (DOLS-ICARDO *et al*., 2014), in *C9orf72* expansion positive patients we

have noted the significantly higher values of the largest detected repeat in patients who had ALS in the family. However, considering the modest sample sizes of our cohorts, and the fact that only 3 *C9orf72* expansion carriers had ALS in the family, these results should be verified on larger sample sizes and family studies with ALS and/or dementia in the family. In ALS patients with and without the expansion in our study, we did not register any significant correlation of the size of the repeats with the age of onset and no difference in repeat size among spinal and bulbar patients as previously reported (DOLS-ICARDO *et al*., 2014, RUTHERFORD *et al*., 2012).

The main limitation of our study is the small sample size of our cohort and a certain imprecision of family history data in patients who reported no positive family history. Due to missing medical records, family history was not taken into account if the diagnoses of family members were not confirmed. Also, with our Southern blot method, we were not able to obtain very large repeat sizes as reported in other studies.

CONCLUSION

In conclusion, this is the first detailed study of *C9orf72* hexanucleotide repeats including allele sizing in ALS patients from Serbia. Our results emphasize the need for *C9orf72* genetic screening in ALS patients, especially with FTD and executive dysfunction.

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C9ORF72 GENETIČKA ANALIZA KOD BOLESNIKA SA AMIOTROFIČNOM LATERALNOM SKLEROZOM IZ SRBIJE

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Izvod

Heksanukleotidna ekspanzija u genu *C9orf72* je najčešći uzrok familijarnih i sporadičnih slučajeva amiotrofične lateralne skleroze (ALS) u Evropi. U ovoj studiji smo imali za cilj da odredimo veličinu i distribuciju *C9orf72* alela i istražimo moguću povezanost veličine ponovaka sa nekoliko kliničkih parametara kod ALS bolesnika iz Srbije. Bolesnici su prikupljani u periodu 2011-2021 i analizirani koristeći fragmentnu analizu i Southern Blot. Od 383 ALS bolesnika registrovali smo 31 (8.09%) bolesnika sa ekspanzijom ponovaka. U ukupnoj ALS kohorti, kliničko preklapanje sa frontotemporalnom demencijom (FTD) registrovano je kod 17 (4.44%) bolesnika, i među njima, 5 (29.41%) bolesnika je bilo nosilac ekspanzije. Nije uočena razlika u godinama početka bolesti, godinama na pregledu, ili trajanju bolesti, polu i učestalosti spinalnog i bulbarnog početka među bolesnicima sa i bez *C9orf72* ekspanzije. Prisustvo pozitivne porodične istorije (34.48% *vs*. 15.65%) i FTD (16.13% vs. 3.41%) je bilo znatno češće kod bolesnika sa ekspanzijom *vs*. bolesnici bez ekspanzije. Kod bolesnika sa ekspanzijom, značajno veće vrednosti najvećeg registrovanog ponovka su pronađene kod bolesnika koji su imali ALS u porodici a kod bolesnika bez ekspanzije, veća vrednost medijane manjeg alela je registrovana kod bolesnika sa pozitivnom porodičnom istorijom na ALS, demenciju i oba u poređenju sa ostatkom grupe. Korelacija veličine ponovaka nije registrovana sa godinama početka bolesti kod bolesnika bez ekspanzije kao ni kod bolesnika sa ekspanzijom. Ovo je prva detaljna studija određivanja veličine *C9orf72* kod ALS bolesnika u Srbiji. Naši rezultati naglašavaju potrebu za *C9orf72* genetičkom analizom kod ALS bolesnika sa i bez FTD.

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