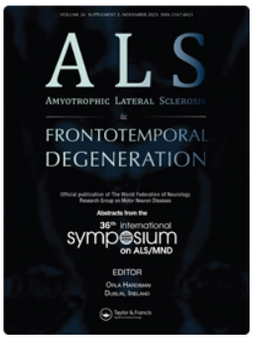

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Unraveling the genetic landscape of ALS in Greece: identification of known and novel causative variants in a 353-patient cohort

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RESEARCH ARTICLE

Unraveling the genetic landscape of ALS in Greece: identification of known and novel causative variants in a 353-patient cohort

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Abstract

Background: Amyotrophic lateral sclerosis (ALS) is an adult-onset, progressive, fatal neurodegenerative disorder characterized by progressive loss of motor neurons. Approximately 15% of individuals diagnosed with ALS have a known genetic variant that contributes to disease. Herein, we present clinical and genetic data of a large Greek ALS cohort. *Patients and Methods*: The cohort consisted of 353 Greek consecutive index patients with ALS, including 16 patients with related motor neuron disease (MND) subtypes (nine with PLS, four with PBP, and three with PMA). Next generation sequencing raw data (obtained from the NYGC ALS Consortium) were further analyzed and used to screen for causative variants in known implicated genes. Repeat expansions in *C9ORF72* and *ATXN2* were investigated using ExpansionHunter software, repeat-primed PCR and fragment analysis. *Results*: Pathogenic repeat expansions in *C9ORF72* were detected in 41 patients (11.6%). In addition, 30 patients (8.5%) carried a causative variant in one of the genes studied. Known causative variants were identified in 27 cases (nine in *SQSTM1*, seven in *TARDBP*, five in *SOD1*, three in *NEK1* and one each in *SETX*, *VCP*, *FUS*), whereas novel causative variants were identified in three cases (*SOD1*, *FIG4*, *TBK1*). In total, 71 cases received a molecular genetic diagnosis (20.1%). Additionally, seven cases (2.0%) carried an intermediate repeat expansion (30–33 CAG) in *ATXN2*. *Conclusion*: Our results reveal the distinct genetic profile of Greek ALS patients. These findings will have an impact on genetic counseling, the design of diagnostic gene panels for the Greek population and on genotype-specific therapeutic interventions. Understanding the genetic causes of ALS in different populations is becoming increasingly important, especially with the advent of personalized medicine.

Abbreviations: AAO: age at onset; ACMG: American College of Medical Genetics and Genomics; AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; BQSR: Base quality score recalibration; BRFAA: Biomedical Research Foundation of the Academy of Athens; BWA: Burrows-Wheeler Aligner; fALS: familial ALS; FTD: frontotemporal dementia; GATK: Genome Analysis Toolkit; GOF: gain-of-function; HRE: hexanucleotide repeat expansion; LOF: loss-of-function; MAF: minor allele frequency; MND: motor neuron disease; NGS: next-generation sequencing; NMD: nonsense-mediated mRNA decay; NYGC: New York Genome Center; PBP: progressive bulbar palsy; PD: Parkinson's disease; PLS: primary lateral sclerosis; PMA: progressive muscular atrophy; RP-PCR: repeat-primed PCR; sALS: sporadic

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ALS; UBA: ubiquitin-associated; VCF: variant calling file; VUS: variant of uncertain significance; WES: whole-exome sequencing; WGS: whole-genome sequencing

Keywords: *Amyotrophic lateral sclerosis (ALS), next generation sequencing (NGS), causative variants, C9ORF72 repeat expansions, Greek population*

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by the selective impairment of motor neurons in the motor cortex, brainstem and spinal cord. ALS is rapidly progressive, leading to death from respiratory failure in 3–5 years after symptom onset (1). Approximately 5%–10% of cases are considered familial ALS (fALS) and inherited in either an autosomal dominant, autosomal recessive, or X-linked mode, while the remaining are sporadic (sALS) (2).

As technology has advanced, molecular genetic techniques have been increasingly applied to ALS research. More than 50 potentially causative or disease-modifying genes have been identified. A pathogenic repeat expansion in *C9ORF72*, and pathogenic variants in *SOD1*, *TARDBP*, and *FUS* occur most frequently, with disease causing variants in other genes being relatively uncommon (3). Understanding the genetic architecture of ALS within a population is becoming more significant, especially in the advent of personalized medicine (4).

Studies implementing either targeted genetic testing or next-generation sequencing (NGS) in order to investigate the genetic profile of Greek ALS patients are sparse and limited only to a small number of cases, more specifically whole-genome sequencing (WGS) in 6 Greek ALS patients (5), whole-exome sequencing (WES) in 58 Greek ALS patients (6), and Sanger sequencing in 32 sALS cases (7). To the best of our knowledge, this is the first comprehensive molecular genetic investigation of a large Greek ALS cohort. Thus, the present study aimed to investigate the genetic profile of ALS patients in Greece, providing insights regarding rare known and novel gene variants and clinical characteristics of Greek ALS cases.

2. Patients and methods

2.1. Recruitment of patients

From 1994 to 2019, 353 Greek ALS index patients [including nine with primary lateral sclerosis (PLS), four with progressive bulbar palsy (PBP), and three with progressive muscular atrophy (PMA)] were referred to Neurogenetics Unit, 1st Department of Neurology, National and Kapodistrian University of Athens, Eginitio Hospital, Athens, Greece for molecular diagnosis. The mean age at onset (AAO) was 58.8 ± 12.1 years (range from 14 to 84). Age at onset under 40 years old was defined as early-onset in this study. Twenty-seven patients (7.6%) were early-onset ALS. Regarding the family history of the patients, 22 cases (6.2%) were familial and 331 cases (93.8%) isolated cases. Patients with fALS were defined as having a self-reported family history of suspected ALS defined as the presence of at least one first-degree relative. The clinical phenotypes considered for the classification of disease onset were limb, bulbar, and both (limb and bulbar). The majority of cases were classified as limb ($n = 220$, 62.3%) whereas bulbar onset was less common among our patients ($n = 88$, 24.9%). Moreover, cases with concomitant frontotemporal dementia (FTD) represented a minority of cases ($n = 8$, 2.3%).

Clinical diagnosis was established based on the Revised El Escorial Criteria (8). Three hundred thirty-seven patients fulfilled criteria for ALS (95.5%) while the rest of them were categorized in one of the following phenotypes: nine in PLS, four in PBP, and three in PMA. Table 1 lists the clinical and demographic details of this cohort. Written informed consent was obtained from all

Table 1. Basic demographic and clinical characteristics of the Greek ALS cohort.

N	353
Sex (%)	
Male	224 (63.5)
Female	129 (36.5)
Age (years)	60.8 ± 12.3 (15–95)
AAO (years)	58.8 ± 12.1 (14–84)
Onset type (%)	
Limb	220 (62.3)
Bulbar	88 (24.9)
Both	14 (4.0)
Unknown	31 (8.8)
Family history (%)	
Familial	22 (6.2)
Sporadic	331 (93.8)
ALS-FTD (%)	8 (2.3)
Revised El Escorial 2015 (%)	
ALS	337 (95.5)
PLS	9 (2.6)
PBP	4 (1.1)
PMA	3 (0.8)

Note: Data are mean ± SD (range).

Abbreviations: AAO: age at onset; ALS: amyotrophic lateral sclerosis; FTD: frontotemporal dementia); PLS: primary lateral sclerosis; PMA: progressive muscular atrophy; PBP = progressive bulbar palsy.

patients. The study protocol was approved by the Eginitio Hospital Ethics Committee.

2.2. Next generation sequencing

Genomic DNA was extracted from peripheral blood lymphocytes using the salting-out method at the Athens Neurogenetics Unit. WGS was performed at the New York Genome Center (NYGC). Libraries were prepared using the TruSeq DNA PCR-free Library Preparation Kit (Illumina) in accordance with the manufacturer's instructions. Sequencing was performed on an Illumina NovaSeq V1 sequencer using 2x150bp cycles. Paired-end 150 bp reads were aligned to the GRCh38 human reference using the Burrows-Wheeler Aligner (BWA-MEM v0.7.15) and processed using the Genome Analysis Toolkit's (GATK) best-practices workflow. Duplicates were marked using the Picard software package (v2.4.1, http://picard.sourceforge.net) and base quality score recalibration (BQSR) was performed via Genome Analysis Toolkit (GATK v3.5) (PMID: 20644199, 21478889). HaplotypeCaller was implemented on each sample separately to produce the variant calling files (VCFs). Repeat expansions (*C9ORF72* and *ATX2*) were called using the ExpansionHunter v2.5.5 (https://github.com/Illumina/ExpansionHunter), which estimates the number of copies of repeated short unit sequence by performing a targeted search through a BAM/CRAM file for reads that span, flank, or are fully contained in each repeat.

2.3. Variant analysis

The WGS VCFs were further processed in the Biomedical Research Foundation, Academy of Athens (BRFAA) via a whole-exome capture algorithm, effectively reducing the WGS data to WES VCFs. The downstream analysis of WES VCFs thus produced was performed at the Athens Neurogenetics Unit. More specifically, variant annotation and filtering was performed in Franklin by Genoox (https://franklin.genoox.com/). Variants were analyzed based on a virtual *in silico* panel of 38 genes associated with ALS, downloaded from panelApp [Amyotrophic lateral sclerosis/motor neuron disease (Version 1.73)] (Table 2). Variants were filtered based on their minor allele frequencies (MAFs) and read-depth. Only variants with MAF $< 1\%$ from the GnomAD database (gnomad.broadinstitute.org/) and read-depth >20X were retained. The detected variants were classified in accordance with the guidelines of the American College of Medical Genetics and Genomics (ACMG) (9) as likely benign, uncertain significance (VUS), likely pathogenic, or pathogenic. Only pathogenic or likely pathogenic variants were reported.

Table 2. ALS-associated genes analyzed in our cohort.

ALS2	*OPTN*	*TARDBP*	*ATXN2*	*UNC13A*
ANG	*PFN1*	*TBK1*	*C9ORF72*	*VEGFA*
DCTN1	*SETX*	*TUBA4A*	*CHCHD10*	*SPG11*
FIG4	*SIGMAR1*	*UBQLN2*	*CHMP2B*	*SQSTM1*
FUS	*SLC52A2*	*VAPB*	*ERBB4*	*MATR3*
HNRNPA1	*SLC52A3*	*VCP*	*GRN*	*ANXA11*
KIF5A	*SOD1*	*NEFH*	*HFE*	
NEK1	*SPAST*	*AR*	*SLC52A1*	

Repeat expansions in *C9ORF72* and *ATXN2* were investigated using ExpansionHunter software. The results were validated using conventional repeat-primed PCR (RP-PCR) and fragment analysis at the Athens Neurogenetics Unit. As per *C9ORF72* hexanucleotide repeat expansion (HRE) the cutoff of 30 repeats was considered pathogenic. The size of the repeat expansion is highly variable but the cutoff of 30 repeats has been suggested as the lowest pathogenic limit (10). Intermediate repeat expansions in *ATXN2* are a known risk factor for ALS. In the present study, 30–33 repeats in *ATXN2* were considered intermediate. This range is most strongly associated with ALS according to a meta-analysis of 9042 ALS cases (11). We defined as oligogenic patients carrying 2 or more variants with unambiguous evidence supporting their pathogenicity.

2.4. Statistical analysis

All statistical analyses were performed using Rstudio version 4.3.1 (RStudio Team, 2023). Comparisons between means were made with the unpaired two-tailed Student *t*-test, whereas comparison between categorical variables was made with the Fisher's exact test. A p-value < 0.05 was considered significant.

3. Results

3.1. Genetic findings

We analyzed the 38 ALS-associated genes in a cohort of 353 Greek patients with ALS including 16 patients with related motor neuron disease (MND) subtypes (PLS, PBP, or PMA). Of the 353 cases examined, 41 carried the *C9ORF72* HRE, representing 11.6% of the entire group and 54.5% of the familial cases. An additional 30 patients carried causative variants, representing a further 8.5% of the overall cohort. A total of 71 cases received a genetic diagnosis (20.1%) considering all MND subtypes. Excluding non-ALS MND subtypes a genetic diagnosis was reached in 21% of the cohort. As per *ATXN2* repeats, seven cases (2.0%) carried an intermediate repeat expansion (30–33 CAG).

3.2. C9ORF72 HREs

The most common pathogenic genetic alteration in our ALS cohort was the *C9ORF72* HRE. Of the 41 *C9ORF72* positive cases, 12 had fALS (27.5%) and 29 sALS (70.7%) with a mean AAO estimated at 56.10 ± 10.14 years. Limb-onset was observed in 18 cases (43.9%), bulbar-onset (primarily dysarthria) in 13 cases (31.7%), both limb and bulbar-onset in five cases (12.2%) whereas motor onset type was unknown in the remaining 5 cases (12.2%). All familial cases had at least one first degree relative with ALS. Four of the *C9ORF72* positive cases had concomitant FTD (9.8%). It should be noted that 1 patient with ALS in our cohort carried the *C9ORF72* HRE along with the variant c.1124G > A in *TARDBP* (ALS430). It was a sporadic case with AAO of 52 years.

According to the results of ExpansionHunter, our *C9ORF72* positive patients carried from 117 to 465 repeats (Figure 1B). All cases were validated using an orthogonal method (fragment analysis and RP-PCR) and the results were concordant.

3.3. Identification of causative ALS-associated variants

We identified known causative variants in 27 cases (nine in *SQSTM1*, seven in *TARDBP*, five in *SOD1*, three in *NEK1*, one in *SETX*, one in *VCP*, and one in *FUS*) and novel variants in 3 cases (1 in *SOD1*, 1 in *FIG4*, 1 in *TBK1*). All 3 novel variants were absent in population databases (gnomAD, ExAC, 1000 G). Both the c.394_395insCA in *SOD1* and c.1859del in *FIG4* are frameshift variants resulting in termination codon. The c.1179_1189 + 8del in *TBK1* is a splice junction loss variant with unknown effect on the protein. All 30 variants were detected in heterozygous state consistent with autosomal dominant inheritance pattern. Figure 1(A) shows the mutational frequency of the genes identified in our cohort. Finally, we identified three carriers of *SPG11* pathogenic variants (c.7155T > A in one patient and c.5381T > C in two patients).

The clinical features of patients carrying pathogenic or likely pathogenic variants are summarized in Table 3. The mean AAO was 58.17 ± 11.39 years, ranging from 37 to 84 years. Two out of five *SOD1* positive patients were early onset ALS. Among the 30 patients carrying pathogenic or likely pathogenic variants, 21 cases presented with limb-onset, five cases exhibited bulbar-onset, whereas motor onset type was unknown in the remaining four cases. None of the patients had concomitant FTD. Three cases had a family history of suspected ALS. The first one (ALS178), carrying the c.143T > C variant in *SOD1* had three brothers and sisters with ALS. The second one (ALS453), carrying the c.1055A > G variant in *TARDBP* had a father with ALS. Finally, the third one (ALS224), carrying the c.1574C > T variant in *FUS*, had a monozygote sister with anterior horn cell disorder.

3.4. Intermediate repeat expansions in ATXN2

Intermediate repeat expansions in *ATXN2* are an established risk factor for ALS. We identified seven

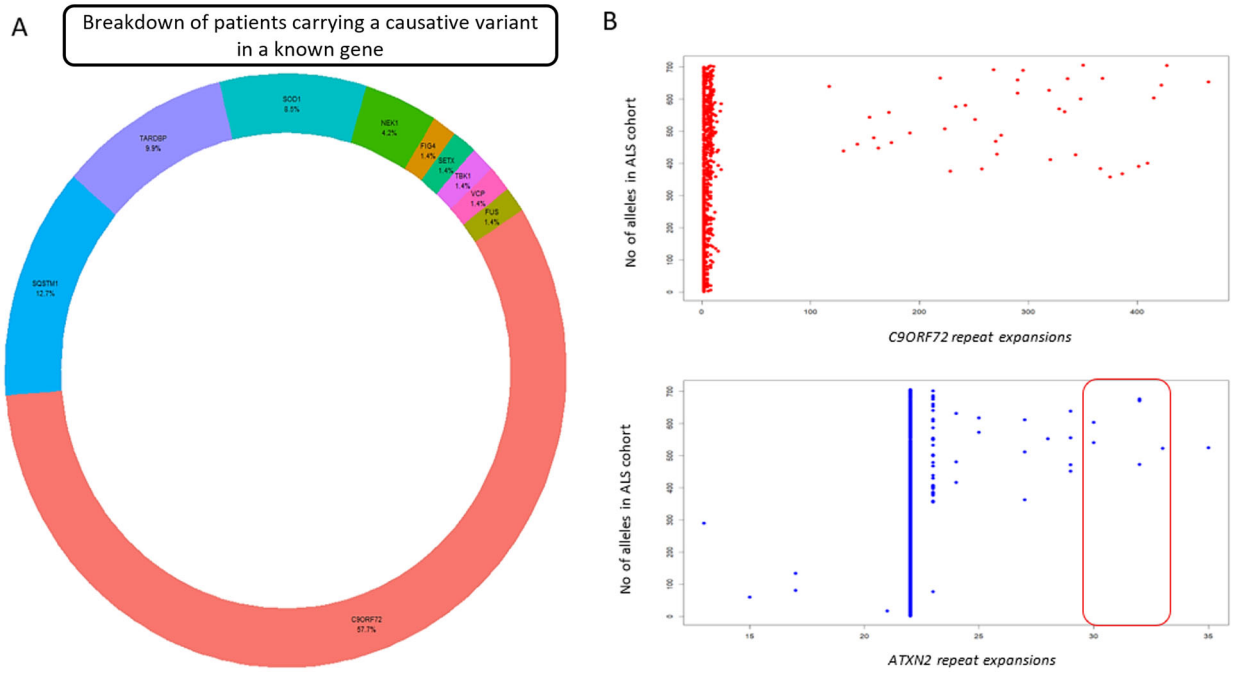

Figure 1. (A) Relative contribution of ALS-associated genes to the total estimate of patients carrying a causative variant. (B) Expansion repeat size in ALS cases for *C9ORF72* and *ATXN2* genes. Scatter plots showing distribution and frequency of repeat sizes, indicated by circles. The red box highlights intermediate ATXN2 repeat expansions (30–33 CAG).

Table 3. Overview of pathogenic or likely pathogenic variants and corresponding clinical features.

Patient ID	Gene	Nucleotide change	AA change	MAF	dbSNP ID	Zygosity	ACMG	C9ORF72 exp	Interm. ATXN2	Sex	AAO	El escorial	ALS-FTD	Site of onset	FH
ALS242	*SOD1*	c.197A > G	p.N66S	<0.01%	rs1568810275	Het	P	No	No	M	50	ALS	No	N/A	No
ALS309	*SOD1*	c.341T > C	p.I114T	<0.01%	rs121912441	Het	P	No	No	M	49	ALS	No	Limb	No
ALS285	*SOD1*	c.338T > C	p.I113T	N/A	rs74315452	Het	P	No	No	M	43	ALS	No	Limb	No
ALS436	*SOD1*	c.394_395insCA	p.N132Tfs*19	N/A	N/A	Het	LP	No	No	M	37	ALS	No	Limb	No
ALS252	*SOD1*	c.317C > T	p.S106L	N/A	rs1378590183	Het	LP	No	No	M	39	ALS	No	N/A	No
ALS178	*SOD1*	c.143T > C	p.V48A	N/A	rs1568809169	Het	LP	No	No	M	68	ALS	No	N/A	Yes
ALS331	*TARDBP*	c.1009A > G	p.M337V	N/A	rs80356730	Het	P	No	No	M	70	PBP	No	Bulbar	No
ALS142	*TARDBP*	c.1009A > G	p.M337V	N/A	rs80356730	Het	P	No	No	F	53	ALS	No	Bulbar	No
ALS75	*TARDBP*	c.1055A > G	p.N352S	<0.01%	rs80356734	Het	P	No	No	F	68	ALS	No	Limb	No
ALS319	*TARDBP*	c.1055A > G	p.N352S	<0.01%	rs80356734	Het	P	No	No	F	63	ALS	No	Limb	No
ALS453	*TARDBP*	c.1055A > G	p.N352S	<0.01%	rs80356734	Het	P	No	No	F	51	ALS	No	Limb	Yes
ALS106	*TARDBP*	c.1147A > G	p.I383V	<0.01%	rs80356740	Het	LP	No	No	M	61	ALS	No	Limb	No
ALS430	*TARDBP*	c.1124G > A	p.S375N	<0.01%	rs1233784457	Het	LP	Yes	No	M	52	ALS	No	Limb	No
ALS115	*FIG4*	c.1859del	p.N620Ifs*3	N/A	N/A	Het	LP	No	No	M	60	ALS	No	Limb	No
ALS251	*NEK1*	c.481C > T	p.R161*	0.01%	rs202115635	Het	P	No	No	M	57	ALS	No	Limb	No
ALS88	*NEK1*	c.481C > T	p.R161*	0.01%	rs202115635	Het	P	No	No	M	67	ALS	No	Limb	No
ALS45	*NEK1*	c.1984_1985del	p.Glu662Lysfs*11	<0.01%	rs760878322	Het	LP	No	No	M	65	ALS	No	Bulbar	No
ALS414	*SETX*	c.5540C > T	p.Thr1847Met	<0.01%	rs763859485	Het	LP	No	No	F	57	ALS	No	Bulbar	No
ALS260	*TBK1*	c.1179_1189 + 8del	p.?	N/A	N/A	Het	LP	No	No	F	53	ALS	No	Bulbar	No
ALS241	*VCP*	c.290G > A	p.Gly97Glu	<0.01%	rs864309502	Het	P	No	No	F	N/A	ALS	No	N/A	No
ALS224	*FUS*	c.1574C > T	p.Pro525Leu	N/A	rs886041390	Het	P	No	No	F	42	ALS	No	Limb	Yes
ALS208	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	F	66	ALS	No	Limb	No
ALS20	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	M	58	ALS	No	Limb	No
ALS72	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	M	44	ALS	No	Limb	No
ALS448	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	M	84	ALS	No	Limb	No
ALS231	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	F	65	ALS	No	Limb	No
ALS14	*SQSTM1*	c.1175C > T	p.Pro392Leu	0.14%	rs104893941	Het	P	No	No	M	69	ALS	No	Limb	No
ALS153	*SQSTM1*	c.286C > T	p.Arg96*	<0.01%	rs886039782	Het	P	No	No	M	77	ALS	No	Bulbar	No
ALS174	*SQSTM1*	c.823_824del	p.Ser275Phefs*17	<0.01%	rs1273214757	Het	P	No	No	M	66	ALS	No	Limb	No
ALS155	*SQSTM1*	c.714_716del	p.Lys238del	<0.01%	rs796052214	Het	LP	No	No	M	53	ALS	No	Limb	No

Abbreviations: AA: amino acid; MAF: minor allele frequency; N/A: not available; Het: heterozygous; ACMG: American College of Medical Genetics; AD: autosomal dominant; AR: autosomal recessive; P: pathogenic; LP: likely pathogenic; AAO: age at onset; ALS: amyotrophic lateral sclerosis; FTD: frontotemporal dementia; FH: family history.

patients (2.0%) with expansions lying within the 30–33 range strongly associated with ALS. The majority of cases had 22 or 23 repeats (range 13–35) (Figure 1(C)). Interestingly, one case (ALS453) had a full *ATXN2* expansion, that is, 35 repeats, along with the c.1055A > G variant in *TARDBP*. Finally, there was a familial ALS case (ALS97) with a pathogenic *C9ORF72* HRE (415 repeats) and an intermediate *ATXN2* repeat expansion (30 repeats) with AAO at 39 years. All cases were validated using an orthogonal method (fragment analysis) and the results were concordant.

Discussion

In this study, we investigated the genetic profile and clinical characteristics of a 353-patient Greek ALS cohort, including 16 patients with related MND subtypes (PLS, PBP, or PMA). To the best of our knowledge, this is the first comprehensive study carried out in a large number of cases from Greece. We used NGS in order to estimate the frequency of known causative variants and discover novel variants. Moreover, we screened our cohort for *C9ORF72* and *ATXN2* repeat expansions. Our findings are important for clinical testing as well as the design of gene-specific trials. Moreover, novel pathogenic or likely pathogenic variants reported in this work could be the starting point for further molecular research.

Our findings should also be considered in the context of the broader Mediterranean genetic landscape. Recent studies have highlighted notable geographical variability in the genetic architecture of ALS across this region. In North Africa, particularly Tunisia, causative variants in *TARDBP* appear most prevalent (~9.4%), whereas *C9ORF72* HREs are less common (~3.9%) compared to European cohorts (12). In contrast, *C9ORF72* HREs are the most frequent genetic cause of ALS in Italy and Spain, accounting for approximately 3.7%–6.7% of sporadic cases in continental Italy (13) and up to 27% of familial cases in Spain (14). Notably, in Sardinia, a founder variant in *TARDBP* (A382T) is highly prevalent, even among apparently sporadic cases (15). These regional differences also reflect phenotypic variability: ALS patients from North Africa often show earlier disease onset and, in some reports, reduced survival (16), whereas carriers of *C9ORF72* HREs - more frequent in Southern Europe – are more likely to exhibit frontotemporal cognitive impairment and more rapid disease progression (17).

The frequency of the causative variants in patients with ALS has been extensively investigated in populations of different ethnic origins. The most frequent genetic alteration was the *C9ORF72* HRE detected in 41 patients (11.6%) of this cohort. Our results are in accordance with the majority of the studies (18–25) except the Italian (26), German (27), Cypriot (28), and Turkish (29) (Table 4). These differences may stem from distinct genetic backgrounds and population history. The frequency of *C9ORF72* HRE in Greek patients with ALS has been studied in detail in previous reports of our group investigating the genotypic and phenotypic profile of *C9ORF72*-related disorders in Greece (31,32).

As per SNVs, causative variants in *SQSTM1* were most frequently detected in our cohort ($n = 9$, 2.5%). *SQSTM1* variants have been reported in less than 1% of ALS patients worldwide (33) (Table 4). According to literature, *SQSTM1* variants are either VUS or detected along with another variant. The gene was originally associated with Paget's disease of bone. However, there are reports linking *SQSTM1* variants with the ALS-FTD spectrum and FTD cases (34,35). Interestingly none of our 9 patients carrying variants in *SQSTM1* had concomitant FTD. Six out of nine patients harbored the c.1175C > T variant in exon 8 located in the C-terminal ubiquitin-associated (UBA) end of the sequestome 1 protein. This is a known variant that has previously been reported in cases of ALS (34).

TDP-43 has been found in inclusion bodies of multiple neurological disorders, including ALS, FTD, Parkinson's disease (PD) and Alzheimer's disease (AD). Variants in the TDP-43 encoding gene, *TARDBP*, have been subsequently reported in sporadic and familial ALS patients (36). Causative variants in *TARDBP* were the third most frequently mutated gene in our cohort, in accordance with literature (3). All variants identified ($n = 7$, 2.0%) were located in exon 6 of the gene with p.M337V being the most common one (carried by two patients in our cohort).

Identified in 1993, *SOD1* was the first gene associated with ALS (37). Across mixed ALS cohorts, *SOD1* variants account for approximately 2% of cases (38). In our cohort, causative variants in *SOD1* gene were identified in six patients (1.7%), a rate comparable to other studies in European populations (21,26,27). Our results are in statistical concordance with all European studies, except from the German cohort detecting a high frequency of *SOD1* causative variants ($n = 34$, 11.3%). This is likely due to the fact that the German study included only fALS cases. Of note, the Maltese (19), Hungarian (20), and Cypriot (28) cohorts reported no patients with *SOD1* pathogenic variants (Table 4). The present study identified a novel frameshift variant c.394_395insCA in *SOD1* predicted to be loss-of-function (LOF). Although *SOD1* variants are primarily associated with toxic gain-of-function (GOF)

Table 4. Comparison of frequencies of SNVs and *C9ORF72* HREs in different populations.

Population	NGS type	Cohort	Genes analyzed	SNVs positive	*C9ORF72* positive	*SQSTM1* positive	*TARDPB* positive	*SOD1* positive	*NEK1* positive	*p*-Value SNVs	p-value *C9ORF72*	p-value *SQSTM1*	p-value *TARDBP*	p-value *SOD1*	p-value *NEK1*
Italian (26)	WGS	959	40	34	71	0	12	17	0	0.0002	0.0094	5.018×10^{-6}	0.2944	1.0000	0.0174
German (27)	WES	301	35	35	75	0‡	11‡	34‡	0‡	0.2946	3.52×10^{-5}	0.0041	0.2425	6.349×10^{-7}	0.2512
Portuguese (30)	NGS*	371	25	8	31	0	1	3	0	7.745×10^{-5}	0.1058	0.0012	0.0307	0.3218	0.1073
Maltese (19)	WGS	24	58	10	0	0	0	0	0	5.782×10^{-5}	0.0917	1.0000	1.0000	1.0000	1.0000
Hungarian (20)	WES	107	30	39	10	0‡	0	0	0‡	1.889×10^{-10}	0.4898	0.122	0.204	0.3432	1.0000
Turkish (29)	WES	477	5	30	29	1†	5	11	0	0.1743	0.0033	0.0003	0.2505	0.8044	0.0706
Norwegian (21)	WES	279	30	31	19	0	0	10	0	0.4166	0.0288	0.0049	0.0180	0.2049	0.2554
UK (22)	Targeted panel	100	44	21	10	0‡	0‡	5	0‡	0.0021	0.7228	0.2203	0.3594	0.1361	1.0000
Chinese (23)	Targeted panel	45	27	29	NA	0	2	11	0‡	3.269×10^{-16}	NA	0.6066	0.2871	1.352×10^{-7}	1.0000
S. Africans (24)	WGS	103	44	62	7	0‡	0‡	5	0‡	2.2×10^{-16}	0.1497	0.1244	0.2077	0.1392	1.0000
Indian (25)	Targeted panel	154	25	13	NA	1	2	2	0	1.0000	NA	0.1829	0.7265	1.0000	0.5553
Cypriot (28)	WES	89	122	5	20	0	1	0	0	0.39	0.0173	0.2143	1.0000	0.3515	1.0000
Present Study	WES	337	38	30	41	9	7	6	3	NA	NA	NA	NA	NA	NA

Note: All studies cited in the table implemented NGS technology and analyzed a panel of ALS-associated genes in order to identify causative variants, similar to our study. Only pathogenic or likely pathogenic variants in the genes most commonly mutated in our study are reported in comparisons. The numbers concern index cases carrying variants. Patients with non-ALS MND subtypes were excluded.

*: not specified NGS type; §: Fisher's exact test; †: along with another variant in *TRPM7* gene; ‡: excluding VUS; NA: not applicable.

mechanisms, frameshifts variants that result in truncated protein and escape nonsense-mediated mRNA decay (NMD) can contribute to cytoplasmic aggregation and toxicity (39). Importantly, patients carrying a *SOD1* causative variant are presently potentially eligible for therapeutic intervention (40).

NEK1 causative variants were the fourth most common mutated SNVs in our cohort ($n = 3$, 0.8%), unlike other European cohort studies that consider *NEK1* as a minor ALS gene (14,19–22, 26–28,30,41) (Table 4). All three cases harbored loss-of-function variants (one frameshift and two stop-gain). In *NEK1* gene haploinsufficiency is a known molecular genetic mechanism of toxicity (42).

Finally, we identified causative variants in *FIG4*, *SETX*, *TBK1*, *VCP*, and *FUS* in single cases (<0.5%). *FUS* is a major ALS gene with high frequency across Europe (43). Being detected in a single case in our cohort underscores the marked differences that exist between ethnic groups and geographical regions with respect to genes commonly implicated in ALS. The c.1574C > T in *FUS* is a missense variant in a mutational hotspot corresponding to a protein functional domain. It has been reported in the Chinese (44) cohort in two sporadic patients and the Turkish (29) cohort in a juvenile ALS de novo case. Our patient (ALS224) carrying the c.1574C > T variant in *FUS* was a familial case with a monozygote sister with anterior cell disorder and AAO 42 years (Table 3). Both *FIG4* and *TBK1* variants detected in two sporadic cases were novel. *SETX* and *VCP* genes are primarily linked to other diseases (ataxia and FTD, respectively) and are considered minor ALS genes (45,46).

SPG11 has been associated with juvenile-onset ALS under a recessive disease model (47). In this context, since we detected variants in *SPG11* gene solely in heterozygous state and in patients with adult-onset ALS, it is likely that these alleles were not disease-causing. Regarding oligogenic mode of inheritance, only one patient (ALS430) was detected to carry a causative variant in two different major ALS genes (*C9ORF72* and *TARDBP*) (Table 3). Our case was a male patient with AAO 53 years and without family history. According to literature, co-occurrence of multiple variants is most frequently observed in patients who carry the *C9ORF72* HRE (48). In addition, it has been described that oligogenic inheritance is also associated with an earlier AAO and rapid disease progression (48).

Intermediate-length repeats in *ATXN2* have been associated with increased risk of ALS (11). Herein we identified seven patients (7/353, 2.0%) with expansions lying within the 30–33 range strongly associated with ALS. This is in line with

an Italian (15/801, p-value = 1.000) (49), and a Chinese population study (17/1067, p-value = 0.636) (50), but statistically significantly different from a Belgian/Netherland (10/1948, p-value = 0.0097) (51) and a French-Canadian study (40/556, p-value = 0.0006) (52). Differences in genotyping arise from the fact that many studies accept a broader range of intermediate length repeat expansions (27–33 CAG) (49,50,53) while others a stricter one (30–33 CAG) (51) strongly associated with increased risk for ALS. Two cases, ALS453 and ALS97, harbored a causative variant in an ALS-associated gene and either a full (35 CAG) or an intermediate (30 CAG) *ATXN2* repeat expansion consistent with oligogenic nature of disease.

An important limitation of the present study is the absence of a healthy control group to compare the frequency of intermediate *ATNX2* repeat expansions and occurrence of novel variants. Furthermore, we screened a limited number of ALS-associated genes and reported only pathogenic or likely pathogenic variants. Nonetheless, our study is the first to screen for genetic aberrations in a large cohort of Greek ALS patients. A limitation of our cross-cohort comparison (Table 4) is that different studies analyzed varying numbers of ALS-associated genes, which may affect the observed SNV frequencies. While all studies included the four most frequently mutated genes (*C9ORF72*, *SOD1*, *TARDBP*, and *FUS*), differences in panel size could influence the detection of additional variants. As this study was retrospective, formal cognitive assessments were not available for all patients, limiting detailed characterization of cognitive function in the cohort.

Our results reveal the distinct genetic profile of Greek ALS patients. These findings will have an impact on genetic counseling, the design of diagnostic gene panels for the Greek population and on genotype-specific therapeutic interventions. Understanding the genetic causes of ALS in different populations is becoming increasingly important, especially with the advent of personalized medicine.

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Contributors: CKar performed bioinformatic analysis, interpretation of the data and wrote the manuscript; ZK, NR, IS, MP performed bioinformatic analysis, interpretation of the data and revised the manuscript; TL and DV performed bioinformatic analysis and revised the manuscript; PM participated in study design and revised the manuscript; CKon, SA, EK, AD, MR, EK, MP examined patients clinically and revised the manuscript; LS examined patients clinically, coordinated NYGC ALS Consortium research activity and revised the manuscript; Gkou examined patients clinically and along with GKar designed the study, supervised the project and critically revised the manuscript. **Collaborators**: WGS of all ALS cases was performed at the NYGC (The NYGC ALS Consortium). The members of the NYGC ALS Consortium include Hemali Phatnani, Justin Kwan, Dhruv Sareen, James R. Broach, Zachary Simmons, Ximena Arcila-Londono, Edward B. Lee, Vivianna M. Van Deerlin, Neil A. Shneider, Ernest Fraenkel, Lyle W. Ostrow, Frank Baas, Noah Zaitlen, James D. Berry, Andrea Malaspina, Pietro Fratta, Gregory A. Cox, Leslie M. Thompson, Steve Finkbeiner, Efthimios Dardiotis, Timothy M. Miller, Siddharthan Chandran, Suvankar Pal, Eran Hornstein, Daniel J. MacGowan, Terry Heiman-Patterson, Molly G. Hammell, Nikolaos. A. Patsopoulos, Oleg Butovsky, Joshua Dubnau, Avindra Nath, Robert Bowser, Matt Harms, Eleonora Aronica, Mary Poss, Jennifer Phillips-Cremins, John Crary, Nazem Atassi, Dale J. Lange, Darius J. Adams, Leonidas Stefanis, Marc Gotkine, Robert Baloh, Suma Babu, Towfique Raj, Sabrina Paganoni, Ophir Shalem, Colin Smith, Bin Zhang, University of Maryland Brain and Tissue Bank, NIH NeuroBioBank, and Brent T. Harris.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Data availability statement

Anonymized data will be shared on request by any qualified investigator.

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