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Re-analysis of the Hungarian amyotrophic lateral sclerosis population and evaluation of novel ALS genetic risk variants

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a presently incurable neurodegenerative disease. Some genes have a causal relationship to ALS, others act as susceptibility and/or risk factors. We aimed to elucidate the role of 14 ALS-related genes in the Hungarian ALS population of 183 patients. Mutation screening of major ALS genes was performed. *SMN1* and *SMN2* genes were examined by multiplex ligation-dependent probe-amplification assay; intermediate repeat expansions in the *ATXN1* and *ATXN2* genes were analyzed by fragment analysis. Additional variants in putative ALS genes were screened from previously acquired next generation sequencing data. We confirmed the repeat expansion of the *C9orf72, ATXN1* and *ATXN2* genes as ALS risk factors in this Hungarian cohort. Additionally, we identified a pathogenic *SOD1* mutation and suggested its founder effect. A likely pathogenic variant in the *MFSD8* gene was detected, and variants of interest were uncovered in the *ANXA11* and *GLT8D1* genes. We provide valuable data as part of the growing body of work on population-specific aspects of the genetic background of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease which is characterized by the loss of lower and upper motor neurons (LMN and UMN, respectively). Initial symptoms usually occur around the age of 50–60 years. Approximately one in ten cases shows a positive family history (fALS), whereas the other 90% remains sporadic (sALS) (Renton, Chiò, & Traynor, 2014). Patients usually develop fatal respiratory failure within 2–4 years of disease onset (Hobson and McDermott, 2016).

The first gene associated with ALS, namely *SOD1,* was reported in 1993 (Rosen et al., 1993). Currently more than 130 genes are linked to the disease, that are categorized either as major or definitive ALS genes or as minor or candidate genes. Due to vast amounts of data generated in recent years with large-scale, next generation sequencing studies, many new genes and variants have been proposed as risk or susceptibility factors for ALS (for more information please visit: "Amyotrophic Lateral Sclerosis Online Database (ALSoD)" – https://www.alsod.ac.uk) (Abel et al., 2012). In 40%–80% of fALS cases and in 5%–15% of sALS, a genetic variant may be identified (van Es et al., 2017).

Genetic factors investigated in our current study include the *survival of motor neuron 1* (*SMN1*) and *2* (*SMN2)* genes, that play a causative role in the pathogenesis of spinal muscular atrophy (SMA). ALS and SMA have been observed to occur together in families, thus intensive research aimed to uncover the role of the *SMN1* and *SMN2* genes in ALS has commenced (Corcia et al., 2002).

CAG repeat expansions in the *ataxin 1 (ATXN1)* and *ataxin 2 (ATXN2)* genes are causative of spinocerebellar ataxia type 1 and 2 (SCA1 and SCA2) respectively. Normal alleles of *ATXN1* contain 6–35 repeats, whereas more than 39 repeats are linked to SCA1 (Quan et al., 1995). Less than 33 repeats are considered normal in the *ATXN2* gene, whereas expanded alleles can include more than 50 repeats on the locus (Pulst et al., 1996). *ATXN1* encodes an RNA-binding protein that has been shown to disrupt the nucleocytoplasmic transport of TDP-43, thus facilitating its aggregation (Tazelaar et al., 2020). Ataxin-2 proteins tend to mislocalize in spinal cord neurons in ALS patients (Elden et al., 2010).

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Table 1
Summary of the studies carried out by our workgroup before.

Year of study	Population size	Aim of study	Methodology	Main results	Reference
2017	28 ALS patients and 50 healthy control individuals	investigating the coding region of 3 Mendelian genes: *FUS*, *SETX*, and *C9orf72*	high-throughput next generation sequencing	a novel heterozygous missense mutation (c.791A > G, p.N264S) of the *SETX* gene	Tripolszki et al., 2017a
2017	66 ALS patients (including previously investigated patients)	elucidating the frequency of the *SOD1* and *C9orf2* variants in the Hungarian ALS population	direct DNA sequencing for the *SOD1* gene and 2 step protocol consisting of fragment length analysis and repeat-primed PCR for the *C9orf72*	*SOD1* gene: three known heterozygous missense mutations (c.43G > A p.Val14Met; c.272A > C p.Asp90Ala; c.435G > C p.Leu144Phe) and 1 novel mutation (c.275_276delAA, p.Lys91ArgfsTer8)	Tripolszki et al, 2017b
				C9orf72 gene: identified the GGGGCC repeat expansion in 1 out of 66 ALS patients (1.51%) with the Finnish risk haplotype	
2019	136 ALS patients (including previously investigated patients)	evaluating *ANG* gene variants detected in the Hungarian ALS patients and characterizing their significance and functional properties	direct DNA sequecing, molecular dynamics simulation by AMBER14, visual molecular dynamics, and ribonucleolytic activity assay, nuclear translocation assay	*ANG* p.M-24I mutation (c.3G > T) was detected in 2 patients a missense variant (c.379G > A; V103I) was identified in 1 patient	Tripolszki et al., 2019a
				the p.R33W variant, located in the nuclear localization signal was identified in 1 patient	
				based on molecular dynamics simulations and functional assay the p.R33W variant displayed 57.4% of the ribonucleolytic activity compared to wild-type angiogenin, and the variant exhibits decreased nuclear translocation activity	
2019	107 ALS patients (including previously investigated patients) and 184 neurogically healthy individuals	detecting population-specific variants in major and minor and/or susceptibility ALS genes, and genes related to other neurogenetic diseases	two step protocol mentioned above for the *C9orf72*	*C9orf72* gene: repeat expansions were present in 9.3% of patients	Tripolszki et al, 2019b
			whole exome sequencing for 21 patients	29 variants and repeat expansions in 14 major ALS genes were observed, we detected a probable causal variant in 36.45% (39/107) of the ALS patients	
			next generation sequencing of a gene panel consisting of 247 genes	most frequently mutated genes were *NEK1* (6/107, 5.6%), *NEFH, SQSTM1* (4/107, 3.7%), *KIF5A, SPG11* (3/107, 2.8%), *ALS2, CCNF, FUS, MATR3, TBK1*, and *UBQLN2* (2/107, 1.9%)	

ALS:amyotrophic lateral sclerosis; *FUS*: Fused in sarcoma gene; *SETX:* Senataxin gene; *C9orf72:* Chromosome 9 open reading frame 72 gene; *SOD1:* superoxide dismutase 1 gene; *ANG:* Angiogenin gene; *NEK1:* NimA-Related Protein Kinase 1 gene; *NEFH:* Neurofilament Heavy Chain gene; *SQSTM1:* Sequestosome 1 gene; *KIF5A:* Kinesin Family Member 5A gene; *SPG11:* Spatacsin gene; *ALS2:* Alsin Rho Guanine Nucleotide Exchange Factor gene; *CCNF:* Cyclin F gene; *MATR3:* Matrin 3 gene; *TBK1:* TANK Binding Kinase 1 gene; *UBQLN2:* Ubiquilin 2 gene.

The p.M719V variant of the *CYLD* gene segregates with ALS and interferes with 2 important roles of the enzyme: it enhances the deubiquitinase activity and impairs the activity of the NF-kB signaling pathway. Evidence suggests that the variant promotes the formation of TDP-43 aggregates (Dobson-Stone et al., 2020). Latest studies presume that not only deubiquitinase domain variants could contribute to the development of disease (Gu et al., 2021).

Some variants in the *annexin A11* (*ANXA11*) gene are thought to contribute to neurodegeneration by forming aggregates inside cells, whilst other variants cause slower breakdown of stress granules (Smith et al., 2017). Recently, ALS associated variants in the gene have been found to lead to increased intracellular calcium levels, which is a key component in neurodegeneration (Nahm et al., 2020).

The genetic background of ALS shows a great variability between different populations; thus, it is extremely important to describe the hereditary factors contributing to ALS in varied ethnic groups to map the genetic heterogeneity behind ALS. Comprehensive data colligating Hungary have not been published yet. Table 1 contains a brief recapitulation of our studies conducted to this day aiming to explore the genetic background of ALS in the Hungarian population (Tripolszki et al., 2019a, 2019b, 2017a, 2017b).

In our present study, we aimed to further expand our investigations to provide an even more detailed description of the genetic background of ALS in the Hungarian population. Within the frameworks of the current study, we performed the variant screening of major ALS genes in the cases of the 29 newly recruited patients. We carried out the screening of *SMN1, SMN2, ATXN1, ATXN2* genes and the screening of 2 exons in the *ANXA11* gene and 1 exon in the *CYLD* gene in all ALS patients and control subjects. Furthermore, were assessed novel ALS associated genes, such as the *ARPP21, GLT8D1, TIA1, MFSD8, CYLD, LRP10, SH2B3, S1R,* and *CYP1A2* genes in the formerly acquired WES data of 21 ALS patients.

Risk factor genes were selected based on a thorough scientific literature search. ALSoD gene classification was taken as a guideline, several “tenuous” and “moderate evidence” classified genes were investigated in our study (Abel et al., 2012). We favored those genes that have been not investigated in our latest study because the involvement of these genes in the pathomechanism of ALS was not known at the time. In addition, we opted for analyzing the genes that have been reported from a cohort of ALS patients, rather than from computer simulation studies or from animal

Table 2
Clinical characteristics of the recruited ALS patients.

Number of patients recruited for the current study	183
Male: Female (ratio)	73:110 (0.66)
Mean age at onset in years (SD in years)	62.99 (10.12)
Minimum age at onset (years)	36
Maximum age at onset (years)	86
Number of healthy age and sex matched control individuals	204

experiments. Table 3 summarises the genes investigated in this study and their classification based on ALSoD (Abel et al., 2012).

2. Patients and methods

2.1. Patients

Patient recruitment was performed by senior clinicians at the Department of Neurology, University of Szeged, Albert Szent-Györgyi Medical School. The study was conducted in concordance with the Declaration of Helsinki and approved by the Ethical Board of the University of Szeged (2013/556). Written informed consent was obtained from all patients. All patients fulfilled the revised El Escorial and the Awaji-Shima criteria for ALS (Carvalho and Swash, 2009; Ludolph et al., 2015). Table 2 shows the main characteristics of the patient cohort.

One patient reported a first-degree relative that had also been diagnosed with ALS. Two additional patients had a relative with an unconfirmed diagnosis of an ALS-like disease. Furthermore, 4 patients (4 of 183, 2.19%) had a positive family history for other neurodegenerative diseases, such as Alzheimer's or Parkinson's disease.

2.2. Methods

In the flowchart (Fig. 1), we present the approach we used in the study.

2.3. DNA extraction

Genomic DNA was extracted from venous blood mixed with EDTA for anticoagulation with the DNeasy Blood & Tissue Kit (QIAGEN, Gödöllő, Hungary). As sample collection was performed over a span of 8 years, not all DNA samples were of sufficient quality to perform all test; hence, the number of samples varied from test to test.

2.4. Sanger sequencing

One hundred thirty-three samples had previously been screened for mutations and variants in the major ALS genes and for repeat expansions of the *C9orf72* gene (DeJesus-Hernandez et al., 2012). In the case of the 29 newly recruited patients we performed a routine diagnostic screening of the following major ALS genes by Sanger sequencing: *SOD1, ANG, TARDBP, FUS, UBQLN2* and *NEK1*. The pathogenic hexanucleotide repeat expansion in the *C9orf72* gene was also examined in the 29 newly recruited patients according to the 2-step protocol we described in our 2019 study (Akimoto et al., 2014; Tripolszki et al., 2019b).

Furthermore, we examined the fourth and seventh exons of the *ANXA11* gene and the fifteenth exon of the *CYLD* gene with the help of specific primers by polymerase chain reaction and subsequent Sanger sequencing.

2.5. Multiplex ligation-dependent probe-amplification assay

Duplications of the *SMN1* and deletions of the *SMN2* genes were examined with the help of multiplex ligation-dependent probe-amplification (MLPA) assays in 148 ALS patients and in 148 healthy control subjects. The testing was carried out according to standard protocols using the SALSA MLPA SMA carrier probemix P060 kit (MRC Holland), which contains probes specific for *SMN1* and *SMN2* as well as control probes specific for different loci.

2.6. Fragment-length analysis

CAG trinucleotide repeats expansions in the *ATXN1* and *ATXN2* genes were examined by fragment-length analysis in 182 and 153 ALS patients and in 178 and 195 healthy control individuals, respectively. Primer sequences described by Annesi *et al.,* (1997), and Pulst *et al.,* (1996) were used. The forward primers were marked with 6-FAM fluorophore. The number of CAG repeats were determined using capillary electrophoresis.

2.7. Next generation sequencing

We previously performed whole exome sequencing (WES) on 21 selected ALS samples (Tripolszki et al., 2019b). The workflow of variant calling and data analysis is well described (Tripolszki et al., 2019b).

In this study we reanalyzed the WES data to identify additional variants in the entire length of the *ANXA11* and *CYLD* genes. Furthermore, we examined the full coding sequence of the following ALS susceptibility and/or risk factor genes: *ARPP21, GLT8D1, TIA1, MFSD8, CYLD, LRP10, SH2B3, S1R* and *CYP1A2*. The variants were validated by bidirectional Sanger sequencing in case the coverage of the region was below 25 reads per base or the allele balance was lower than 0.3.

2.8. Statistical analysis

We used descriptive and comparative statistical methods to analyze our data. Statistical tests were carried out on GraphPad Prism 8. To compare the ALS and the control population Student's t-tests, Fisher's exact test and χ^2 statistics were used and an odds ratio was also determined. The level of significance was set at $p < 0.05$.

3. Results

In this study, we report the results of the mutational screening of 29 newly recruited ALS patients. Furthermore, copy number variation analysis of *SMN1* and *SMN2* genes and repeat expansion assessment of *ATXN1* and *ATXN2* genes could be performed in a high proportion of our ALS cohort. Additionally, we assessed genetic risk factors and/or susceptibility genes for ALS by reanalyzing previously acquired WES data of 21 ALS patients. We highlight and discuss genetic variants in both well-established major ALS genes and in potential new ALS genes. Main findings of our study are presented in Table 4 and 5.

3.1. Variants identified in definitive ALS genes

We identified a repeat expansion in heterozygous form in the non–coding region of the *C9orf72* gene in 3 out of the 29 newly diagnosed patients (3 of 29, 10.3%). The expanded allele contained more than 145 repeats in all patients.

A pathogenic variant in the *SOD1* gene was discovered during the major ALS gene screening in 1 patient out of the 29 newly recruited patients. The p.L145F variant (rs1482760341) was found

Table 3
Gene panel used to identify ALS-related variants.

routine diagnostic screening of every patient diagnosed with ALS		*C9orf72*
		FUS
		NEK1
		SOD1
		TARDBP
		UBQLN2
		ANG
additional ALS risk factor and/or susceptibility genes screened for research purposes in our current study	definitive ALS genes	*ANXA11*
	clinical modifier genes	*ATXN2*
	strong evidence genes	*ATXN1*
	moderate evidence genes	*SMN1*
	tenuous genes	*ARPP21*
		GLT8D1
		SMN2
		TIA1
	genes not mentioned in ALSoD	*CYLD*
		MFSD8
		LRP10
		SH2B3
		S1R
		CYP1A2
Gene classification according to ALSoD (https://alsod.ac.uk) (Abel et al., 2012)		

ALS: amyotrophic lateral sclerosis; *C9orf72:* Chromosome 9 open reading frame 72 gene; *FUS:* Fused in sarcoma gene; *NEK1:* NimA-Related Protein Kinase 1 gene; *SOD1:* superoxide dismutase 1 gene; *TARDBP:* TAR DNA Binding Protein gene; *UBQLN2:* Ubiquilin 2 gene; *ANXA11:* Annexin A11 gene; *ATXN2:* Ataxin 2 gene; *ATXN1:* Ataxin 1 gene; *SMN1:* Survival Of Motor Neuron 1, Telomeric gene; *ARPP21:* cAMP Regulated Phosphoprotein 21 gene; *GLT8D1:* Glycosyltransferase 8 Domain Containing 1 gene; *SMN2:* Survival Of Motor Neuron 2, Centromeric gene; *TIA1:* TIA1 Cytotoxic Granule Associated RNA Binding Protein gene; *CYLD:* CYLD Lysine 63 Deubiquitinase gene; *MFSD8:* Major Facilitator Superfamily Domain Containing 8 gene; *LRP10:* LDL Receptor Related Protein 10 gene; *SH2B3:* SH2B Adaptor Protein 3 gene; *S1R:* Sigma 1 receptor gene; *CYP1A2:* Cytochrome P450 Family 1 Subfamily A Member 2 gene; ALSoD: ALS Online Database.

Table 4
Summary of our own results concerning copy number variation of *SMN1* and *SMN2* genes and intermediate repeat expansions in the *ATXN1* and *ATXN2* genes and overview of literature.

Gene	Variant	Number of patients and/or controls in this study	Frequency in ALS patients and/or controls described by other groups (population size)	References	First report	Published functional studies
SMN1	duplication	6/148 (4.05%)/ 7/148 (4.73%)	7.8%/ 1.8% (167 ALS patients and 167 healthy controls); 5.1%/2.6% (433 ALS patients and 454 healthy controls)	Corcia et al., 2002; Corcia et al, 2006	Corcia et al, 2002	
SMN2	homozygous deletion	7/148 (4.73%)/ 10/148 (6.76%)	20%/ 2% (25 ALS patients and 25 healthy controls); 5.8%/7.4% (502 ALS patients and 502 healthy controls)	Lee et al., 2012; Corcia et al, 2012	Lee et al., 2012	
ATXN1	intermediate length CAG trinucleotide expansion	16/182 (8.79%)/ 2/178 (1.12%)	7.07%/ 2.38% (418 ALS patients and 296 healthy individuals); 5.84%/ 2.75% (411 ALS patients and 436 healthy control individuals)	Conforti et al, 2012; Gonçalves et al, 2020	Conforti et al, 2012	(Tazelaar, G.H., et al., 2020).
ATXN2	intermediate length CAG trinucleotide expansion	28/153 (18.3%)/ 18/195 (9.23%)	3%/ 0% (232 ALS patients and 395 healthy control individuals) 7.2%/ 5.1% (471 ALS cases and 556 healthy controls)	(Corrado et al., 2011); Daoud et al., 2011	(Elden et al., 2010)	Elden et al, 2010.

ALS: amyotrophic lateral sclerosis; *SMN1:* Survival Of Motor Neuron 1, Telomeric gene; *SMN2:* Survival Of Motor Neuron 2, Centromeric gene; *ATXN1:* Ataxin 1 gene; *ATXN2:* Ataxin 2 gene.

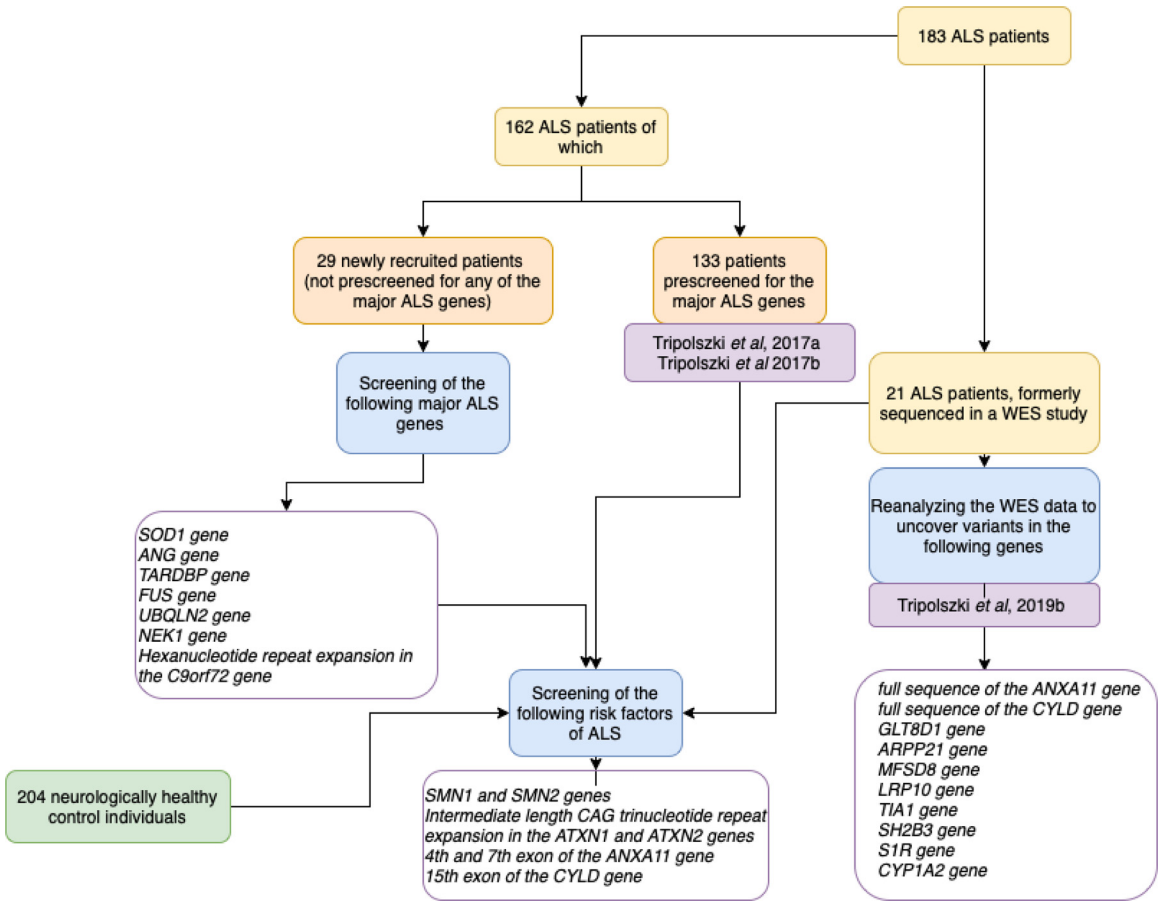

Fig. 1. Workflow of the extended genetic analysis of the Hungarian ALS patient cohort. ALS: amyotrophic lateral sclerosis; *SOD1:* superoxide dismutase 1 gene; *ANG:* Angiogenin gene; *TARDBP:* TAR DNA Binding Protein gene; *FUS:* Fused in sarcoma gene; *UBQLN2:* Ubiquilin 2 gene; *NEK1:* NimA-Related Protein Kinase 1 gene; *C9orf72:* Chromosome 9 open reading frame 72 gene; *SMN1:* Survival Of Motor Neuron 1. Telomeric gene; *SMN2:* Survival Of Motor Neuron 2, Centromeric gene; *ATXN1:* Ataxin 1 gene; *ATXN2:* Ataxin 2 gene; *ANXA11:* Annexin A11 gene; *CYLD:* CYLD Lysine 63 Deubiquitinase gene; WES: whole exome sequencing; *GLT8D1:* Glycosyltransferase 8 Domain Containing 1 gene; *ARPP21:* cAMP Regulated Phosphoprotein 21 gene; *MFSD8:* Major Facilitator Superfamily Domain Containing 8 gene; *LRP10:* LDL Receptor Related Protein 10 gene; *TIA1:* TIA1 Cytotoxic Granule Associated RNA Binding Protein gene; *SH2B3:* SH2B Adaptor Protein 3 gene; *S1R:* Sigma 1 receptor gene; *CYP1A2:* Cytochrome P450 Family 1 Subfamily A Member 2 gene.

in heterozygous form in a 70-year-old woman. The patient's complaints started 8 months before her clinical ALS diagnosis: she initially experienced weakness of the left leg, subsequently, the weakness spread to all her limbs. On examination she presented pseudobulbar and UMN signs of ALS as well, but her symptoms were predominantly of LMN origin. She did not report any family members suffering either from ALS or from other neurodegenerative diseases.

In 2017 Smith *et al.,* published an elegant stringent analysis proving that the rare *ANXA11* p.D40G variant segregates with ALS (Smith et al., 2017). However, we did not identify the mentioned variant in our 183 patients. A different variant, p.E369K in the *ANXA11* gene was detected in 1 patient during the re-analysis of the WES data from our 21 samples. The variant is reported to have a MAF of 0.0041 in the ExAc database. Other than SIFT (0.001) and Polyphen (0.99), Varity (0.74), MutationTaster (1), GenoCanyon (1) and fitCons (0.78) suppose its deleterious effect. We also identified a common recurring missense benign variant in the *ANXA11* gene. The p.R230C variant was found in 117 patients (117 of 176 is 66.5%).

3.2. Results of copy number variation assays of SMN1 and SMN2 genes

The copy number variation of *SMN1* and *SMN2* genes has been investigated using MLPA assay in 148 ALS samples and 148 neurologically healthy control individuals. We identified duplications of the *SMN1* gene in 6 ALS patients (6 of 148, 4.05%) and in 7 control samples (7 of 148, 4.73%). Several studies reported the duplications as risk factor of ALS, with a frequency of 5–10% (Blauw et al., 2012; Corcia et al., 2006). Carrying more than 2 copies of the *SMN1* gene increases risk of developing the diseases 2-fold (Blauw et al., 2012).

Homozygous deletions of the *SMN2* gene were found in 7 ALS patients (7 of 148, 4.73%) and in 10 control individuals (6.76%).

3.3. Investigations on the intermediate repeat expansion of the ATXN1 and ATXN2 genes

The length of the CAG trinucleotide tract in the *ATXN1* gene was examined in 182 ALS patients and 178 healthy control individuals by amplicon-fragment analysis. Distribution and characteristics of the repeats can be seen in Fig. 2. Based on the data, we conclude that the allele distribution is similar in both groups. Twenty-five patients carried homozygous repeat numbers: 16 patients had 27/27, 8 patients 28/28, whereas only 1 patient had 30/30 repeats. One of the patients carried a homozygous intermediate length repeat expansion: her genotype was 32/35 repeats. Eighteen control individuals were homozygous when considering repeat length (1 person had 25/25 and 1 had 26/26 repeats, 5 people had 27/27 and 11 people had 28/28 repeats). The average repeat number among patients was 28.03, whereas healthy control individuals possessed a mean repeat number of 27.62, and this difference is significant

Table 5
Variants identified in our study. Known benign variants are not listed in this table.

Gene	Variant	Number of patients in this	Classification according to the ACMG guidelines	MAF in ExAc/gnomAD	Predictions by bioinformatic tools SIFT/PolyPhen/REVEL/CADD PHRED	First report	Published functional studies
SOD1	p.L145F (rs1482760341)	1/29 (3.45%)	pathogenic	0/0.000016	0/ n.a./ 0.92/ 24	Deng et al., 1993	Gal et al., 2016
ANXA11	p.E369K (rs34414015)	1/21 (4.76%)	benign	0.004036/ 0.003508	0/ 1/ 0.43/ 53	Nahm et al., 2020.	-
GLT8D1	p.K5E	1/21 (4.76%)	VUS	0/0	0/ n.a./ 0.23/ 26.1	not reported before in ALS	-
ARPP21	p.V521A	1/21 (4.76%)	VUS	0/0	0.34/ n.a./ 0.07/ 24.2	not reported before in ALS	-
MFSD8	p.Q304X	1/21 (4.76%)	likely pathogenic	0/0	n.a./ n.a./ n.a./ 35	not reported before in ALS	-

ACMG: American College of Medical Genetics and Genomics; MAF in ExAc/ gnomAD: minor allele frequency in ExAc aggregated database/ gnomAD genome databases; SIFT: Sorting Intolerant from Tolerant (https://sift.bii.a-star.edu.sg), PolyPhen: Polymorphism Phenotyping (http://genetics.bwh.harvard.edu/pph2/); REVEL: Rare Exome Variant Ensemble Learner (https://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg19&g=revel); CADD PHRED: Combined Annotation Dependent Depletion (https://cadd.gs.washington.edu/snv); *SOD1:* superoxide dismutase 1 gene; n.a.: not reported; *ANXA11:* Annexin A11 gene; *GLT8D1:* Glycosyltransferase 8 Domain Containing 1 gene; *ARPP21:* cAMP Regulated Phosphoprotein 21 gene; *MFSD8:* Major Facilitator Superfamily Domain Containing 8 gene.

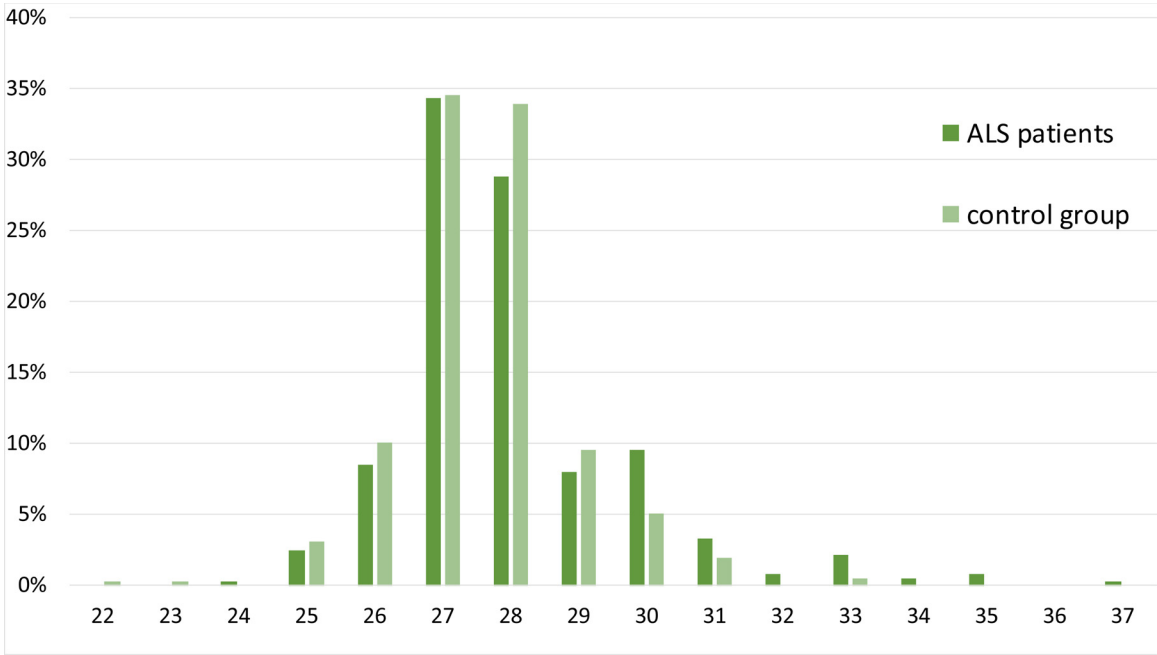

Fig. 2. Distribution of *ATXN1* allele frequency between ALS patients and control group. ALS: amyotrophic lateral sclerosis; *ATXN1:* Ataxin 1 gene.

($p = 0.0014$, 95% confidence interval (CI): 0.16–0.6589).To examine the intermediate-length repeat expansion in the *ATXN1* gene, we used the repeat cutoff value proposed by Conforti *et al.*, which was determined to be ≥32 repeats via receiver operating characteristic curve analysis (Conforti et al., 2012). Sixteen of the ALS patients harbored an allele of 32 repeats or more (16 of 182, 8.79%). In comparison, only 2 control individuals were found to have an intermediate length repeat expansion (2 of 178, 1.12%). This difference was significant ($p = 0.001$), and we calculated an odds ratio of 8.482 (95% CI: 2.052–37.56).

One hundred and fifty-three ALS patients and 195 healthy control individuals were screened for the intermediate length repeat expansion in the *ATXN2* gene. Fig. 3 shows the similarities and differences in terms of distribution of repeats between patients and the control group. Twenty-six ALS patients were found to be heterozygous for the locus (26 of 153, 16.99%). Of these 26 people, 9 carried a heterozygous intermediate-length repeat expansion. One hundred twenty-seven ALS patients were homozygous, and 2 of these carried the repeat expansion. Eighteen healthy control individuals were heterozygous (18 of 195, 9.23%) and none of these carried the repeat expansion. Thus, we detected a significant difference in the distribution of the zygosity between the ALS and control groups: heterozygosity is 2 times more likely to occur among ALS patients ($p = 0.035$, odds ratio: 2.013, 95% CI: 1.07–3.792). Our findings are the first to our knowledge to highlight the association of *ATXN2* intermediate repeat length expansion heterozygosity and ALS.

The average number of *ATXN2* repeats among ALS patients was found to be 23.28 repeats, while the mean number of repeats was 23.11 in the control group: thus, a significant difference in average allele length was observed ($p = 0.0409$, 95% CI: 0.007215–0.3395). We also examined the relationship between intermediate length repeat expansions in the *ATXN2* gene and ALS. After considering 5 different cutoff numbers, ≥24, ≥27, ≥29, ≥30 and ≥32 repeats, proposed by 4 different studies performed on ALS patients of different European ethnicities (Corrado et al., 2011; Elden et al., 2010;

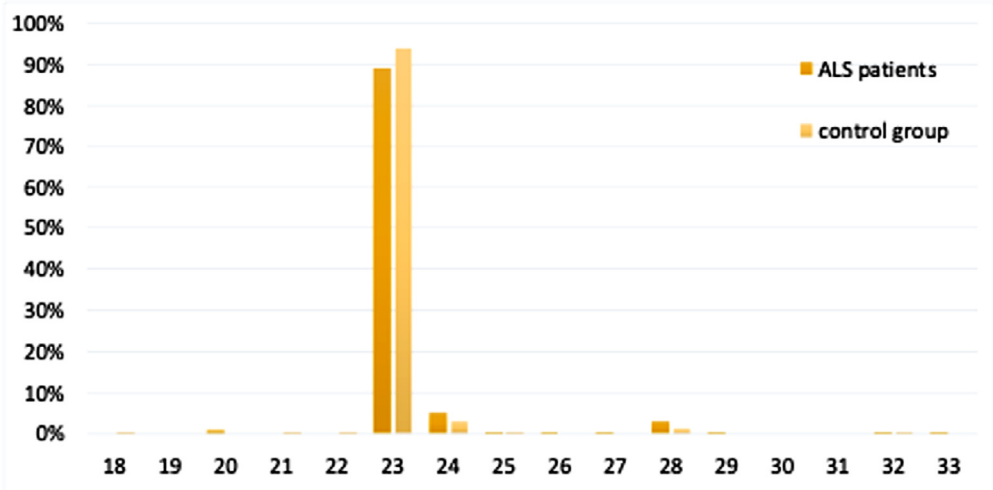

Fig. 3. Distribution of *ATXN2* allele frequency between ALS patients and control group. ALS: amyotrophic lateral sclerosis; *ATXN2:* Ataxin 2 gene.

Sproviero et al., 2017; Van Damme et al., 2011), since population specific number for Hungarian patients has not been determined yet, we opted for ≥24, as Elden *et al.* originally defined intermediate length alleles. By using this value, 28 of 153 (18.3%) ALS patients were considered to carry an intermediate length repeat expansion, whereas 18 of 195 (9.23%) controls carried a glutamine tract of 24 repeats or more ($p = 0.0164$). One patient had 24/24 and 2 patients had 28/28 homozygous genotype. Fourteen patients had an allele of 24 repeats, 5 patients carried a heterozygous allele with 28 repeats. 25, 26, 27, 29 and 32 repeats were represented by only 1 patient. Similar to other studies, we identified more than 2fold increase of risk for developing ALS associated with an intermediate-length repeat expansion (OR: 2.203, 95% CI: 1.196–4.098) (Tavares de Andrade et al., 2018).

3.4. Novel genes associated with ALS reveal some variants of interest

As intensive research is still required to fully understand and describe the genetic background of ALS, several novel genes are reported to be associated with ALS every year. In 2018 our research group performed WES of 21 ALS samples (Tripolszki et al., 2019b). By reanalyzing our former WES data, we were able to re-assess those genes that have been associated with ALS since late 2018. All variants are interpreted according to the 2015 guideline of the American College of Medical Genetics (Richards et al., 2015).

We have detected a variant of uncertain significance in the *GLT8D1* gene, which encodes a member of the glycosyltransferase family. The p.K5E variant affects the cytoplasmic domain GLT8D1 protein, which includes the Golgi localization signal as well.

We detected 2 different variants in the *ARPP21* gene. One patient carried the p.V521A variant, and the p.L273V variant was observed in 2 patients.

A likely pathogenic variant in the *MFSD8* gene was also identified. In the case of the p.Q304X variant, a wild type glutamine codon is replaced by a premature stop codon, which terminates translation early. The variant affects the eighth transmembrane domain of the protein.

In our study we identified a missense variant, p.R562H in the *LRP10* gene. The variant affects an arginine-rich domain of the protein, which is localized to the cytoplasm.

In the low complexity domain of *TIA1* we identified 2 of the rare variants described by Baradaran-Heravi *et al.* One patient carried the p.Q31R variant, whereas the p.N357S variant was detected in 2 of our samples. Both variants, p.Q31R and p.N357S, were found in healthy control individuals as well, and, thus, they are not likely to be deleterious (Baradaran-Heravi et al., 2018). Furthermore, 3 missense variants were also detected in the gene outside of the low-complexity domain, which we assume to be benign polymorphisms. The p.G12S and p.G31V variants are involved in the formation of the first RNA recognition motif of the protein. The p.E242G variant is located inside the third RNA recognition motif.

In a recent linkage disequilibrium study, a haplotype with increased risk for ALS was identified. The haplotype includes SNPs from both the *SH2B3* and the *ATXN2* genes (Lahut et al., 2012). We have detected 1 missense variant in the *SH2B3* gene that is a part of the risk haplotype. Thirteen of our patients (13 of 21 is 61.9%) carried the variant in either in a homozygous form (5 of 21, 23.8%) or in a heterozygous form (8 of 21, 38.1%), which makes it unlikely that the presence of the p.W60R (rs3184504) variant itself increases the risk of developing ALS.

We did not detect the p.M719V variant in the *CYLD* gene in our 166 ALS patients. Furthermore, by re-analyzing the WES data in 21 patients, we did not find any variant in the coding sequence of the gene.

Furthermore, we examined the *S1R* gene and the *CYP1A2* gene, as both are known to modify the age of onset for ALS (Couly et al., 2020; Siokas et al., 2021). However, we did not find any relevant variants in either of these genes.

4. Discussion

In our current study we performed the comprehensive genetic analysis of 29 newly diagnosed ALS patients. Copy number variation of *SMN1* and *SMN2* genes and intermediate repeat expansions of the *ATXN1* and *ATXN2* genes were evaluated. Furthermore, we conducted the re-assessment of our 2018 whole exome sequencing study and thus explored ALS associated risk factor and/or susceptibility genes.

During the analysis of the 29 newly recruited patients we identified the GGGGCC hexanucleotide expansion in the *C9orf72* gene in 3 patients. This is concordant with the frequency of repeat expansions in *C9orf72* gene reported in our 2019 study (9.3% vs. 10.3%). The mean age of the patients carrying the repeat expansion in the *C9orf72* gene was 58.3 years, which is lower than the mean age in our cohort (62.99 years vs. 58.33 years). All patients presented at the Department of Neurology less than 6 months after the initial symptoms appeared. All of them displayed signs of severe damage to the LMN, 2 of the 3 also had bulbar and UMN manifestations.

The *C9orf72* hexanucleotide repeat expansion is less prevalent in Asian populations: from Japanese and Iranian sporadic ALS cohorts less than 4% and 1.6% were reported respectively (Alavi et al., 2014; Konno et al., 2013). Repeat expansions may be more common among Hungarian ALS patients than in non–Finnish Europeans, where it has been reported to account for around 8% of sporadic ALS cases (Pliner et al., 2014).

The p.L145F variant in the *SOD1* gene was identified in heterozygous form in 1 patient. The variant has been previously reported in familial ALS from an Istro-Rumanian community living on the Istrian peninsula, Croatia, and from an area of the former Yugoslavia. The variant is associated with a later disease onset and slower progression due to the moderate decrease in the *SOD1* enzyme activity. Furthermore, cognitive impairment was also reported in association with this variant (Ferrera et al., 2003; Masè et al., 2001). The patient displayed modest memory problems and some attention deficit issues. However, the Mini-Mental State Examination test (MMSE) score was 28 of 30. Our results confirm both the later onset of disease and slower progression, although we could not confirm the cognitive impairment. By conducting a haplotype analysis, we could determine whether the mutation arose de novo or the patient may be related to the abovementioned family. However, the fact that the patient claimed to not have any affected relatives perhaps it raises the possibility of our theory of the variant being a founder mutation.

In the *ANXA11* gene a missense variant, p.E369K was revealed. Even though the mutant amino acid (position 369) is not involved in forming one of the annexin repeat domains, it could be pathogenic, as C-terminal *ANXA11* variants are thought to disrupt essential calcium-dependent intracellular processes (Nahm et al., 2020). Our results further confirm that the *ANXA11* gene can be considered as an ALS candidate gene. Nonetheless, its disease-causing variants are very rare among Hungarian ALS patients.

Our MLPA assay results indicate that duplication of the *SMN1* gene is similar for the ALS and control groups (Veldink et al., 2005). Reports have been inconsistent as to whether homozygous deletion of the *SMN2* gene is a risk factor for motor neuron diseases or even protective against them (Corcia et al., 2012, 2006; Lee et al., 2012). Based on our results a tendency might be observed, that neither *SMN1* nor *SMN2* play important role as a risk factor for ALS in the Hungarian population.

Intermediate length CAG repeats expansions of the *ATXN1* and *ATXN2* genes were assessed by amplicon fragment length analysis. We confirm the role of intermediate length *ATXN1* CAG repeat expansions as a risk factor of ALS in the Hungarian population. *ATXN1* intermediate length repeat expansions were found to be more common than in other ethnically homogenous cohorts. In a Brazilian study, the frequency of the intermediate repeat expansion was found to be 5.84% among ALS patients (OR: 2.191), and 7.07% of Italian patients carried an intermediate-length allele (Conforti et al., 2012; Gonçalves et al., 2020). The mean age at onset of the patients bearing the intermediate length repeat expansion was 60.18 years. At first presentation, these patients had been experiencing symptoms on average for 18 months. All patients had both UMN and LMN signs, and the majority also showed symptoms of bulbar impairment as well. None of our patients presented with ataxia. Two of the 16 patients carrying the intermediate length repeat expansion in the *ATXN1* gene also had a variant in a major ALS gene. One female harbored the variant p.R84H in the *UBQLN2* gene, which we previously reported (Tripolszki et al., 2019b).

Lattante *et al*., proposed that *ATXN1* intermediate-length repeat expansions are associated with the well-known repeat expansion in the *C9orf72* gene (Lattante et al., 2018). One patient in our cohort was detected to carry both the *ATXN1* intermediate length repeat expansion and the repeat expansion in the *C9orf72* gene. She presented at the Department of Neurology at the age of 70, complaining of a 3-month long history of weakness and dysphagia. Notably, she scored the lowest on the ALS-Functional Rating Scale (37 points) of patients with the intermediate-length repeat expansion in the *ATXN1* gene, and cognitive impairment was not detected (Mini Mental State Exam: 29 of 30 points). Due to the limited size of our cohort, we cannot conclude the link between *ATXN1* and *C9orf72* repeats, but we support the theory of the 2 genes contributing to neurodegeneration via a common pathway (Lattante et al., 2018). None of the patients carrying an intermediate length repeat expansion in the *ATXN2* gene displayed clinical signs of SCA2. Furthermore, no effect of the intermediate CAG repeats could be observed on the phenotype of our patients, most of them presented the typical ALS signs and symptoms. We could not identify an association between *ATXN2* intermediate length alleles and frontotemporal dementia (Lattante et al., 2014). In the last decade, the determination of the cutoff value of intermediate length alleles has undergone an evolutionary process in itself, and guidelines have been published about the topic (Van Damme et al., 2011). To reach a consensus we need studies that identify the intermediate-length CAG repeats in the *ATXN2* gene as a susceptibility factor for ALS from as many ethnically diverse populations as possible. Notwithstanding the limited evidence provided by our study, we strongly support considering the number of intermediate length repeat expansions in the *ATXN2* gene as a risk factor for ALS.

By re-analyzing the formerly acquired WES data from 21 ALS patients we detected several variants of interest.

Abnormal ganglioside metabolism has been known to be associated ALS, and the corresponding genes may modulate the course of the disease (Desport et al., 2006; Stevens et al., 1993). Glycosylation of the gangliosides normally takes place while the proteins pass through the Golgi network. We hypothesize that the p.K5E variant of the *GLT8D1* gene interferes with this process as the proteins might be unable to move across the Golgi network due to the abruption of the Golgi localization signal. In the same article in which Cooper-Knock *et al.,* reported *GLT8D1* as a candidate gene for ALS, they also proposed that variants in the *ARPP21* gene might act synergistically with *GLT8D1* variants, leading to a more severe phenotype (Cooper-Knock et al., 2019). We did not detect any synergistically cooperating variants in our patients; thus, we cannot confirm a synergistic relationship between the 2 genes.

A stop gain variant, p.Q304X was uncovered in the *MFSD8* gene. To our knowledge, this variant has previously not been reported either for ALS or frontotemporal dementia patients. We suppose that the truncated protein is not able to fulfill its role as a transport protein, causing lysosomal dysfunction, possibly contributing to the development of ALS (Geier et al., 2019).

Variants in the *LRP10* gene have long been associated with neurodegenerative diseases, including Parkinson's disease and Lewy body dementia (Quadri et al., 2018). Although the exact function of the protein is yet to be confirmed, its experimental overexpression leads to the formation of fibrillary aggregates in other neurodegenerative diseases (α-synuclein, tau, Aβ) ((Quadri et al., 2018). Based on this knowledge we assume that variants in the *LRP10* gene may contribute to the development of ALS as it also is a neurodegenerative disease.

TIA1 was prioritized as a candidate gene for ALS after identifying several rare variants segregating with the disease. All described variants were located in the low-complexity domain of the protein, which plays an essential role in stress granule assembly. Variants in this domain are believed to lead to neurodegeneration by disrupting the physiological breakdown process of stress granules (Mackenzie et al., 2017). However, *TIA1* variants are not a common cause of ALS among the patients of European origin (Baradaran-

Heravi et al., 2018). Our results do not support the recategorization of the *TIA1* gene as a candidate gene for ALS.

5. Conclusion

With our results, we contribute to the body of work about the genetic heterogeneity of the complex ALS disease. It is known that different genetic factors linked to ALS have a different importance in various ethnic groups (Zou et al., 2017). By characterizing these genetic factors in multiple populations, we hope to understand the role of the proteins encoded by these genes and their varying involvement in the pathomechanism of ALS. Understanding the genetic background of the disease may bring us closer to developing therapeutic approaches personalized to the genetics of each patient (van Es et al., 2017). An oligogenic mode of inheritance has been proposed for ALS, as several variants with high penetrance and many variants with reduced penetrance contribute to ALS (Blitterswijk et al., 2012). Although much data has accumulated, further research is needed to elucidate the role of the novel genes associated with ALS.

Our cohort comprises the most comprehensive sample collection of ALS patients of Hungarian origin. This is the first thorough study to assess the highest number of risk factors for ALS in one specific population. Possible differences from the findings of other groups can be attributed to properties of the Hungarian ALS patients and variations in their genetic background.

In Hungary, a country of less than 10 million inhabitants, it is challenging to collect a larger number of samples than reported in this study. The reported population of ALS patients was collected over more than a decade, and many of the included patients are not alive and not available to establish possible genotype–phenotype correlations. The number of samples may be considered the main limitation of our study; too far-fetched generalized conclusions might not be drawn from our results. However, we stand by collecting data from different ethnicities groups and thus highlighting the distinctive characteristics of each population.

Previously not reported variants identified by our study cannot be considered pathogenic until functional studies confirm their involvement in ALS associated pathologic processes. Thus, our future research will be focused on conducting functional studies to strengthen the link between the newly described variants and ALS.

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CRediT authorship contribution statement

Zsófia Flóra Nagy: Conceptualization, Methodology, Investigation, Writing – original draft. **Margit Pál:** Conceptualization, Methodology, Investigation, Writing – review & editing. **András Salamon:** Resources, Writing – review & editing. **Gloria Kafui Esi Zodanu:** Investigation. **Dalma Füstös:** Investigation. **Péter Klivényi:** Resources. **Márta Széll:** Conceptualization, Methodology, Writing – review & editing, Supervision.

References

Abel, O., Powell, J.F., Andersen, P.M., Al-Chalabi, A., 2012. ALSoD: A user-friendly online bioinformatics tool for amyotrophic lateral sclerosis genetics. Hum. Mutat. 33, 1345–1351. doi:10.1002/humu.22157.

Akimoto, C., Volk, A.E., van Blitterswijk, M., Van den Broeck, M., Leblond, C.S., Lumbroso, S., Camu, W., Neitzel, B., Onodera, O., van Rheenen, W., Pinto, S., Weber, M., Smith, B., Proven, M., Talbot, K., Keagle, P., Chesi, A., Ratti, A., van der Zee, J., Alstermark, H., Birve, A., Calini, D., Nordin, A., Tradowsky, D.C., Just, W., Daoud, H., Angerbauer, S., DeJesus-Hernandez, M., Konno, T., Lloyd-Jani, A., de Carvalho, M., Mouzat, K., Landers, J.E., Veldink, J.H., Silani, V., Gitler, A.D., Shaw, C.E., Rouleau, G.A., van den Berg, L.H., Van Broeckhoven, C., Rademakers, R., Andersen, P.M., Kubisch, C., 2014. A blinded international study on the reliability of genetic testing for GGGGCC-repeat expansions in C9orf72 reveals marked differences in results among 14 laboratories. J. Med. Genet. 51, 419–424. doi:10.1136/jmedgenet-2014-102360.

Alavi, A., Nafissi, S., Rohani, M., Shahidi, G., Zamani, B., Shamshiri, H., Safari, I., Elahi, E., 2014. Repeat expansion in C9ORF72 is not a major cause of amyotrophic lateral sclerosis among iranian patients. Neurobiol. Aging 35, 267.e1–267.e7. doi:10.1016/j.neurobiolaging.2013.07.016.

Annesi, G., Muglia, M., Conforti, F.L., Leone, O., Grandinetti, C., Imbrogno, E., Gabriele, A.L., Naso, F., Brancati, C., 1997. A Simple and rapid nonisotopic method for sizing cag repeats in the sca1 gene. Hum. Hered. 47, 47–51. doi:10.1159/000154389.

Baradaran-Heravi, Y., Dillen, L., Nguyen, H.P., Van Mossevelde, S., Baets, J., De Jonghe, P., Engelborghs, S., De Deyn, P.P., Vandenbulcke, M., Vandenberghe, R., Van Damme, P., Cras, P., Salmon, E., Synofzik, M., Heutink, P., Wilke, C., Simon-Sanchez, J., Rojas-Garcia, R., Turon-Sans, J., Lleó, A., Illán-Gala, I., Clarimón, J., Borroni, B., Padovani, A., Pastor, P., Diez-Fairen, M., Aguilar, M., Gelpi, E., Sanchez-Valle, R., Borrego-Ecija, S., Matej, R., Parobkova, E., Nacmias, B., Sorbi, S., Bagnoli, S., de Mendonça, A., Ferreira, C., Fraidakis, M.J., Diehl-Schmid, J., Alexopoulos, P., Almeida, M.R., Santana, I., Van Broeckhoven, C., van der Zee, J., Goeman, J., Nuytten, D., Sieben, A., De Bleecker, J.L., Santens, P., Versijpt, J., Michotte, A., Ivanoiu, A., Deryck, O., Bergmans, B., Willems, C., De Klippel, N., Peeters, D., Archettim, S., Bonomi, E., Piaceri, I., Ferrari, C., Simões do Couto, F., Verdelho, A., Miltenberger-Miltényi, G., 2018. No supportive evidence for TIA1 gene mutations in a European cohort of ALS-FTD spectrum patients. Neurobiol. Aging 69, 293.e9–293.e11. doi:10.1016/j.neurobiolaging.2018.05.005.

Blauw, H.M., Barnes, C.P., Van Vught, P.W.J., Van Rheenen, W., Verheul, M., Cuppen, E., Veldink, J.H., Van Den Berg, L.H., 2012. SMN1 gene duplications are associated with sporadic ALS. Neurology 78, 776–780. doi:10.1212/WNL.0b013e318249f697.

Blitterswijk, M. Van, Es, M.A. Van, Hennekam, E.A.M., Dooijes, D., Rheenen, W. Van, Medic, J., Bourque, P.R., Schelhaas, H.J., Kooi, A.J. Van Der, Visser, M. De, Bakker, P.I.W. De, Veldink, J.H., Berg, L.H. Van Den, 2012. Evidence for an oligogenic basis of amyotrophic lateral sclerosis 21, 3776–3784. doi:10.1093/hmg/dds199.

Carvalho, M.D.E., Swash, M., 2009. Awaji diagnostic algorithm increases sensitivity of El Escorial criteria for ALS diagnosis 53–57. doi:10.1080/17482960802521126.

Conforti, F.L., Spataro, R., Sproviero, W., Mazzei, R., Cavalcanti, F., Condino, F., Simone, I.L., Logroscino, G., Patitucci, A., Magariello, A., Muglia, M., Rodolico, C., Valentino, P., Bono, F., Colletti, T., Monsurrò, M.R., Gambardella, A., La Bella, V., 2012. Ataxin-1 and ataxin-2 intermediate-length polyQ expansions in amyotrophic lateral sclerosis. Neurology 79, 2315–2320. doi:10.1212/WNL.0b013e318278b618.

Cooper-Knock, J., Moll, T., Ramesh, T., Castelli, L., Beer, A., Robins, H., Fox, I., Niedermoser, I., Van Damme, P., Moisse, M., Robberecht, W., Hardiman, O., Panades, M.P., Assialioui, A., Mora, J.S., Basak, A.N., Morrison, K.E., Shaw, C.E., Al-Chalabi, A., Landers, J.E., Wyles, M., Heath, P.R., Higginbottom, A., Walsh, T., Kazoka, M., McDermott, C.J., Hautbergue, G.M., Kirby, J., Shaw, P.J., 2019. Mutations in the glycosyltransferase domain of GLT8D1 are associated with familial amyotrophic lateral sclerosis. Cell Rep 26, 2298–2306.e5. doi:10.1016/j.celrep.2019.02.006.

Corcia, P., Camu, W., Halimi, J.M., Vourc'h, P., Antar, C., Vedrine, S., Giraudeau, B., De Toffol, B., Andres, C.R., 2006. SMN1 gene, but not SMN2, is a risk factor for sporadic ALS. Neurology 67, 1147–1150. doi:10.1212/01.wnl.0000233830.85206.1e.

Corcia, P., Ingre, C., Blasco, H., Press, R., Praline, J., Antar, C., Veyrat-Durebex, C., Guettard, Y.O., Camu, W., Andersen, P.M., Vourc'h, P., Andres, C.R., 2012. Homozygous SMN2 deletion is a protective factor in the Swedish ALS population. Eur. J. Hum. Genet. 20, 588–591. doi:10.1038/ejhg.2011.255.

Corcia, P., Mayeux-Portas, V., Khoris, J., De Toffol, B., Autret, A., Müh, J.P., Camu, W., Andres, C., 2002. Abnormal SMN1 gene copy number is a susceptibility factor for amyotrophic lateral sclerosis. Ann. Neurol. 51, 243–246. doi:10.1002/ana.10104.

Corrado, L., Mazzini, L., Oggioni, G.D., Luciano, B., Godi, M., Brusco, A., Alfonso, S.D., 2011. ATXN-2 CAG repeat expansions are interrupted in ALS patients. Hum. Genet. 130, 575–580. doi:10.1007/s00439-011-1000-2.

Couly, S., Khalil, B., Viguier, V., Roussel, J., Maurice, T., Liévens, J., 2020. Sigma-1 receptor is a key genetic modulator in amyotrophic lateral sclerosis. Hum Mol Genet 29, 529–540. doi:10.1093/hmg/ddz267.

Daoud, H., Belzil, V., Martins, S., Sabbagh, M., Provencher, P., Lacomblez, L., Meininger, V., Camu, W., Dupré, N., Dion, P. A., Rouleau, G. A., 2011. Association of long ATXN2 CAG repeat sizes with increased risk of amyotrophic lateral sclerosis. Arch Neurol 68(6), 739–742. doi:10.1001/archneurol.2011.111.

DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Gilmer, F., Adamson, J., Kouri, N., Wojtas, A., Sengdy, P., Hsiung, G.R., Karydas, A., Seeley, W.W., Josephs, K.A., Geschwind, D.H., Wszolek, Z.K., Feldman, H., Petersen, R., Miller, B.L., Dickson, D., Boylan, K., 2012. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 72, 245–256. doi:10.1016/j.neuron.2011.09.011.Expanded.

Deng, H.X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W. Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., 1993. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science 261(5124), 1047–1051. doi:10.1126/science.8351519.

Desport, J.C., Torny, F., Lacoste, M., Preux, P.M., Couratier, P., 2006. Hypermetabolism in ALS: correlations with clinical and paraclinical parameters. Neurodegener. Dis. 2, 202–207. doi:10.1159/000089626.

Dobson-Stone, C., Hallupp, M., Shahheydari, H., Ragagnin, A.M.G., Chatterton, Z., Carew-Jones, F., Shepherd, C.E., Stefen, H., Paric, E., Fath, T., Thompson, E.M., Blumbergs, P., Short, C.L., Field, C.D., Panegyres, P.K., Hecker, J., Nicholson, G., Shaw, A.D., Fullerton, J.M., Luty, A.A., Schofield, P.R., Brooks, W.S., Rajan, N., Bennett, M.F., Bahlo, M., Shankaracharya, Landers, J.E., Piguet, O., Hodges, J.R., Halliday, G.M., Topp, S.D., Smith, B.N., Shaw, C.E., McCann, E., Fifita, J.A., Williams, K.L., Atkin, J.D., Blair, I.P., Kwok, J.B., 2020. CYLD is a causative gene for frontotemporal dementia - Amyotrophic lateral sclerosis. Brain 143, 783–799. doi:10.1093/brain/awaa039.

Elden, A.C., Kim, H.J., Hart, M.P., Chen-Plotkin, A.S., Johnson, B.S., Fang, X., Armakola, M., Geser, F., Greene, R., Lu, M.M., Padmanabhan, A., Clay-Falcone, D., McCluskey, L., Elman, L., Juhr, D., Gruber, P.J., Rüb, U., Auburger, G., Trojanowski, J.Q., Lee, V.M.Y., Van Deerlin, V.M., Bonini, N.M., Gitler, A.D., 2010. Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. Nature 466, 1069–1075. doi:10.1038/nature09320.

Ferrera, L., Caponnetto, C., Marini, V., Rizzi, D., Bordo, D., Penco, S., Amoroso, A., Origone, P., Garrè, C., 2003. An Italian dominant FALS Leu144Phe SOD1 mutation: genotype-phenotype correlation. Amyotroph. Lateral Scler. Other Mot. Neuron Disord. 4, 167–170. doi:10.1080/aml.4.3.167.170.

Gal, J., Kuang, L., Barnett, K. R., Zhu, B. Z., Shissler, S. C., Korotkov, K. V., Hayward, L. J., Kasarskis, E. J., Zhu, H., 2016. ALS mutant SOD1 interacts with G3BP1 and affects stress granule dynamics. Acta Neuropathol 132(4), 563–576. doi:10.1007/s00401-016-1601-x.

Geier, E.G., Bourdenx, M., Storm, N.J., Cochran, J.N., Sirkis, D.W., Hwang, J.H., Bonham, L.W., Ramos, E.M., Diaz, A., Van Berlo, V., Dokuru, D., Nana, A.L., Karydas, A., Balestra, M.E., Huang, Y., Russo, S.P., Spina, S., Grinberg, L.T., Seeley, W.W., Myers, R.M., Miller, B.L., Coppola, G., Lee, S.E., Cuervo, A.M., Yokoyama, J.S., 2019. Rare variants in the neuronal ceroid lipofuscinosis gene MFSD8 are candidate risk factors for frontotemporal dementia. Acta Neuropathol 137, 71–88. doi:10.1007/s00401-018-1925-9.

Gonçalves, J.P.N., de Andrade, H.M.T., Cintra, V.P., Bonadia, L.C., Leoni, T.B., de Albuquerque, M., Martins, M.P., de Borba, F.C., Couteiro, R.E.D., de Oliveira, D.S., Claudino, R., Gonçalves, M.V.M., Dourado, M.E., de Souza, L.C., Teixeira, A.L., de Godoy Rousseff Prado, L., Tumas, V., Oliveira, A.S.B., Nucci, A., Lopes-Cendes, I., Marques, W., França, M.C., 2020. CAG repeats $\geq$ 34 in Ataxin-1 gene are associated with amyotrophic lateral sclerosis in a Brazilian cohort. J. Neurol. Sci. 414, 15–18. doi:10.1016/j.jns.2020.116842.

Gu, X., Chen, Y., Wei, Q., Hou, Y., Cao, B., Zhang, L., Ou, R., Lin, J., Liu, K., Zhao, B., Shang, H., 2021. Rare CYLD variants in chinese patients with amyotrophic lateral sclerosis. Front. Genet. 12, 1–6. doi:10.3389/fgene.2021.740052.

Hobson, E.V., McDermott, C.J., 2016. Supportive and symptomatic management of amyotrophic lateral sclerosis. Nat. Rev. Neurol. 12, 526–538. doi:10.1038/nrneurol.2016.111.

Konno, T., Shiga, A., Tsujino, A., Sugai, A., Kato, T., Kanai, K., Yokoseki, A., Eguchi, H., Kuwabara, S., Nishizawa, M., Takahashi, H., Onodera, O., 2013. Japanese amyotrophic lateral sclerosis patients with GGGGCC hexanucleotide repeat expansion in C9ORF72. J. Neurol. Neurosurg. Psychiatry 84, 398–401. doi:10.1136/jnnp-2012-302272.

Lahut, S., Ömür, Ö., Uyan, Ö., Ağim, Z.S., Özoğuz, A., Parman, Y., Deymeer, F., Oflazer, P., Koç, F., Özçelik, H., Auburger, G., Başak, A.N., 2012. ATXN2 and its neighbouring gene SH2B3 are associated with increased ALS risk in the Turkish population. PLoS One 7. doi:10.1371/journal.pone.0042956.

Lattante, S., Millecamps, S., Stevanin, G., Rivaud-Péchoux, S., Moigneu, C., Camuzat, A., Da Barroca, S., Mundwiller, E., Couarch, P., Salachas, F., Hannequin, D., Meininger, V., Pasquier, F., Seilhean, D., Couratier, P., Danel-Brunaud, V., Bonnet, A.M., Tranchant, C., Le Guern, E., Brice, A., Le Ber, I., Kabashi, E., 2014. Contribution of ATXN2 intermediary polyQ expansions in a spectrum of neurodegenerative disorders. Neurology 83, 990–995. doi:10.1212/WNL.0000000000000778.

Lattante, S., Pomponi, M.G., Conte, A., Marangi, G., Bisogni, G., Patanella, A.K., Meleo, E., Lunetta, C., Riva, N., Mosca, L., Carrera, P., Bee, M., Zollino, M., Sabatelli, M., 2018. ATXN1 intermediate-length polyglutamine expansions are associated with amyotrophic lateral sclerosis. Neurobiol. Aging 64, 157.e1–157.e5. doi:10.1016/j.neurobiolaging.2017.11.011.

Lee, J.B., Lee, K.A., Hong, J.M., Suh, G.I., Choi, Y.C., 2012. Homozygous SMN2 deletion is a major risk factor among twenty-five Korean sporadic amyotrophic lateral sclerosis patients. Yonsei Med. J. 53, 53–57. doi:10.3349/ymj.2012.53.1.53.

Ludolph, A., Drory, V., Hardiman, O., Nakano, I., Ravits, J., Robberecht, W., Shefner, J., 2015. A revision of the El Escorial criteria - 2015. Amyotroph. Lateral Scler. Front. Degener. 16, 291–292. doi:10.3109/21678421.2015.1049183.

Mackenzie, I.R., Nicholson, A.M., Sarkar, M., Messing, J., Purice, M.D., Pottier, C., Annu, K., Baker, M., Perkerson, R.B., Kurti, A., Matchett, B.J., Mittag, T., Temirov, J., Hsiung, G.Y.R., Krieger, C., Murray, M.E., Kato, M., Fryer, J.D., Petrucelli, L., Zinman, L., Weintraub, S., Mesulam, M., Keith, J., Zivkovic, S.A., Hirsch-Reinshagen, V., Roos, R.P., Züchner, S., Graff-Radford, N.R., Petersen, R.C., Caselli, R.J., Wszolek, Z.K., Finger, E., Lippa, C., Lacomis, D., Stewart, H., Dickson, D.W., Kim, H.J., Rogaeva, E., Bigio, E., Boylan, K.B., Taylor, J.P., Rademakers, R., 2017. TIA1 mutations in amyotrophic lateral sclerosis and frontotemporal dementia promote phase separation and alter stress granule dynamics. Neuron 95, 808–816.e9. doi:10.1016/j.neuron.2017.07.025.

Masè, G., Ros, S., Gemma, A., Bonfigli, L., Carraro, N., Cazzato, G., Rolfo, M., Zanconati, F., Sepcic, J., Jurjevic, A., Pirulli, D., Boniotto, M., Zezlina, S., Crovella, S., Amoroso, A., 2001. ALS with variable phenotypes in a six-generation family caused by leu144phe mutation in the SOD1 gene. J. Neurol. Sci. 191, 11–18. doi:10.1016/S0022-510X(01)00625-6.

Nahm, M., Lim, S.M., Kim, Y.E., Park, J., Noh, M.Y., Lee, S., Roh, J.E., Hwang, S.M., Park, C.K., Kim, Y.H., Lim, G.T., Lee, J., Oh, K.W., Ki, C.S., Kim, S.H., 2020. ANXA11 mutations in ALS cause dysregulation of calcium homeostasis and stress granule dynamics. Sci. Transl. Med. 12, 1–16. doi:10.1126/scitranslmed.aax3993.

Pliner, H.A., Mann, D.M., Traynor, B.J., 2014. Searching for Grendel: origin and global spread of the C9ORF72 repeat expansion. Acta Neuropathol 127, 391–396. doi:10.1007/s00401-014-1250-x.

Pulst, S.M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., Dejong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C., Sahba, S., 1996. Moderate expansion of a normally biallelic trinucleotide repeat in spinooerebellar ataxia type. Nat. Genet. 14, 269–276. doi:10.1038/ng1196-269.

Quadri, M., Mandemakers, W., Grochowska, M.M., Masius, R., Geut, H., Fabrizio, E., Breedveld, G.J., Kuipers, D., Minneboo, M., Vergouw, L.J.M., Carreras Mascaro, A., Yonova-Doing, E., Simons, E., Zhao, T., Di Fonzo, A.B., Chang, H.C., Parchi, P., Melis, M., Correia Guedes, L., Criscuolo, C., Thomas, A., Brouwer, R.W.W., Heijsman, D., Ingrassia, A.M.T., Calandra Buonaura, G., Rood, J.P., Capellari, S., Rozemuller, A.J., Sarchioto, M., Fen Chien, H., Vanacore, N., Olgiati, S., Wu-Chou, Y.H., Yeh, T.H., Boon, A.J.W., Hoogers, S.E., Ghazvini, M., IJpma, A.S., van IJcken, W.F.J., Onofrj, M., Barone, P., Nicholl, D.J., Puschmann, A., De Mari, M., Kievit, A.J., Barbosa, E., De Michele, G., Majoor-Krakauer, D., van Swieten, J.C., de Jong, F.J., Ferreira, J.J., Cossu, G., Lu, C.S., Meco, G., Cortelli, P., van de Berg, W.D.J., Bonifati, V., 2018. LRP10 genetic variants in familial Parkinson's disease and dementia with Lewy bodies: a genome-wide linkage and sequencing study. Lancet Neurol 17, 597–608. doi:10.1016/S1474-4422(18)30179-0.

Quan, F., Janas, J., Popovich, B.W., 1995. A novel CAG repeat configuration in the SCA1 gene: implications for the molecular diagnostics of spinocerebellar ataxia type 1. Hum. Mol. Genet. 4, 2411–2413. doi:10.1093/hmg/4.12.2411.

Renton, A.E., Chiò, A., Traynor, B.J., 2014. State of play in amyotrophic lateral sclerosis genetics. Nat Neurosci 17, 17–23. doi:10.1038/nn.3584.State.

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W.W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., Rehm, H.L., 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. Genet. Med. 17, 405–424. doi:10.1038/gim.2015.30.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Berger, R., Tanzi, R.E., Halperin, J.J., Herzfeldt, B., Bergh, Den, Van, R., Hung, W.Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Laing, N.G., Soriano, E., Pericak-Vance, M.A., Haines, J., Rouleau, G.A., Gusella, J.S., Horvitz, H.R., Brown, R.H., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62. doi:10.1038/362059a0.

Siokas, V., Karampinis, E., Aloizou, A.M., Mentis, A.F.A., Liakos, P., Papadimitriou, D., Liampas, I., Nasios, G., Bogdanos, D.P., Hadjigeorgiou, G.M., Dardiotis, E., 2021. CYP1A2 rs762551 polymorphism and risk for amyotrophic lateral sclerosis. Neurol. Sci. 42, 175–182. doi:10.1007/s10072-020-04535-x.

Smith, B.N., Topp, S.D., Fallini, C., Shibata, H., Chen, H.J., Troakes, C., King, A., Ticozzi, N., Kenna, K.P., Soragia-Gkazi, A., Miller, J.W., Sato, A., Dias, D.M., Jeon, M., Vance, C., Wong, C.H., De Majo, M., Kattuah, W., Mitchell, J.C., Scotter, E.L., Parkin, N.W., Sapp, P.C., Nolan, M., Nestor, P.J., Simpson, M., Weale, M., Lek, M., Baas, F., De Jong, J.M.V., Ten Asbroek, A.L.M.A., Redondo, A.G., Esteban-Pérez, J., Tiloca, C., Verde, F., Duga, S., Leigh, N., Pall, H., Morrison, K.E., Al-Chalabi, A., Shaw, P.J., Kirby, J., Turner, M.R., Talbot, K., Hardiman, O., Glass, J.D., De Belleroche, J., Maki, M., Moss, S.E., Miller, C., Gellera, C., Ratti, A., Al-Sarraj, S., Brown, R.H., Silani, V., Landers, J.E., Shaw, C.E., 2017. Mutations in the vesicular trafficking protein Annexin A11 are associated with amyotrophic lateral sclerosis Bradley N. Smith. Sci. Transl. Med. 9, 1–16. doi:10.1126/scitranslmed.aad9157.

Sproviero, W., Shatunov, A., Stahl, D., Shoai, M., van Rheenen, W., Jones, A.R., Al-Sarraj, S., Andersen, P.M., Bonini, N.M., Conforti, F.L., Van Damme, P., Daoud, H.,

Del Mar Amador, M., Fogh, I., Forzan, M., Gaastra, B., Gellera, C., Gitler, A.D., Hardy, J., Fratta, P., La Bella, V., Le Ber, I., Van Langenhove, T., Lattante, S., Lee, Y.C., Malaspina, A., Meininger, V., Millecamps, S., Orrell, R., Rademakers, R., Robberecht, W., Rouleau, G., Ross, O.A., Salachas, F., Sidle, K., Smith, B.N., Soong, B.W., Sorarù, G., Stevanin, G., Kabashi, E., Troakes, C., van Broeckhoven, C., Veldink, J.H., van den Berg, L.H., Shaw, C.E., Powell, J.F., Al-Chalabi, A., 2017. ATXN2 trinucleotide repeat length correlates with risk of ALS. Neurobiol. Aging 51, 178.e1–178.e9. doi:10.1016/j.neurobiolaging.2016.11.010.

Stevens, A., Weller, M., Wietholter, H., 1993. A characteristic ganglioside antibody pattern in the CSF of patients with amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 56, 361–364. doi:10.1136/jnnp.56.4.361.

Tavares de Andrade, H.M., Cintra, V.P., de Albuquerque, M., Piccinin, C.C., Bonadia, L.C., Duarte Couteiro, R.E., Sabino de Oliveira, D., Claudino, R., Magno Gonçalves, M.V., Dourado, M.E.T., de Souza, L.C., Teixeira, A.L., de Godoy Rousseff Prado, L., Tumas, V., Bulle Oliveira, A.S., Nucci, A., Lopes-Cendes, I., Marques, W., França, M.C., 2018. Intermediate-length CAG repeat in ATXN2 is associated with increased risk for amyotrophic lateral sclerosis in Brazilian patients. Neurobiol. Aging 69, 292.e15–292.e18. doi:10.1016/j.neurobiolaging.2018.04.020.

Tazelaar, G.H., Boeynaems, S., De Decker, M., JFA van Vugt, J., Kool, L., Stephan Goedee, H., McLaughlin, R.L., Sproviero, W., Iacoangeli, A., Moisse, M., Jacquemyn, M., Daelemans, D., Dekker, A.M., van der Spek, R.A., Westeneng, H.-J., Kenna, K.P., Assialioui, A., Da Silva, N., Millecamps, S., Vourc, P., Corcia, P., Couratier, P., Morrison, K.E., Shaw, P.J., Shaw, C.E., Jeroen Pasterkamp, R., Landers, J.E., Van Den Bosch, L., Robberecht, W., Al-Chalabi, A., van den Berg, L.H., Van Damme, P., Veldink, J.H., van Es, M.A., 2020. ATXN1 repeat expansions confer risk for amyotrophic lateral sclerosis and contribute to TDP-43 mislocalization Running Title: ATXN1 repeat expansions in ALS Authors and affiliations. Brain Commun 11, 10.

Tripolszki, K., Csányi, B., Nagy, D., Ratti, A., Tiloca, C., Silani, V., Kereszty, É., Török, N., Vécsei, L., Engelhardt, J.I., Klivényi, P., Nagy, N., Széll, M., 2017a. Genetic analysis of the SOD1 and C9ORF72 genes in Hungarian patients with amyotrophic lateral sclerosis. Neurobiol. Aging 53, 195.e1–195.e5. doi:10.1016/j.neurobiolaging.2017.01.016.

Tripolszki, K., Danis, J., Padhi, A.K., Gomes, J., Bozó, R., Nagy, Z.F., Nagy, D., Klivényi, P., Engelhardt, J.I., Széll, M., 2019a. Angiogenin mutations in Hungarian patients with amyotrophic lateral sclerosis: clinical, genetic, computational, and functional analyses. Brain Behav 9, 1–10. doi:10.1002/brb3.1293.

Tripolszki, K., Gampawar, P., Schmidt, H., Nagy, Z.F., Nagy, D., Klivényi, P., Engelhardt, J.I., Széll, M., 2019b. Comprehensive genetic analysis of a Hungarian amyotrophic lateral sclerosis cohort. Front. Genet. 10. doi:10.3389/fgene.2019.00732.

Tripolszki, K., Török, D., Goudenége, D., Farkas, K., Sulák, A., Török, N., Engelhardt, J.I., Klivényi, P., Procaccio, V., Nagy, N., Széll, M., 2017b. High-throughput sequencing revealed a novel SETX mutation in a Hungarian patient with amyotrophic lateral sclerosis 7, 1–7. doi:10.1002/brb3.669.

Van Damme, P., Veldink, J.H., Van Blitterswijk, M., Corveleyn, A., Van Vught, P.W.J., Thijs, V., Dubois, B., Matthijs, G., Van Den Berg, L.H., Robberecht, W., 2011. Expanded ATXN2 CAG repeat size in ALS identifies genetic overlap between ALS and SCA2. Neurology 76, 2066–2072. doi:10.1212/WNL.0b013e31821f445b.

van Es, M.A., Hardiman, O., Chio, A., Al-Chalabi, A., Pasterkamp, R.J., Veldink, J.H., van den Berg, L.H., 2017. Amyotrophic lateral sclerosis. Lancet 390, 2084–2098. doi:10.1016/S0140-6736(17)31287-4.

Veldink, J.H., Kalmijn, S., Van Der Hout, A.H., Lemmink, H.H., Groeneveld, G.J., Lummen, C., Scheffer, H., Wokke, J.H.J., Van Den Berg, L.H., 2005. SMN genotypes producing less SMN protein increase susceptibility to and severity of sporadic ALS. Neurology 65, 820–825. doi:10.1212/01.wnl.0000174472.03292.dd.

Zou, Z.Y., Zhou, Z.R., Che, C.H., Liu, C.Y., He, R.L., Huang, H.P., 2017. Genetic epidemiology of amyotrophic lateral sclerosis: a systematic review and meta-analysis. J. Neurol. Neurosurg. Psychiatry 88, 540–549. doi:10.1136/jnnp-2016-315018.