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Genetic landscape of ALS in Malta based on a quinquennial analysis

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ABSTRACT

Genetic risk for amyotrophic lateral sclerosis (ALS) is highly elevated in genetic isolates, like the island population of Malta in the south of Europe, providing a unique opportunity to investigate the genetics of this disease. Here we characterize the clinical phenotype and genetic profile of the largest series of Maltese ALS patients to date identified throughout a 5-year window. Cases and controls underwent neuromuscular assessment and analysis of rare variants in ALS causative or risk genes following whole-genome sequencing. Potentially damaging variants or repeat expansions were identified in more than 45% of all patients. The most commonly affected genes were *ALS2, DAO, SETX* and *SPG11*, an infrequent cause of ALS in Europeans. We also confirmed a significant association between *ATXN1* intermediate repeats and increased disease risk. Damaging variants in major ALS genes *C9orf72, SOD1, TARDBP* and *FUS* were however either absent or rare in Maltese ALS patients. Overall, our study underscores a population that is an outlier within Europe and one that represents a high percentage of genetically explained cases.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, fatal neurodegenerative disease. Clinical signs of upper and/or lower motor neuron degeneration are typically found on onset and patients usually present with weakness in either the bulbar or limb muscles, and, infrequently, both regions simultaneously. Death typically occurs around 3 years after clinical onset, mostly due to respiratory failure. A small percentage of patients with ALS also experience progressive cognitive decline and are eventually co-diagnosed with frontotemporal dementia (FTD) (Brown and Al-Chalabi, 2017; van Es et al., 2017). ALS is classified as sporadic (sALS) in the absence of a clear family history of the disease and familial (fALS) when this is present. Genetics plays a strong role in ALS etiology, either a directly causative one in case of fALS or through a complex interplay with environment and lifestyle factors in case of sALS. To date, more than 40 different genes have been associated with the disease (Gregory et al., 2020; Smukowski et al., 2022). In admixed populations, a sizable fraction of fALS cases and a small number of sALS cases can be explained by dominant highly-penetrant rare causal variants residing in *C9orf72, SOD1, TARDBP* and *FUS* genes (Zou et al., 2017). Nonetheless, the frequency of ALS-linked variants in these major ALS genes varies in diverse populations or geographical regions with marked differences mostly observed in isolated and, therefore, relatively homogenous populations (Borg et al., 2021; Borghero et al., 2014).

Malta, one of the smallest countries in Europe (total area 316 km²), is an archipelago of 3 inhabited islands in the middle of the Mediterranean Sea. The population of Malta, which presently numbers around half a million, represents a genetic isolate. The pop-

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale-revised; CK, creatine kinase; fALS, familial amyotrophic lateral sclerosis; FTD, frontotemporal dementia; gnomAD, Genome Aggregation Database; Indel, small insertion and deletion; MAF, minor allele frequency; OR, odds ratio; PCA, principal component analysis; sALS, sporadic amyotrophic lateral sclerosis; SNV, single nucleotide variant.

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ulation was initially founded in the sixth millennium BC by settlers that crossed the sea to Malta from the neighboring island of Sicily. Re-founding events from the same direction probably occurred multiple times in the centuries that followed to replace and/or bolster the population (Cassar, 2000). Although influences by different colonizers can be hypothesized, genetic evidence indicates that this was minimal (Capelli et al., 2006; Caruana, 2012; Cassar et al., 2008). The isolation of Maltese from the rest of mainland Europe is apparent since Neolithic times based on analysis of ancient genomes revealing genomic insularity (Ariano et al., 2022). At 2.5/100,000 person-years (Borg et al., 2021), incidence of ALS in Malta is marginally above the European median (2.1/100,000 person-years) (Chio et al., 2013) and much higher than that reported for South Europe (1.49/100,000 person-years) (Xu et al., 2020). Interestingly, we have recently reported that deleterious variants in *C9orf72, SOD1, TARDBP* or *FUS* genes are rare or absent in ALS patients of Maltese ancestry. Nonetheless, the rate of fALS in Malta is higher than the European median and most Maltese ALS patients were found to harbor deleterious variants in genes that are an infrequent cause of ALS in continental Europe (Borg et al., 2021; Farrugia Wismayer et al., 2022).

Detailed knowledge of the genetic landscape of ALS in specific populations is valuable for genetic counselling, the design of ALS multigene diagnostic panels, prognosis or participation in clinical trials for genotype-specific treatments. With this aim, here we describe the clinical characteristics and genetic profile of a larger series of Maltese ALS patients and matched controls investigated by our center during the past 5 years.

2. Materials and methods

2.1. Patients and controls

Fifty-two ALS patients of Maltese ancestry, except for 1 fALS case (British origin) and 2 sALS cases (British and half-Dutch origin), were identified between 2017 and 2022. The patients were diagnosed with probable or definite ALS according to the revised El Escorial criteria for ALS (Brooks et al., 2000; Ludolph et al., 2015) and were mostly referred to our centre by consultant neurologists and neurophysiology units. Patients with fALS were identified as having a self-reported family history of ALS, or probable ALS, as defined by the presence of at least 1 first-degree relative affected by the disease. Sixty-six subjects of Maltese ancestry that were not affected by neurological disorders were also randomly identified to roughly match patients for age, sex and geographical location. Ethical approval for the study was given by the Research Ethics Committee of the University of Malta and written informed consent was obtained from each study participant or the next of kin or legal guardian if the patient was either a minor or too severely disabled.

2.2. Neuromuscular assessment

In addition to a core dataset collected via a structured questionnaire that included age, sex, site of onset, date of disease onset, family history and ALS Functional Rating Scale-revised (ALSFRS-R) score, a clinical examination was performed to determine neuromuscular function. To this end, each subject was assessed for muscle tone of both upper and lower limbs, strength of different muscle groups graded according to the MRC scale and status of reflexes. Creatine kinase (CK) levels at recruitment were also measured via a biochemical assay as a marker of muscle damage.

2.3. Whole genome sequencing

DNA was extracted from whole EDTA-containing venous blood samples using the QIAamp DNA Blood Mini Kit and was whole-genome sequenced by the BGISEQ-500 platform (BGI, Hong Kong, China) to generate 100 bp paired-end reads with an average depth of 40X. Reads were aligned to the GRCh37 (hg19) or GRCh38 (hg38) reference genome using Burrows–Wheeler Aligner software. Single nucleotide variant (SNV) and small insertion and deletion (indel) calling and quality filtering were performed using the Genome Analysis Toolkit (GATK).

3.4. Genetic analysis

In order to estimate the genetic ancestry of patient and control samples in relation to the European reference map derived from 1385 European samples (Novembre et al., 2008), principal component analysis (PCA) was performed on LASER with results plotted using the LASER Server plot facility (https://laser.sph.umich.edu/). The ExpansionHunter v5.0.0 tool was used to analyse repeat sizes of *ATXN1* (NM_000332.3:c.589_591CAG), *ATXN2* (NM_002973.3:c.496_498CAG), *C9orf72* (NM_001256054.2:c.-45+163GGGGCC), and *NIPA1* (NM_144599.4:c.24_26GGC) (Dolzhenko et al., 2017). Genome mining was restricted to protein-coding and splice-site altering SNVs and indels in 62 established ALS causative or risk genes (*Supplementary Table 1*) that were present in ALS patients but absent in controls. A European minor allele frequency (MAF) threshold of <0.01 was adopted. Where available, variants were then annotated with information from the dbSNP database including European-specific MAF estimates from the Genome Aggregation Database (gnomAD). The superior ensemble-based MetaSVM prediction tool was utilized to determine variant pathogenicity. Indels, nonsense and splice-site acceptor/donor variants were automatically classified as deleterious. Variants and the associated phenotypes have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) with accession numbers SCV002555576-SCV002555583. New variants detected in the Maltese case-control cohort were submitted to the dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) with the submission SNP IDs of ss2137544388, ss5981325820 and ss5981325821.

3.5. Statistics

Comparisons between continuous variables were made with the unpaired Mann–Whitney's test, the unpaired Kolmogorov-Smirnov test or the Wilcoxon signed-rank test. The χ^2 test was used to investigate for differences between categorical variables. Odds ratio (OR) and associated 95% confidence interval were estimated where appropriate and the two-sided Fisher's exact test was utilized to determine associations. A p value <0.05 was considered significant. Statistical analysis was carried out with GraphPad Prism v9 software (La Jolla, CA, USA).

3. Results

3.1. Baseline features

Over a period of 5 years (2017–2022) we identified an ALS case-control cohort of 120 individuals with Maltese ancestry from a population that according to Malta's National Statistics Office stood at 516,100 inhabitants in 2020. Key features of patients and controls are detailed in Table 1. ALS cases were predominantly male and mean age of symptom onset occurred around mid-age, with this being slightly lower in females (58.3 $\pm$ 12.3 S.D.) compared

Table 1
Baseline characteristics of Maltese ALS patients and controls

	ALS (n = 52)	Controls (n = 68)
Genetically analyzed, % (n)	90.4 (47)	98.5 (67)
Gender		
Male, % (n)	69.2 (36)	48.5 (33)
Female, % (n)	30.8 (16)	51.5 (35)
Age at recruitment		
Mean year ± SD (range)	62.73 ± 13.8 (12–83)	67.4 ± 11.3 (27–95)
Age at onset		
Mean year ± SD (range)	59.4 ± 15.5 (1.5–81)	-
Site of onset		
Spinal, % (n)	78.8 (41)	-
Bulbar, % (n)	17.3 (9)	-
Both, % (n)	3.8 (2)	-
Type		
Familial, % (n)	11.5 (6)	-
Sporadic, % (n)	88.5 (46)	-
Deceased, % (n)	50 (26)	-
Survival, mean months ± SD (range)[a]	44.5 ± 61.1 (4–312)	-
Cognitive status		
Normal, % (n)	92.3 (48)	100 (66)
Impaired, % (n)	5.8 (3)	0 (0)
ALSFRS-R score, mean ± SD (range)	30.2 ± 10.8 (11.5–34.5)	47.7 ± 0.7 (44–48)
CK levels at recruitment, IU/L[b]		
Mean in males ± SD (range)	304.1 ± 226 (15–878)	-
Mean in females ± SD (range)	211.2 ± 141.2 (38–557)	-

Key: ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; CK, Creatine Kinase
[a] Survival data available for 26 patients
[b] Normal CK range = 39-308 U/L (males), 26-192 U/L (females)

to males (60 ± 16.8 S.D.). Five patients (9.6%) had an age of onset below 40 years including 1 male subject whose symptoms first appeared at an age of one and a half years, hence, diagnosed with juvenile ALS. Late onset (≥80 years) was observed in 4 patients (7.7%) that were all male. The majority of ALS cases had spinal onset of symptoms. Bulbar onset ALS had a marginally higher occurrence in females (54.5%). A family history of ALS was recorded for only a minority of cases (Table 1). Half of the patients recruited died within our 5 year study window, with survival from symptom onset till death being on average less than 4 years (Table 1). A shorter survival was observed in male patients (40.5 ± 73.3 S.D. months) compared to females (50.8 ± 36.8 S.D. months), and in patients with bulbar onset (35.6 ± 22.4 S.D. months) compared to those with spinal onset (48.7 ± 72.3 S.D. months). In patients that remained alive, more than half (57.9%) had a disease duration ≥5 years.

The mean ALSFRS-R score in ALS recruits was more than 15 points lower than that recorded for control subjects ($p < 0.0001$). On average, the levels of creatine kinase (CK) in the patient cohort were either close to or above the upper reference limit in case of males and females, respectively, indicating either acute or chronic muscle injury (Table 1). Differences in muscle strength, tone and reflex status of different muscle groups were observed in patients compared to controls (*Supplementary Fig.1* and *Fig.2*). Geographic distribution of the identified cases and controls is displayed in Fig. 1. A cluster of cases is apparent in the center and southeast of mainland Malta with this reflecting a higher population density in these regions. For a population size of 34,563 inhabitants based on Malta's National Statistics Office data in 2020, a higher than expected number of cases were registered on the island of Gozo.

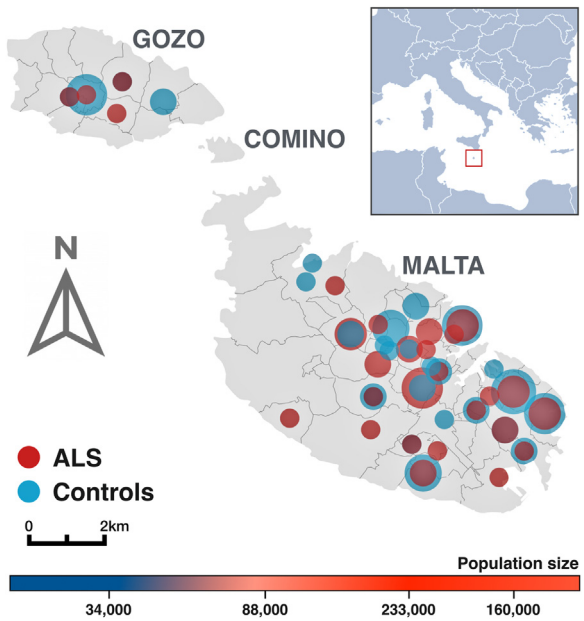

Fig. 1. Geographical distribution of incident ALS cases (2017–2022) and matched controls resident in the archipelago of Malta. Size of circles reflects size of sample. The majority of incident cases cluster in centre and southeast of mainland Malta, reflecting the high population density of these regions. Population size is based on NSO data in 2020. Symbol map was generated by Datawrapper. Abbreviations: ALS, amyotrophic lateral sclerosis.

3.2. Genetic ancestry

PCA analysis of the Maltese case-control cohort determined that ALS cases and controls with Maltese ancestry were within one standard deviation of the mean for the combined samples along principal component 1 (PC1) and 2 (PC2), hence ensuring genetic matching. Mapping either the ALS patient or control samples to the reference PCA coordinates of the European Population Reference (POPRES) samples demonstrates an overlap with samples derived from the South of Europe (Italy) (Fig. 2), thus indicating similarity of genetic structure that is supported by historical records.

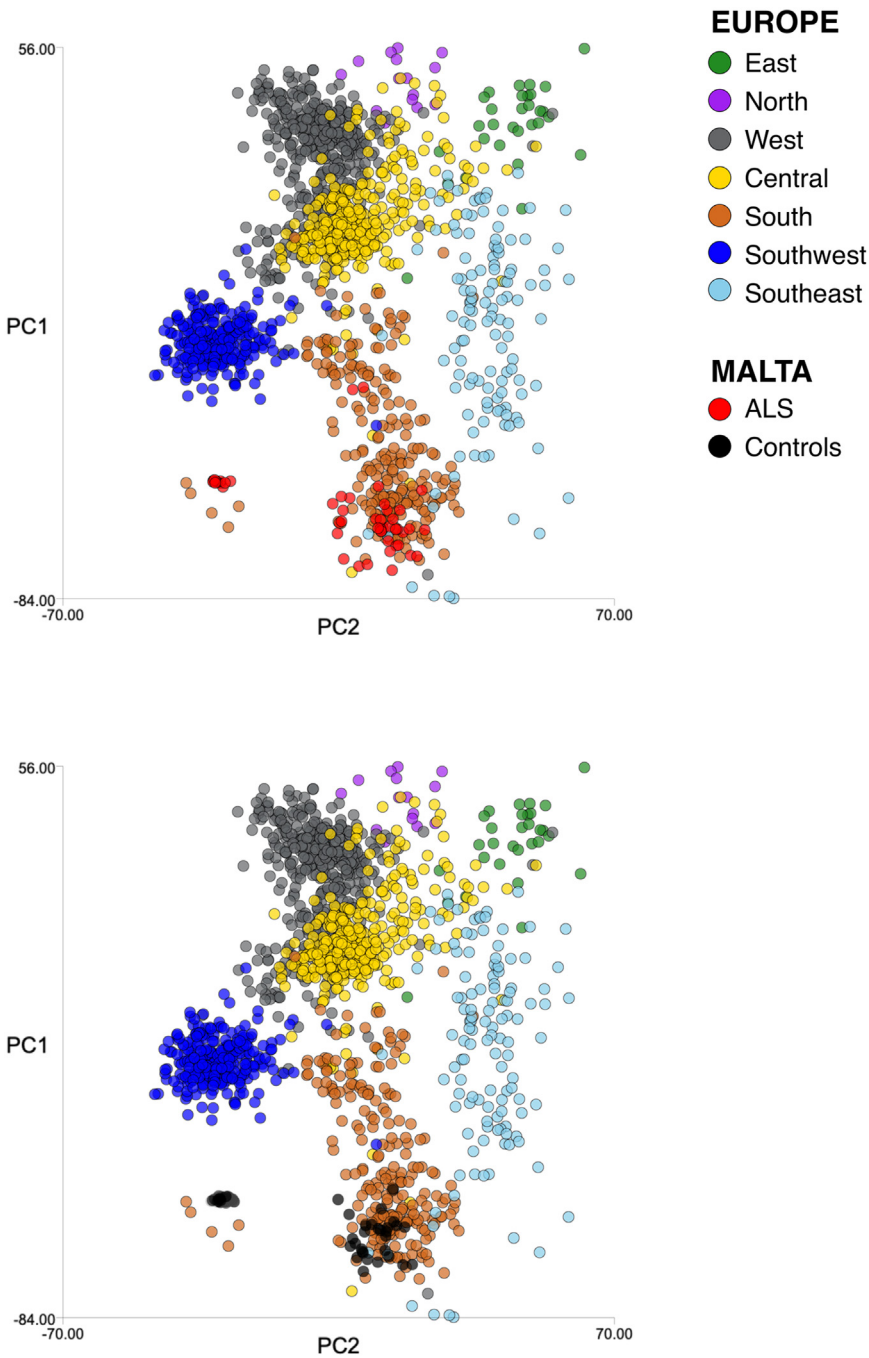

Fig. 2. Genetic ancestry of Maltese ALS cases and controls compared to the European Population Reference panel. Principal components analysis of genetic data from European and Maltese samples based on principal component axis 1 (PC1) and axis 2 (PC2). European samples were colour coded according to geography. Abbreviations: ALS, amyotrophic lateral sclerosis; PC1, principal component 1; PC2, principal component 2.

3.3. *Repeat expansions in* C9orf72, ATXN1, ATXN2 *and* NIPA1 *genes*

In contrast to ALS patients in European populations (Renton et al., 2011; Smith et al., 2013; Zou et al., 2017), pathogenic hexanucleotide (GGGGCC) repeat expansions (≥24) in *C9orf72* are rare in Maltese ALS patients. We encountered only 1 case with a repeat length above threshold (32 repeats) (Fig. 3). The patient who was a female developed spinal-onset ALS at an age of 55 years. Family history was unremarkable except for her mother who had dementia. Interestingly, during our study period, we came across 2 male asymptomatic individuals (ALSFRS-R score = 48) within a family with a history of bulbar- and spinal-onset ALS that had in excess of 100 repeats (Fig. 3 and *Supplementary Fig. 3*). The index subject (III:7) who is now in his early fifties (53 years) is a carrier of a *C9orf72* allele with 248 repeats. He has a deceased mother (II:3) who was diagnosed with bulbar-onset ALS at 48 years of age, dying after a survival period of 2 years and 3 older siblings that are also asymptomatic. Two of his maternal uncles developed ALS. At 55 years of age, 1 (II:2) experienced weakness in the lower limbs that progressed to the upper limb and bulbar muscles. The other (II:9) had bulbar-onset ALS at 69 years. Both died within a few months of

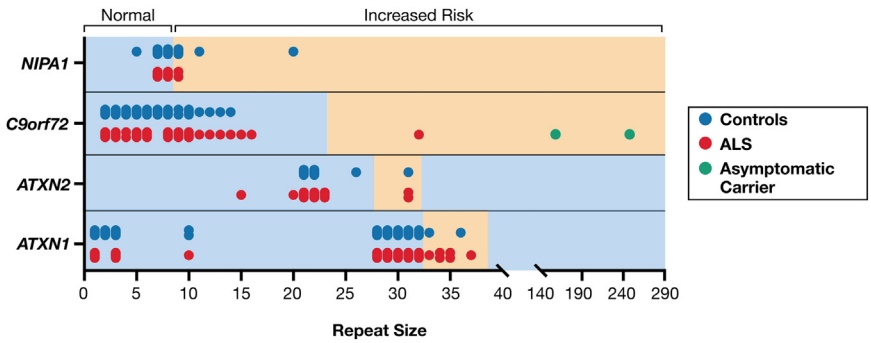

Fig. 3. Expansion repeat size in ALS cases and controls for *NIPA1, C9orf72, ATXN2* and *ATXN1* genes. Scatter plot showing distribution and frequency of allele repeat sizes, indicated by dots. Each dot corresponds to 1 individual. Abbreviations: ALS, amyotrophic lateral sclerosis

Table 2
Rare variants predicted as damaging in Maltese ALS patients

Gene	cDNA change	Protein change	dbSNP[141] ID	European gnomAD MAF	No. of patients	ALS type	Zygosity
ALS2	NM_020919.4:c.2221C>T	p.(Arg741*)[a]	rs759408917	0.00001771	1	fALS	het
ALS2	NM_020919.4:c.3624+1G>A	Splice variant[a]	NA	NA	1	fALS	het
ALS2	NM_020919.4:c.142C>G	p.(Leu48Val)	NA	NA	1	sALS	het
DAO	NM_001917.4:c.250G>A	p.(Ala84Thr)	rs781658657	0.00001	2	sALS	het
DCTN1	NM_004082.4:c.1864A>T	p.(Ile622Phe)	rs1328116832	0.000004[b]	1	sALS	het
ERBB4	NM_005235.2:c.3814G>A	p.(Gly1272Arg)	rs371332509	0.00001	1	sALS	het
FUS	NM_004960.4: c.515_523+3delGAGGTGGAGGTG	p.(Gly173_Gly175del)[a]	rs772959031	NA	1	fALS	het
KANK1	NM_001256876.1:c.149A>T	p.(Asp50Val)	rs61737971	0.00947	1	sALS	het
NEFH	NM_021076.4:c.1321G>A	p.(Glu441Lys)	rs145061116	0.000030	1	sALS	het
SETX	NM_015046.5:c.5308_5311del	p.(Glu1770Ilefs*15)[a]	rs750959420	0.000036	1	sALS	het
SETX	NM_015046.7:c.3229G>A	p.(Asp1077Asn)	rs145097270	0.001233	1	sALS	het
SOD1	NM_000454.5:c.272A>C	p.(Asp91Ala)	rs80265967	0.00228	1	sALS	het
SPG11	NM_025137.3:c.1618C>T	p.(Arg540Cys)	rs758046989	0.00001	1	sALS	het
SPG11	NM_025137.4:c.5381T>C	p.Leu1794Pro	rs201689565	0.0001166	1	sALS	het
SQSTM1	NM_003900.5:c.769A>G	p.(Ile257Val)	rs778679611	0.00001	1	sALS	het

Key: dbSNP, Single Nucleotide Polymorphism database; gnomAD, Genome Aggregation Database; MAF, minor allele frequency; NA, not available; het, heterozygote
[a] Indels, nonsense and splice variants were automatically considered as deleterious.
[b] Data from Trans-Omics for Precision Medicine (TopMed) Program.

disease onset. Based on DNA availability and subject consent, we determined that a male descendant (III:1) of the former (II:2) is a carrier of a *C9orf72* allele that has an expansion repeat size of 158.

Compared to controls, a higher frequency of ALS patients had polyglutamine repeat expansions in the *ATXN1* and *ATXN2* genes, within the intermediate-length range that has been associated with an increased disease risk (Conforti et al., 2012; Elden et al., 2010; Tazelaar et al., 2020) (Fig. 3). In the total cohort of ALS patients, we found that 17% possessed *ATXN1* ALS-associated trinucleotide repeat expansions compared to 4.5% of controls (OR = 4.3, 95% CI 1.2–15.5, $p < 0.05$). Intermediate-length *ATXN2* repeats known to increase ALS risk (31 repeats) were found in 2 male patients with spinal-onset ALS and 1 control. Similarly, 2 of our ALS patients having spinal onset of symptoms carried polyalanine expansions in *NIPA1* greater than 8 repeats, a threshold that has been previously found to increase predisposition to ALS (Blauw et al., 2012). Nonetheless, we find that several control subjects also had repeat lengths higher than this threshold (Fig. 3).

3.4. Rare damaging variants in known ALS genes

In contrast to European populations (Zou et al., 2017), we find that damaging variants in the most commonly mutated ALS genes other than *C9orf72*, are either rare or absent in Maltese ALS patients. Hence, in our patient cohort, we identified only 2 patients that carried a damaging variant in either *SOD1* or *FUS* and none that were positive for deleterious variants in *TARDBP* (Table 2). Interestingly, these 2 patients were derived from the small population inhabiting the island of Gozo. The male patient having the *SOD1* D91A variant has been described previously (Farrugia Wismayer et al., 2022). The *FUS* deletion G173-G175del was found in a recently diagnosed male patient with a late disease onset (79 years), who started experiencing fasciculations and bilateral weakness in the upper limbs. The patient has a positive family history and remains alive. We next examined 59 ALS-associated genes in our patient and control cohort, and identified 13 rare (European MAF <0.01) variants in 9 genes predicted as damaging that were present in Maltese ALS patients but absent in controls (Table 2). Two SNVs in *ALS2* were not found in the dbSNP (v155) database and have since been submitted. Two patients were identified to have the same NM_001917.4:c.250G>A; p.(Ala84Thr) variant in the *DAO* gene. Both developed dysarthria in addition to upper limb weakness. It is noteworthy that in 1 patient, who also possessed a deleterious variant in the *DCTN1* gene, disease onset occurred at an earlier age.

Different variants in the *ALS2, SETX* and *SPG11* genes were identified in more than 1 patient (Table 2). The only patient with juvenile ALS was a male compound heterozygote for the splice-site altering NM_020919.4:c.3624+1G>A and nonsense NM_020919.4:c.2221C>T; p.(Arg741*) variants in the *ALS2* gene. He is now in his 12th year and has since lost the ability to walk in addition to experiencing dysarthria (ALSFRS-R = 21). Another rare *ALS2* variant predicted to be damaging was detected in the heterozygous state in a male patient that developed spinal-onset ALS at 60 years of age. Multiple *SETX* and *SPG11* damaging variants

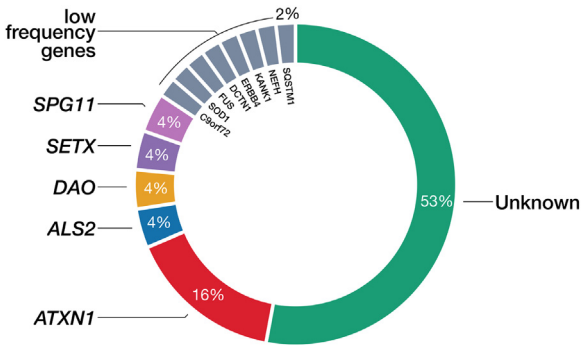

Fig. 4. Percentage of Maltese ALS patients carrying rare damaging variants or pathogenic repeat expansions in known ALS genes. Variants in the major ALS genes *C9orf72, SOD1* and *FUS* are present at a very low frequency or are totally absent in case of *TARDBP*.

were identified in patients with spinal-onset of symptoms. Rare non-deleterious variants that were unique to patients are listed in *Supplementary Table 2*. Two SNVs, one in *FIG4* and another in *GGNBP2*, not found in the dbSNP (v155) database have since been submitted. Seventeen of 42 apparently sALS cases had rare and potentially deleterious variants (absent in controls) or pathogenic repeat expansions (absent or low frequency in controls) in at least 1 ALS-associated gene, leading us to estimate that the genetic risk for ALS in the Maltese population is 40.5%. Only 40% of patients with fALS carried pathogenic variants in known ALS genes, spurring further work on discovery of novel causative genes in this unique population.

4. Discussion

In our study, we investigated the clinical characteristics and genetic profile of the largest cohort of ALS patients identified to date from the Maltese archipelago, that is home to a population that experienced relative socio-cultural isolation over the centuries. Population-specific aspects of Maltese ALS cases are similar to those reported for the larger Mediterranean islands of Sardinia (Borghero et al., 2022; Pugliatti et al., 2013), Sicily (Ragonese et al., 2012) and Cyprus (Demetriou et al., 2017) including male preponderance, mid-age of onset, a 3 year mean survival period from symptom onset till death, a predominance of cases with spinal-onset, and a higher rate of bulbar-onset in female patients. Using stringent criteria, the percentage of cases with fALS in Malta (11.5%) is above that reported by pooled data (5%) from prospective population-based registries across the world (Byrne et al., 2011). Although higher than that of Cyprus (4%) (Demetriou et al., 2017) and the South of Italy including Sicily (7%) (Ungaro et al., 2021), the rate of familial cases in Malta nears that recently registered for Sardinia (15%) (Borghero et al., 2022). Linking to this, we found that a high percentage (>45%) of all Maltese ALS cases carry a rare damaging allele or pathogenic repeat expansion in an ALS-associated gene (Fig. 4), similar to findings described for the ALS population in Sardinia (Borghero et al., 2014). This highlights that a large portion of ALS cases in Malta are genetically determined.

Importantly, using a larger cohort of ALS patients we confirmed our earlier findings showing that rare deleterious variants in the major ALS genes, including *C9orf72, SOD1, TARDBP*, and *FUS*, are either absent or have an extremely low frequency in Maltese ALS patients (Borg et al., 2021; Farrugia Wismayer et al., 2022), making the population of Malta an outlier within the European continent (Zou et al., 2017). It is interesting that the only patients with *SOD1* and *FUS* variants previously reported to be ALS causing (Kwiatkowski et al., 2009; Robberecht et al., 1996) were identified on the minuscule island of Gozo (total area 67 km^2). Across centuries, Gozo experienced population size fluctuations, unrelated to those of mainland Malta (Bezzina, 2002), including conquest-driven depopulations and eventual repopulations by families with mostly Sicilian ancestry. The *SOD1* D91A and *FUS* G173-G127del variants, which were also identified in Southern Italian ALS patients (Ungaro et al., 2021), likely reflect a founder effect specifically operating within the Gozitan population. We also identified 2 related individuals on the island of Malta that were asymptomatic carriers of expanded *C9orf72* alleles and were members of a family with bulbar-onset ALS. Although DNA of family members that died of ALS was not available, the presence of pathogenic expansions in descendants and studies indicating that *C9orf72* expansions lead to a higher frequency of bulbar-onset ALS (reviewed in Van Mossevelde et al., 2017), all support the possibility that in this family, ALS is due to damaging *C9orf72* expansions. Our study underscores that the frequencies of deleterious variants in genes that are commonly implicated in ALS vary greatly by ethnicity or geographic origin (Zou et al., 2017). Thus, it is highly likely that the genetic architecture of ALS in Malta is influenced by the genomic insularity resulting from geographical isolation. This is despite the fact that our PCA analysis indicated a related genetic ancestry with Southern European populations in which a large number of cases can be explained by genetic alterations in the 4 major ALS genes (Ungaro et al., 2021).

A relatively higher frequency of rare damaging variants was found in genes that are an infrequent cause of ALS in European populations including *ALS2, DAO, SETX* and *SPG11*. The *ALS2* and *SPG11* genes, which encode proteins with a role in vesicular trafficking, are known to cause juvenile-onset ALS under a recessive disease model (Daoud et al., 2012; Hadano et al., 2001; Hentati et al., 1994; Orlacchio et al., 2010). To this end, we identified a child that was a compound heterozygote for 2 rare variants predicted as deleterious in the *ALS2* gene. It is interesting to note that *ALS2* was originally identified as an ALS causing gene in a family from the Mediterranean country of Tunisia (Hentati et al., 1994), which is only a few hundred kms south of Malta. In other patients, *ALS2* and *SPG11* variants were observed solely in heterozygous configurations and in patients with adult-onset ALS. In these cases, it is highly likely that these alleles are themselves not disease causing on their own and, most probably, other genetic or non-genetic factors, including a history of strenuous activity as a result of the patients' former occupation (Farrugia Wismayer et al., 2021), play a role in disease manifestation.

It is noteworthy that some degree of overlap exists in the clinical phenotype of patients identified to have damaging variants in *DAO* or *SETX*. Both genes, which encode enzymes involved in different pathways, have been previously associated with ALS having an autosomal dominant mode of inheritance (Chen et al., 2004; Mitchell et al., 2010). In our population and in others (Hirano et al., 2011; Kenna et al., 2013; Saracchi et al., 2014; Tripolszki et al., 2017; Zhao et al., 2009), *SETX* alleles predicted as damaging lead to adult-onset ALS with symptoms appearing in the limbs at onset followed by a slow rate of disease progression. In contrast, patients with loss of function variants in the *DAO* gene appear to be more prone to develop bulbar signs in addition to limb involvement. Simulation studies on *DAO* variants with a genetic location near those identified in Maltese ALS patients indicate that due to conformational changes, enzymatic activity is likely to be compromised (Padhi and Zhang, 2020), similar to what was demonstrated for the originally identified ALS-associated *DAO* variant R199W (Mitchell et al., 2010). Both DAO and SETX proteins form homodimers and it remains to be seen whether the identified variants form heterodimers with the wild-type protein, with negative con-

sequences on enzymatic activity due to a potential dominant negative effect.

Our findings based on a quinquennial analysis of a large cohort of ALS patients relative to its population size, give a comprehensive picture of the genetic landscape of ALS in the isolated island population of Malta. We confirmed that damaging variants in major ALS genes are either absent or present at a very low frequency, which contrasts heavily with frequencies reported for European populations including those neighboring Malta (Ungaro et al., 2021). Notably, more than half of Maltese patients with fALS and close to two thirds of apparently sALS cases cannot be explained by genetic alterations in known ALS genes, hence, spurring further studies to determine factors that are either causative or increase disease risk. It is interesting to highlight the significant association identified here between *ATXN1* repeat expansions and disease risk in Maltese ALS cases mirroring earlier studies identifying this gene as a risk factor in Southern Italian ALS cohorts (Conforti et al., 2012; Ungaro et al., 2021). Genes like *ALS2, DAO, SETX* and *SPG11* appear to be more relevant to ALS pathoaethiology in the Maltese population compared to genes which are a major cause of ALS in other populations. Further work is therefore warranted to confirm causation and to better understand how loss of or a change in their function leads to disease, and generation of novel animal models is probably a good starting point (Aquilina and Cauchi, 2018). This is ultimately important for the development of treatments that will be more valuable to specific populations relative to others.

CRediT author statement

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Disclosure statement

The authors declare no competing interests.

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Supplementary materials

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