Journal of Neurology (2020) 267:3578–3592
https://doi.org/10.1007/s00415-020-10042-y

ORIGINAL COMMUNICATION

Targeted next-generation sequencing study in familial ALS-FTD Portuguese patients negative for *C9orf72* HRE

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Received: 8 May 2020 / Revised: 25 June 2020 / Accepted: 30 June 2020 / Published online: 7 July 2020

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with clinical and etiological heterogeneity and a complex genetic contribution. Clinical, neuropathological, and genetic evidence revealed that ALS and frontotemporal dementia (FTD) are in part of a single disease continuum. Genetic causes have been identified in sporadic (SALS) and familial patients (FALS) and the recurrent genetic factor underlying ALS and FTD is the *C9orf72* hexanucleotide repeat expansion (HRE). However, in our population, the concomitance of ALS and FTD cannot be explained by *C9orf72* HRE in many FALS and SALS cases. Our aim is to further understand the genetic basis of ALS in Portuguese patients. 34 patients with FALS or SALS-FTD, negative for *C9orf72* HRE, were screened for rare variants in a panel of 29 relevant genes by next-generation sequencing. We detected 15 variants in 11 genes, one classified as pathogenic in *TARDBP*, two as likely pathogenic in *TARDBP* and *PRPH,* and the others as variants of unknown significance (VUS). Gene variants, including VUS, were found in 41.2% FALS patients and 40% SALS-FTD. In most patients, no potential pathogenic variants were found. Our results emphasize the need to enhance the efforts to unravel the genetic architecture of ALS-FTD.

Keywords Amyotrophic lateral sclerosis · Frontotemporal dementia · Next-generation sequencing · Genetic screening · Gene variants

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset, progressive neurodegenerative disease, characterized by the loss of both upper motor neurons (UMN) and lower motor neurons (LMN) at spinal cord and bulbar levels. Additionally, cognitive and/or behavioural impairment due to the involvement of prefrontal cortex is detected in 30–50% of ALS patients, and 13% of patients fulfil the clinical diagnostic criteria for frontotemporal dementia (FTD) [1]. Conversely, up to 50% of FTD patients develop signs of motor involvement with about 15% meeting the diagnostic criteria of ALS [2]. Thus, based on clinical, neuropathological, and genetic links, ALS and FTD are now recognized to be extremes in the phenotypic spectrum of a single disease: the ALS–FTD continuum [1].

Although most ALS patients have sporadic disease, in which no family history can be discernible (SALS), the population-based rate of familial ALS (FALS) is about 20% [3, 4]. In our ALS population, FALS was disclosed in 11.6% of patients [5].

Population-based frequency of the mutation rate in ALS European populations estimates that variants in four genes account for 51% of FALS and 6.2% of SALS, being the most common the *C9orf72* hexanucleotide repeat expansion (FALS 39.6%, SALS 4.6%), followed by *SOD1* (FALS 6.9%, SALS 0.8%), *TARDBP* (FALS 2.7%, SALS 0.6%), and *FUS* mutations (FALS 1.8%, SALS 0.2%) [6]. In FTD,

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the frequency of the *C9orf72* expansions in Western Europe was about 10%, with 18.5% in familial and 6.3% in sporadic FTD patients [7]. Moreover, the *C9orf72* HRE accounts for up to 88% of familial ALS–FTD patient series and is thus the major genetic cause of the FTD–ALS spectrum [8–11]. In addition to *C9orf72*, other genes in ALS/FTD spectrum include: *CCFN, CHCHD10, CHMP2B, OPTN, SQSTM1, TBK1, UBQLN2, and VCP* [12, 13].

A large population-based pedigree study confirms that up to 50% of variance in ALS has a genetic basis and estimate the overall mean lifetime heritability of ALS in *C9orf72*-negative patients to be 36.9% (95%CI, 19.8–53.9%) [14]. More than 30 genes have been described to confer a higher risk for ALS [15–17] and their contribution is complex. ALS has a monogenic component of rare high-penetrant variants, an oligogenic component of rare intermediate penetrant variants, and a multifactorial component of common risk variants, possibly all under the influence of gene–environment interactions [18]. A multistep process has been hypothesized as necessary for the onset of the disease [19, 20] and the number of steps required is reduced in patients with genetic variants [21].

We have previously found that, in the Portuguese population, *C9orf72* HRE accounts for 4.6% of sporadic ALS and 37.5% of FALS, while *SOD1* mutations are rare (0.83%). As expected, patients with *C9orf72* HRE have a higher prevalence of FTD however, but the concomitance of ALS and FTD cannot be explained by *C9orf72* HRE in 42% of FALS and 83% of sporadic cases. Also, two ALS patients with FTD onset were *C9orf72* HRE negative. Not only a positive family history for ALS or FTD, but also the presence of comorbid FTD increases the chance of having a genetic variant by 3.5 [22]. Thus, genetic screening is indicated in patients manifesting both diseases without the *C9orf72* HRE, regardless of family history of disease [23, 24].

Thus, whenever possible, our patients with familial ALS and/or FTD negative for *C9orf72* HRE were investigated for rare variants in a panel of ALS genes by next-generation sequencing (NGS). Our aim is to further understand the genetic basis of ALS in Portuguese patients.

Methodology

Patients

Patients were recruited by MdC in the Neuromuscular Unit, Department of Neurosciences, Hospital de Santa Maria-CHULN, the main ALS center in Portugal. ALS Patients met the revised El-Escorial criteria [25], including eligible patients with PMA (progressive muscular atrophy) and PLS (primary lateral sclerosis). Diagnosis was supported by neurophysiological evaluation [26]. All included patients had disease progression.

Clinical information was recorded following a structured assessment tool, incorporating neurological examination [27]. FALS was defined based on familial history, and was classified as definitive, probable, or possible according to the criteria of Byrne and co-workers [28].

The presence of comorbid FTD on clinical judgment (always by the same clinician, MdC) was supported by the International consensus criteria for possible behavioural FTD, i.e., presence of at least three of six clinically discriminating features (disinhibition, apathy/inertia, loss of sympathy/empathy, perseverative/compulsive behaviours, hyperorality, and dysexecutive neuropsychological profile) [29]. The presence of language dysfunction was clinically appreciated and selected cases were evaluated by ECAS (Edinburgh Cognitive and Behavioural ALS Screen) to confirm clinical diagnosis.

The functional rate of decay (ΔFS) was calculated as follows: (48-total ALSFRS-R at diagnosis)/duration in months from weakness onset to diagnosis. We considered the time since motor onset, because some patients had previous FTD symptoms and the Revised ALS Functional Rating Scale (ALSFRS-R) only evaluates motor functional impairment [30].

All patients that agreed to be tested and that signed inform consent were tested for *C9orf72* HRE in GenoMed-Diagnósticos de Medicina Molecular, S.A, as previously described [31]. We excluded those with marked signs of respiratory insufficiency, and who were psychologically stressed or had major depression. From a total of 282 patients tested, 265 (94%) were negative for *C9orf72* HRE (less than 30 GGG GCC hexanucleotide repeats in the first intron of *C9orf72* gene).

In the group of 265 *C9orf72-negative* patients, there were 19 unrelated FALS patients (7.2% of the total), of which six had concomitant FTD, and 23 SALS with concomitant FTD (8.7% of the total). From this group, enough DNA quantity and quality for further genetic testing with Next-Generation Sequencing were available from 32 patients (Fig. 1). Additionally, two family members with ALS of two FALS patients (a brother and a nephew) were also sequenced. All patients were Caucasians of European origin (Portugal), except for one with African origin.

Targeted gene enrichment and sequencing

Genomic DNA was isolated from whole blood samples using a standard in-house salting-out method. Whole-exome libraries were prepared using the SureSelect Human All Exon v6 kit (Agilent). Target regions were sequenced (paired-end) on an Illumina platform (NovaSeq 6000) with 150 base read length, with a medium read depth of at least

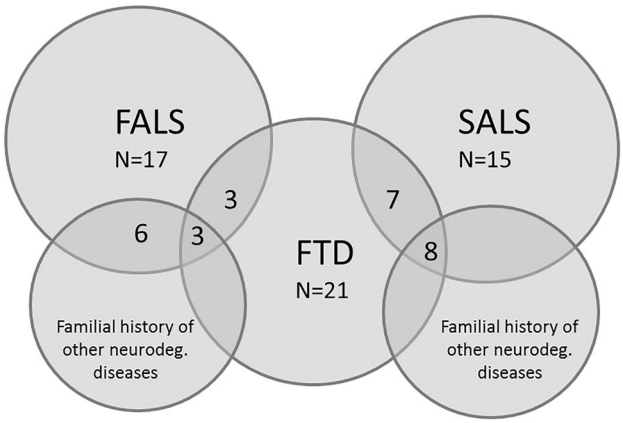

Fig. 1 Schematic diagram with the categorization of the studied patients; *FALS* familial amyotrophic lateral sclerosis, *SALS* sporadic amyotrophic lateral sclerosis, *FTD* frontotemporal dementia, other neurodegenerative disorders include Alzheimer disease, Parkinson disease, and FTD and unspecified dementia

60x. The exonic and splice-site DNA sequence regions of 29 ALS relevant genes were analysed in 34 patients, all negative for *C9orf72*: *ANG, ATXN2, CCNF, CHCHD10, CHMP2B, DCTN1, ERBB4, FIG4, FUS, hnRNPA1, hnRNPA2B1, KIF5A, MATR3, NEFH, OPTN, PFN1, PRPH, SIGMAR1, SOD1, SQSTM1, SPG11, TARDBP, TBK1, TREM2, TRPM7, TUBA4A, UBQLN2, VAPB*, and *VCP*.

In four patients, the following additional genes were studied: *ALS2, ANXA11, ATP13A2, BSCL2, CHCHD2, DAO, ELP3, ERLIN1, EWSR1, GLE1, GLT8D1, GNE, GRN, HSBP1, MAPT, NEK1, PLEKHG5, SETX, SLC52A2, SLC52A3, TAF15, TIA1, TRPV4, UNC13A*, and *VRK1*. No variants were detected in none of them.

The coverage of the gene panel was higher than 98% for variants with a read depth of > 20X. Thus, only those variants with insufficient quality or coverage are confirmed by Sanger sequencing. Genetic analysis was performed at GenoMed—Diagnósticos de Medicina Molecular, S.A.

Bioinformatic data analysis

Raw sequencing data in fastq format were assessed for quality with fastp software (version 0.19.8) and aligned with BWA (version 0.7.17) against the human reference genome GRCh37.

For variant calling, we used GATK-HaplotypeCaller [32] (version 4.1.1.0), FreeBayes [33] (version 1.1.0.46), and SAMtools mpileup [34] (version 1.9). While the first two apply a haplotype-based approach, the latter use an alignment-based approach. By taking advantage of both approaches, variants located in coding regions, including the splice site, called by at least two of the callers with a read depth of 20X or more were selected for further analysis. Variants with minor allele frequency (MAF) > 0.01 were filtered out. Reported variants are below the highest disease-specific maximum credible population allele frequency (AF) for which the observed allele count is compatible with pathogenicity [35]. AF in control populations and ALS cases was determined using variant frequencies from The Genome Aggregation Database (gnomAD, version 2.1.1) and from project MinE data browser (https://databrowser.projectmine.com/) which currently contains WGS data from 4366 ALS cases and 1832 controls [36]. Variants were also searched at the ALS Data Browser v3, ALSdb, New York City, New York (URL: https://alsdb.org) [date (03, 2020) accessed], a catalogue of genetic variants identified from 3317 Caucasian ALS patients [37]. The clinical impact of coding variants was annotated with the National Center for Biotechnology Information (NCBI) ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) and classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines [38]. Therefore, synonymous variants were reported if computational evidence suggested a possible impact on splicing [38]. To assess the potential functional consequences of detected variants, we used well-established computational methods based on evolutionary conservation, protein structure, and/or sequence homology: MutationTaster [39], PolyPhen-2 HVAR [40], and SIFT [41], that predict the effect of the change on protein function; the ensemble methods, Combined Annotation Dependent Depletion (CADD), Rare Exome Variant Ensemble Learner (REVEL) [42], MutationAssessor [43], Meta LR [44], as well as conservation scores as computed with the Genomic Evolutionary Rate Profiling (GERP) [45]. Thus, the Variant Effect Predictor tool (Ensembl.org Release 99) [46] and the tools available from the dbNSFP database v3.0 (MetaSVM, MetaLR, PROVEAN, SIFT, Polyphen2, MutationTaster, MutationAssessor) [47], and LoFtool [48] were used to predict the effect of each variant on the corresponding protein. The probabilities of a variant being splice altering were predicted with two ensemble learning methods, adaptive boosting (AdaBoost, ada score) and random forests (rf score) [49].

Results

Thirty-two unrelated patients, plus two family members, totalizing 34 patients (2 PLS, 7 PMA and 25 ALS) negative for the presence of *C9orf72* HRE and with a positive family history for ALS and/or with concomitant FTD were analysed with targeted NGS. Thus, considering only unrelated patients, 53% were classified as FALS (17/32). The studied population consisted of 14 males and 20 females with an average age of disease onset of 65.9 ± 13.4 years. Fourteen patients had spinal-onset, nine had bulbar-onset, one had respiratory-onset, two had a generalized-onset,

and 8 had FTD-onset. Besides, 13 other patients developed FTD during disease progression. They all presented the behavioural phenotype of FTD, but in a few language changes were associated at onset; in most of them, language dysfunction was clear over disease progression, as identified by abnormalities in writing (agrammatism and limited vocabulary). In the patients with milder cognitive changes at onset, ECAS confirmed abnormal results, and in all patients, cognition deteriorated over disease progression. Eighteen patients had predominant LMN signs, 15 had predominant UMN signs, and in one, there was no UMN vs. LMN predominance.

The targeted NGS data showed pathogenic variants, likely pathogenic variants and those of uncertain significance (VUS) in the following 11 genes: *CCNF* (Cyclin F), *CHMP2B* (Charged Multivesicular Body Protein 2B), *ERBB4* (Erb-B2 Receptor Tyrosine Kinase 4), *FUS* (FUS RNA Binding Protein), *NEFH* (Neurofilament Heavy), *PRPH* (Peripherin), *SPG11* (Vesicle Trafficking Associated, Spatacsin), *TARDBP* (TAR DNA Binding Protein), *TBK1* (TANK Binding Kinase 1), *TREM2* (Triggering Receptor Expressed On Myeloid Cells 2), and *TRPM7* (Transient Receptor Potential Cation Channel Subfamily M Member 7).

The 15 gene variants, including VUS, detected in 13 Caucasian unrelated patients are listed in Table 1. All the variants were found in a heterozygous state.

Based on the American College of Medical Genetics and Genomics (ACMG) variant classification, one of the 15 detected gene variants was categorized as pathogenic, two as likely pathogenic and the remaining 12 as VUS.

Three genetic alterations found in our study had already been previously reported in ALS: *TARDBP* (c.1144G > A, p.Ala382Thr), a genetic alteration classified as pathogenic, that we found in an FALS patient and confirmed its presence in her affected brother (pedigree 1106). This variant is presented in population databases and was found in project MinE patients' dataset but not in controls. This variant has been reported in association with both familial and sporadic ALS [50].

Also, the likely pathogenic variant in *PRPH* (c.421G > T, p.Asp141Tyr) and the VUS found in *FUS* (c.1292C > T, p.Pro431Leu) have already been described in ALS [51–53]. The *PRPH* gene variant is found in population databases and in both ALS patients and controls from project MinE, and has conflicting interpretations of pathogenicity at ClinVar database. The *FUS* gene variant is found in population databases but only in ALS patients from project MinE (absent in controls) and it is classified as uncertain significance at ClinVar database.

Additionally, a novel variant c.1154G > T, (p.Trp385Leu) classified as likely pathogenic was found in a mutational hotspot for ALS-linked *TARDBP* variants (exon 6). Another variant in the same amino acid (p.Trp385Gly) has already been described in ALS [54] and most bioinformatic prediction tools indicate pathogenicity.

Clinical characteristics of patients with pathogenic and likely pathogenic variants

The clinical characteristics of patients harbouring gene variants are presented in Table 2.

Pathogenic and likely pathogenic variants were all found in FALS patients. In both pedigrees (1106 and 1363) with *TARDBP* variants, genetic anticipation was observed (i.e., appeared at an earlier age in the succeeding generation), although disease progression was not more severe, reflected in a similar rate of decay and long survival in both cases (Table 2). These patients from the second generation were not tested with NGS. To note, their disease onset characteristics were similar in sex, age, site, and UMN vs. LMN predominance. While #1106 nephew developed FTD after long-disease duration, no cognitive alterations were noted in #1363 son, who survived long time dependent of non-invasive ventilation (NIV). Among members of family 1106, clinical heterogeneity was observed.

The FALS patient with the *PRPH* (p.Asp141Tyr) is a female with bulbar-onset disease and concomitant FTD and LMN predominance.

Variants uncertain significance (VUS)

Variants classified as VUS were found in the following genes: *NEFH, TRPM7, CHMP2B, ERBB4, TREM2, SPG11,* and *CCNF*. Those found in *TRPM7*, *CHMP2B, TBK1,* and one of *NEFH* (p.Lys741Gln) are novel variants; absent from population databases and from the current dataset of project MinE containing WGS data from 4366 ALS cases and 1832 controls. In silico predictions suggest pathogenicity for the *TBK1* (c.682C > T, p.Arg228Cys), but are inconclusive regarding the *CHMP2B* (c.287 T > C, p.Met96Thr), and suggest that the *NEFH* (c.2221A > C, p.Lys741Gln) is non-pathogenic. The intronic variant (c.322-2A > C) in *TRPM7* gene is predicted to be splice altering (ada score: 1; rf score: 0.936) and thus pathogenic. The rare variants found in *CCNF* (p.Asn87Lys), both of *ERBB4* (p.Phe414Leu) and (p.Ile910Val) and one of *TREM2* (p.Glu151Lys), are not reported in ClinVar database. In silico prediction tools do not suggest them to be pathogenic, except for the *CCNF*. In fact, the *CCNF* (p.Asn87Lys) is presented in patients from project MinE, but was not found in controls, though it is found in population databases with very low MAF. The *TREM2* (p.Glu151Lys) is reported in population databases as well as in project MinE database. Both *TREM2* variants (p.Glu151Lys) and (p.Arg47His) have been associated with the susceptibility to AD and most bioinformatic predictions suggest that they are likely benign. This variant is reported

Table 1 Summary of identified gene variants, their classification, and characterization

Patient ref	Gene	Exon	Nucleotide change	Amino acid change	dbSNP	ACMG classif	In silico pred	ClinVar ID: classif	MAF gnomAD_total	MAF gnomAD_NFE	AF_MiNE cases	AF_MiNE controls	CADD/GERP + + RS/MutationTaster/PolyPhen-2/SIFT/REVEL/Meta LR/MutationAssessor/Meta SVM/PROVEAN/LoF tool
1106	TARDBP	6	c.1144G > A	p.Ala382Thr	rs367543041	P		21,474: LP	3.04E–05	6.62E–05	2.29E–04	0	LB/3.87/D/B/T/LDC/D/L/D/N/_
1363	TARDBP	6	c.1154G > T	p.Trp385Leu		LP	P		0	0	0	0	LD/5.51/D/PossD/D/LDC/D/M/D/D/_
1228	PRPH	1	c.421G > T	p.Asp141Tyr	rs58599399	LP	P	13,707: CIP	3.29E–03	5.73E–03	5.96E–03	6.55E–03	LB/4.68/D/PossD/D/LDC/D/N/D/N/PossD
1316	FUS	12	c.1292C > T	p.Pro431Leu	rs186547381	VUS	P	37,069: U Sig	1.18E–04	1.50E–04	3.44E–04	0	LD/3.57/D/ProD/D/LDC/T/L/T/D/ProD
1093	SPG11	33	c.6250C > T	p.Leu2084Phe	rs754874642	VUS	P	406,515: U Sig	2.48E–05	1.41E–04	0	0	LB/4.74/D/ProD/D/LB/D/M/D/N/PossD
719	TRPM7	Intron 4	c.322-2A > G			VUS	P		0	0	0	0	LD/_/D/_/_/_/_/_/_/B
1353	TBK1	6	c.682C > T	p.Arg228Cys		VUS	P		0	0	0	0	LD/4.82/D/ProD/D/LB/D/M/D/D/PossD
1201	CCNF	3	c.261C > A	p.Asn87Lys	rs752507974	VUS	P	Not reported	6.45E–05	1.42E–04	2.29E–04	0	LB/2.1/D/PossD/D/LB/T/M/T/D/PossD
1170	CHMP2B	3	c.287T > C	p.Met96Thr		VUS	Inc		0	0	0	0	LB/5.02/D/B/T/LDC/T/N/T/N/PossD
1132	ERBB4	11	c.1240T > C	p.Phe414Leu	rs1428712079	VUS	Inc	Not reported	7.96E–06	0	0	0	LB/6.02/D/B/T/LB/T/N/T/N/ProD
1096	ERBB4	23	c.2728A > G	p.Ile910Val	rs754195521	VUS	Inc	Not reported	1.42E–05	7.77E–06	0	0	LB/6.05/D/B/T/LB/T/N/T/N/ProD
719	NEFH	2	c.1054C > A	p.Arg352Ser	rs149955255	VUS	Inc	66,725: CIP	2.31E–03	3.87E–03	6.55E–03	2.30E–03	LB/3.16/D/ProD/D/LDC/D/M/D/D/ProD

Table 1 (continued)

Patient ref	Gene	Exon	Nucleotide change	Amino acid change	dbSNP	ACMG classif	In silico pred	ClinVar ID: classif	MAF gnomAD_total	MAF gnomAD_NFE	AF_MiNE cases	AF_MiNE controls	CADD/GERP++RS/MutationTaster/PolyPhen-2/SIFT/REVEL/Meta LR/MutationAssessor/Meta SVM/PROVEAN/LoF tool
1316	NEFH	4	c.2221A>C	p.Lys741Gln		VUS	LB		0	0	0	0	LB/1.54/N/B/LB/T/M/T/N/ProD
1106	TREM2	2	c.140G>A	p.Arg47His	rs75932628	VUS	Inc	195,350: LB	2.48E–03	2.47E–03	0	0	LB/4.56/D/ProD/D/LB/T/M/T/D/B
1147	TREM2	3	c.451G>A	p.Glu151Lys	rs79011726	VUS	LB	Not reported	1.42E–04	1.78E–04	5.46E–04	2.91E–04	LB/2.77/N/B/T/LB/T/M/T/N/B

ACMG classif: American College of Medical Genetics classification, *gnomAD* Genome Aggregation Database, *MAF* Minor allele frequency, *NFE* Non-Finnish European, *AF* allele frequency, *P* pathogenic, *LP* likely pathogenic, *VUS* variant of unknown significance, *Inc* inconclusive, *CIP* conflicting interpretations of pathogenicity, *U Sig* uncertain significance, *LB* likely benign

CADD scores above 30 classified as likely deleterious (LD) and scores below as likely benign (LB). GERP++RS positive scores represent highly conserved positions, while negative scores represent highly variable positions. Scores range from − 12.3 to 6.17. Mutation Taster—D: disease causing; N: polymorphism. Polyphen-2 score > 0.908: Probably Damaging (ProbD); > 0.446 and ≤ to 0.908: Possibly Damaging (PossD); ≤ to 0.446: Benign (B). SIFT score < 0.05 are called deleterious (D) and all others are tolerated (T). REVEL score > 0.5 likely disease causing (LDC) and < 0.5 likely benign (LB). MetaLR score between 0 and 1, higher scores are more likely to be deleterious, and cut-off is 0.5.: Tolerated (T) or Damaging (D). Mutation Assessor rank score between 0 and 1 where variants with higher scores are more likely to be deleterious N: neutral; L: low; M: medium; and H: high. Meta SVM—score between 0 and 1, higher scores are more likely to be deleterious, cut-off is 0.5.: Tolerated (T) or Damaging (D). PROVEAN–D: damaging; N: neutral. LoF tool is a rank of genic intolerance, the lower the LoFtool gene score percentile, the most intolerant is the gene to functional variation, ProbD: Probably Damaging; PossD: Possibly Damaging; B-Benign

Table 2 Demographic and clinical characteristics of ALS patients with gene variants and of other affected family members with available information

Patient Ref	Variant	ACMG classif	In silico pred	Sex	Onset Age	Onset site	UMN vs LMN	Disease duration (months)	ΔFS @ diagnosis	FALS	FTD	Other familial Neurodeg. Disorders	Atypical Features
1106	*TARDBP* p.Ala382Thr	Pathog	Pathog	F	75	B	UMN	25.2 (d)	0.34	Yes (brother, nephew)	No	No	Familial phenotypic heterogeneity
	TREM2 p.Arg47His	VUS											
1106 brother	*TARDBP* p.Ala382Thr	Pathog	Pathog	M	66	LL	LMN	41.1 (d)	1.61		No	No	
1106 nephew	Not tested			M	36	UL	LMN	130.0 (a)	0.25		Yes	No	Very long survival
1363	*TARDBP* p.Trp385Leu	Likely Pathog	Pathog	M	70	UL	LMN (PMA)	22.0 (a)	0.25	Yes (son)	No	No	Onset after his son
1363 Son	Not tested			M	37	UL	UMN & LMN	76.9 (d)	0.38		No	No	Long survival on NIV (24 h/day)
1228	*PRPH* p.Asp141Tyr	Likely Pathog	Pathog	F	68	B	LMN	45.0 (a)	0.55	Yes (mother, aunt)	Yes	Dementia (mother, aunt)	
1093	*SPG11* p.Leu2084Phe	VUS	Pathog	F	71	UL	LMN	71.8 (d)	0.17	Yes (mother)	Yes	AD (sister)	
719	*TRPM7* c.322-2A > G	VUS	Pathog	F	72	LL	UMN	92.6 (a)	0.63	Yes (father)	No	AD (mother)	Very long survival
	NEFH p.Arg352Ser	VUS	Inconcl										
1170	*CHMP2B* p.Met96Thr	VUS	Inconcl	F	82	B	LMN	30.1 (d)	0.45	Yes (nephew)	No	No	Late-onset
1170 nephew	No variants found			M	39	LL	LMN	62.0 (a)	0.39		No	Probable AD (grandmother)	
1147	*TREM2* p.Glu151Lys	VUS	Likely benign	F	82	LL	LMN (PMA)	41.3 (d)	0.32	Yes (aunt)	No	PD (mother)	Familial phenotypic heterogeneity
1147 aunt	Not tested			F	54	B	UMN (PLS)	60.7 (d)	0.30		Yes	N.A	
1160	*TREM2* p.Glu151Lys	VUS	Likely benign	F	87	B	UMN	10.6 (d)	5.44	No	Yes	No	Late-onset; very short survival
1096	*ERBB4* p.Ile910Val	VUS	Inconcl	M	59	LL	UMN	61.1 (a)	0.23	No	Yes	No	

Table 2 (continued)

Patient Ref	Variant	ACMG classif	In silico pred	Sex	Onset Age	Onset site	UMN vs LMN	Disease duration (months)	ΔFS @ diagnosis	FALS	FTD	Other familial Neurodeg. Disorders	Atypical Features
1132	*ERBB4* p.Phe414Leu	VUS	Inconcl	M	71	FTD—UL	UMN	81.5 (d)	0.12	No	Yes, onset	No	FTD onset 4y before motor symptoms
1353	*TBK1* p.Arg228Cys	VUS	Pathog	F	61	FTD—LL	UMN	37.7 (a)	0.56	No	Yes, onset	PD (father)	FTD onset 1y before motor symptoms
1201	*CCNF* p.Asn87Lys	VUS	Pathog	M	57	FTD—B	UMN	33.7 (d)	0.22	No	Yes, onset	AD (mother)	FTD onset 8 m before motor symptoms
1316	*FUS* p.Pro431Leu	VUS	Pathog	F	75	FTD—B	UMN	51.7 (d)	0.53	No	Yes, onset	Dementia (mother, brothers)	Late-onset; FTD onset 17 m before motor symptoms
	NEFH p.Lys741Gln	VUS	Likely benign										

ACMG classif American College of Medical Genetics classification, *VUS* variant of unknown significance, *Pathog* pathogenic, *Inconcl* inconclusive, *F* female, *M* male, *B* bulbar, *UL* upper limb, *LL* lower limb, *FTD* frontotemporal dementia, *UMN* upper motor neuron, *LMN* lower motor neuron, *PMA* progressive muscular atrophy, *PLS* primary lateral sclerosis, *(a)* alive, *(d)* dead, *ΔFS* (48-total ALSFRS-R at diagnosis)/duration in months from motor disease onset to diagnosis, *FALS* familial amyotrophic lateral sclerosis, *AD* Alzheimer disease, *PD* Parkinson disease, *N.A.* not available, *NIV* non-invasive ventilation

in population databases and in ALSdb with similar frequencies, but is not found in MinE database.

The *NEFH* (p.Arg352Ser) is presented in both population databases as well as in MinE dataset and has conflicting interpretations of pathogenicity.

The *SPG11* (p.Leu2084Phe) is present in population databases, but was not found in MinE dataset, either in patients or controls, or in ALSdb. This variant is reported in ClinVar database associated with spastic paraplegia 11, autosomal recessive and classified with uncertain significance. A potential negative impact is suggested by bioinformatics tools; however, the available evidence is inconclusive. Moreover, the conditions related to *SPG11* variants are inherited in an autosomal recessive pattern and we found it in heterozygosity.

The co-occurrence of multiple variants may be underestimated in our study, since those patients who were positive for *C9orf72* HRE were not further analysed with the NGS gene panel. Nevertheless, three patients harboured two rare variants in different genes (8.8%, 3/34): Patient #1106, besides the pathogenic *TARDBP* (p.Ala382Thr) variant, also harboured the *TREM2* (p.Arg47His) variant. This later VUS was not found in her affected brother. Unlike the other family members, patient #1106 was the only with bulbar-onset and UMN predominance, the oldest at disease onset, and the one with the shortest survival (Table 2).

Patient #719 has a novel splice acceptor variant in gene *TRPM7* and another VUS in gene *NEFH* (p.Arg352Ser). This FALS patient has a very long survival and is still alive.

Patient #1316, who had a VUS in *FUS* (p.Pro431Leu) and another in *NEFH* (p.Lys741Gln), had late FTD-onset disease that after 17 months progressed to the bulbar region.

In the majority of the analysed patients, no potential pathogenic variants were found (59.4%, 19/32). Gene variants, including VUS, were found in 7 of the 17 FALS patients (41.2%) and in 6 of the 15 SALS with concomitant FTD (40%). Among those with FTD-onset, VUS were detected in 50% (4/8).

Discussion

In our study, we found 15 gene variants in 13 unrelated ALS patients negative for *C9orf72* HRE, and those variants were found in 11 genes: *TARDBP*, *PRPH*, *SPG11, TRPM7,* and *CHMP2B* in FALS patients; *ERBB4, FUS, TBK1,* and *CCNF* in SALS/FTD patients, and *TREM2* and *NEFH* in both SALS/FTD and FALS patients. Nevertheless, in 58.8% of FALS and in 60% of SALS/FTD, no variants were identified in any of the analysed genes. This highlights that efforts, like those of project MinE [55], are still needed to identify additional genes influencing ALS/FTD risk.

Besides the major ALS genes (*C9orf72*, *SOD1*, *TARDPB*, and *FUS*), recent extensive genetic screenings of ALS patients in European populations have identified variants in more rarely mutated genes in only about 4–5% of cases [56–59]. Moreover, those results reflect the ALS genetic diversity, even among European populations, since those variants are found in different genes and not even the major ALS genes are found or predominant in all cohorts, except the *C9orf72* HRE, confirming it as still the main genetic cause of ALS/FTD.

We found variants in *FUS* and *TARDBP*, but not in *SOD1*, a gene that is rarely found mutated in Portuguese ALS patients [5].

Variants classified as pathogenic or likely pathogenic were only found in FALS patients in genes *TARDBP* and *PRPH*.

Both *TARDBP* variants were found in the mutational hotspot in exon 6 [50]. The p.Ala382Thr variant was initially described in two FALS patients of French origin [60]. Subsequently, large studies carried out on patients of Italian and French origin have identified this variant in FALS and SALS patients with or without FTD (reviewed in [50]). The incomplete penetrance of the disease in carriers at 70 years, calculated to be 74% in males and 42.5% in females [61], justifies why a 73-year-old female from family 1106 (sister and mother) is asymptomatic to date. We also found a new *TARDBP* p.Trp385Leu variant that is absent from population and ALS datasets; however, another variant in the same amino acid, c.1153T > G, p.Trp385Gly, was described in two affected brothers, one of which with very long-disease duration [54]. We found it in a PMA patient with disease onset long after his son who survived over 6 years, although for more than 2 years fully dependent on Non-Invasive Ventilation (NIV).

Disease anticipation was observed in both families and the patients from second generation (#1106 nephew and #1363 son) had similar disease onset characteristics. Despite that, considerable intrafamilial phenotypic differences in age, site of onset, and survival were observed in the families carrying the *TARDBP* variants, as previously reported [54], meaning that ALS manifestation is influenced by other genetic and environmental factors [62].

PRPH p.Asp141Tyr was the first described variant related to ALS in this gene and was found in a homozygous sporadic patient with limb onset at 42 years old who died nearly 3 years after without cognitive changes [52]. It was also found in heterozygosity in 4/342 patients and 2/550 controls [51, 63]. This variant does not abolish the ability of peripherin to assemble into filaments, but leads to the formation of aggregates, even in heterozygous state [52]. Although this functional evidence upholds that this could be a low penetrance variant involved in ALS susceptibility, the excess of its frequency in MinE controls and similar frequencies in

ALS patients and in population databases argues against it, but there is also the possibility of having pre-symptomatic individuals in control populations. We found this variant in an FALS patient with bulbar-onset disease at 68-years old and concomitant FTD that is still alive with almost 4 years of disease duration, different from the phenotype previously described [52] not confirming a genotype–phenotype relation for this variant.

In FALS patients, other variants of unknown significance were found in genes *SPG11*, *TRPM7*, *CHMP2B, TREM2,* and *NEFH*.

The patient with the *SPG11* p.Leu2084Phe missense variant also had concomitant FTD, a familial history of AD and upper limb onset with predominant LMN signs. Although *SPG11* variants, mainly truncating mutations, have previously been associated with autosomal recessive juvenile ALS [64], several heterozygous missense variants in *SPG11* have been reported in ALS, but attributed to the large size of the gene coding region and interpreted as unlikely deleterious [57, 59, 65–67].

The FALS patients with variants in *CHMP2B, TRPM7*, *NEFH,* and *TREM2* had no signs of FTD. However, variants in *NEFH* and *TREM2* were also found in SALS/FTD patients.

The novel variant in *CHMP2B* p.Met96Thr was found in an FALS patient with late bulbar-onset disease without FTD. A variant in the C-terminus of *CHMP2B* was originally reported in a Danish pedigree with autosomal dominant FTD [68]. Other variants were later found in ALS and PMA in English and Dutch populations, [69, 70] and in one Indian SALS patient [71]. We did not find segregation of the variant with the disease in the other ALS patient of the family, what provides evidence against its pathogenicity.

The *TRPM7* (c.322-2a > G) slice acceptor intronic variant is predicted to be pathogenic and is not reported in population or ALS databases. A missense variant in *TRPM7* has been described to confer susceptibility to Guamanian ALS and Parkinsonian dementia [72]. The discrepancy in TRPM7 channel function and expression leads to various neuronal diseases such as AD and PD. Furthermore, it is a key factor in anoxic neuronal death and *TRPM7* mutations may play a crucial role in neurotransmitter release in ALS [73]. We found the *TRPM7* variant along with a VUS in *NEFH* p.Arg352Ser, in female patient with very long-disease survival (92.6 months, still alive) and whose father had ALS and mother had AD.

Neither *NEFH* variants were described in ALS; in fact, the p.Lys741Gln is a new variant not reported in population databases, but predicted to be likely benign by bioinformatic tools. Previously described clinical features observed in individuals with the *NEFH* (c.1054C > A, p.Arg352Ser) variant include: hypertonia, abnormality of coordination, morphological abnormality of the central nervous system, and neurophysiological changes (NCBI. ClinVar; [VCV000066725.3], https://www.ncbi.nlm.nih.gov/clinvar/variation/VCV000066725.3 (accessed Feb. 20, 2020).

Further studies are needed to conclude about the pathogenicity of the detected VUS. Both were detected in patients who also had another variant. The *NEFH* p.Arg352Ser was detected along with the *TRPM7* c.322-2A > G in an FALS patient with very long survival. The *NEFH* p.Lys741Gln was found in a patient with FTD-onset that also harboured a variant in *FUS* p.Pro431Gln.

TREM2 gene variants were reported to increase the risk of AD and other neurodegenerative diseases [74]. Recent meta-analysis studies have confirmed *TREM2* p.Arg47His to be significantly associated with AD and PD risk in North Americans, but not PD in Europeans or ALS [74] and to be gene a risk factor for FTD in Caucasian populations [75]. The *TREM2* p.Arg47His variant has also been associated with a neuroinflammatory processes, especially microglial activation [76, 77].

The *TREM2* p.Glu151Lys was described before as risk factor for AD [78] and was found in a demented patient with LMN signs and Parkinsonism who was positive for the *C9orf72* HRE [79]. We detected the *TREM2* p.Glu151Lys variant in patients with late-onset disease (above the age of 80), one of which was a PMA patient with positive familial history, and the other was an SALS/FTD patient with UMN predominance and very short survival. The FALS patient with the *TREM2* p.Arg47His variant also harboured the pathogenic *TARDBP* p.Ala382Thr, but in her brother, the *TREM2* variant was not detected. The diverse neurodegenerative phenotypes associated with *TREM2* variants indicate a role in neurodegeneration, although its risk effect in ALS remains elusive.

In the sporadic patients with FTD-onset, we found variants in genes *FUS*, *TBK1*, *CCNF* and *ERBB4*.

Since 2009, when the first *FUS* mutations were associated with ALS [80, 81], more than 50 variants have been identified in this gene [50, 82] with a frequency in European populations of about 2.8% in FALS and 0.3% in SALS [6]. Most pathogenic *FUS* variants are located in the C-terminal within, or completely deleting, the nuclear localization signal, thereby impairing nuclear import of FUS [83]. We found a variant at the zinc-finger motif, a rarely mutated site that has a predominant role in RNA recognition [84, 85]. This variant was first described in a patient with hereditary essential tremor-4 (ETM4; 614,782) whose lymphoblastoid cells showed lower expression of mutant FUS mRNA than did cells from patients with ALS due to *FUS* variants [86]. It was later found in an SALS Caucasian male patient with spinal-onset at the age of 60 [53], a quite different phenotype from our patient: a 75-year-old female with FTD-onset that evolved to the bulbar region after 17 months. It is known that certain *FUS* genetic variants do result in very

different phenotypes [83, 87] emphasizing the influence of other factors in disease manifestation which, in this case, could be related with the presence of the *NEFH* variant p.(Lys741Gln), even if it is predicted to be likely benign. Also, the lack of significant numbers of non-nuclear localization signal variants preclude the description of their phenotypes properly [83]. *FUS* mutation carriers usually manifest earlier symptom onset, a higher rate of bulbar-onset, and shorter disease duration. Nonetheless, FTD has rarely been reported among ALS patients with *FUS* variants [87–89].

Whole-exome sequencing and a gene-based rare variant analysis identified *TBK1* as an ALS gene [37, 90]. In ALS–FTD patients, most reported pathogenic *TBK1* variants are loss-of-function (LoF) mutations [90–92] and functional *TBK1* missense variants have been proposed to be considered high risk rather than causal variants [92]. However, in vitro tests have demonstrated that at least some missense variants have functional deficits and, therefore, may be disease-causing [90, 93]. Furthermore, a recent meta-analysis has shown that LoF mutations are less frequent than missense variants in ALS–FTD and both are associated with an increased risk for ALS–FTD spectrum. However, while *TBK1* LoF mutations were associated with a significant increased risk, missense variants of *TBK1* were only related to a moderately increased susceptibility [94]. We found a missense variant predicted to be pathogenic by in silico tools in a patient with FTD-onset that progressed to lower limbs 1 year after and whose father had PD.

CCNF was found as ALS causative gene in a genome-wide linkage analysis in large ALS–FTD kindred [95]. The analysis of a replication cohort suggested its role in both FALS and SALS pathogenesis through abnormal ubiquitination and accumulation of ubiquitinated proteins, including TDP-43 [95]. We found a rare variant, predicted to be pathogenic, in an SALS patient with FTD-onset disease that progressed to the bulbar region after 8 months and whose mother had AD/dementia.

Rare genetic variants in the *ERBB4*, a member of the epidermal growth factor subfamily of receptor tyrosine kinases, were detected in different pedigrees of FALS patients by whole-genome sequencing and parametric linkage analysis [96]. Clinical presentations of those individuals were characterized by the involvement of both UMN and LMN, a lack of obvious cognitive dysfunction, and relatively slow progression. *ERBB4* variants were later found in patients with ALS with concomitant FTD [23, 97] some evidencing incomplete penetrance [97]. Both our patients with *ERBB4* variants had UMN predominance and concomitant FTD; one of them presented with FTD 4 years before upper limb symptoms. No familial history of ALS or any other neurodegenerative disease was uncovered.

Although both found variants are rare and in silico predictions are inconclusive, p.Ile910Val is located within the tyrosine kinase domain and p.Phe414Leu within a receptor domain, suggesting that these variants might impact receptor ligand interaction and kinase activity, key functions of ErbB4.

In summary, regarding the VUS found in *FUS, SPG11, TRPM7, TBK1, CCNF, ERBB4, NEFH,* and *TREM2,* no definite conclusion regarding its pathogenicity can be drawn and further functional, genetic, and clinical studies in large independent cohorts, which are required to fully understand and establish their contribution for ALS.

In agreement with several studies that have shown an oligogenic basis of ALS [12, 53, 57, 98–100] as the number of patients with multiple ALS-associated variants is higher than what can be expected by chance, based on the individual mutation frequencies of the respective genes, we also found three patients carrying two potential disease-causing variants. Thus, certain variants alone may not cause disease and the simultaneous analysis of disease genes is highly important [59].

For those variants that had already been described in ALS, we observed some consistency of phenotypes with those previously reported, supporting possible genotype–phenotype relation, but mainly, we found distinctive characteristics highlighting the influence of other genetic and non-genetic factors in ALS manifestation.

A recent population-based parent–offspring heritability study showed that inherited and non-inherited factors contribute approximately equally toward ALS, and that even in a population devoid of known gene mutations, genetic factors account for almost 40% of the risk [14].

All the above emphasize the need to continue and enhance the efforts to unravel the genetic architecture of ALS-FTD mindful that only a better knowledge of the disease will give us the basis to fight it.

Author contributions Conceptualization, MG and MdC; methodology, ACPL and JT; software, MG and JT; validation, DA; formal analysis, MG, AMC, and RR; data curation, MG, AMC, RR, and DA; writing—original draft preparation, MG; writing—review and editing, MG and MdC; supervision, MdC.

Funding This project was supported by FTC—Fundação para a Ciência e Tecnologia—through project "Comprehensive evaluation of circulating MicroRNA as diagnostic and prognostic biomarkers in Amyotrophic Lateral Sclerosis"—PTDC/MEC-NEU/31195/2017.

Availability of data and material Further data are available upon request.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval This project was approved by the Local Ethics Committee (ID number 215/15). The study conformed to the standards

defined in the latest revision of the Declaration of Helsinki. All patients and controls signed a written informed consent. Databases were properly treated for privacy.

Consent to participate All listed authors have approved the manuscript before submission, including the names and order of authors.

Consent for publication All listed authors have approved the version to be published.

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