

# Association of Copresence of Pathogenic Variants Related to Amyotrophic Lateral Sclerosis and Prognosis

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## Abstract

### Background and Objectives

Despite recent advances, it is not clear whether the various genes/genetic variants related to amyotrophic lateral sclerosis (ALS) interact in modifying patients' phenotype. The aim of this study was to determine whether the copresence of genetic variants related to ALS has interactive effects on the course of the disease.

### Methods

The study population includes 1,245 patients with ALS identified through the Piemonte Register for ALS between 2007 and 2016 and not carrying superoxide dismutase type 1, TAR DNA binding protein, and fused in sarcoma pathogenic variants. Controls were 766 Italian participants age-matched, sex-matched, and geographically matched to cases. We considered Unc-13 homolog A (*UNC13A*) (rs12608932), calmodulin binding transcription activator 1 (*CAMTA1*) (rs2412208), solute carrier family 11 member 2 (*SLC11A2*) (rs407135), and zinc finger protein 512B (*ZNF512B*) (rs2275294) variants, as well as ataxin-2 (*ATXN2*) polyQ intermediate repeats ( $\geq 31$ ) and chromosome 9 open reading frame 72 (*C9orf72*) GGGGCC intronic expansions ( $\geq 30$ ).

### Results

The median survival time of the whole cohort was 2.67 years (interquartile range [IQR] 1.67–5.25). In univariate analysis, only *C9orf72* (2.51 years, IQR 1.74–3.82;  $p = 0.016$ ), *ATXN2* (1.82 years, IQR 1.08–2.33;  $p < 0.001$ ), and *UNC13A*<sup>C/C</sup> (2.3 years, IQR 1.3–3.9;  $p < 0.001$ ) significantly reduced survival. In Cox multivariable analysis, *CAMTA1* also emerged to be independently related to survival (hazard ratio 1.13, 95% CI 1.001–1.30,  $p = 0.048$ ). The copresence of 2 detrimental alleles/expansions was correlated with shorter survival. In particular, the median survival of patients with *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> alleles was 1.67 years (1.16–3.08) compared with 2.75 years (1.67–5.26) of the patients not carrying these variants ( $p < 0.001$ ); the survival of patients with *CAMTA1*<sup>G/G+G/T</sup> alleles and *ATXN2* <sup>$\geq 31$</sup>  intermediate polyQ repeats was 1.75 years (0.84–2.18) ( $p < 0.001$ ); the survival of patients with *ATXN2* <sup>$\geq 31$</sup>  polyQ repeats and *UNC13A*<sup>C/C</sup> allele was 1.33 years (0.84–1.75) ( $p < 0.001$ ); the survival of patients with *C9ORF72* <sup>$\geq 30$</sup>  and *UNC13A*<sup>C/C</sup> allele was 1.66 years (1.41–2.16). Each pair of detrimental alleles/expansions was associated to specific clinical phenotypes.

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## Glossary

ALS = amyotrophic lateral sclerosis; ALSFRS-R = Revised ALS Functional Rating Scale; *ATXN2* = ataxin-2; *C9orf72* = chromosome 9 open reading frame 72; *CAMTA1* = calmodulin binding transcription activator 1; FTD = frontotemporal dementia; *FUS* = fused in sarcoma; GP = general practitioner; HR = hazard ratio; IQR = interquartile range; *SLC11A2* = solute carrier family 11 member 2; *SOD1* = superoxide dismutase type 1; *TARDBP* = TAR DNA binding protein; TDP-43 = TAR DNA binding protein-43; *UNC13A* = Unc-13 homolog A; WGS = whole genome sequencing; *ZNF512B* = zinc finger protein 512B.

## Discussion

We showed that gene variants acting as modifiers of ALS survival or phenotype can act on their own or in unison. Overall, 54% of patients carried at least 1 detrimental common variant or repeat expansion, emphasizing the clinical impact of our findings. In addition, the identification of the interactive effects of modifier genes represents a crucial clue for explaining ALS clinical heterogeneity and should be considered when designing and interpreting clinical trials results.

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder of the CNS, characterized by the involvement of upper motor neurons and lower motor neurons, as well as the cortical neurons of the frontotemporal cortices. ALS is considered a multifactorial disorder caused by an interaction between genetics and the environment.<sup>1</sup> While relatively little is known about the environmental contributions to ALS, pathogenic variants in more than 30 genes have been linked to the disease, the most common being chromosome 9 open reading frame 72 (*C9orf72*), superoxide dismutase type 1 (*SOD1*), TAR DNA binding protein (*TARDBP*), and fused in sarcoma (*FUS*). Overall, the genetic etiology is known for approximately 70% of patients with ALS with a familial history of ALS and 10% of patients with apparently sporadic ALS.<sup>2</sup>

In addition to disease-causing genes, several other genes have been reported to be modifiers of ALS phenotype, especially patients' survival. Among these, the most relevant are Unc-13 homolog A (*UNC13A*) (rs12608932 variant),<sup>3,4</sup> calmodulin binding transcription activator 1 (*CAMTA1*) (rs2412208 variant),<sup>5</sup> ataxin-2 (*ATXN2*) (intermediate polyQ repeats),<sup>6,7</sup> solute carrier family 11 member 2 (*SLC11A2*) (rs407135 variant),<sup>8</sup> and zinc finger protein 512B (*ZNF512B*) (rs2275294 variant).<sup>9</sup> Interestingly, *UNC13A* variant has been demonstrated to be also a modifier of the response to drugs.<sup>10</sup> These observations are important from the clinical trial perspective. Not only does it provide additional new targets for drug development, but it also suggests that these data should be incorporated into the clinical trial design; their effect on survival often equals the anticipated therapeutic effect, meaning balancing of genotypes in the treatment and placebo arms is needed to avoid false positive findings.

Despite these advances, it is not clear whether the various ALS genes/genetic variants interact in modifying the phenotype of patients. This study aimed to determine whether the co-presence of variants related to disease has interactive effects on the course of ALS in a population-based cohort.

## Methods

The study population includes the patients with ALS diagnosed between 2007 and 2016 and identified through an Italian prospective population-based register (Piemonte and Valle d'Aosta Register for ALS).<sup>11,12</sup> All patients were diagnosed as definite, probable, probable laboratory-supported, or possible ALS according to El Escorial revised criteria.<sup>13</sup> More details concerning the epidemiologic register are reported in eMethods (links.lww.com/WNL/C799). Controls were randomly identified from the lists of patients' general practitioners (GPs) and matched to the cases by sex and age ( $\pm 5$  years). Because the list of GP assisted persons is by definition in the same community of their assisted patients with ALS, a geographical matching was ensured.

## Whole Genome Analysis

Whole genome sequencing (WGS) methods are reported in detail as eMethods (links.lww.com/WNL/C799). WGS of 1,029 patients with ALS and 766 controls have already been reported.<sup>14</sup> An additional 290 ALS cases underwent WGS as described elsewhere.<sup>15</sup>

## *ATXN2* CAG and *C9orf72* Repeat Analysis

*C9ORF72* intronic expansions were determined using an established repeat-primed PCR method. *ATXN2* polyQ repeat in exon 1 (NM\_002973.3) was amplified using a fluorescent primer and sized by capillary electrophoresis on an ABI3130 genetic analyzer (Applied Biosystems, Foster City, CA). Both methodologies are described in detail as eMethods and eAppendix 1 (links.lww.com/WNL/C799).

## Survival Modifiers Genes

For this study, we considered the following genes reported to be related to ALS outcome: *UNC13A*, *CAMTA1*, *SLC11A2*, and *ZNF512B*. We also considered the interaction between these genes and *C9orf72* repeat expansion and *ATXN2* polyQ intermediate repeats, which also affects the survival of patients with ALS. Gene variants were dichotomized as follows: *UNC13A*<sup>C/C</sup> vs *UNC13A*<sup>A/A+A/C</sup>;

**Table 1** Demographic and Clinical Characteristics of Patients and Controls

	Cases (n = 1,245)	Controls (n = 766)	p Value
Age at onset, y, median (IQR)	68.0 (60.1–74.3)	65.6 (57.4–72.1)	0.36
Sex (female), n (%)	556 (44.7)	368 (48.0)	0.14
Site of onset (bulbar), n (%)	2 (10)	NA	—
Time from onset to diagnosis, mo, median (IQR)	9.0 (5.1–14.0)	NA	—
Education, median (IQR) <sup>a</sup>	8 (5–11)	8 (5–11)	0.87
ALSFERS-R at diagnosis, median (IQR)	42 (37–45)	NA	—
FVC% at diagnosis, median (IQR) <sup>b</sup>	90 (71–104)	NA	—
BMI at diagnosis, median (IQR) <sup>c</sup>	24.1 (21.9–26.8)	NA	—
ΔALSFERS-R, points per month, median (IQR)	0.68 (0.33–1.35)	NA	—
ΔWeight, kg/mo, median (IQR) <sup>c</sup>	0.27 (0–0.97)	NA	—
ALS-FTD, n (%) <sup>d</sup>	146 (16.1)	NA	—
King's stage (1/2/3/4A+4B) at diagnosis, n	524/392/283/46	NA	—
MiToS stage (0/1/2/3/4) at diagnosis, n	822/368/41/12/2	NA	—
ΔKing's	0.2 (0.11–0.34)	NA	—

Abbreviations: ALS = amyotrophic lateral sclerosis; ALSFRS-R = Revised ALS Functional Rating Scale; BMI = body mass index; FVC = forced vital capacity; FTD = frontotemporal dementia; IQR = interquartile range; MiToS = Milano-Torino staging; NA = not available.

<sup>a</sup> Available for 1,238 patients.

<sup>b</sup> Available for 1,162 patients.

<sup>c</sup> Available for 1,223 patients.

<sup>d</sup> Available for 909 patients.

*CAMTA1*<sup>G/G+G/T</sup> vs *CAMTA1*<sup>T/T</sup>; *SLC11A2*<sup>A/C+C/C</sup> vs *SLC11A2*<sup>A/A</sup>; *ZNF512B*<sup>C/C+C/T</sup> vs *ZNF512B*<sup>T/T</sup>. The first allele(s) reported here is the detrimental one. All dichotomies were based on the original articles reporting the gene in ALS or subsequent studies<sup>3–5,8,9</sup> and were confirmed in our cohort (data not shown).

### Clinical Variables

The mean monthly decline of Revised ALS Functional Rating Scale (ALSFERS-R) (ΔALSFERS-R) was calculated using the following formula: (48 – ALSFRS-R score at diagnosis)/(time from onset to diagnosis, in months). Similarly, the mean monthly decline of weight (ΔWeight) was calculated as (weight at diagnosis – healthy body weight)/(time from onset to diagnosis, in months). Finally, to have a proxy of disease spread, the mean monthly decline of King's staging (ΔKing's) was calculated as (King's staging at diagnosis)/(time from onset to diagnosis, in months).

A total of 909 patients underwent cognitive assessment at the time of diagnosis using an extensive test battery. Cases were classified into 5 categories according to the Consensus Criteria for diagnosing frontotemporal cognitive and behavioral syndromes in ALS.<sup>16</sup> The battery that assessed visuospatial function, language, executive function, memory, and social cognition, as well as anxiety and depression, is reported in detail as eMethods (links.lww.com/WNL/C799).<sup>17</sup>

### Statistical Analysis

Hardy-Weinberg equilibrium was calculated for all the considered variants. The effect of survival of each gene was firstly evaluated in isolation. Second, all genes were assessed together in Cox multivariable analysis. Third, the interaction of alleles on survival and other phenotypic characteristics was evaluated by pair of genes. Differences between continuous variables were assessed with the Mann-Whitney *U* test. Differences between discrete variables were assessed with the  $\chi^2$  test. Kaplan-Meier curves were used to calculate survival and were compared with the log-rank test, setting the onset date as day 0 and the date of death or tracheostomy as the end point. The last day of follow-up for censored cases was December 31, 2021.

Multivariable analysis for survival was performed with the Cox proportional hazards model (stepwise backward) with a retention criterion of a *p* value <0.1. In the final model, we considered significant a *p* value <0.05. Besides the examined genes, the following variables were included in the model: age at onset (continuous), time from onset to diagnosis (continuous), genetic sex (male vs female), site of onset (bulbar vs spinal), King's staging, ΔALSFERS-R (continuous), forced vital capacity % at diagnosis (continuous), ΔWeight (continuous), ΔKing's (continuous), and chronic obstructive pulmonary disease (yes vs no). The SPSS 28.0 statistical package was used for the analyses (SPSS, Chicago, IL).

**Table 2** Cox Multivariable Analysis

Factors	Values	HR (95% CI)	p Value
Age at onset, y	Per each year of age at onset	1.029 (1.023–1.036)	<0.001
Time from onset to diagnosis	Per each month	0.955 (0.946–0.964)	<0.001
Site of onset	Spinal	1 (reference)	<0.001
	Bulbar	1.484 (1.288–1.709)	
$\Delta$ ALSFERS-R	Per each point loss/month	1.329 (1.267–1.394)	<0.001
$\Delta$ King's	Per each point loss/month	1.663 (1.259–2.197)	<0.001
$\Delta$ Weight	Per each kg loss/month	1.071 (1.028–1.116)	<0.001
C9orf72 repeats	C9orf72 <sup>≤29</sup>	1 (reference)	<0.001
	C9orf72 <sup>≥30</sup>	1.645 (1.302–2.079)	
ATXN2 polyQ repeats	ATXN2 <sup>≤30</sup>	1 (reference)	0.003
	ATXN2 <sup>≥31</sup>	1.645 (1.181–2.292)	
UNC13A	UNC13A <sup>VA+VC</sup>	1 (reference)	0.005
	UNC13A <sup>CC</sup>	1.309 (1.087–1.578)	
COPD	No	1 (reference)	0.011
	Yes	1.330 (1.068–1.656)	
CAMTA1	CAMTA1 <sup>T/T</sup>	1 (reference)	0.048
	CAMTA1 <sup>G/G+G/T</sup>	1.135 (1.001–1.287)	

Abbreviations: ALSFRS-R = Revised Amyotrophic Lateral Sclerosis Functional Rating Scale; ATXN2 = ataxin-2; C9orf72 = chromosome 9 open reading frame 72; CAMTA1 = calmodulin binding transcription activator 1; COPD = chronic obstructive pulmonary disease; HR = hazard ratio; UNC13A = Unc-13 homolog A.

## Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Ethics Committees of the ALS Expert Centers of Torino and Novara (Comitato Etico Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino, and Comitato Etico Azienda Ospedaliero-Universitaria Maggiore della Carità, Novara #0038876). Patients and controls provided written informed consent before enrollment. The databases were anonymized according to Italian law for the protection of privacy.

## Data Availability

The individual-level sequence data are available on the database of Genotypes and Phenotypes (accession number: phs001963.v1.p1). Phenotypic data will be available on motivated request by interested researchers.

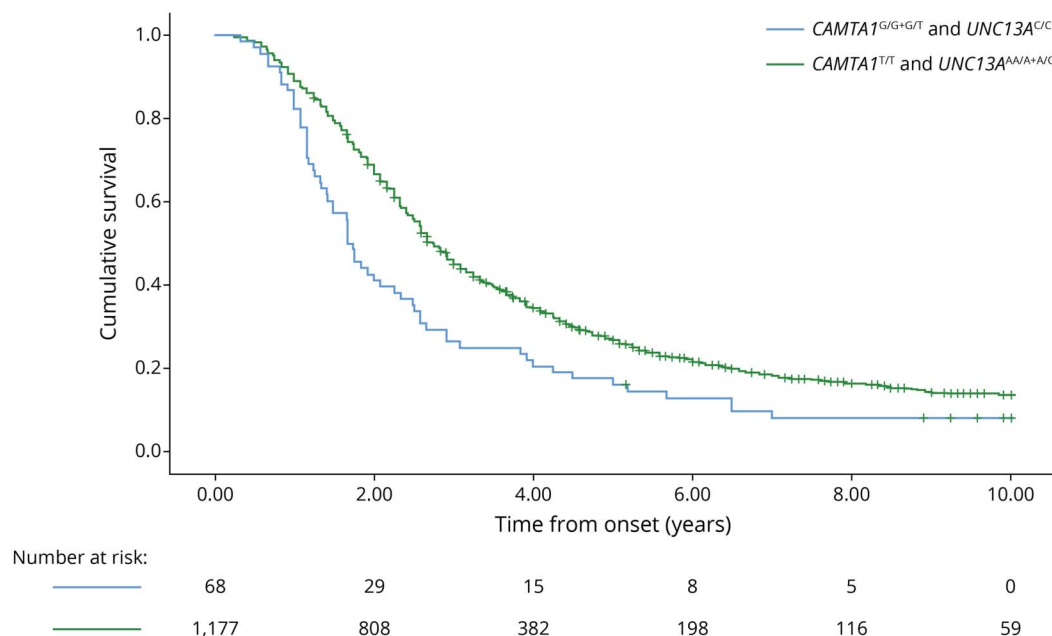
## Results

During the 2007–2016 period, a total of 1,445 patients were diagnosed with ALS in the study area. Of these, 1,319 (91.2%) had available DNA and were therefore whole genome sequenced. A total of 74 patients carrying SOD1 (n = 45), TARDBP (n = 22), and FUS (n = 7) pathogenic variants were excluded from the analysis because of the heterogeneous

clinical course of the different missense and nonsense pathogenic variants of these genes.<sup>18</sup> Therefore, the final study population included 1,245 patients (n = 689 male patients [55.3%], median age at onset = 68.0 years [interquartile range (IQR) 60.3–74.3]). A flow diagram summarizing the patient selection is reported as eFigure 1 ([links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)). A total of 766 matched controls were included in the study. The clinical and demographic characteristics of cases and controls are detailed in Table 1.

The frequency of the alleles of the examined variants is reported in eTable 1 ([links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)). For UNC13A, the allele frequency was significantly different among cases and controls (p = 0.037); the allele frequency did not deviate from the Hardy-Weinberg Equilibrium among controls (p = 0.29), while a deviation observed among patients (p = 0.015), reflecting an increase of risk associated with the C allele. In addition, for ZNF512B, allele frequency was significantly different among cases and controls (p = 0.027); however, for patients and controls, the allele frequency of ZNF512B did not deviate from the Hardy-Weinberg Equilibrium. CAMTA1 and SLC11A2 allele frequencies were not different among cases and controls (p = 0.60 and p = 0.33, respectively), and allele frequency did not deviate from the Hardy-Weinberg Equilibrium both in

**Figure 1** Survival Curves (Kaplan-Meier) for the Interaction Between *CAMTA1* rs2412208 Variant and *UNC13A* rs12608932 Variant



Median survival time: *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> (68 cases, blue line) 1.67 years (1.16–3.08), *CAMTA1*<sup>T/T</sup> and *UNC13A*<sup>A/A+A/C</sup> (1,177 cases, green line) 2.75 years (1.67–5.26),  $p < 0.001$ . Ticks represent censored patients. *CAMTA1* = calmodulin binding transcription activator 1; *UNC13A* = Unc-13 homolog A.

cases and in controls. A total of 40 patients (3.2%) had *ATXN2* polyQ repeats  $\geq 31$ , and 91 patients (7.3%) carried the *C9orf72* repeat expansion. The frequency of the combination of genetic variants and expansions did not deviate from the expected figures.

The median survival time of the entire cohort was 2.67 years (IQR 1.67–5.25). The examined variants in *C9orf72* (median survival 2.51 years, IQR 1.74–3.82;  $p = 0.016$ ), *ATXN2* (median survival 1.82 years, IQR 1.08–2.33;  $p < 0.001$ ), and *UNC13A*<sup>C/C</sup> (median survival 2.3 years, IQR 1.3–3.9;  $p < 0.001$ ) were significantly related to shorter survival in univariate analysis, while the variants in *CAMTA1*<sup>G/G+G/T</sup> (median survival 2.58 years, IQR 1.59–5.08;  $p = 0.231$ ), *SLC11A2*<sup>A/C+C/C</sup> (median survival 2.66 years, IQR 1.59–5.58,  $p = 0.665$ ), and *ZNF512B*<sup>C/C+C/T</sup> (median survival 2.66 years, IQR 1.66–5.16;  $p = 0.325$ ) did not influence ALS outcome (eFigures 2–7, [links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)). In the Cox multivariable analysis, *C9orf72* (hazard ratio [HR] 1.65, 95% CI 1.30–2.08,  $p < 0.001$ ), *ATXN2* (HR 1.65, 95% CI 1.18–2.29,  $p = 0.003$ ), *UNC13A* (HR 1.31, 95% CI 1.09–1.58,  $p = 0.005$ ), and *CAMTA1* (HR 1.13, 95% CI 1.001–1.30,  $p = 0.048$ ) were independently related to survival (Table 2). Therefore, we assessed the combined effects of *C9orf72*, *ATXN2*, *UNC13A*, and *CAMTA1* on ALS outcome in patients with deleterious alleles or expansions compared with those without, on a pairwise basis.

When assessing the interaction by pairs of genes, we found that, in most gene pairs, the presence of both detrimental alleles/repeat expansion was correlated with significantly

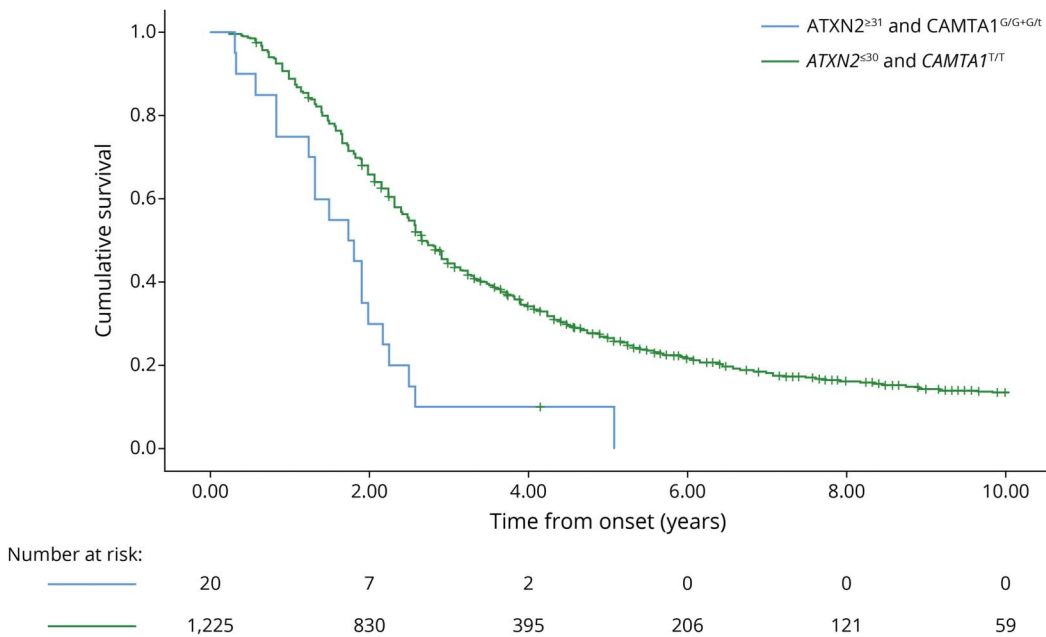
shorter survival compared with other cases. The partial exception was the interaction between *C9orf72* and *CAMTA1*, which was only marginally significant ( $p = 0.052$ ).

Specifically, a total of 68 cases (5.5%) carried the *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> alleles. Their median survival was 1.67 (1.16–3.08) years compared with 2.75 (1.67–5.26) for patients who did not carry detrimental alleles at both genes ( $p < 0.001$ ) (Figure 1). From the phenotypic perspective, patients with both *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> alleles were characterized by a 4-year older age at onset, a higher  $\Delta$ Weight, and a more frequent bulbar onset (eTable 2, [links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)).

A total of 20 cases (1.6%) carried the *CAMTA1*<sup>G/G+G/T</sup> alleles and the *ATXN2* <sup>$\geq 31$</sup>  intermediate polyQ repeats. Their median survival was 1.75 (0.84–2.18) years compared with 2.67 (1.67–5.25) for patients who did not carry detrimental alleles at both genes ( $p < 0.001$ ) (Figure 2). The phenotype of patients with both *CAMTA1*<sup>G/G+G/T</sup> alleles and *ATXN2* <sup>$\geq 31$</sup>  CAG repeats was characterized by a more frequent bulbar onset and a higher  $\Delta$ ALSFRS-R and  $\Delta$ King’s (eTable 3, [links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)).

A total of 38 cases (3.1%) carried both the *CAMTA1*<sup>G/G+G/T</sup> alleles and the *C9ORF72* repeat expansion. Their median survival was 2.33 (1.49–3.84) years compared with 2.67 (1.67–5.25) for patients who did not carry any of these detrimental alleles ( $p = 0.052$ ) (Figure 3). Patients with *CAMTA1*<sup>G/G+G/T</sup> alleles and *C9ORF72* <sup>$\geq 30$</sup>  had an 8-year younger

**Figure 2** Survival Curves (Kaplan-Meier) for the Interaction Between *ATXN2* polyQ Repeats and *CAMTA1* rs2412208 Variant

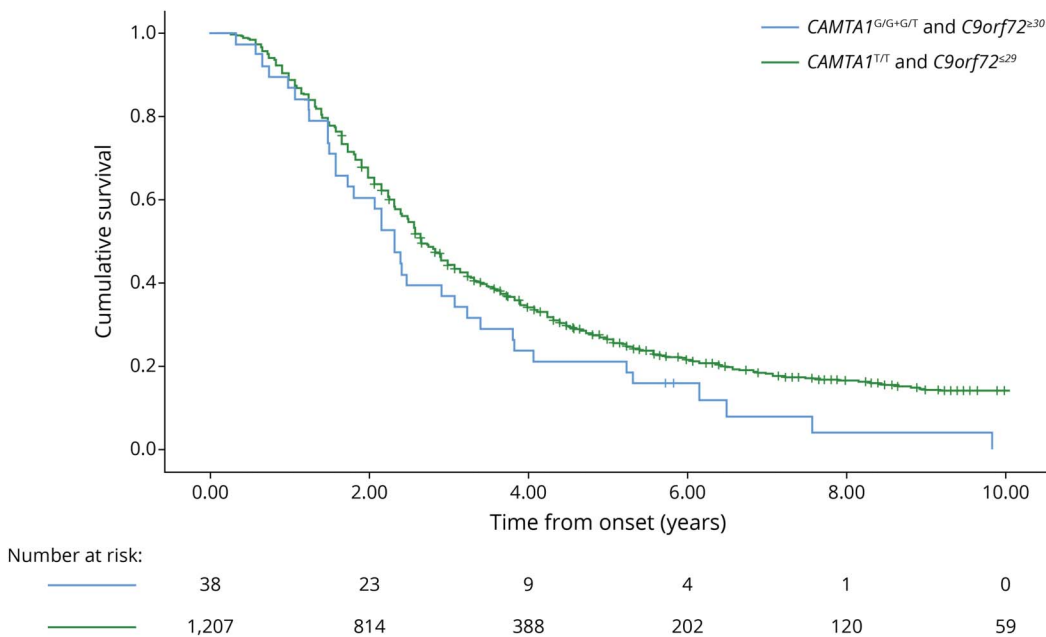


Median survival time: *ATXN2*<sup>≥31</sup> and *CAMTA1*<sup>G/G+G/T</sup> (20 cases, blue line) 1.75 years (0.84–2.18), *ATXN2*<sup>≤30</sup> and *CAMTA1*<sup>T/T</sup> (1,225 cases, green line) 2.67 years (1.67–5.25),  $p < 0.001$ . Ticks represent censored patients. *ATXN2* = ataxin-2; *CAMTA1* = calmodulin binding transcription activator 1.

age at onset and were more frequently affected by comorbid frontotemporal dementia (FTD) (34.4% vs 15.3%) (eTable 4, [links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)).

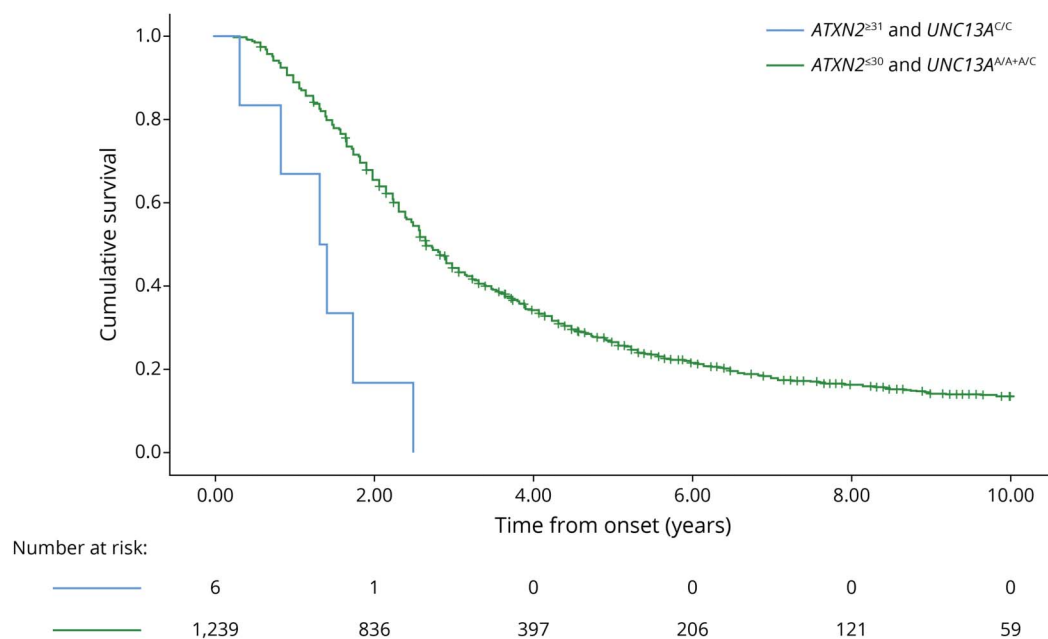
Six patients (0.5%) carried both *ATXN2*<sup>≥31</sup> polyQ repeats and *UNC13A*<sup>C/C</sup> variant; their median survival time was 1.33 (0.84–1.75) vs 2.67 (1.67–5.25) for those who carried

**Figure 3** Survival Curves (Kaplan-Meier) for the Interaction Between *CAMTA1* rs2412208 Variant and *C9orf72* GGGGCC Expansion



Median survival time: *CAMTA1*<sup>G/G+G/T</sup> and *C9orf72*<sup>≥30</sup> (38 cases, blue line) 2.33 years (1.49–3.84), *CAMTA1*<sup>T/T</sup> and *C9orf72*<sup>≤29</sup> (1,207 cases, green line) 2.67 years (1.67–5.25),  $p = 0.052$ . Ticks represent censored patients. *C9orf72* = chromosome 9 open reading frame 72; *CAMTA1* = calmodulin binding transcription activator 1.

**Figure 4** Survival Curves (Kaplan-Meier) for the Interaction Between *ATXN2* polyQ Repeats and *UNC13A* rs12608932 Variant



Median survival time: *ATXN2*<sup>≥31</sup> and *UNC13A*<sup>C/C</sup> (6 cases, blue line) 1.33 years (0.84–1.75) *ATXN2*<sup>≤30</sup> and *UNC13A*<sup>A/A+A/G</sup> (1,239 cases, green line) 2.67 years (1.67–5.25),  $p < 0.001$ . Ticks represent censored patients. *ATXN2* = ataxin-2; *UNC13A* = Unc-13 homolog A.

*ATXN2*<sup>≤30</sup> CAG repeats and *UNC13A*<sup>A/A+A/G</sup> ( $p < 0.001$ ) (Figure 4).

Five patients (0.4%) carried both *C9ORF72*<sup>≥30</sup> and *UNC13A*<sup>C/C</sup> alleles; their median survival time was 1.66 (1.41–2.16) vs 2.67 (1.67–5.25) for those who carried *C9ORF72*<sup>≥30</sup> and *UNC13A*<sup>A/A+A/G</sup> ( $p < 0.019$ ) (Figure 5).

Finally, in the present cohort, no cases with *C9orf72* expansion carried also an *ATXN2* intermediate repeat expansion.

We also evaluated the effect on ALS outcome in patients with 1, 2, or 3 deleterious alleles. The 573 patients (46.0%) with no deleterious allele had a median survival time of 3.0 years (1.67–5.92) compared with 2.67 years (1.75–5.0) for those carrying 1 deleterious variants/expansion (543 cases, 43.6%), 1.84 years (1.25–3.25) for those carrying 2 deleterious variants/expansions (125 cases, 10.0%), and 0.84 years (0.33–1.33) for the 4 (0.3%) patients carrying 3 variants ( $p < 0.001$ ). The corresponding survival curves are reported in eFigure 8 ([links.lww.com/WNL/C799](https://links.lww.com/WNL/C799)). Clinical details of the 4 patients carrying 3 deleterious alleles are reported in eTable 5. These patients are characterized by an age at onset over 70 years, a short time from onset to diagnosis (3–8 months), a wide range of ALSFRS-R scores at diagnosis (10–42) and a rapid disease, as indicated by the  $\Delta$ ALSFRS-R and the  $\Delta$ King's. Three of the 4 patients had comorbidity for dyslipidemia. Only 1 was a former cigarette smoker. Owing to these characteristics, it is likely that patients carrying multiple

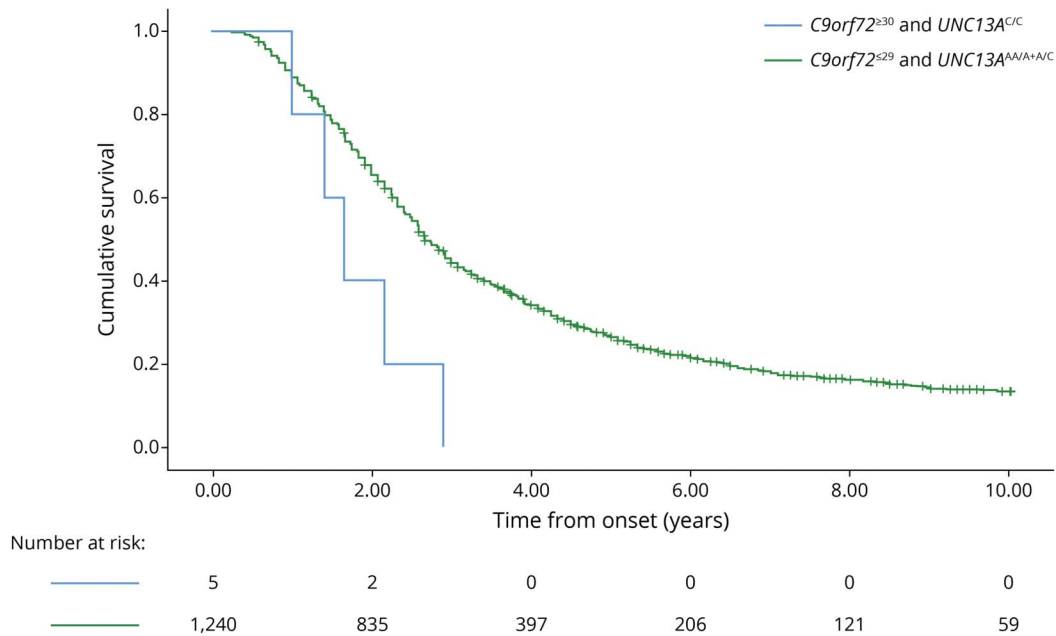
deleterious variant can be missed due to their extremely rapid clinical course.

## Discussion

In our cohort, we have found that the copresence of selected detrimental alleles at common variants or repeat expansions that individually are detrimental to survival in patients with ALS has an additive effect. In particular, the copresence of *CAMTA1*<sup>G/G+G/T</sup> variants with either *UNC13A*<sup>C/C</sup> variant or *ATXN2* polyQ intermediate expansion or *C9ORF72* expansion was related to a significantly worse patients' outcome. This effect was also found when assessing the copresence of *UNC13A*<sup>C/C</sup> variant with either *C9orf72* GGGGCC expansion or *ATXN2* polyQ intermediate expansion. In our cohort, 672 patients (54%) carried at least 1 deleterious variant/expansion.

Genetic modifiers of ALS phenotype have been generally studied in isolation.<sup>3-5,8,9</sup> Notable exceptions are 2 studies reporting that the co-occurrence of the *C9orf72* repeat expansion and *UNC13A*<sup>C/C</sup> variant significantly worsened the prognosis of patients with ALS.<sup>19,20</sup> However, identifying the mechanisms underlying the wide phenotypic heterogeneity of ALS, which hinders the discovery of effective therapies,<sup>2</sup> remains one of the significant unmet goals of ALS research. ALS heterogeneity is likely due to an interplay between genetics, age, sex,<sup>1,21</sup> and environmental factors, both related to lifestyle

**Figure 5** Survival Curves (Kaplan-Meier) for the Interaction Between *C9orf72* GGGCC Expansion and *UNC13A* rs12608932 Variant



Median survival time: *C9orf72*<sup>≥30</sup> and *UNC13A*<sup>C/C</sup> (5 cases, blue line) 1.66 years (1.41–2.16), *C9orf72*<sup>≤29</sup> and *UNC13A*<sup>A/A+A/C</sup> (1,240 cases, green line) 2.67 years (1.67–5.25),  $p < 0.019$ . Ticks represent censored patients. *C9orf72* = chromosome 9 open reading frame 72; *UNC13A* = Unc-13 homolog A.

(i.e., physical activity, smoking)<sup>22,23</sup> and metabolic factors (i.e., lipid metabolism, gut microbiome).<sup>24–26</sup> In this study, we have shown that another element determining ALS phenotypic heterogeneity is the copresence of 2 or more different genetic modifiers of survival.

We did not confirm the prognostic role of 2 of the examined variants, *SLC11A2* (rs407135) and *ZNF512B* (rs2275294). The prognostic effect of *SLC11A2* has been reported in only 1 study on a small cohort and has never been replicated afterward.<sup>8</sup> Similarly, *ZNF512B* has been evaluated in only 2 small cohorts of patients of Asian ancestry, accounting for a total of 388 participants.<sup>9,27</sup> Therefore, for both of these genes, further study is needed to explore their possible prognostic role on ALS.

*CAMTA1*<sup>G/G+G/T</sup> alleles in our cohort seem to interact with all other examined genes in shortening ALS survival. The interaction between *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> variants, accounting for 68 patients (5.5%), besides the strong negative effect on survival, is also phenotypically characterized by an older age at onset, a more frequent bulbar onset, and a higher reduction of weight ( $\Delta$ Weight). The interaction between *CAMTA1*<sup>G/G+G/T</sup> and *ATXN2*<sup>≥31</sup> (20 cases, 1.6%) is characterized by an increased  $\Delta$ ALSFRS-R and  $\Delta$ King’s, indicating a faster spreading and worsening of motor symptoms. Finally, patients with both *CAMTA1*<sup>G/G+G/T</sup> and *C9ORF72*<sup>≥30</sup> were younger, had an increased  $\Delta$ King’s, and had a higher frequency of FTD. The number of cases with the interaction between *UNC13A*<sup>C/C</sup> and *C9ORF72*<sup>≥30</sup> (5 cases, 0.4%) and

*UNC13A*<sup>C/C</sup> and *ATXN2*<sup>≥31</sup> (6 cases, 0.5%) was too low to detect any significant phenotypic difference.

The biological reasons for these interactions remain to be elucidated. The proteins encoded by these genes may interact at a molecular level. It has been reported that TAR DNA binding protein-43 (TDP-43), cytoplasmic inclusions of which are a pathologic hallmark of the disease, enhances translation of *CAMTA1* and midline-1-interacting G12-like protein through a gain-of-function mechanism operating through their 5′ untranslated regions<sup>28</sup>; however, this article did not assess if the occurrence of *CAMTA1*<sup>G/G+G/T</sup> variant differentially influences the observed effect. More recently, it has been shown that TDP-43 represses a cryptic exon-splicing event in *UNC13A*, causing a reduction in *UNC13A* protein expression.<sup>29,30</sup> In addition, the C/C genetic variation in *UNC13A* promotes cryptic exon inclusion on nuclear depletion of TDP-43.<sup>28,29</sup> Independently from the previously reported mechanism, *CAMTA1* was found to be a relevant “Master Regulator” of neurodegenerative disease transcriptional programs in a cultured motor neuron-based ALS model.<sup>31</sup> Biological studies on preclinical models are therefore necessary to understand how these genes do interact.

This study is not without limitations. First, not all patients were tested for cognitive function, reducing the possibility of assessing the genetic interactions on cognition. However, the clinical and demographic characteristics of tested and non-tested patients were similar, limiting the possible selection bias. Second, very few patients carried both *ATXN2*<sup>≥31</sup> CAG



repeats and *UNC13A*<sup>C/C</sup> detrimental alleles or *C9ORF72*<sup>≥30</sup> and *UNC13A*<sup>C/C</sup> detrimental alleles, reducing the possibility of assessing their phenotype and limiting the power of these analyses. Similarly, owing to the reduced number of patients carrying more than 2 variants, we could not evaluate the effect of more than 2 variants. Larger patient cohorts are necessary to analyze these combinations and to calculate a polygenic risk score for survival. In addition, external replication of our finding would be necessary to confirm them in other populations. A remarkable aspect of our study is its population-based nature because it included some 90% of the incident cohort patients in the Piemonte and Valle d'Aosta regions.<sup>14</sup> It has been demonstrated that prevalent and incident populations strongly differ from the clinical point of view, including survival, supporting the notion that studies derived from incident, population-based cohorts better represent the ALS population.<sup>32,33</sup>

We demonstrated that gene variants and expansions acting as genetic modifiers of ALS survival can act on their own or in unison. Overall, 54% of patients carried at least 1 detrimental allele at common variant or repeat expansion, highlighting the clinical impact of our findings. This observation has several implications. First, identifying the interactive effects of modifier genes represents a crucial clue for explaining ALS clinical heterogeneity. Second, the interactive effect of these variants is likely to have profound effects on clinical trial design and interpretation, in particular for relatively common combinations, such as the association of *CAMTA1*<sup>G/G+G/T</sup> and *UNC13A*<sup>C/C</sup> variants which accounted in our series for 5.5% of patients and reduced patients' survival by more than 1 year. Third, our study indicates that variants acting as phenotypic modifiers should be included in ALS genetic panels to provide patients and their families with a better prediction of the course of the disease and improve the planning of therapeutic interventions.

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## Disclosure

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Continued

## Appendix (continued)

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## Appendix (continued)

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