Journal of Neurology (2023) 270:877–890
https://doi.org/10.1007/s00415-022-11426-y

ORIGINAL COMMUNICATION

Factors predicting disease progression in C9ORF72 ALS patients

Jessica Mandrioli[1,2] · Elisabetta Zucchi[1,2] · Ilaria Martinelli[2,3] · Laura Van der Most[1,2] · Giulia Gianferrari[1] · Cristina Moglia[4] · Umberto Manera[4] · Luca Solero[4] · Rosario Vasta[4] · Antonio Canosa[4,5] · Maurizio Grassano[4,5] · Maura Brunetti[5] · Letizia Mazzini[6] · Fabiola De Marchi[6] · Cecilia Simonini[2] · Nicola Fini[2] · Rossella Tupler[1] · Marco Vinceti[1,7,8] · Adriano Chiò[4,5] · Andrea Calvo[4,5]

Received: 13 July 2022 / Revised: 10 October 2022 / Accepted: 13 October 2022 / Published online: 25 October 2022

Abstract

Objective To unveil clinical features, comorbidities, disease progression and prognostic factors in a population-based cohort of ALS patients carrying C9ORF72 expansion (C9 + ALS).

Methods This is a retrospective observational study on ALS patients residing in Emilia Romagna and Piedmont-Valle D'Aosta regions whose data are available through population based registers. We analysed patients who underwent genetic testing, focusing on C9 + ALS subgroup.

Results Among 2204 genotyped patients of the two registers, 150 were C9 + ALS. In comparison with patients without mutation, a higher proportion of family history (12.85 vs 68%, $p < 0.001$) and frontotemporal dementia (3.93% vs 10.67%, $p < 0.001$) was detected in C9 + ALS. C9 + ALS presented a faster disease progression as measured by monthly decline in ALS Functional Rating Scale-Revised (1.86 ± 3.30 vs 1.45 ± 2.35, $p < 0.01$) and in forced vital capacity (5.90 ± 5.24 vs 2.97 ± 3.47, $p < 0.01$), a shorter diagnostic delay (8.93 ± 6.74 vs 12.68 ± 12.86 months, $p < 0.01$) and earlier onset (58.91 ± 9.02 vs 65.04 ± 11.55 years, $p < 0.01$). Consistently, they reached death or tracheostomy earlier than other patients (31 vs 37 months, HR = 1.52, 95% C.I. 1.27–1.82, $p < 0.001$). With respect to other genotyped patients, C9 + ALS patients did not present a significantly higher prevalence of concomitant diseases. Independent prognostic factors of survival of C9 + ALS included sex, age, progression rate, presence of frontotemporal dementia and thyroid disorders, with the latter being associated with prolonged ALS survival (43 vs 29 months, HR = 0.42, 95% C.I. 0.24–0.74, $p = 0.003$).

Conclusion Even in the context of a more aggressive disease, C9 + ALS had a longer survival in presence of thyroid disorders. This finding may suggest protective pathogenic pathways in C9 + ALS to be explored, looking for therapeutic strategies to slow disease course.

Keywords Amyotrophic lateral sclerosis · C9ORF72 · Population-based register · Survival · Prognostic factor · Thyroid disorders

Jessica Mandrioli, Elisabetta Zucchi, Adriano Chiò and Andrea Calvo contributed equally to the work.

✉ Jessica Mandrioli
jessica.mandrioli@unimore.it; mandrioli.jessica@aou.mo.it

[1] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

[2] Department of Neurosciences, Azienda Ospedaliero-Universitaria di Modena, Modena, Italy

[3] Clinical and Experimental Medicine Ph.D. Program, University of Modena and Reggio Emilia, Modena, Italy

[4] "Rita Levi Montalcini" Department of Neuroscience, ALS Centre, University of Torino, Turin, Italy

[5] SC Neurologia 1U, AOU Città della Salute e della Scienza of Torino, Turin, Italy

[6] ALS Center, Neurology Unit, AOU Maggiore della Carità and University of Piemonte Orientale, Novara, Italy

[7] Department of Science of Public Health, Research Centre in Environmental, Genetic and Nutritional Epidemiology, University of Modena and Reggio Emilia, Modena, Italy

[8] Department of Epidemiology, Boston University School of Public Health, Boston, MA, USA

Introduction

Hexanucleotide repeat expansion (GGGGCC) in the first intron of *C9ORF72* gene accounts for up to 40% of familial and 5–10% of sporadic forms of amyotrophic lateral sclerosis (ALS) depending on the studied population [1].

Notwithstanding the advances obtained by cellular and animal models, the exact mechanism of *C9ORF72*-mediated pathogenesis of ALS is not fully elucidated and encompasses the toxicity of bi-directionally transcribed repetitive RNA transcripts and/or dipeptide repeats (DPRs) derived from non-conventional translation known as repeat-associated non-ATG (RAN) [2] and possibly a loss of function effect because of aborted RNA transcripts [3]. Quite surprisingly, *C9ORF72* knock-out mice models do not display a motor neuron loss phenotype; instead, they present profound immune system dysregulation with progressive splenomegaly and lymphadenopathy, age-related neuroinflammation, abnormal leukocyte expansion with neutrophilia and increased cytokine expression and autoantibody production resulting in autoimmune diseases and early death [4, 5].

In ALS patients, while previous epidemiological studies suggested a link between ALS and autoimmune diseases independently of the genetic background [6], more recent works have investigated heterozygotes patients for *C9ORF72* expanded (*C9*+) alleles in frontotemporal dementia (FTD) and motor neuron disease (MND) cohorts, with conflicting results. More specifically, some studies reported no difference in non-thyroid autoimmune diseases between *C9*+ and non-*C9*+ FTD and FTD/MND [7], whereas others, unexpectedly, found that *C9*+ have even a lower incidence of these conditions [8]. On the other hand, intermediate length expansions in *C9ORF72* were found prevalent in systemic lupus erythematosus (SLE) and rheumatoid arthritis patients [9]. A significantly higher number of *C9ORF72* expansions than expected was found also in a cohort of patients with multiple sclerosis that later developed ALS, showing a particularly fast progression rate [10]. Nevertheless, a further Sardinian study did not confirm a higher prevalence of *C9ORF72* expansion among patients with multiple sclerosis [11].

Recent studies suggest that effects of *C9ORF72* loss-of-function may be modulated by environmental factors as the intestinal microbiota, which commands upon systemic and neural immune surveillance system, resulting in separate survival outcome in mice models with this genetic background [12].

In the present study, we aimed at analyzing clinical features and the genotype–phenotype correlates of a cohort of Italian *C9ORF72* ALS patients accrued by the Emilia Romagna (ERRALS) and Piemonte and Valle D'Aosta (PARALS) Registers.

Furthermore, gathering medical history from two ALS Italian regional registries, our study is meant to clarify (1) whether *C9ORF72* expansion carriers have increased odds for autoimmune diseases or other comorbidities compared to the general ALS population, suggesting separate autoinflammatory profiles; and (2) whether comorbidities and in particular autoimmune diseases influence ALS progression in patients carrying *C9ORF72* expansion.

Methods

Patients' data collection

This is a retrospective observational study. The study population includes adult ALS patients (age ≥ 18 years) residing in Emilia Romagna and Piedmont regions, enrolled by ERRALS and PARALS registers [13, 14].

Both registers enrol patients affected by ALS at the time of diagnosis. Caring physicians collect a detailed phenotypic description of each ALS patient, including age at onset and diagnosis, gender, residence, employment history, site and time of onset, affected body regions, upper and lower motor neuron signs, El Escorial-revised classification, clinical phenotype (classic ALS, bulbar ALS, upper motor neuron predominant ALS, flail arm ALS, and flail leg ALS, respiratory ALS)[15], molecular findings, presence of dementia or extrapyramidal signs, family history, diagnostic tests, drugs history (including Riluzole), forced vital capacity (FVC), ALS Functional Rating Scale Revised (ALSFRS-R), the use of artificial enteral nutrition and non-invasive or invasive ventilatory support, and the date, place and cause of death [16, 17].

Registration of data on DNA analysis includes at least presence/absence of *SOD1, FUS, TARDBP* mutations and *C9ORF72* expansion. Depending on the results of DNA analyses and on the presence of family history of ALS and/or FTD, further genes are also explored in a subgroup of patients. *C9ORF72* status is determined by repeat primed PCR as described previously (with individual laboratory-based validation and quality control by Southern blot analyses) [18, 19].

Comprehensive comorbid medical history was accounted by categorizing concurrent conditions in psychiatric diseases, hypertension and cardiovascular diseases, diabetes, thyroid disorders (including both hypo and hyperthyroidism), metabolic alterations (including hypertriglyceridemia, hypercholesterolemia, hyperhomocysteinemia, hyperuricemia and gout, obesity), chronic obstructive pulmonary disease (COPD) and other

respiratory disorders, gastrointestinal, urological, haematological, autoimmune, neoplastic diseases[20]. According to EFNS guidelines [21], patients undergo a regular multidisciplinary follow-up at least every 3–4 months, with regular data collection on disease progression and procedures. When patients are no longer able to reach the ALS centers of Emilia Romagna and Piedmont and Valle D'Aosta, home monitoring is carried out. Information about the adoption of non-invasive ventilation (NIV), tracheostomy and invasive ventilation (IV), percutaneous endoscopic gastrostomy (PEG) and death are retrieved either directly from the patients and their caregivers, then confirmed through the query of administrative data [22, 23].

Disease progression is measured by ALSFRS-R considering the total score at diagnosis, at first and last follow up visit.

For this study, King's staging system was calculated from ALSFRS-R at diagnosis [24] as we previously described [25].Progression rate at diagnosis has been defined accordingly to Kimura et al. [26] by the following formula:

$$\text{Progression rate at diagnosis} = \frac{(48 - \text{ALSFRS-R total score at diagnosis})}{(\text{months from onset to diagnosis})}$$

Progression rate has been defined also from first to last visit as:

$$\text{Progression rate from first to last visit} = \frac{(\text{ALSFRS-R total score at diagnosis} - \text{ALSFRS-R total score at last visit})}{(\text{months from diagnosis to last visit})}$$

"Weight loss at diagnosis" was defined as the difference in kilograms between the body weight during healthy status and at the time of diagnosis.

Forced vital capacity assessed by spirometry was available at diagnosis and during follow up in a limited number of patients. Cognitive and behavioural impairment in the FTD disease spectrum was evaluated according to Strong criteria with single centers validated neuropsychological testing batteries [27, 28](which considered memory, language, visuospatial skills, attention, executive function, praxis and social cognition) [29].

Statistics

We assessed differences across ALS patients' groups by using *T* test, ANOVA, Chi-square tests as appropriate. We reported missing data as a separate category in the dataset and each variable has been described with frequencies of "not known" values.

Adjusted analyses for each outcome included cox proportional hazard models for time-to-event outcomes and generalized linear models for longitudinal outcomes.

Cox regression analysis has been used to estimate the hazard ratio (HR) and corresponding 95% confidence interval (95% CI) for the independent variables and ALS tracheostomy-free survival.

Missing data were handled by using multiple imputation (MI) analysis [30]. Among the MI predictors, the outcome (death or tracheostomy), sex, time to diagnosis, and age at onset were known for all patients, while other variables of interest were not available for all patients and were estimated by MI using linear regression in 30 imputation datasets.

Data analysis was performed using the STATA statistical package 15 (StataCorp. 2017. College Station, TX: StataCorp LLC).

Results

Patients' clinical features

The two Italian registers (ERRALS and PARALS) accrued a population of 4486 ALS patients, of whom 2204 (49.13%) underwent genetic testing. Of these, 150 patients (6.8%) carried *C9ORF72* expansion (*C9* + patients) (Fig. 1), 72 males and 78 females, with a male to female ratio of 0.92.

Table 1 shows the key clinical features of *C9ORF72* patients in comparison with patients without mutations/

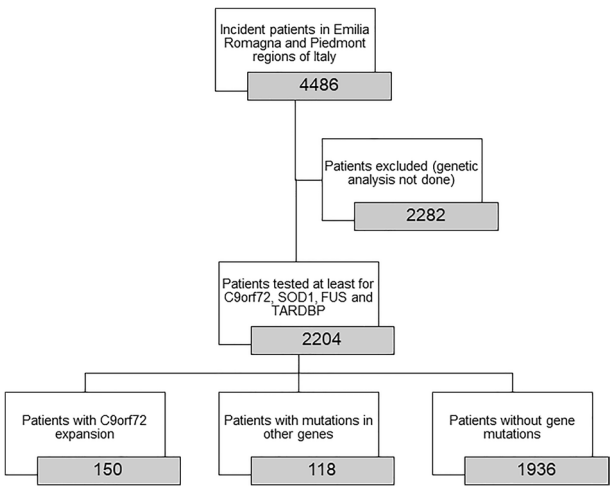

Fig. 1 Study diagram showing patients who were included in the study

Table 1 Clinical features of *nmALS* patients, *C9+* patients, and those carrying other gene mutations

Clinical features**	*nmALS* (*n* = 1936) *N* (%), mean [SD]	C9orf72 ALS (*n* = 150) *N* (%), mean [SD]	Others[&] (*n* = 118) *N* (%), mean [SD]	*p* value
Gender, male	1082 (55.85)	72 (48)	58 (49.15)	0.074
Age at onset, years	65.04 [11.55]	58.91 [9.02]	60.11 [13.33]	< 0.001
Diagnostic delay, months	12.68 [12.86]	8.93 [6.74]	15.24 [15.47]	< 0.001
BMI at diagnosis, kg/m^2	24.61 [4.13]	23.90 [4.47]	24.42 [4.05]	0.147
Weight loss at diagnosis, kg	3.85 [5.87]	3.60 [4.76]	2.86 [5.41]	0.231
ALSFRS-R at diagnosis	39.13 [9.05]	39.43 [9.53]	39.05 [9.07]	0.922
Progression rate at diagnosis*	1.45 [2.35]	1.86 [3.30]	1.53 [2.58]	0.130
Progression rate – first to last visit[#]	1.28 [1.47]	2.05 [1.49]	1.23 [1.21]	0.010
FVC at diagnosis, %	91.59 [67.48]	88.60 [26.78]	91.17 [30.27]	0.971
Monthly FVC decline[§]	2.97 [3.47]	5.90 [5.24]	3.18 [3.67]	0.002
PEG positioning	685 (40.60)	69 (48.94)	30 (30.30)	0.015
Non-invasive ventilation	809 (46.93)	55 (39.29)	50 (49.50)	0.181
Invasive Ventilation	297 (16.49)	24 (16.67)	16 (14.41)	0.845
Death	1461 (75.50)	126 (84.00)	78 (66.67)	0.004
Time from ALS onset to PEG, months	28.88 [20.60]	22.79 [12.10]	40.48 [29.91]	< 0.001
Time from onset to NIV, months	29.43 [24.44]	24.50 [13.92]	36.62 [36.94]	0.043
Time from onset to IV, months	34.05 [22.81]	24.33 [13.63]	31.62 [25.95]	0.121
Time from onset death or tracheostomy or last observation, months	48.29 [41.22]	33.95 [21.60]	63.17 [52.41]	< 0.001
Riluzole treatment	733 (88.10)	69 (90.79)	51 (91.07)	0.643
FTD	76 (3.93)	16 (10.67)	3 (2.54)	< 0.001
Parkinsonism	16 (0.84)	1 (0.69)	2 (1.78)	0.569
Family history of ALS/FTD	90 (12.85)	34 (68.00)	18 (40.91)	< 0.001

BMI body mass index, *FVC* forced vital capacity, *PEG* percutaneous endoscopic gastrostomy, *NIV* non-invasive ventilation, *IV* invasive ventilation, *FTD* frontotemporal dementia, *SD* standard deviation

[*]Progression rate at diagnosis is calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset

[**]FVC at diagnosis was available for 448 patients, monthly FVC decline for 374 patients; information on PEG positioning was available for 1927 patients, data on NIV was available for 1965 patients, and data on IV for 2056 patients. ALSFRS-R monthly decline was available for 2190 patients. Family history was available for 794 patients, riluzole data for 964 patients, presence of parkinsonism for 2148 patients

[#]Progression rate first to last visit is calculated as monthly decline of ALSFRS-R score from the first to the last available visit or from the first visit to the first time when a total score of 0 was recorded (whichever came first)

[§]Monthly decline of FVC has been calculated considering first FVC% value, the last available FVC% value, and time between the first and last FVC

[&]Other gene mutation include: SOD1 (*n* = 57 patients), FUS (*n* = 22 patients), TARDBP (*n* = 28 patients), TUBA4A (*n* = 2 patients), MATR3 (*n* = 2 patients), OPTN (*n* = 2 patients), VCP (*n* = 1 patients), CHMP2B (*n* = 1 patients), C21ORF2 (*n* = 1 patients), MFN2 (*n* = 1 patients), TBK1 (*n* = 1 patients)

expansions in genes related to ALS ("*nmALS*") and with patients with other genes mutations.

Patients with *C9ORF72* expansion reached ALS diagnosis before the other patients, due to an earlier disease onset (58.91 ± 9.02 vs 65.04 ± 11.55 years, $p < 0.01$) and a shorter diagnostic delay (8.93 ± 6.74 vs 12.68 ± 12.86 months, $p < 0.01$), in comparison with *nmALS* patients. Moreover, *C9+* patients showed a faster disease progression, as shown by a steeper ALSFRS-R and FVC monthly decline (1.86 ± 3.30 vs 1.45 ± 2.35, $p < 0.01$ and 5.90 ± 5.24 vs 2.97 ± 3.47, $p < 0.01$, respectively), by a shorter time to undergo NIV, IV, and PEG positioning, and also by a higher mortality (Table 1).

Among *nmALS* patients, at the time of diagnosis the majority of patients were at initial stages of the disease, although there was also a significant quote in advanced stage (King's stage 4): 1023 (53.62%) were in King's stage 1, 436 (22.85%) in stage 2, 222 (11.64%) in stage 3, and 204 (10.69%) in stage 4. Among *C9+* patients at diagnosis 83 (55.70%) were in King's stage 1, 40 (26.85%) in stage 2, 15 (10.07%) in stage 3, and 8 (5.37%) in stage 4; among patients carrying other gene mutations

Table 2 demographic and clinical features of C9 + patients according to sex

Clinical features**	Women ($n=78$) *N* (%), mean [SD]	Men ($n=72$) *N* (%), mean [SD]	Total ($n=150$) *N* (%), mean [SD]	*p* value
Mean age at onset, years	58.58 [9.29]	59.26 [8.77]	58.91 [9.02]	0.648
Mean diagnostic delay, months	9.29 [6.34]	8.54 [7.17]	8.93 [6.74]	0.496
Weight loss at diagnosis, kg	4.07 [5.03]	3.10 [4.45]	3.60 [4.76]	0.239
BMI diagnosis	23.14 [4.82]	24.73 [3.92]	23.90 [4.47]	0.037
Site of onset				0.491
Bulbar	37 (47.44)	27 (37.50)	64 (42.67)	0.247
Spinal, UL	18 (23.08)	18 (25.00)	36 (24.00)	0.849
Spinal, LL	22 (28.21)	24 (33.33)	46 (30.67)	0.594
Respiratory	0 (0.00)	2 (2.78)	2 (1.33)	0.228
Phenotype				0.369
Bulbar	35 (44.87)	25 (34.72)	60 (40.00)	0.246
Classic	32 (41.03)	30 (41.67)	62 (41.33)	1.000
Flail arm	1 (1.28)	4 (5.56)	5 (3.33)	0.192
Flail leg	6 (7.69)	5 (6.94)	11 (7.33)	1.000
UMN-p	4 (5.13)	5 (6.94)	9 (6.00)	0.742
Respiratory	0 (0.00)	2 (2.78)	2 (1.33)	0.225
FTD	10 (12.82)	6 (8.33)	16 (10.67)	0.374
Parkinsonism	0 (0.00)	1 (1.41)	1 (0.69)	0.309
Family history of ALS/FTD	22 (75.86)	12 (57.14)	34 (68.00)	0.161
ALSFRS-R score at diagnosis	40.20 [7.95]	38.59 [11.01]	39.43 [9.53]	0.314
Progression rate at diagnosis*	1.42 [2.82]	2.33 [3.71]	1.86 [3.30]	0.090
Progression rate – first to last visit#	1.73 [1.09]	2.44 [1.84]	2.05 [1.49]	0.161
FVC at diagnosis	96.71 [27.44]	77.12 [22.02]	88.60 [26.78]	0.050
Monthly FVC decline§	6.13 [5.72]	5.55 [4.79]	5.90 [5.24]	0.814
Riluzole treatment	34 (85.00)	35 (97.22)	69 (90.79)	0.066
PEG	35 (47.94)	34 (50.00)	69 (48.94)	0.807
NIV	24 (32.88)	31 (46.27)	55 (39.29)	0.105
IV	8 (10.67)	16 (23.19)	24 (16.67)	0.044
Time from ALS onset to PEG, months	22.00 [9.17]	23.62 [14.62]	22.79 [12.10]	0.583
Time from onset to NIV, months	25.83 [12.33]	23.52 [15.12]	24.50 [13.92]	0.552
Time from onset to IV, months	31.00 [11.84]	21.00 [13.56]	24.33 [13.63]	0.090
Time from onset death or tracheostomy or last observation, months	39.13 [22.93]	28.35 [18.64]	33.96 [23.97]	0.002

Spinal UL spinal upper limb, *Spinal LL* spinal lower limb, *UMN-p* upper motor neuron predominant, *FTD* frontotemporal dementia, *FVC* forced vital capacity, *PEG* percutaneous endoscopic gastrostomy, *NIV* non-invasive ventilation, *IV* invasive ventilation, *SD* standard deviation

*Progression rate at diagnosis is calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset

**FVC at diagnosis was available for 29 patients, monthly FVC decline for 20 patients; information on PEG positioning was available for 141 patients, data on NIV was available for 140 patients, and data on IV for 144 patients. ALSFRS-R monthly decline was available for 149 patients. Family history was available for 50 patients, riluzole data for 76 patients, presence of parkinsonism for 144 patients. Phenotypes were available for 149 patients, and site of onset for 148 patients

#Progression rate first to last visit is calculated as monthly decline of ALSFRS-R score from the first to the last available visit or from the first visit to the first time when a total score of 0 was recorded (whichever came first)

§Monthly decline of FVC has been calculated considering first FVC% value, the last available FVC% value, and time between the first and last FVC

60 (51.72%) were in King's stage 1, 30 (25.86%) in stage 2, 13 (11.21%) in stage 3, and 6 (5.17%) in stage 4 ($p=0.205$). From comparisons among the three patients' cohorts, there was a significant difference only in the frequency of patients in stage 4, that was higher in *nmALS* with respect to *C9 + ALS* ($p=0.023$).

A family history of ALS or FTD was more frequent in *C9+* patients in comparison with *nmALS* (12.85 vs 68%, $p < 0.001$). Also FTD was more frequently detected in *C9+ALS* subgroup (3.93 vs 10.67%, $p < 0.001$) than in *nmALS*.

Table 2 shows overall demographic and clinical features of *C9+* patients, stratified by sex.

There were 83 patients under 60 years at diagnosis, 55 patients between 60 and 70 years and 12 patients over 70 years.

We observed that progression rate at diagnosis was on average higher in males than in females, though with a wide variation and without reaching statistical significance, especially among patients < 60 years at diagnosis (3.03 ± 4.35 in men versus 1.58 ± 3.51 in women, $p = 0.096$) and among the eldest (i.e., patients > 70 years at diagnosis) (0.95 ± 0.61 in men versus 0.37 ± 0.21 in women, $p = 0.054$), although without reaching statistical significance. Interestingly, age alone did not impact on progression rate at diagnosis, regardless of sex ($p = 0.224$).

The same trend was observed also for progression rate measured from first to last visit, and for FVC decline (data not shown).

Patients' comorbidities

Table 3 shows the prevalence of comorbidities in the analysed cohort. Considering diabetes, respiratory, cardiac, autoimmune, thyroid, haematological, psychiatric, neoplastic, urologic, metabolic disorders, we didn't find any statistical difference between the three groups. Prevalence of hypertension and gastrointestinal diseases was more frequent among *nmALS* patients, while COPD was less frequent in *C9+* patients.

Next, we analyzed whether differences existed in terms of number of comorbidities among the three groups: among *nmALS* patients, 439 (22.68%) had no comorbidities, 534 (27.58%) had one comorbidity, 490 (25.31%) had two comorbidities, 273 (14.10%) had three comorbidities, and 200 (10.33%) had four or more comorbidities. Patients harboring mutations had the following frequency of comorbidities: 52 (34.67%) C9+ patients and 42 (35.59%) other mutation carriers had no comorbidities, 43 (28.67%) C9+ and 30 (25.52%) other mutation carriers

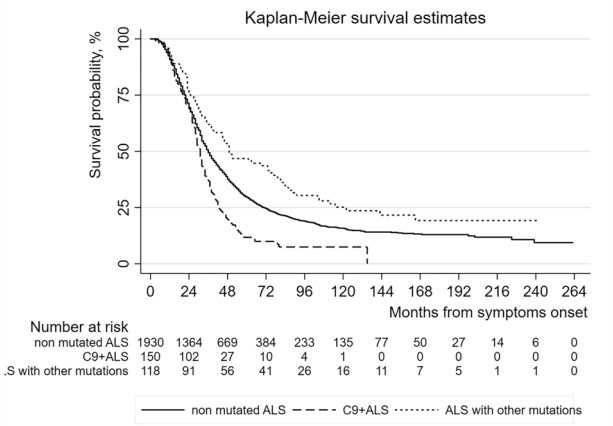

Fig. 2 Kaplan–Meier analysis of time to tracheostomy-free survival from symptom onset comparing *C9+* ALS, *nmALS* and ALS with other gene mutations

Table 3 Comorbidities distribution in *C9+* patients, *nmALS* patients and the population carrying another ALS associated gene mutation

Comorbidities	*nmALS* ($n = 1936$) *N* (%), mean [SD]	*C9+ALS* ($n = 150$) *N* (%), mean [SD]	Other mutations ($n = 118$) *N* (%), mean [SD]	*p* value
Hypertension	928 (47.93)	56 (37.33)	41 (34.75)	0.001
COPD	145 (7.49)	1 (0.67)	7 (5.94)	0.006
Other respiratory diseases	124 (6.40)	7 (4.67)	6 (5.08)	0.608
Heart diseases	264 (13.64)	10 (6.67)	15 (12.71)	0.051
Autoimmune disorders	138 (7.13)	12 (8)	6 (5.08)	0.633
Diabetes	194 (10.02)	12 (8)	6 (5.08)	0.165
Thyroid diseases	219 (11.31)	23 (15.33)	11 (9.32)	0.248
Psychiatric disorders	129 (6.66)	9 (6)	8 (6.78)	0.949
Haematological disorders	63 (3.25)	1 (0.67)	1 (0.85)	0.075
Neoplastic disease	257 (13.27)	14 (9.33)	15 (12.71)	0.382
Urologic diseases	101 (5.22)	3 (2)	5 (4.24)	0.202
Gastrointestinal diseases	332 (17.16)	15 (10)	14 (11.86)	0.029
Metabolic disorders*	301 (15.55)	14 (9.33)	14 (11.86)	0.075

*Metabolic disorders included dyslipidemia, hyperhomocysteinemia, hyperuricemia and gout, and obesity; patients with dyslipidemia were 264 (13.64%) among non-mutated ALS, 13 (8.66%) among C9orf72 ALS, and 11 (9.32%) among patients carrying other mutations ($p = 0.125$)

Table 4 univariate Cox regression analysis of survival in *C9* + ALS and in *nmALS* patients

Variable	C9 + ALS ($n = 150$)		nmALS ($n = 1936$)	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Gender				
Female	1		1	
Male	1.75 (1.23–2.48)	0.002	1.00 (0.91–1.11)	0.942
Site of onset	0.93 (0.77–1.12)	0.458	0.79 (0.74–0.84)	< 0.001
Bulbar			1	
Upper limbs			0.64 (0.57–0.73)	< 0.001
Lower limbs			0.57 (0.50–0.64)	< 0.001
Respiratory			1.35 (0.96–1.91)	0.082
Phenotype	1.03 (0.90–1.18)	0.678	0.89 (0.87–0.92)	< 0.001
Classic			1	
Bulbar			1.63 (1.45–1.85)	< 0.001
Flail arm			0.85 (0.60–1.23)	0.395
Flail leg			0.87 (0.74–1.02)	0.088
UMN-predominant			0.53 (0.44–0.63)	< 0.001
Respiratory			1.95 (1.40–2.74)	< 0.001
FTD	2.61 (1.49–4.58)	0.001	1.61 (1.19–2.17)	0.002
Parkinson	15.13 (1.53–149.50)	0.020	0.79 (0.46–1.36)	0.413
Weight loss at diagnosis	1.03 (0.99–1.08)	0.147	1.04 (1.03–1.05)	< 0.001
BMI at diagnosis	1.00 (0.96–1.04)	0.894	0.97 (0.96–0.98)	< 0.001
ALSFRS-R score at diagnosis	0.98 (0.96–0.99)	0.010	0.98 (0.97–0.98)	< 0.001
FVC at diagnosis	1.00 (0.99–1.00)	0.318	1.00 (0.99–1.00)	< 0.001
Progression rate at diagnosis	1.11 (1.06–1.15)	< 0.001	1.10 (1.08–1.12)	< 0.001
Age, years	1.05 (1.02–1.07)	< 0.001	1.03 (1.03–1.04)	< 0.001
Diagnostic delay	0.95 (0.92–0.98)	< 0.001	0.96 (0.95–0.96)	< 0.001
Riluzole	1.07 (0.57–1.99)	0.839	1.24 (1.01–1.50)	0.032
Family history of ALS/FTD	0.76 (0.46–1.26)	0.289	1.03 (0.84–1.26)	0.758
Hypertension	1.22 (0.86–1.75)	0.271	1.29 (1.17–1.43)	< 0.001
COPD	5.02 (0.68–36.97)	0.113	1.40 (1.17–1.68)	< 0.001
Other respiratory diseases	1.33 (0.62–2.85)	0.468	0.92 (0.74–1.12)	0.391
Heart diseases	1.04 (0.51–2.14)	0.905	1.35 (1.17–1.55)	< 0.001
Autoimmune disorders	1.02 (0.55–1.91)	0.946	1.14 (0.94–1.38)	0.173
Diabetes	1.22 (0.66–2.27)	0.529	1.09 (0.93–1.29)	0.283
Thyroid diseases	0.50 (0.29–0.84)	0.009	0.95 (0.81–1.12)	0.552
Psychiatric disorders	0.89 (0.36–2.19)	0.801	1.08 (0.88–1.32)	0.465
Haematological disorders	0.42 (0.06–3.00)	0.384	1.34 (1.03–1.75)	0.031
Neoplastic disease	1.13 (0.64–2.00)	0.679	1.16 (1.01–1.35)	0.042
Urologic diseases	0.94 (0.30–2.97)	0.921	1.31 (1.05–1.62)	0.015
Gastrointestinal diseases	1.46 (0.84–2.55)	0.181	1.14 (1.00–1.30)	0.056
Metabolic disorders	1.40 (0.79–2.49)	0.253	0.96 (0.83–1.10)	0.539

UMN-p upper motor neuron predominant, *FTD* frontotemporal dementia, *BMI* body mass index, *ALSFRS-R* ALS functional rating scale—revised, *COPD* chronic obstructive pulmonary disease, *HR* hazard ratio, *CI* confidence interval

*A separate analysis for dyslipidemia was carried out; the presence of dyslipidemia was not associated to a different survival both in C9ORF72 ALS (HR 1.30, 95% CI 0.71–2.36, $p = 0.396$) and in non-mutated ALS (HR 0.93, 95% CI 0.79–1.08, $p = 0.325$)

had one comorbidity, 39 (26.00%) C9 + and 26 (22.03%) other mutations carriers had two comorbidities, 12 (8.00%) C9 + and 14 (11.86%) other mutation carriers had three comorbidities, 4 (2.67%) C9 + and 6 (5.08%) other mutation carriers had four or more comorbidities ($p < 0.001$). From comparisons among the three patients' cohorts, there

was a significant difference in the frequency of patients without comorbidities, that was lower in *nmALS* with respect to *C9+ALS* and patients with other gene mutations ($p < 0.001$), and in the frequency of patients with four or more comorbidities, that was higher in *nmALS* with respect to *C9+ALS* and other mutations carriers ($p = 0.002$).

C9ORF72, disease progression and survival

C9+ patients showed a shorter survival with respect to other genotyped patients: median survival was 31 months from disease onset in *C9+* ALS and 37 months for other ALS patients (HR = 1.50, 95% C.I. 1.25–1.79, $p < 0.001$) (Fig. 2).

Factors influencing survival differed between *C9+* and *nmALS* patients. Table 4 shows univariate Cox regression analysis of survival in the two populations.

In *nmALS* patients, multivariate analysis of survival showed that independent prognostic factors for tracheostomy free survival were weight loss at diagnosis (kg) (HR = 1.03, 95% C.I: 1.02–1.04, $p < 0.001$), BMI at diagnosis (HR = 0.98, 95% C.I: 0.97–0.99, $p < 0.001$), ALSFRS-R score at diagnosis (1 point) (HR = 0.98, 95% C.I: 0.97–0.98, $p < 0.001$), age at onset (years) (HR = 1.03, 95% C.I: 1.03–1.04, $p < 0.001$), diagnostic delay (months) (HR = 0.95, 95% C.I: 0.95–0.96, $p < 0.001$), FTD (presence) (HR = 1.48, 95% C.I: 1.09–2.01, $p = 0.012$), site of onset (HR = 0.92, 95% C.I. 0.86–0.98, $p = 0.007$), phenotype (HR = 0.92, 95% C.I: 0.89–0.95, $p < 0.001$), cardiovascular diseases (presence) (HR = 1.16, 95% C.I: 1.01–1.35, $p = 0.040$).

In *C9+* ALS patients, multivariate analysis of survival showed that independent prognostic factors for tracheostomy free survival were gender (male, worse prognosis) (HR = 1.87, 95% C.I: 1.28–2.72, $p = 0.001$), presence of FTD (worse prognosis) (HR = 4.01, 95% C.I: 2.21–7.26, $p < 0.001$), age at onset (years, worse prognosis with increasing age) (HR = 1.06, 95% C.I: 1.03–1.08, $p < 0.001$), progression rate at diagnosis (monthly decline, worse prognosis with higher monthly decline) (HR = 1.12, 95% C.I: 1.07–1.17, $p < 0.001$), presence of thyroid disorders (median survival for patients with thyroid disorders 43 months, median survival for patients without thyroid disorders 29 months; HR = 0.50, 95% C.I: 0.28–0.87, $p = 0.016$) (Fig. 3).

Patients with thyroid disorders (23 out of 150 *C9+ALS*) had a phenotypic profile very similar to other C9 + ALS patients, except for a higher frequency of female patients (Table 5). For 16 patients a detailed description of thyroid disorder was available, with a medical report of hypothyroidism for 12 of them.

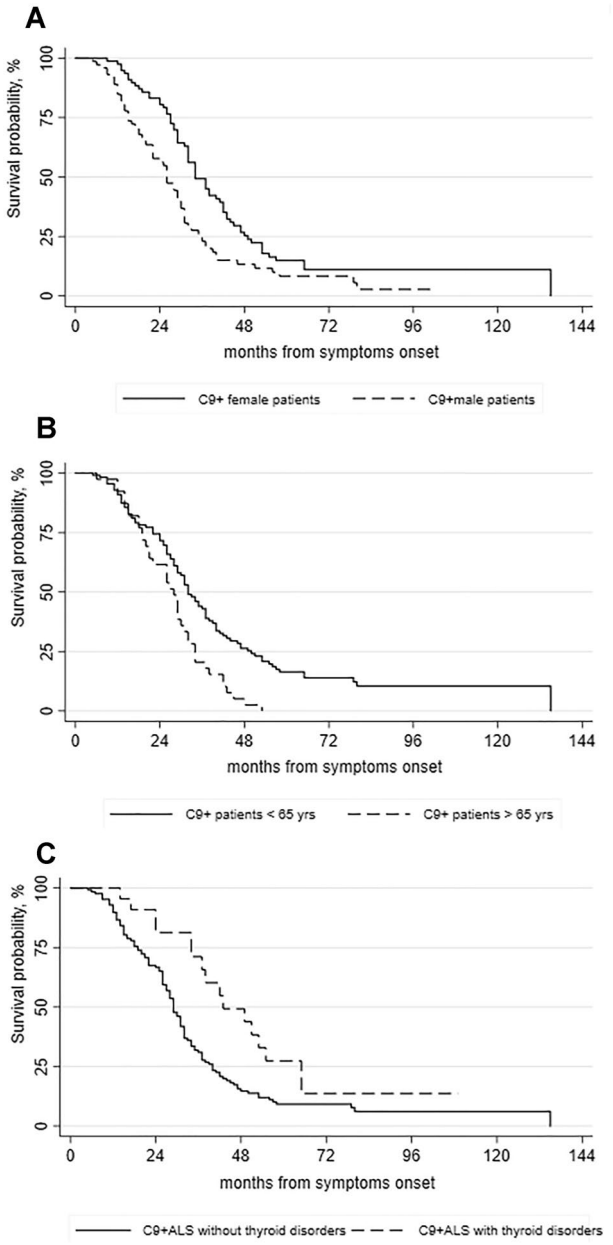

Fig. 3 Kaplan–Meier analysis of time to tracheostomy-free survival from symptom onset in *C9+ALS* patients by gender (**A**), age classes (< or ≥ 65 years) (**B**), presence or absence of thyroid disorders (**C**)

Discussion

This is the first population-based study based on prospectively collected data from two Italian registries on *C9ORF72* expansion carriers analyzing the unique clinical and prognostic profile of this peculiar ALS population.

Table 5 demographic and clinical features of C9 + patients according to presence or absence of thyroid disorders

Clinical features	Presence of thyroid disorders *N* (%), mean [SD]	Absence of thyroid disorders *N* (%), mean [SD]	Total *N* (%), mean [SD]	*p* value
Sex, male	3 (13.04)	69 (54.33)	72 (48.00)	< 0.001
Mean age at onset, years	56.79 [9.45]	59.29 [8.92]	58.91 [9.02]	0.222
Mean diagnostic delay, months	9.61 [7.00]	8.81 [6.71]	8.93 [6.74]	0.603
Weight loss at diagnosis, kg	4.47 [5.15]	3.47 [4.73]	3.60 [4.76]	0.411
BMI diagnosis	24.02 [5.35]	23.87 [4.33]	23.90 [4.47]	0.899
Site of onset				0.653
Bulbar	10 (43.48)	54 (42.52)	64 (42.67)	0.932
Spinal, UL	6 (26.09)	30 (23.62)	36 (24.00)	0.799
Spinal, LL	6 (26.09)	40 (31.50)	46 (30.67)	0.605
Respiratory	0 (0.00)	2 (1.57)	2 (1.33)	0.716
Unknown	1 (4.35)	1 (0.79)	2 (1.33)	0.303
Phenotype				0.513
Bulbar	9 (39.13)	51 (40.16)	60 (40.00)	0.926
Classic	10 (43.48)	52 (40.94)	62 (41.33)	0.820
Flail arm	2 (8.70)	3 (2.36)	5 (3.33)	0.149
Flail leg	0 (0.00)	11 (8.66)	11 (7.33)	0.143
UMN-p	2 (8.70)	7 (5.51)	9 (6.00)	0.630
Respiratory	0 (0.00)	2 (1.57)	2 (1.33)	0.716
Unknown	0 (0.00)	1 (0.79)	1 (0.67)	0.836
FTD	2 (8.70)	12 (9,45)	14 (9.33)	0.909
Parkinsonism	0 (0.00)	1 (0.81)	1 (0.69)	0.687
Family history of ALS/FTD	9 (81.82)	25 (64.10)	34 (68.00)	0.266
ALSFRS-R score at diagnosis	40.33 [5.05]	39.28 [10.11]	39.43 [9.53]	0.641
Progression rate at diagnosis*	2.38 [5.08]	1.77 [2.91]	1.86 [3.30]	0.428
Progression rate – first to last visit#	1.28 [081]	2.26 [1.58]	2.05 [1.49]	0.102
FVC at diagnosis	100 [20.63]	85.63 [27.77]	88.60 [26.78]	0.249
Monthly FVC decline§	6.83 [6.05]	5.59 [5.14]	5.90 [5.24]	0.658
Riluzole treatment	11 (78.57)	58 (93.55)	69 (90.79)	0.080
PEG	5 (22.73)	64 (53.78)	69 (48.94)	0.007
NIV	10 (43.48)	45 (38.46)	55 (39.29)	0.652
IV	1 (4.35)	23 (19.01)	24 (16.67)	0.084
Total	23 (15.33)	127 (84.67)	150 (100)	

Spinal UL spinal upper limb, *Spinal LL* spinal lower limb, *UMN-p* upper motor neuron predominant, *FTD* frontotemporal dementia, *FVC* forced vital capacity, *PEG* percutaneous endoscopic gastrostomy, *NIV* non-invasive ventilation, *IV* invasive ventilation, *SD* standard deviation

*Progression rate at diagnosis is calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset

#Progression rate first to last visit is calculated as monthly decline of ALSFRS-R score from the first to the last available visit or from the first visit to the first time when a total score of 0 was recorded (whichever came first)

§Monthly decline of FVC has been calculated considering first FVC% value, the last available FVC% value, and time between the first and last FVC

In this cohort of 150 patients carrying the *C9ORF72* hexanucleotide expansion, bulbar phenotype was more frequent than in the general ALS population (40%) and equally represented as the classic phenotype, as already reported [31]. Our study confirmed also that *C9* + ALS patients have a worse prognosis: they present a higher rate of disease progression as measured by ALSFRS-R and FVC monthly declines, a shorter diagnostic delay and an earlier onset [10]. They reach PEG, NIV and death or tracheostomy earlier than other patients [32]. As expected, there was a high proportion of patients with FTD and with family history for ALS and FTD than in other gene mutation carriers [33, 34].

nmALS patients at diagnosis were more frequently in advanced stages (King's college stage 4) compared to *C9+ALS*, probably due to their longer diagnostic delay.

Part of the shorter diagnostic delay in ALS mutation carriers might be explained by the higher frequency of a family history with a better recognition of motor symptoms typical of ALS within family member [35].

Progression rate in *C9+*ALS was higher in male patients, especially among the elderly, in line with literature [36–38], possibly because of an early respiratory dysfunction in male *C9+ALS* who in facts presented with lower baseline values of FVC, a higher FVC monthly decline and more patients undergoing IV.

As a novelty of this study, we attempted to investigate whether other salient clinical features and comorbid conditions may suggest for the presence of C9 expansion in ALS patients before the genetic results.

Despite its great neurological phenotypic variability and the clinical picture in C9-ALS animal models, *C9+ALS* patients did not have a significantly higher prevalence of concomitant diseases with respect to other genotyped patients. We also failed to find a significantly higher prevalence of autoimmune diseases, diabetes, thyroid, hematological and even psychiatric diseases in *C9+ALS* patients compared to *nmALS*. On the contrary, a minor burden of concomitant diseases was found in *C9+ALS* patients, probably due to their younger age.

This is one of the largest population-based studies analyzing factors related to survival in *C9+ALS*. Male gender was already associated with a worse prognosis in *C9+ALS* [36, 38], differently from *nmALS* patients. However, in our multivariate analysis in the two independent cohorts, we found that *nmALS* patients with spinal onset survived longer than patients with bulbar or respiratory site onset, whereas survival in *C9+ALS* patients did not vary across different site of onset. This suggests that it is the genotype (together with age and sex) that influences disease phenotype and progression [39] and not the other way around.

In previous studies weight loss was demonstrated to be a strong and independent negative prognostic factor in ALS population [40], possibly in relation to a hypermetabolic state or a catabolic metabolism [41], a higher impairment of bulbar function (with dysphagia and reduced diet income) and a ventilatory dysfunction (causing inappetence).

We did not find a prognostic role of weight loss in our cohort of *C9+ALS*, but thyroid disorders resulted as an independent variable affecting survival. Consistently, mean ALSFRS-R monthly decline from first to last visit was decreased in *C9+ALS* patients with thyroid disorders, although not achieving statistical significance (1.28 points/month versus 2.26 points/month, $p=0.102$). Thyroid disorders are associated with a longer survival exclusively in *C9+ALS* patients. In line with our observation, a previous study focusing on comorbidities in a single-center cohort of ALS patients (without genotypization), found that thyroid disorders together with other diseases were associated with a delayed age at onset, and hypothesized the role of hypervigilant regulation in disease onset [42].

The neutral impact of weight loss/BMI at diagnosis in our population could argue against the slowing effect of hypothyroidism on metabolism and its protective role in ALS progression. Still, weight control is multifactorial in ALS, including higher waste of energy because of muscle fasciculations, increasing respiratory efforts, hypermetabolism and decreased food intake due to depression [43], all factors that could not be punctually and quantitively measured in each patient.

In our cohort of patients suffering from thyroid disorders the vast majority presented hypothyroidism (12 out of 16 patients). These findings may suggest that the metabolic status may play a role for the prognosis of C9-ALS: if a catabolic attitude may worsen the disease progression and decline, on the opposite side an anabolic attitude may slow down the disease progression. Differences between *C9+ALS* patients and other ALS patients from a metabolic point of view, even years before disease onset, have been recently reported [36]. Nevertheless, the mechanisms by which thyroid disorders or in general thyroid function may influence ALS remains elusive.

As a proxy of an altered metabolism, we also examined dyslipidemia, but we could not find a role for it as a prognostic factor neither in *C9+ALS*, nor in the other patients. Nevertheless, complex interactions among environmental factors including diet, gut microbiome and genetic factors may have an effect on dyslipidemia [44].

Previous basic research demonstrate that hypermetabolism could exacerbate the rate of motor neuron degeneration by increasing the production of reactive oxygen species in mitochondria [45], and an exploratory trial with thyrotropin releasing hormone (TRH) intrathecal infusion, determining a catabolism secondary to drug-induced hypermetabolism, did not induce clinical improvement in ALS patients [46].

Nevertheless, very early studies in ALS did not find alteration in thyroid function in ALS patients with respect to controls [47] or in correlation with survival [48], suggesting that thyroid hormone by itself did not represent a prognostic factor for ALS.

Although a pharmacologic approach with methimazole leading to drug-induced hypothyroidism does not alter the disease course in the SOD1-G93A ALS mouse [49] it would be of interest to perform the same treatment in *C9ORF72* mouse model, reflecting a distinct disease pathogenesis among patients with different genetic background. Interestingly, the protein μ-crystallin (CRYM) which is a key regulator of thyroid hormone transportation and a reductase of

sulfur-containing cyclic ketimines, is expressed in the corticospinal tract, and in human ALS brains was found to be markedly reduced, suggesting that later in life, CRYM may perform cell-specific functions in selected neuronal populations through its interactions with T3 or ketimines in these cells and organs [50]. Recently, an interesting point linking TDP-43 pathology and thyroid function has been revealed. Nelson et al. demonstrate that, combined with the evidence that several genetic factors could modulate T3 and T4 levels in brain parenchyma, the dysregulation of thyroid hormone signaling may play a role in age-related TDP-43 proteinopathy [51].

Finally, a pathological involvement of epithelial hormone-producing cells of the pituitary gland and of hypothalamic pituitary hormone-stimulating nuclei have been documented especially in *C9+ALS* and DPR pathology, whereas pTDP-43 aggregates modestly affected hypothalamic–pituitary axis. Whether this pituitary involvement may interfere with hormone regulation and secretion could itself contribute in ALS pathogenesis, remains to be elucidated [52].

The main limitations of this study are represented by the retrospective nature of our analysis, the small sample size of some analyzed patients' subgroup (i.e., *C9+ALS* patients with thyroid disorders), and by the lack of systematic analysis of other genetic variants associated to *C9ORF72* expansion. Furthermore, excluding those patients who did not undergo genetic testing may have biased the control cohort's features (e.g., family history prevalence). Since our finding regarding thyroid comorbidity's role on survival has never been described in literature, it deserves to be further explored, in larger samples and prospectively, in order to overcome the instability of data due to the small number of *C9+ALS*. Should the data be confirmed, it could suggest new pathogenic pathways of ALS associated to *C9ORF72* expansion, as well as point out new therapeutic possibilities to slow down the disease progression.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00415-022-11426-y.

Acknowledgements The authors thank all the ERRALS and all the PARALS members.

Author contributions Conception and design of the study: JM, EZ, IM, RT, MV, ACh, ACal. Acquisition and analysis of data: JM, EZ, IM, LVDM, GG, CM, UM, LS, RV, ACan, GM, MB, LM, FDM, CS, NF, VM. Drafting a significant portion of the manuscript or figures: JM, EZ, RT, ACh, ACal. Funding: JM, LM, ACh, Aca. All authors had access to all the study data and read, contributed to, reviewed and approved the submission of the manuscript for final publication.

Funding The Emilia Romagna Registry for ALS (ERRALS) is supported by a Grant from the Emilia Romagna Regional Health Authority. This work was in part supported by the Italian Ministry of Health (Ministero della Salute, Ricerca Sanitaria Finalizzata, grant RF-2016-02,362,405), the European Commission's Health Seventh Framework Programme (FP7/2007–2013 under grant agreement 259,867), the Italian Ministry of Education, University and Research (Progetti di Ricerca di Rilevante Interesse Nazionale, PRIN, grant 2017SNW5MB), the Joint Programme—Neurodegenerative Disease Research (ALS-Care, Strength and Brain-Mend projects), granted by the Italian Ministry of Education, University and Research, the Horizon 2020 Programme (project Brainteaser under grant agreement 101,017,598). This study was performed under the Department of Excellence grant of the Italian Ministry of Education, University and Research to the 'Rita Levi Montalcini' Department of Neuroscience, University of Torino, Italy and to the AGING Project for Department of Excellence at the Department of Translational Medicine (DIMET), Università del Piemonte Orientale, Novara,. Italy.

Data availability Data are available from the authors upon reasonable request.

Declarations

Conflict of interest The authors have no conflict of interest.

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