ATXN2 polyQ intermediate repeats are a modifier of ALS survival.

ATXN2 polyQ intermediate repeats are a modifier of ALS survival

Adriano Chiò, MD, FAAN, Andrea Calvo, MD, PhD, Cristina Moglia, MD, Antonio Canosa, MD, Maura Brunetti, BSc, Marco Barberis, BSc, Gabriella Restagno, MD, Amelia Conte, MD, Giulia Bisogni, MD, Giuseppe Marangi, MD, Alice Moncada, MD, Serena Lattante, MD, Marcella Zollino, MD, Mario Sabatelli, MD, Alessandra Bagarotti, PhD Lucia Corrado, PhD, Gabriele Mora, MD, Enrica Bersano, MD, Letizia Mazzini, MD; Sandra D'Alfonso, PhD for the PARALS*

From: the ALS Center, 'Rita Levi Montalcini' Department of Neuroscience, University of Torino, Torino, Italy (Chiò, Calvo, Moglia, Canosa, Brunetti, Barberis); the Azienda Ospedaliero Universitaria Città della Salute e della Scienza di Torino, Torino, Italy (Chiò, Calvo); the Neuroscience Institute of Torino (NIT), Torino, Italy (Chiò, Calvo); the Department of Neurosciences, Ophthalmology, Genetics, Rehabilitation and Child Health, University of Genoa (Canosa); the Laboratory of Molecular Genetics, Azienda Ospedaliero Universitaria Città della Salute e della Scienza di Torino, Torino (Brunetti, Barberis, Restagno); the Neurological Institute, Catholic University of the Sacred Heart, Rome, Italy (Conte, Bisogni, Sabatelli); the Institute of Medical Genetics, Catholic University of the Sacred Heart, Rome, Italy (Marangi, Moncada, Lattante, Zollino); the Department of Health Sciences, Interdisciplinary Research Center of Autoimmune Diseases (IRCAD), 'Amedeo Avogadro' University of Eastern Piedmont, Novara, Italy (Bagarotti, Corrado, D'Alfonso); the Salvatore Maugeri Foundation, IRCSS; Scientific Institute of Milan, Milan, Italy (Mora); the Department of Neurology, 'Amedeo Avogadro' University of Eastern Piedmont and Azienda Ospedaliera Universitaria Maggiore della Carità, Novara, Italy (Bersano, Mazzini).

* A list of the members of PARALS is provided in the Co-investigator Appendix

Abstract word count: 247

Text work count: 2643

Character count for title: 63

Number of references: 40

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

Running title: *ATXN2* polyQ repeats in ALS

Disclosures

Dr. Chiò serves on the editorial advisory board of Amyotrophic Lateral Sclerosis and has received research support from the Italian Ministry of Health (Ricerca Finalizzata), Regione Piemonte (Ricerca Finalizzata), University of Torino, Federazione Italiana Giuoco Calcio, Fondazione Vialli e Mauro onlus, and the European Commission (Health Seventh Framework Programme); he serves on scientific advisory boards for Biogen Idec and Cytokinetics. Dr. Calvo has received research support from the Italian Ministry of Health (Ricerca Finalizzata). Dr. Moglia reports no disclosures. Dr. Canosa reports no disclosures. Dr. Brunetti reports no disclosures. Dr. Barberis reports no disclosures. Dr. Restagno has received research support from the Italian Ministry of Health (Ricerca Finalizzata) and Regione Piemonte (Ricerca Finalizzata). Dr. Conte reports no disclosures. Dr. Bisogni reports no disclosures. Dr. Marangi reports no disclosures. Dr. Moncada reports no

disclosures. Dr. Lattante reports no disclosures. Dr. Zollino reports no disclosures. Dr. Sabatelli reports no disclosures. Dr. Bagarotti reports no disclosures. Dr. Corrado reports no disclosures. Dr. Mora reports no disclosures. Dr. Bersano reports no disclosures. Dr. Mazzini reports no disclosures. Dr. D'Alfonso reports no disclosures.

Author Contributions: *Study concept and design:* Chiò, Calvo, Restagno, Zollino, Sabatelli, Mora, Mazzini, D'Alfonso. *Acquisition of data:* Moglia, Canosa, Brunetti, Barberis, Conte, Bisogni, Marangi, Moncada, Lattante, Bagarotti, Corrado, Bersano. *Analysis and interpretation of data:* Chiò, Calvo, Restagno, Zollinjo, Sabatelli, Mora, Mazzini, D'Alfonso. *Drafting of the manuscript:* Chiò, Restagno, Sabatelli, Mora, Mazzini, D'Alfonso. *Critical revision of the manuscript for important intellectual content:* Chiò, Calvo, Restagno, Zollinjo, Sabatelli, Mora, Mazzini, D'Alfonso, Moglia, Canosa, Brunetti, Barberis, Conte, Bisogni, Marangi, Moncada, Lattante, Bagarotti, Corrado, Bersano. *Obtained funding:* Chiò, Sabatelli. *Administrative, technical, and material support:* Moglia, Canosa, Brunetti, Barberis, Conte, Bisogni, Marangi, Moncada, Lattante, Bagarotti, Corrado, Bersano. *Study supervision:* Chiò, Restagno, Sabatelli, Mora, Mazzini, D'Alfonso.

Adriano Chiò had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have approved the submitted version of the paper.

Abstract

Objective. To analyze the frequency and clinical characteristics of ALS patients with intermediate-length (CAG) expansion (encoding 27-33 glutamines, polyQ) in the *ATXN2* gene, in a population-based cohort of Italian ALS patients (discovery cohort), and to replicate the findings in an independent cohort of consecutive patients from an ALS tertiary center (validation cohort).

Methods. PolyQ repeats were assessed in 672 ALS patients incident in Piemonte and Valle d'Aosta regions, Italy, in the 2007-2012 period (discovery cohort); controls were 509 neurologically healthy age- and gender-matched subjects resident in the study area. The validation cohort included 661 ALS patients consecutively seen between 2001 and 2013 in the ALS Clinic Center of the Catholic University in Rome, Italy.

Results. In the discovery cohort the frequency of ≥31 polyQ *ATNX2* repeats was significantly more common in ALS cases (19 patients *vs*. 1 control, p=0.0001; odds ratio 14.8, 95% confidence interval, 1.9-110.8). Patients with an increased number of polyQ repeats had a shorter survival than those with <31 repeats (median survival, polyQ ≥31, 1.8 years, interquartile range [IQR] 1.3-2.2; polyQ <31, 2.7 years, IQR 1.6-5.1) (p=0.001). An increased number of polyQ repeats remained independently significant also at multivariable analysis. In the validation cohort, patients with ≥31 polyQ repeats had a shorter survival than those with <31 repeats (median survival, polyQ ≥31, 2.0 years, IQR 1.5-3.4; polyQ <31, 3.2 years, IQR 2.0-6.4; p=0.007).

Conclusions. *ATXN2* polyQ intermediate-length repeat is a modifier of ALS survival. Disease-modifying therapies targeted to *ATXN2* represent a promising therapeutic approach for ALS.

Keywords: Amyotrophic Lateral Sclerosis; Ataxin2 gene; prognosis; phenotype

Amyotrophic lateral sclerosis (ALS) is characterized by a progressive degeneration of upper and lower motor neurons, leading to loss of motor function and, eventually, to complete palsy of limb muscles, loss of speech and swallowing, and respiratory failure, usually within 2 to 3 years after onset. Several prognostic factors are known in ALS, in particular age at onset, bulbar onset, diagnostic delay, and cognitive impairment.

With the exception of riluzole, which slightly increases survival, no disease-modifying drug is available. The cause of ALS is still unknown, but at least 20 genes have been related to the disease; the most common in Caucasian populations are *C9ORF72, SOD1, TARDBP* and *FUS*, which are present in about 10% of patients. In 2010, an intermediate-length (CAG) expansion (encoding 27-33 glutamines, polyQ) in the *ataxin 2* (*ATXN2*) gene, already known as the cause of spinocerebellar ataxia type 2 (SCA2),[1,2,3] was reported to be associated to an increased risk of developing ALS.[4] The association was subsequently confirmed in clinically-based series,[5-16] but the clinical characteristics of patients with this expansion still remain to be fully investigated.

The aim of this paper was to analyze the frequency of intermediate polyQ expansion in the *ATXN2* gene in a population-based cohort of ALS patients, with an in-depth assessment of their clinical and prognostic characteristics. The study findings would then be replicated in an independent replication cohort of consecutive patients from an ALS tertiary center.

Methods

Discovery cohort. The study population included all ALS cases diagnosed in Piemonte and Valle d'Aosta, Italy, during the 6-year period January 1 2007, to December 30 2012. ALS cases were recruited through the PARALS, a prospective epidemiologic register involving all the neurologic departments of the two regions of northern Italy. Epidemiologic data regarding the 1995–2004 period have been published elsewhere.[17] Both familial and apparently sporadic ALS patients were

included in the present study. The diagnosis of ALS was based on El Escorial revised criteria.[18] Patients with definite, probable, and probable laboratory-supported ALS were included in the register. Controls were (1) 395 regionally matched unrelated Italian subjects, mainly blood donors, already reported in a previous paper;[6] (2) 114 matched subjects identified through the patients' general practitioners (population-based controls). The two series of controls were similar for demographic characteristics and had a substantially similar frequency of ATXN2 intermediate polyQ expansions (0.4% and 0.3%, respectively).

Validation cohort. This cohort included 661 patients consecutively admitted between January 2001 and December 2013 to the ALS Clinic Center of the Catholic University in Rome, Italy. All patients were resident in the central or southern regions of Italy. Diagnostic criteria were identical to those of the discovery cohort. Data on 528 of the patients were already reported in a study on genetics of ALS patients.[19]

Genetic analysis. Genomic DNA was isolated from peripheral blood lymphocytes using a standard protocol. The ATXN-2 CAG repeat in exon 1 (Ref Seq NM_002973.3) was amplified using a fluorescent primer and sized by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystem, Foster City, CA, USA).[20] As reported in recent guidelines for molecular genetic testing of SCA,[21] capillary electrophoresis is the preferred method . This method allows to size alleles accurately and to resolve alleles one triplet apart in size. As a quality control, 20 samples have been genotyped in the different laboratories that performed the molecular genetic testing for the present study. The comparison of the results showed a consistent allele assignment for all the samples. Receiver operating characteristics (ROC) analysis showed that a cutoff ≥31 polyQ repeats in ATXN2 had the greatest sensitivity and specificity for discriminating ALS patients versus controls. However, also data related to a repeat size 27-30 were assessed.

All ALS cases of both cohorts were also tested for *SOD1* (all exons), *TARDBP* (exon 6), *FUS* (exons 14 and 15), *ANG* and *C9ORF72* using the methodology described elsewhere.[19,22] Familial ALS patients were also tested for *OPTN* (exons 5, 9, 12 and 14).

Statistical methods. Two-tailed Fisher's exact test was used to evaluate the genetic association between *ATXN2* polyQ repeat size and ALS. Survival was calculated from onset to death, tracheostomy or censoring date (December 31, 2013), using the Kaplan-Meier method, and compared with the log-rank test. No patients were lost to follow-up. Multivariable analysis was performed with the Cox proportional hazards model (stepwise backward) with a retention criterion of $p<0.1$. Significance level was set at $p<0.05$. Data were processed using SPSS statistical package version 21 (IBM Corporation, Chicago, IL, USA).

Ethical approval. The study was approved by the ethical committees of the participating centers. All patients and controls signed a written informed consent. Databases were treated with due respect for Italian privacy regulations.

Results

Discovery cohort

Out of a total of 869 ALS cases incident in the period January 1st 2007 to December 31st 2012, 672 (77.3%) patients were included in the study and tested for DNA. Data on 28 of them had already been reported in a previous paper.[6] Of the 197 patients not included in the study, 59 did not give their consent for genetic analysis, 60 died before blood sampling and 78 were found only through secondary sources and were therefore not tested for DNA. The patients not included in the genetic analysis had an older mean age at onset (67.9 [SD 10.7] vs. 65.6 [SD 10.8] years, $p<0.02$), but their site of onset and clinical phenotype were similar (data not shown). A total of 71 (10.6%) patients

carried a mutation of one of the ALS-related genes (*C9ORF72*=43; *SOD1*=15; *TARDBP*=10; *FUS* =2; *OPTN*=1).

ATXN2 polyQ repeat size at risk. The size of the *ATXN2* repeats in ALS patients compared to the control group is reported in Figure 1. The more common alleles (22 and 23) were identified in 98.9% of controls' chromosomes and 95.9% of cases' chromosomes. *ATXN2* repeats ≤30 were similarly distributed between ALS cases and controls, while those ≥31 were significantly more common in cases (19 cases and 1 control, p=0.0001); the odds ratio for ALS of having ≥31 repeats was 14.8 (95% confidence interval, 1.9-110.8) (Figure 1, insert). The second allele in patients with ≥27 repeats was 22 in all but one case, who had 29/24.

Clinical characteristics of patients according to ATXN2 polyQ repeat size. Demographic and clinical characteristics of patients with *ATXN2* polyQ repeats ≥31 vs. patients with polyQ repeats <31 are presented in Table 1. No significant differences were found in gender, age at onset, and presence of co-morbid FTD. Conversely, bulbar onset was present in only one case with *ATXN2* polyQ repeats ≥31 (~5%) versus about one third of patients with polyQ repeats <31 (p=0.0001). No patients with *ATXN2* polyQ repeats ≥31 had a positive family history for ALS or FTD. Of the 71 patients carrying a known genetic mutation, 69 were homozygous (22-22 repeats), one p.D90N *SOD1* patient had 22-24 repeats and one *C9ORF72* patient had 22-27 repeats. Clinical characteristics of ALS patients with ≥31 polyQ repeats of the ATXN2 gene are detailed in Table e-1. The single patient carrying 39 polyQ repeats had features of both ALS and SCA. She developed gait ataxia at the age of 55 and spinal ALS 12 years later; her mother was also affected by SCA. She underwent percutaneous endoscopic gastrostomy 12 months after the onset of ALS and died of respiratory failure at age 69.

Patients with ≥31 polyQ repeats had a shorter median survival than those with <31 repeats (1.8 years, interquartile range [IQR] 1.3-2.2 vs. 2.7 years, IQR 1.6-5.1; p=0.001) (Figure 2A). An increased number of polyQ repeats remained independently significant also at multivariable

analysis (Table 2). A second analysis was performed assessing separately the survival of patients with 27-30 polyQ repeats: this group had an intermediate survival time between those with ≥31 and those with <27 polyQ repeats, indicating a possible dose-response relationship (Figure 2C).

Replication cohort

Clinical and demographic characteristics of the 661 ALS patients included in this cohort are reported in Table 1. All were tested for ALS genes and 80 (12.1%) carried a mutation of one of the ALS-related genes (*C9ORF72*=34; *SOD1*=20; *TARDBP*=13; *ANG*=5, *FUS*=4; *OPTN*=3; *VCP*=1). Most of these cases were reported in a previous paper.[19] The more common alleles (22 and 23) were identified in 95.8% of chromosomes (Figure e-1). Sixteen cases had ≥31*ATXN2* polyQ repeats. The second allele in patients with ≥27 repeats was 22 in all cases.

Of the 80 patients carrying a known genetic mutation, 63 were homozygous (22-22 repeats); one *C9ORF72* patient and one p.I380V *TARDBP* patient had 19-22 repeats; two *C9ORF72* patients had 21-22 repeats; 3 *C9ORF72* patients, one p.R521C *FUS* patient, one p.D11Y *SOD1* patient and one p.S134N *SOD1* patient had 22-23 repeats; one heterozygous D90A *SOD1* patient had 22-26 repeats; one p.N87K *ANG* patient had 22-27 repeats; one p.G93D *SOD1* patient had 22-28 repeats; two *C9ORF72* patients had 22-29 repeats; and one p.N390S *TARDBP* patient had 22-30 repeats.

Out of the 16 patients with ≥31*ATXN2* polyQ repeats, 2 had a bulbar onset and 2 had a positive family history for ALS or FTD (Table e-1). Cognitive impairment was not systematically assessed in patients of this series. Patients with ≥31polyQ repeats had a shorter median survival than those with <31 repeats (2.0 years, IQR 1.5-3.4 vs. 3.2 years, IQR 2.0-6.4; p=0.007) (Figure 2B). Multivariable analysis confirmed that ≥31polyQ repeats was an independent prognostic factor (data not shown). Also in this cohort, patients with 27-30 polyQ repeats had an intermediate survival time between those with ≥31 and those with <27 polyQ repeats, indicating a possible dose-response relationship (Figure 2D).

Discussion

In this population-based study, which included ~80% of ALS patients incident in Piemonte/Valle d'Aosta over a 6-year period, we found that ≥31 polyQ repeats in the *ATXN2* gene represents a risk factor for ALS, with an odds ratio of 14.8. All but one patient with intermediate-length polyQ repeats had a spinal onset and all the patients who were positive for mutations of ALS-related genes had normal size polyQ repeats. To the best of our knowledge, the effect of an increased number of polyQ repeats in ALS outcome has never been studied. In our series, the presence of ≥31 polyQ repeats reduced the median survival by ~1 year, patients with 27-31 repeats showing an intermediate survival time between that of those with ≥31 and those with <27 polyQ repeats, and this effect persisted after adjusting for other known prognostic factors at multivariable analysis. These survival data were confirmed in a validation cohort of 661 consecutive patients from a referral ALS center in central Italy.

The correlation between *ATXN2* intermediate polyQ repeats and a shorter survival, observed in both our series, is further supported by finding that almost all patients in both series have a spinal onset. In fact, spinal onset is considered a positive prognostic factor in ALS,[23] and therefore these patients were expected to have a better prognosis.

Besides the correlation with spinal onset, *ATXN2* intermediate polyQ repeats in both our series did not influence the age at onset of ALS, differently from the original observation of a lower age at onset observed in the first report of ATXN2 in ALS,[4] but in keeping with all subsequent papers[5-16]

In the last few years several papers have highlighted the phenotypic variability of ALS, characterized by a wide range of age at onset, different clinical phenotypes, variable involvement of upper and lower motor neuron signs, possible impairment of cognitive function, and an extremely variable length of survival.[22-24] An increased understanding of the genetic background of ALS has

partly clarified its pathogenetic mechanisms, but has provided few clues about its clinical heterogeneity, with the possible exception of the frequent cognitive involvement in patients with *C9ORF72* mutations and the young age at onset and severe course of most patients carrying *FUS* mutations. The search for genes modulating the clinical expression of ALS has produced few, albeit interesting, findings. For example, polymorphisms of *Unc-13 homolog A* (*UNC13A*)[25,26] and *Non-Imprinted in Prader–Willi/Angelman syndrome 1* (*NI-PA1*)[27] have been associated to a shorter survival, while a locus on 1p34.1[28] has been associated to a younger age at onset. In our population-based series we found that intermediate-length polyQ repeats in the *ATXN2* gene are a strong modifier of ALS outcome in an Italian subgroup, reducing survival by one year, without modifying age at onset. This finding was replicated in an independent validation cohort from an ALS tertiary center. In addition, ALS patients carrying ≥31 polyQ repeats had more frequently a spinal onset. A meta-analysis of published case series (Table 3) revealed that 70 (88.6%) cases had a spinal onset, significantly more than the expected 65%.[29]

The mechanism underlying the increased risk due to an intermediate-length polyQ repeat in the *ATXN2* gene in ALS is still unclear. It was initially hypothesized that *ATXN2* intermediate-length polyQ repeats interact with TDP-43 augmenting its toxicity[4] or increase the translocation of TDP-43 and mutant FUS to the cytoplasm enhancing their pathogenicity.[30,31] More recent studies found that *ATXN2* intermediate-length polyQ repeats may interact with the NADPH oxidase enzyme, increasing ROS production, and may also interfere with RNA metabolism, sequestering essential RNA-binding proteins.[32]

≥31 polyQ repeats in the *ATNX2* gene have also been identified in patients with FTD (4/641, 0.6%),[33] but in no patients in a small series of subjects with FTD-ALS.[15] In our discovery cohort, behavioral FTD was found in two patients (10.5%) with ≥31 polyQ repeats, compared to 76 patients (12.4%) with <31 repeats (p=n.s.), confirming that cognitive impairment can be present also in association to intermediate-length polyQ repeat expansions in ALS, but the frequency is similar to

that found in general in ALS.[24,34] Due to incomplete cognitive testing in part of our patients, we could not include in the multivariable analysis intermediate forms of cognitive impairment, in particular isolate dysexecutive function.[24,34]

A meta-analysis of the published literature (Table 3) provides some clues regarding a possible different frequency of polyQ expansions, in particular a north-to-south increasing trend, ranging from <1% in patients of northern European ancestry[7,8,12,15] to ~2% in French/French Canadian[5] and ~2.5% in Italian patients.[6,10,11,13] In Turkish[14] and in Chinese,[9,16] the frequency of patients with ≥31 polyQ repeats is ~1%. These ethnic differences in polyQ repeats parallel the different frequencies of other ALS genes, such as the north-to-south decreasing gradient of *C9ORF72* in Europe[35] or the rarity of *SOD1* mutations in Irish[36] and Dutch[37] compared to Scandinavian patients.[38] Interestingly, a north-to-south increasing gradient of the frequency of full-length polyQ repeats of the *ATXN2* gene has been also described in SCA2.[39]

A recent multicenter paper performed on an US population has assessed polyQ intermediate repeats of *ATXN2* in a series of 331 patients carrying a *C9ORF72* expansion compared to 376 control subjects and found the *ATXN2* expansion was more significantly frequent in *C9ORF72* mutation carriers with ALS or ALS-FTD phenotype but not in those with pure FTD phenotype.[40] Combining our series, out of 79 ALS patients with *C9ORF72* mutation, 3 (3.8%) had ≥ 27 polyQ repeats, not significantly different from patients not carrying the *C9ORF72* mutation (7.4%) or controls (1.6%). This finding reinforce the differential characteristics of *ATXN2* in population with different ethnic background.

A strength of our discovery cohort is that it includes ~80% of patients diagnosed in Piemonte/Valle d'Aosta in the 2007-2011 period. Moreover, all patients were prospectively followed and none was lost. Finally, all patients were assessed for major ALS genes (*C9ORF72, SOD1, TARDBP, FUS, ANG* and *OPTN*). The validation cohort included a large series of ALS patients consecutively seen

in an ALS referral center, and, although not population-based, it is fairly representative of the ALS population.

We found that an *ATXN2* intermediate-length polyQ repeat is a significant risk factor for ALS, is correlated to a spinal phenotype and associated to shorter survival. Disease-modifying therapies targeted to *ATXN2* represent a promising therapeutic approach for a devastating disease such as ALS; possible strategies may be the use of antisense oligonucleotides, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats.[32]

Acknowledgment

The authors thank the patients and their families for their collaboration in this study.

Study Funding. This work was in part supported by the Italian Ministry of Health (Ministero della Salute, Ricerca Sanitaria Finalizzata, 2010, grant RF-2010-2309849), the European Community's Health Seventh Framework Programme (FP7/2007-2013 under grant agreement 259867), the Joint Programme - Neurodegenerative Disease Research (Italian Ministry of Education and University) (Sophia and ALS-Care Projects), the Agenzia Italiana per la Ricerca sulla SLA (ARISLA) (RepeatALS project), the Associazione Piemontese per l'Assistenza alla SLA (APASLA), Torino, Italy, the Uniti per la Ricerca sulla Sclerosi Laterale Amiotrofica (URSLA) Association, Novara, Italy, and the Fondazione Mario e Anna Magnetto, Alpignano, Torino

References

1. Imbert G, Saudou F, Yvert G, et al. Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. Nat Genet 1996; 14:285-291.

2. Pulst SM, Nechiporuk A, Nechiporuk T, et al. Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. Nat Genet 1996; 14:269-276.

3. Sanpei K, Takano H, Igarashi S, et al. Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. Nat Genet 1996; 14:277-284.

4. Elden AC, Kim HJ, Hart MP, et al. Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. Nature 2010; 466:1069-1075.

5. Daoud H, Belzil V, Martins S, et al. Association of long ATXN2 CAG repeat sizes with increased risk of amyotrophic lateral sclerosis. Arch Neurol 2011; 68:739-742.

6. Corrado L, Mazzini L, Oggioni GD, et al. *ATXN-2* CAG repeat expansions are interrupted in ALS patients. Hum Genet 2011; 130:575-80.

7. Van Damme P, Veldink JH, van Blitterswijk M, et al. Expanded ATXN2 CAG repeat size in ALS identifies genetic overlap between ALS and SCA2. Neurology 2011; 76:2066-2072.

8. Lee T, Li YR, Ingre C, et al. Ataxin-2 intermediate-length polyglutamine expansions in European ALS patients. Hum Mol Genet 2011; 20:1697-700.

9. Chen Y, Huang R, Yang Y, et al. Ataxin-2 intermediate-length polyglutamine: a possible risk factor for Chinese patients with amyotrophic lateral sclerosis. Neurobiol Aging 2011; 32:1925.e1-5.

10. Sorarù G, Clementi M, Forzan M, et al. ALS risk but not phenotype is affected by ataxin-2 intermediate length polyglutamine expansion. Neurology 2011; 76: 2030-2031.

11. Gellera C, Ticozzi N, Pensato V, et al. ATAXIN2 CAG-repeat length in Italian patients with amyotrophic lateral sclerosis: risk factor or variant phenotype? Implication for genetic testing and counseling. Neurobiol Aging 2012; 33:1847.e15-21.

12. Gispert S, Kurz A, Waibel S, et al. The modulation of Amyotrophic Lateral Sclerosis risk by ataxin-2 intermediate polyglutamine expansions is a specific effect. Neurobiol Dis 2012; 45:356-361.

13. Conforti FL, Spataro R, Sproviero W, et al. Ataxin-1 and ataxin-2 intermediate-length PolyQ expansions in amyotrophic lateral sclerosis. Neurology 2012; 79:2315-2320.

14. Lahut S, Ömür Ö, Uyan Ö, et al. ATXN2 and its neighboring gene SH2B3 are associated with increased ALS risk in the Turkish population. PLoS One 2012; 7:e42956.

15. Van Langenhove T, van der Zee J, Engelborghs S, V, et al. *Ataxin-2* polyQ expansions in FTLD-ALS spectrum disorders in Flanders-Belgian cohorts. Neurobiol Aging 2012; 33:1004.e17-20.

16. Liu X, Lu M, Tang L, et al. *ATXN2* CAG repeat expansions increase the risk for Chinese patients with amyotrophic lateral sclerosis. Neurobiol Aging 2013; 34:2236.e5-8

17. Chiò A, Logroscino G, Hardiman O, et al. Prognostic factors in ALS: a critical review. Amyotr Lateral Sclerosis 2009; 10:310-323.

18. Brooks BR, Miller RG, Swash M, et al. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor Neuron Disord 2000; 1:293–299.

19. Lattante S, Conte A, Zollino M, et al. Contribution of major amyotrophic lateral sclerosis genes to the etiology of sporadic disease. Neurology 2012; 79:66-72.

20. Cancel G, Dürr A, Didierjean O, et al. Molecular and clinical correlations in spinocerebellar ataxia 2: a study of 32 families. Hum Mol Genet 1997; 6:709–715

21. Sequeiros J, Seneca S, Martindale J. Consensus and controversies in best practices for molecular genetic testing of spinocerebellar ataxias. Eur J Hum Genet 2010;18:1188-1195

22. Chiò A, Calvo A, Mazzini L, et al. Extensive genetics of ALS: a population-based study in Italy. Neurology 2012; 79:1983-1989.

23. Chiò A, Logroscino G, Hardiman O, et al. Prognostic factors in ALS: A critical review. Amyotroph Lateral Scler 2009; 10:310-23.

24. Phukan J, Elamin M, Bede P, et al. The syndrome of cognitive impairment in amyotrophic lateral sclerosis: a population-based study. J Neurol Neurosurg Psychiatry 2012; 83:102-108.

25. Diekstra FP, van Vught PW, van Rheenen W, et al. *UNC13A* is a modifier of survival in amyotrophic lateral sclerosis. Neurobiol Aging 2012; 33:630.e3-8.

26. Chiò A, Mora G, Restagno G, et al. *UNC13A* influences survival in Italian amyotrophic lateral sclerosis patients: a population-based study. Neurobiol Aging 2013; 34:357.e1-5.

27. Blauw HM, van Rheenen W, Koppers M, et al. *NIPA1* polyalanine repeat expansions are associated with amyotrophic lateral sclerosis. Hum Mol Genet 2012; 21:2497-2502.

28. ALSGEN Consortium, Ahmeti KB, Ajroud-Driss S, Al-Chalabi A, et al. Age of onset of amyotrophic lateral sclerosis is modulated by a locus on 1p34.1. Neurobiol Aging 2013; 34 :357.e7-19.

29. Logroscino G, Traynor BJ, Hardiman O, et al. Incidence of amyotrophic lateral sclerosis in Europe. J Neurol Neurosurg Psychiatry 2010; 81:385-390.

30. Farg MA, Soo KY, Warraich ST, et al. Ataxin-2 interacts with FUS and intermediate-length polyglutamine expansions enhance FUS-related pathology in amyotrophic lateral sclerosis. Hum Mol Genet 2013; 22:717-28.

31. Nihei Y, Ito D, Suzuki N. Roles of ataxin-2 in pathological cascades mediated by TAR DNA-binding protein 43 (TDP-43) and Fused in Sarcoma (FUS). J Biol Chem 2012; 287:41310-41323.

32. van den Heuvel DM, Harschnitz O, van den Berg LH, Pasterkamp RJ. Taking a risk: a therapeutic focus on ataxin-2 in amyotrophic lateral sclerosis? Trends Mol Med 2014; 20:25-35.

33. Ross OA, Rutherford NJ, Baker M, et al. Ataxin-2 repeat-length variation and neurodegeneration. Hum Mol Genet 2011; 20:3207-3212.

34. Montuschi A, Iazzolino B, Calvo A, et al. Cognitive correlates in amyotrophic lateral sclerosis: a population-based study in Italy. J Neurol Neurosurg Psychiatry 2014 Apr 25. doi: 10.1136/jnnp-2013-307223.

35. Majounie E, Renton AE, Mok K, et al. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. Lancet Neurol 2012; 11323-330.

36. Kenna KP, McLaughlin RL, Byrne S, et al. Delineating the genetic heterogeneity of ALS using targeted high-throughput sequencing. J Med Genet 2013; 50:776-783.

37. van Blitterswijk M, van Es MA, Hennekam EA, et al. Evidence for an oligogenic basis of amyotrophic lateral sclerosis. Hum Mol Genet 2012; 21:3776-84.

38. Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the *CuZn superoxide dismutase* gene. Curr Neurol Neurosci Rep 2006; 6:37-46.

39. Schöls L, Bauer P, Schmidt T, et al. Autosomal dominant cerebellar ataxias: clinical features, genetics, and pathogenesis. Lancet Neurol 2004; 3:291-304.

40. van Blitterswijk M, Mullen B, Heckman MG, et al. Ataxin-2 as potential disease modifier in C9ORF72 expansion carriers. Neurobiol Aging. 2014 May 2. doi: 10.1016/j.neurobiolaging.2014.04.016.

Table 1. Demographic and clinical characteristics of patients according to *ATXN2* repeat size

Factor	Discovery cohort			Validation cohort		
	<31 (n=653)	**≥31 (n=19)**	**p**	**<31 (n=645)**	**≥31 (n=16)**	**p**
Age at onset (years, SD)	65.5 (10.8)	68.8 (8.1)	0.19	60.6 (12.1)	62.2 (10.4)	0.61
Gender, female (%)	300 (45.9%)	8 (42.1%)	0.92	274 (42.5%)	6 (37.5%)	0.45
Site of onset, bulbar (%)	217 (33.2%)	1 (5.3%)	0.005	167 (25.9%)	2 (12.5%)	0.18
Positive family history for ALS or FTD	59 (9.0%)	0	0.17	64 (9.9%)	2 (12.5%)	0.74

Table 2. Cox Multivariable analysis

Factor	**Level**	**Hazard ratio**	**p**
Age at onset	20-49	1	0.0001
	50-59	1.49 (0.90-2.33)	
	60-69	1.90 (1.23-2.90)	
	70-79	2.38 (1.54-3.67)	
	80-99	4.00 (2.39-6.72)	
Site of onset	Spinal	1	0.0001
	Bulbar	1.55 (1.25-1.90)	
ATXN2 polyQ repeats	<31	1	0.0001
	≥31	2.79 (1.67-4.64)	

Table 3. Frequency, odds ratio and clinical presentation of patients with ≥31 CAG repeats in the *ATXN2* gene

Reference	**N. of ALS cases**	**N. of expanded cases (≥31)**	**% of expanded cases**	**N. of controls**	**N. of expanded controls (≥31)**	**Odds ratio (95% c.i.)**	**N. of spinal onset**	**N. of bulbar onset**	**Ethnic origin**
North European									
[9]	1294	13	1.00	679	0	6.9 (0.9-52.8)	NS	NS	Mostly north European
[7]	1948	10[§]	0.51	2002	2	5.2 (1.1-23.6)	9	1	Dutch/Flemish Belgian
[12]	559	4	0.72	1369	2	5.0 (0.9-26.9)	NS	NS	German
[15]	72	1	1.39	810	1	11.4 (0.7-184.1)	NS	NS	Flemish Belgian
North European	**3873**	**28**	**0.72**	**4860**	**5**	**7.1 (2.7-18.3)**	**9**	**1**	
French/French Canadian									
[6]	556	11	1.98	471	1	9.8 (1.2-73.7)	8[+]	0	French/French Canadian
French	**556**	**11**	**1.98**	**471**	**1**	**9.8 (1.2-73.7)**	**8**	**0**	
South European									
[4] #	232 #	7 #	3.02	395 #	0 #	12.3 (1.5-100.5)	6 #	1 #	Northern Italian

[24]	418	13	3.11	296	1	9.5 (1.2-72.8)	10	3	Southern Italian
[11]	801	15	1.87	551	1	10.5 (1.4 -79.7)	NS	NS	Northern Italian
[10]	247	5	2.02	356	2	3.7 (0.7-19.0)	NS	NS	Northern Italian
Present paper – discovery cohort	672	19	2.83	509	1	14.8 (1.9-110.8)	18	1	Northern Italian
Present paper – validation cohort	661	16	2.42	-	-	-	14	2	Central Italian
South European	**3014**	**73**	**2.47**	**1712**	**5**	**8.5 (3.9-21.1)**	**48**	**7**	
Turkish									
[14]	236	4	1.69	420	0	7.2 (0.8-65-2)	2	2**	Turkish
Turkish	**236**	**4**	**1.69**	**420**	**0**	**7.2 (0.8-65-2)**	**2**	**2**	
Chinese									
[9]	345	4	1.16	350	0	4.1 (0.5-36.9)	NS	NS	Chinese
[16]	1067	17	1.59	506	0	8.2 (1.1-61.7)	13*	1	Chinese
Chinese	**1412**	**21**	**1.49**	**856**	**0**	**12.9 (1.7-96.5)**	**13**	**1**	
Overall									

Overall	**9640**	**147**	**1.52**	**14908**	**20**	**11.5 (7.2-18.4)**	**80**	**11**	

[+]unknown in 1 case; # cases and controls also included in the present paper are excluded from the total count; *unknown in 3 cases; **1 mixed (spinal and bulbar); [§]≥32 repeats; NS: not stated

Figure legends

Figure 1. Discovery cohort. The distribution of ATXN-2 polyQ repeat lengths in ALS and control cases. In the insert, data concerning cases and controls with ≥27 repeats are magnified. PolyQ lengths ≥31 are significantly more frequent in ALS cases (p=0.0001) (blue, ALS patients; red, controls).

Figure 2. Kaplan-Meier survival estimates from onset to death/tracheostomy. **A.** Discovery cohort. Blue line, <31 polyQ repeats; green line, ≥31 polyQ repeats. p=0.0001. **B.** Validation cohort. Blue line, <31 polyQ repeats; green line, ≥31 polyQ repeats. p=0.009. **C**. Discovery cohort. Kaplan-Meier survival estimation from onset to death/tracheostomy. Blue line, <27 polyQ repeats; red line, 27-30 polyQ repeats green line, ≥31 polyQ repeats. p=0.0001. **D.** Validation cohort**.** Kaplan-Meier survival estimation from onset to death/tracheostomy. Blue line, <27 polyQ repeats; red line, 27-30 polyQ repeats green line, ≥31 polyQ repeats. p=0.003.

Figure 1

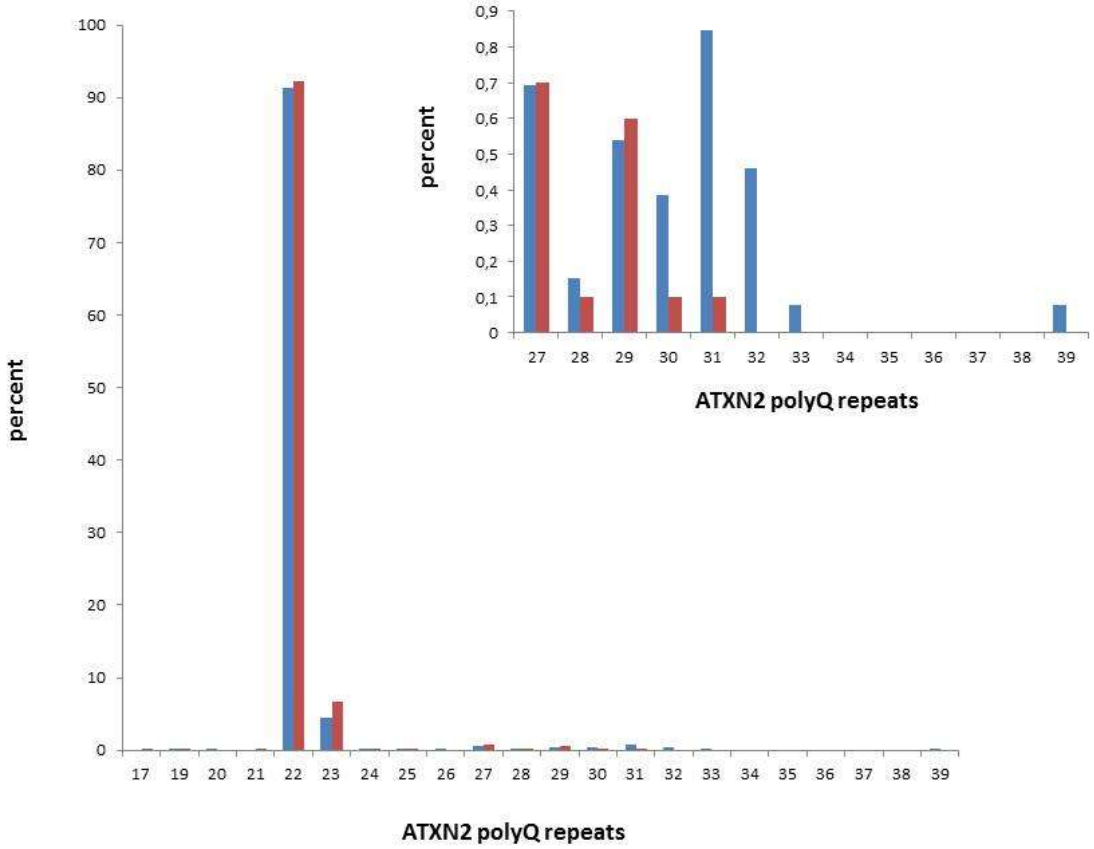
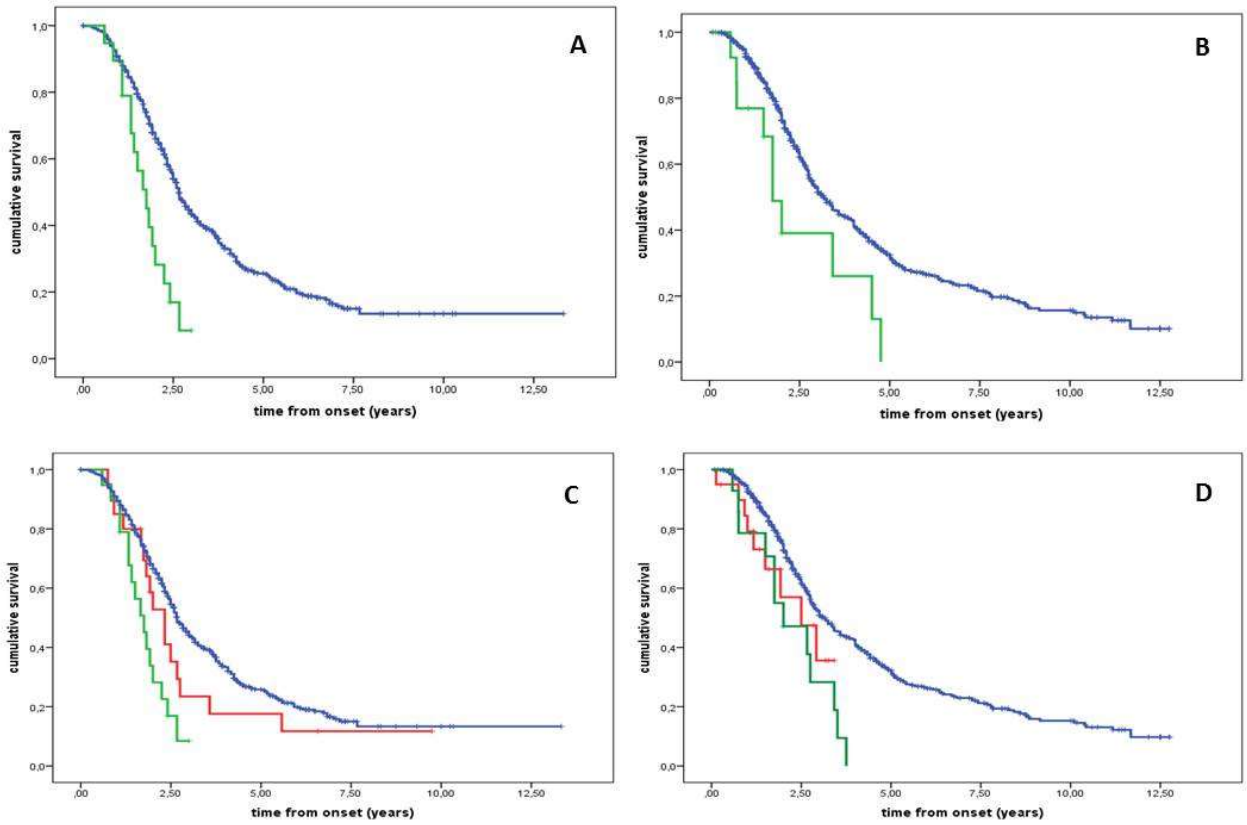

percent
100
90
80
70
60
50
40
30
20
10
0
17 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
ATXN2 polyQ repeats
percent
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
27 28 29 30 31 32 33 34 35 36 37 38 39
ATXN2 polyQ repeats

Figure 2


A
B
C
D
cumulative survival
time from onset (years)
1,0
0,8
0,6
0,4
0,2
0,0
,00
2,50
5,00
7,50
10,00
12,50