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Genetic architecture of ALS in Sardinia

Giuseppe Borghero^{a,1}, Maura Pugliatti^{b,1}, Francesco Marrosu^a, Maria Giovanna Marrosu^c, Maria Rita Murru^c, Gianluca Floris^a, Antonino Cannas^a, Leslie D Parish^b, Patrizia Occhineri^b, Tea B. Cau^d, Daniela Loi^d, Anna Ticca^e, Sebastiano Traccis^f, Umberto Manera^g, Antonio Canosa^{g,h}, Cristina Moglia^g, Andrea Calvo^{g,i}, Marco Barberis^{g,i}, Maura Brunetti^{g,i}, Hannah A Pliner^j, Alan E Renton^j, Mike A Nalls^k, Bryan J Traynor, MD^{j,l}, Gabriella Restagno^j, Adriano Chiò^{g,h,m,*}, and for the ITALSGEN and SARDINALS consortia

^aDepartment of Neurology, Azienda Universitario Ospedaliera di Cagliari and University of Cagliari, Cagliari, Italy

^bDepartment of Clinical and Experimental Medicine, University of Sassari, Italy (Pugliatti, Parish, Occhineri)

^cMultiple Sclerosis Center Laboratory, University of Cagliari, Cagliari, Italy (Maria G Marrosu, Murru)

^dAzienda Sanitaria Locale n. 2, Olbia-Tempio, Italy

^eDepartment of Neurology, Azienda Ospedaliera San Francesco, Nuoro, Italy

^fDepartment of Neurology, Ospedale Antonio Segni, Ozieri, Italy

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*Corresponding author: Prof. Adriano Chiò, 'Rita Levi Montalcini' Department of Neuroscience, Via Cherasco 15, I-10126 Torino, Italy, achio@usa.net.

¹These authors contributed equally to this work.

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²See Appendix or the other members of ITALSGEN and SARDINALS

Authors contributions: *Study concept and design:* Borghero, Pugliatti, Traynor, Restagno, Chiò. *Acquisition of data:* Murru, Floris, Cannas, Parish, Occhineri, Cau, Loi, Ticca, Manera, Canosa, Miglia, Calvo, Barberis, Brunetti, Pliner, Renton, Nalls. *Analysis and interpretation of data:* Borghero, Pugliatti, F. Marrosu, M.G: Marrosu, Murru, Renton, Nalls, Traynor, Restagno, Chiò. *Drafting of the manuscript:* Borghero, Pugliatti, Traynor, Chiò. *Critical revision of the manuscript for important intellectual content:* Borghero, Pugliatti, F. Marrosu, M.G Marrosu, Murru, Floris, Cannas, Parish, Occhineri, Cau, Loi, Ticca, Manera, Canosa, Moglia, Calvo, Barberis, Brunetti, Pliner, Renton, Nalls, Traynor, Restagno, Chiò. *Obtained funding:* Borghero, Pugliatti, Restagno, Chiò. *Administrative, technical, and material support:* Murru, Floris, Cannas, Parish, Occhineri, Cau, Loi, Ticca, Manera, Canosa, Moglia, Calvo, Barberis, Brunetti, Pliner, Renton, Nalls. *Study supervision:* Borghero, Pugliatti, Traynor, Restagno, Chiò. Adriano Chiò has full access to data. The corresponding author confirm that all authors have read and approved the final draft of the manuscript and given written permission to include their names in the manuscript.

Conflict of interest: Adriano Chiò serves on a scientific advisory board for Biogen Idec, Cytokinetics, and Italfarmaco. Borghero, Pugliatti, F. Marrosu, M.G Marrosu, Murru, Floris, Cannas, Parish, Occhineri, Cau, Loi, Ticca, Manera, Canosa, Moglia, Calvo, Barberis, Brunetti, Pliner, Renton, Nalls, Traynor, Restagno report no conflicts of interest. The sponsor organizations had no role in data collections and analysis and did not participate to writing and approving the manuscript. The information reported in the manuscript has never been reported elsewhere.

The data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

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^gALS Center, 'Rita Levi Montalcini' Department of Neuroscience, University of Turin, Turin, Italy

^hDepartment of Neurosciences, Ophthalmology, Genetics, Rehabilitation and Child Health, University of Genoa, Italy

^hAzienda Ospedaliero Universitaria Città della Salute e della Scienza, Turin, Italy

ⁱLaboratory of Molecular Genetics, Azienda Ospedaliero Universitaria Città della Salute e della Scienza, Turin, Italy Torino, Italy

^jNeuromuscular Diseases Research Section, Laboratory of Neurogenetics, National Institute of Aging, Bethesda, USA

^kMolecular Genetics Section, Laboratory of Neurogenetics, National Institute of Aging, Bethesda, USA

^lBrain Science Institute, Department of Neurology, Johns Hopkins University, Baltimore, Maryland, USA

^mNeuroscience Institute of Torino (NIT), Turin, Italy

Abstract

Conserved populations, such as Sardinians, displaying elevated rates of familial or sporadic ALS provide unique information on the genetics of the disease. Our aim was to describe the genetic profile of a consecutive series of ALS patients of Sardinian ancestry. All ALS patients of Sardinian ancestry, identified between 2008 and 2013 through the ITALSGEN consortium, were eligible to be included in the study. Patients and controls underwent the analysis of *TARDBP*, *C9ORF72*, *SOD1*, and *FUS* genes. Genetic mutations were identified in 155 out of 375 Sardinian ALS cases (41.3%), more commonly the p.A382T and p.G295S mutations of *TARDBP*, and the GGGGCC hexanucleotide repeat expansion of *C9ORF72*. One patient had both p.G295S and p.A382T mutation of *TARDBP* and eight carried both the heterozygous p.A382T mutation of *TARDBP* and a repeat expansion of *C9ORF72*. Patients carrying the p.A382T and the p.G295S mutations of *TARDBP* and the *C9ORF72* repeat expansion shared distinct haplotypes across these loci. Patients with co-occurrence of *C9ORF72* and *TARDBP* p.A382T missense mutation had a significantly lower age at onset and shorter survival. More than 40% of all cases on the island of Sardinia carry a mutation of an ALS-related gene, representing the highest percentage of ALS cases genetically explained outside of Scandinavia. Clinical phenotypes associated with different genetic mutations show some distinctive characteristics, but the heterogeneity between and among families carrying the same mutations implies that ALS manifestation is influenced by other genetic and non-genetic factors.

Keywords

Amyotrophic Lateral Sclerosis; Sardinia; phenotype; genetics; penetrance; prognosis

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of the adult life, characterized by a progressive deterioration of motor function, causing death due to

respiratory failure within 2 to 4 years after onset. In Caucasian populations, 90% of patients appear sporadically (sporadic ALS, SALS), while ~10% of patients have a family history positive for ALS or frontotemporal dementia (FTD) (familial ALS, FALS). The commonest genes implied in ALS so far are *C9ORF72*, *SOD1*, *TARDBP* and *FUS* (Renton et al, 2014), with marked differences between ethnic groups and geographical regions. Examples of this diversity include the virtual absence of *SOD1* mutations in Ireland and the Netherlands (van Blitterswijk et al, 2012; Kenna et al, 2013), the extremely high frequency of *C9ORF72* repeat expansions in Scandinavia (Majounie et al, 2012; Smith et al, 2012), and the high frequency of *OPTN* mutations combined with the relative scarcity of *C9ORF72* repeat expansions observed in Japan (Maruyama et al, 2010; Konno et al, 2013).

Sardinia, the second largest Mediterranean island, represents a genetic isolate, characterized by a high frequency of autoimmune disorders (such as multiple sclerosis and diabetes mellitus type 1) and monogenic diseases (such as Wilson's disease). As might be expected, this population displays decreased genetic and allelic heterogeneity. We and others have reported that ALS patients of Sardinian ancestry have a higher frequency than expected of the *TARDBP* p.A382T missense mutation and of the rate of familial ALS (Chiò et al, 2011; Orry et al, 2011). Despite this, the incidence of ALS in Sardinians retrospectively investigated seems to be within the range of European studies (Pugliatti et al, 2013).

In this report, we describe the genetic profile of a larger series of ALS patients of Sardinian ancestry and extend our analysis to include other ALS genes (*C9ORF72*, *SOD1*, *TARDBP* and *FUS*).

2. Methods

Patients

All ALS patients of Sardinian ancestry (i.e., defined as subjects with both parents of Sardinian origin) were eligible to be included in the study. Patients were identified between 2008 and 2013 through the ITALSGEN consortium, which includes sixteen ALS centers in Italy (Chiò et al, 2012a). Clinical information, including cognitive status, were collected on all patients. ALS patients met the EL Escorial revised criteria for definite, probable, probable laboratory-supported, or possible ALS (Brooks et al, 2000). The genetics of *TARDBP* and *SOD1* for 135 cases has been published elsewhere (Chiò et al, 2011).

Controls

DNA samples obtained from 700 subjects of Sardinian ancestry not affected by neurodegenerative disorders were screened for *TARDBP* mutations. This cohort included 604 control samples that were previously reported (Cannas et al, 2013). DNA samples obtained from 262 subjects of Sardinian ancestry not affected by neurodegenerative disorders were screened for *SOD1*, *FUS* and *C9ORF72* mutations. This cohort included 166 control samples that were previously reported (Chiò et al, 2012a). Control subjects were collected at the Department of Neurology, University of Cagliari, and were spouses or non-blood relatives of patients diagnosed with ALS or multiple sclerosis.

Classification of familial ALS

Patients were classified according to the current revised classification of familial ALS (Byrne et al, 2011; Chiò et al, 2014).

Mutational screening

The following exons and 50 base pair (bp) flanking intron-exon boundaries were screened for mutations by PCR amplification, sequencing using the Big-Dye Terminator v3.1 kit (Applied Biosystems Inc.), and analysis on an ABIPrism 3130 genetic analyzer: (a) all five coding exons of *SOD1*, (b) exon 6 of *TARDBP*, and (c) exons 14 and 15 of *FUS*. These exons were selected as the vast majority of known pathogenic variants lie within these mutational hotspots. A repeat-primed PCR assay was used to screen for the presence of the GGGGCC hexanucleotide expansion in the first intron of *C9ORF72* (Renton et al, 2011; DeJesus-Hernandez et al, 2011). A cut-off of 30 repeats combined with a typical sawtooth pattern was considered pathological.

Haplotype analysis

For haplotype analysis, we analyzed genome-wide single-nucleotide polymorphism (SNP) data from patients carrying the same mutation. A custom PERL software script was used to compare unphased sample genotype data.

Statistical analysis

Differences between groups were analyzed using t-tests for continuous variables (such as age at symptom onset) and χ -square for discrete variable (such as gender, site of onset, presence of frontotemporal dementia [FTD]). Comparison between series of means was performed with ANOVA. Survival was calculated using Kaplan-Meier curves and the log-rank test was used to compare survival across groups. The last day of follow-up was March 31, 2014, and none of the patients were lost to follow-up. Significance was set at $p < 0.05$, two-tail test. Statistical Package for the Social Sciences (SPSS) version 21 was used (SPSS Inc, IBM, Somers, New York, USA).

Standard Protocol Approvals, Registrations, and Patient Consents

The study design was approved by the Ethical Committees of all the involved centers. Patients and controls signed written informed consent. The study was conducted in line with the Italian ethical rules for data collection for statistical or scientific purposes and for data protection.

3. Results

We identified a cohort of 375 ALS cases with Sardinian ancestry, 236 men and 139 women with a mean (SD) age at symptom onset of 61.1 (12.1) years. Ninety-nine patients (26.4%) had bulbar-onset disease, whereas the remaining 276 (73.6%) cases reported spinal onset of symptoms. A positive family history for ALS, FTD or both was reported in 100 cases (26.7%).

Genetics of Sardinian ALS cases

We identified mutations in one or more genes in 155 cases (41.3%) of our Sardinian ALS cohort (Table 1). Of these, 75 patients (20.0%) were heterozygous for the p.A382T mutation of *TARDBP*, three patients (0.8%) were homozygous for the p.A382T mutation, ten patients (2.7%) were heterozygous for the p.G295S mutation of *TARDBP*, one patient (0.3%) was homozygous for the p.G295S mutation, and one patient (0.3%) carried both p.G295S and p.A382T mutations of *TARDBP*. Fifty-one patients (13.6%) carried a GGGGCC hexanucleotide repeat expansion of the *C9ORF72* gene. Eight patients (2.1%) carried both the heterozygous p.A382T mutation of *TARDBP* gene and a pathogenic repeat expansion of *C9ORF72* (two of these patients have been described elsewhere) (Chiò et al, 2012b). Four patients (1.1%) had a missense mutation of the *SOD1* gene (three FALS patients with p.A95G and one FALS patient with p.A4T). Two patients carried a p.T622A missense mutation of the *MATR3* gene (reported by us in the original paper as pedigree ITALS#10) (Johnson et al, 2014). No *FUS* mutations were found.

Genetic mutations in Sardinian controls

A *TARDBP* p.A382T heterozygous missense mutation was detected in eight of the 700 control samples (1.1% of control cohort, age range 60 to 86 years). Since 86 out of the 366 ALS cases (23.5%) in this series carried the p.A382T mutation, the relative risk of developing ALS in a subject carrying this mutation was 66.2 (95% confidence interval (c.i.): 32.9–141.9). Based on our data, the penetrance of this mutation at 70 years was found to be 66.1% (95% c.i. 48.3–83.9) among women and 80.4% (95% c.i., 70.0–90.8) among men. The p.G295S mutation of *TARDBP* and the pathological *C9ORF72* repeat expansions were not found in the Sardinian control population.

Haplotype analysis

Genotype data showed that ALS patients carrying the p.A382T mutation of *TARDBP*, the p.G295S mutation of *TARDBP* and the *C9ORF72* repeat expansion shared distinct haplotypes across these loci (Table 2).

Geographic distribution of ALS cases carrying mutations

We observed a different distribution of the two most common ALS-related mutations across the island ($p=0.0001$, Figure 1 and Supplementary Table 1). The *TARDBP* p.A382T missense mutation was most frequent in the two central-western provinces, where it accounts for ~30% of cases. The *C9ORF72* mutation is most frequent in northern and eastern Sardinia, where it accounts for ~20% of cases. Geographical clustering was not observed for the p.G295S *TARDBP* missense mutation.

Clinical characteristics of ALS cases (Table 3)

Subjects carrying the *C9ORF72* repeat expansion had a higher frequency of bulbar-onset disease. Age at symptom onset also differed between genetic subgroups; in particular, patients with co-occurrence of *C9ORF72* and the *TARDBP* p.A382T missense mutation and patients with *SOD1* mutations had a significantly lower age at onset (Figure 2). FTD was identified in 51 patients (13.6%) and was more frequent in patients with *C9ORF72*

mutations, while it was rarer in patients with no detected mutations and in those with *SOD1* mutations.

Ten patients (2.7%) showed extrapyramidal signs manifesting as muscle rigidity and resting tremor. Of these, two patients carried *C9ORF72* mutation, three carried a *TARDBP* p.A382T heterozygous missense mutation, one patient had a *TARDBP* p.A382T homozygous missense mutation (Borghero et al, 2011), and four did not carry a known mutations. Eight of these patients (80.0%) had comorbid FTD ($p=0.0001$).

The overall median survival of Sardinian ALS patients was 4.2 years (interquartile range [IQR] 2.3–10.0). Survival diverged significantly between ALS patients carrying different mutations ($p=0.0001$, Figure 3). Patients with *C9ORF72* had a median survival of 2.7 years (IQR 1.9–3.8), patients carrying both *C9ORF72* and *TARDBP* p.A382T mutation had median survival of 3.1 years (IQR 1.8–3.9), and patients with a *TARDBP* p.A382T heterozygous mutation had the longest survival (median 6.5 years, IQR 3.3–10.5).

4. Discussion

In our study of a large cohort of Sardinian ALS patients, a causative genetic mutation was identified in more than 40% of cases. The most common mutations were the p.A382T missense mutation in *TARDBP*, the pathogenic repeat expansion of *C9ORF72*, and the p.G295S missense mutation of *TARDBP*. Moreover, several patients carried a double mutation, more commonly the combination of *C9ORF72* and *TARDBP* p.A382T missense mutation, and a smaller number were homozygous for *TARDBP* missense mutations. Overall, detected mutations accounted for 75% of FALS and 30% of apparently SALS patients. This represents one of the largest proportion of ALS that is genetically explained in a population (Majounie et al, 2012). This likely reflects a founder effect operating within the Sardinian population and highlights the value of genetic studies of island isolates.

To confirm the founder nature operating on Sardinians, we demonstrated that ALS patients carrying the *TARDBP* p.A382T missense mutation and the p.G295S missense mutation share common haplotypes across the gene loci and showed that patients with the *C9ORF72* repeat expansion carry the Finnish haplotype (Mok et al, 2012).

We observed distinct clinical and demographic phenotypic among patients carrying different mutations. First, patients carrying a double *C9ORF72/TARDBP* mutation have a significantly younger age at onset, i.e., 20 years lower than other Sardinian cases. Second, patients with *C9ORF72* and p.G295S *TARDBP* mutations have more frequently a bulbar-onset disease. Third, a positive family history for ALS or FTD was more frequently found in patients with *C9ORF72* mutation (~65%). Fourth, patient with *C9ORF72* mutation had the shortest survival, whereas those with p.A382T heterozygous missense *TARDBP* mutation had the longest one.

The co-morbidity of ALS and FTD in patients carrying *TARDBP* mutations in the present series is similar to that expected in the general ALS population (Phukan et al, 2012; Montuschi et al, 2014). Interestingly, there are reports of pure FTD cases of Sardinian

ancestry carrying this mutation with a positive family history of ALS (Synofzik et al, 2014; Floris G, personal communication), indicating that this association warrants further analyses.

The geographical distribution of the genetic mutations in Sardinia may provide clues on the possible origin of the founders. For example, the concentration of the p.A382T *TARDBP* cases in central-western Sardinia suggests that it originated in that area, while the *C9ORF72* mutation probably originated in northern Sardinia.

The rate of positive family history for ALS or FTD in our series varies according to the type of genetic mutations involved. The highest rate was observed among patients with *SOD1* (100%) and *C9ORF72* mutation (~70%), and the lowest in those with *TARDBP* p.A382T and p.G295S mutations (~30%), suggesting that *SOD1* and *C9ORF72* mutations are more penetrant within the Sardinian population. This finding is confirmed by the identification of *TARDBP* mutations in about 1% of Sardinian controls, many of whom were in their sixth decade of life without manifesting symptoms. It is noteworthy that the penetrance of the *TARDBP* p.A382T mutation is significantly lower in women than in men, indicating that other genetic, hormonal or environmental factors may influence the onset of the disease (Orrù et al, 2011).

In summary, we found that over 40% of all ALS Sardinian cases carry a mutation of an ALS-related gene, representing the highest percentage of cases that are genetically explained outside of Scandinavia, and largely higher than in other Caucasian populations (van Blitterswijk et al, 2012; Chiò et al, 2012c; Kenna et al, 2013). More than 10% of Sardinian cases without a known mutation show a positive family history for ALS or FTD, indicating that one or more new genes still have to be detected within this population. Clinical phenotypes associated with different genetic mutations showed some distinctive characteristics, but the heterogeneity within and among families carrying the same mutations may also imply the role of other genetic or non-genetic factors in the disease manifestation. The complex genetic interactions are clearly highlighted by the cases with double genetic mutations of the *C9ORF72* and *TARDBP* genes, who present an early age at onset and have a shorter survival than patients carrying either mutation alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Conserved populations, such as Sardinians, displaying elevated rates of familial or sporadic ALS provide unique information on the genetics of the disease. Our aim was to describe the genetic profile of a consecutive series of ALS patients of Sardinian ancestry. More than 40% of a series of 375 consecutive cases of Sardinian ancestry carry a mutation of an ALS-related gene, representing the highest percentage of ALS cases genetically explained outside of Scandinavia. Clinical phenotypes associated with different genetic mutations show some distinctive characteristics, but the heterogeneity between and among families carrying the same mutations implies that ALS manifestation is influenced by other genetic and non-genetic factors.

Figure 1.

Geographic distribution of area of origin of parents of Sardinian ALS cases according to more common mutations. The two *TARDBP* p.A382T homozygous patients and the single patients with double p.A382T and p.G295S heterozygous mutations are included in the *TARDBP* figures. The 8 patients with double mutation (*C9ORF72* & p.A382T *TARDBP*) were included in both *C9ORF72* and *TARDBP* figures.

Figure 2.

Cumulative probability of ALS onset according to genetic status of Sardinian patients. Brown, no detected mutations; violet, *TARDBP* p.A382T heterozygous mutations; green, *C9ORF72* mutations; red, *TARDBP* p.G295S heterozygous mutations; yellow, double *C9ORF72* and *TARDBP* p.A382T heterozygous mutations.

Figure 3.

Kaplan-Meier curves (tracheostomy-free survival) of ALS Sardinian patients with different genetic mutations. Brown, no detected mutations; violet, *TARDBP* p.A382T heterozygous mutations; green, *C9ORF72* mutations; red, *TARDBP* p.G295S heterozygous mutations; yellow, double *C9ORF72* and *TARDBP* p.A382T heterozygous mutations.

Table 1

ALS in Sardinia: frequency of mutations according to presence or absence of positive family history for ALS or FTD

Mutation	FALS (n=100)	SALS (n=275)	FALS+SALS (n=375)
Wild type	26 (26.0%)	194 (70.5%)	220 (58.7%)
<i>TARDBP</i> (p.A382T, hetero- and homozygous)	25 (25.0%)	53 (19.3%)	88 (23.5%)
<i>TARDBP</i> (p.G295S, hetero- and homozygous)	3 (3.0%)	8 (2.9%)	11 (2.9%)
<i>TARDBP</i> (p.A382T and p.G295S double mutation)	1 (1.0%)	-	1 (0.3%)
<i>C9ORF72</i>	33 (33.0%)	18 (6.5%)	51 (13.6%)
<i>C9ORF72</i> and <i>TARDBP</i> (p.A382T)	6 (6.0%)	2 (0.7%)	8 (2.1%)
<i>SOD1</i>	4 (4.0%)	-	4 (1.1%)
<i>MATR3</i>	2 (2.0%)	-	2 (0.5%)

Table 2

Haplotype analysis of genotype data across the TARDBP and C9ORF72 loci

Mutation	Shared segment	Length	Number of shared SNPs	Number of analysed cases
p.A382T <i>TARDBP</i>	11,025,493 to 11,856,378	831 Kb	257	60
p.G285S <i>TARDBP</i>	10,799,577 to 12,779,618	1,980 Kb	571	7
<i>C9ORF72</i> repeat expansion	27,536,894 to 27,574,515	38 Kb	73*	25

* 4 of these 73 SNPs were the same as the previously reported Finnish founder risk haplotype,^{4,25} indicating that the Sardinian patients carrying the pathogenic GGGGCC repeat expansion also shared the Finnish founder risk haplotype, at least in part.

Table 3

Clinical-demographic characteristics of Sardinian ALS cases

	Number of cases*	Gender (female, %)	Site of onset (bulbar, %)	Age at onset (median, IQR)	FALS (%)	FTD (%)
Wild type	220	85 (38.6%)	55 (25.0%)	63.2 (55.9–71.4)	26 (11.8%)	19 (8.6%)
<i>TARDDBP</i> (p.A382T)	75	25 (33.3%)	18 (24.0%)	61.5 (53.6–68.6)	23 (30.7%)	9 (12.0%)
<i>C9ORF72</i>	51	20 (39.2%)	18 (35.3%)	62.3 (55.0–67.4)	33 (64.7%)	16 (31.4%)
<i>TARDDBP</i> (p.A382T) & <i>C9ORF72</i>	8	1 (12.5%)	3 (37.5%)	43.8 (38.2–46.4)	6 (75.0%)	5 (62.5%)
<i>TARDDBP</i> (p.G295S)	10	6 (60%)	5 (50%)	62.8 (56.2–74.9)	3 (30.0%)	1 (10.0%)
<i>SOD1</i>	4	0	0	57.1 (45.7–64.5)	4 (100%)	0
Overall	368	137 (37.2%)	99 (26.9%)	62.5 (54.4–69.2)	96 (26.1%)	50 (13.6%)
<i>P</i>	-	n.s.	n.s.	0.0001	0.0001	0.0001

* Seven cases are not included in the table: one case with a double missense mutation of the *TARDDBP* gene (p.G295S and p.A382T), 3 cases with homozygous p.A382T *TARDDBP*, one case with homozygous p.G295S *TARDDBP* and 2 cases with MATR3 mutation.

FALS, familial amyotrophic lateral sclerosis; FTD, frontotemporal dementia according to Neary's criteria; IQR, interquartile range