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Genetic epidemiology of amyotrophic lateral sclerosis in Cyprus: a population-based study

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Amyotrophic lateral sclerosis (ALS) is a devastating, uniformly lethal degenerative disease of motor neurons, presenting with relentlessly progressive muscle atrophy and weakness. More than fifty genes carrying causative or disease-modifying variants have been identified since the 1990s, when the first ALS-associated variant in the gene *SOD1* was discovered. The most commonly mutated ALS genes in the European populations include the *C9orf72*, *SOD1*, *TARDBP* and *FUS*. Understanding the genetic causes of ALS within a population is becoming more significant, especially in light of the possible development of personalized medicine. Here, we provide clinical and genetic data on familial and sporadic ALS patients in a Greek-Cypriot population-based cohort. Eighty-nine ALS patients, including 21 familial ALS (fALS) (23.6%) and 68 sporadic ALS (sALS) (76.4%), provided the cohort for variant screening of the most common ALS-associated genes. Moreover, next-generation sequencing (NGS) was also performed to identify rare ALS variants, and in silico prediction tools were applied to predict the downstream effect of the variants detected in our study. The pathogenic hexanucleotide G_4C_2 repeat expansion in *C9orf72* was the predominant genetic cause (22.47%) of ALS in our population, while variants in six additional ALS-associated genes were identified, including *ALS2*, *TARDBP*, *FIG4*, *TBK1*, *GLT8D1*, and *BICD2*.

Keywords Genetics, Epidemiology, Amyotrophic lateral sclerosis, Cyprus, Population-based study

ALS was initially defined by the neurologist Jean-Martin Charcot in 1873 as a fatal, rapidly progressive motor neuron disease (MND) characterized by degenerative changes in both upper and lower motor neurons[1]. Changes are responsible for the selective loss of motor neurons, leading to an inability of neuronal transmission from the brain and spinal cord to the muscles[2]. Nowadays, ALS is considered a multisystem neurodegenerative disease with heterogeneity at the clinical and genetic levels. It is the most common adult MND, with an incidence reported between 0.6 and 3.8 per 100,000 person/year[3–6], whereas prevalence is at 4.1–8.4 per 100,000 persons/year in Europe[3,7,8]. However, geographical differences do exist[9]. The disease is separated into two categories: sporadic/idiopathic ALS (sALS) and inherited/familial ALS (fALS). Sporadic ALS accounts for 85–90% of all cases with no prior family history, while the remaining 15–10% of familial ALS follows Mendelian inheritance. In sALS, patients tend to have a late disease onset, accounting for approximately 51–66 years, while fALS cases tend to have an earlier disease onset. However, in extremely rare cases, it can appear in individuals below 25 years of age; this is known as juvenile ALS (jALS)[10]. Despite decades of research, the pathogenic mechanisms responsible for the cause are still unclear. Presently, it is unquestionable that the development and progression of the disease are influenced by multiple factors and not by a single initiating event. Numerous disease mechanisms have been proposed due to the many genes and cellular processes involved[11]. The application of different molecular genetic techniques has allowed for understanding the genetic cause of fALS. Since the discovery of the first ALS gene in 1993, *SOD1*[12], a large number of additional causative or disease-modifying genes have been discovered, with pathogenic variants in *C9orf72* (23%), *SOD1* (19%), *TARDBP* (3%), and *FUS* (3%) which are considered the most common variants observed in fALS[13,14]. Inheritance of ALS mainly occurs in autosomal dominant, with

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	fALS *n* = 21 (23.6%)	sALS *n* = 68 (76.4%)	Total *n* = 89
Sex	*n* = 21	*n* = 68	*n* = 89
Male, n (%)	8 (38.1)	36 (52.94)	44 (49.4)
Females, n (%)	13 (61.9)	32 (47.06)	45 (50.6)
Age of onset	n = *20*	n = *68*	n = *88*
Mean (± SD)	55 (12.24)	59 (10.39)	58.51 (11)
Classification of disease	*n* = 20	*n* = 68	*n* = 88
Early-onset, n (%)*	4 (20)	13 (19.12)	17 (19.32)
Late-onset, n (%)**	16 (80)	55 (80.88)	71 (80.68)
Site of onset	*n* = 20	*n* = 68	*n* = 88
Limb onset, n (%)	13 (65)	58 (85.29)	71 (80.68)
Bulbar onset, n (%)	6 (30)	8 (11.76)	14 (15.9)
FTD–ALS onset, n (%)	1 (5)	2 (2.94)	3 (3.4)
Asymptomatic carrier, n (%)	1 (4.76)	0	1 (1.14)
Cognitive status	*n* = 20	*n* = 68	*n* = 88
Normal, n (%)	19 (95)	67 (98.53)	86 (97.7)
Impaired, n (%)	1 (5)	1 (1.47)	2 (2.3)

Table 1. Clinical characteristics of ALS cases. *<50 years of age; **≥50 years of age.

Gene	cDNA change	Protein change	Patients	Incidence in Cyprus (%)	Gender, *n*	ALS type, *n*	Site of onset, *n*	Varsome classification	Franklin classification
C9orf72	GGGGCC expansion		20	22.47	Male, 7	Familial, 12	Limb, 16	–	–
					Female, 13	Sporadic, 8	Bulbar, 2		
							FTDAL, 2		
TARDBP	c.800 A > G	p.Asn267Ser	1	1.12	Female, 1	Sporadic, 1	Limb, 1	Likely benign	Likely pathogenic
TBK1	c.1760 + 1G > A	-	1	1.12	Female, 1	Familial, 1	Bulbar, 1	Likely pathogenic	Likely pathogenic
Figure 4	c.295G > T	p.Val99Phe	1	1.12	Male, 1	Sporadic, 1	Limb, 1	VUS	VUS
GLT8D1	c.713 C > G	p.Thr238Ser	1	1.12	Male, 1	Sporadic, 1	Limb, 1	VUS	VUS
BICD2	c.1180 C > A	p.Leu394Met	1	1.12	Female, 1	Familial, 1	Limb, 1	VUS	VUS

Table 2. ALS-associated variants in greek-cypriot ALS patients.

autosomal recessive or X-linked manner rarely observed[15]. However, in cases of sALS, diagnostic developments have only contributed to explaining a few cases, suggesting the implication of a complex interplay of various genetic and environmental risk factors[16]. To our knowledge, this is the first genetic epidemiology investigation of ALS in the Greek-Cypriot population. Thus, this current study aimed to investigate the genetic profile of ALS patients in Cyprus and provide clinical characteristics of Cypriot ALS cases.

Results

Patient cohort

The incidence and prevalence of ALS in Cyprus were estimated according to the population Census published by the National Statistics of Cyprus. On December 31st 2023, the crude prevalence of ALS in the government-controlled area of Cyprus was estimated at 6.6/100,000, while the annual incidence rate for ALS in the 2022–2023 period was 1.38/100,000.

In this cohort (Table 1), twelve families (Supplementary Table S1) have a family history of ALS. A notable higher frequency of female cases was observed in fALS cases (61.9%), whereas in sALS cases, an increased frequency in males (52.94%) was identified. Three clinical phenotypes were considered for the classification of disease onset: limb-onset, bulbar-onset, and FTD-ALS-onset. Patients who initially presented upper/lower limb weakness, were classified with limb-onset (*n* = 71, 80.68%), whereas bulbar-onset was considered the second most common phenotype among patients (*n* = 14, 15.9%). Also, individuals with FTD-ALS-onset represent the minority of cases (*n* = 3, 3.4%), with cognitive impairment affecting only 2.3% of all cases.

Genetic findings

In summary of the genetic investigation study, variants in six ALS-associated genes were identified in the Greek-Cypriot population (Table 2; Fig. 1). In total, twenty-five cases (28.1%), were found with either *C9orf72* pathogenic repeat expansion or at least one pathogenic, likely pathogenic or VUS variant in the identified genes. No variants were detected in *FUS* or *SOD1*, neither intermediate CAG repeats in *ATXN2* nor *SMN1* duplication.

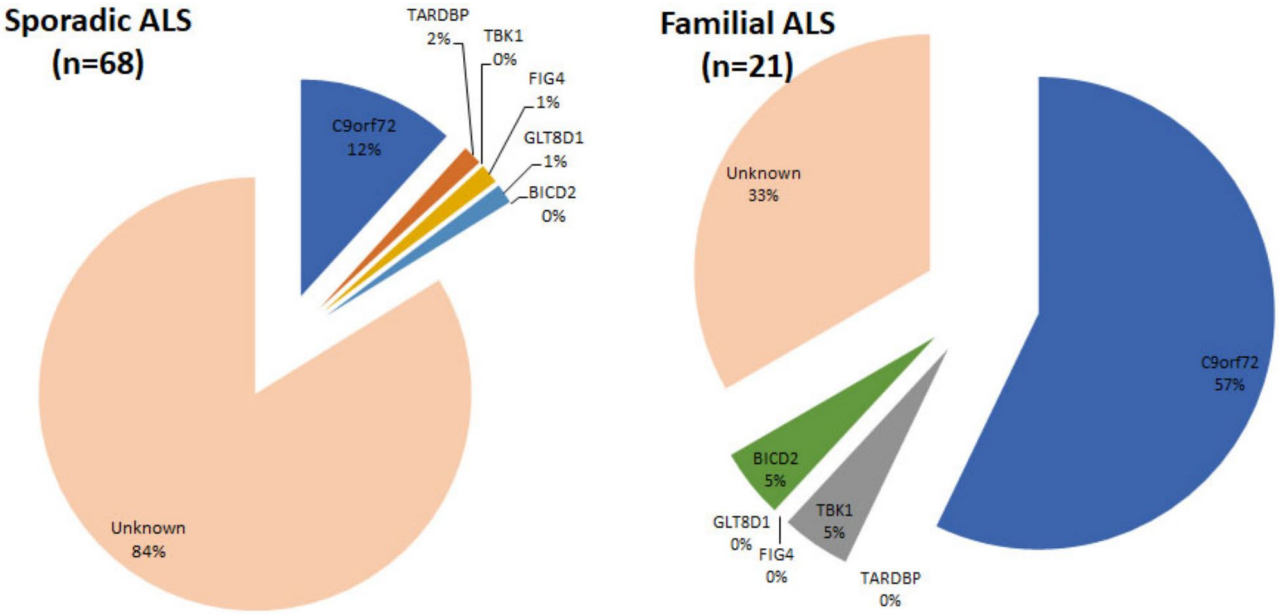

Fig. 1. Summary of genetic findings among familial and sporadic ALS patients in Cyprus.

C9orf72 hexanucleotide repeat expansions

The *C9orf72* repeat expansion is the most predominant ALS genetic cause in Cyprus, with a frequency of 22.47% ($n = 20$). Of the 20 *C9orf72*-positive cases, 12 have fALS (60% of fALS) and eight sALS (12.12% of sALS), with a mean age of onset estimated at 59.73 ± 7.87. Seven ALS families (58.33%) have at least two family members with an expanded *C9orf72* repeat allele. In addition, one asymptomatic carrier of *C9orf72* expansion with a family history of ALS was also reported. The male-to-female ratio is 1:2, with females being over-represented, with 13 *C9orf72*-positive cases (65%), and only seven male cases (35%).

Identification of possible ALS-associated variants

Five additional variants in the genes *TARDBP*, *TBK1*, *FIG4*, *GLT8D1*, and *BICD2* were identified in the Greek-Cypriot population (Table 2). The interpretation of sequence variants identified in this cohort was based on the American College of Medical Genetics and Genomics (ACMG) guidelines. The frequency of the variants in the identified genes was estimated at 1.12%, with a single patient harbouring each variant. The c.800 A > G (p.Asn267Ser) variant in the *TARDBP* gene was identified in one female patient with sALS and is classified as likely pathogenic and likely benign according to Franklin and VarSome databases, respectively. Additionally, the c.1760 + 1G > A variant in the *TBK1* gene, also classified as likely pathogenic, was found in a female fALS case. Similarly, the c.1180 C > A VUS variant in the *BICD2* gene (p.Leu394Met) was found in another female fALS patient, while the c.295G > T and the c.713 C > G variants in the *FIG4* and *GLT8D1* genes, both classified as VUS, were identified in a male sALS patient, respectively.

Discussion

Here, we studied the characteristics of Greek-Cypriot ALS cases, analyzed their genetic profile, and determining the incidence and prevalence of ALS in Cyprus. Based on our findings, the incidence of ALS in Cyprus was 1.38/100,000 person-year, slightly lower than the European median rate (1.47–2.43)[24]. According to other population-based registers, a slightly higher incidence was observed in Norway and Italy (Friuli-Venezia Giulia)[3]. To date, Ecuador has the lowest incidence (0.26/100,000 person-years), while Japan has the highest incidence of ALS (23.46/100,000 person-years), worldwide[25]. The crude prevalence in Cyprus was estimated at 6.6/100,000 persons, comparable to the European median rate (4.06–7.89)[24]. A significant difference was observed in the prevalence of Malta 3.44/100,000[26], while the prevalence of Sicily 6.0/100,000 was close to our findings[27]. The frequency of ALS in the Greek-Cypriot population was equal (1:1) in males and females, even though the male gender has long been considered a risk factor for ALS[3]. Other population-based studies also corroborate that the male-to-female ratio is greater in males compared to females[26–30]. However, data from recent studies suggests that the ratio of men to women has decreased, potentially as a result of women's changing lifestyles over time and their increased exposure to exogenous risk factors[31]. Nevertheless, among the fALS cases, there was a significantly increased frequency in females ($n = 13$, 61.9%, M: F 0.6:1), suggesting that a genetic variant may be more prevalent in females than males. We also observed that our study cohort appeared younger than other studies[9], suggesting that it might be due to the high incidence of fALS cases in our population, decreasing the mean age of onset for the total ALS population. Moreover, the proportion of familial cases (23.6%) in our population is remarkably higher compared to other epidemiological studies[6,27–30,32–34], including populations such as Malta (11.5%)[34], Sicily (7%)[35], and the recently registered rate for Sardinia (15%)[36]. This highlights that the Greek-Cypriot population has the highest frequency of fALS cases ever reported. In addition, a large proportion of ALS

cases in Cyprus are influenced by genetic factors (Fig. 1), since disease-causing variants or pathogenic repeat expansion in ALS-associated genes were identified in 28.1% of all recruited patients, including 56% fALS and 44% sALS cases. Nonetheless, a recent study in Malta reported an even greater number of genetically influenced ALS patients (> 45%), similar to results reported from Sardinia's ALS population[37].

One major finding was the identification of the G_4C_2 repeat expansion in the *C9orf72* gene as the predominant genetic cause of ALS in Cyprus, accounting for 22.47% of cases. This frequency is higher than in some other populations[38,39], potentially due to population isolation and racial admixture with Northern European countries[39,40].

Surprisingly, we did not find any variants in *SOD1* or *FUS*, which are common genetic causes of ALS in Europe[40]. Similar results were also observed in Malta, where variants in *SOD1* and *FUS* were not identified in the Maltese ALS population[26]. The frequencies of ALS-linked variants, relating to these main ALS genes, vary among populations and geographical regions. The absence of pathogenic variants in these common ALS genes in our population and the high frequency of fALS cases without genetic aetiology, might support the presence of unknown novel genes that are specific to the Greek-Cypriot population but are not yet discovered. Additionally, CAG intermediate-length repeats in *ATXN2* and duplication in *SMN1* were also absent from our cohort, indicating that these genes may not be significant ALS-causative or risk factors in the Greek-Cyprus population.

The known c.800 A > G variant in *TARDBP* gene, which has been previously linked with ALS having an autosomal dominant mode of inheritance, was present in our population. This variant was initially identified in an Italian cohort with *TARDBP* frequency estimated at 2.7%[42]. Nonetheless, in our cohort, the frequency of *TARDBP* was 1.09%, similar to Northern European populations[42], and within the range of the average European frequency rate in sALS cases (0.2–1.5%)[40]. Moreover, the proportion of *TBK1* variants in Cyprus (1.12%) was also within the range of the total ALS population (0.4–4%), with more than 90 ALS-linked variants found worldwide[43,44]. Frameshift, splice-site and nonsense variants causing the generation of premature termination codons are responsible for the loss of function through the loss of mutant transcript and protein. Moreover, rare missense variants as well as single amino acid deletions have been previously identified[45].

In this study, VUS were also identified in *FIG4*, *GLT8D1*, and *BICD2*, with a reported frequency of 1.12%, each. Pathogenic variants in the *FIG4* gene were first linked to ALS in Caucasian populations in 2009, where authors identified *FIG4* variants in 2% of patients with ALS, suggesting that *FIG4* pathogenic variants were another genetic contributor to ALS[46]. According to our findings, the *FIG4* variant frequency in our population is significantly less than in previous studies on Caucasian populations[47]. On the other hand, *GLT8D1* has a wide ALS frequency range among populations, ranging from 0.2 to 4.76%[48–53]. In vitro, cytotoxicity and ALS-like motor deficits in zebrafish were both exhibited by mutant *GLT8D1*[54], indicating its pathogenicity in ALS. Additionally, the *BICD2* gene is also present in the Greek-Cypriot ALS population. Although *BICD2* variants have been associated with SMA, novel *BICD2* mutations have been recently discovered in ALS cases[55,56]. So far, only in the Norwegian ALS population, *BICD2* have been investigated[29], sharing similar results with our findings (1.1%). Finally, in silico prediction tools were used for the identified VUS variants, supporting a pathogenic effect of each identified variant in this cohort.

In addition, a previous study in Cyprus had identified the pathogenic splice-site variant c.2980–2 A > G in *ALS2* in a consanguineous Cypriot family[17]. All three affected individuals of the family had the same phenotype of juvenile Primary Lateral Sclerosis (jPLS). Patients who exhibit pathogenic variants of *ALS2* are characterized by jPLS, HSP, and ALS suggesting a common pathway that highlights the significance of the *ALS2* product in the process of neurodegeneration[41]. In the Maltese ALS population, a relatively higher frequency of rare damaging variants was found in genes that are an infrequent cause of ALS in European populations including *ALS2*[34]. However, in our ALS population, no variants were identified for *ALS2*.

An important limitation of this genetic study is the small sample size of patients analyzed with WES. Better conclusions regarding the genetic profile of cases could have been drawn if all genetically undiagnosed ALS patients had been analyzed with WES. Furthermore, DNA unavailability from relatives of fALS cases was another major limitation. In some fALS cases, relatives were either deceased or abroad, and therefore segregation analysis to determine whether there is evidence that the identified variant underlies the development of ALS, was not performed.

In conclusion, our study provides a complete picture of the genetic landscape of ALS in Cyprus, based on the investigation of a large cohort of ALS patients. We concluded that pathogenic variants in major ALS genes are either absent or present at a very low frequency, which differs significantly from frequencies reported for other European populations. Our findings revealed important regional variations and found pathogenic ALS variants in both familial and sporadic ALS cases, with *C9orf72* being the most common causative ALS gene. Genes like *TBK1*, *FIG4*, *GLT8D1* and *BICD2* appear to be more relevant to ALS pathoaetiology in the Greek-Cypriot population compared to genes which are a major cause of ALS in other populations, including *SOD1* and *FUS*. Results have also shown that the male-to-female ratio of ALS in Cyprus was equal and male gender was not considered a risk factor in Cyprus. It is important to mention that a significantly higher frequency of familial ALS cases was observed in the Greek-Cypriot population, compared with other populations. This could support that unknown novel genes that are not yet found might be specific in our population. Finally, findings should encourage the genetic screening of additional patients using advanced technologies, as well as the creation of animal models in order to establish causality and gain a deeper understanding of disease mechanisms.

Methods

Participants

For the period 2020–2023, eighty-nine Greek-Cypriot ALS patients were included in the study. Patients were recruited from the Cyprus Institute of Neurology and Genetics (CING), the only referral centre for ALS in Cyprus, and evaluated by Neurologists. Inclusion criteria included (i) a definitive diagnosis of ALS according to

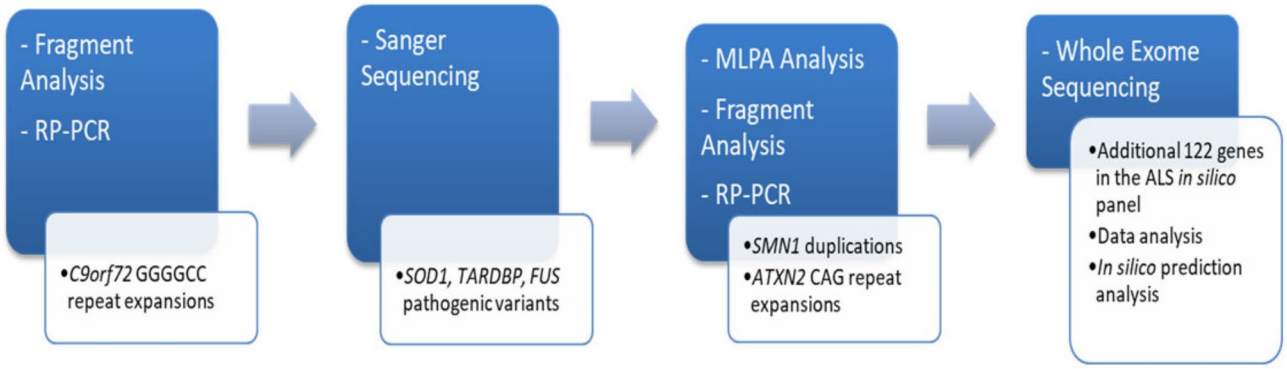

Fig. 2. Flow chart of the procedures for genetic screening in the cohort ALS population.

ALS2, ANG, ANXA11, ATXN2, C9orf72, CCNF, CHCHD10, CHMP2B, CYLD, DAO, DCTN1, ELP3, ERBB4, FIG4, FUS, GLT8D1, HNRNPA1, KIF5A, MATR3, NEFH, NEK1, OPTN, PFN1, PRPH, SBMA, SCA2, SETX, SIGMAR1, SMN1, SMN2, SOD1, SPG11, SPTLC1, SQSTM1, SS18L1, TARDBP, TBK1, TIA1, TUBA4A, UBQLN2, UNC13A, VAPB, VCP, VEGFA, KIFAP3, FLJ10986, WDR7, HTT, CAV1, AIFM1, AR, ARPP21, ASAH1, ASCC1, ATL1, ATP7A, BICD2, BSCL2, C21orf2/CFAP410, CAPN14, DDX20, DNAJB2, DNAJC7, DYNC1, H1, EPHA4, ERLIN1, EWSR1, EXOSC8, GBE1, GGNBP2, GLE1, GNE, GPX3, GRN, HEXA, HEXB, HNRNPA2B1, IGHMBP2, LAS1L, LGALSL, MAPT, MOBP, NIPA1, PLEKHG5, REEP1, RNF13, SARM1, SCFD1, SLC52A1, SLC52A2, SLC52A3, SPART, SPAST, SPG7, TAF15, TRIP4, TRPV4, UBA1, UBQLN4, VRK1, NDUFS4, ZC3H7B, CCDC59, TXNP1/INPP5F, TOP2A, THRAP3, TRPM3, ATP10A, FAM184B, NCS1, COX5A, SLF1, LIPH, CAMLG, ACSL5, KANK1, CAMK1G, ZNF512B, CYP27A1, DPP6, ITPR2.

Table 3. Genes included in the NGS in silico ALS-Panel.

the El Escorial criteria and (ii) Greek-Cypriot nationality. Blood samples and clinical data were obtained from all study participants. All patients who fulfilled the criteria were included and had given written informed consent prior to study participation. The study was ethically approved by the Cyprus National Bioethics Committee (ΕΕΒΚ/ΕΠ/2013/28).

Genetic analyses

Whole blood samples were collected, and genomic DNA was extracted using the Gentra Puregene DNA purification kit (Qiagen Sciences, USA), according to the manufacturer's protocol. Genomic DNA (gDNA) of the clinically diagnosed ALS patients was screened for the four most commonly mutated genes in European populations, including *C9orf72*, *SOD1*, *TARDBP*, and *FUS*. The AB3500xl Genetic analyzer (Applied Biosystems, US) was used for sequencing analyses. Figure 2 summarizes the procedures for genetic screening in this cohort population. All ALS patients were initially examined for *C9orf72* hexanucleotide repeat expansions using Fragment and Repeat-Prime (RP)-PCR analyses. *C9orf72*-negative cases were screened for disease variants in the remaining three genes (*SOD1*, *TARDBP*, and *FUS*) by Sanger sequencing. Next, two additional genes, *ATXN2* and *SMN1*, were selected for further investigation in genetically undiagnosed ALS patients. They were mainly screened for intermediate-length *ATXN2* repeats by Fragment and RP-PCR analyses, while Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed to identify *SMN1* duplications. Fragment analysis results were interpreted with the AB GeneMapper Analysis System Software. Sanger sequencing and MLPA interpretation were achieved using the SeqScape System Software and Coffalyser software, respectively. Finally, twenty-four patients (37.5%) negative for the above genes were further examined by whole exome sequencing (WES). Those patients were selected according to heritability status (familial ALS cases) and early age of disease onset (< 50 years). Following the manufacturer's instructions, the Illumina DNA Prep with Exome 2.5 Enrichment kit was used for library preparation. Sequencing was performed on the Illumina NextSeq 2000 system (Illumina, USA) using P3 reagents (200 cycles). BWA was used to map the reads to the reference sequence (GRCh37/hg19). One hundred twenty-two genes (Table 3) associated with ALS were included in the in silico gene panel, and variants were filtered during the data analysis. Variants were filtered according to the population frequency < 0.001 (1/1,000) and the pathogenicity class (based on ACMG criteria).

In silico prediction analysis

In silico tools were used to predict the downstream effect of the missense variants of uncertain significance (VUS) identified with WES, which have conflicting pathogenicity interpretations based on the American College of Medical Genetics and Genomics (ACMG) guidelines. These include the Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/index.shtml)[18], MutationTaster (https://www.mutationtaster.org/)[19], MutationTaster2021 (https://www.genecascade.org/MutationTaster2021/#transcript)[20], MutPred2 (http://mutpred.mutdb.org/)[21], PANTHER (https://www.pantherdb.org/tools/csnpScoreForm.jsp?)[22] and Combined Annotation Dependent Depletion (CADD) (https://cadd.gs.washington.edu/) (Supplementary Table S2).

Data availability

The datasets generated and/or analysed during the current study are available in the ClinVar repository, with accession numbers [SCV005184308, SCV005184319, SCV005184320, SCV005184321, SCV005184322] or data provided within the supplementary information files.

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Author contributions
E.M., P.N., E.Z.P., and K.C.: conception and design of the study; C.C.: contribution to the study design; E.Z.P., and K.K.: acquisition of data; E.M., P.N., P.K., A.G., C.V., and K.C.: analysis of data; E.M., P.N., P.K., E.Z.P., and K.C.: interpretation of data; E.M.: drafting text and figures; E.M., P.N., E.Z.P., K.C., and C.C.: review of the manuscript. All the authors have read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Study approval statement
The study was reviewed and ethically approved by the Cyprus National Bioethics Committee (EEBK/ΕΠ/2013/28) and conducted in accordance with the Declaration of Helsinki.

Consent to participate
A written informed consent was obtained from participants. For deceased patients, information was collected from their next of kin, if possible.

Additional information
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