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UNC13A confers risk for Sporadic ALS and influences survival in a Spanish cohort

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ABSTRACT

Objective. To investigate the association of functional variants of the human UNC13A gene with the risk of ALS, survival and the disease progression rate in a Spanish ALS cohort.

Methods. 136 sporadic ALS (sALS) patients and 487 healthy controls were genotyped for the UNC13A rs12608932 variant. Clinical characterization of ALS patients included gender, age at first symptom, initial topography, disease progression rate, and survival. Genetic association was analyzed under five inheritance models.

Results. The sALS patients with the rs12608932^{CC} genotype had an increased risk of ALS under a recessive genetic model (OR=2.16; 95% CI [1.23, 3.8], p=0.009; corrected p =0.028). Genotypes with a C allele are also associated with increased risk (OR=1.47; 95% CI [1.11, 1.95]; p=0.008; corrected p =0.023) under an additive model. sALS patients with a C/C genotype had a shorter survival than patients with A/A and A/C genotypes (HR=1.44; (95% CI [1.11, 1.873]; p=0.007) under a recessive model. In an overdominant model, heterozygous patients had a longer survival than homozygous patients (HR=0.36 (95% CI [0.22, 0.59]; p=0.001). The rs12608932 genotypes modify the progression of symptoms measured using the ALSFRS-R. No association with age of onset, initial topography or rate of decline in FVC was found.

Conclusion. Our results show that rs12608932 is a risk factor for ALS in the Spanish population, and replicate the findings described in other populations. The rs12608932 is a modifying factor for survival and disease progression rate in our series. Our results also corroborated that it did not influence the age of onset.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most frequent degenerative disorder affecting motor neurons in adults. Phenotypic heterogeneity is a characteristic feature of the disease, with different survival times, ages of onset, types of onset, gender predominance, degrees of upper and lower motor neuron signs, and rates of progression. This heterogeneity is relevant in clinical trials, as a significant number of atypical phenotypes may lead to bias in the results. Many authors are undecided as to whether ALS should be considered a single disease or a syndrome [1, 2].

Like most neurodegenerative disorders, environmental and genetic factors have been described as determining the broad variability of the ALS phenotype. Over the last two decades, ALS research has focused on the search for genetic factors which would account for this phenotypic heterogeneity. New gene screening methodologies, especially genome-wide association studies (GWAS), genomic structural variation studies, and whole exome sequencing (WES), have led to the identification of 32 causing genes and 8 different genetic loci with dominant, recessive and X-linked patterns of inheritance for familial ALS. An increasing number of susceptibility and modifier loci have also been suggested for sporadic (sALS) and familial ALS cases. Gene-gene and gene-environment interactions also seem to play an important role in the appearance and phenotype of the disease [3–6].

Ascertaining genetic susceptibility and determining gene-environment interactions for ALS susceptibility loci that contribute to sALS can improve our understanding of the underlying pathogenic mechanisms of ALS. Genome-wide association studies (GWAS) have provided evidence that various modifier loci and associated genes are related to complex diseases including sALS [5, 6].

UNC13A gene has been shown to be a powerful modifying gene of survival and progression in sALS in Western populations, but not in Asian groups [7–16]. In order to confirm whether this is indeed the case, larger independent studies of other populations that replicate these findings are needed to establish their definite role in ALS. This gene has never been investigated in the Spanish ALS population. In this study, we examine whether *UNC13A* has disease-modifying effects in a series of 136 Spanish sporadic ALS patients.

MATERIAL AND METHODS

Study population and clinical data

A cohort of 136 sALS patients meeting the revised El Escorial criteria for Clinically Definite ALS, Clinically Probable ALS, or Clinically Probable ALS – Laboratory-supported was included in this study [17]. A control group of 750 non-affected and non-related subjects was recruited from the Spanish National DNA Bank (Salamanca, Spain). A subset of 487 controls was randomly selected to assess the risk of disease, matching for age and sex. The control and sALS patients were Spanish citizens of European origin.

The patients were clinically characterized, monitored and received quarterly follow-up in the Hospital Universitari Vall d'Hebron ALS Unit. Gender, age at onset, initial topography and survival time (defined as the time between the onset of clinical symptoms and the date of death, or the date of tracheostomy ventilation, or the date when non-invasive ventilation of more than 23 h/day was required) were ascertained for each patient using the homogeneous criteria of a unique observer (JG) (see Table 1 for a detailed demographic description). All the patients had previously been screened for *C9orf72* expansions and *SOD1* mutations [18, 19] which are the most common genetic defects in sALS patients in our population.

The patients were classified as (i) bulbar, spinal or respiratory onset, and (ii) according to the rate of progression. The mean slopes for ALSFRS-R decline and for FVC decline reported in the historical control groups in the minocycline in ALS trial (MINO), used in a lithium trial, were applied as a tool for classifying the patients' rate of progression in our ALS cohort [20, 21]. The ALSFRS-R is the most commonly used outcome marker of disease progression in clinical practice and research, and evaluates symptoms related to bulbar, limb, and respiratory function. The rate of change in ALSFRS-R provides useful guidance on patients' progression and may predict survival and indirectly measure the disease's progression from a statistical point of view [22-24]. Distribution in tertiles was used to classify patients in three subgroups: fast progression (subgroup P1, first tertile for ALSFRS-R and FVC values); normal progression (subgroup P2, second tertile for ALSFRS-R and FVC values); and slow progression (subgroup P3, third tertile for ALSFRS-R and FVC values).

DNA purification and Genotyping

Genomic DNA samples were extracted from whole blood samples of patients using the QIAamp DNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions.

The UNC13A rs12608932 variant was genotyped using the KASPar SNP Genotyping System (LGC Genomics, UK) according to the provider's standard procedures using the following primer set (UNC13A_A: 5'ATCCA TCCAC CCATC AATTT ATCCAA3'; UNC13A_C: 5'CCATC CACCC ATCAA TTTAT CCAC3'; and UNC13A_rev: 5'GAGTG GCTGG TGGAC AGACG AA3'. Fluorescence was measured at 25ºC in a 7300 real time PCR System (Applied Bioscience, USA). Genotype calling was carried out using 7300 system SDS software v1.4 (Applied Bioscience, USA) and Klustercaller software (KBioscience, UK).

Statistical Design, Analysis and Power

The study design was based on a pragmatic, case-control, retrospective clinical study. The samples analyzed were 136 sALS patients and 487 controls (three controls per case). UNC13A rs12608932 genotypes were used as predictors of the progression rate of the symptoms, the survival time, the risk of suffering ALS and the age at onset.

The odds ratio (OR) and the 95% Confidence Interval (95% CI) were computed using generalized linear models (for either quantitative or binary traits), as implemented in the SNPassoc package from R Software [25]. Analyses were performed using five different inheritance models: additive, codominant, dominant, overdominant and recessive. The Hardy-Weinberg Equilibrium (HWE) was calculated using Fisher's exact test implemented in the SNPassoc package. Ordinal logistic regression was carried out using SPSS software (IBM, Spain). Analyses were performed adjusted by sex, age at onset and topographic clinical onset, based on previous studies by other groups (see table 2) [12, 14]. p-values were corrected by a factor of 3 to correct for multiple comparisons using 5 different genetic models [25–27]. Using this criterion, the uncorrected level for statistical significance was established at $p < 0.017$. The uncorrected and corrected p-values are shown.

Survival analysis was performed using Kaplan-Meier curves and COX multivariable regression (SPSS software), including all the dead patients and those alive and receiving either non-invasive ventilation for more than 23 hours a day or ventilation by tracheostomy.

Sample size and statistical power were calculated using Quanto software [28]. This study has a prior statistical power of 80% (alpha=5%) for detecting a difference of 15 months in survival between groups of patients (main population survival= 49.27±41.6 months, minor allele frequency= 0.307), and a beta of 98% (alpha=5%) for detecting a difference of >21 months. For the risk of suffering ALS, this study has a power of 80% (alpha=5%) for detecting an OR>1.5 between cases and controls.

RESULTS

We obtained the genotype of UNC13A rs12608932 polymorphism in 136 sALS patients and 487 controls of the Spanish population. The genotyping results indicated that these genotypes were in HWE (p = 0.5104) in cases (p = 0.2788) and controls (p = 1.0)

Association analysis of this polymorphism with the risk of suffering ALS under different models of inheritance has shown that the SNP rs12608932 is a risk marker for ALS in our Spanish ALS series. Patients with the CC genotype had an increased risk of suffering from ALS with an OR of 2.16 (95% CI [1.23, 3.8]), a result that was statistically significant (p=0.009; corrected p-value= 0.028) under a recessive model (table 2 and 3). The genotypes with the C allele were also associated with an increased disease risk under an additive model (OR=1.47; 95% CI [1.11, 1.95]; p=0.008; corrected p-value= 0.023). The same risk was observed for sALS patients with spinal topographic onset under recessive (OR = 1.91; 95% CI [1.7, 3.43]; p=0.035) and additive (OR = 1.40; 95% CI [1.05, 1.86]; p=0.024) models.

As regards the survival of ALS patients, we grouped sALS patients under different genetic models and performed a Kaplan Meyer Survival curve and Cox regression analyses. Under a recessive model, the results showed statistical differences between patients with *UNC13A* A/A and A/C genotypes (median=57.0 months, 95% CI [48.9, 65.1]) and a C/C genotype (median=24.0 months, 95% CI [21.4, 26.6]) with a long Rank=0.011 and HR=1.44 (95% CI [1.11, 1.873]; p-value=0.007; Table 4; Figure 1A). This result indicated that a CC genotype would be a risk factor for shorter survival. Under an overdominant model, there are also statistical differences between patients with a *UNC13A* A/C (median=90.0 months, 95% CI [43.1, 136.9]) and A/A genotype or C/C genotypes (median=39.0 months, 95% CI [16.1, 61.9]) with a long Rank=0.001 and HR=0.36 (95% CI [0.22, 0.59]; p-value=0.001; Table 4; Figure 1B). The dominant model also showed statistical differences (Table 4). Under this model, AC genotype would be a genetic factor for longer survival.

As regards the progression of ALS symptoms: the ALSFRS-R scores of patients with A/C (2.562 ± 0.149) and C/C (2.923 ± 0.143) genotypes were lower than those for wild-type genotypes (3.109 ± 0.118). Under a dominant model, the difference (2.562 ± 0.149 vs 3.056 ± 0.094) was statistically significant (diff= -0.431; 95% CI [-0.747, -0.115]; p=0.008; corrected p-value= 0.024). We also detected differences under an overdominant model (diff= -0.460; 95% CI [-0.770, -0.149]; p=0.004; corrected p-value= 0.012; table 5). No statistical differences were observed on the FVC between genotypes under the different genetic models (table 2).

Measuring the ALS symptom progression ranked the patients by ALSFRS-R and FVC scores by tertiles: we found no associations under any genetic models.

We also observed no statistical association between the genetic marker UNC13A rs12608932 and the age of onset, gender or the topographic onset in our series of Spanish ALS patients (Table 2).

DISCUSSION

Here we report the association analysis of the SNP rs12608932 in the UNC13A gene as a disease risk and modifying genetic factor in a cohort of Spanish sALS patients. Our results showed that this variant is a risk factor for the development of ALS in our sALS population. Furthermore, our results indicated that the UNC13A gene could be a modifying factor for the progression of the disease's symptoms and survival time. We found no evidence of rs12608932 affecting the age at onset of the disease or its topographic onset.

This is the first time that the genetic analysis of rs12608932 has been performed in Spanish ALS cases. Our disease risk results are consistent with the previous results describing UNC13A as a risk factor for ALS in North American, Dutch and Belgian ALS populations [7, 8, 12, 15]. All these results indicate that this marker is a genetic risk factor for suffering from sporadic ALS in most of the European populations. However, this risk has not been found in other populations from Italy, China and Japan [11, 14, 16].

Our results indicate that patients with the UNC13A rs12608932 C/C genotype have shorter survival times (33 months) than A/A or A/C patients under a recessive model. This effect is identical to that reported for the Italian [14] and Dutch populations [12]. In the Spanish ALS population, the CC genotype would be a risk factor for survival. However, an alternative aggrupation of patients under an overdominant genetic model showed that the patients with A/C genotype have a longer survival than patients with AA or CC. Consequently, the heterozygous genotype would be a protective factor. This is the first reported analysis of the overdominant model of the UNC13A rs12608932 polymorphism in association with ALS survival. This is also the first time that the analysis of rs12608932 has been performed using five genetic models, including co-dominant and overdominant models. Our results using these five models introduce the hypothesis of the heterozygote's advantage role in the resulting phenotype of ALS patients.

To the best of our knowledge, this is the first time the UNC13A rs12608932 variant has been associated with the disease's progression of symptoms, as measured by the ALSFRS-R scale. Our results indicate that the genotypes containing the C allele are risk factors for decline in the ALSFRS-R score. This enables us to propose that the UNC13A rs12608932 could be a new modifying factor for disease progression.

Finally, although the UNC13A rs12608932 variant has been proposed as a modifying gene for the age of onset [29] in different populations from USA and Europe, we did not observe this association in the Spanish series analyzed in our study.

The role of UNC13A in ALS pathogenesis has been proposed due to its involvement in the release of neurotransmitters such as glutamate by neuronal presynaptic vesicles [7]. Mice lacking *UNC13A* suffer from disrupted glutamatergic neurotransmission due to arrested synaptic vesicle maturation [30]. It has been argued that the UNC13A gene could be involved in glutamate-mediated excitotoxicity in ALS, with evidence suggesting glutamatergic excitotoxicity as a significant mechanism for motor neuron death in ALS [7, 31]. A link between neuroinflammation and excitotoxicity has been proposed in the etiopathogenesis of ALS [32–34]. Neuroinflammation is one of the pathological hallmarks of ALS [4]. Microglia cells have a major role in neuroinflammation and their activation and crosstalk with immune cells appear to play a significant role in neuronal death in *in vivo* and *in vitro* studies [35–37]. In ALS,

microglial activation correlates with disease progression and symptoms, and might therefore modify the outcome of this devastating disease [22, 38–41]. A GEO dataset search indicated that UNC13A gene is expressed in human microglia [42, 43]. The mouse ortholog of human UNC13A, the Munc13-1 gene, is expressed in mouse microglia [Josep Saura, personal communication] and in peripheral mouse macrophages [44]. In macrophages, Munc13-1 has been proposed as a membrane fusion regulating protein that interacts with Rab37. In activated macrophages, both proteins colocalized in vesicles containing TNF-α and knockdown of Munc13-1 by siRNA significantly decreased TNF-α secretion. These data indicate that Munc13-1 (UNC13A) is related to the control of TNF-a secretion in activated macrophages and the inflammatory process [44]. No data about this effect related to microglia has been published to date.

Our study has some specific strong points. It is the only one that analyzes the association using the five different inheritance models: additive, codominant, dominant, overdominant and recessive. It is the first to use the mean estimated slope for ALSRFS-R and FVC decline observed in the control patients in the MINO trial to measure functional and respiratory deterioration. In addition, there is homogeneity in the criteria for evaluating the clinical variables, because the clinical data were collected by a unique observer in a unique center. This study is also well powered (beta>95%, alpha=5%) for detecting changes in the survival in sALS patients with different UNC13A genotypes. However, these findings must be interpreted with caution due to the small number of patients (the major limitation of the study). Although the cohort is relatively small – consistent with the incidence in Catalonia being among the lowest rates in Europe [18, 45] - this study is sufficiently well powered (beta>95%, alpha=5%) to replicate the disease risk association reported in different Caucasian populations and to detect changes in the survival of sALS patients with different UNC13A genotypes. It also fails to take into consideration dietary factors, lifestyle, physical activity, occupational and other environmental risk factors which were found to be significant in other populations [46].

The identification of biomarkers associated with different rates of progression and survival time could provide new insights into the pathophysiological determinants of disease progression in ALS. Our results show that rs12608932 is a risk factor for ALS in the Spanish population, and replicate the findings previously described in other western populations. The rs12608932 variant is a modifying factor for survival and disease progression in our series. These results suggest that UNC13A could be an interesting potential target for developing strategies for controlling neurodegeneration in ALS.

ETHICAL STATEMENT

This genetic study was approved by the local IRBs at the Vall d'Hebron Research Institute and the Spanish National DNA Bank. The study has been conducted according to the principles expressed in the Declaration of Helsinki. All patients gave their informed written consent to participation in the study prior to their inclusion in the study, and a blood sample for genetic analysis was obtained from all of them.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Figure Captions

Figure 1. Kaplan Meyer Survival Curves grouped by rs12608932_UNC13A genotypes under recessive (A) and overdominant (B) genetic models

Figure 1
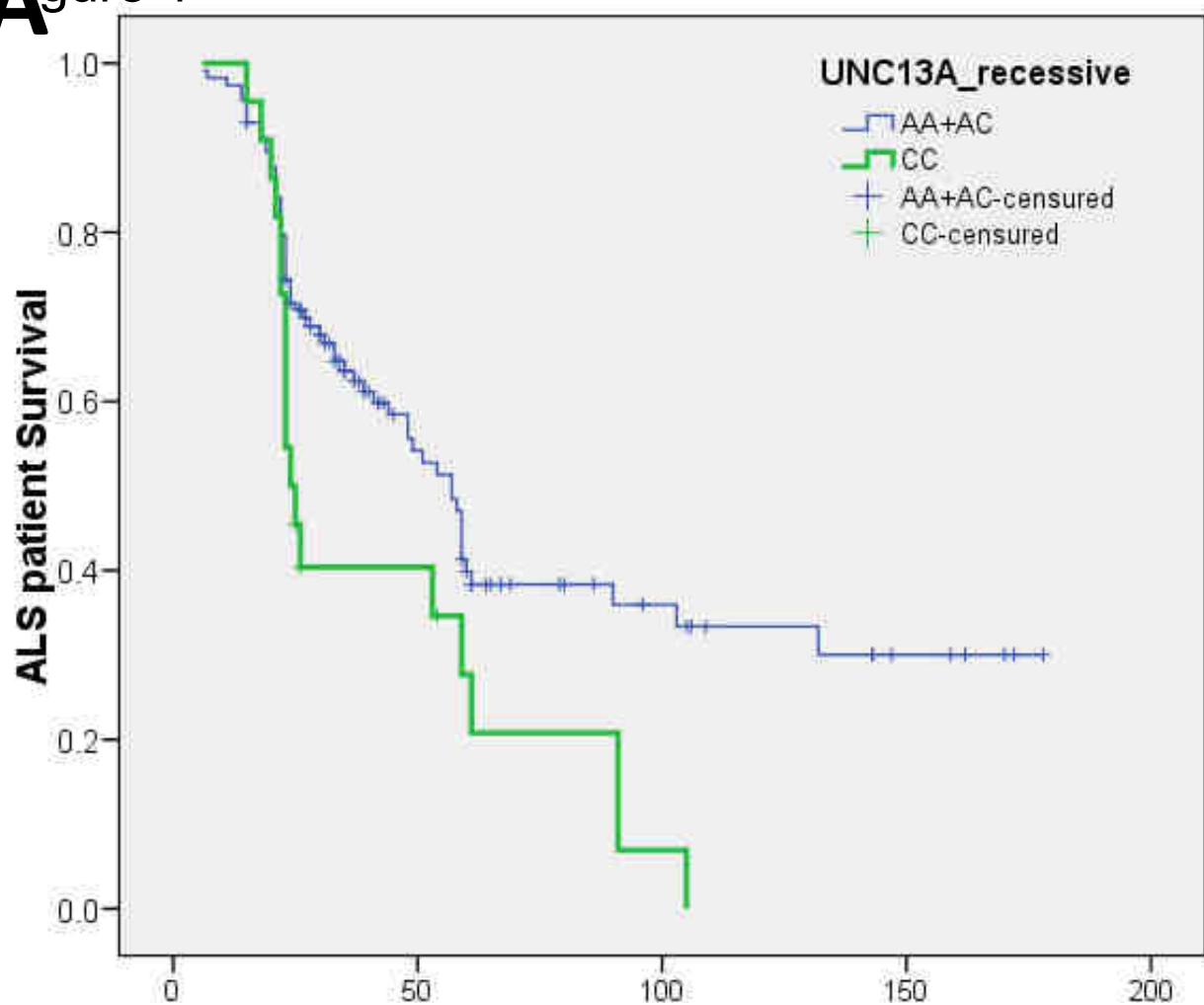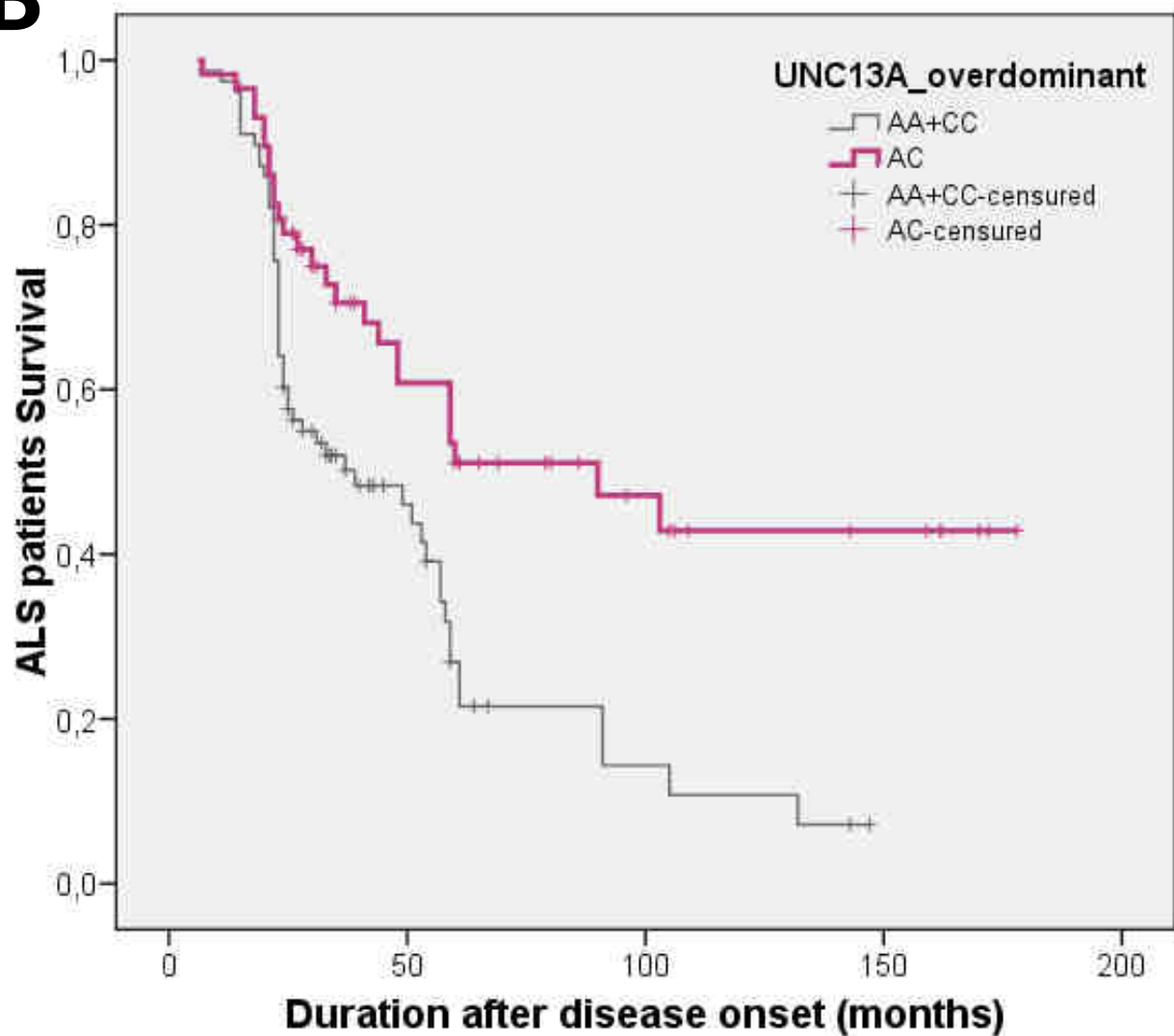
Table 1

Table 1. Demography and clinical data

	Control	sALS
Subjects	487	136
Sex: female	225	65
male	262	71
Mean age (SD)	57.81 (14.10)	61.24 (12.92)
Mean age at onset (SD)	-	57.24 (13.79)
Median age at onset (range)	-	58.5 (22, 85)
Spinal onset	-	99
Bulbar onset	-	35
Respiratory onset	-	2

Table 2

Table 2. Association of p-values (non-corrected) of the rs12608932 variant of the UNC13A gene for several phenotypes according to different inheritance models

Phenotype	Genetic models					adjusted by[b]
	additive	dominant	recessive	co-domin.	over-domin.	
Disease Risk	**0.008**	0.053	**0.009**	0.018	0.766	S+A
Age at onset	0.461	0.662	0.400	0.696	0.843	S+T
Survival[a]	0.483	0.025	**0.007**	0.875	**0.001**	S+AO+T
Symptom progression	0.679	0.218	0.412	0.184	**0.066**	S+T
ALSFRS-R	0.105	**0.008**	0.748	0.012	0.004	S+AO+T
FVC	0.295	0.491	0.269	0.520	0.882	S+AO+T
Topography	0.302	0.401	0.380	0.584	0.862	S+AO

[a] Cox Regression p-value. [b] S=sex, A=age, AO= age at onset, T=topography

Table 3

Table 3. Effect of the rs12608932_UNC13A variant on the risk of suffering ALS calculated for different genetic models (adjusted by sex and age)

Genetic model	genotype	Control (n)	%	Case (n)	%	OR	95% CI	p-value
Additive	0,1,2 [a]	487	78.2	136	21.8	1.47	1.11, 1.95	**0.008**
Dominant	A/A	247	50.7	56	41.2	1.46	0.99, 2.16	0.053
	A/C+C/C	240	49.3	80	58.8			
Recessive	A/A+A/C	447	91.8	114	83.8	2.16	1.23, 3.8	**0.009**
	C/C	40	8.2	22	16.2			
Overdominant	A/A+C/C	287	58.9	78	57.4	1.06	0.72, 1.56	0.766
	A/C	200	41.1	58	42.6			
Codominant	A/A	247	50.7	56	41.2	1	-	
	A/C	200	41.1	58	42.6	1.27	0.84, 1.92	**0.018**
	C/C	40	8.2	22	16.2	2.43	1.33, 4.42	

[a] Number of C allele in the genotype

Table 4

Table 4. Cox multivariable survival analysis of the rs12608932_UNC13A[*]

Genetic model	Factor	HR	95% CI	p-value	p-value model
Dominant	sex	1.067	1.672, 0.681	0.777	
	Age at onset	0.955	0.974, 0.936	**0.0001**	**0.0001**
	topo	0.802	1.247, 0.516	0.328	
	UNC13A_C+	0.585	0.936, 0.366	0.025	
Recessive	sex	0.957	1.493, 0.614	0.848	
	Age at onset	0.958	0.977, 0.939	**0.0001**	**0.0001**
	topo	0.755	1.200, 0.475	0.234	
	UNC13A_CC	1.439	1.873, 1.105	**0.007**	
Over-dominant	sex	1.088	1.698, 0.696	0.712	
	Age at onset	0.951	0.971, 0.932	**0.0001**	**0.0001**
	topo	0.744	1.151, 0.481	0.784	
	UNC13A_ AC	0.359	0.594, 0.217	**0.001**	

[*] Calculated for deceased and equivalent (n=80) and for different genetic models (adjusted by sex and age at onset and initial topography)

Table 5

Table 5. Effect of the rs12608932_UNC13A variant on the ALSFRS-R scale calculated for different genetic models (adjusted by sex, age at onset and topography)

Genetic model	genotype	n	mean	se	Diff.	95% CI	p-value
Dominant	A/A	55	3.109	0.118	0		
	A/C-C/C	80	2.661	0.116	-0.431	-0.747, -0.115	**0.008**
Overdominant	A/A-C/C	77	3.056	0.094	0		
	A/C	58	2.562	0.149	-0.460	-0.770, -0.149	**0.004**
Codominant	A/A	55	3.109	0.118	0		
	A/C	58	2.562	0.149	-0.519	-0.857, -0.181	0.012
	C/C	22	2.923	0.143	-0.201	-0.651, 0.248	