ATNX2 is not a regulatory gene in Italian ALS patients with C9ORF72 GGGGCC expansion

Adriano Chiò, MD, FAAN;\textsuperscript{a,b} Gabriele Mora, MD;\textsuperscript{c} Mario Sabatelli, MD;\textsuperscript{d} Claudia Caponnetto, MD;\textsuperscript{e} Christian Lunetta; MD\textsuperscript{f} Bryan J. Traynor, MD, PhD;\textsuperscript{g} Janel O. Johnson, PhD;\textsuperscript{g,h} Mike A. Nalls, PhD;\textsuperscript{i} Andrea Calvo, MD, PhD;\textsuperscript{a,b} Cristina Moglia, MD;\textsuperscript{a} Giuseppe Borghero, MD;\textsuperscript{j} Francesca Trojsi, MD;\textsuperscript{k} Vincenzo La Bella, MD;\textsuperscript{l} Paolo Volanti, MD;\textsuperscript{m} Isabella Simone, MD;\textsuperscript{n} Fabrizio Salvi, MD;\textsuperscript{o} Francesco O. Logullo, MD;\textsuperscript{p} Nilo Riva, MD, PhD;\textsuperscript{q} Paola Carrera, BSc PhD;\textsuperscript{r} Fabio Giannini, MD;\textsuperscript{s} Jessica Mandrioli, MD;\textsuperscript{t} Raffaella Tanel, MD;\textsuperscript{u} Margherita Capasso. MD;\textsuperscript{v} Lucio Tremolizzo, MD, PhD;\textsuperscript{w} Stefania Battistini, MD, PhD;\textsuperscript{x} Maria Rita Murru, BSc;\textsuperscript{y} Paola Origone, MD;\textsuperscript{z} Marcella Zollino, MD;\textsuperscript{a} Silvana Penco, PhD;\textsuperscript{aa} ITALSGEN consortium;§ SARDINALS consortium;\wedge Letizia Mazzini, MD;\textsuperscript{ab} Sandra D’Alfonso, PhD;\textsuperscript{ac} Gabriella Restagno, MD;\textsuperscript{a,ad} Maura Brunetti, MSc;\textsuperscript{a,ad} Marco Barberis, MSc;\textsuperscript{a,ad} Francesca L. Conforti, PhD\textsuperscript{ac}

\textsuperscript{a}ALS Center, ‘Rita Levi Montalcini’ Department of Neuroscience, Neurology II, University of Torino

\textsuperscript{b}Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino, Italy

\textsuperscript{c}Department of Neurological Rehabilitation, Fondazione Salvatore Maugeri, IRCCS, Istituto Scientifico di Milano, Milano, Italy

\textsuperscript{d}Centro Cinico NEMO-Roma. Neurological Institute, Catholic University and I.C.O.M.M. Association for ALS Research, Rome, Italy

\textsuperscript{e}Department of Neurosciences, Ophthalmology, Genetics, Rehabilitation, Maternal and Child Health, IRCCS Azienda Ospedaliero-Universitaria San Martino IST, Genoa, Italy
1Neuromotor Omnicenter, Serena Onlus Foundation, Milan

2Neuromuscular Diseases Research Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

3Department of Neurology, Neurological Institute, Neuromuscular Center, Cleveland Clinic, Cleveland, OH, USA

4Molecular Genetics Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA.

5Department of Neurology, Azienda Universitario Ospedaliera di Cagliari and University of Cagliari, Cagliari, Italy

6Department of Medical, Surgical Neurological Metabolic and Aging Sciences, Second University of Naples, Naples, Italy

7ALS Clinical Research Center, Bio. Ne. C., University of Palermo, Palermo, Italy

8Neurorehabilitation Unit/ALS Center, Salvatore Maugeri Foundation, IRCCS, Scientific Institute of Mistretta, Mistretta, Italy

9Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari, Bari, Italy

10Center for Diagnosis and Cure of Rare Diseases, Department of Neurology, IRCCS Institute of Neurological Sciences, Bologna, Italy

11Neurological Clinic, Marche Polytechnic University, Ancona, Italy.

12Department of Neurology and Institute of Experimental Neurology (INSPE), IRCCS San Raffaele Scientific Institute, Milan, Italy
Division of Genetics and Cell Biology; Unit of Genomics for human disease diagnosis, IRCCS San Raffaele Scientific Institute, Milan, Italy

Department of Medical, Surgical and Neurological Sciences, University of Siena, Siena, Italy

Department of Neuroscience, S. Agostino- Estense Hospital, and University of Modena and Reggio Emilia, Modena, Italy

Department of Neurology, Santa Chiara Hospital, Trento, Italy

Department of Neurology, University of Chieti, Chieti, Italy

Neurology Unit, School of Medicine and Surgery and NeuroMI, University of Milano-Bicocca, Monza, Italy

Multiple Sclerosis Centre, ASL 8, Cagliari/Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Italy

Institute of Medical Genetics, Catholic University, Rome, Italy.

Department of Laboratory Medicine, Medical Genetics, Niguarda Ca’ Granda Hospital, Milan, Italy

Department of Health Sciences, Interdisciplinary Research Center of Autoimmune Diseases (IRCAD), ‘Amedeo Avogadro’ University of Eastern Piedmont, Novara, Italy

ALS Center, Department of Neurology, Azienda Ospedaliera Universitaria Maggiore della Carità, Novara, Italy

Laboratory of Molecular Genetics, Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino, Italy

Institute of Neurological Sciences, National Research Council, Mangone, Cosenza, Italy
*Corresponding author: Prof. Adriano Chiò, ‘Rita Levi Montalcini’ Department of Neuroscience, Via Cherasco 15, I-10126 Torino, Italy, E-mail: achio@usa.net
Other members of the ITALSGEN consortium are: Giancarlo Logroscino (Bari and Tricase, LE); Ilaria Bartolomei (Bologna); Gianluigi Mancardi, Paola Mandich (Genova); Kalliopi Marinou, Riccardo Sideri (Milan, Maugeri Foundation); Lorena Mosca (Milan, Niguarda Ca' Granda Hospital); Giuseppe Lauria Pinter (Milan, Besta Neurological Institute), Massimo Corbo (Milan, Casa di Cura del Policlinico); Nicola Fini, Antonio Pasano (Modena); Alessandro Arosio, Carlo Ferrarese (Monza); Gioacchino Tedeschi, Maria Rosaria Monsurrò, Giovanni Piccirillo, Cinzia Femiano (Napoli); Anna Bersano, Lucia Corrado, Alessandra Bagarotti (Novara); Rossella Spataro, Tiziana Colletti (Palermo); Amelia Conte, Marco Luigetti, Serena Lattante, Giuseppe Marangi (Rome, Catholic University of Sacred Heart); Marialuisa Santarelli (Rome, San Filippo Neri Hospital); Antonio Petrucci (Rome, San Camillo Forlanini Hospital); Claudia Ricci, Michele Benigni (Siena); Federico Casale, Giuseppe Marrali, Giuseppe Fuda, Irene Ossola, Stefania Cammarosano, Antonio Ilardi, Umberto Manera, Davide Bertuzzo (Torino), Raffaella Tanel (Trento); Fabrizio Pisano (Veruno, NO).

Other members of the SARDINIALS consortium are: Emanuela Costantino, Carla Pani, Roberta Puddu, Carla Caredda, Valeria Piras, Stefania Tranquilli, Stefania Cuccu, Daniela Corongiu, Maurizio Melis, Antonio Milia, Francesco Marrosu, Maria Giovanna Marrosu, Gianluca Floris, Antonino Cannas, Stefania Cuccu e Stefania Tranquilli (Cagliari), Anna Ticca (Nuoro), Maura Pugliatti, Angelo Pirisi, Leslie D. Parish, Patrizia Occhineri (Sassari), Enzo Ortu (Ozieri), Tea B. Cau, Daniela Loi (Tempio-Olbia).
Abstract

There are indications that both familial ALS and sporadic ALS phenotype and prognosis are partly regulated by genetic and environmental factors, supporting the theory that ALS is a multifactorial disease. The aim of this paper was to assess the role of ATXN2 intermediate length repeats in a large series of Italian and Sardinian ALS patients and controls carrying a pathogenetic C9ORF72 GGGGCC hexanucleotide repeat. A total of 1972 ALS cases were identified through the database of the Italian ALS Genetic consortium (ITALSGEN), a collaborative effort including 18 ALS centers throughout Italy. The study population included: (1) 276 Italian and 57 Sardinian ALS cases who carried the C9ORF72 expansion; (2) 1340 Italian and 299 Sardinian ALS cases not carrying the C9ORF72 expansion. A total of healthy 1043 controls were also assessed. Most Italian and Sardinian cases and controls were homozygous for 22/22 or 23/23 repeats or heterozygous for 22/23 repeats of the ATXN2 gene. ATXN2 intermediate length repeats alleles (≥28) were detected in 3 (0.6%) Italian ALS cases carrying the C9ORF72 expansion, in none of the Sardinian ALS cases carrying the expansion, in 60 (4.3%) Italian cases not carrying the expansion, and in 6 (2.0%) Sardinian ALS cases without C9ORF72 expansion. Intermediate length repeat alleles were found in 12 (1.5%) Italian controls and 1 (0.84%) Sardinian controls. Therefore, ALS patients with C9ORF72 expansion showed a lower frequency of ATXN2 polyQ intermediate length repeats than both controls (Italian cases, p=0.137; Sardinian cases, p=0.0001) and ALS patients without C9ORF72 expansion (Italian cases, p=0.005; Sardinian cases, p=0.178). In our large study on Italian and Sardinian ALS patients with C9ORF72 GGGGCC hexanucleotide repeat expansion, compared to age-, gender- and ethnic-matched controls, ATXN2 polyQ intermediate length does not represent a modifier of ALS risk, differently from non-C9ORF72 mutated patients.
1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder of the central nervous system, almost invariably fatal, characterized by a loss of cortical, bulbar and spinal motor neurons. In 10-15% of cases it is genetically transmitted (familial ALS, fALS), while in the remaining cases it appears sporadically in the population (sporadic ALS, sALS) (Renton et al, 2014). More than 20 major genes have been related to ALS, the most common in the Caucasian population being C9ORF72, SOD1, TARDBP and FUS (Renton et al, 2014). However, there are now indications that both familial ALS and sporadic ALS phenotype and prognosis are partly regulated by genetic and environmental factors, supporting the theory that ALS is a multifactorial disease (Al Chalabi et al, 2014).

ATXN2 intermediate length repeats have been identified as a risk factor for ALS (Neuenschwander et al, 2014) and their presence are additionally associated with reduced survival in ALS patients (Chiò et al, 2015). More recently, it has been reported that ATXN2 is also a risk factor for ALS patients carrying the GGGGCC hexanucleotide repeat in the first intron of the C9ORF72 gene (van Blitterswijk et al, 2014a). This gene accounts for 40% of familial ALS and 7% sporadic ALS in European and American series (Majounie et al, 2012). Phenotypes associated with this repeat expansion include ALS and/or frontotemporal dementia (FTD), psychotic symptoms (hallucinations and delusions), and extrapyramidal signs. The wide and heterogeneous symptomatology related to C9ORF72 has yet not been fully explained (Roher et al, 2015).

The aim of this paper was to assess the role of ATXN2 intermediate length repeats in a large series of Italian and Sardinian ALS patients and controls carrying a pathogenetic C9ORF72 GGGGCC hexanucleotide repeat.
2. Methods

2.1 Patients

A total of 1972 ALS cases were identified through the database of the Italian ALS Genetic consortium (ITALSGEN), a collaborative effort including 18 ALS centers throughout Italy. The study population included: (1) 276 Italian and 57 Sardinian ALS cases who carried the \textit{C9ORF72} expansion; (2) 1340 Italian and 299 Sardinian ALS not carrying the \textit{C9ORF72} expansion.

2.2 Controls

The 1043 controls were included in the analysis. This included: (1) 686 regionally-matched, unrelated Italian subjects, reported in previous papers (Corrado et al 2011; Conforti et al, 2012). These individuals were predominantly blood donors; (2) 243 regionally-matched, unrelated Sardinian subjects; (3) 114 matched subjects identified through the patients’ general practitioners (population-based controls) (Chiò et al, 2015).

2.3. Genetic analysis

Genomic DNA was isolated from peripheral blood lymphocytes using a standard protocol. The \textit{ATXN2} CAG repeat in exon 1 (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) (Cancel et al, 1997). As reported in recent guidelines for molecular genetic testing of Spinocerebellar Ataxias (SCA), capillary electrophoresis is the preferred method to size alleles as it allows resolution of alleles that are one triplet apart (Sequeiros et al, 2010). As a quality control, 20 samples have been genotyped in the six laboratories that performed the molecular genetic testing for the present study. The results showed a consistent allele assignment for all the samples.

To compare our findings to those of van Blitterswijk and colleagues (2014a), we used a threshold of 28 repeats (or greater) as the definition of intermediate size repeats. However, data using a
threshold of 27 repeats (the most common used cut-off for \textit{ATXN2} intermediate length repeats in the literature) are reported as supplementary table.

All ALS cases were also tested for \textit{SOD1} (all exons), \textit{TARDBP} (exon 6), \textit{FUS} (exons 14 and 15), and \textit{C9ORF72} using the methodology described elsewhere (Chiò et al, 2012a).

\textbf{2.4. Statistical analysis}

The difference between \textit{ATXN2} polyQ intermediate length repeats in cases and controls was assessed with Fisher’s exact test.

\textbf{2.5. Standard protocol approvals and patient consents}

The study was approved by the Ethical committees of participating centers. Patients and controls signed a written informed consent.

\textbf{3. Results}

The demographic and clinical characteristics of patients and controls are reported in Table 1. Most Italian and Sardinian cases, as well as most Italian and Sardinian controls, were homozygous for 22/22 or 23/23 repeats or heterozygous for 22/23 repeats of the \textit{ATXN2} gene. \textit{ATXN2} intermediate length repeats alleles (≥28) were detected in 3 (0.6%) Italian ALS cases carrying the C9ORF72 expansion, in none of the Sardinian ALS cases carrying the expansion, in 60 (4.3%) Italian cases not carrying the expansion, and in 6 (2.0%) Sardinian ALS cases without \textit{C9ORF72} expansion. Intermediate length repeat alleles were found in 12 (1.5%) Italian controls and 1 (0.84%) Sardinian controls. Therefore, ALS patients with \textit{C9ORF72} expansion showed a lower frequency of \textit{ATXN2} polyQ intermediate length repeats than both controls (Italian cases, p=0.137; Sardinian cases, p=0.0001) and ALS patients without \textit{C9ORF72} expansion (Italian cases, p=0.005; Sardinian cases, p=0.178). In patients with \textit{C9ORF72} expansion the presence of \textit{ATXN2} polyQ intermediate length
repeats did not modify the age at onset of ALS (*ATXN2* expanded, 55.8 years [SD 13.7]) vs. non-expanded, 58.0 years [9.1], p=0.56).

4. Discussion

In our large series of Italian and Sardinian ALS patients, we did not find evidence of increased occurrence of *ATXN2* polyQ intermediate length repeats in patients with *C9ORF72* hexanucleotide repeat expansion. In contrast, we confirmed that in patients without *C9ORF72* expansion *ATXN2* polyQ intermediate length repeats are associated with a higher risk of ALS.

*C9ORF72* GGGGCC expansions have been related to a quite wide spectrum of clinical presentations, going from pure ALS to pure FTD, but also including psychotic and extrapyramidal signs and symptoms (Rohrer et al, 2015). This wide range of clinical expressions is reflected by neuropathological, MRI and PET studies, showing in *C9ORF72* patients an extension of TDP43 pathology extends toward non-motor areas including prefrontal cortex, cingulate cortex, basal ganglia and cerebellum (Cooper-Knock et al, 2012; Bede et al, 2013; Cistaro et al, 2014). The widespread diffusion of alterations is considered a hallmark of *C9ORF72* mutations in neuroimaging and functional studies.

The reasons of the heterogeneous symptom constellation in patients carrying a GGGGCC expansion on the first intron of the *C9ORF72* gene are still unclear. All studies up to date have shown that the expansion pattern of GGGGCC in different brain areas was not related to the clinical picture and that no correlation was found between expansion size in frontal lobe and occurrence of cognitive impairment (van Blitterswijk et al, 2013a; Dols-Icardo et al, 2104; Nordin et al, 2015).

Another possibility could be an interaction between the presence of *C9ORF72* expansion and one or more regulatory genes. The presence of mutations of other ALS- and FTD-related genes (*GRN*, *MAPT*, *TARDBP*, *FUS*, *SOD1*) in patients carrying the *C9ORF72* expansion has been reported as possible modifiers of patients’ clinical picture (Chiò et al, 2012b; van Blitterswijk et al, 2014b; van
Blitterswijk et al 2013b). A study on 36 common genetic variants found that three variants were significantly associated with age at onset (rs7018487, UBAP1; rs6052771, PRNP; and rs7403881, MT-Ie) and six variants were significantly associated with survival after onset (rs5848, GRN; rs7403881, MT-Ic; rs13268953, ELP3; the ε4 allele of APOE; rs12608932, UNC13A; and rs1800435, ALAD) (van Blitterswijk et al, 2014b). Finally, it has been shown that TMEM106B protect C9ORF72 expansion carriers from developing FTD (van Blitterswijk et al., 2014c).

Our data contrasts a recent publication based on 331 U.S. patients and 376 U.S. controls reporting that ATXN2 polyQ intermediate length repeats act as a disease modifier in C9ORF72 carriers (van Blitterswijk et al, 2014a). This discrepancy may be explained by (1) the larger size of the control cohort in our series, reducing the risk of false-negative association, and (2) the ethnic-matching of patients and controls, avoiding a possible mismatch related to the different frequency of ATXN2 polyQ intermediate length repeats according to ethnic background (Chiò et al, 2015).

In our large study on Italian and Sardinian ALS patients with C9ORF72 GGGGCC hexanucleotide repeat expansion, compared to age-, gender- and ethnic-matched controls, ATXN2 polyQ intermediate length does not represent a modifier of ALS risk, differently from non-C9ORF72 mutated patients. Our findings highlight the importance of having complete genetic information on ALS patients when assessing putative genetic modifiers.

**Study Funding.** This work was in part supported by the Italian Ministry of Health (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, Strength and ALS-Care Projects), the Agenzia Italiana per la Ricerca sulla SLA (ARISLA) (Sardinials and RepeatALS projects), 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.

Intramural Research Program of the NIH.
References


Table 1. Clinical characteristics of ALS cases

<table>
<thead>
<tr>
<th></th>
<th>Italian cases (n=1616)</th>
<th>Sardinian cases (n=356)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female)</td>
<td>743 (46%)</td>
<td>128 (36%)</td>
</tr>
<tr>
<td>Mean age at onset (years)</td>
<td>61.5 (11.8)</td>
<td>61.3 (11.5)</td>
</tr>
<tr>
<td>Site of onset (bulbar)</td>
<td>465 (28.8%)</td>
<td>86 (24.2%)</td>
</tr>
</tbody>
</table>
Table 2. ATXN2 polyQ intermediate length repeats (<28 vs. ≥28) in C9ORF72 and non-C9ORF72 cases

<table>
<thead>
<tr>
<th></th>
<th>Italian cases</th>
<th></th>
<th>Sardinian cases</th>
<th></th>
<th>Overall</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C9ORF72 cases</td>
<td>Non-C9ORF72 cases</td>
<td>Controls</td>
<td>C9ORF72 cases</td>
<td>Non-C9ORF72 cases</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C9ORF72 cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;28</td>
<td>549</td>
<td>2620</td>
<td>1588</td>
<td>114</td>
<td>592</td>
<td>485</td>
</tr>
<tr>
<td>≥28</td>
<td>3</td>
<td>60</td>
<td>12</td>
<td>0</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value (cases vs</td>
<td>0.62</td>
<td>0.0001</td>
<td>-</td>
<td>0.63</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>controls)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |
|                   |               |                     |                 |                     |         |                     |