ATXN2 is a modifier of phenotype in ALS patients of Sardinian ancestry

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

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

[a]ALS Center, 'Rita Levi Montalcini' Department of Neuroscience, Neurology II, University of Torino

[b]Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino, Italy

[c]Department of Neurological Rehabilitation, Fondazione Salvatore Maugeri, IRCCS, Istituto Scientifico di Milano, Milano, Italy

[d]Centro Cinico NEMO-Roma. Neurological Institute, Catholic University and I.C.O.M.M. Association for ALS Research, Rome, Italy

[e]Department of Neurosciences, Ophthalmology, Genetics, Rehabilitation, Maternal and Child Health, IRCCS Azienda Ospedaliero-Universitaria San Martino IST, Genoa, Italy

[f]NEuroMuscular Omnicenter, Serena Onlus Foundation, Milan

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

[h]Department of Neurology, Neurological Institute, Neuromuscular Center, Cleveland Clinic, Cleveland, OH, USA

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

[j]Department of Neurology, Azienda Universitario Ospedaliera di Cagliari and University of Cagliari, Cagliari, Italy

[k]Department of Medical, Surgical Neurological Metabolic and Aging Sciences, Second University of Naples, Naples, Italy

[l]ALS Clinical Research Center, Bio. Ne. C., University of Palermo, Palermo, Italy

[m] Neurorehabilitation Unit/ALS Center, Salvatore Maugeri Foundation, IRCCS, Scientific Institute of Mistretta, Mistretta, Italy

[n]Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari, Bari, Italy

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

[p]Neurological Clinic, Marche Polytechnic University, Ancona, Italy.

[q]Department of Neurology and Institute of Experimental Neurology (INSPE), IRCCS San Raffaele Scientific Institute, Milan, Italy

[r]Division of Genetics and Cell Biology; Unit of Genomics for human disease diagnosis, IRCCS San Raffaele Scientific Institute, Milan, Italy

[s]Department of Medical, Surgical and Neurological Sciences, University of Siena, Siena, Italy

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

[u]Department of Neurology, Santa Chiara Hospital, Trento, Italy

[v]Department of Neurology, University of Chieti, Chieti, Italy

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

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

[z]Institute of Medical Genetics, Catholic University, Rome, Italy.

[aa]Department of Laboratory Medicine, Medical Genetics, Niguarda Ca' Granda Hospital, Milan, Italy

[ab]Department of Health Sciences, Interdisciplinary Research Center of Autoimmune Diseases (IRCAD), 'Amedeo Avogadro' University of Eastern Piedmont, Novara, Italy

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

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

[ae]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 Fasano (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).

ATNX2 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 *C9ORF72* expansion; (2) 1340 Italian and 299 Sardinian ALS not carrying the *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 *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 *ATXN2* intermediate length repeats in the literature) are reported as supplementary table.

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

2.4. Statistical analysis

The difference between *ATXN2* polyQ intermediate length repeats in cases and controls was assessed with Fisher's exact test.

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.

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 *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 patients with *C9ORF72* expansion the presence of *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-Ie; 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

Al-Chalabi, A., Calvo, A., Chiò, A., Colville, S., Ellis, C.M., Hardiman, O., Heverin, M., Howard, R.S., Huisman, M.H., Keren, N., Leigh, P.N., Mazzini, L., Mora, G., Orrell, R.W., Rooney, J., Scott, K.M., Scotton, W.J., Seelen, M., Shaw, C.E., Sidle, K.S., Swingler, R., Tsuda, M., Veldink, J.H., Visser, A.E., van den Berg, L.H., Pearce, N. 2014. Analysis of amyotrophic lateral sclerosis as a multistep process: a population-based modelling study. Lancet Neurol 13, 1108-1113.

Bede, P., Elamin, M., Byrne, S., McLaughlin, R.L., Kenna, K., Vajda, A., Pender, N., Bradley, D.G., Hardiman, O. 2013. Basal ganglia involvement in amyotrophic lateral sclerosis. Neurology. 81, 2107-2115.

Cancel, G., Dürr, A., Didierjean, O., Imbert, G., Bürk, K., Lezin, A., Belal, S., Benomar, A., Abada-Bendib, M., Vial, C., Guimarães, J., Chneiweiss, H., Stevanin, G., Yvert, G., Abbas, N., Saudou, F., Lebre, A.S., Yahyaoui, M., Hentati, F., Vernant, J.C., Klockgether, T., Mandel, J.L., Agid, Y., Brice, A. 1997. Molecular and clinical correlations in spinocerebellar ataxia 2: a study of 32 families. Hum Mol Genet. 6, 709-715.

Chiò, A., Calvo, A., Mazzini, L., Cantello, R., Mora, G., Moglia, C., Corrado, L., D'Alfonso, S., Majounie, E., Renton, A., Pisano, F., Ossola, I., Brunetti, M., Traynor, B.J., Restagno, G., On behalf of PARALS. 2012. Extensive genetics of ALS: a population-based study in Italy. Neurology. 79, 1983-1989.

Chiò, A., Calvo, A., Moglia, C., Canosa, A., Brunetti, M., Barberis, M., Restagno, G., Conte, A., Bisogni, G., Marangi, G., Moncada, A., Lattante, S., Zollino, M., Sabatelli, M., Bagarotti, A., Corrado, L., Mora, G., Bersano, E., Mazzini, L., D'Alfonso, S.; PARALS. 2015. *ATXN2* polyQ intermediate repeats are a modifier of ALS survival. Neurology. 84, 251-258.

Chiò, A., Restagno, G., Brunetti, M., Ossola, I., Calvo, A., Canosa, A., Moglia, C., Floris, G., Tacconi, P., Marrosu, F., Marrosu, M.G., Murru, M.R., Majounie, E., Renton, A.E., Abramzon, Y., Pugliatti, M., Sotgiu, M.A., Traynor, B.J., Borghero, G.; SARDINIALS Consortium. 2012b. ALS/FTD phenotype in two Sardinian families carrying both C9ORF72 and TARDBP mutations. J Neurol Neurosurg Psychiatry. 83, 730-733.

Cistaro, A., Pagani, M., Montuschi, A., Calvo, A., Moglia, C., Canosa, A., Restagno, G., Brunetti, M., Traynor, B.J., Nobili, F., Carrara, G., Fania, P., Lopiano, L., Valentini, M.C., Chiò, A. 2014. The metabolic signature of C9ORF72-related ALS: FDG PET comparison with non-mutated patients. Eur J Nucl Med Mol Imaging 41, 844-852.

Conforti, F.L., Spataro, R., Sproviero, W., Mazzei, R., Cavalcanti, F., Condino, F., Simone, I.L., Logroscino, G., Patitucci, A., Magariello, A., Muglia, M., Rodolico, C., Valentino, P., Bono, F., Colletti, T., Monsurrò, M.R., Gambardella, A., La Bella, V. 2012. Ataxin-1 and ataxin-2 intermediate-length PolyQ expansions in amyotrophic lateral sclerosis. Neurology. 79, 2315-2320.

Cooper-Knock, J., Hewitt, C., Highley, J.R., Brockington, A., Milano, A., Man, S., Martindale, J., Hartley, J., Walsh, T., Gelsthorpe, C., Baxter, L., Forster, G., Fox, M., Bury, J., Mok, K., McDermott, C.J., Traynor, B.J., Kirby, J., Wharton, S.B., Ince, P.G., Hardy, J., Shaw, P.J. 2012. Clinico-pathological features in amyotrophic lateral sclerosis with expansions in C9ORF72. Brain. 135, 751-764.

Corrado, L., Mazzini, L., Oggioni, G.D., Luciano, B., Godi, M., Brusco, A., D'Alfonso, S. 2011. ATXN-2 CAG repeat expansions are interrupted in ALS patients. Hum Genet. 130, 575-580.

Dols-Icardo, O., García-Redondo, A., Rojas-García, R., Sánchez-Valle, R., Noguera, A., Gómez-Tortosa, E., Pastor, P., Hernández, I., Esteban-Pérez, J., Suárez-Calvet, M., Antón-Aguirre, S., Amer, G., Ortega-Cubero, S., Blesa, R., Fortea, J., Alcolea, D., Capdevila, A., Antonell, A., Lladó, A., Muñoz-Blanco, J.L., Mora, J.S., Galán-Dávila, L., Rodríguez De Rivera, F.J., Lleó, A.,

Clarimón, J. 2014. Characterization of the repeat expansion size in *C9orf72* in amyotrophic lateral sclerosis and frontotemporal dementia. Hum Mol Genet. 23, 749–754.

Majounie, E., Renton, A.E., Mok, K., Dopper, E.G., Waite, A., Rollinson, S., Chiò, A., Restagno, G., Nicolaou, N., Simon-Sanchez, J., van Swieten, J.C., Abramzon, Y., Johnson, J.O., Sendtner, M., Pamphlett, R., Orrell, R.W., Mead, S., Sidle, K.C., Houlden, H., Rohrer, J.D., Morrison, K.E., Pall, H., Talbot, K., Ansorge, O.; The Chromosome 9-ALS/FTD Consortium; The French research network on FTLD/FTLD/ALS; The ITALSGEN Consortium, Hernandez, D.G., Arepalli, S., Sabatelli, M., Mora, G., Corbo, M., Giannini, F., Calvo, A., Englund, E., Borghero, G., Floris, G.L., Remes, A.M., Laaksovirta, H., McCluskey, L., Trojanowski, J.Q., Van Deerlin, V.M., Schellenberg, G.D., Nalls, M.A., Drory, V.E., Lu, C.S., Yeh, T.H., Ishiura, H., Takahashi, Y., Tsuji, S., Le Ber, I., Brice, A., Drepper, C., Williams, N., Kirby, J., Shaw, P., Hardy, J., Tienari, P.J., Heutink, P., Morris, H.R., Pickering-Brown, S., Traynor, B.J. 2012. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. Lancet Neurol. 11, 323-330.

Neuenschwander, A.G., Thai, K.K., Figueroa, K.P., Pulst, S.M. 2014. Amyotrophic Lateral Sclerosis Risk for Spinocerebellar Ataxia Type 2 *ATXN2* CAG Repeat Alleles. A Meta-analysis. JAMA Neurol. 71, 1529-1534.

Nordin, A., Akimoto, C., Wuolikainen, A., Alstermark, H., Jonsson, P., Birve, A., Marklund, S.L., Graffmo, K.S., Forsberg, K., Brännström, T., Andersen, P.M. 2015. Extensive size variability of the GGGGCC expansion in C9orf72 in both neuronal and non-neuronal tissues in 18 patients with ALS or FTD. Hum Mol Genet. 24, 3133-3342.

Renton, A.E., Chiò, A., Traynor, B.J. 2014. State of play in amyotrophic lateral sclerosis genetics. Nat Neurosci. 17:17-23.

Rohrer, J.D., Isaacs, A.M., Mizielinska, S., Mead, S., Lashley, T., Wray, S., Sidle, K., Fratta, P., Orrell, R.W., Hardy, J., Holton, J., Revesz, T., Rossor, M.N., Warren, J.D. 2015. C9orf72 expansions in frontotemporal dementia and amyotrophic lateral sclerosis. Lancet Neurol. 14, 291-301.

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

van Blitterswijk, M., Baker, M.C., DeJesus-Hernandez, M., Ghidoni, R., Benussi, L., Finger, E., Hsiung, G.Y., Kelley, B.J., Murray, M.E., Rutherford, N.J., Brown, P.E., Ravenscroft, T., Mullen, B., Ash, P.E., Bieniek, K.F., Hatanpaa, K.J., Karydas, A., Wood, E.M., Coppola, G., Bigio, E.H., Lippa, C., Strong, M.J., Beach, T.G., Knopman, D.S., Huey, E.D., Mesulam, M., Bird, T., White, C.L. 3rd, Kertesz, A., Geschwind, D.H., Van Deerlin, V.M., Petersen, R.C., Binetti, G., Miller, B.L., Petrucelli, L., Wszolek, Z.K., Boylan, K.B., Graff-Radford, N.R., Mackenzie, I.R., Boeve, B.F., Dickson, D.W., Rademakers, R. 2013b. *C9ORF72* repeat expansions in cases with previously identified pathogenic mutations. Neurology. 81, 1332-1341.

van Blitterswijk, M., Mullen, B., Heckman, M.G., Baker, M.C., DeJesus-Hernandez, M., Brown, P.H., Murray, M.E., Hsiung, G.Y., Stewart, H., Karydas, A.M., Finger, E., Kertesz, A., Bigio, E.H., Weintraub, S., Mesulam, M., Hatanpaa, K.J., White, C.L. 3rd, Neumann, M., Strong, M.J., Beach, T.G., Wszolek, Z.K., Lippa, C., Caselli, R., Petrucelli, L., Josephs, K.A., Parisi, J.E., Knopman, D.S., Petersen, R.C., Mackenzie, I.R., Seeley, W.W., Grinberg, L.T., Miller, B.L., Boylan, K.B., Graff-Radford, N.R., Boeve, B.F., Dickson, D.W., Rademakers, R. 2014a. Ataxin-2 as potential disease modifier in C9ORF72 expansion carriers. Neurobiol Aging. 35, 2421.e13-7.

van Blitterswijk, M., Mullen, B., Wojtas, A., Heckman, M.G., Diehl, N.N., Baker, M.C., DeJesus-Hernandez, M., Brown, P.H., Murray, M.E., Hsiung, G.Y., Stewart ,H., Karydas, A.M., Finger, E., Kertesz, A., Bigio, E.H., Weintraub, S., Mesulam, M., Hatanpaa, K.J., White, C.L. 3rd, Neumann,

M., Strong, M.J., Beach, T.G., Wszolek, Z.K., Lippa, C., Caselli, R., Petrucelli, L., Josephs, K.A., Parisi, J.E., Knopman, D.S., Petersen, R.C., Mackenzie, I.R., Seeley, W.W., Grinberg, L.T., Miller, B.L., Boylan, K.B., Graff-Radford, N.R., Boeve, B.F., Dickson, D.W., Rademakers, R. 2014b. Genetic modifiers in carriers of repeat expansions in the *C9ORF72* gene. Mol Neurodegener. 9:38.

van Blitterswijk M, van Es MA, Hennekam EA, Dooijes D, van Rheenen W, Medic J, Bourque PR, Schelhaas HJ, van der Kooi AJ, de Visser M, de Bakker PI, Veldink JH, van den Berg LH. 2012. Evidence for an oligogenic basis of amyotrophic lateral sclerosis. Hum Mol Genet. 21, 3776-3784.

van Blitterswijk, M., DeJesus-Hernandez, M., Niemantsverdriet, E., Murray, M.E., Heckman, M.G., Diehl, N.N., Brown, P.H., Baker, M.C., Finch, N.A., Bauer, P.O., Serrano, G., Beach, T.G., Josephs, K.A., Knopman, D.S., Petersen, R.C., Boeve, B.F., Graff-Radford, N.R., Boylan, K.B., Petrucelli, L., Dickson, D.W., Rademakers, R. 2013a. Association between repeat sizes and clinical and pathological characteristics in carriers of C9ORF72 repeat expansions (Xpansize-72): a cross-sectional cohort study. Lancet Neurol 12, 978–988.

van Blitterswijk, M., Mullen, B., Nicholson, A.M., Bieniek, K.F., Heckman, M.G., Baker, M.C., Dejesus-Hernandez, M., Finch, N.A., Brown, P.H., Murray, M.E., Hsiung, G.Y., Stewart, H., Karydas, A.M., Finger, E., Kertesz, A., Bigio, E.H., Weintraub, S., Mesulam, M., Hatanpaa, K.J., White Iii, C.L., Strong, M.J., Beach, T.G., Wszolek, Z.K., Lippa, C., Caselli, R., Petrucelli, L., Josephs, K.A., Parisi, J.E., Knopman, D.S., Petersen, R.C., Mackenzie, I.R., Seeley, W.W., Grinberg, L.T., Miller, B.L., Boylan, K.B., Graff-Radford, N.R., Boeve, B.F., Dickson, D.W., Rademakers, R. 2014c. *TMEM106B* protects *C9ORF72* expansion carriers against frontotemporal dementia. Acta Neuropathol 127, 397-406.

Table 1. Clinical characteristics of ALS cases

	Italian cases (n=1616)	**Sardinian cases (n=356)**
Gender (female)	743 (46%)	128 (36%)
Mean age at onset (years)	61.5 (11.8)	61.3 (11.5)
Site of onset (bulbar)	465 (28.8%)	86 (24.2%)

Table 2. *ATXN2* polyQ intermediate length repeats (<28 vs. ≥28) in *C9ORF72* and non-*C9ORF72* cases

	Italian cases			**Sardinian cases**			**Overall**		
	C9ORF72 cases	Non-*C9ORF72* cases	Controls	*C9ORF72* cases	Non-*C9ORF72* cases	Controls	*C9ORF72* cases	Non-*C9ORF72* cases	controls
<28	549	2620	1588	114	592	485	663	3212	2073
≥28	3	60	12	0	6	1	3	66	13
p value (cases vs controls)	0.62	0.0001	-	0.63	0.10	-	0.61	0.0001	-