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“The ALS-FTLD continuum: clinical, genetic  
and neuropsychological aspects”

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# **1. The ALS-FTLD continuum**

## **1.1 Clinical and epidemiological aspects**

*Amyotrophic Lateral Sclerosis (ALS)* is a fatal neurodegenerative disease affecting both upper and lower motor neurons, leading to progressive palsy of voluntary muscles. It may manifest with dysarthria, dysphagia and dysphonia (bulbar onset) or with limb weakness (spinal onset). Along the disease course, every voluntary motor function may be affected. Median survival is 2-4 years from disease onset (Chiò et al, 2013), being respiratory failure the main cause of death.

Incidence and prevalence rate are similar across Western Countries. Population-based data from Piemonte and Valle d'Aosta, Northern Italy, show an incidence of 2.9/100000/year in the period 1995-2004 and a prevalence of 7.9/100000 at December 31, 2004. The peak of incidence is around 70 years of age (Chiò et al, 2009).

Approximately 10% of cases are familial. The major genes related to familial ALS (fALS) are *SOD1*, *TARDBP*, *FUS* and *c9orf72* (Renton et al, 2014).

ALS diagnosis is based on the El Escorial Diagnostic Criteria revised in 2000 (Brooks et al, 2000).

*Frontotemporal Lobar Degeneration (FTLD)* is a heterogeneous group of disorders characterized by progressive damage of frontal and temporal lobes, with onset before 65 years of age in 75-80% of cases and a familial recurrence in 30-50% of cases. The major genes related to familial FTLD are *c9orf72*, *MAPT* and *PGRN* (Sieben et al, 2012). Epidemiological estimates vary considerably across studies, with an incidence ranging from 2.7 to 4.1/100000/year and a prevalence of 15-22/100000 (Onyike et al, 2013). This variability is

probably due to different measures (point versus cumulative prevalence), different ascertainment methods, and heterogeneity of study populations.

FTLD diagnosis is based on the diagnostic criteria published by Neary and colleagues in 1998 (Neary et al, 1998). FTLD includes three different phenotypes, defined according to their distinctive clinical features.

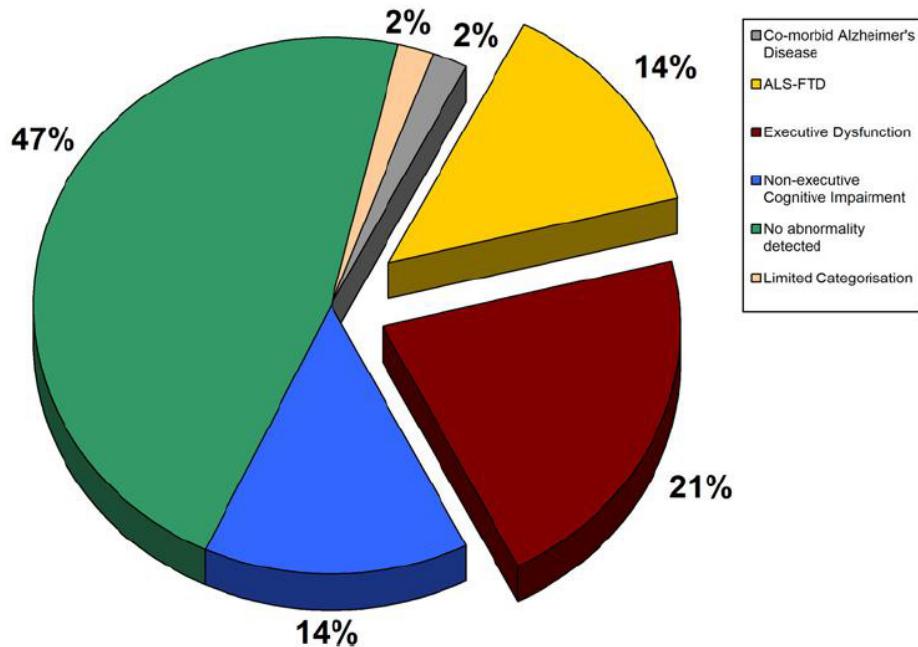
The *behavioral variant (bvFTD)* is characterized by the subtle onset of abnormalities of social and personal conduct, loss of insight and emotional blunting. This entity may manifest with disinhibition, perseverative and stereotyped behavior, mental rigidity and inflexibility, distractibility, hyperorality, decline in personal hygiene and apathy.

*Progressive Non Fluent Aphasia (PNFA)* displays as core features a non-fluent spontaneous speech, hampered by agrammatism, anomia and phonemic paraphasias.

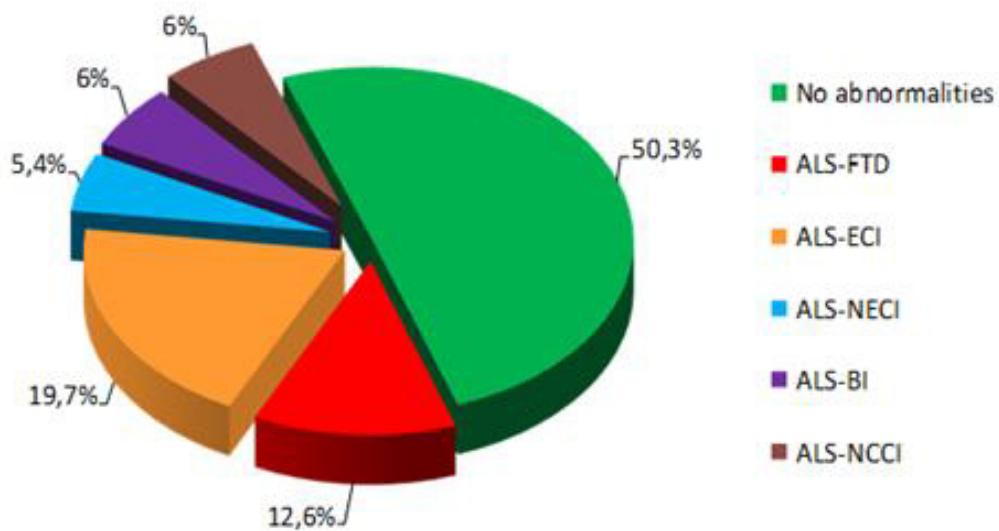
*Semantic Dementia (SD)* is a language disorder characterized by a fluent, empty, spontaneous speech, with impaired naming and comprehension, semantic paraphasias, prosopagnosia and alteration of objects recognition.

In recent years the concept of ALS as a pure motor disorder has been progressively challenged. Cognitive and behavioral abnormalities falling within the spectrum of frontotemporal syndromes are commonly detected in ALS patients. Behavioral changes often include disinhibition, blunting of emotions, apathy, lacking of concern for disability, over-reactivity for sensory stimuli, behavioral stereotypes, and gluttony. Cognitive deficits mainly affect executive planning, attention, verbal and non-verbal fluency (Strong, 2008). Two population-based studies, from the Irish and the Piemonte and Valle d'Aosta Registers for ALS respectively, have demonstrated that approximately 15% of ALS patients show comorbid full blown FTD, about 35% display more subtle cognitive and/or behavioral

changes, only 50% being cognitively normal (Phukan et al, 2012 – fig. 1; Montuschi et al, 2014 – fig. 2).



**Fig. 1** Cognitive profile of a population-based series of ALS patients from the Irish Register for ALS (From Phukan et al, 2012)



**Fig. 2** Cognitive profile of a population-based series of ALS patients from the Piemonte and Valle d'Aosta Register for ALS (data from Montuschi et al, 2014)

The study of cognitive and behavioral abnormalities in ALS patients is of crucial importance, since the presence of comorbid dementia and even executive dysfunction alone is related to worse prognosis (Olney et al, 2005; Elamin et al, 2011). Besides, the presence of cognitive impairment constitutes a strong if not the main factor of caregivers' burden (Chiò et al, 2010; Lillo et al, 2012).

## **1.2 Genetic and neuropathological aspects**

There are many genetic and neuropathological data supporting the overlap of ALS and FTLD. A milestone in this research field was the identification of the protein TDP-43 as the major component of neuronal cytoplasmic ubiquitin-positive inclusions both in FTLD and ALS (Neumann et al, 2006). TDP-43 is a transcriptional regulator involved in RNA splicing and stability, encoded by the *TARDBP* gene. In physiological conditions TDP-43 shuttles between the nucleus and the cytoplasm, but it is mostly localized in the nucleus. In pathological conditions it is mislocalized in the ubiquitinated cytoplasmic inclusions (Neumann et al, 2006). Mutations of *TARDBP* gene have been detected in sporadic and familial ALS (Sreedharan et al, 2008), in pure FTLD (Borroni et al, 2010), in FTD patients developing motor neuron disease (FTD-MND) (Benajiba et al, 2009), in ALS cases with FTD (ALS-FTD) (Chiò et al, 2010).

Fused in sarcoma (FUS) is a protein involved in transcription processes, mRNA splicing and transport. Mutation of *FUS* gene were found to be causative in approximately 4% of familial ALS. The FUS protein was found in cytoplasmic aggregates in such patients (Kwiatkowski et al., 2009). In pathological conditions FUS nuclear staining is often reduced, with increased

cytoplasmic accumulation (Dormann et al, 2011). An accumulation of FUS protein in neuronal nucleus and cytoplasm is also found in some FTLD subtypes (Sieben et al, 2012).

Valosin Containing Protein (VCP) is a protein with a role in multiple cellular functions, including protein degradation via ubiquitin-proteasome system and autophagy (Yamanaka et al, 2012). Notably, VCP mutations cause mitochondrial uncoupling, leading to reduced ATP production (Bartolome et al, 2013). Mutations in the *VCP* gene have been identified in families with the IBMPFD phenotype (Inclusion Body Myopathy, Paget disease of bone, Frontotemporal Dementia) (Watts et al, 2004), in 1-2% of cases of familial ALS (Johnson et al, 2010) e in less than 1% of sporadic ALS patients (Abramzon et al, 2012). FTLD with *VCP* mutations is associated with TDP-43 proteinopathy (Weihs et al, 2011).

A crucial step in the comprehension of the ALS-FTD overlap was the discover in 2011 of a hexanucleotide repeat expansion of the first intron of the *C9orf72* gene as the most frequent cause of familial ALS and FTD, accounting for 30-40% of fALS cases and about 12% of familial FTD (DeJesus-Hernandez et al, 2011; Renton et al, 2011). Such expansion also causes ~7% of sporadic ALS and ~6% of sporadic FTD (Majounie et al, 2012). FTLD in *C9orf72* expansion carriers is characterized by TDP-43-positive neuronal and glial inclusion bodies (Sieben et al, 2012).

P62 is another multifunctional protein involved both in ALS and FTLD pathology. It is encoded by the *SQSTM1* gene and intervenes in the autophagy pathway. Mutations of *SQSTM1* have been identified as a cause of fALS and sALS (Fecto et al, 2011) and in FTLD patients (Rubino et al, 2012). P62-positive inclusions can be detected in *c9orf72* expansion carriers, with or without TDP-43 proteinopathy (Bennion Callister et al, 2014).

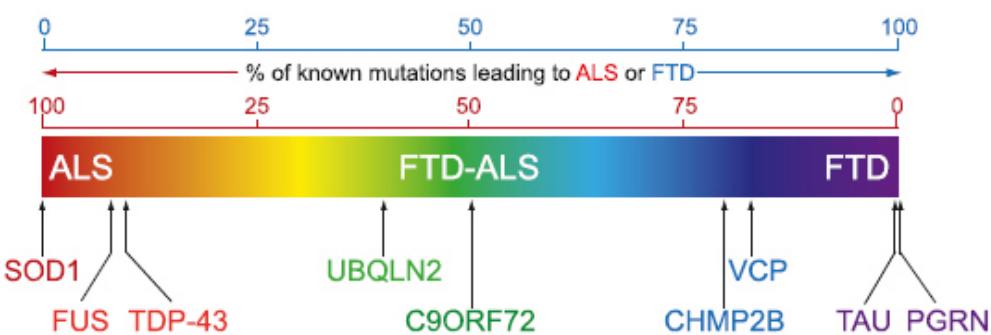
The *UBQLN2* gene encodes Ubiquilin2, a protein involved in the degradation of ubiquitinated proteins. Mutations in *UBQLN2* have been identified as a rare cause of dominant-inherited X-linked ALS and ALS-FTLD. Interestingly, ubiquilin2-positive inclusions were found in spinal motor neurons of mutation carriers, with immunoreactivity for FUS, ubiquitin and p62. Noteworthy, the ubiquilin2-proteinopathy is detectable also in patients without *UBQLN2* mutations (Deng et al, 2011).

*SOD1* is the first gene demonstrated to cause familial ALS (Rosen et al, 1993). Notably, cognitive impairment is extremely rare among *SOD1* mutations carriers (Millecamps et al, 2012). The frequency of *SOD1* gene mutations in fALS patients varies across populations, from 0% in Ireland (Kenna et al, 2013) to 13.6% in Italy (Chiò et al, 2008) and 23.5 % in Scandinavia (Andersen et al, 1997). It is worthy of note that *SOD1* mutations carriers show ubiquitin-positive, TDP-43-negative neuronal inclusions (MacKenzie et al, 2007).

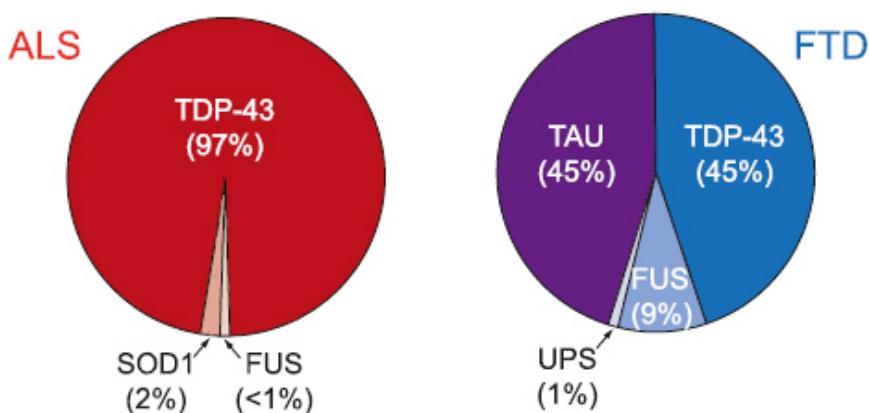
### **1.3 The ALS-FTLD continuum hypothesis**

The increasing literature demonstrating a clinical, neuropathological and genetic overlap between ALS and FTLD has led to the formulation of the hypothesis that these two conditions constitute the extremities of the same disease spectrum, defined as the ALS-FTLD continuum. On one side of the spectrum we find pure motor degeneration (motor neuron disease), on the other one a pure cognitive phenotype (frontotemporal lobar degeneration), with a florid middle ground including motor neuron disease associated with FTLD (see Fig. 3).

### A Genetics of ALS and FTD



### B Pathological inclusions in ALS and FTD



**Fig. 3** The ALS-FTD continuum: genetics (A) and neuropathological features (B) (from Ling et al, 2013).

The study of the common underlying pathomechanisms is of outstanding importance since the association of motor and cognitive impairment predicts a worse prognosis and a harder burden of care compared to pure phenotypes (Olney et al, 2005; Elamin et al, 2011; Chiò et al, 2010; Lillo et al, 2012). This clinical overlap is also worthy of consideration in the definition of outcome measures in clinical trials, that are currently centered on motor performance. Besides, the elucidation of genetic factors common to ALS and FTLD warrants a new concept of familial disease. In fact, some genes are associated with a phenotypic heterogeneity and may cause pure ALS, pure FTLD or ALS-FTLD in different patients even within the same

pedigree. This is particularly relevant in the field of genetic counselling: the presence of subjects affected by dementia in the kindred of an ALS case should arise the possibility of a genetic cause and viceversa, with important consequences on the clinical management.

#### **1.4 Aims of the present project**

The research activity was aimed to study the overlap of ALS and FTLD from a clinical, genetic and neuropsychological point of view. The study population included the incident ALS cases afferent to the Piemonte and Valle d'Aosta Register for ALS (PARALS), a clinic-based series of ALS patients of the ALS Centre of Turin ("Rita Levi Montalcini" Department of Neuroscience, University of Torino, Torino, Italy), and a series of ALS patients of Sardinian ancestry recruited through the ITALSGEN Consortium, which includes sixteen Italian ALS Centers. The PARALS sample has the quality to allow population-based studies of high value from the epidemiological point of view. The Sardinian sample has the extraordinary characteristic to be part of a genetic isolate. As other conserved populations, it provides valuable information about genetic diseases, and amyotrophic lateral sclerosis as well. It is well known that in Sardinia the p.A382T mutation of *TARDBP* and the rate of familial ALS are higher than expected (Chiò et al, 2011; Orrù et al, 2012), even with an overall ALS incidence equal to other European Countries (Pugliatti et al, 2013).

The three study included in the research project have explored different aspects of the ALS-FTLD continuum. The first one describes the cognitive correlates of a novel mutation of the *SOD1* gene in a familial ALS case. Notably, cognitive impairment is extremely rare in *SOD1* mutations carriers. The second one provides preliminary evidence of the applicability of the cognitive reserve hypothesis to the frontotemporal syndromes of ALS patients, being the first

study ever performed on this topic. The third survey is centered on the genetic architecture of ALS in Sardinia, highlighting the frequency of different genes mutations and their association with FTD. The background, the methods and the results of the single studies are discussed below in the specific chapters.

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## **2. A Familial ALS case carrying a novel p.G147C SOD1 heterozygous missense mutation with non-executive cognitive impairment**

### **2.1 Introduction**

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease causing progressive muscle weakness and wasting. Death usually occurs within 3-5 years from respiratory failure. Approximately 10% of cases are familial (fALS) (Chiò et al, 2012). The frequency of *SOD1* gene mutations in fALS patients varies among populations, from 0% in Ireland (Kenna et al, 2013) to 13,6% in Italy (Chiò et al, 2008) and 23,5 % in Scandinavia (Andersen et al, 1997). *SOD1* encodes for the Cu/Zn Superoxide Dismutase 1. Mutations are usually autosomal dominant, but the p.D90A and the p.D96N may be autosomal recessive (Andersen et al, 1995; Hand et al, 2001).

### **2.2 Methods**

Performing the genetic screening of an Italian fALS series, we found a novel c.442g>t heterozygous missense mutation of *SOD1* leading to a substitution of cysteine for glycine (p.G147C). Such mutation was absent in healthy controls (n=130). The patient provided written informed consent.

### **2.3 Case report**

The index case displayed progressive weakness and wasting of both hands when he was 52. One year after the onset he also exhibited tongue hypotrophy with fasciculations, spastic paraparesis, impairment of feet extension and brisk jaw jerk and lower limbs reflexes. Plantar response was absent bilaterally. He referred diffuse cramps and fasciculations. Dysphagia, dysarthria, dysphonia and dyspnea were absent. The ALS FRS - R was 44/48. Needle EMG showed chronic and active denervation in bulbar and spinal regions. Forced vital capacity was 106%. The neuropsychological evaluation showed an impaired performance in the Rey-Osterrieth Complex Figure Test (ROCF), both in copy and recall task, while other tests had normal scores. Brain MRI showed selective atrophy of the right supramarginal gyrus (Figure 1A), slight hyperintensity of the corticospinal tracts in T2-weighted scans, reduced Fractioned Anisotropy along the right corticospinal tract in DTI scans. Cervical cord MRI was normal. <sup>18</sup>F-FDG cerebral PET revealed reduced uptake ( $p=0,001$ ) in the right supramarginal gyrus (BA 40) (Figure 1B). Brain MRI and <sup>18</sup>F-FDG PET and neuropsychological assessment were repeated six months later. The focal atrophy of the right supramarginal gyrus resulted unchanged at MRI. <sup>18</sup>F-FDG PET showed slight extension of the area of hypometabolism previously observed at this site and the appearance of a new area in the right fronto-polar region ( $p=0.01$ ) (Figure 1C). A relative reduction of the uptake in the right caudate nucleus, probably due to deafferentation, seems to support the signifiativity of the fronto-polar hypometabolism. The neuropsychological evaluation confirmed the deficit in the ROCF and demonstrated a reduction of the scores of MMSE, TMT-A and -B, Clock Test and FAB, although they were still normal.

Two proband's brothers died from ALS. The former showed muscle weakness and wasting at upper limbs when he was 46. He had a rapid worsening and died from respiratory failure 10 months after the onset. The latter reported cramps at lower limbs when he was 48, followed

by weakness of the left upper limb. He showed a classic ALS phenotype and died from respiratory failure 27 months after the onset. Two siblings were 46 and 50 and healthy. His father died when he was 76 from chronic kidney failure without any neurological impairment; his mother was 83 and healthy. No other relatives with neurological impairment were reported. The index case showed a p.G147C missense mutation of *SOD1* (mutations of other ALS-related genes were excluded). The same mutation was found in the latter affected sibling. DNA of other family members was unavailable. A diagnosis of clinically definite fALS with genetic confirmation was made. The patient is still alive, 24 months after the onset. He also shows slight dysarthria and occasional dysphagia. The disease is slowly progressive and respiratory involvement is absent. The autonomy in daily living activities is good. ALS FRS - R is 41/48.

## 2.4 Discussion

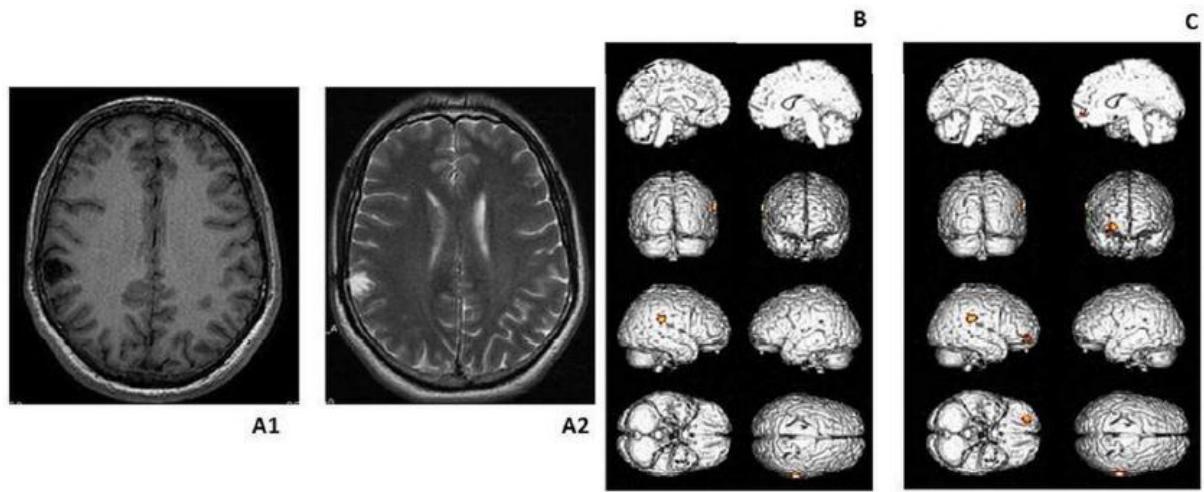
Three other missense mutations of codon 147 of *SOD1* have already been found. Andersen and colleagues reported an Icelandic case with a p.G147R mutation and a patient carrying a p.G147D mutation (Andersen et al, 2003). Two families with a p.G147D mutation were identified in a series of French fALS cases: age of onset ranged from 45 to 73, with spinal onset for three of four; tested subjects had no cognitive impairment; disease duration ranged from 10 to 49 months (Millecamps et al, 2010). The same mutation was found in Chinese familial cases with a fast course (Niu et al, 2011). The p.G147S mutation was described in an apparently sporadic case with bulbar onset at the age of 56 and death from respiratory failure in 8 months. Codon 147 encodes a highly conserved aminoacidic residue across species. This change was predicted to affect protein function by molecular modelling studies (Origone et al, 2012).

We report the first ALS case carrying a p.G147C heterozygous missense mutation of *SOD1*. Noteworthily, the impairment of the ROCF is in agreement with the reduced uptake of the tracer in the right supramarginal gyrus (BA 40) and the right middle frontal gyrus (BA 10) at <sup>18</sup>F-FDG PET and with the atrophy at MRI in the right supramarginal gyrus (BA 40). A <sup>18</sup>F-FDG PET study in probable AD patients showed a significant positive association between ROCF performance and cortical metabolism in the following regions: the widest area was situated in the posterior part of the right hemisphere and included the supramarginal gyrus; other significant clusters were found in the right frontal lobe and included the middle frontal gyrus (Melrose et al, 2013).

Cognitive impairment is extremely rare in *SOD1* mutations carriers (Millecamp et al, 2010; Millecamp et al, 2012). *SOD1* fALS subjects seem less vulnerable to cognitive dysfunction than non-*SOD1* fALS (Wicks et al, 2009). The follow up including neuropsychological tests and <sup>18</sup>F-FDG PET showed a trend to progression of cognitive impairment with frontal lobe involvement. This finding supports the hypothesis that cognitive impairment is related to ALS rather than to other causes (i.e. developmental anomalies).

Most of the cases carrying mutations of codon 147 show a rapid worsening, while our patient displays a slower trend. His affected siblings had a faster course. We need further data to establish possible genotype-phenotype correlations.

Although genetic data were unavailable for one of the affected siblings and healthy relatives, the presence of the p.G147C mutation in two ALS cases supports the hypothesis that it is pathogenic. Nevertheless, the presence of healthy aged parents and the absence of other affected relatives raises the possibility of an incomplete penetrance. Further studies are necessary to highlight its pathogenic role and its clinical manifestation.



**Figure 1.** A. MRI axial T1 (A1) and T2 (A2) images show a focal atrophy of the right supramarginal gyrus (Brodmann area [BA] 40). B.  $^{18}\text{F}$ -FDG cerebral PET parametric study confirmed this finding, showing a reduced uptake ( $p=0.001$ ) in the right supramarginal gyrus (BA 40). C. A  $^{18}\text{F}$ -FDG cerebral PET parametric study performed six months after the previous study showed an extension of reduced uptake in the middle frontal gyrus (BA 10) (at  $p=0.01$ ).

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### **3. Cognitive reserve in amyotrophic lateral sclerosis – frontotemporal dementia: a population-based study**

#### **3.1 Abstract**

The cognitive reserve hypothesis has been suggested by the observation that, for a given level of cognitive impairment, patients with high degree of education and occupational skills show a more sizeable cerebral damage load, as if they had a greater ability to resist the neurodegeneration. This hypothesis has been confirmed in patients with Alzheimer's Disease, Frontotemporal Dementia and other conditions characterized by cognitive impairment, but data about the frontotemporal syndromes associated to Amyotrophic Lateral Sclerosis are lacking. We analyzed a population-based cohort (n=183) (Discovery Cohort) of ALS incident cases collected through the Piemonte and Valle d'Aosta Register for ALS (PARALS) between January 1<sup>st</sup> 2009 and December 31<sup>st</sup> 2011. Furthermore we examined a Validation Cohort including ALS patients diagnosed in the same period at our Centre who were not resident in Piemonte and Valle d'Aosta (n=40), and cases identified through the PARALS between January 1<sup>st</sup> 2012 and June 30<sup>th</sup> 2013 (n=73). Subjects underwent neuropsychological and genetic testing. Years of schooling and a Reserve Index (RI) (2-12) calculated from education and occupation were considered as cognitive reserve proxies. Patients' cognitive status was classified into the following 7 categories: 1) ALS with normal cognition; 2) ALS with frontotemporal dementia (ALS-FTD); 3) ALS with other dementias; 4) ALS with executive cognitive impairment (ALS-ECI); 5) ALS with non-executive cognitive impairment (ALS-NECI); 6) ALS with behavioral impairment (ALS-Bi); 7) ALS with non-classifiable cognitive impairment (ALS-NCCI). In the Discovery Cohort, ALS-FTD patients had lower

education (4.7 years, SD 1.9) and RI (4.9, SD 1.3) than other groups ( $p=0.0001$ ). In the Validation Cohort, ALS-FTD patients (7.0 years, SD 2.6) had education equal to ALS-NECI (7.0, SD 1.4), that was lower than other groups ( $p=0.003$ ). ALS-FTD patients had lower RI (5.7, SD 1.6) than other groups but ALS-NECI ( $p=0.003$ ). Results were independent from sex, age, site of onset and were confirmed among *c9orf72*-mutated patients ( $p=0.012$ ). Subdividing our patients according to low-, intermediate- and high-score RI groups, we found a significant positive correlation between RI and some neuropsychological tests evaluating frontal functions.

Our data support the hypothesis that cognitive reserve may play a role in the cognitive impairment related to ALS, in particular in full-blown FTD, may have an influence also in patients carrying genetic mutations, such as *c9orf72*, and may be related to frontal lobe functioning. Further studies are required to correlate these data to neuroimaging measures of cerebral lesion load to better identify the structural and functional basis of cognitive reserve in ALS patients with cognitive impairment.

### **3.2 Introduction**

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive disease characterized by a degeneration of both upper and lower motor neurons. Patients show progressive muscle weakness and wasting involving bulbar and spinal regions, with death usually occurring within 3 years due to respiratory failure (Hardiman et al., 2011). Besides motor impairment, extramotor abnormalities in ALS may include cognitive and behavioral changes falling within the frontotemporal lobar degeneration (FTLD) spectrum. Two population-based studies in Ireland and Italy have shown that ~15% of ALS patients display a full-blown frontotemporal

dementia (FTD) while ~35% have more subtle cognitive alterations involving executive and non-executive domains (Phukan et al., 2012; Montuschi et al., 2014).

In the area of degenerative dementias a growing interest is developing about the cognitive reserve hypothesis. This concept is based on the observation that, for a given degree of dementia, there is an inverse correlation between educational and occupational skills level on one side and cerebral lesion load on the other, as if educated and skilled subjects were able to cope better with brain pathology. The neurobiological basis underlying cognitive reserve is still poorly understood. The elucidation of factors capable of increasing such reserve might have a key role in prevention strategies. Education can be measured as years of schooling, while several algorithm have been proposed to assess occupational attainment (Garibotto et al., 2008; Spreng et al., 2010; Spreng et al., 2011). Several studies suggest a role of cognitive reserve in Alzheimer's disease (AD) (Garibotto et al., 2008; Garibotto et al., 2012; Stern et al., 2012; Meng et al., 2012), in amnestic mild cognitive impairment (aMCI) converters (Garibotto et al., 2008; Morbelli et al., 2013) and in preclinical AD (Ewers et al., 2013). Although its relevance in the FTLD spectrum has been supported by various papers (Perneczky et al., 2007; Perneczky et al., 2007; Borroni et al., 2009; Spreng et al., 2010; Spreng et al., 2011), to the best of our knowledge, there are no studies specifically designed to establish the validity of the cognitive reserve hypothesis for the frontotemporal syndromes observed in ALS patients.

The aim of our study is to verify the applicability of the cognitive reserve assumption in a population-based cohort of ALS patients, fully characterized from the clinical, neuropsychological and genetic point of view, and to replicate the results in a clinical-based validation cohort.

### **3.3 Materials and methods**

#### *Patients*

First, we analyzed a population-based cohort (n=183) composed of ALS incident cases collected through the Piemonte and Valle d'Aosta Register for ALS (PARALS) between January 1<sup>st</sup> 2009 and December 31<sup>st</sup> 2011. Patients with history of neurological disorders affecting cognition (major stroke, severe head injuries, mental retardation), alcohol-dependence and drug-dependence, severe mental illness and use of high-dose psychoactive medications were not enrolled in the study. Patients resident in the area but who were not of Italian mother tongue were excluded from the study. This sample represented our discovery cohort.

Furthermore we examined a sample including ALS patients diagnosed in the same period at our Centre who were not resident in Piemonte and Valle d'Aosta (n=40), and cases identified through the PARALS between January 1<sup>st</sup> 2012 and June 30<sup>th</sup> 2013 (n=73). Exclusion criteria were the same adopted for the discovery cohort. This sample (n=113) was conceived as a validation cohort.

All ALS cases met the revised El Escorial diagnostic criteria (Brooks et al., 2000) for definite, probable and probable laboratory-supported ALS. Disease severity was assessed with the amyotrophic lateral sclerosis functional rating scale - revised (ALS FRS - R) (Cedarbaum et al., 1999). A respiratory function assessment was carried out for every subject within 4 weeks before or after neuropsychological testing. Patients were asked to participate to the study at the time of diagnosis or less frequently during the first follow up visit (usually 2 months later). Anyway, in all cases the neuropsychological battery was administered within 12

months after diagnosis. Neuropsychological evaluation was performed at the ALS Center or at patients' home.

#### *Genetic analysis*

All the coding exons and 50bp of the flanking intron-exon boundaries of *SOD1*, of exon 6 of *TARDBP*, and of exons 14 and 15 of *FUS/TLS* and exons 5, 9, 12 and 14 of *OPTN* and the single exon of *ANG* have been PCR amplified, sequenced using the Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems Inc.), and run on an ABI Prism 3130 genetic analyzer. In patients with positive family history for ALS or FTD all the coding exons of *VCP* have also been sequenced. These exons were selected as they contain the vast majority of the known mutational hotspots. A repeat-primed PCR assay was used to search for the presence of the GGGGCC hexanucleotide repeat expansion in the first intron of the *c9orf72* gene (Renton et al., 2011). A cut-off of  $\geq 30$  repeats was considered pathological.

#### *Neuropsychological assessment*

The selection of the neuropsychological tests, evaluating executive function, memory, visuo-spatial function and language, was based on the Clinical Diagnostic Criteria for Frontotemporal Lobar Degeneration (Neary et al., 1998), and the ALS-FTD Consensus Criteria (Strong et al., 2009). The neuropsychological battery included: Mini Mental State Examination; Wisconsin Card Sorting Test; Trail Making A and B; Stroop Colour-Word Interference Test; letter and category fluency test; Wechsler Memory Scale II - revised (Form 2); Rey-Osterrieth Complex Figure Test; Token test; Wechsler Adult Intelligence Scale revised; Raven's Progressive Colored Matrices; Frontal Assessment Battery. Neurobehavioral dysfunction was assessed through the direct observation and the patient's history, and with the Frontal Systems Behavior Scale, using the Family-form completed by a close relative (scores:

normal  $\leq$ 59, borderline 60–64; pathological  $\geq$ 65). If a subject showed scores corresponding to a frontal systems abnormality both in the premorbid and in the postillness forms, he/she was considered pathological only if there was an increase of  $\geq$ 10 points at the T score between the two forms. The evaluation of anxiety and depression was based on the Hospital Anxiety and Depression Scale. The battery was administered following the same sequence for every patient in order to avoid eventual differential interferences of the answers of one test over the others. The administration of the battery required approximately 2 hours, and was usually performed in the morning. If the subject felt too tired, a further session was scheduled to complete the battery, within 2 weeks after the first one. At the time of evaluation none of the patients of either cohorts showed oxygen saturation  $<$ 92% at pulse oximetry.

#### *Cognitive classification*

Clinical diagnosis and cognitive classification were obtained by a team of neurologists and neuropsychologists expert in ALS and FTD. Patients' cognitive status was classified into the following 7 categories:

- 1) *ALS with normal cognition*;
- 2) *ALS with frontotemporal dementia (ALS-FTD)* → the diagnosis of frontotemporal dementia was defined according to Clinical Diagnostic Criteria for Frontotemporal Lobar Degeneration (Neary et al., 1998);
- 3) *ALS with other dementias* → non-FTD dementias were diagnosed according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders-IV-TR (DSM IV-TR) and those of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (McKhann et al., 1984);

- 4) *ALS with executive cognitive impairment (ALS-ECI)* → patients with ALS who did not meet criteria for FTD or other types of dementia, but who had an impairment in two tests of executive dysfunction compared with healthy controls, were classified as ALS with executive cognitive dysfunction. A more conservative cut-off than that proposed by the ALS-FTD Consensus Criteria (Strong et al., 2009) was used (2.3<sup>rd</sup> centile) (Phukan et al., 2012);
- 5) *ALS with non-executive cognitive impairment (ALS-NECI)* → patients with impairment in two non-executive domains, particularly visuopraxic abilities, and no impairment in executive function.
- 6) *ALS with behavioral impairment (ALS-Bi)* → patients with predominant behavioural disturbances and with impairment in none or only one test of executive dysfunction and no impairment in non-executive domains.
- 7) *ALS with non-classifiable cognitive impairment (ALS-NCCI)* → patients with impairment in one executive and/or one non-executive test, sometimes associated with smooth behavioural changes.

#### *Cognitive reserve evaluation*

The identification of education and occupation as proxies of cognitive reserve in AD and FTD has been reported by various studies (Garibotto et al., 2008; Spreng et al., 2010; Spreng et al., 2011; Garibotto et al., 2012). We looked for a possible correlation between such variables and the cognitive profiles of ALS patients to verify the applicability of the cognitive reserve hypothesis in the ALS population. Occupations were classified through the six-rank system proposed in the general NEST-DD Project protocol (Garibotto et al., 2008):

1. no occupation;
2. unskilled laborer;
3. housewife;

4. skilled laborer, tradesman, lower level civil servant, employee, self-employed small business, office or sales personal;
5. mid-level civil servant or management, head of a small business, academician or specialist in a subordinate position;
6. senior civil servant or management, senior academic position, self-employed with high degree of responsibility.

Unlike the NEST-DD Project protocol, we did not consider only the last employment but the higher-ranked one, provided that it covered at least one fourth of the whole work history. The occupational rank score was established blindly by three independent raters. Discrepant scores were resolved through discussion among raters. In case of persistent disagreement a fourth independent rater has been consulted. Education was rated considering the number of years of schooling, adding the possible years of apprenticeship only when formal education was present. As previously proposed (Garibotto et al., 2008), we performed a six-rank transformation of years of education, similar to the six-rank classification of occupations. We propose the following ranking:

1. 0-4 years;
2. 5-7;
3. 8-10;
4. 11-12;
5. 13-16;
6.  $\geq 17$ .

Since occupation and education are highly related, their rank was summed to compose a score from 2 to 12 called Reserve Index (RI) (Garibotto et al., 2008). RI has been hypothesized to be a better estimate of cognitive reserve than education level alone (Garibotto et al., 2008).

### *Statistical analysis*

Comparisons between categorical variables were made with  $\chi^2$  test; comparisons between continuous variables were performed with Kruskall-Wallis test or analysis of variance (ANOVA). Since multiple comparisons were performed, p level was corrected according to Bonferroni (see corresponding tables). All tests were two-tailed. Statistical analyses were carried out using the SPSS 21.0 statistical package (SPSS, Chicago, IL, USA).

### *Standard Protocol Approvals, Registrations and Patient Consents*

The study was approved by the Institutional Ethical Committee of our Center and every patient provided a written informed consent before the enrollment. Data were kept according to the Italian law for the protection of privacy.

## **3.4 Results**

### *Discovery cohort*

Demographic and clinical data of the patients included in the discovery cohort are reported in Table 1. The neuropsychological assessment was always performed within 12 months after diagnosis (median time: 1.9 months; interquartile range [IQ]: 1.2-3.8).

### *Cognitive classification.*

Patients were assigned to the different cognitive categories as follows: 23 (12.6%) ALS-FTD, 36 (19.7%) ALS-ECI, 10 (5.5%) ALS-NECI, 11 (6%) ALS-Bi, and 11 (6%) ALS-NCCI; 91 (49.7%) patients were cognitively normal. One patient showed co-morbid AD and therefore was excluded from subsequent analyses.

### *Cognitive reserve*

Mean educational levels and RI scores according to patients' cognitive impairment are reported in Table 2. The comparison of the educational level among the different cognitive categories showed that patients with ALS-FTD had a significantly lower education level (4.7 years, SD 1.9) than all other groups. Similarly, ALS-FTD patients had significantly lower RI score (4.9, SD 1.3) than all other groups ( $p=0.0001$ ).

### ***Validation cohort***

Demographic and clinical data of the 113 patients of the validation cohort are reported in Table 1. Patients included in the validation cohort were significantly younger and had a higher mean educational level than those of the discovery cohort, as expected for a non-epidemiological sample. All the patients of this cohort underwent neuropsychological assessment within 12 months after diagnosis (median time: 3.8 months; IQ range: 2.3-6.3).

### *Cognitive classification*

Patients were assigned to the cognitive categories as follows: 18 (15.9%) ALS-FTD, 21 (18.6%) ALS-ECI, 4 (3.5%) ALS-NECI, 7 (6.2%) ALS-Bi, and 6 (5.3%) ALS-NCCI; 57 (50.5%) patients were cognitively normal.

### *Cognitive reserve*

The number of years of schooling and RI scores according to cognitive classification are reported in Table 2. Patients with ALS-FTD (7.0 years of education, SD 2.6) had the same education level of ALS-NECI (7.0 [SD 1.4]), that was significantly lower than that observed in all other cognitive categories ( $p=0.003$ ). ALS patients with a full-blown FTD had lower RI score (5.7, SD 1.6) than all other groups but those with ALS-NECI ( $p=0.003$ ).

### ***Whole study population***

To verify the possible impact of age, gender, site of onset and genetic characteristics on the correlation between education and RI and cognitive classification, we considered together the two cohorts. We adopted the same approach to evaluate which neuropsychological tests were better related to RI.

### ***Demographic and clinical subgroups***

Subdividing patients according to gender, ALS-FTD cases showed significantly lower education and RI score than all other groups both in males and females (data not shown). The same findings were obtained considering patients with bulbar and spinal onset separately (data not shown) and subdividing patients according to age (<60 and ≥60 years) (data not shown).

### ***Genetic data***

A total of 44 patients (14.9%) carried a mutation of one ALS gene (*c9orf72*=25; *TARDBP*=10; *SOD1*=6; *FUS*=2; *OPTN*=1). For the scope of this paper, we focused on *c9orf72* expansion carriers since this was the only sample to have an adequate size. Eleven patients with *c9orf72* expansion were classified as ALS-FTD (44%), 6 as ALS-ECI (24%), and 8 were cognitively normal (32%). As already observed in the whole population, also among patients carrying *c9orf72* mutation those with ALS-FTD had a RI (5.5, SD 1.6) significantly lower than those with ALS-ECI (6, SD 1.4) and normal cognition (7.6, SD 1.6) (p=0.037). The number of years of education was slightly higher in ALS-FTD patients (7.1, SD 2.9) than in ALS-ECI patients (6.7, SD 2.9), but for both groups it was significantly lower than in cognitively normal patients (11.5, SD 2.9) (p=0.012).

### *Correlation between neuropsychological tests and RI*

We evaluated whether specific tests were related to RI score (Table 3). For this purpose we subdivided patients into three categories: low RI (3-5), intermediate RI (6-8), high RI (9-12). A  $p \leq 0.002$  was considered significant after correction for multiple comparisons. A significant positive correlation was found with Trail Making Test (TMT) B ( $p=0.0001$ ), TMT B-A ( $p=0.0001$ ), Stroop Colour - Word Interference Test (Stroop) ( $p=0.0001$ ), Wechsler Adult Intelligence Scale revised (WAIS-R) Block Design ( $p=0.0001$ ), Wechsler Memory Scale - revised (WMS-R-Form 2) ( $p=0.0001$ ), Raven's Colored Progressive Matrices (CPM) A ( $p=0.0001$ ), B ( $p=0.0001$ ), and AB ( $p=0.0001$ ), CPM total score ( $p=0.001$ ), and Frontal Assessment Battery (FAB) total score ( $p=0.0001$ ).

### **3.5 Discussion**

In our population-based series of ALS patients we have found that a lower educational level and a worse RI are related to a higher risk of FTD, indicating that the hypothesis that a higher cognitive reserve is protective for the development of cognitive impairment also holds for ALS. This finding has been confirmed in an independent validation cohort including patients seen in our center but not resident in Piemonte and patients resident in Piemonte but seen after the completion of the epidemiological survey. Cognitive reserve seems to play a role also in patients carrying *c9orf72* repeat expansion, a gene which is *per se* strictly linked with FTD. To the best our knowledge, our study is the first one to have assessed the correlation between ALS cognitive profile and patients' education and occupational skills level.

The cognitive reserve hypothesis has been supported by the observation that, for a given level of cognitive impairment, patients with high degree of education and occupational skills show

a more sizeable cerebral damage load at  $^{18}\text{FDG-PET}$  or SPECT, as if they had a greater ability to resist the neurodegeneration. This finding has been reported in several dementing diseases, such as in Alzheimer's Disease (AD) (Garibotto et al., 2008; Garibotto et al., 2012; Stern et al., 2012; Meng et al., 2012), preclinical AD (cognitively healthy subjects with low CSF levels of  $\text{A}\beta_{1-42}$ ) (Ewers et al., 2013), amnestic mild cognitive impairment converters (Garibotto et al., 2008; Morbelli et al., 2013), FTD (Perneczky et al., 2007; Borroni et al., 2009; Spreng et al., 2011), multiple sclerosis (Sumowski et al., 2014) and traumatic brain injury (Schneider et al., 2014).

In a population-based study on cognitive impairment in ALS, ALS-FTD patients and non-demented patients showed no significant differences in Full Scale IQ (FSIQ) and number of years of formal education (Phukan et al., 2012). However, patients with executive dysfunction and subjects with non-executive cognitive impairment showed significantly lower education and estimated premorbid FSIQ compared to patients without cognitive abnormalities.

Minor differences have been found between the two cohorts of our study. First, in the discovery cohort ALS-FTD patients had the lowest scores compared to all other cognitive groups, while in the validation cohort ALS-FTD and ALS-NECI had similar values both in educational level and RI, however still lower than all other groups. Second, in the discovery cohort, the ALS-NCCI group displayed the highest education level and RI score, but this observation has not been confirmed in the validation cohort. The partial discrepancy between the two cohorts may be due to different factors. First, the discovery cohort is an epidemiological series, while the validation cohort is a clinic-based series. Consequently, the discovery cohort includes significantly older patients, with a lower educational level. Second, the characterization of ALS-NECI and ALS-NCCI as separate entities needs to be defined more precisely. Third, low scores of ALS-NECI in the validation cohort may be related to the

small number of cases with this cognitive classification. Nevertheless, taken together, our data suggest that ALS patients with comorbid FTD tend to have lower education and worse occupational skills than non-demented patients. This trend is independent from patients' gender, age and site of onset.

ALS is considered a complex disease in which genetic and environmental factors contribute to disease susceptibility (Simpson et al., 2006). Environmental factors might play a role also in ALS cases due to mutations of major ALS genes, contributing to their phenotypic variability. To verify whether a strong genetic background could be influenced by educational and occupational achievements, we assessed the 25 patients who carried a *c9orf72* expansion, the most common gene related to ALS and FTD (Renton et al., 2011). We found that among *c9orf72* expansion carriers, those with ALS-FTD had a lower RI than those with ALS-ECI or normal cognition, and an education level similar to that of ALS-ECI but much lower than that of cognitively normal patients. These data suggest that cognitive reserve may play a role also in patients with a strong genetic background. Our findings are in keeping with the observation that education and occupation are proxies for cognitive reserve in both *apoE ε4* carrier and non-carrier patients with probable AD (Garibotto et al., 2012). Similarly, studies involving FTLD patients confirm the validity of the cognitive reserve hypothesis also in patients with positive family history (Premi et al., 2012) and *GRN* gene mutations (Premi et al., 2013), albeit with partially different mechanisms.

A study involving prodromal AD patients (amnestic MCI converting to AD) showed that high-education (HE) patients had a more relevant brain lesion load at <sup>18</sup>FDG-PET in areas typically affected in AD than low-education (LE) prodromal AD, strengthening the cognitive reserve hypothesis. Besides, the authors observed a more intense and extensive functional connectivity of the dorso-lateral frontal cortex with other areas in HE prodromal AD than in

LE prodromal AD and HE controls (Morbelli et al., 2013). This findings suggest that in pathological conditions the cerebral circuitry function may change through a more intense activation of preexisting networks and the recruitment of new, compensatory pathways.

Undoubtedly, neuropsychological tests have limited spatial resolution in the exploration of brain functions and provide partial information about the function of brain networks.

Nevertheless, it is noteworthy that, subdividing our patients into low, intermediate and high RI groups, we found a significant positive correlation between RI and Trail Making Test (TMT), Stroop (Word), Wechsler Adult Intelligence Scale revised (WAIS-R) Block design, Wechsler Memory Scale (WMS), Raven's coloured progressive matrices (CPM) and Frontal Assessment Battery (FAB). Strikingly, such tests mostly explore frontal executive functions. In this context, the association with FAB, a global measure of executive abilities, seems to be relevant. The relation between RI and visuo-perceptive and spatial abilities evaluated by WAIS Block Design appears less clear, since they are influenced by executive functioning. Likewise the correlation with memory is probably less relevant, since WMS provides a global evaluation of memory encompassing other cognitive functions.

Some limitations of this study should be considered. First, the six-rank system for classifying occupations attainments is not based on the analysis of the different abilities required for each occupation (Spreng et al., 2010); a more precise characterization of occupational abilities could be useful to evaluate whether education and occupation have a different weight to provide cognitive reserve. Moreover, RI does not take into account leisure activities, that are reported to contribute to cognitive reserve (Stern et al., 2012). Although their precise measure can be difficult, they are worthy of study since they are in the game from the earliest stages of life. Second, we need to correlate education and occupational attainment with brain lesion load (i.e. brain  $^{18}\text{FDG-PET}$  or functional MRI) at different degrees of cognitive impairment.

The elucidation of cognitive reserve mechanisms might allow the elaboration of prevention strategies and rehabilitation protocols based on cognitive exercise. Besides, the identification of the underlying molecular pathways might provide targets for enviromimetics drugs, namely therapeutic agents aiming to mimic and/or enhance the effects of environmental stimulation (Nithianantharajah et al., 2009). However, this scenario is still largely unexplored.

Our data provide an evidence that cognitive reserve may play a role in the cognitive impairment related to ALS, in particular in full-blown FTD, and may have an influence also in patients carrying genetic mutations, such as *c9orf72*. Further studies are required to correlate these data to neuroimaging measures of cerebral lesion load to better identify the structural and functional basis of cognitive reserve in ALS patients with cognitive impairment.

**Table 1.** Demographic and clinical characteristics of ALS patients (discovery and validation cohorts)

	<b>Discovery cohort (n=183)</b>	<b>Validation cohort (n=113)</b>	<b>p</b>
Mean age at onset (years, SD)	67.0 (9.9)	60.1 (10.7)	0.0001*
Gender (female, %)	76 (41.5%)	38 (33.6%)	0.11
Education (number of years, SD)	8.3 (4.1)	9.2 (3.6)	0.02*
Site of onset (bulbar, %)	62 (33.9%)	46 (40.7%)	0.11

\* Kruskal-Wallis test

**Table 2.** Mean years of education and mean reserve index (RI) score according to cognitive status in the two cohorts

	Discovery cohort			Validation cohort		
	#	Education	RI	#	Education	RI
Cognitively normal	91 (50.0%)	8.6 (3.7)	7.0 (1.9)	58 (51.3%)	10.4 (3.3)	7.6 (1.9)
ALS-FTD	23 (12.6%)	4.7 (1.9)	4.9 (1.3)	18 (15.9%)	7.0 (2.6)	5.7 (1.6)
ALS-ECI	36 (19.8%)	7.8 (4.0)	6.6 (2.0)	21 (18.6%)	8.2 (4.4)	6.9 (1.9)
ALS-NECI	10 (5.5%)	9.5 (5.1)	7.4 (2.0)	4 (3.5%)	7.0 (1.4)	5.3 (0.5)
ALS-Bi	11 (6.0%)	9.9 (5.2)	6.8 (2.6)	7 (6.2%)	9.6 (3.3)	7.3 (1.6)
ALS-NCCI	11 (6.0%)	12.4 (4.4)	8.5 (2.4)	5 (4.4%)	8.4 (2.9)	6.8 (1.1)
Overall	182	8.3 (4.1)	6.7 (2.1)	113	9.2 (3.6)	7.0 (1.9)
p-value	-	0.0001	0.0001	-	0.003	0.003

One patient with co-morbid Alzheimer's disease (discovery cohort) is not included in the Table. FALS, familial ALS; FTD, frontotemporal dementia; ECI, executive cognitive impairment; NECI, non-executive cognitive impairment; Bi, behavioural impairment; NCCI, non-classifiable cognitive impairment; RI, reserve index. P-value is calculated with ANOVA.

**Table 3.** Results of cognitive tests according to level of Reserve Index

	Low Reserve Index (3 to 5)	Intermediate Reserve Index (6 to 8)	High Reserve Index (9 to 12)	p
FrSBe CG - Apathy	73.3 (17.9)	67.1 (16.0)	60.9 (15.6)	0.004
FrSBe CG - Disinhibition	58.5 (14.4)	55.1 (14.5)	56.9 (18.0)	0.47
FrSBe CG - Executive	63.9 (14.5)	60.3 (13.8)	59.1 (17.9)	0.26
FrSBe CG - Total score	67.0 (15.2)	62.1 (14.7)	61.3 (20.1)	0.15
MMSE	27.3 (3.9)	28.5 (2.7)	28.9 (1.4)	0.02
Phonemic Fluency	26.3 (9.8)	31.6 (11.1)	31.3 (10.7)	0.02
Categorical Fluency	19.4 (8.1)	24.4 (10.8)	24.6 (9.4)	0.011
RCFT - Copy	29.4 (7.9)	32.2 (6.0)	33.9 (3.1)	0.005
RCFT - Delayed Recall	12.8 (5.7)	14.2 (6.0)	15.2 (7.4)	0.24
TMT-A	52.3 (32.0)	46.6 (36.5)	31.6 (13.2)	0.01
TMT-B	178.9 (134.4)	105.9 (112.7)	59.6 (37.3)	<b>0.0001</b>
TMT B-A	118.7 (109.2)	58.9 (91.6)	29.2 833.5)	<b>0.0001</b>
Stroop W	7.8 (3.3)	10.0 (3.7)	11.6 (4.4)	<b>0.0001</b>
Stroop C	6.5 (2.8)	7.4 (2.7)	8.5 (3.5)	0.03
Stroop CW	7.8 (4.3)	8.4 (4.1)	9.5 (3.7)	0.20
Token Test	30.4 (3.3)	31.6 (3.5)	32.6 (2.3)	0.01
WAIS-R Block design	14.9 (8.7)	21.7 814.7)	28.1 (11.1)	<b>0.0001</b>
WMS	93.1 (9.6)	100.5 (8.5)	109.8 (9.8)	<b>0.0001</b>
WCST - number of categories	2.5 (1.9)	2.8 (2.1)	4.0 (1.9)	0.004

WCST Total Score	78.2 (25.5)	77.2 (28.0)	70.3 (36.5)	0.44
CPM A	8.3 (2.5)	9.7 (1.7)	10.8 (1.3)	<b>0.0001</b>
CPM AB	7.3 (2.6)	8.2 (2.5)	10.4 (1.3)	<b>0.0001</b>
CPM B	5.9 (2.0)	6.8 (2.4)	9.0 (2.4)	<b>0.0001</b>
CPM total score	24.8 (6.4)	27.6 (5.1)	29.2 (3.8)	<b>0.001</b>
FAB total score	11.9 (3.4)	15.8 (1.8)	16.6 (1.3)	<b>0.0001</b>

CPM, Raven's coloured progressive matrices; FAB, Frontal Assessment Battery; FrSBe, Frontal Systems Behavior Scale; MMSE, Mini Mental State Examination; RCFT, Rey-Osterrieth Complex Figure Test; Stroop, Stroop Colour-Word Interference Test; TMT, Trail making test; WAIS-R, Wechsler Adult Intelligence Scale revised; WCST, Wisconsin Card Sorting Test; WMS, Wechsler Memory Scale II – revised. A p-value <0.002 was considered significant after Bonferroni correction. Significant p are in bold.

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## **4. Genetics of Amyotrophic Lateral Sclerosis in Sardinia, insular Italy**

### **4.1 Abstract**

Sardinians are a conserved population, constituting a genetic isolate and showing elevated rates of familial and sporadic ALS. The study of genetic isolates provides striking information on the genetic basis of human diseases. Therefore, we aimed to characterize the genetic profile of a consecutive series of Sardinian ALS patients. The study population was composed of all ALS patients of Sardinian ancestry, identified through the ITALSGEN consortium between 2008 and 2013. Genetic screening was performed both in patients and Sardinian healthy controls looking for mutations of *TARDBP*, *c9orf72*, *SOD1*, and *FUS* genes. 155 out of 375 Sardinian ALS cases (41.3%) carried genetic mutations, more commonly the p.A382T and p.G295S mutations of *TARDBP*, and the GGGGCC hexanucleotide repeat expansion of *c9orf72*. One patient displayed a double mutation of *TARDBP* (both p.G295S and p.A382T mutation) and eight carried both the heterozygous p.A382T mutation of *TARDBP* and a repeat expansion of *c9orf72*. Patients carrying the p.A382T and the p.G295S mutations of *TARDBP* and the *c9orf72* repeat expansion shared distinct haplotypes across these loci. The co-occurrence of *c9orf72* and *TARDBP* p.A382T missense mutation was related to a significantly lower age at onset and shorter survival. Among Sardinians we can find the highest percentage of genetically explained ALS cases outside of Scandinavia, since more than 40% of all cases coming from such island carry a mutation of an ALS-related gene. Different genetic mutations show some distinctive phenotype correlates, but the clinical heterogeneity within

and among families carrying the same mutations suggests that other genetic and environmental factors are involved in determining ALS clinical presentation.

## 4.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of the adult life, causing progressive loss of upper and lower motor neurons and leading to death due to respiratory failure within 2 to 4 years after the onset. The rate of patients with a family history positive for ALS or frontotemporal dementia (FTD) (familial ALS, FALS) is approximately 10% in Caucasian populations. The remaining 90% of cases appear sporadically (sporadic ALS, SALS). *c9orf72*, *SOD1*, *TARDBP* and *FUS* are the major ALS-related genes identified so far (Renton et al, 2014), with evident differences among ethnic groups and geographical regions. This heterogeneity includes the virtual absence of *SOD1* mutations in Ireland and the Netherlands (van Blitterswijk et al, 2012; Kenna et al, 2013), the extremely high frequency of *c9orf72* repeat expansions in Scandinavia (Majounie et al, 2012; Smith et al, 2012), and the high frequency of *OPTN* mutations associated with the relative rarity of *c9orf72* repeat expansions in Japan (Maruyama et al, 2010; Konno et al, 2013).

Sardinia is the second largest Mediterranean island and represents a genetic isolate, displaying a high frequency of disorders related to immune system disregulation (such as multiple sclerosis and diabetes mellitus type 1) and monogenic diseases (such as Wilson's disease and thalassaemia). This population shows reduced genetic and allelic heterogeneity, as expected for a genetic isolate. It is well known that ALS patients of Sardinian ancestry have a higher frequency than expected of the *TARDBP* p.A382T missense mutation and of the rate of familial ALS (Chiò et al, 2011; Orrù et al, 2012). Nevertheless, the incidence of ALS in

Sardinians retrospectively investigated seems to be within the range of European studies (Pugliatti et al, 2013).

This survey describes the genetic profile of a large series of ALS patients of Sardinian ancestry and extend the analysis to other ALS genes (*c9orf72*, *SOD1*, *TARDBP* and *FUS*).

### 4.3 Methods

**Patients.** All ALS cases of Sardinian ancestry, defined as patients with both parents of Sardinian origin) were considered eligible for the study. The study population was collected between 2008 and 2013 through the ITALSGEN consortium, which is composed of sixteen Italian ALS centers (Chiò et al, 2012a). All patients underwent a comprehensive clinical evaluation, including cognitive status assessment. ALS diagnosis was made according the El Escorial revised criteria for definite, probable, probable laboratory-supported, or possible ALS (Brooks et al, 2000).

**Controls.** 700 subjects of Sardinian ancestry not affected by neurodegenerative disorders were screened for *TARDBP* mutations. 262 subjects of Sardinian ancestry not affected by neurodegenerative disorders were screened for *SOD1*, *FUS* and *C9ORF72* mutations. The recruitment of controls was completed at the Department of Neurology, University of Cagliari. They were spouses or non-blood relatives of patients diagnosed with ALS or multiple sclerosis.

**Classification of familial ALS.** Patients were considered familial according to the current revised classification (Byrne et al, 2011; Chiò et al, 2014).

**Mutational screening.** The following exons and 50 base pair (bp) flanking intron-exon boundaries were screened for mutations by PCR amplification, sequencing through the Big-Dye Terminator v3.1 kit (Applied Biosystems Inc.), and analysis on an ABI Prism 3130 genetic analyzer: (a) all five coding exons of *SOD1*, (b) exon 6 of *TARDBP*, and (c) exons 14 and 15 of *FUS*. The selection of these exons was based on the evidence that the vast majority of known pathogenic variants lie within these mutational hotspots. A repeat-primed PCR assay was performed to search for the presence of the GGGGCC hexanucleotide expansion in the first intron of *c9orf72* (Renton et al, 2011; Dejesus-Hernandez et al, 2011). A cut-off of  $\geq 30$  repeats combined with a typical sawtooth pattern was considered pathological.

**Haplotype analysis.** We analyzed genome-wide single-nucleotide polymorphism (SNP) data from patients carrying the same mutation for haplotype analysis. A custom PERL software script was used to compare unphased sample genotype data.

**Statistical analysis.** The analysis of the differences between groups were performed using t-tests for continuous variables (such as age at symptom onset) and  $\chi^2$  for discrete variable (such as gender, site of onset, presence of frontotemporal dementia [FTD]). Comparison between series of means was performed through ANOVA. Kaplan-Meier curves were employed to calculate survival and the log-rank test was used to compare survival across groups. The end of follow-up was established on March 31<sup>st</sup>, 2014. None of the patients was lost to follow-up. Significance was set at p<0.05, two-tail test. Statistical Package for the Social Sciences (SPSS) version 21 was used (SPSS Inc, IBM, Somers, New York, USA).

**Standard Protocol Approvals, Registrations, and Patient Consents.** The Ethical Committees of all the involved centers approved the study protocol. A written informed consent was signed by all patients and controls before the enrollment. The survey was performed in

accordance with the Italian ethical rules for data collection for statistical or scientific purposes and for data protection.

#### 4.4 Results

We collected a cohort of 375 ALS cases with Sardinian ancestry, including 236 men and 139 women, with a mean (SD) age at onset of 61.1 (12.1) years. The site of onset was bulbar for ninety-nine patients (26.4%), while the others (276 [73.6%]) had a spinal onset disease. 100 cases (26.7%) reported a positive family history for ALS, FTD or both.

**Genetics of Sardinian ALS cases.** 155 cases (41.3% of our Sardinian ALS cohort) showed mutations in one or more genes (Table 1). Considering this group, 75 patients (20.0%) displayed a heterozygous p.A382T mutation of *TARDBP*, three patients (0.8%) were homozygous for the p.A382T mutation, ten patients (2.7%) were heterozygous for the p.G295S mutation of *TARDBP*, one patient (0.3%) was homozygous for the p.G295S mutation, and one patient (0.3%) carried both p.G295S and p.A382T mutations of *TARDBP*. Fifty-one patients (13.6%) carried a GGGGCC hexanucleotide repeat expansion of the *c9orf72* gene. Eight patients (2.1%) carried both the heterozygous p.A382T mutation of *TARDBP* gene and a pathogenic repeat expansion of *c9orf72*. Four patients (1.1%) with a missense mutation of *SOD1* were identified (three FALS with a p.A95G and one FALS with a p.A4T mutation). Two patients carried a p.T622A missense mutation of the *MATR3* gene (reported in the original paper as pedigree ITALS#10) (Johnson et al, 2014). No *FUS* mutations were detected.

**Genetic mutations in Sardinian controls.** Eight of the 700 control samples carried a p.A382T heterozygous missense mutation of *TARDBP* (1.1% of the control cohort, age 60-86 ys).

Given that 86 out of the 366 ALS cases (23.5%) in this series carried the p.A382T mutation, the relative risk of developing ALS in a subject carrying this mutation was 66.2 (95% CI: 32.9-141.9). The penetrance of this mutation at 70 years was calculated to be 66.1% (95% CI 48.3-83.9) among women and 80.4% (95% CI 70.0-90.8) among men in our cohort. Otherwise, the p.G295S mutation of *TARDBP* and the GGGGCC repeat expansions of *c9orf72* were absent in the Sardinian control population.

**Haplotype analysis** ALS patients displaying the p.A382T mutation of *TARDBP*, the p.G295S mutation of *TARDBP* and the *C9ORF72* repeat expansion shared distinct haplotypes across these loci (Table 2).

**Geographic distribution of ALS cases carrying mutations.** The two most common ALS-related mutations had a different distribution across the island ( $p=0.0001$ , Figure 1). The *TARDBP* p.A382T missense mutation showed the highest frequency in the two central-western provinces, where it covers approximately 30% of cases. The Northern and Eastern Sardinia had the higher rate of the *c9orf72* repeat expansion, with about 20% of cases. The p.G295S *TARDBP* missense mutation was not characterized by any geographical clustering.

**Clinical characteristics of ALS cases (Table 3).** Patients with the *c9orf72* repeat expansion showed a higher frequency of bulbar onset. Focusing on age at onset, patients with co-occurrence of *c9orf72* and the *TARDBP* p.A382T missense mutation and patients with *SOD1* mutations had a significantly lower age at onset (Figure 2). Moving to cognitive characteristics, a diagnosis of full-blown FTD was established in 51 patients (13.6%). Its frequency was higher in patients with *c9orf72* expansions, while it was rarer in patients without genetic mutations and in *SOD1* mutations carriers.

Our cohort underwent a specific clinical evaluation to detect the eventual presence of extrapyramidal signs: ten patients (2.7%) showed muscle rigidity and/or resting tremor. Of

these, two patients carried *c9orf72* mutation, three carried a *TARDBP* p.A382T heterozygous missense mutation, one patient had a *TARDBP* p.A382T homozygous missense mutation (Borghero et al, 2011), and four did not carry any known mutation. Eight of these patients (80.0%) had co-morbid FTD ( $p=0.0001$ ).

The overall median survival of Sardinian ALS patients was 4.2 years (interquartile range [IQR] 2.3-10.0). Survival was significantly different among groups carrying distinct mutations ( $p=0.0001$ , Figure 3). Patients with *c9orf72* expansion had a median survival of 2.7 years (IQR 1.9-3.8), patients with both *c9orf72* expansion and *TARDBP* p.A382T mutation showed a median survival of 3.1 years (IQR 1.8-3.9), and patients with a *TARDBP* p.A382T heterozygous mutation displayed the longest survival (median 6.5 years, IQR 3.3-10.5).

#### 4.5 Discussion

Our survey was conceived to analyze a large series of ALS patients coming from Sardinia, a genetic isolate. More than 40% of patients of the study population displayed a genetic mutation thought to be causative of ALS. The most common mutations were the p.A382T heterozygous missense mutation of *TARDBP*, the hexanucleotide repeat expansion of *c9orf72*, and the p.G295S heterozygous missense mutation of *TARDBP*. Noteworthily, several patients carried a double mutation, more commonly the *c9orf72* expansion and the p.A382T missense mutation of *TARDBP*. Less cases carried homozygous *TARDBP* missense mutations. Overall, 75% of FALS and 30% of apparently SALS patients displayed a genetic mutation. This represents one of the largest proportion of genetically explained ALS ever detected in a single population (Majounie et al, 2012). This is probably due to a founder effect acting within the Sardinian population and underlines the striking value of genetic studies of genetic isolates.

To confirm the hypothesis of a founder effect among Sardinians, we demonstrated that ALS patients carrying the *TARDBP* p.A382T missense mutation and the *TARDBP* p.G295S missense mutation share common haplotypes across the gene loci. Similarly we detected that patients carrying the *c9orf72* repeat expansion display the Finnish haplotype (Mok et al, 2012).

Distinct clinical and demographic characteristics have been found among patients carrying mutations of different genes. First, patients carrying a double *c9orf72/TARDBP* mutation show a significantly earlier onset, namely 20 years before other Sardinian cases. Second, patients carrying the *c9orf72* expansion and the p.G295S *TARDBP* mutations have higher frequency of bulbar onset. Third, patients with *c9orf72* mutation had the highest rate of positive family history for ALS or FTD (~65%). Fourth, patient with *c9orf72* mutation had the shortest survival, whereas those with p.A382T heterozygous missense *TARDBP* mutation had the longest one.

The frequency of frank FTD in patients carrying *TARDBP* mutations in the Sardinian population is similar to that expected in the general ALS population (Phukan et al, 2012; Montuschi et al, 2014). Noteworthily, there are reports of pure FTD cases of Sardinian ancestry carrying this mutation with a positive family history of ALS (Synofzik et al, 2014), suggesting that this association needs further analysis.

The possible origin of the founders might be hypothesized evaluating the geographical distribution of the genetic mutations across the island. For example, the p.A382T *TARDBP* cases are clustered in central-western Sardinia, suggesting that it originated in that area, while the *c9orf72* mutation probably originated in northern Sardinia.

The rate of positive family history for ALS or FTD in our series varies according to the gene involved. The highest rate was observed among patients with *SOD1* (100%) and *C9ORF72*

mutation (~70%), and the lowest in those with *TARDBP* p.A382T and p.G295S mutations (~30%). This is probably due to a higher penetrance of *SOD1* and *c9orf72* mutations within the study population. The identification of *TARDBP* mutations in approximately 1% of Sardinian controls, many of whom were in their sixties without any neurological disturbance, confirmed our finding. It is noteworthy that the penetrance of the *TARDBP* p.A382T mutation is significantly lower in women than in men, indicating that other genetic, hormonal or environmental factors may influence the onset of the disease (Orrù et al, 2011).

In conclusion, we found that more than 40% of all ALS Sardinian patients carry a mutation of an ALS-related gene. This is the highest percentage of genetically explained cases outside of Scandinavia, and largely higher than in other Caucasian populations (van Blitterswijk et al, 2012; Chiò et al, 2012b; Kenna et al, 2013). The percentage of Sardinian cases without a known mutation showing a positive family history for ALS or FTD is over 10%, suggesting that one or more new genes still have to be detected within this population. Some distinctive phenotypic characteristics are associated with mutations of different genes, but the heterogeneity within and among families carrying the same mutations may also imply the role of other genetic or non-genetic factors in the disease manifestation. The complex genetic interactions are clearly highlighted by the cases with double genetic mutations of the *c9orf72* and *TARDBP* genes, who present an early age at onset and have a shorter survival than patients carrying either mutation alone.

**Table 1.** ALS in Sardinia: frequency of mutations according to presence or absence of positive family history for ALS or FTD

Mutation	FALS (n=100)	SALS (n=275)	FALS+SALS (n=375)
Wild type	26 (26.0%)	194 (70.5%)	220 (58.7%)
<i>TARDBP</i> (p.A382T, hetero- and homozygous)	25 (25.0%)	53 (19.3%)	88 (23.5%)
<i>TARDBP</i> (p.G295S, hetero- and homozygous)	3 (3.0%)	8 (2.9%)	11 (2.9%)
<i>TARDBP</i> (p.A382T and p.G295S double mutation)	1 (1.0%)	-	1 (0.3%)
<i>C9ORF72</i>	33 (33.0%)	18 (6.5%)	51 (13.6%)
<i>C9ORF72</i> and <i>TARDBP</i> (p.A382T)	6 (6.0%)	2 (0.7%)	8 (2.1%)
<i>SOD1</i>	4 (4.0%)	-	4 (1.1%)
<i>MATR3</i>	2 (2.0%)	-	2 (0.5%)

**Table 2.** Haplotype analysis of genotype data across the *TARDBP* and *C9ORF72* loci

Mutation	Shared segment	Length	Number of shared SNPs	Number of analysed cases
p.A382T <i>TARDBP</i>	11,025,493 to 11,856,378	831 Kb	257	60
p.G285S <i>TARDBP</i>	10,799,577 to 12,779,618	1,980 Kb	571	7
<i>C9ORF72</i> repeat expansion	27,536,894 to 27,574,515	38 Kb	73*	25

\* 4 of these 73 SNPs were the same as the previously reported Finnish founder risk haplotype, indicating that the Sardinian patients carrying the pathogenic GGGGCC repeat expansion also shared the Finnish founder risk haplotype, at least in part.

**Table 3.** Clinical and demographic characteristics of Sardinian ALS cases

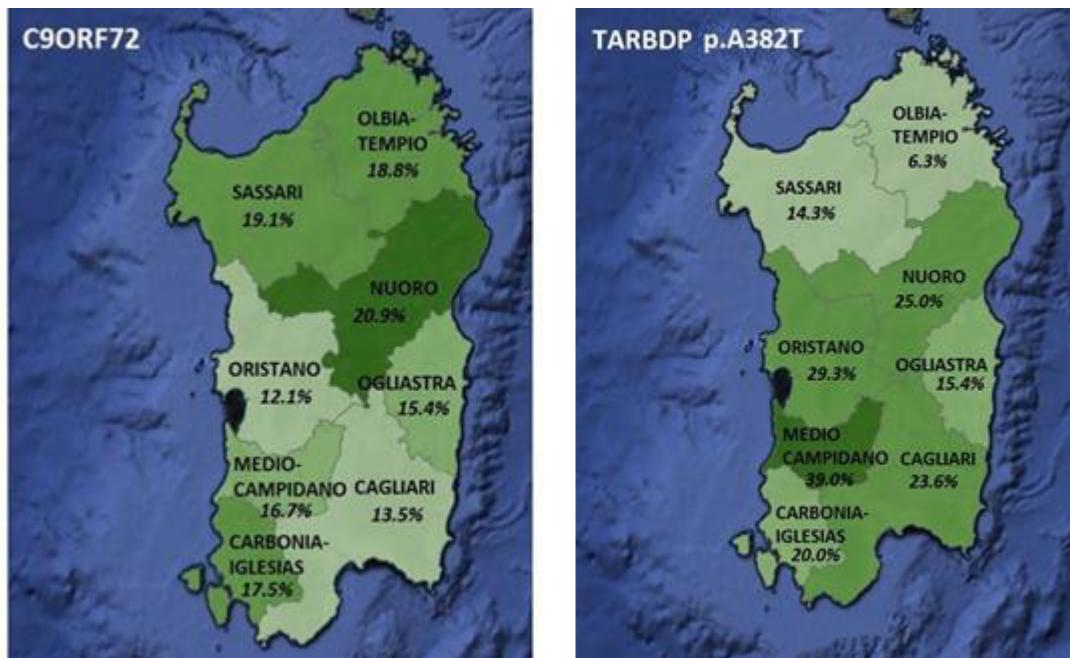
	Number of cases*	Gender (female, %)	Site of onset (bulbar, %)	Age at onset (median, IQR)	FALS (%)	FTD (%)
Wild type	220	85 (38.6%)	55 (25.0%)	63.2 (55.9-71.4)	26 (11.8%)	19 (8.6%)
<i>TARDBP</i> (p.A382T)	75	25 (33.3%)	18 (24.0%)	61.5 (53.6-68.6)	23 (30.7%)	9 (12.0%)
<i>C9ORF72</i>	51	20 (39.2%)	18 (35.3%)	62.3 (55.0-67.4)	33 (64.7%)	16 (31.4%)
<i>TARDBP</i> (p.A382T) & <i>C9ORF72</i>	8	1 (12.5%)	3 (37.5%)	43.8 (38.2-46.4)	6 (75.0%)	5 (62.5%)
<i>TARDBP</i> (p.G295S)	10	6 (60%)	5 (50%)	62.8 (56.2-74.9)	3 (30.0%)	1 (10.0%)
<i>SOD1</i>	4	0	0	57.1 (45.7-64.5)	4 (100%)	0
Overall	368	137 (37.2%)	99 (26.9%)	62.5 (54.4-69.2)	96 (26.1%)	50 (13.6%)
<i>P</i>	-	n.s.	n.s.	0.0001	0.0001	0.0001

\*Seven cases are not included in the table: one case with a double missense mutation of the *TARDBP* gene (p.G295S and p.A382T), 3 cases with homozygous p.A382T missense mutation of *TARDBP*, one case with homozygous p.G295S mutation of *TARDBP* and 2 cases with MATR3 mutation.

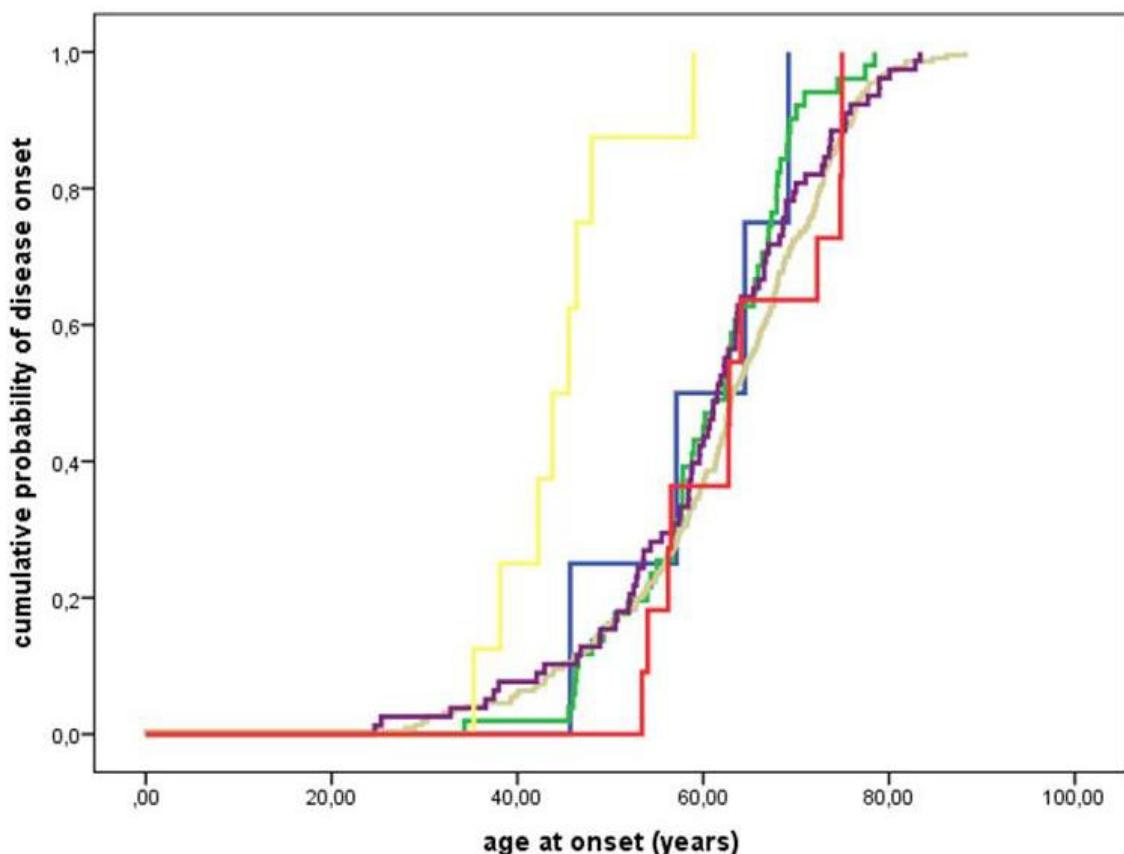
FALS, familial amyotrophic lateral sclerosis; FTD, frontotemporal dementia according to Neary's criteria; IQR, interquartile range

**Figure 1.** Geographic distribution of the areas of origin of parents of Sardinian ALS cases according to more common mutations. The two *TARDBP* p.A382T homozygous patients and the single patients with double p.A382T and p.G295S heterozygous mutations are included in the *TARDBP* figure.

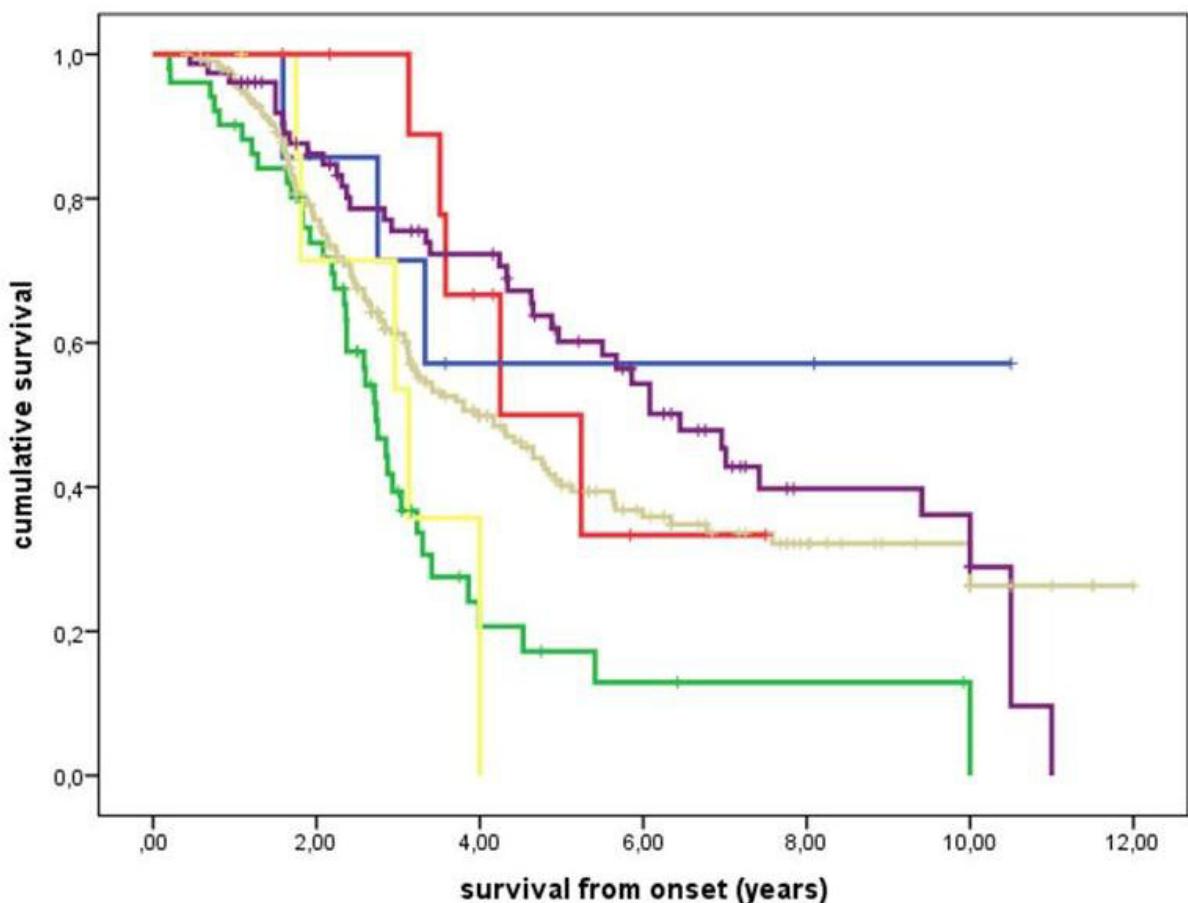
The 8 patients with double mutation (*C9ORF72* & p.A382T *TARDBP*) were included in both *C9ORF72* and *TARDBP* figures.



**Figure 2.** Cumulative probability of ALS onset according to genetic status of Sardinian patients. Brown, no detected mutations; violet, *TARDBP* p.A382T heterozygous mutation; green, *c9orf72* mutation; red, *TARDBP* p.G295S heterozygous mutation; yellow, double *C9ORF72* and *TARDBP* p.A382T heterozygous mutations.



**Figure 3.** Kaplan-Meier curves (tracheostomy-free survival) of ALS Sardinian patients with different genetic mutations. Brown, no detected mutations; violet, *TARDBP* p.A382T heterozygous mutation; green, *c9orf72* mutation; red, *TARDBP* p.G295S heterozygous mutation; yellow, double *c9orf72* and *TARDBP* p.A382T heterozygous mutations.



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## 5. Conclusions

### 5.1 Discussion of the results of the present project

“She was very clever, she seemed to understand perfectly all the questions that I did.

However, she responded with great difficulty and in a completely incomprehensible way.

Particularly, swallowing was impaired, food often penetrated in the larynx, causing dangerous fits of suffocation.

Madame Aubel also showed brisk tendon reflexes and very relevant fibrillar movements at the tongue. I also observed the lack of sensory deficits and sphincter dysfunction.” (Charcot & Joffroy, 1869).

With these words in 1869 Jean Martin Charcot reported a patient affected by ALS, underlining the exclusive motor impairment and the integrity of cognitive functions. Since the original description of ALS as a pure motor disease, the motor neuron disease scenario has deeply changed. Several papers have reported patients with clinical overlap of motor neuron disease and frontotemporal dementia over the years (Neary et al, 1990; Caselli et al, 1993; Vercelletto et al, 1999; Lomen-Hoerth et al, 2002). Two population-based studies have recently assessed that approximately 15% of ALS patients display a frank FTD, while about 35% show more subtle cognitive and behavioral changes (Phukan et al, 2012; Montuschi et al, 2014). A milestone in the field of the ALS-FTD spectrum was placed with the identification of a linkage of familial ALS with FTD to chromosome 9p.21 (Hosler et al, 2000), that has started a hunt ended in 2011 with the finding of the *c9orf72* gene (Renton et al, 2011; DeJesus-Hernandez et al, 2011). In the last decade some other genes have been found as

shared genetic factors involved in both ALS and FTD: *TARDBP* (Sreedharan et al, 2008), *FUS* (Kwiatkowski et al, 2009), *VCP* (Johnson et al, 2010), *UBQLN2* (Deng et al, 2011), *SQSTM1* (Fecto et al, 2011), and other less common genes.

Taken together, clinical, genetic and neuropathological data have led to the formulation of the ALS-FTLD continuum hypothesis, suggesting that these pathological conditions are situated at the extremes of the same disease spectrum, encompassing a broad area of clinical, subclinical, and neuropathological overlap.

The study of the frontotemporal syndromes affecting approximately half of ALS patients is of outstanding importance because of their substantial impact on prognosis (Olney et al, 2005) and burden of care (Chiò et al, 2010). Besides, the elucidation of the pathomechanisms common to ALS and FTLD might be essential for the identification of targets for future therapeutic strategies.

During the development of the research project we detected a novel p.G147C heterozygous missense mutation of *SOD1* gene in a fALS case, associated with non-executive cognitive impairment. The index case showed an impaired performance in the Rey-Osterrieth Complex Figure Test in agreement with a reduced uptake of the tracer in the right supramarginal gyrus (BA 40) and the right middle frontal gyrus (BA 10) at <sup>18</sup>F-FDG PET and with a focal atrophy at MRI in the right supramarginal gyrus (BA 40). Noteworthily, we demonstrated a progression of the cognitive deficit at the neuropsychological assessment and <sup>18</sup>F-FDG PET. Three aspects make this case notable. First, the patient carried a novel mutation, broadening the field of *SOD1* mutations. Second, the presence of cognitive impairment is rare in *SOD1* mutations carriers (Millecamp et, 2012), so this report provides original information to better characterize this uncommon eventuality. Third, the trend to progression of the cognitive

deficit is worthy of note, since data about the natural course of cognitive impairment in ALS are limited.

The second study presented here was aimed to evaluate the applicability of the cognitive reserve hypothesis to the frontotemporal syndromes of ALS patients. The cognitive reserve hypothesis is based on the observation that, for a given degree of cognitive impairment, subjects with high levels of education, occupational skills and leisure abilities, show a more sizeable cerebral lesion load at neuroimaging or neuropathological studies, as if they were able to cope better with neurodegeneration. This theory has been proved valid for cognitive impairment ascribable to several dementing pathologies: Alzheimer's Disease (Garibotto et al, 2012), Frontotemporal Dementia (Spreng et al, 2011), Multiple Sclerosis (Sumowski et al, 2014), Brain Trauma (Schneider et al, 2014). As proxies of cognitive reserve, we considered education, in terms of years of schooling, and the Reserve Index, a parameter encompassing education and occupational skills level. In a population-based series of ALS patients we have found that a lower education and a worse RI are associated with a higher risk of FTD, supporting the hypothesis that cognitive reserve is protective for the development of cognitive impairment in ALS patients. This finding has been replicated in an independent clinic-based validation cohort. Notably, cognitive reserve seems to act as a protective factor also in patients carrying the hexanucleotide expansion of *c9orf72*, a gene with a strong link with FTD. To the best of our knowledge, our study is the first one to provide a preliminary evidence that the cognitive reserve hypothesis may be valid for the cognitive impairment associated with ALS, in particular in patients with frank FTD, and may have an influence also in patients carrying genetic mutations, such as *c9orf72*. Nevertheless, further studies are necessary to correlate our epidemiological data to neuroimaging measures of cerebral lesion load. The mechanisms of cognitive reserve are worthy of study, as their comprehension might

be the basis for prevention strategies and rehabilitation treatments based on cognitive exercise. Furthermore, the elucidation of the underlying molecular pathways might offer potential targets for enviromimetics drugs, that constitute a novel field of therapeutic research. These are treatments mimicking and/or enhancing the action of environmental factors (Nithianantharajah et al, 2009).

The third study of our project was focused on the genetic architecture of ALS in Sardinia, insular Italy. The Sardinian population is a genetically conserved population, displaying a high rate of ALS cases due to genetic mutations (Chiò et al, 2011). Therefore it represents a valuable tool to investigate ALS genetics.

In our Sardinian population more than 40% of patients carried a genetic mutation in one of the major ALS-related genes. The frequency of genetic mutations was 75% among fALS and 30% among apparently sALS patients. This is the highest percentage of genetically explained cases outside of Scandinavia. The p.A382T heterozygous missense mutation of *TARDBP*, the hexanucleotide repeat expansion of *c9orf72*, and the p.G295S heterozygous missense mutation of *TARDBP* were the most frequent findings. The p.A382T and p.G295S carriers shared common haplotypes across the gene loci, while patients with *c9orf72* repeat expansion showed the Finnish haplotype (Mok et al, 2012). Therefore, the haplotype analysis supports the hypothesis of a founder effect. Notably, we detected several cases showing a double pathogenetic mutation. The most common finding was the presence of the *c9orf72* expansion with the p.A382T missense mutation of *TARDBP*: these patients showed an earlier age at onset and a shorter survival than patients carrying either mutation alone. Homozygous *TARDBP* missense mutations resulted less common. The presence of multiple mutations in ALS-related genes has been detected also in Dutch FALS cases: van Blitterswijk and

colleagues reported this finding in excess of what is to be expected by chance, suggesting the concept of ALS as an oligogenic disease (van Blitterswijk et al, 2012).

Patients with *c9orf72* mutation had the highest rate of positive family history for ALS or FTD (~65%). The percentage of cases with full-blown FTD among Sardinian *TARDBP* mutation carriers is comparable to that observed in the general ALS population (Phukan et al, 2012; Montuschi et al, 2014). This percentage rises up to ~31% in the *c9orf72* group and to ~62% in double mutation carriers (p.A382T of *TARDBP/c9orf72*).

Overall, more than 10% of Sardinian cases without a known genetic mutation display a positive family history for ALS or FTD, supporting the hypothesis that one or more novel genes linked to ALS and FTD are still hidden within this population.

Our results confirm the value of genetic isolates for the study of the genetic basis of human diseases. In this context, the Sardinian population is considered particularly helpful for the investigation of the genetic factors underlying ALS-FTD, the interaction between different genes in the same subject and the phenotypic heterogeneity among different subjects within the same family. Besides, new causative genes for ALS and FTD might arise from the deep analysis of the genetic characteristics of the Sardinian population.

In conclusion, the development of the present project has encompassed several aspects of the ALS-FTLD continuum. We have elucidated the cognitive correlates of genetic mutations in ALS patients through the study of a genetic isolate (a highly conserved population from Sardinia, insular Italy), and the recruitment of a population-based series afferent to the Piemonte and Valle d'Aosta Register for ALS and a clinic-based series afferent to the ALS Centre of Turin. Likewise, also environmental factors such as education and occupation have been investigated as potential modifier factors of the development of cognitive impairment in ALS. Cognitive deficit in ALS is detectable in up to 50% of cases, involving mostly executive

functions. Our data suggest that it is certainly related to genetic factors, the strongest being the *c9orf72* repeat expansion, but its development is probably influenced by environmental factors. Taken together, our results are in keeping with the concept of ALS as complex disease, in which both genetic and environmental factors play a role (Simpson & Al Chalabi, 2006), and as one of the two sides of a disease continuum with FTLD (Ling et al, 2013). These data should warrant further investigations, looking for potential molecular targets for therapeutic interventions acting on both actors of the ALS-FTLD continuum, and valuable strategies aimed to prevent the manifestation of cognitive impairment.

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